1117

Acetylcholine Receptor Channel Structure Probed in Cysteine-Substitution Mutants, M.H. Akabas, D.A. Stauffer, M. Xu, and A. Karlin, Center for Molecular Recognition, Departments of Biochemistry & Molecular Biophysics, Physiology & Cellular Biophysics, Medicine, and Neurology, College of Physicians & Surgeons, Columbia University, New York, NY 10032.

In a systematic approach to the identification of residues lining the cation-conducting channel of the acetylcholine receptor, we have mutated nine consecutive residues in the alpha-subunit M2 -membrane-spanning segment to cysteine, expressed the mutants in Xenopus oocytes, and determined the accessibility of these cysteines to small, charged, sulfhydryl-specific reagents. Alpha Ser248, Leu250, Ser252, and Thr254 are exposed in the channel and may form a beta-strand. The channel gate is closer to the cytoplasm than any of these residues. On channel opening, Leu251 also becomes exposed, reflecting a change in channel structure.

1118

Structure and Function of Outer Membrane Channels.
Bing K. Jap, Thomas N. Earnest, Peter J. Walian and Kalle Gehring
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Porins are a family of outer membrane channels of the Gram-negative bacteria. These channels serve as pathways for waste products and nutrients for transportation out of or into the cells. Porin channels have been shown, *in vitro*, to be voltage-gated. The three-dimensional structure of PhoE porin reconstituted into a lipid bilayer has been determined to a resolution of 3.4 Å by electron crystallographic techniques. The 3-D map shows that the basic structural design of porin consists of a trimer of elliptically shaped, cylindrical walls. The wall of each cylinder is formed by sixteen β-sheet strands having major fraction of strands tilted at about 55 degrees from membrane plane. Within each elliptical cylinder, there is an additional polypeptide that forms a lining, located near the β-sheet wall that is furthest away from the three-fold symmetry axis of the trimer and therefore situated away from the center of the elliptical cylinder. This lining is a part of the structure at the constricted region of the channel and plays an important role in the channel selectivity. The presence of the lining provides an answer as to how deletions of segments of polypeptide, which are found in certain mutants, can result in an actual increase in the channel size in response to the growth environments. However, the presence of a rather large channel opening observed in our structure provides no clear clue as to the closing structural stage of the channel. Lipopolysaccharide is required for the closing and opening of the channel to occur, and the closing and opening of a trimer of channels has been reported to be cooperative. This would suggest that lipopolysaccharide is located between monomeric channels of a trimer and plays a role in the closing and opening of the channel. In fact, the presence of lipopolysaccharide at the three fold symmetric axis has earlier been proposed to exist for *E. coli* porins in their native state.

Poster Sessions: Neurotransmitters, Peptides, and Receptors (1119-1122)

1119

Chemotactic Response of Growth Cones in A Gradient of Acetylcholine. J.Q. Zheng, M. Felder, Z.W. Zheng, A. Lohof, M.M Poo, Department of Biological Sciences, Columbia University, New York, NY 10027.

With a micropipette and repetitive pulsed ejection, a microscopic gradient of acetylcholine (ACh) was produced near the growth cone of embryonic *Xenopus* spinal neurons in cell culture. Facing such an extracellular gradient of ACh, the growth cone of cultured *Xenopus* spinal neurons exhibited a chemotactic response toward the source of ACh, resulting a turning of the neurite. The sucrose gradient did not have any effect on neurite orientation. Similar gradients of glutamate, however, inhibited the growth and caused the neurite retraction. In these experiments, the ACh pipette was positioned 100 μm away from the growth cone with 45°C angle from the direction of neurite extension, and ACh was ejected into bathing solution at 2 Hz frequency. The concentration of ACh reaching the growth cone was estimated in the micromoler range (typically about 100 μM). When applied uniformly in the bathing solution, such concentration of ACh was found to increase the rate of neurite extension, suggesting a trophic effect of ACh on the neurite extension. Apparently, the chemotactic response of the growth cone to the gradient of ACh was mediated by neuronal nicotinic ACh receptors since it was inhibited by d-tubocurarine. Intracellular calcium, as examined using fluorescence probe fluo3, was found to increase under the application of ACh. Thus cytosolic calcium ions may serve as second messenger mediating this ACh-induced turning response. Taken together, our results indicate that neurotransmitters, besides serving as messengers at the chemical synapse, are potential neurotrophic and neurotropic agents during development of the nervous system.

1120

Purification, Cloning and Expression of the GH_4C_1 pituitary cell Somatostatin receptor. J. R. Hadcock C. M. Eppler, and J. Strnad. American Cyanamid Company, Agricultural Research Division, PO Box 400, Princeton, NJ 08543-0400.

Somatostatin receptors are members of the G-protein-linked receptor superfamily and exert their biological effects by binding to specific high-affinity cell-surface receptors. Activation of somatostatin receptors inhibits adenylyl cyclase activity and decreases intracellular cyclic AMP levels. Somatostatin is a potent inhibitor of secretion from many pituitary cells including somatotrophs, lactotrophs, thyrotrophs, and corticotrophs. Somatostatin inhibits secretion of insulin, glucagon, many gut hormones and digestive enzymes. The somatostatin receptor from GH_4C_1 pituitary somatotrophs has been purified to homogeneity. Cyanogen Bromide digestion of the purified receptor yielded five major peptide fragments. Peptide sequence analysis of the fragments resulted in amino acid sequences ranging from 6 to 25 residues. Several of these contained consensus sequences found in many G-protein-linked receptors. Using oligonucleotide primers based on most frequently used codons, a 501 base pair cDNA fragment was amplified via PCR. The full-length GH_4C_1 receptor cDNA has been cloned, sequenced, and expressed in Chinese Hamster Ovary (CHO) cells. On the basis of homology to the GH_4C_1 SSTR, the rat gut/brain SSTR has also been cloned. The ability of somatostatin to inhibit forskolin-stimulated cyclic AMP accumulation was examined in the transfected CHO cells. Whereas wild-type CHO cells showed no detectable response to somatostatin, somatostatin inhibited forskolin-stimulated cyclic AMP accumulation in cells transfected with the GH_4C_1 receptor and the gut/brain receptor (63 and 75%, respectively).

1121

Role of Nitric Oxide in NMDA Receptor-Mediated Release of Neurotransmitters and ADP-Ribosylation of a 36 kDa Protein. L.M. Brumley, M.J. Friedlander, P.R. Montague, C.D. Gancayco, and R.B. Marchase. Department of Cell Biology and Neurobiology Research Center, University of Alabama at Birmingham, Birmingham, AL 35294.

Nitric oxide (NO) is a non-polar gas made in the mammalian brain upon N-methyl-D-aspartate (NMDA) receptor activation. NO may serve as a diffusible signal that communicates the status of postsynaptic sites to neighboring pre- and postsynaptic sites. We investigated the role of NO in synaptosomes prepared from anesthetized adult guinea pigs, and found that NMDA caused release of three neurotransmitters - norepinephrine, glutamate, and aspartate (EC_{50} = 10-30 μM, n=25). This effect was blocked in zero calcium, by 2-amino-5-phosphonovaleric acid, by preincubation with the NO synthase inhibitors monomethyl-L-arginine and N^G-nitro-L-arginine, and by inclusion of the NO scavenger hemoglobin (Hb). Activation of the NMDA receptors on one group of synaptosomes induced neurotransmitter release from a second group that were inhibited in their production of NO and therefore in their response to NMDA. This effect was also blocked by Hb. We have also begun an investigation of the ADP-ribosylation of synaptosomal proteins, since NO may exert its effects via this posttranslational modification. We have demonstrated that the radiolabeling of a 36 kDa protein in synaptosomal homogenates by [^{32}P]NAD^+ is enhanced by the NO-producing compound sodium nitroprusside. We have also demonstrated that a 36 kDa protein is radiolabeled in intact synaptosomes preincubated with $^{32}PO_4$. These findings suggest that NMDA receptor activation can cause neurotransmitter release from neighboring synaptic elements via NO production, and that the ADP-ribosylation of a 36 kDa protein may be involved in the regulation of synaptic transmission by NO. Supported by NIH EY06714 (RBM) and EY05116 (MJF).

1122

A Unique Serine Carboxyl-Terminal Adrenocorticotropin-Cleaving Enzyme in Bovine Intermediate Lobe Secretory Vesicle Membranes. T.C. Friedman, H.C. Chen and Y.P. Loh, LDN, NICHD, NIH, Bethesda, MD 20892. (Spon. E.A. Neale.)

Adrenocorticotropin (ACTH) is a 39-amino acid peptide synthesized in the pituitary as part of the precursor molecule, pro-opiomelanocortin (POMC). In this study, a unique enzyme from bovine intermediate lobe secretory vesicle membranes which functions both as a dipeptidyl carboxypeptidase by removing the carboxyl-terminal Glu-Phe dipeptide from $ACTH^{1-39}$ as well as a carboxypeptidase by removing the carboxyl-terminal phenylalanine residue from ACTH was characterized. Kinetic studies indicate that the formation of $ACTH^{1-37}$ occurred initially within minutes, while the formation of $ACTH^{1-38}$ was slower, within hours. These enzymatic activities both had a pH optima of 5.5 and were inhibited by serine and thiol but not metallo- or aspartic protease inhibitors. The similar pH optimum, and inhibitor profile suggest that one enzyme is responsible for both enzymatic activities. Bovine anterior lobe pituitary homogenates were also found to contain a large amount of $ACTH^{1-37}$ and a smaller amount of $ACTH^{1-38}$ in addition to $ACTH^{1-39}$. Although the biological activity of $ACTH^{1-37}$ and Glu-Phe are not known, these results suggest that this unique serine dipeptidyl carboxypeptidase functions as a converting enzyme *in vivo*.

1123

Effect of ACTH 1-39 on neuritogenesis of developing chick retina. M.J. Perone, M.E. Vazquez, L. Guelman, N.G.Carri and F.E. Estivariz. Dept. of Neurosc, IMBICE, CC 403, 1900 La Plata, Argentina.

The hormone ACTH is a peptide derived from a major precursor protein: POMC (pro-opiomelanocortin). The peptide is present in the pituitary in high quantities; however, ACTH-immunoreactive cell bodies are located in rat brain. The aim of this study was to check the effect of ACTH 1-39 on neuritogenesis. The assay consisted of retinal explants (White Leghorn E6) grown on collagenous substrates and incubated for 4 days with trophic support (optic lobe extract E18). Controls were performed in flat media. Experiments were performed with ACTH (picomolar concentrations) and it was found that the peptide clearly stimulated neuritogenesis and outgrowth. Fascicles grew radially with a good density and a high elongation rate (12 um/h). Maximum neurite outgrowth reached 1.16 mm and the effect of different concentrations showed that stimulation followed a dose-response pattern. The minimum effective dose used was 5 pg/ml. Our results suggested that this molecule could have trophic activity easily comparable with neurotrophines. We could postulate that this peptide or any other ACTH-related peptide may act on the developing chick retina.

1124

Polyamines & NMDA Receptors Modulate Cold-Injury Induced Pericapillary Astrocytosis in Rat Cerebrum

J.J. Trout, H. Koenig, C.Y. Lu & A.D. Goldstone, Depts. of Neurology, Cellular Molecular & Developmental Biology, Northwestern Univ. & Research Services, VA Lakeside Medical Center, Chicago, IL 60611

Previously we have shown that polyamines (PA) regulate cryo-injury (CI) induced transcellular transport in capillary endothelium & that PA & N-methyl-D-aspartate receptor (NMDA-R) modulate excitatory amino acid induced cytotoxicity in embryonic (15 d) chick retina. In this study we report the role of polyamines & NMDA-R in the development of astrocytosis following (CI). Pericapillary astrocytes were examined by TEM 4 h after CI. The area examined was a circle of radius equal to the average of the longest & shortest diameter of each capillary (ratio>8.5). The percentage of pericapillary space (PS) occupied by astrocytic profiles as determined by morphometrically using a grid with point-point spacing of 1.0 cm was as follows: Sham=11.41 ± .87, CI=17.39 ± 1.21, CI + DFMO= 11.38 ± .78, CI + MK-801=11.21 ± .71, CI + DFMO + putrescine (PUT)=22.08 ± 1.18. CI caused a significant increase in the percentage of the PS occupied by astrocytes, which was reduced to basal levels by addition of either a specific inhibitor of PA synthesis, α-difluoromethylornithine (DFMO), or the specific NMDA-R inhibitor, MK-801. PUT reversed the DFMO effect & raised the area occupied by astrocytic profiles significantly above the CI levels. These data suggest that both ODC (via synthesis of PA) and NMDA receptors play a role in astrocytic swelling observed following CI. (Supported by the VA Research Service)

1125

Fibroblast and Neuronal Bradykinin (BK) B_2 Receptors Are Tyrosine-Phosphorylated. **Y.-J. I. Jong, L.R. Dalemar, and N.L. Baenziger, Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110**

WI-38 human lung fibroblasts express bradykinin B_2 receptors activating PGE_2 production, in three affinity forms identifiable by biological activity and ^{3}H-BK binding: high (H, K_D 440 pM), intermediate (I, K_D 5.6 nM), and low (L, K_D 42 nM). We have generated monoclonal antibodies (MAbs) directed against BK B_2 receptor biological activity. Certain MAbs selectively distinguish I-form or L-form receptors, and other MAbs recognize epitopes common to both. All the MAbs immunoblot an M_r 73-78 KDa protein band in fibroblast membranes. The 73 KDa BK receptor isolated by immunoaffinity chromatography on an anti-BK receptor MAb column is also blotted by the Py20 MAb recognizing phosphotyrosine (Tyr-P). Anti-Bk receptor MAbs blot the same band in membranes from the human neuroblastoma line SH-SY5Y and rat pheochromocytoma line PC-12, indicating fibroblast and neuronal B_2 BK receptors share antigenic determinants. The neuronal receptor also blots with the Py20 MAb, and thus has Tyr-P. The Tyr kinase inhibitor genistein blocks BK-mediated PGE_2 production with an ED_{50} of 8 μM, indicating a role for a Tyr phosphorylation step in the signal transduction path leading to prostaglandin synthesis.

1126

HISTAMINE 3 (H_3) RECEPTOR ANTAGONISTS POTENTIATE BRAIN MAST CELLS (MC) AND NEURONAL SEROTONIN (5HT) AND HISTAMINE SECRETION. Iacek I. Rozniecki, Richard J. Letourneau, Miltiadis K. Sugiultzoglu and Theoharis C. Theoharides. Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

Vasoactive mediators of brain mast cells, as well as neuronal biogenic amines, seem to play an important role in brain pathophysiology. As we showed before (FASEB Journal, 6, 1559, 1992) the H_3 receptor agonist - N-alpha-methyl histamine inhibited brain mast cell and neuronal serotonin secretion. In the present study, we investigated the effect of the H_3 receptor antagonist - thioperamide on the some test system. Rat thalamic and hypothalamic slices were loaded with ^{3}H-5HT ($5x10^{-7}$ M) for 15 min at 37 °C, washed, placed in 2 ml wells of a perfusion chamber and washed again with saturated 5% CO_2/ 95% O_2 Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4, 37 °C) containing 10^{-4} M imipramine (IMI) for 60 min. The slices were then perfused for 36 min with thioperamide (10^{-6} M), followed by 24 min stimulation by 100 μg/ml of C48/80 in KRB+IMI to stimulate MC. The slices were then washed for 20 min, followed by 40 mM K^+ to cause neuronal depolarization. Control samples were not treated with thioperamide, which did not have any effect on its own. Aliquots of collected fractions were assayed for ^{3}H-5-HT by liquid scintillation counting and histamine by radioimmunoassay. After each experiment, perfused brain slices were fixed in 2.5% glutaraldehyde with 0.5% tannic acid in 0.1 M phosphate buffer for 24 hours at 4°C. The samples were that washed, postfixed in 2% osmium tetroxide, dehydrated in graded alcohols, embedded in Epon and observed under electron microscope. Thioperamide potentiated 5HT, as well as histamine release, both from brain mast cells and neurons by more than 50%. Ultrastructural changes of brain mast cells showed much more pronounced degranulation and loss of electron-dense contents from thioperamide-treated cells, as compared to controls. These data confirm the presence of H_3 receptors on brain mast cells and indicate that they may have an important role in the regulation of their secretion. (Supported by Kos Pharmaceuticals, FL).

1127

Developmental Regulation of Glucose Consumption in Cerebellar Neurons in Primary Culture. A. Torreblanca, M.T. Fernández, S. Gascón and A. Novelli. Department of Functional Biology, University of Oviedo, School of Medicine, Oviedo 33006, Spain.

Cerebellar neurons in primary culture are undergoing morphological, ultrastructural, biochemical and electrophysiological differentiation. No data are available on whether the achievement of full differentiation may involve changes in the cellular energy requirements. On the other hand, it is known that toxicity by excitatory amino acids (EAAs) in fully differentiated cultured cerebellar neurons is dependent upon cellular energy charge. Here we report that glucose consumption presented two linear phases during days in culture (DIC), changing from ~1 μmol glucose/10^6 cells x DIC from 3 to 12 DIC (phase A) to ~2 μmol glucose/10^6 cells x DIC from 12 to 20 DIC (phase B). The number of neurons in culture decreased by ~40% from 0 to 3 DIC and then remained constant at ~10^6 neurons /35 mm dish. The number of non neuronal cell was limited to ~3% of total by the addition of ARA-C (10 μM) at 1 DIC. Protein content doubled from 3 to 12 DIC, and then remained constant at ~200μg/10^6 cells thereafter. Thus, when glucose consumption was calculated with respect to protein content, the ratio between phase B and phase A was ~4. We investigated whether phase B of glucose consumption could be dependent upon generic energy-dependent enzimatic activities, such as NAD(P)H-dependent reductase, or could be specifically related to glutamatergic neurotransmission. NAD(P)H-dependent reductase activity, as measured by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, increased from ~400 nmol formazan/mg protein at 3 DIC to ~800 nmol formazan/mg protein at 12 DIC and remained constant thereafter, thus excluding a correlation between phase B of glucose consumption and reductase activity. On the other hand, antagonists of the NMDA-type of EAA receptors, such as aminophosphonovaleric acid (APV) and MK-801, significatively reduced phase B of glucose consumption, while they did not affect phase A. We conclude that at least part of phase B of glucose consumption in cerebellar neurons in primary culture may be specifically related to the glutamatergic nature of these neurons.

1128

THE UNIQUE FEATURES OF THE SIGNAL SEQUENCE OF RAT DOPAMINE ß-HYDROXYLASE Z. Feng*[1], R.H. Angeletti[2], and E.L. Sabban[1]. [1]Dept. Biochem. & Mol. Biol. N.Y. Med. Coll. Valhalla, NY 10595 and [2]Dept. Dev. Biol. & Cancer, Albert Einstein Sch. Med. Bronx, NY 10461.

Dopamine ß-hydroxylase (DBH) which catalyzes the synthesis of norepinephrine is present in multi-forms in neurosecretory vesicles. Since the cloning of human, bovine and rat DBH revealed a single open reading frame and an unusually long putative signal sequence, considerable progress has been made in elucidating the mechanism of the difference between these forms, but the role of signal sequence is controversial. To investigate how they may arise from translation of a single mRNA and the function of signal sequence, a full length and two 5' truncated rat DBH cDNAs were used to generate mRNA with a full length, partially and completely deleted signal sequence. Truncating transcription was also used to generate 3' truncated mRNA. All of these DBH mRNAs were capped and translated in a rabbit reticulocyte lysate cell-free system in the presence or absence of pancreatic microsomal membranes. The results indicate that the newly synthesized DBH in the cell free system undergoes glycosylation and incorporation into microsomal membranes without cleavage of the amino-terminal "signal" sequence. Partial deletion of the amino-terminal signal sequence and the deletion of carboxy-terminal sequence did not affect membrane insertion and glycosylation, but as expected, they were abolished by complete deletion of signal sequence. Unique features of the signal sequence of rat DBH will be discussed.

1129

Identification of Cyclic Nucleotide Regulated Phosphoproteins in Motile Rod Photoreceptor Inner/Outer Segments. K. Pagh-Roehl, E. Han and B. Burnside. Department of Molecular/Cell Biology, University of California, Berkeley

In teleost retinas, rod photoreceptors elongate in the light and shorten in the dark through a redistribution of cytoskeletal actin. To understand how the actin cytoskeleton is regulated, we have been using isolated rod inner/outer segments (RIS-ROS) which elongate when cultured in the light. Since increasing intracellular cGMP and cAMP inhibits RIS-ROS elongation and promotes shortening via effects on protein kinase activity (Liepe & Burnside, *JCB 115:210a*,1991), we have begun to characterize cyclic nucleotide-dependent phosphoproteins in RIS-ROS. Homogenates incubated with $^{32}P_\gamma ATP$ in the presence of 8BrcAMP (≥ 100 nM) showed increased phosphorylation of proteins at 67, 35, 33, 23-25 and 17 kDa. Identical proteins were phosphorylated in the presence of 8BrcGMP at 1000-fold higher concentration. Both 8BrcGMP- and 8BrcAMP-stimulated phosphorylation were inhibited by PKI, a specific inactivator of cAMP-dependent protein kinase (PKA), implying that cGMP is acting via PKA rather than PKG in these assays. The 35, 33 and 17 kDa phosphoproteins were retained in cytoskeletal fractions of RIS-ROS, although they could be eluted from the cytoskeletons by washing; an abundant light-regulated phosphoprotein, opsin, was not retained in cytoskeletons. The identity of these phosphoproteins is being further investigated by microsequencing and immunoblotting. Using an antibody against phosducin, the major cAMP-regulated phosphoprotein found in mammalian photoreceptors and also shown to bind photoreceptor Gβ and Gγ (Lee et al, *Biochemistry 26*:3983, 1987; antibody from Dr. R. Lee), we found that teleost RIS-ROS also contain phosducin, which comigrates with the 33/35 kDa phosphoproteins. Our results suggest that both cGMP and cAMP regulate rod motility through PKA modulation of target phosphoproteins which act upon the rod actin cytoskeleton. [*Supported by EY03575*]

1130

MODULATION OF THE cGMP-GATED CHANNELS OF ROD PHOTORECEPTOR CELLS BY CALMODULIN. Y.T. Hsu and R.S. Molday. (SPON :N. A. Auersperg). Dept. of Biochemistry, Univ. of British Columbia, Vancouver, B.C., V6T 1Z3.

Photoreceptor rod outer segments (ROS) are highly specialized organelles that mediate phototransduction under dim light condition. This phototransduction process occurs when the light-activated rhodopsin initiates a series of enzymatically catalyzed reactions leading to a decrease in the cGMP level and the closure of the cGMP-gated channels in the plasma membrane of ROS. The recovery phase of this process involves the deactivation of rhodopsin, transducin, and phosphodiesterase as well as the resynthesis of cGMP by guanylate cyclase and the reopening of the cGMP-gated channels in response to elevated level of cGMP. Cytoplasmic calcium plays a role in this recovery process by modulating the guanylate cyclase activity through a calcium binding protein recoverin. We have recently identified a 17 kDa protein from the EDTA extract of ROS membranes as calmodulin, a calcium binding protein known to regulate the activity of various enzymes. In the presence of Ca^{2+}, calmodulin binds to a 240 kDa protein which has been shown to be tightly associated with the cGMP-gated channels of mammalian rod photoreceptors. Kinetic analysis of cGMP-dependent cation influx into ROS membrane vesicles indicates that calmodulin increases the Km of the channel for cGMP without affecting its cooperativity. This effect can be reversed by either decreasing the Ca^{2+} level or by the use of calmodulin inhibitor mastoparan. On the basis of these studies, this calmodulin modulation may represent a novel calcium dependent regulation of the photorecovery and light adaptation processes.
These studies were supported by grants from MRC and NIH.

1131

Expression of MGSA/gro by Human Retinal Pigment Epithelial (RPE) Cells
G.J. Jaffe, R. Shattuck*, W. Roberts, A. Richmond* Duke University, Durham, NC. and *Vanderbilt University and Department of Veterans Affairs, Nashville, TN.

Cytokines produced by RPE cells are important in the pathogenesis of ocular diseases such as proliferative vitreoretinopathy (PVR) and uveitis. The cytokines MGSA/gro α, β and γ are members of a supergene family with growth regulatory and inflammatory activities. The role of MGSA/gro in ocular disease is unknown. Herein, we determined the mechanism for induction of MGSA/gro mRNA in human RPE and whether these cells produce MGSA/gro protein. To measure mRNA expression, total RNA was extracted from cultured human RPE cells, reverse-transcribed to cDNA, and amplified with PCR using primers specific for MGSA/gro α. RPE cells were transfected with MGSA/groα350/CAT fusion gene DNA to determine transcriptional activation of MGSA/gro. Protein secretion was determined by immunoprecipitation of RPE cell-conditioned medium. Minimal MGSA/groα was expressed under basal conditions; however, MGSA/groα mRNA was markedly induced within 1 hour after cells were exposed to IL-1β (5 U/ml) or after serum-starved cells were refed serum. IL-1β caused a 57.6-fold increase in CAT conversion, and actinomycin D completely inhibited IL-1β induction of MGSA/gro mRNA expression, demonstrating that IL-1 transcriptionally activates the MGSA/gro gene. MGSA/gro protein secretion paralleled mRNA expression; unstimulated cells did not secrete MGSA/gro, but secretion was strongly induced by IL-1β. We conclude that MGSA/gro is produced by human RPE in response to cytokines involved in inflammation and wound healing; thus, MGSA/gro may be an important cellular mediator of ocular wound healing and inflammatory diseases such as PVR and uveitis, respectively.

1132

Repair of Single Cell Wounds in Retinal Pigment Epithelial Cells *in Situ*. H.Nagai and V.I.Kalnins, Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario, Canada.

Since retinal pigment epithelial (RPE) cells do not normally multiply after birth, the loss of cells with increasing age leads to an increase in the size of remaining ones. The mechanism by which the integrity of RPE is maintained after cell death has not been established. In this study, we describe a series of stages in the repair process after the death of single RPE cells in 8 to 15 day chick embryos. Whole mounts of RPE showed that among the great majority of RPE cells which were regular in size and hexagonal in shape, were scattered irregularly shaped degenerating cells containing inclusion bodies. The regions occupied by the dying or dead cells, whose residual bodies could often be observed above the wounds, were repaired by the spreading of the surrounding hexagonal cells. A study of the distribution of microfilaments (MF) in these cells by confocal laser scanning microscopy revealed both a decrease in size and staining intensity of circumferential MF bundles and the formation of fine MF bundles running parallel to the wound edge in each of the surrounding cells on the side facing the wound. These MF bundles give a spider-web like appearance to the wounded region. Similar MF bundles running parallel to the wound edge were also seen in RPE cells in organ culture spreading into larger wounds. These findings indicate that during repair of spontaneous single cell wounds there is a dramatic reorganization of the cytoskeleton. This type of repair by spreading alone which results in an increase in the average size of the RPE cells in the monolayer with age would not be expected to trigger cell proliferation which in these cells is linked to cell migration (Hergott *et al., Exp. Cell Res.* 1991;195:307).
Supported by the RP Eye Research Foundation of Canada.

Neural Development I (1133-1134)

1133

Neuro-2a Neuroblastoma Cells Internalize Gold-Labeled Cholera Toxin-B in a Temperature Dependent Manner. I.H. Fentie and F.J. Roisen. Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, KY 40292.

Murine Neuro-2a neuroblastoma cells undergo enhanced neuritogenesis *in vitro* when exposed to exogenous ganglioside GM1. Our previous studies examined the effects of Taxol (a microtubule stabilizer) and Colcemid (for microtubule disassembly) on the sur-face distribution and internalization of endogenous GM1 labeled with cholera toxin B-colloidal gold. Taxol-treated Neuro-2a cells possessed more surface GM1 than cells treated with Colcemid. In contrast, Colcemid-treated cells internalized 3.5 times more label than Taxol-treated cells. A preferential shuttling of GM1 to the plasma membrane occurred during Taxol-mediated neuritogenesis. Perhaps this movement allows GM1 to play a modulatory role in neuritogenesis. In this study the effects of temperature on GM1 internalization and surface distribution are reported. Following 24 hr normal growth, cells were exposed to gold labeled cholera toxin-B under different experimental conditions for 1 hr prior to fixation. Cells were (1) exposed to label and incubated at 35°C, (2) cold equilibrated (4°C), exposed to label and retained in the cold, or (3) cold equilibrated, exposed to label and returned to 35°C. Perikaryal and neuritic areas as well as their respective perimeters were determined morphometrically. The numbers of gold particles in these regions were determined per unit area or surface length. This study indicated that neurites possessed greater levels of endogenous GM1 (membranebound and internalized) than perikarya in all treatment groups. Cells incubated at 35°C internalized large quantities of GM1-positive label. Cold-adapted cells internalized the least label. The internalized label remained close to the plasma membrane; these cells had the most surface label of all treatments. Compared to cells maintained at 35°C, the level of internalized GM1 increased when cells were exposed to the cold prior to exposure to 35°C. These results demonstrate that the rate of internalization of GM1 in Neuro-2a cells is temperature dependent. This preferential location of GM1 in neurites further suggests that gangliosides may play a role in neuritogenesis.

1134

Two Regulatory Genes Share Common Functions in *Drosophila* CNS and Head Development. L.L. Restifo, V.K.L. Merrill*, and R. Page, ARL Division of Neurobiology, University of Arizona, Tucson AZ 85721, and *Dept. of Biology, Brandeis University, Waltham MA 02254. (Spon. by C.A. Boswell.)

During the metamorphosis of *Drosophila melanogaster*, the central nervous system (CNS) is reorganized and the adult epidermis develops *de novo*. Both events require activity of the *Broad Complex* (*BR-C*), a steroid hormone-regulated gene (Restifo and White, *Dev. Biol.* 148:174-194, 1991; Kiss *et al., Genetics* 118:247-259, 1988). Here we provide anatomical and genetic evidence that *BR-C* and *Deformed* (*Dfd*), a homeotic selector gene (Merrill *et al., Dev. Biol.* 122:379-395, 1987), participate in common events in CNS and head morphogenesis. Like *BR-C* mutants, *Dfd* mutants dying during metamorphosis manifest failure to separate the subesophageal ganglion (SEG) from the thoracic ganglion (TG), with concomitant defects in cervical connective formation. Failure of SEG-TG separation maps to the *Dfd* locus. We used a temperature-sensitive allele of *Dfd* to ask when during development (larval life, metamorphosis, or both) *Dfd* is required for SEG morphogenesis. Unlike viability, SEG-TG separation does not have a discrete temperature-sensitive period. Rather, permissive temperature during either larval life or metamorphosis allows SEG-TG separation. Scanning electron microscopy demonstrates that *BR-C* and *Dfd* mutants also share defects of the maxillary palps and proboscis, head structures innervated by the SEG. The phenotypic overlap is not complete, with the palps being much more sensitive to lack of *Dfd* activity than to loss of *BR-C* function. Using a variety of *BR-C* and *Dfd* alleles, we asked whether these two vital loci interact genetically. Neither gene enhances or suppresses the lethality associated with mutations of the other. However, in specific *BR-C--Dfd* double mutants, the *Dfd* head phenotype is enhanced. This enhancement, seen with a *BR-C* viable allele that does not cause head defects by itself, appears to be allele- or complementation group-specific.

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Expression of the HuD Neuronal Regulatory Protein During Development. M.F. Marusich[1], H.M. Furneaux[2], and J.A. Weston[1], [1]Institute of Neuroscience, University of Oregon, Eugene, OR 97403; [2]Laboratory of Molecular Neuro-Oncology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. (Spon. by M.F. Marusich.)

HuD is a neuronal protein that has been suggested to be involved in mRNA processing vital for neuronal development and maintenance (Szabo et al., 1991, *Cell*, 67, 325-333). HuD pre-mRNA is alternatively spliced yielding three isoforms called HuD, HuDpro, and HuDmex. To test whether these isoforms are expressed differentially in discrete classes of neurons or at different times in development, we have developed monoclonal antibodies specific for epitopes unique to either HuD or HuDmex. Synthetic peptides whose sequences span the predicted alternative splicing site, and that share a six amino acid sequence were used as immunogens. Of 198 Anti-peptide MAbs generated to date, 8 are $HuD^+/HuDmex^-$, 6 are $HuD^-/HuDmex^+$, and the remainder bind both peptides. Two of these (MAb 16A11 and MAb 12G10) react well with intact HuD protein (both human and avian) in western blots and show an Anti-Hu-like pattern of neuron-specific immunoreactivity in fixed avian embryos (Anti-Hu is the human autoantibody specific for the Hu-antigens). Whereas MAb 16A11 binds to peptide HuD but not peptide HuDmex, MAb 12G10 binds to both peptides. Except for slight differences in background, the two MAbs show identical patterns of reactivity both in terms of tissue specificity and developmental expression. In particular, both MAbs show early expression of immunoreactivity in neurons of the dorsal root ganglia, sympathetic ganglia and spinal cords of avian embryos. We conclude therefore, that HuD (revealed by MAb 16A11) is expressed early in the neuronal tissues studied, and appears to be expressed in all neurons studied. At present, we cannot establish whether or not HuDmex is also expressed early in all HuD^+ neurons, since the marker for HuDmex (MAb 12G10) also reacts with HuD. Although additional study will be required to determine if the two proteins are coordinately expressed in all neuronal tissues, it is clear that MAbs 16A11 and 12G10, as well as additional MAbs specific for other Hu-isoforms, will be valuable reagents for studies of neurogenesis, neuronal maintenance, neuropathy and small cell lung carcinoma. Supported by NSF grant DCB-8820070 and NIH grant NS29438.

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Effects of Retinoic Acid on Avian Trunk Neural Crest Cells.
P. D. Henion, V. A. Larson, and J. A. Weston, Institute of Neuroscience, University of Oregon, Eugene, OR 97403

The neural crest of vertebrate embryos gives rise to a variety of cell types during development, including both neuronal and non-neuronal derivatives. Although numerous studies have implicated different growth factors in the overt differentiation and subsequent survival of specific types of neural crest-derived peripheral neurons, comparatively little is known about environmental cues that regulate the initial phenotypic choices of early neural crest cells, and in particular, the choice between neuronal and non-neuronal fates. We have utilized cultures of early quail neural crest cells to test the possibility that retinoic acid (RA) affects this lineage decision. We have found that treatment of crest cultures with nanomolar concentrations of RA appears to cause increased cell proliferation and precocious expression of an early neuronal marker by a subpopulation of cells. Subsequently, RA treated cultures exhibit a dramatic increase in the proportion of cells which express neuronal markers compared to controls. In our culture system, neuronal differentiation normally requires the presence of E 10 chick embryo extract (CEE). We have found, however, that RA can at least partially substitute for CEE-derived factors in the initial expression of neuronal properties by neural crest cells. Our results indicate that RA, either directly or indirectly, causes the early appearance and specific increase in neuronal cells in crest cultures, and can induce neuronal differentiation in the absence of CEE. Taken together with the findings of others that subpopulations of avian neural crest and crest-derived cells express RA receptors and RA binding proteins, these results raise the possibility that RA and/or other retinoids may play a role in proliferation of neuronal precursors and neuronal differentiation during development *in vivo*. Supported by NS29438 and NS09031.

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A Novel Ankyrin is Required for Axon Guidance in *C. elegans*. A. J. Otsuka, P. Boontrakulpoontawee, Y.-Y. Zhang, L. Z. Tang, and A. Sobery, Department of Biological Sciences, Illinois State University, Normal, IL 61761-6901.

Mutations in the *Caenorhabditis elegans unc-44* gene result in aberrant guidance of neurons, including several sensory neurons that normally target the central nervous system, and in fasciculation of these neurons with inappropriate partners [Hedgecock, E. M., Culotti, J. G., Thomson, J. N., and Perkins, L. A., *Dev. Biol., 111*, 158-170 (1985)]. In order to determine the nature of the defect, the Tc*1* transposon insertion in *unc-44(rh1042)* was used as a physical marker to clone the gene. Six transposon-induced alleles were localized to two regions, designated sites A and B, in a single complementation group. DNA sequence analyses of cDNA and genomic clones from site A reveal putative proteins with all three ankyrin domains: 1) the ankyrin repeat domain, 2) the spectrin-binding domain, and 3) the regulatory domain. Comparison of cDNA and genomic sequences indicates alternative splicing of the linker between the ankyrin repeat and spectrin binding domains, as well as of the regulatory domain. The ankyrin repeat domain is composed of twenty three repeats of approximately 33 amino acids, clustered in six groups, rather than located on individual exons. The repeats of the *unc-44* product can be assigned to their vertebrate equivalents. It appears that a novel form(s) of ankyrin, encoded by transcripts that span sites A and B, is involved in the neural defects in *C. elegans*. This proposal is based on positions of the insertion mutations, the complementation results, and the detection of RNA's by site A and B cDNA's, in the 14 kb range, that differ between wild type and transposon insertion mutants. The site B cDNA fragment appears to encode a protein that is different from the large form of ankyrin discovered in the neonatal rat brain [Kunimoto, M., Otto, E., and Bennett, V., *J. Cell Biol., 115*, 1319-1331 (1991)]. It will be of considerable interest to identify the proteins with which the novel ankyrin interacts.

1138

L2-4C A Growth Cone-Associated Protein Bearing the L2-Carbohydrate Epitope with Similarity to Ankyrins. G.P. Owens, M. Horan, C. Burger and W.E. Hahn. Department of Cellular and Structural Biology, University of Colorado, School of Medicine, Denver, CO 80262.

Many of the adhesion molecules expressed in the mammalian nervous system share a carbohydrate epitope defined by the monoclonal antibodies L2/HNK-1 (Kruse *et al.* Nature 311:153). To identify novel L2-glycoproteins with the L2 epitope expressed during neurodevelopment, we screened an expression library prepared from 5 day old mouse cerebellar mRNA with a polyclonal sera (Rest L2 provided by M. Schachner) directed against affinity purified L2 containing glycoproteins. A cDNA clone, L2-4C, was isolated that corresponds to a multiform protein whose presence and abundance changes relative to postnatal brain development. Antibodies raised against the L2-4C fusion protein recognize proteins of about 450 kD, 320 kD, 230 kD and 150 kD. The 450 kD and 320 kD species are most prominent from late embryonic through early postnatal development, whereas the 230 kD protein is most abundant in adult brain. L2-4C (320) is highly enriched in purified growth cone fractions.

Immunoglobulin from antisera made against L2-4C fusion protein immunoprecipitates a 450 kD protein that reacts with the L2 mAb. In immunoblots of proteins from growth cone fractions, a 320 kD protein that reacts with the L2 mAb is also observed. No evidence for glycosylation of the 230 kD protein has been obtained. L2-4C has a domain that is ~ 80% homologous with the C-terminal regulatory domain of human brain ankyrin II. Therefore it might represent mouse brain ankyrin or be a member of the ankyrin family. If so, the larger L2-4C proteins may have extracellular and intracellular domains that couple signal transduction with changes in the cell cytoskeleton.

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Expression of Cadherins in Developing Mouse Brain. A.M. Fannon and D.R. Colman, Department of Anatomy & Cell Biology, Columbia University, 630 168th St., New York, NY 10032

The cadherins are a family of calcium dependent cell adhesion molecules which act via homophilic interactions and appear to be important mediators of morphogenesis. The "classical" cadherins, the neural (N)-, epithelial (E)-, and placental (P)-cadherin, consist of five extracellular putative calcium binding domains, a transmembrane domain and a highly conserved cytoplasmic carboxy terminal domain. Since our laboratory is interested in cell adhesion, myelinogenesis and the function of myelin proteins, it was decided to investigate the possible role of cadherins in myelin. The initial approach was to find cadherins which were enriched in white matter using PCR.

Degenerate PCR primers which annealed to the putative calcium binding sequence and a conserved region in the carboxy terminus of the classical cadherins were used to amplify cDNAs from whole mouse brain or human optic nerve, an enriched source of white matter. The PCR products were subcloned, sequenced and determined to be E-cadherin and the newly described neural cadherin-6 (N_6-cadherin).

Since E-cadherin and N_6-cadherin showed extensive homology, the extracellular domain 5 of each was used for riboprobes. The extracellular 5 domain demonstrates the least amount of sequence homology both at the cDNA and protein level. Northern blot analyses of postnatal day 5, 10, 15, 20 and adult brain showed that both E-cadherin and N_6-cadherin messages were present at all ages. However, E-cadherin messages appeared to be more abundant in the adult while N_6-cadherin messages appeared to be more abundant in five and ten day old mice. The tissue localization of these cadherins during myelinogenesis is currently being examined.

1140

The Role of Substrate and Calcium in Neurite Retraction of Leech Neurons Following Depolarization. M.D. Neely and M. Gesemann, Biocenter Univ. Basel, 4056 Basel, Switzerland

The substrate has a crucial influence on the development of leech neurons in culture. The aim of these experiments was to analyze how depolarization influences neurite outgrowth of leech neurons growing on different substrates. Leech neurons growing on leech extracellular matrix substrate (ECM) were depolarized by raised extracellular K^+ for 30 minutes, which induced retraction in a subset of neurites, a response comparable to that observed after electrical stimulation (S. Grumbacher-Reinert and J. Nicholls, J. Exp. Biol. 167:1-14, 1992). This retraction was reversible and inhibited by raised extracellular Mg^{2+}, suggesting a mechanism dependent on calcium. Cells plated on Concanavalin A (Con A), however, did not retract their neurites after depolarization, but continued to extend processes. The growth cones of cells on ECM revealed retraction of lamellipodial and filopodial structures after depolarization. In cells on Con A no differences were observed between growth cones of cells exposed to high K^+ and those of control cells. Immunofluorescence analysis of the effect of depolarization on the cytoskeleton revealed changes in the microfilaments in cells on ECM. No alteration in the organization of microfilaments or microtubules was observed in cells on Con A after high K^+ application. Time lapse studies using microfilament and microtubule stabilizing and destabilizing drugs confirmed an involvement of microfilaments in the depolarization-induced neurite retraction. These results demonstrate the key role of substrate molecules in defining the morphology of a neuron.

Supported by the Swiss Nationalfond No. 31 27 814.89

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Characterization of a Novel Glial Cell Adhesion Molecule, G-CAM, in Normal and Injured CNS. T.P. Murphy, M.H. Irwin, and E.E. Geisert, Jr. Department of Cell Biology and Neurobiology Research Center, University of Alabama at Birmingham, Birmingham, AL 35294-0019.

Using a monoclonal antibody, AMP1, we have identified a 106 kDa glycoprotein that is involved in rat glial cell-cell adhesion, tentatively termed "glial cell adhesion molecule" (G-CAM). Immunostaining with AMP1 reveals a discrete pattern of labelling at cell-cell contacts in confluent monolayers of rat astrocytes. AMP1 does not label cultured embryonic neurons, nor does it reorganize under Thy-1 negative neurons plated on astrocyte monolayers. Treatment of living monolayers of astrocytes with AMP1 reversibly disrupted cell-cell contacts, while treatment with control antibodies had little effect. G-CAM is first expressed at low levels in E18 choroid plexus and ependyma; as the CNS matures, G-CAM expression is upregulated, becoming widely distributed throughout the adult brain and spinal cord. G-CAM appears to be involved in CNS wound healing, with more than a 100-fold increase in immunoreactivity in the region of the glial scar, as compared to surrounding brain tissues. Taken together, these data indicate that G-CAM plays an important role in the maintenance of glial cell-cell contacts and in the formation and stabilzation of the glial scar. (Supported by the Spinal Cord Society.)

1142

BDNF Signal Transduction in Developing Rat Septal Neurons Grown in Bilaminar Cultures. L.M.Mudd and H.C.Palfrey. Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637. (Spon. by M.Rao)

Recent work has demonstrated that brain-derived neurotrophic factor (BDNF) promotes the survival of septal cholinergic neurons in the basal forebrain and that the trkB gene product is the receptor tyrosine kinase responsible for BDNF action. We used a bilaminar culture technique to characterize BDNF signal transduction events in the developing rat septum. Embryonic day 16 septal neurons were grown in defined medium at a distance of 1 mm from a removable glial plane. Both the neuronal and glial planes were greater than 99% pure as determined by staining for neurofilament protein and glial fibrillary acidic protein, respectively. Antibodies directed against amino acids 794-808 of the predicted sequence of the mouse trkB gene product recognized a ~120 kDa protein in embryonic septal neurons, as well as in adult septum and hippocampus, but not in the astrocytic plane. Correspondingly, the presence of this protein in about 73% of septal neurons was detected immunocytochemically, whereas only~15% of the cells stained positively for the low-affinity nerve growth factor receptor. Immunoreactivity was found in both cell bodies and processes of the neurons. After removal of the glial layer, signal transduction was examined in the pure neuronal population. Treatment with BDNF (10ng/ml) resulted in a generalized increase in tyrosine phosphorylation of a number of substrates. Immunoblotting with anti-phosphotyrosine antibodies showed a widespread increase in immunoreactive bands with a maximum at 2-5 min, followed by a decline within 10 min. Proteins of~85, 110, 128, and 160 kDa were responsive while proteins of ~120 and 140 kDa were not. Microtubule-associated protein (MAP) 2 kinase activity also appeared to increase in this same time period as demonstrated by a transient increase in the phosphorylation of myelin basic protein by cytoplasmic extracts from cells treated with the trophic factor. In addition, BDNF increased the expression of Fos, with nuclear staining increasing from 1.1% of cells to a maximum of 66% of cells at 4 h following treatment. The percentage of Fos-positive neurons declined after 6 h incubation and returned to basal levels within 24 h of treatment. Thus BDNF initiates a signal transduction cascade in embryonic septal neurons that may underlie its trophic effects on this cell population.

1143

On the Origin of Glia in the Embryonic CNS of Drosophila. G. Udolph, J. Sohn, and G.M. Technau, Institut für Genetik, Zellbiologie, Universität Mainz, Saarstraße 21, 6500 Mainz, Germany. (Spon. by R. Benavente.)

In the CNS of Drosophila and other insects, glia represent a prominent fraction of the cells. In order to study the anatomy and distribution as well as the origin and development of glia we use the following approaches. 1) By injecting HRP we map the distribution of presumptive glia-precursors in the neurogenic region of the ectoderm. 2) We transplant single labelled neuroectodermal cells (isotopically and heterotopically) to disclose their lineages and to test their state of determination. 3) We screen enhancer-trap lines for glia-specific expression patterns (Gal4). We show that glia-precursors segregate from sites at all dorsoventral levels of the ventral neurogenic region. However, depending on their site of segregation from the neurogenic ectoderm, precursors give rise to characteristic types of glial cells. The analysis of clones derived from cells of the early gastrula reveals that, in addition to neuroblasts and glioblasts, there are also common precursors for both neurons and glia. Following heterotopic transplantation neuroectodermal cells develop clones according to their new position.

1144

A Versatile Herpes Amplicon Vector System Has Been Used to Deliver Foreign Genes into Neurons in Culture and *in vivo*. M.E. Fotaki, R. J. Dunn, Center for Research in Neuroscience, Montreal General Hospital, McGill Univ., Montreal, Canada.

The manipulation of neuronal cell gene expression by introducing foreign genes may alter neuronal growth and regeneration. Herpes amplicon expression vectors have been used to mediate the gene transfer. The amplicon vector is a plasmid based vector that carries viral sequences which allow for the packaging of multiple copies of vector DNA into viral capsids in the presence of helper virus . We have constructed a series of amplicon vectors, pHermes, which include the human cytomegalovirus major early promoter to drive the expression of the foreign gene. The vector has been packaged into viral progeny by cotransfecting plasmid DNA and DNA of the replication defective helper virus d120 into E5 cells . Our first viral amplicon, Hermes-lacZ, expressed the *E. coli* lacZ gene upon infection of stable cell lines (Vero and PC12) and primary neuronal cultures (chick retinal ganglion cells). This vector is now being used for *in vivo* expression of the reporter gene in the adult rat brain. A modification of the original vector allows for the simultaneous expression of the reporter gene, lacZ , and the gene for the rat myelin associated glycoprotein (MAG). This vector increases the sensitivity of the system by marking the cells that express MAG. Infection of myelinating cultures of rat DRG sensory neurons with this vector is expected to show the effect of ectopic expression of MAG on the myelination process.

1145

Homophilic Binding and Phosphatidylinositol Linkage of the Limbic System-Associated Membrane Protein (LAMP) are Consistent with a Role in Specific Recognition Events in the Developing Brain. V. Zukhareva and P. Levitt, Department of Anatomy and Neurobiology, The Medical College of Pennsylvania, Philadelphia, PA 19129.

The limbic system-associated membrane protein (LAMP) is a 64-68 kilodalton membrane glycoprotein (Zacco, et al, 1990 J. Neurosci. 10:73-90), whose functioning is required for the formation of limbic circuits, such as the septo-hippocampal projection (Keller, et al, 1989, Neuron 3:551-561.) The mechanism by which LAMP regulates the formation of this circuit is unclear, but because of its location transiently both pre- and postsynaptically during development, we predicted that interaction could occur through homophilic binding. In the present experiments, we used detergent-solubilized, immunoaffinity-purified LAMP that was linked to fluorescent Covaspheres (Duke Scientific) to assess the ability of this glycoprotein to mediate adhesion. Rapid, reversible aggregation occurred between LAMP-coated beads, and interaction could be partially blocked by anti-LAMP monoclonal antibodies. In order to determine the specific molecular interactions of LAMP and neuronal membranes, additional studies were performed. In the rat hippocampus, LAMP is anchored to the cell surface through a phosphatidylinositol (PI) linkage, as demonstrated by the ability of PI-specific phospholipase C to liberate almost all of the antigen in soluble form. LAMP joins the family of putative membrane recognition molecules with PI linkages and the ability to self-interact during critical periods of neural development, suggesting a possible signal transduction mechanism for recognition between limbic neurons. Supported by NIMH grant MH45507.

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Enhancement of Spinal Neuron Axonal Outgrowth by Target-Derived Astroglia Is Mediated through Protein Kinase C Second Messenger System. J. Qian*, H. Y. Wang**, E. Freidman**, I. Fischer* and P. Levitt*. *Dept. of Anatomy & Neurobiology, and **Psychiatry, The Medical College of Pennsylvania, Philadelphia, PA 19129.

Astroglial cells participate in a variety of developmental events during neuronal morphogenesis. We have shown that axonal outgrowth of spinal cord neurons can be promoted by a diffusible factor(s) secreted from cerebellar astroglia in vitro in comparison with spinal astroglia. This glial effect seems to be mediated via protein kinase C (PKC) since: (1) inhibition of PKC activity by sphingosine greatly reduced the mean axonal length of spinal neurons cultured in medium conditioned by cerebellar astroglia (SCn-CBg), while activation of PKC by the phorbol ester TPA was not additive to the glial effect and (2) activation of PKC by TPA promoted axon growth of spinal neurons cultured in medium conditioned by spinal astroglia (SCn-SCg). We used quantitative Western Blotting and PKC activity assays to provide further evidence of the involvement of PKC in the specific axon-promoting effect by target glia. The data show that there is increased PKC activity and protein levels (in particular, PKCβ) in SCn-CBg cultures, which correlates with enhanced axon growth. This can be inhibited by sphingosine treatment. In SCn-SCg cultures, phorbol ester activation of PKC increased both activity and protein levels of both PKCα and PKCβ. This activation correlated with stimulated axonal outgrowth. These results suggest that the glial signalling that regulates axonal outgrowth is mediated in part by the PKC system. Supported by NIMH grant MH45507 and NIH Program grant NS24707.

1147

Characterization of Primary Cultures of Developing Neurons from Specific Areas of Brain. S.E. Poduslo, P. Coates*, W. Chen, R. Seelow, P. Decker, and P. Thomas. Dept. of Neurology and Dept. of Cell Biology and Anatomy*, Texas Tech University Health Sciences Center, Lubbock, TX 79430.

Primary cultures of neurons were prepared from dissected cortical, striatal, and midbrain areas of embryonic rats. The dissected tissue was subjected to trypsination in isolation medium and the softened tissue was passed through nylon screens to obtain a single cell suspension. The cells were plated onto polyethylenimine coated flasks in Dulbecco's high glucose medium with zinc-free insulin (Squibb), 1% BSA, and 10% fetal calf serum (Hyclone). After one hour the medium was changed to remove cell fragments. Over the next 24-48 hours, the cultures consisted primarily of neurons extending long processes. Choline acetyltransferase and tyrosine hydroxylase enzymatic activities were analyzed and immunofluorescence staining was performed on the primary neurons after two days in culture. The enzyme assays were modified to measure activities on small quantities of cells. After two days in culture, neurons from midbrain had an activity of 20 pmol/min/mg soluble protein for tyrosine hydroxylase and 313 pmol/min/mg protein for choline acetyltransferase. Levels of mRNA for the dopamine D1 and D2 receptors were quantitated using the polymerase chain reaction. Neurons grown in T25 flasks were concentrated and total RNA was prepared. Then cDNA was made using an oligo $(dT)_{15}$ primer and AMV reverse transcriptase. After amplification in the presence of [α-^{32}P]dCTP, the samples were separated by acrylamide electrophoresis. The radiolabeled bands were stained, excised, and mRNA levels quantitated using a control standard. Co-cultures of target areas of striatal and midbrain were also made to evaluate the effects of target innervation on the cited parameters. This neuronal culture system can be used as a model system to measure the effects of drugs on neuronal function during development. Supported by NIH grant DA07366.

1148

Expression of myelin proteolipid protein and basic protein mRNAs during development of normal and jimpy cultures using nonradioactive in situ hybridization histochemistry. A.A.Maki, R.P. Skoff and P.E. Knapp. Dept. of Anat. & Cell Biol., Wayne State Univ., Detroit, MI 48201. (Spon. by K.Palmer)

The jimpy (jp) mouse is an X-linked recessive mutant with severe CNS dysmyelination due to a point mutation in the gene coding for myelin proteolipid protein (PLP). In vivo and in vitro, PLP protein is not detectable in jp and in vivo, PLP-mRNA is reduced by 80-95% of normal level. Myelin basic protein (MBP) and its message are also drastically affected, although not to the extent of PLP and its message. Transcription of PLP and MBP mRNA has not been investigated in jp cultures. The objective of this study is to compare the developmental expression of mRNAs for PLP and MBP in jimpy and age-matched normal mice in primary cultures. cDNA inserts specific for PLP or MBP-mRNAs (obtained from Dr. A.T. Campagnoni) were separated from their cloning vector by using appropriate restriction enzymes. The cDNAs were labelled with digoxigenin conjugated to dUTP using a Genius random priming kit (Boehringer-Mannheim). An in situ hybridization histochemical procedure was employed on cultures at 6 and 14 days in vitro (DIV). The hybridized cells were detected using an antibody to digoxigenin conjugated to alkaline phosphatase in the presence of enzyme substrates. In jp cultures, at 14 DIV, the number of cells labelled for MBP and PLP-mRNAs were reduced by 20% and 33% respectively. At 6 DIV, preliminary observations indicate that the percent reduction in labeled cells is even less than at 14 DIV. Immunocytochemistry on sister coverslips showed weak staining for MBP. In both jp and normal cultures, cells labeled for message were heavily stained. These results suggest that transcription of myelin messages is much less affected by the jp mutation than is translation. (supported by NS18883 & NS15338).

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Expression and secretion of iron regulatory proteins and Transferrin mRNA in neuroglial cells in culture. J.R. Connor, S.L. Menzies and B.S. Snyder. Department of Neuroscience & Anatomy, M.S. Hershey Medical Center, Penn State College of Medicine, Hershey PA 17033.

Recent studies investigating iron and iron regulatory proteins in the nervous system indicate that neuroglial cells are responsible for maintaining brain iron homeostasis. The purpose of this experiment is two-fold: (i) determine whether Tf is secreted by neuroglial cells in culture and (ii) identify the neuroglial cells which express iron regulatory proteins and/or transcripts. Brains from neonatal mice were removed, stripped of the meninges and prepared for tissue culture according to standard procedures. Cells were plated at high density on poly-L lysine coated coverslips. The cells were grown to confluence (~ 5 days) in a medium containing 10% fetal calf serum. Upon reaching confluence, the cultures were either changed to a defined medium which contained 50 µg/ml of transferrin or continued in the initial medium. Immunohistochemical analysis for transferrin (Tf), ferritin, and the transferrin receptor, in situ hybridization for transferrin mRNA and a histochemical stain for iron were performed. Proteins undergoing synthesis in culture were labeled with ^{35}S-methionine. Tf secretion was demonstrated in the mixed glial cultures and enriched microglial cultures using immunoprecipitation. By combining immunohistochemistry with in situ hybridization the cells capable of synthesizing Tf were identified as oligodendrocytes and microglial cells. A subpopulation of cells resembling astrocytes (but not all GFAP+) also express the mRNA for Tf. Oligodendrocytes and microglial cells also express the transferrin receptor in these culture conditions. Ferritin and iron staining are only found in microglial cells. These experiments form the baseline for future studies on the dynamics of iron metabolism in neuroglia. Supported by NS22671, NMSS RG2118.

Developmental Control of Gene Expression (1150-1151)

1150

A Transgenic Model Distinguishes Cellular Commitment and Differentiation in the Developing Pulmonary Epithelium. B.P Hackett and J.D. Gitlin. Dept. of Ped.,Wash. Univ. School Med., St. Louis, MO. (Spon. by P. Shackelford).

To define the mechanisms of cell-specific gene expression in the bronchiolar epithelium we generated a series of transgenic mice using Clara cell secretory protein (CCSP) - human growth hormone chimeric gene constructs. These experiments revealed that *cis*-acting elements within 2.25 kB of the rat CCSP gene 5' flanking sequence were sufficient to direct tissue and cell-specific gene expression. CCSP gene expression is also developmentally regulated being expressed at E17 in the mouse and increasing in abundance during late fetal and early postnatal life concomitant with epithelial cell differentiation within the developing pulmonary acinus. In contrast transgene expression was detectable by E15 and was abundantly expressed within the developing bronchiolar epithelium prior to endogenous CCSP gene expression. Therefore, despite an absolute conservation of tissue and cell-type specificity of CCSP gene expression in these transgenic animals such expression was not developmentally conserved. These data are not due to a unique effect of growth hormone since the developmental timing of endogenous CCSP gene expression is unaltered in these same mice. The conservation of cell-type specific expression during development in this model allows identification of pulmonary epithelial cells committed to but not yet differentiated towards this cell phenotype. This model permits characterization of pulmonary epithelial cell progenitors at defined stages of early lung development. In addition the developmental uncoupling of endogenous and transgene expression suggests the presence of unique *cis*-acting elements which transduce signals for developmental timing within the pulmonary epithelium.

1151

Identification of Developmentally Regulated Genes in the Developing Mouse Heart. C.H. Mjaatvedt and J.D. Gearhart. Department of Gynecology and Obstetrics, Johns Hopkins School of Medicine, Baltimore, MD 21205 (spon. by D.M. Raben)

The stage-specific molecules and mechanism of signal transduction which regulate the tissue-specific gene expression of mouse heart morphogenesis remain largely to be elucidated. The purpose of this study is to identify and characterize these molecular components of heart morphogenesis through screens of stage-specific cDNA libraries at critical stages of heart development. The remodeling of the single primitive heart tube into a multichambered adult heart is dependent on the formation of the cardiac mesenchyme. By day 10.5 *p.c.* the mouse heart contains a mixed population of cardiac mesenchyme in various states of differentiation derived from both the neural crest and endothelium. A heart cDNA library has been constructed from mRNA of day 10.5 *p.c.* mouse embryonic hearts. Three sub-libraries of stage-specific clones have been obtained from this embryonic heart cDNA library using subtractive hybridization. Thirty-one clones were randomly picked from the three separate subtracted heart libraries, the inserts were partially sequenced and compared with those found in the Genbank/EMBL databases. Among these, nine clones showed significant homology with known genes. Specifically, one has significant homology (90%) with a high mobility group DNA binding protein HMG-17. HMG-17 is a member of the HMG-14/17 family of non-histone proteins (mw 10-12 kD) found in the nucleus. Others had homology (78.2%) with bovine inositol monophosphatase and mouse Ki-*ras* cellular oncogene (89.4%). Sixteen of the subtracted clones showed no significant homology to sequences in the databases and thus appear to represent novel genes involved in mouse heart development.

1152

A system for isolation of genes that repress myogenesis. I.Pieri and B.B.Olwin
Department of Biochemistry, University of Wisconsin, Madison, WI, 53706.

We are interested in understanding the mechanisms involved in bFGF-mediated muscle differentiation. We modified BC3H1 muscle cells by transfection with a plasmid containing a neomycin resistance gene under the control of SV40 promoter and the hygromycin resistance gene under the control of the muscle creatine kinase (mCK) enhancer/promoter (mCK is only expressed in differentiated cells). For differentiation, BC3H1 cells are exposed to low concentration of serum (0.5%) and selected with 200 ug/ml of hygromycin. Anti-myosin antibody staining showed that 90% of the cells were differentiated compared to 60% without selection. Following selection, BC3H1 cells reenter the cell cycle (or dedifferentiate) in response to mitogenic stimulation (15% serum, bFGF). Reentry into the cell cycle requires new protein synthesis, suggesting new gene transcription occurs. A subtractive library method (Wang & Brown, 1991, PNAS, 88, 11505-11509) is being used to isolate FGF-regulated genes involved in repression of myogenesis. Isolation of these genes is in progress.

1153

The Bovine *Myf-5* gene can activate all of the endogenous myogenic factors except the endogenous *Myf-5* gene in 10T1/2-derived myoblasts. Robert A. Worrell, Todd Arnold and Robert Ivarie. Department of Genetics, The University of Georgia, Athens, GA, 30602.

We have previously described a DD3 myoblast cell line carrying the *bmyf* gene under the control of the glucocorticoid-inducible MMTV promoter. In proliferating DD3 cells, *myogenin* is also dexamethasone inducible but lags 4 hrs behind the induction of *bmyf*. *Mrf-4* can be induced with a lag of 20-30 min. We now demonstrate that *bmyf* is still dexamethasone inducible in differentiating DD3 cell cultures, but that the peak of expression was at ~6 hrs rather than the 1.5-2 hrs seen in proliferating cultures. *Myogenin* was similarly induced. *MyoD* was barely detectable in proliferating cultures but was at least 10-fold higher under differentiation conditions. The *MyoD* expression in differentiating cultures was significantly reduced within 3 hrs of dexamethasone induction. The endogenous *Myf-5* gene, as determined by a RNAse protection assay sufficiently sensitive to detect mouse *myf-5* transcripts at 10^{-8} sensitivity of detection, was not activated under proliferation or differentiation conditions. *MyoD*, constitutively expressed from the MSV LTR in 10T1/2-derived myoblasts, also failed to activate the endogenous *Myf-5* gene. We conclude from these experiments that, since all four myogenic factors are expressed, none is sufficient for the activation of the mouse *Myf-5* gene in these cells.

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Temporal Expression of Myogenic Regulatory Genes in Cultured Rat Skeletal Muscle Satellite Cells. M.J. Janney[1], C.K. Smith II[1] and R.E. Allen[2]. [1]Animal Science Discovery Research, Lilly Research Laboratories, Eli Lilly and Company, Greenfield IN 46140 and [2]Department of Animal Sciences, University of Arizona, Tucson AZ 85721.

Utilizing polymerase chain reaction amplification of cDNA from reverse transcribed RNA, we examined the temporal pattern of expression of *myf5*, *myogenin*, *MRF4/herculin*, and *myoD* from rat myogenic satellite cells grown in culture. Cells were prepared from 9 month old rats. The rats were not growing and were not injured; therefore, satellite cells were assumed to be quiescent. Expression was examined at 0, 24, 48, 72 and 96 hours and in fused cultures (144-168 hours). In our system, satellite cells reenter the cell cycle within approximately the first 36 hours, proliferate between 36 and 96 hours, then differentiate and fuse beginning around 96 hours. None of the myogenic regulatory factor genes were expressed in 0 hour cells. *Myf-5* and *myoD* expression were first detected at 24 hours. *MRF4/herculin* expression was first detected at 48 hours. By 72 hours in culture, mRNA for all four genes was present and remained so throughout the duration of the experiment. Thus it appears that quiescent satellite cells are maintained in the myogenic lineage without expression of the myogenic regulatory genes. These genes, however, are apparently involved in activation, proliferation and differentiation of mammalian skeletal muscle satellite cells.

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Expression of the Rat Intestinal Lactase-Phlorizin Hydrolase Gene along the Proximal to Distal Axis and during Postnatal Development. S.D. Krasinski, G. Estrada, K.Y. Yeh, M. Yeh, E.H.H.M. Rings, M. Verhave, H.A. Buller, R.K. Montgomery and R.J. Grand. New Engl. Med. Ctr. and Tufts Univ. School of Med., Boston, MA 02111, LSU Med. Ctr., Shreveport, LA 71130, and Academic Med. Ctr., Univ. of Amsterdam, The Netherlands.

The enzyme activity of lactase-phlorizin hydrolase (LPH), an intestinal brush border enzyme, is known to be regulated along the proximal to distal (horizontal) axis and during postnatal development. It is unclear and remains controversial, however, whether this regulation occurs at the transcriptional or posttranscriptional level. To investigate transcriptional regulation of LPH expression, we quantified LPH mRNA using RNase protection assays. LPH mRNA in the 14 day old rat was expressed at high levels throughout the intestine. In contrast, LPH mRNA in the adult animal was most abundant in the proximal jejunum, but was barely detectable in the duodenum and distal ileum. In an extensive analysis of 5 postnatal age groups (n=4 rats in each group), LPH mRNA in mid-jejunum declined approximately 3-fold from the preweaning to postweaning period. The most proximal and distal segments, however, displayed a more prominant postweaning decline (10- and 25-fold, respectively) in LPH mRNA abundance. The pattern of expression of the LPH pre-mRNA, as determined by an intron I antisense RNA probe, was similar in all respects to that of the mRNA, but 20- to 100-fold less abundant. Measurement of LPH activity and quantitation of LPH protein by rocket immunoelectrophoresis both showed a postweaning decline which paralleled the decline in mRNA abundance. Immunostaining of intestinal segments for LPH also demonstrated a postweaning decline. Conclusion: LPH appears to be transcriptionally regulated along the horizontal axis and during postnatal development. The postweaning decline in LPH transcription is region-specific since the most striking decrease occurs in the duodenum and distal ileum.

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Molecular Biology of Ecdysone and Juvenile Hormone Action. E.M. Berger, Biology Dept., Dartmouth College, Hanover,NH 03755

The development of holometabolous insects is regulated through the interaction of two hormones; ecdysterone, the steroid moulting hormone, and juvenile hormone, a sesquiterpene. In an effort to understand the mechanisms by which these hormones modulate the transcriptional activity of target genes a hormone responsive, Drosophila, continuous cell culture system has been developed and analyzed using DNA mediated gene transfer and a transient transfection assay.

The small heat shock protein genes are inducible in response to either high temperature or ecdysterone treatment, and their expression shows a parallel pattern of regulation in vivo during imaginal disc development. Regulatory sequences required for both heat shock and ecdysterone induction are located in the 5' untranscribed region of each gene. The heat shock regulatory elements are composed of a head to head array of redundant elements (HSEs) containing the basic motif NGAAN. The nature and location of ecdysterone response elements (EcREs) is less certain but the EcREs appear to contain a 15bp dyad in which two 7bp half-elements (TGANCPyPy) are separated by a single base pair spacer. The EcRE is the binding site for the ecdysterone receptor. Binding of a ligand free receptor to the EcRE leads to transcriptional repression, and the addition of ecdysterone and its binding to the receptor, converts the complex into a transcriptional activator.

The juvenile hormone analogue, methoprene, inhibits the ecdysterone inducible expression of small hsps, but has no effect on the expression following heat shock. Model of methoprene action are discussed.

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Characterization of the Signal Transduction Pathways and cis-Acting Sequences Involved in α-Mannosidase Transcriptional Induction in Dictyostelium discoideum. J. Schatzle, S. Titus, and J. Cardelli, Dept of Microbiology, LSUMC, Shreveport, LA, 71130

The D. discoideum lysosomal α-mannosidase gene is transcriptionally induced under two different conditions: during early development (initiated by starvation), and approximately three generations before logarithmically growing cells using bacteria as a food source reach stationary phase. The induction of transcription during growth is the result of the accumulation and response to a secreted glycoprotein termed the prestarvation response factor (PSF). Northern blot analysis has indicated that, at least for the Ax3 wild-type strain, the PSF dependent induction pathway requires de novo protein synthesis while in contrast the starvation induced increase in α-mannosidase mRNA occurs to the same extent in cycloheximide treated cells compared to control cells. Furthermore, preliminary evidence involving examination of a Gα2 null mutant suggests that both the response to starvation and to PSF are mediated through the plasma membrane-localized Gα2 protein. The Gα2 mutant is, however, capable of producing and secreting PSF. Interestingly, although the Gα2 protein has been shown to be coupled to the cAMP receptor1 (cAR1), α-mannosidase mRNA accumulates to wild-type levels during growth and starvation of mutant cells containing a disrupted cAR1 gene. This is consistent with our earlier report indicating that α-mannosidase expression was regulated independently of the cAMP signalling pathway. Promoter deletion constructs of an internal deletion clone of the α-mannosidase gene were used in transformation experiments to identify cis-acting sequence elements required for transcriptional induction during growth and starvation. These studies revealed a 145 bp sequence element (-509 to -364) in the α-mannosidase promoter that was required for transcriptional induction under both conditions. This sequence element also contained sequences similar to the TTG box identified as an important regulatory element in the promoter of another prestarvation response gene, the discoidin I gene. Therefore, the TTG box may represent a consensus sequence element important in the induction of the entire class of prestarvation response genes.

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Characterization of a Developmentally Regulated Glycoprotein Complex of Dictyostelium discoideum. N. Watson and S. Alexander, Division of Biological Sciences, University of Missouri, Columbia, MO 65211.

In order to learn the roles of various glycoproteins in D. discoideum morphogenesis and development, monoclonal antibodies have been produced which recognize carbohydrate epitopes on developmentally regulated glycoproteins. The use of such monoclonal antibodies and the isolation of mutants deficient in these modifications has led to the identification of several classes of novel glycoproteins which appear to have O-linked sugar groups. O-linked glycans are present on proteins in the cytoplasm and nuclei of cells of a number of different organisms, but the structure, mode of synthesis and function of these glycans are not understood. The genetic and molecular biological techniques applicable to D. discoideum provide a powerful system for studying these interesting glycoproteins and their role in the developing organism. We have identified a soluble multiprotein complex which is expressed at the slug stage of D. discoideum. The complex is immunoprecipitated by a monoclonal antibody specific for a particular prespore glycoprotein, PsB, which contains an O-linked glycan. The complex is large and is primarily composed of six different proteins. The proteins are co-ordinately expressed in the complex with maximal synthesis at the slug stage. Pulse-chase experiments indicate that the proteins of the complex are unique gene products and do not represent different stages of processing of one protein. The glycosylated protein of the complex partitions into the aqueous phase after Triton X-114 extraction, indicating that it is not membrane associated. This glycoprotein is much more polymorphic than the other proteins in the complex. The characterization of this complex will allow us to study the processing and sorting of cytoplasmic O-glycosylated glycoproteins. Supported by NSF DBC 8818687 and a UMC Molecular Biology Predoctoral Fellowship.

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Developmental Regulation of a *Dictyostelium* Spore Germination Cellulase Gene and Functional Organization of the Enzyme. R. Ramalingam, J.E. Blume, and H.L. Ennis. Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

Dictyostelium discoideum, a simple eukaryote, is a suitable model to study regulation of gene expression during development. Similar to other stages of its development, spore germination is accompanied by developmentally regulated changes in the synthesis of macromolecules. A number of genes expressed exclusively during spore germination have been isolated and sequenced. One of them, Cel A, was found to encode a cellulase, an enzyme thought to be important in digesting the cellulose-containing spore wall. Both Cel A and its mRNA are synthesized only during spore germination. Neither the mRNA or enzyme are found in dormant spores, vegetative amoebae, or any multicellular stage of the life cycle. The temporal regulation of Cel A gene expression is being studied. A region of the 5' flanking sequences of the gene lying between 770 and 584 bases upstream of the initiator AUG is necessary for spore germination-specific expression. Further deletions of this region have been constructed to accurately define the sequence element necessary for transcriptional regulation. Expression vectors containing the entire Cel A coding sequence or portions of the sequence were constructed. These constructs produce full-length or truncated proteins when transformed into amoebae, and the proteins have been purified. In this way the 705 amino acid Cel A was shown to be made up of three functional domains: a 462 amino acid N-terminal catalytic domain, a contiguous 90 residue "hinge" region composed of a threonine-glutamic acid-threonine-proline repeat, which is connected to the 153 amino acid C-terminal cellulose binding domain. The requirements for each of the domains in digesting cellulose is being examined.

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The Characterization of the Cyclic AMP Inducible, Prespore Specific Gene, PL3 and its Protein Product in the Cellular Slime Mould *Dictyostelium discoideum*. B.K. Yoder and D.D. Blumberg, Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD. 21228.

The PL3 cDNA clone was one of the first probes used to define the expression of a group of coordinately regulated,cAMP inducible prespore genes. I have isolated and sequenced the genomic fragment which hybridizes to the PL3 cDNA. The sequence predicts a protein of 72kdals containing many internal repeats and three potential N-linked glycosylation sites. Several of these repeats are centered around conserved cysteine motifs and show homology to repeats found in *Dictyostelium* spore coat proteins. Primer extension and RNase mapping reveal three exons which agree with the apparent size of the mRNA observed by Northern analysis. To localize cells expressing the PL3 protein, I have created polyclonal antibodies to a PL3-β-galactosidase fusion protein. The polyclonal antisera recognize a prespore specific protein with an apparent molecular weight of 92kdals. The protein is first detectable at tight mounds concurrent with mRNA expression and continues to be expressed in late culminants where the PL3 message is repressed. Using immunofluorescent microscopy, I have shown that cells expressing the PL3 protein follow the known morphological changes that occur in the prespore zone during development. The PL3 protein appears to surround individual spore cells in the culminant suggesting that PL3 encodes a spore coat protein. Transformation of *Dictyostelium* cells with deletions of the 5' PL3 promoter region fused to the E.coli lac Z gene identified a 818bp fragment upstream of the start site of transcription which is sufficient to confer correct temporal, spatial, and cAMP inducibility. Cells expressing the PL3 promoter driven β-galactosidase protein colocalize with those cells expressing the PL3 protein as determined by immunofluorescence.

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Developmental Regulation of Cyclic AMP Response Element Binding Protein (CREB) Phosphorylation in the Embryonic Murine Palate. W.M. Weston and R.M. Greene, Department of Anatomy and Developmental Biology, Thomas Jefferson University, School of Medicine, Philadelphia, PA 19107.

The CREB protein is a transcription factor whose activity is regulated by several molecular species, such as cyclic AMP, calcium, and the transforming growth factors β. We have been examining the developmental behavior of this protein in the embryonic murine palate, as cAMP has been demonstrated to regulate numerous critical steps in palatal ontogeny. CREB is present in palatal tissue throughout its developmental period (gestational days 12 through 14). Western blot analysis of staged palatal tissue extracts using an anti-CREB antiserum (obtained from Marc Montminy, Salk Institute) revealed 2 immunoreactive bands: one at 43 kD, which is the expected molecular weight for monomeric CREB, and a second at approximately 100 kD. The intensity of both bands remained relatively constant throughout development, indicating that CREB is constituitively expressed during palatal development. Southwestern blot analysis using an end-labeled oligonucleotide probe containing the CRE demonstrated that both proteins recognized by the antiserum bind to the CRE, showing that the two proteins recognized by the antiserum are functionally as well as immunologically related. Immunofluorescence microscopy revealed uniform distribution of the CREB protein throughout the palatal mesenchyme and epithelium. Metabolic labeling of staged palatal shelves with inorganic $^{32}PO_4$ followed by immunopreciptation revealed that phosphorylation of the protein represented by the 43 kD band increased steadily during palatal development. The 100 kD band did not appear to be immunoprecipitated by the antiserum. These data are consistent with previous observations in this laboratory that certain genes that have been shown to contain a CRE in their promotor, such as TGF-β2 and TGF-β3, are expressed at steadily increasing levels during palate development. Taken together, these data indicate that CREB activity in the developing palate is most likely to be regulated at the level of protein phosphorylation rather than at the level of CREB gene expression. Supported in part by NIH grants DE05550 and DE08199. WMW supported by NRSA DE05593.

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Cell Type Specific Repression of AFP Transcription in the Adult Mouse. L.A. Cirillo, J. Vacher*, and A.L. Tyner. Department of Genetics, University of Illinois at Chicago, Chicago, IL 60612, and *Department of Molecular Biology, Princeton University, Princeton, NJ 08540.

The gene encoding α-fetoprotein (AFP), the major serum protein expressed in the mammalian fetus, is transcribed in the fetal yolk sac, liver and gut. The level of AFP expression in both the liver and gut peaks shortly before birth, after which transcription of the AFP gene is repressed. It was recently determined that the region of the AFP promoter between -250 and -800 contains a negative cis-acting element that mediates repression of AFP transcription in the adult liver and gut. Transgenic mice carrying an AFP minigene in which this element is deleted transcribe high levels of AFP mRNA in these tissues. In order to determine the specific liver and gut cell types in which the AFP minigene is being expressed, we have performed in situ hybridization experiments using adult liver and small intestine obtained from these mice. While all hepatocytes in the fetal liver express AFP, only hepatocytes in the cell layers immediately surrounding the central veins express the AFP minigene in the adult. Similarly, while almost all epithelial cells lining the fetal gut express AFP, transcription of the AFP minigene is restricted to single differentiated cells in adult transgenic animals. Additional in situ hybridizations performed on liver and small intestine obtained from transgenic mice at various timepoints after birth indicate that the switch from the fetal to adult pattern of AFP minigene expression occurs between the second and third week after birth throughout the liver. In contrast, this switch occurs in a gradient along the length of the small intestine, initiating proximally one week after birth. Our results suggest that the factors capable of maintaining AFP transcription in the adult liver and gut are produced in specific subsets of cells in both of these tissues.

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Transcriptional Regulation of Cytoskeletal Gene Expression During Postnatal Development of the Rat Brain. Paula F. Moskowitz and Monica M. Oblinger. Dept. of Cell Biology and Anatomy, Chicago Medical School, North Chicago, Il 60064

The neuronal cytoskeleton performs many diverse functions including neurite outgrowth and maintenance of neuronal cytoarchitecture. During development of the mammalian brain dramatic alterations in the expression of cytoskeletal genes have been documented, however, very little is known about the level at which cytoskeletal genes are regulated during development. Changes in cytoskeletal mRNA levels may be due to alterations in the rate of gene transcription, selective alterations in mRNA stability or some combination thereof. In order to explore these possibilities, the transcriptional activity of various cytoskeletal genes including those coding for the neurofilament triplet proteins, NF-L, NF-M and NF-H, α-tubulin and β-tubulin were studied during postnatal development of the rat brain using an *in vitro* transcription assay. Nuclei from rat cortex were isolated at postnatal days 2,5,10,18, 35 and 90 (adult), nuclear run-off assays were performed and labelled RNA transcripts were isolated. Labelled RNA from different developmental stages was hybridized to slot blots containing equal amounts of cytoskeletal cDNA's. Robust changes in the transcription rate of several cytoskeletal genes were observed. For example, both α and β tubulin exhibited a major decrease in the rate of transcription during the course of postnatal development. These findings indicate that alterations in transcription are involved in the dramatic changes in cytoskeletal mRNA that occur during the postnatal interval in mammalian brain.

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SELECTION INDUCTION OF GENE EXPRESSION IN RA RESISTANT MURINE EMBRYONAL CARCINOMA CELLS. ELLEN J. PURPUS AND PETER A. MCUE. DEPARTMENT OF PATHOLOGY AND CELL BIOLOGY, THOMAS JEFFERSON UNIVERSITY, PHILADELPHIA, PA 19107

Embryonal carcinoma cells, the malignant puripotent stem cells of teratocarcinomas, are able to differentiate in response to several compounds including retinoic acid. Our laboratory has isolated several differentiation-defective EC cell mutants selected for their resistance to RA. Additionally, the line nulli $(RA)^{-1}$ has been shown to be unresponsive to other inducers such as DMSO and HMBA as well. In the presence of 10^{-6}M RA, these cells retain their EC phenotype, do not lose the cell surface antigen SSEA-1 and show no induction of plasminogen activator or cRABP activity. However, we have found that some RA responsiveness is retained at the level of mRNA expression for selected genes. Upon treatment with 10^{-6}M RA, increases in RA receptors alpha and beta expression are observed by northern blot analysis. Also, messages for the cytoplasmic isoforms of actin are induced. This selective gene induction might be considered "partial differentiation" and could indicate a specific block to an hierarchy of events and/or cascade of gene expression resulting in what is typically considered terminal differentiation of EC cells.

1165

Expression of hsp70 transcripts during steroid hormone-regulated mycelial branching. G.Kyriakopoulou, M.Borkar, V.Armavil, S.Brunt and J.C. Silver. Division of Life Sciences, University of Toronto, Scarborough, Ontario, Canada M1C 1A4.

In the filamentous oomycete fungus Achlya, HSP70 proteins are encoded by a family of seven or eight sequence-related genes. Three different Achlya genomic hsp70 sequences were cloned. Transcript populations recognized by each of the three Achlya hsp70 sequences were found to be regulated by the steroid hormone, antheridiol, a fungal steroid hormone which mediates the development of specialized lateral branches (antheridia) involved in mating and several subsequent developmental events. The different transcript populations recognized by each of the three hsp70 sequences respectively, show a complex temporal expression pattern relative to one another and relative to the branching response. One of the three hsp70 sequences cloned, recognizes transcripts regulated also by glucose-starvation, tunicamycin and calcum ionophore A23187 treatments, suggesting that this clone represents the gene encoding the ER luminal protein, GRP78 or alternatively, the putative glucose-regulated mitochondrial protein, GRP75. The remaining two Achlya hsp70 sequences each recognize transcripts which are expressed constitutively and up-regulated further by heat shock. These latter two hsp70 sequences exhibit expression patterns expected for genes encoding cytoplasmic HSP70 proteins similar to the yeast SSA genes. Our results suggest that HSP70 proteins play a role in the mycelial branching observed in response to the hormone. (Supported by Natural Sciences and Engineering Research Council of Canada)

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Preliminary Identification and Analysis of Heat Shock and Steroid Hormone Regulatory Sequences in the Steroid-Inducible Hsp85 Genes of the fungus Achlya. S.A. Brunt and J.C. Silver, Division of Life Sciences, University of Toronto, Scarborough, Ontario, Canada M1C 1A4.

Sexual differentiation in the male mating type of the fungus Achlya ambisexualis is mediated by the steroid hormone antheridiol. One of the prominent antheridiol-induced proteins has the identical electrophoretic behaviour and is antigenically related to Achlya HSP85, a member of the HSP90 family of proteins. Two distinct but highly similar hsp85 gene sequences have been isolated from Achlya genomic libraries. Northern analyses have shown that there are two hsp85 message populations. A 2.8 kB transcript is constitutively expressed in vegetative mycelia and is up-regulated in both heat-shocked and hormone-treated cells. A 2.9 kB transcript is inducible only in heat-shocked cells. Sequence data indicate that the 5' non-transcribed region of both of the cloned hsp85 gene sequences contain a number of copies of a sequence motif proposed to be conserved (Riddihough and Pelham, 1987) in many steroid response elements from Drosophila to mammals. In addition each hsp85 sequence contains at least four 15bp heat shock elements, some of which overlap the putative steroid response element motifs. The identification of functional steroid response elements in fungal genomes has implications for fungal pathogenicity in both plants and animals. (Supported by NSERC Canada)

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Regulation of U3 snRNA Gene Expression. Y. Hitti, R. Savino and S.A. Gerbi, Division of Biology and Medicine, Brown University, Providence, RI 02912

Sequencing of two U3 snRNA genes from a genomic library of Xenopus laevis revealed consensus motifs common to other U snRNA genes: a distal sequence element (DSE, octamer motif at -222 to -215), a proximal sequence element (PSE, at -62 to -52) and a 3' Box (15 or 16 bp downstream of the U3 genes). Nonetheless, there is differential regulation of transcription from U3 snRNA genes compared to the spliceosomal snRNA genes. How is this achieved? The "U3 Box" is an inverted CCAAT motif in the DSE of mammals specific for U3 snRNA genes, and we find this is conserved in amphibian U3 snRNA genes. The Xenopus U3 Box is exactly one helical turn further upstream of the octamer than its mammalian counterpart, suggesting interaction of putative transcription factors bound to these motifs. We mutated the U3 Box and part of an adjacent Sp1 site and found transcription was greatly depressed after injection into Xenopus oocytes, implying a role for the U3 Box in transcriptional efficiency of U3 snRNA. Electrophoretic mobility shift assays using nuclear extract from Xenopus oocytes implicate transcription factor binding to the U3 Box. Supported by PHS GM20261 to S.A.G.

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The Initial Transcribed Sequence of The Rat Osteocalcin Gene Contains a Negative Regulatory Element. B. Frenkel, J. Milnes, J.L. Stein, J.B. Lian, and G.S. Stein, Department of Cell Biology, University of Massachusetts Medical Center, Worcester, MA 01655.

Osteocalcin (OC) is a bone specific, vitamin D-responsive protein, which is associated with differentiation in both *in vivo* and *in vitro* model systems. Basal gene transcription is controlled by the TATA and CCAAT box elements, while vitamin D responsiveness is mediated by an upstream responsive element. We identified a strong negative regulatory element within the sequences of the rat osteocalcin gene (+23/+147) designated TIS (transcribed inhibitory sequence). Deletion of the TIS from an OC promoter-CAT reporter construct resulted in a 30-fold increased expression in either normal osteoblasts or ROS 17/2.8 cells. The repressor activity was conserved following deletion of the ATG initiation codon and a splicing site, both present within the native sequence, thus confirming the presence of a repressor element. Activity of the OC-TIS is orientation-independent. In addition, the OC-TIS was capable of trans-activity, following co-transfection of either normal or transformed osteoblasts with OC-CAT constructs together with the isolated subcloned TIS element. This trans-inhibitory activity of the vector containing the TIS alone suggests that the negative regulation is at the transcriptional level. Both normal osteoblasts and ROS 17/2.8 cells contain nuclear factors that bind the OC-TIS element, as shown by gel mobility- shift assays. Formation of a unique sequence-specific protein-DNA complex is well correlated with basal OC transcription, exhibiting marked reduction in proliferating normal diploid osteoblasts that do not express the OC gene. In conclusion, the presence of a negative regulatory element within the OC-TIS is indicated by deletion analysis,, co-transfection experiments, and protein-DNA interaction studies. Furthermore, the OC-TIS contains C>T-rich stretches, identical or highly similar to sequences within silencers previously identified in several genes that include rat type II collagen, insulin, growth hormone and chicken lysozyme. Thus, the putative mechanism involved in the developmental regulation of OC in osteoblasts may be related to general silencing mechanisms, operative at the onset of differentiation-related gene expression.

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Developmental Commitment of Mouse Enamel Organ Epithelium to *Amelogenin* Transcription. R.L. Couwenhoven and M.L. Snead, Ctr. for Craniofac. Mol. Bio., Univ. of So. Calif. School of Dentistry, Los Angeles, CA 90033.

Cyto- and biochemical-differentiation of inner enamel epithelium requires temporal- and spatial-specific interactions of enamel organ epithelium with dental papilla mesenchyme and extracellular matrix. Biosynthesis of *amelogenin* gene products has been associated with the differentiation of inner enamel epithelium. The purpose of the present study was to determine: *i*) the developmental stage when mouse enamel organ epithelium initiates *amelogenin* transcription, and *ii*) the developmental stage when mouse enamel organ becomes determined for *amelogenin* transcription in the absence of dental papilla mesenchyme. Mandibular first molars were dissected from developmentally-staged mouse embryos. Enamel organ epithelium was separated from mesenchyme by protease digestion, and enamel organs were cultured either in Matrigel, on laminin-coated filters or on plastic. Total RNA was recovered from cultured and control tissues by the guanidinium isothiocyanate/cesium chloride method. RNA phenotyping was accomplished by reverse transcription-polymerase chain reaction amplification (RT-PCR) utilizing oligonucleotide primers specific for *amelogenin*, *collagen* $\alpha 1(I)$ and β-*actin* DNA sequences. RT-PCR of RNA from enamel organ cultures failed to amplify *collagen* $\alpha 1(I)$ sequences, indicating that mesenchymal cells were not detected in the enamel organ cultures. Enamel organs cultured in Matrigel demonstrated the presence of *amelogenin* transcripts. *Amelogenin* transcription is initiated at the early cap stage of odontogenesis and epithelial cells within bud stage enamel organs are committed to *amelogenin* transcription. Supported by DE-06988 and DE-10150.

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Construction and Characterization of Immortalized Enamel Organ Epithelial Cells. L.S. Chen, W. Luo and M.L. Snead. The University of Southern California, Center for Craniofacial Molecular Biology, School of Dentistry, 2250 Alcazar Street, Los Angeles CA 90033.

Mammalian tooth development is dependent upon exchange of instructive signals between the dental epithelium and neural crest derived ectomesenchyme. Determined dental epithelium differentiates to ameloblasts with de novo expression of a unique family of hydrophobic proteins termed amelogenins. Amelogenins are secreted into the extracellular space and subsequently removed, a process believed to regulate the formation of enamel hydroxyapatite crystallites. Characterization of these events has proven enigmatic due in part to the absence of defined cell lines through which amelogenin expression and crystallite formation could be characterized. To address these shortcomings, we used retroviral vectors containing the SV40 large T-antigen and a selectable marker to immortalize dental epithelium in order to identify cell clones which express amelogenin gene products. Based upon either reverse transcription followed by polymerase chain amplification or immunodetection, several ameloblast-like clones have been identified which express both amelogenin messenger RNA and polypeptides. In contrast, other independent cell clones express high levels of TGF-beta messenger RNA and polypeptide but not amelogenin, suggesting their origin from the stratum intermedium, a unique cell population of unknown function. Identifying clones with distinctive phenotypes suggests that specific populations of odontogenic cells were transformed by the retroviral constructs. Cloned ameloblast-like cell lines now afford opportunities to examine protein-mineral interaction required for enamel formation, as well as appropriate host cells required for a detailed investigation of molecular mechanism involved in the regulation of amelogenin gene transcription. Supported by NIH, NIDR DE06988.

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Characterization of Cytokine Gene Expression in the PMA-Induced Human Histocytic Lymphoma Cell Line, U-937, using RT-PCR. R.R. Buchner and D.T. McKenzie. Stratagene Cloning Systems, La Jolla, CA 92037.

Cytokines play a central role in mediating both immune responses and inflammatory reactions. The central role of the cytokine in the immune system has been described as inducing differentiation and proliferation of leucocytes, and in regulating production or function of other cytokines. We were interested in performing a comprehensive characterization of the production of inflammatory cytokines in the human histocytic cell line, U-937. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) for 0, 3, 6, and 24 hours. Total RNA was isolated and first strand was amplified using reverse transcriptase-mediated polymerase chain reaction (RT-PCR). In these experiments, we examined the kinetics of cytokine mRNA expression and demonstrated that there is an induction of cytokine mRNA for IL-8, IL-6, GM-CSF, TGF-β1 and IL-6 receptor by PMA. In contrast, the message for cytokines IL-1α and IL-10 was constitutive and displayed no variation in expression over the time points examined. Gene expression for the cytokines IL-2 and IL-4, important in the development of T and/or B cells were negative. Based on these results, it may be of interest to monitor both inducible and constitutively-produced cytokine gene expression for their relation to tumor progression and/or immunosuppression.

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Control of Gene Expression in Eukaryotic Cells Using a Modified *E.coli lac* Repressor System. D.L. Wyborski, L.C. DuCoeur, and J.M. Short, Stratagene Cloning Systems, La Jolla, CA 92037.

The *E.coli lac* repressor system has been modified for the control of gene expression in eukaryotic cells, and potentially transgenic animals. The SV40 large T antigen nuclear localization signal (NLS) has been added to the *lac* repressor gene to direct the protein to the nucleus of mammalian cells. This modified Lac repressor protein was able to specifically repress expression of a luciferase gene linked to *lac* operator sequences. Basal expression of the luciferase in the repressed state amounted to approximately 10 molecules per cell. Following exposure of the cells to β-galactoside inducers, expression of luciferase increased over 50-fold. Coupled with recent data on the pharmacokinetics of *lac* inducers in mice, these experiments suggest that the *lac* repressor system can be used to tightly control gene expression in transgenic animals.

Tissue Specific Gene Expression I (1173-1174)

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A Novel Zinc Finger Encoding Gene Is Expressed in the Organ of Corti. M.N. Rivolta[1], J. Fex[1], N. Slepecky[1,2], and E. Wilcox[1]. [1]Laboratory of Molecular Biology, NIDCD, NIH, Bethesda, MD 20892 USA. [2]Institute For Sensory Research, Syracuse University, Syracuse, NY 13244 USA.

Transcriptional regulatory proteins play major roles in cells during the events leading to their differentiated states and in keeping the genes responsible for a steady-state phenotype "turned on". Transcription factors function by binding to specific DNA sequences regulating gene expression. Several transcription factor families have been described, and homologous proteins are included in a family based on the structure of the domain involved in binding to DNA. These include, for example, the homeodomain, the paired domain, the zinc finger domain, etc. The zinc finger domain was originally dicovered in TFIIIA, a 5S gene-specific transcription factor from Xenopus, where each finger contains a pair of cysteines separated from a pair of histidines by a stretch of around twelve amino acid residues. Each finger is folded around a tetrahedrally coordinated zinc ion bound by the cysteines and histidines. In the present study, we characterize a zinc finger-bearing gene cloned from the organ of Corti, as a step towards understanding the molecular mechanisms that produce and maintain the particular phenotype of this sensory epithelium. Total RNA was purified from microdissected guinea pig organ of Corti and reverse transcribed to cDNA. Degenerate primers were synthesized using highly conserved regions within the Cys_2-His_2 type zinc finger motif (Pellegrino et al., PNAS. 88: 671-675, 1991) and a band of 158 bp was amplified by PCR. It was cloned and sequenced and showed a consensus with two consecutive zinc finger sequences. The purified fragment was utilized to screen an organ of Corti cDNA library. Positives clones were sequenced and results indicate the presence of at least nine tandem zinc fingers, towards the carboxy terminus of the protein. A Northern blot probed with a 700 bp fragment outside the zinc finger region of one clone, showed hybridization to a 4.3 Kb transcript in the organ of Corti. This transcript is also detected in other tissues including stria vascularis, auditive nerve, tongue, retina, cerebellum and kidney. This transcript represents the first putative regulatory gene cloned directly from the organ of Corti. Further characterization and manipulation of this gene may provide us with a valuable tool which will help us to understand the development and differentiation of the auditory epithelium.

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Isolation of progesterone-induced genes from rhesus monkey endometrium by sequential subtractive hybridization and PCR amplification. C.I. Ace and W.C. Okulicz, Dept. of Ob/Gyn, University of Massachusetts Medical School, Worcester, MA 01655.

The steroid sex hormone progesterone (P) induces the expression of a variety of genes through a signal transduction pathway mediated by the P receptor, a DNA-binding activator of transcription. In-situ hybridization and immunohistochemical analysis of endometrial tissue isolated from ovariectomized rhesus monkeys in artificial menstrual cycles (AMC) reveal a complex spatial and temporal pattern of P-dependent gene expression. PolyA+ RNA was isolated from both P-dominant (day 21-23 of AMC's) and estrogen (E)-dominant (day 9-13) endometrium. The two classes of RNA were converted to cDNA, ligated to EcoRI adaptors and amplified by PCR using a primer homologous to the adaptor. E-dominant cDNA (EcDNA) was labelled with biotin and hybridized in excess to P-dominant cDNA (PcDNA). Sequences common to both classes of cDNA were subtracted by phenol/chloroform extraction of streptavidin-treated hybrid products. The remaining P-dependent cDNAs were amplified by PCR. After multiple rounds of hybridization/amplificaiton PcDNA was cloned into a bacterial expression plasmid vector. The subtracted library contained more than 90% P-dependent cDNA and minimal sequences common to EcDNA as detected by Southern blot and colony hybridization. This approach will be highly useful for further identification of P-induced genes. (Supported by HD-20290)

1175

In Situ Localization of Gonadotropin-Releasing Hormone Precursor mRNA in First Trimester Human Placental Tissues Using Laser Scanning Confocal Microscopy. T.M. Duello, R.L. Knowles, and P.J. Van Ess, Department of Obstetrics and Gynecology, University of Wisconsin, Madison, WI 53706.

It has been hypothesized that human placental gonadotropin-releasing hormone (GnRH)is produced by the cytotrophoblast and acts on the adjacent syncytiotrophoblast in a paracrine manner to regulate the secretion of human chorionic gonadotropin (hCG). However, immunocytochemical studies have localized GnRH to the cytotrophoblast and/or the syncytiotrophoblast of human placental tissues. To determine which cells are capable of GnRH synthesis, *in situ* hybridization studies were undertaken using first trimester human placental tissues, because it is at this time that maternal serum [hCG] is elevated. Placental tissues were fixed in 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2, cryoprotected, and frozen in isopentane in a liquid N_2 bath. Twelve micron sections were cut, mounted on chrom-alum/diethylpyrocarbonate-treated slides, and hybridized to a biotinylated RNA probe to the precursor of gonadotropin-releasing hormone (JL Roberts). Control slides were first hybridized with a 200-fold excess of unlabeled probe prior to hybridization with the biotinylated RNA probe. For detection, sections were first incubated with mouse anti-biotin-Cy.5 followed by donkey anti-mouse IgG-Cy.5 (Abs from Jackson Immunoresearch). Using confocal microscopy, a portion of the cytotrophoblast cells demonstrated intense fluorescence, while the remainder did not label at all. Those that labeled were adjacent to one another suggesting that all of the cells transcribing GnRH precursor mRNA were associated with a single syncytial unit. Labeling of the syncytiotrophoblast was equivocal. If consecutive laser scanned confocal images were accumulated, faint labeling was seen, but approximated that of fibroblasts in the negative control. These findings are consistent with the hypothesis that the cytotrophoblasts are responsible for human placental GnRH synthesis. (NSF DCB-9106102)

1176

A Novel Transcriptional Enhancer is Involved in the Prolactin- and Extracellular Matrix-Dependent Regulation of β-casein Gene Expression. C. Schmidhauser*, G. F. Casperson+, C. A. Myers*, K. T. Sanzo+, S. Bolten+, and M. J. Bissell*, *Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, U. of California, Berkeley, California 94720; +Monsanto Corporate Research, AA3C, Chesterfield Village Parkway, Chesterfield, MO 63198

Lactogenic hormones and extracellular matrix (ECM) act synergistically to regulate β-casein expression in culture. We have developed a functional subpopulation of the mouse mammary epithelial cell strain COMMA-1D (designated CID 9), which expresses high level of β-casein, forms alveolar structures when plated onto the EHS tumor-derived matrix, and apically secretes β-casein. We have further shown that ECM- and prolactin-dependent regulation of β-casein occurs mainly at the transcriptional level and that 5' sequences play an important role in these regulations. To address the question of the nature of the DNA sequence requirements for such regulation, we analyzed the bovine β-casein gene promoter in these cells. We now have located a 160-bp transcriptional enhancer (BCE1) within the 5' flanking region of the β-casein gene. Using functional assays, we show that BCE1 contains responsive elements for prolactin- and ECM-dependent regulation. BCE1 placed upstream of a truncated and inactive β-casein promoter (the shortest extending from -89 to +42 bp with regard to the transcription start site) reconstitutes a promoter even more potent than the intact promoter, which contains BCE1 in its normal context more than 1.5 kb upstream. This small fusion promoter also reconstitutes the normal pattern of regulation, including a requirement for both prolactin and ECM and a synergistic action of prolactin and hydrocortisone. By placing the milk promoter with a heterologous viral promoter, we show that BCE1 participates in the prolactin- and ECM-mediated regulation.

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Up-Regulation of Androgen Receptor Expression by Cyclic Adenosine 3',5'-Monophosphate in Human Hepatoma Cells.

L.-X. Shan, C.W.Bardin, and O.A. Jänne The Population Council and The Rockefeller University, 1230 York Avenue, New York, NY 10021. (Spon. by J. Catterall)

The effects of 8-bromo cyclic AMP (8-Br-cAMP) and forskolin on androgen receptor (AR) mRNA and protein levels were investigated in cultured human hepatoma (HepG2) cells. AR mRNA concentrations were quantified by Northern blotting with a single-stranded cRNA probe, and a polyclonal antibody raised against the N-terminal region of the receptor (residues 14-32) was employed in immunological analyses of the receptor protein content. Incubation of HepG2 cells for 6 h with increasing concentrations of 8-Br-cAMP (0.1-100 μM) elevated AR mRNA levels 1.7- to 3.3-fold above those in control HepG2 cells. Maximal response was achieved within 6 h after addition of 50 μM 8-Br-cAMP. A cAMP-elevating agent, forskolin, also brought about an increase in the AR mRNA concentration in a time- and dose-dependent fashion, with the maximal response being achieved with a 25 μM forskolin exposure for 6 h. Similar, but less marked, changes occurred in the AR protein content in HepG2 cells under identical conditions, in that both 8-Br-cAMP and forskolin increased immunoreactive receptor concentration. These data thus indicate that both exogenously added and endogenously formed cAMP is capable of up-regulating AR mRNA and protein accumulation in HepG2 cells. This work was supported in part by grants from the NIH (grant Nos. HD13541 and DK37692), the Medical Research Council of the Finnish Academy and the Finnish Life and Pension Insurance Companies.

1178

Purification and Characterization of a Male- and Liver-specific Estrogen Binding Protein. N.J. Barsic, R.P. Hughey, and P.K. Eagon, VAMC and Depts. of Medicine and Molecular Genetics & Biochemistry, University of Pittsburgh, Pittsburgh, PA 15261.

The liver of sexually mature male rats contains a unique estrogen binding protein (MEB) which constitutes at least 99% of the total cytosolic estradiol binding activity and has properties which distinguish it from the classical estrogen receptor. The MEB protein binds estradiol with moderate affinity (Kd=32nM) and has a strict specificity for steroidal estrogens and certain estrogenic metabolites; the protein does not bind nonsteroidal estrogens or other steroid hormones. In males, MEB is induced by androgen at puberty, and requires neonatal androgen imprinting and an intact pituitary for full expression. In females, MEB can be partially induced by androgen treatment after ovariectomy. The exact role of MEB is unknown, but it is postulated to act as a scavenger of estrogens and/or estrogenic metabolites. Using several chromatographic steps, a partial purification (150-fold) of the protein has been achieved. This partially purified MEB preparation was used to produce polyclonal antibodies. On Western blots, these polyclonal antibodies react with a 31K band in a sex- and liver-specific manner. To obtain protein sequence, the partially purified MEB preparation was purified further by SDS gel electrophoresis and transferred to nitrocellulose. Attempts to sequence the intact 31K protein were unsuccessful due to a blocked N-terminus. However, using a Cleveland digest, transfer to polyvinylidene difluoride (PVDF) membrane and Edman degradation, protein sequence of 40 residues was obtained, which constitutes approximately 15% of the MEB sequence and appears to be unique, as determined from current Genebank information. From this sequence data, three oligonucleotide probes were constructed to screen a male rat liver cDNA library. Further studies should clarify the regulation and function of this unusual protein.

1179

Heterogeneity of Secretin Receptor Gene Expression in Proliferating Intrahepatic Bile Duct Epithelial Cells in the Rat. G. Alpini, L.D. Pham, C.D. Ulrich, L.J. Miller, and N.F. LaRusso, Mayo Clinic, Rochester, MN 55905

Secretin, a gastrointestinal hormone, stimulates bile secretion by interacting with receptors on epithelial cells (i.e., cholangiocytes) that line the ducts in the liver. In the rat, basal and secretin-induced bile flow increases after relief of bile duct ligation (BDL), a procedure which also induces cholangiocyte proliferation. Although these proliferating cholangiocytes are morphologically diverse and while recent reports suggest differential expression of selected proteins (e.g., blood group antigens) by cholangiocytes, there is no evidence that such apparent diversity is reflected by functional heterogeneity. Thus, using the secretin receptor as a physiologically important phenotype, we began to test the HYPOTHESIS that cholangiocytes are functionally heterogeneous. METHODS: We generated a secretin receptor probe identical to the previously reported cDNA for a secretin-binding protein (EMBO 10:1635, 1991) using PCR with rat pancreatic cDNA as template. We used this probe to study the distribution, expression and transcription rate of the secretin receptor mRNA (SR-mRNA) using Northern blot hybridization and nuclease protection and run-on transcription assays in two subpopulations of morphologically distinct cholangiocytes purified from BDL rats by centrifugal elutriation. [i.e., small cholangiocytes (mean diameter, 8 um) and large cholangiocytes (mean diameter, 13 um)]. RESULTS: Using Northern blot analysis and nuclease protection assays, we detected SR-mRNA only in large cholangiocytes. Similarly, run-on assays using nuclei isolated from large (but not small) cholangiocytes demonstrated active transcription of SR-mRNA. SUMMARY AND CONCLUSIONS: In cholangiocytes isolated from BDL rats, there is differential gene expression of SR-mRNA by two, morphologically distinct, cholangiocyte subpopulations. The data suggest that large cholangiocytes play a specific role in regulating secretin-induced hypercholeresis after BDL and support the hypothesis that cholangiocytes are functionally heterogeneous.

1180

Retinoid X Receptor Binds to and Regulates Liver Specific Enhancer of Hepatitis B Virus. B. Huan and A. Siddiqui, Department of Microbiology and Program in Molecular Biology, University of Colorado Medical School, Denver, CO 80262.(Spon. by W.E. Hahn)

A retinoid X receptor (RXRα) response element is located in the liver-specific enhancer of the hepatitis B virus (HBV) in a region that has been defined by genetic analysis to play a key role in the regulation of enhancer function. Using full length liver-specific RXRα expressed in *E. Coli* as a glutathione S-transferase-fusion protein or its DNA binding domain, we demonstrate binding to a putative retinoic acid response element (RARE) in the HBV enhancer by mobility shift assay and DNase I protection analysis. When cotransfected with an RXRα expression vector, the HBV enhancer/ reporter gene construct responds to induction by retinoic acid (RA). A single base transition of G to A in the HBV RARE motif led to a dramatic reduction in the *in vitro* binding activity of RXRα. Furthermore, this mutation results in a significant decrease in enhancer function and a lack of response to RA induction. Transfection of the HBV-producing cell line 2.2.15, with an RXRα expression plasmid followed by induction with RA or 9-cis RA, led to an increase in viral protein synthesis. The results of these studies implicate potentially important role of RXRα in the liver specific regulation of HBV gene expression and perhaps in the disease pathogenesis associated with infection including hepatocellular carcinoma.

1181

Role of Hepatocyte Nuclear Factor 3α in Clara Cell Specific Gene Expression
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Clara Cell Secretory Protein (CCSP) gene expression is confined to non-ciliated bronchiolar epithelial (Clara) cells of the lung. Using transgenic mice we have shown that *cis*-acting elements within 2.1 kB of the 5' flanking region of the rat CCSP gene confer tissue and cell specific gene expression of a chimeric CCSP-human growth hormone gene construct. We now have utilized DNAase 1 footprinting to identify regions of protein-DNA interaction within this flanking region. These studies have identified a prominent footprint using lung nuclear extract in the region from -127 to -76 in the CCSP gene promoter. Mobility shift assays using nuclear extract from lung or H441 cells reveal a number of specific DNA binding proteins capable of interacting with this promoter region. Nucleotide sequence analysis of this DNA reveals two consensus binding sites for the hepatocyte transcription factor HNF3. Specific protein binding to this region was competed by oligonucleotides containing previously characterized binding sites for HNF3 but not for other transcription factors including NFkB, HNF-4, and C/EBP. Using recombinant proteins and specific antibodies for the various HNF3 family members we have determined that this specific interaction is caused by HNF3α. A role for HNF3α in CCSP gene expression is further supported by Southwestern blotting and transfection studies using HNF3 expression plasmids. These data are the first to demonstrate regulation of pulmonary epithelial cell specific gene expression by this transcription factor and support the concept that members of the *fork head* family of transcription factors may determine tissue specific gene expression in different cell types derived from embryonic gut endoderm.

1182

Gene Expression During Hibernation.
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Liver gene expression is modified during mammalian hibernation. Previous studies in our lab have shown that the plasma protein, α_2-macroglobulin, is induced during hibernation. The amount of α_2-macroglobulin protein and its corresponding activity increases during hibernation. This increase is completely accounted for by an increase in the corresponding mRNA for this protein. We are continuing the study of liver-specific gene expression to determine the extent of repatterning occurring during hibernation.

Qualitative and quantitative differences exist between the mRNA population in liver isolated from active and hibernating ground squirrels. mRNA isolated from the liver of active and hibernating ground squirrels was translated *in vitro* in the presence of ^{35}S-methionine. Two-dimensional gel electrophoresis of the *in vitro* translation products showed many proteins present in both states, however, there are some proteins that are unique to one state or the other. This indicates that there are mRNA species differentially expressed between the active and hibernating states. To identify these mRNAs we will use a subtractive hybridization strategy. This approach is useful because it does not make any presumptions as to what types of genes are important and should lead to the identification of new genes in the liver that are induced during hibernation.

1183

Analysis of Fibrinogen Aα Gene Transcription Following Interleukin-6 Induction
G.M. Fuller, R. Zheng, and J. Shuman, Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294

Interleukin-6 and glucocorticoids (GLC) up-regulate the expression of fibrinogen genes in a highly coordinated manner during the early hours of an acute inflammatory reaction. To characterize the transcriptional response to these signals, we have linked portions of the Aα fibrinogen promoter (-500 to -85) to a luciferase reporter construct. Using transient transfections as an assay (H35 Reuber hepatoma cells), we provisionally assign the IL-6 response to two independent sequence elements within the promoter of this gene. One element (TTNNGNAAT) (-225 to -180), a consensus C/EBP binding site, was shown to be protected in a DNAse I footprint assay using purified recombinant C/EBP. The second element appears to be a consensus type II IL-6 reponse element (CTGGGA) (-129 to -123). Whole cell extracts from primary rat hepatocytes form two complexes with a 30mer oligonucleotide corresponding to the type II IL-6 responsive element. When extracts are prepared from IL-6 treated hepatocytes the faster migrating complex diminishes and the slower one increases in intensity. This change is consistent with a posttranslational modification of a single factor that interacts with the site. The presence of a C/EBP site in the promoter region for a type II IL-6 element was unexpected in view of the lack of response of fibrinogen genes to IL-1. (Supported in part by NIH grant #HL43155)

1184

Cloning of the Gene for Mouse C-Reactive Protein and Tissue-Specific Expression.
Nam-On Ku and R.F. Mortensen, Department of Microbiology, Ohio State University, Columbus, OH 43210.

Although human C-reactive protein (CRP) is a well-documented acute phase protein that increases by 1000 fold, mouse CRP (moCRP) is a minor acute phase reactant. Therefore, the gene for moCRP was cloned to determine the molecular basis for the reported low amounts of moCRP. The mRNA coding for mouse CRP was detected only in mouse liver. Regulation of mouse CRP gene expression might be regulated by multiple nuclear factors that interact with cis-acting DNA sequences if it is similar to the human CRP gene. The promoter sequence responsible for tissue-specific expression has not been determined. A series of transient expression vectors containing progressive deletions of the mouse CRP gene 5'-flanking sequence were fused to the promotorless bacterial chloramphenicol acetyltransferase (CAT) gene. Promoter activity was determined by transfecting hepatoma and non-hepatoma cell lines with pCAT constructs. The 5'Δ-220 construct containing nucleotides from -220 bp to -3 bp from the transcriptional start site expressed almost the same amount of CAT activity as the pCBPX-MCRP construct containing nucleotides from -2.3 kb to -3 bp, suggesting that constitutive expression of the mouse CRP-CAT fusion gene required at least 220 bp of the 5'-flanking sequence. A construct with a 5'Δ-153 displayed about a 75% decrease in transcriptional activity compared with construct 5'Δ-220. A construct of 5'Δ-50 exhibited about an 85% decrease in transcriptional activity compared with the construct 5'Δ-90. The results indicated that the mouse CRP promoter contained at least two cell-specific elements between nucleotides -220 and -153 and between -90 and -50. The moCRP gene did not respond to the inflammatory cytokine IL-6 despite the presence of an IL-6 responsive element at approximately -400 bp. (Supported by USPHS grant CA30015)

1185

Molecular Cloning of Rat P-Selectin and Studies of mRNA Expression in a Model of Acute Inflammation.
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P-Selectin (GMP-140) is a rapidly inducible cell adhesion receptor that is expressed upon the surface of activated platelets and endothelial cells, modulating their interactions with leukocytes. To evaluate its role in rat models of inflammation and thrombosis, the present study aimed to clone P-Selectin and demonstrate its tissue distribution and inducibility in a rat model of endotoxin shock. A bacterial lipopolysaccharide (LPS)-stimulated lung/liver cDNA library was constructed and screened with a canine P-selectin cDNA to isolate a partial rat P-selectin clone. Sequence analysis of this 2060 bp clone demonstrated significant nucleotide identity (74%) with human P-selectin. Alignment of rat P-Selectin with human revealed the rat cDNA comprised 1179 bp of the coding region, including complement repeats (CR) 5-9, the transmembrane domain (TD), the cytoplasmic domain (CD). A 3' untranslated region of 861 bp followed the coding region. The 393-amino acid peptide deduced from the coding sequence exhibited an overall 76% identity and 85% similarity to human P-Selectin, with TD and CD being more highly conserved than the CR. Tissue-specific expression of P-Selectin mRNA was studied in rats administered LPS (5mg/kg, 3h). A cDNA fragment encompassing CR 7-9, TD, CD, and untranslated region was used in Northern blot analysis. In unstimulated rats, P-Selectin mRNA was not detectable in any of the tissues examined. However, 3 hrs after LPS administration elevated levels of P-Selectin mRNA were detected in several tissues, most notable lung, liver, kidney and spleen. These data demonstrate that part of the cellular response *in vivo* to endotoxin shock includes the rapid increase of P-Selectin mRNA levels.

1186

Cloning and Characterization of a Novel Rat Lung mRNA.
A.K. Rishi, M. Joyce-Brady, J.M. Fisher, L.G.Dobbs, J. Floros, and M.C. Williams, Pulmonary Center, Boston Univ. Sch. of Med., Boston, MA 02118

A novel mRNA (T1α) was characterized by sequence analysis of a cDNA obtained from an expression library of adult rat lung. The clone was identified by a monoclonal antibody previously shown to be specific for an adult type 1 alveolar epithelial cell antigen of 40-42 kD (reduced.) The cDNA is 1672 base pairs and identifies a single mRNA species of the same size on Northern blots of adult rat lung. The sequence of cloned DNA was verified by direct overlapping sequencing of native lung mRNA by reverse transcription-PCR amplification (RT-PCR.) The longest open reading frame of the cDNA is 498 bps which would encode a 20kD protein. The encoded protein contains a 29 amino acid hydrophobic domain near the C-terminus but lacks a signal for N-linked sugars. Northern blots and RT-PCR show high expression of T1α mRNA in adult lung with marginally detectable levels in kidney, brain, and intestine. Preliminary Northern analysis shows T1α expression in isolated adult type 1 cells and in type 2 cells cultured on plastic for 2-5 days, but not in freshly isolated type 2 cells or alveolar macrophages. RT-PCR analyses shows expression of T1α in freshly isolated type 1 cells (50-60% purity) but not in freshly isolated type 2 cells or macrophages. Preliminary in situ hybridization shows diffuse labeling over alveolar septae but little/no labeling over bronchiolar epithelium or vasculature. We believe that T1α is primarily expressed in type 1 cells. By RT-PCR T1α is detected in rat lung from day 13.5 of gestation onwards. The mouse T1α has about 90% homology and encodes a protein of 21kD. Homologs have been identified in adult rabbit and human lung. Further characterization of the protein product of T1α and clarification of the discrepancy between the molecular weights of the cDNA product (20kD) and the original antigen (40kD) are in progress.

1187

The Effect of Serum-free Medium on Surfactant Protein Gene Expression in Primary Type II Cell Cultures. K. Guzman, D. W. Hart and G.E.R. Hook. NIEHS/NIH, Research Triangle Park, NC 27709. (Spon. by T. Vollberg)

Pulmonary Type II (T2) cells are reponsible for production of pulmonary surfactant, the substance which is essential for the prevention of alveolar collapse at low lung volumes. Traditionally, maintainance of T2 cells in culture has been difficult since T2 cells rapidly dedifferentiate when cultured on tissue culture plastic with serum-supplemented medium. Recently, a number of paramaters have been described to maintain the T2 cell phenotype *in vitro* (eg. a serum-free defined medium); however, the effect of this medium on surfactant protein (SP) gene expression has not been determined. We have compared the effects of defined medium (DM) and serum supplemented media (SSM) on the production the mRNAs for SPA, SPB, SPC from T2 cells cultured on either tissue culture plastic or Engelbreth-Holm-Swarm tumor basement membrane gels (Matrigel). T2 cells, isolated from the lungs of adult Sprague Dawley rats, were plated on plastic or Matrigel at a concentration of $5x10^5$ cells/cm^2 and incubated in DM (1:1 mixture of Ham's F12 and DMEM supplemented with insulin, dibutyryl cAMP, hydrocortisone, epidermal growth factor, selenium, and albumin/linoleic acid complex) or SSM (DMEM with 5% rat serum). RNA was isolated by the one step guanidiuium-phenol-chloroform procedure and mRNA levels assayed by an RNase protection assay. Data for SP mRNAs were normalized to actin. Production of SP mRNAs was improved when cells were incubated in DM. At 18 hr after plating, SP mRNAs (A, B , and C) had increased by approximately 1.4- to 2.2- fold from T2 cells on Matrigel and 1.7-fold (SPC only) on plastic. Increases in the mRNAs for SPA and SPB were also observed but were not statistically significant. Greater differences between DM and SSM were observed at 4 days after plating. Increases ranged from approximately 3.4- to 4.7-fold in SP mRNAs from T2 cells on matrigel and 1.7- to 1.9- fold from cells on plastic. In conclusion, soluble factors in DM stimulate, or soluble factors in serum inhibit, production of SP mRNAs from T2 cells in primary cell culture.

1188

1.6 kb of 5' Flanking Sequence of the Rat Secretin Gene is Sufficient for Cell-Specific and Developmental Expression of Hybrid Genes in Transgenic Mice. M. J. Lopez, B. Upchurch, and A. Leiter . Departments of Medicine and Pediatrics, Tufts University School of Medicine-New England Medical Center , Boston, MA 02111 (Spon. by D. Jefferson)

The hormone secretin is produced primarily by isolated mucosal endocrine cells, the S-cells, of the proximal small intestine. In addition, we have recently shown that secretin is produced in developing β-cells of the pancreatic islets. Previous work in this laboratory defined a positive cis-active regulatory domain in the secretin gene by DNA transfection studies. We now report on work in transgenic mice which express either SV40 large T antigen (Tag) or human growth hormone (hGH) under the control of 1.6kb of the 5' flanking sequence of the rat secretin gene. Tissue sections of these mice were analyzed by immunohistochemistry with polyclonal antibodies to secretin, hGH, and Tag. Our results show that the small intestine is the major site of expression for both hGH and Tag bearing fusion genes. Double immunofluorescence for hGH and secretin confirmed that transgene expression was restricted to secretin producing cells in both fetal and adult mice. In adult mice expressing Tag, tumors are seen in both the intestine and in pancreatic islets. The intestinal tumors are highly undifferentiated and invasive and do not appear to make hormones. In contrast, the pancreatic islet tumors are more differentiated adenomas and primarily express insulin. Prior to the development of intestinal tumors, Tag expressed in young developing mice frequently colocalizes with secretin. Both transgenes are first expressed at the time in development when secretin first appears. We conclude that 1.6kb of the secretin gene 5' flanking sequence contains the information necessary to direct appropriate cell-specific and developmental expression of secretin. These results also confirm the relationship between the β-cell and the secretin cell in pancreatic islets.

1189

Patterns of Gene Expression in the Mouse Jejunal Epithelium. H. Cheng, L. Ling, M. Bjerknes, and H. Chen. Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario, Canada.

Cellular proliferation, differentiation, and physiology show a high degree of spatial organization in the intestinal epithelium. It seems reasonable to expect that the pattern of gene expression parallels that organization. A few patterns of gene expression have been observed in the villus but little is known about gene expression in the crypt, probably due to the difficulty in isolating crypt-specific cDNA clones. In an attempt to isolate crypt-specific and, in particular, stem-cell related genes, single-stranded cDNA from normal mouse jejunal epithelium was used in subtractive hybridization against single-stranded cDNA from epithelium in which the cycling cells were depleted by 2,000 rads gamma irradiation. Northern analysis showed that 79 of the 83 clones from the subtraction library were of epithelial origin. The localization of 72 clones was determined by *in situ* hybridization, and the identity of 40 clones were matched to previously published genes by partial DNA sequencing. Patterns of expression of the genes represented by the clones could be classified into three categories: (1) expression in the whole epithelium, (2) expression restricted to the villus, and (3) expression restricted to the crypt. Clones in the first two categories could be further divided into three subgroups: (a) those with uniform expression, (b) those with an increasing gradient, and (c) those with a decreasisng gradient of expression along the crypt-villus axis. Five clones were differentially localized to the crypt. Of these, three displayed a uniform distribution in the crypt, one higher in the lower crypt, and the remaining one higher in the crypt base.

1190

A Novel Approach to the Study of Differential Gene Expression along the Crypt-Villus Axis. D.F. Cano-Gauci,* J.C. Lualdi,+ A.J. Ouellette,+ and R.N. Buick,* *Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 500 Sherbourne St. Toronto, Ont. M4X 1K9, Canada and +The Shriners' Burns Institute, 1 Kendall Square, Cambridge, Mass. 12142.

Cell division in the mouse small intestine occurs in the stem cell zone near the crypt base, after which cells differentiate and migrate to the villus tip. Intestinal differentiation can by studied by isolating populations of cells representing successive fractions along the crypt-villus axis. Due to intrinsic limitations with this and other methods, however, gene expression regulating intestinal differentiation is not well understood. A novel approach to the problem is presented here. Single intact crypts were isolated by EDTA incubation and mechanical vibration of everted mouse intestinal segments. Crypts isolated in this manner were suitable for mRNA-directed polymerase chain reaction, thus generating crypt-specific cDNA, representing approx. 300bp at the 3' end of the corresponding transcripts. Crypt specificity was confirmed by Southern blot hybridization of amplified cDNA with the small intestinal Paneth cell-specific cryptdin cDNA but lack of hybridization to the villus-specific fatty acid binding protein cDNA. Crypt cDNA was then used to construct a jejunal crypt cDNA library in λZAPII. The library contains approx. $1.2X10^7$ pfu/uL with an average insert size of 275 bp. Screening the library with a cryptdin cDNA probe confirmed the presence of bona fide cryptdin. Preliminary results of a differential hybridization of the library with crypt versus villus cDNA indicate the presence of several potential crypt-specific transcripts.

Pattern Formation (1191-1192)

1191

The *Drosophila* gene, *tinman*, is a determinant of heart development and is required for the heart-specific expression of other genes. R. Bodmer, Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048.

The homeobox-containing gene *tinman* (previously called *msh-2*) is first expressed in the early mesoderm and expression depends on the mesoderm determinant *twist*. Later, after the first mesodermal subdivision has occurred, expression becomes limited to the developing heart and the visceral mesoderm. Embryos that are deficient for the *tinman* gene develop no heart or cardiac precursors and also lack visceral muscles, but show relatively little somatic muscle defects. These results suggests that *tinman* is involved in the determination of a major subdivision of the mesoderm, namely the heart and visceral mesoderm as opposed to the somatic mesoderm. Since *tinman* appears to be a transcription factor specifically required for heart development, we have been searching for genes that are regulated by *tinman*. Several genes with a potential downstream function of *tinman* have been identified. The heart-specific expression of these genes is dependent on *tinman*. We are now in the process of inducing mutations in these genes to determine their function in heart development.

1192

A Role for a Quail Homeobox Gene in Establishment of the Dorsal Midline Structure. Y. Takahashi+, N. M. Le Douarin. Institut d'Embryology du CNRS, Nogent, France. +:Present address, Institute of Neuroscience, University of Oregon, Eugene, Oregon

The quail homeobox gene, Quox 8, is expressed in a variety of regions which seem to undergo active tissue interactions during development. In the dorsal region of the embryo, Quox 8 mRNA is expressed in the dorsal portion of the neural tube and its overlying mesenchyme and surface ectoderm in a coordinated manner. We have studied the possible developmental significance of Quox 8 expression by manipulating avian embryos. We have found; 1. Quox 8-expressing mesenchyme differentiates into dorsal vertebral cartilage. 2. The neural tube provides an inductive signal to overlying tissues to express Quox 8 mRNA. 3. Dorsally restricted expression of the Quox 8 gene requires the normal polarity of the neural tube. 4. The Quox 8 gene appears to be involved in dorsal vertebral formation. Thus, the Quox 8 gene is regulated by local tissue interactions leading to establishment of the dorsal structure.

1193

Neural Cell Adhesion Molecule (NCAM) and Pancreatic Islet Architecture. V. Cirulli, D. Baetens, U. Rutishauser, P.A. Halban, L. Orci, and D.G. Rouiller. Laboratory Louis-Jeantet and Department of Morphology, CMU, 1211 Geneva 4, Switzerland; Department of Genetics, Case Western Reserve University, Cleveland, Ohio.

Endocrine cell types are non-randomly distributed within pancreatic islets. In the rat, a central core of insulin-secreting B-cells is envelopped by a mantle of non-B-cells, secreting glucagon, somatostatin, and pancreatic polypeptide. To examine whether a differential expression of cell adhesion molecules (CAMs) characterize central and peripheral islet domains, frozen sections of rat pancreata were stained for the Ca2+-dependent CAM E-cadherin, or the Ca2+-independent NCAM, by indirect fluorescence using polyclonal antisera. While E-cadherin staining was equally distributed on the entire islet section, NCAM immunofluorescence clearly predominated on non-B-cells of the islet periphery. This is consistent with our recent finding of higher levels of NCAM on purified islet non-B-cells than B-cells, with similar levels of E-cadherin on both. It suggests that differential expression of NCAM plays a role in the special arrangement of endocrine cells within islets. Islet cells dispersed in culture are known to form aggregates with cellular architecture similar to native islets, thus providing an *in vitro* model of cell type segregation. Purified rat islet B-cells and non-B-cells were obtained by autofluorescence-activated cell sorting. They were differentially labeled with the fluorescent carbocyanines dil (B-cells) and diO (non-B-cells), mixed, and cultured for 5 days to form aggregates. Cell type segregation was studied by confocal microscopy. In the presence of non-CAM-directed anti-islet cell Fab-fragments, non-B-cells segregated to the periphery of the aggregates. In aggregates formed in the presence of anti-NCAM Fab-fragments, however, islet cells remained randomly distributed. In conclusion, the present study demonstrates 1) that NCAM immunostaining predominates on non-B-cells of rat islets *in situ*; 2) that the normal organization of islet cell types within aggregates *in vitro* is prevented by anti-NCAM Fab-fragments. These findings suggest that NCAM is responsible, at least in part, for the non-random organization of cells within islets of Langerhans.

1194

Pattern Development during Pericardial Coelom Formation and Specification of the Cardiomyocyte Cell Population by N-Cadherin and the *Drosophila* Armadillo Protein Homologue in the Early Chick Embryo. K.K. Linask, Y.H. Gui, R. Rasheed, and L. Kwon. Dept. of Pediatrics, Univ. of Pennsylvania School of Medicine and Div. of Cardiology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104.

An immunohistochemical analysis of N-cadherin localization in the heart-forming region (HFR) of HH stage 5-8 chick embryos suggested an important role for N-cadherin in specification of the pattern of pericardial coelom formation that for the first time delineates the cardiomyocyte cell population in the embryo (Linask, K.K. 1992. Dev. Biol. 151:213-224). The transmembrane cell-cell adhesion protein N-cadherin associates via its cytoplasmic domain with three or more proteins whose structure and function are not yet established. Three associated proteins, termed α-, ß-, and γ-catenins (70-100 kD) may form a link to the cytoskeleton, and/or regulate N-cadherin function. Immunoprecipitates of N-cadherin complexes, isolated from early embryos or early tubular hearts, indicate the presence of the three catenins and a 230 kD protein. Western blots indicate antibodies to bovine plakoglobin and the *Drosophila* armadillo protein cross-react with two of the catenin proteins. Immunohistochemical localization of plakoglobin and armadillo protein in the stage 7 chick embryonic HFR indicate different immunostaining patterns, suggesting differing functions for the two catenins. Only the homologue of the armadillo protein, a *Drosophila* segment polarity gene product, is involved in pattern development during pericardial coelom formation. This study extends to vertebrate heart development a role for a *Drosophila* protein homologue. [This work is supported by a Grant-In Aid to KKL from the Southeastern Pennsylvania American Heart Association Affiliate].

1195

Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. G.R. Cunha, P. Young, C. Christov-Tzelkov, R. Guzman, S. Nandi, F. Talamantes, and G. Thordarson. Department of Anatomy, University of California, San Francisco, Ca. 94143, Cancer Research Laboratory, University of California, Berkeley, Ca. 94720, Department of Biology, University of California, Santa Cruz, 95064

The goal of this research was to establish methods for inducting mammary epithelial differentiation from non-mammary epithelium. For this purpose mid-ventral epidermis from 13-day rat embryos was associated with 13-day embryonic mouse mammary mesenchyme (mouse MgM + rat SKE). The resultant mouse MgM + rat SKE recombinants as well as control (homotypic mouse mammary recombinants, homotypic mouse skin recombinants and mouse mammary mesenchyme by itself) were grafted under the renal capsule of syngeneic or athymic female nude mouse hosts. Some of the female hosts were induced to become lactogenic by grafting an adult pituitary which elicited a state of hyperprolactinemia. Grafts of mammary mesenchyme by itself formed a mixed fibrous/adipose connective tissue without epithelial elements. Tissue recombinants of mouse MgM + rat SKE grown for 1 month in vivo formed a hair-bearing keratinized skin from which mammary ductal structures extended into the underlying mesenchyme, while homotypic skin recombinants only formed a hair-bearing keratinized skin. Ducts in mouse MgM + rat SKE recombinants were composed of columnar luminal cells and basal, actin-positive myoepithelial cells. When grown in pituitary-grafted hosts, the ductal epithelial cells expressed casein and α-lactalbumin as judged by immunocytochemistry. The presence of these milk proteins was confirmed by Western blot. The epithelial cells in mouse MgM + rat SKE recombinants expressing milk proteins were shown to be rat cells, while the surrounding connective tissue was composed of mouse cells based upon staining with Hoechst dye 33258. Using mammary-specific markers, these studies confirmed the earlier morphological studies of Propper and unequivocally demonstrated for the first time that mammary mesenchyme can induce morphological and functional mammary differentiation in a non-mammary epithelium.

1196

Retinoic acid regulates expression of late alveolar genes and alters the pattern of growth of the developing lung in vitro. W.V. Cardoso, M.C. Williams, M. Joyce-Brady, A. Rishi, and L.S. Brody. Pulmonary Center, Boston Univ. Sch. Med. Boston, MA. 02118

Retinoic acid (RA) has been shown to influence patterns of morphogenesis and regeneration in a number of vertebrate systems. We studied the effect of RA on the pattern of development and on epithelial cell differentiation in the 13 day rat embryonic lung bud cultured in defined media for periods up to 9 days. Lung development was assessed by morphology and by gene expression using reverse transcribed lung mRNA and PCR with oligonucleotide primers specific for 5 epithelial genes expressed in the lung periphery. In control media the lung bud developed a central to peripheral structure of branching airways and peripheral alveolar sacs and expressed genes associated with epithelial cell differentiation in a fashion similar to that of the in vivo lung. Surfactant protein SP-C , lysozyme and a type 1 cell gene, T1α, were expressed in the early lung bud, while SP-A and SP-B, so called late genes, were expressed after 3-5 days in culture. RA (10^{-5}M) altered the pattern of lung development, favoring growth of proximal airways while suppressing peripheral epithelial buds. RA also prevented expression of late alveolar genes, SP-A and SP-B, which are expressed in the peripheral portions of the lung. The RA effect was reversible and did not appear to be the result of direct suppression of late genes. Rather, RA acted to proximalize the developing lung, possibly through its effect on expression of pattern-determining genes in the lung.

1197

The Level of CRABP-I Expression Influences the Levels and Types of All Trans Retinoic Acid Metabolites in F9 Teratocarcinoma Stem Cells. John F. Boylan and Lorraine J. Gudas. Department of Pharmacology, Cornell University Medical College, NY, NY 10021

F9 teratocarcinoma stem cells represent an attractive model system in which to study the actions of RA. When F9 stem cells are treated with RA, they differentiate into primitive endoderm, an epithilial-like cell type. The mechanism by which RA induces F9 stem cell differentiation involve at least three high affinity nuclear receptors (RAR α, RAR β, RAR γ) and two cytoplasmic RA binding proteins (CRABP-I and CRABP-II). F9 teratocarcinoma stem cells readily metabolize RA to more polar compounds such as 4-oxo-retinoic acid and 4-oxo-16-hydroxy-retinoic acid. The exact mechanism and enzymes involved in this process are not clearly understood. A major metabolic pathway of RA consists of the hydroxylation followed by the oxidation at position C-4 of the cyclohexenyl ring to form 4-oxo-RA. The purpose of this study was to examine whether or not CRABP-I plays a role in the metabolism of RA during F9 teratocarcinoma stem cell differentiation. F9 cell lines which express elevated levels of CRABP-I and F9 cells which contain a reduced level of CRABP-I were examined for their ability to metabolize [^{3}H]-RA. We show that the level of CRABP-I influences both the type and amount of [^{3}H]-RA metabolites in F9 stem cells. In addition, CRABP-I effects the rate at which RA is metabolized. The results presented here support the idea that CRABP-I is indeed involved in the catabolism of intracellular RA, thus providing the mechanism by which CRABP-I can regulate the concentration of intracellular RA.

1198

Deep cytoplasmic rearrangements in first cell cycle Xenopus embryos. B. Brown and M. Danilchik, Dept. Biology, Wesleyan Univ., Middletown, CT 06457

Following fertilization in Xenopus, dramatic rearrangements of the egg cytoplasm relocalize maternally synthesized egg components. During the first cell cycle the vegetal yolk mass rotates relative to the egg surface, toward the sperm entry point (SEP; Vincent et al., 1986), while concomitant deep cytoplasmic rearrangements occur in the animal hemisphere (Danilchik & Denegre, 1991). We have further examined the cytoplasmic swirl pattern in labeled eggs and have found that both the first cell cycle yolk mass rotation and the sperm are involved in producing rearrangements of the animal cytoplasm. Even in UV-irradiated embryos, in which yolk mass rotation is inhibited, a swirl pattern develops during the first cell cycle, suggesting a role for the expanding sperm aster in directing animal cytoplasmic movements. However, in unirradiated control embryos the dorsal (anti-SEP) swirl is larger than in UV-irradiated embryos and often extends into the vegetal hemisphere. Thus the yolk mass rotation may enhance the dorsalward cytoplasmic movement, begun by the sperm aster, enough to induce normal axis formation.

1199

Analysis of mutations which affect early development and fin regeneration in zebrafish. Stephen L. Johnson, Dana Africa, and James A. Weston, Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403.

Many of the genes and the cell biological mechanisms which are employed in early development, including control of cell proliferation, differentiation, migration and pattern formation, may also be employed in the regeneration of adult tissues. We have begun to exploit the rapid regeneration of the caudal fin of adult zebrafish, and the availability of mutations which affect embryonic pigment pattern, to explore the relative roles of these processes in re-establishment of the pigment stripes in the regenerating fin. Two populations of melanocytes can be identified in the regenerating fin by treatment of the animal with phenylthiourea (PTU), a drug which blocks melanin synthesis, and thereby identifies pre-existing melanocytes. First, a PTU-resistant population of melanocytes appears by three days following amputation in the regenerated tissue just distal of the old stripe, suggesting that these cells have migrated in from the old stripe. A second population of melanocytes, whose visualization is PTU-sensitive, and thereby may represent de novo differentiation of melanocytes, then appears uniformly throughout the proximal half of the regenerate (distal to stripe and interstripe alike). Previously identified mutations affecting pigment pattern in embryos or adults were investigated for defects in patterning during fin regeneration. In late embryos mutant for sparse, melanocytes are absent. In adult sparse mutants, pigmentation is normal in the fin, but defective upon regeneration. The PTU-sensitive population of melanocytes fails to appear; only the PTU-resistant population appears in the regenerated tissue distal to the old-stripe. These defects in fin regeneration, and other evidence concerning the development of melanocytes in sparse embryos, suggest that the sparse gene product plays a role in the differentiation or survival of melanocytes. Further screens for mutants that are temperature-sensitive for aspects of fin regeneration are now underway, and may likewise identify defects in embryogenesis. Supported by Grant HD22486, an NIH Postdoctoral Fellowship (HD07470) and the Neurofibromatosis Foundation Inc.

1200

Homeostatic control of bud morphogenesis in *S. cerevisiae*. J.L. Brewster and M.C. Gustin, Dept. Biochem & Cell Biol., Rice U., Houston, TX 77251

We have been studying a MAP kinase-mediated signaling pathway in yeast that mediates cellular responses to osmotic stress. The following results suggest that this pathway is required for cells to maintain normal patterns of bud morphogenesis following osmotic stress. Actin filaments are important in directing growth to the bud and are localized to the bud region. An increase in external osmolarity arrests bud growth and causes actin filaments to be distributed homogeneously throughout the mother cell and bud. When osmoregulatory processes restore the osmotic gradient, actin filaments are again localized to the bud and bud growth resumes. In wild-type cells, resumption of bud growth occurs by continued expansion of buds that were initiated before osmotic stress was applied. The location of new buds after recovery from osmotic stress follows normal spatial patterns: axial budding in haploids, bipolar budding in diploids. Null mutants in the osmo-sensing pathway genes *HOG1* and *HOG4* respond differently to osmotic stress. *HOG1* is a new member of the MAP kinase gene family. *HOG4* is identical to *PBS2*, a putative protein kinase related to *STE7* and *wis1*. Like the wild-type, exposure of cells lacking either *HOG1* or *HOG4* to small increases in external osmolarity causes a temporary arrest of bud growth and delocalization of actin filaments. However, the mutant cells after recovery from osmotic stress abandon the old buds and resume growth by initiating new buds. The location of the new bud does not follow normal spatial patterns. This result suggests that wild-type cells have a *HOG1*- and *HOG4*-dependent homeostatic mechanism for resuming cell morphogenesis at pre-existing sites within the cell following a change in external osmolarity.

1201

Patterning and Segmentation in the Vertebrate Central Nervous System. E. Birgbauer, and S. E. Fraser, Division of Biology, Beckman Institute, California Institute of Technology, Pasadena, CA 91125.

We are examining pattern formation in the vertebrate central nervous system. The rhombencephalon is overtly segmented into rhombomeres which appear on day E2 in chick and then disappear by about day E6. The rhombomeres show a segmental pattern of neurogenesis and expression of certain homeobox-containing genes. Previous analyses of cell lineage by labelling clones with fluorescent dextran on E2 and examining their descendants on E3 show that the rhombomeres are compartments of lineage restriction for at least motor and commissural neurons. We have been examining this lineage restriction in more detail and have obtained results that suggest that rhombomere boundaries are not impenetrable at all stages. Some clones of cells labelled on E2 and examined later on E4 show members that are not restricted to a single rhombomere. This loss of compartmental restriction occurs despite the continued presence of morphological boundaries, the undulations of the neural tube. It remains to be determined if the clones that violate the boundaries do so because the rhombomere boundaries no longer represent barriers to cell crossing by E4, or because only certain cell types are segmentally restricted, leaving other cell types, which are generated later, free to cross.

1202

Stage-Specific Requirement of Myosin during *Dictyostelium* Development. M.L. Springer, B. Patterson, and J.A. Spudich, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305. (Spon. by T. Soldati.)

A *Dictyostelium* strain has been isolated that is cold-sensitive for myosin function (B. Patterson, unpublished). Multicellular development in this strain was compared to that in a myosin-wild-type strain. At non-permissive temperature, the cold-sensitive strain is blocked at the beginning of fruiting body formation, while at permissive temperature, it forms almost-normal fruiting bodies. Shifting this strain between permissive and non-permissive temperatures at various times of development revealed two stages during which myosin function was essential for correct development to proceed. The first myosin-dependent stage is aggregation, during which myosin may play a role in differentiation and sorting of cells. The second stage is the final raising of the spore mass to the top of the stalk, during which myosin may be required as a motor to function against gravity. Calcofluor staining of the structures made at non-permissive temperature revealed the presence of cells possessing spore coats; however, these cells were killed by heat treatment conditions that true spores survive.

1203

A Cell Density Sensing Mechanism in the Eukaryote Dictyostelium Discoideum. Renu Jain, Ita S. Yuen, and Richard H. Gomer, Howard Hughes Medical Institute, Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251-1892.

Throughout development, gene expression in Dictyostelium discoideum is regulated by a variety of factors including cell density. The effect of cell density on gene expression is mediated by a secreted factor referred to as conditioned medium factor (CMF). This factor, added exogenously during starvation to cells at low cell densities, allows them to express prespore and prestalk genes in response to extracellular cAMP. In contrast, cells starved at this density without CMF can not express these genes. Size fractionation of CMF shows two classes with activity: one an 80 kD glycoprotein, the second is composed of polypeptides of molecular weight between 0.5-6 kD which are breakdown products of the 80 kD CMF. The CMF cDNA has been cloned. The predicted molecular mass of the polypeptide backbone is 62.6 kD and it shows no significant similarity to any known proteins. Antisense constructs of the CMF gene were transformed into Dictyostelium cells. This resulted in cells which secrete very little CMF, have lower motility, and do not aggregate. However, the addition of exogenous purified CMF to the CMF antisense transformed cells causes them to develop normally. To examine the distribution of CMF, antibodies were raised against a portion of the bacterially expressed CMF gene and were affinity purified. CMF is present in all vegetative and early developing cells, and is secreted by both the precursors of the stalk and spore cells. We previously observed that the 80 kD CMF breaks down to a 65 kD protein which has CMF activity. The apparent molecular weights of both the 80 and the 65 kD bands were reduced by deglycosylation with various enzymes which remove either N- or O- linked glycosylation, indicating that the 65 kD protein is a proteolytic fragment of the 80 kD CMF. The CMF activity of the bacterially synthesized CMF, which is not glycosylated, was one third that of the purified endogenous protein. We previously observed that the small breakdown products of CMF have very little activity after deglycosylation; the above results thus suggest that glycosylation may be important for stability rather than the function of CMF.

1204

Finite-Element Modelling of Plant Somatic Embryo Development. H. Vits, J.J. Derby, and W-S. Hu, Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455.

A mathematical model for somatic embryo development was developed as a tool to enhance our understanding of the dynamic interactions between environmental factors and the regulation of embryo growth and development occurring via endogenous synthesis of growth regulators and differentiation of tissue. Solutions are obtained with a Galerkin-finite element method with boundary tracking, that allows simulation of morphological histories. The simulation results show that globular to heart transitions can be obtained by imposing simple growth patterns and that surface tension can affect the displacement patterns within the tissue. The latter effect suggests that the interaction between surface and volumetric events has to be considered in the representation of other plant morphogenetic events such as the development of shoot apical meristems.

1205

Expression of Rh(D) antigenicity does not involve its SH groups. K. Suyama, R. Lunn and J. Goldstein Cell Biochemistry Laboratory, New York Blood Center, New York, NY 10021.

One or more SH groups of the Rh(D) polypeptide are thought to be involved in expression of Rh(D) antigenicity. To explore this, we treated intact red cells with membrane permeable NEM(N-ethylmaleimide) or impermeable DTNB [5,5'-dithiobis (2-nitrobenzoic acid)], reagents which specifically bind to the SH group. Hemagglutination titers of these treated cells were determined using human monoclonal and polyclonal anti-D. No significant decrease in hemagglutination titer was detected in Rh positive cells treated with 5mM NEM or DTNB for 1 hr. at room temperature. Increasing the concentrations of DTNB or NEM to the extent of appreciable hemolysis or increasing incubation time does not result in a loss of hemagglutination titer by anti-D, suggesting that cysteine residues are not involved in Rh(D) antigenicity. Rh(D) polypeptide is known to be palmitoylated at its cysteine residues. NEM or DTNB-pretreatment (30 min, room temp., 1.5-5.0mM) of intact red cells completely inhibited uptake of 3H-palmitic acid by Rh(D) polypeptide, indicating that its SH groups are blocked by NEM or DTNB. However, immunoprecipitation of Rh(D) polypeptides from such treated cells with human anti-D was not affected. Thus, SH group involvement in Rh(D) antigentic expression was not observed at either the cellular or protein levels. Supported by Office of Naval Research Contract No. N-00014-84-C-0543.

1206

RBC Membrane Protein Concentration Variances between Normal and Abnormal Red Cells. J. Planas, D. Denson, R. Gomez, H. Gu and D.J. Wilson, Division of Biomedical Sciences, Meharry Medical College, Nashville, TN 37208.

In our laboratory, we have found that the amount of Glycophorin A is decreased in red blood cells (RBC) taken from patients with sickle cell anemia when compared with that found in normal control RBC membranes. An 18-20% gradient SDS polyacrylamide gel demonstrates a 3-fold decrease of the middle protein of the PAS 2 band of erythrocyte ghosts. In the United States, Sickle Cell Disease is a common disease among African Americans and Hispanics from the Caribbean, Central And South America. Glycophorins are the major sialoglcyoproteins found in the membrane of the RBC. Five (5) different glycophorins have been described in the literature. The objective of our research was to determine the macromolecular events responsible for the differences in glycophorin A amounts in these cells. Samples were collected in collaboration with the Comprehensive Sickle Cell Center at Meharry Medical College Hubbard Hospital. Protein isolation was done acording to the hypotonic lysis method. Total protein concentration was determined by the BCA procedure, SDS-PAGE analysis was carried out on 10% discontinuous gels. Glycophorins were stained using Periodic Acid Schiff (PAS) and Dzandu staining procedures. Ghost preparations were also submitted to O- and N-glyconase analysis, as well as to Western blot analysis using several glycophorin-specific antibodies. Densitometric analysis was conducted on a laser densitometer. Results are discussed in terms of the relative concentration of glycophorins found on the RBC membranes of these individuals. This project was supported in part by Minority Biomedical Research Support (MBRS) and National Science Foundation.

1207

Molecular Cloning of the cDNA Encoding the Dense Granule Protein GRA3 from Toxoplasma gondii. D. Bermudes*, J. F. Dubremetz**, and K. A. Joiner*.
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**Unité 42 INSERM, 369 rue Jules Guesde, 95650 Villeneuve d'Ascq, France.

Modification of the parasitophorous vacuole (PV) by *Toxoplasma gondii* occurs by exocytosis from a set of parasite secretory organelles, the dense granules. Four dense granule proteins have been identified by monoclonal antibodies (GRA1, 2, 3 and 4) and characterized by intracellular localization. Of the four dense granule proteins only the 30 kD GRA 3 is preferentially associated with the parasitophorous vacuole membrane (PVM) after exocytosis as determined by immunofluorescence and immunogold EM (Achbarou et al. 1991, Parasitology 103: 321-329). A λgt11 cDNA library of *T. gondii* (kindly provided by J. Boothroyd) was screened using a polyclonal GRA3 antiserum. Six clones were isolated and tested by immunoblot using both the polyclonal and monoclonal antibodies to GRA3. Only one clone produced a β-galactosidase fusion protein which reacted with both antibodies. This fusion protein was then used to affinity purify the polyclonal antisera. On immunoblots these antibodies reacted with a 30 kD band and by immunofluorescence reacted with the PVM and dense granules. Translation of a partial DNA sequence shows a single open reading frame and a hydrophobic region of 22 amino acids flanked by charged residues consistent with a transmembrane domain. No significant homology has been detected to other proteins, including other dense granule proteins. These results are being used to define the mechanism and topology of GRA3 association with the PVM.

1208

Overexpression and Structural Studies of Yeast α-Agglutinin, A Putative Member of the Immunoglobulin Superfamily. M.-H. Chen, Z.-M. Shen, and P.N. Lipke. Department of Biological Sciences, Hunter College, CUNY, 695 Park Avenue, New York, NY 10021

α-agglutinin is a cell adhesion glycoprotein expressed on yeast cells of α mating type. It binds to a-agglutinin, expressed on cells of a mating type, to facilitate mating. The N-terminal half of α-agglutinin contains 3 regions with limited homology to immunoglobulin (Ig) subdomains. The third domain shows significant identity with an Ig domain consensus sequence ($p < 10^{-7}$). All three regions appear to be necessary for α-agglutinin binding to a-agglutinin. The N-terminal half of the α-agglutinin gene was PCR subcloned into a yeast high expression vector (pPGK) to encode a secreted form of α-agglutinin that retains binding activity. The production of α-agglutinin with PGK promoter was about 40 times greater than that with the α-agglutinin promoter. α-agglutinin was purified by concentration of the culture supernatant and chromatography on DEAE-Sephadex and Bio-Gel P-60. The circular dichroism spectrum (CD) of purified α-agglutinin showed a structure rich in ß-sheets. The α-agglutinin CD profile was similar to that of CD2, a T-lymphocyte transmembrane cell adhesion protein that is a member of the immunoglobulin superfamily on the basis of NMR structural studies (Recny et al., 1990. J. Biol. Chem. 265: 8542-8549; Driscoll et al., 1991. Nature 353: 762-765). Preliminary results of peptide sequencing of trypsin-digested α-agglutinin fragments purified by HPLC indicated that there is a disulfide bridge within one of the predicted Ig-like domains.

Supported by grants from NIGMS and NIH-RCMI.

1209

Identification and isolation of membrane-bound and soluble forms of the apical glycoprotein, butyrophilin. L.R.Banghart, L.J.W.Jack, and I.H.Mather, Dept. Animal Sciences, University of Maryland, College Park, MD 20742.

Butyrophilin (BT) is a glycoprotein expressed on the apical surfaces of mammary epithelial cells which may function in the secretion of milk-fat droplets. The sequence derived from cloned cDNA predicts that unprocessed BT has a M_r of 59,260, an amino-terminal hydrophobic leader peptide and a transmembrane domain in the middle. To confirm this, mRNA was prepared from the cDNA and translated *in vitro*. BT was incorporated into microsomes as a 61,000-dalton peptide. As expected, treatment with proteinase K left a 35,000-dalton fragment associated with the membranes which was degraded on the further addition of Triton X-100. Rabbit antibodies to synthetic peptides encompassing residues 125-136 and 328-344, in the exoplasmic and cytoplasmic domains, respectively, specifically bound to all of the isoelectric variants of BT on nitrocellulose blots of fat globule membrane (FGM) proteins. These antibodies were used to determine the distribution of BT in cell fractions of lactating mammary tissue. One immunoreactive species with the same M_r (67,000) as fully processed BT in FGM was detected in Na_2CO_3-washed microsomal membranes. Surprisingly, *soluble* forms of BT of M_r 57,000-64,000 were detected in post-microsomal supernatants that bound to both exoplasmic and cytoplasmic antipeptide antibodies. These could be recovered as a water soluble complex of M_r 204,000 after gel filtration in the *absence of detergent* and purified in soluble forms by immunoaffinity chromatography. The possible function of soluble and membrane-bound BT in the assembly of FGM during lipid secretion will be discussed.

1210

The mechanism of the release of NADase from activated macrophages. U.-H. Kim, and M.K. Han, Department of Biochemistry, Chonbuk National University Medical School, Chonju, 560-182, Korea.

We have previously shown that membrane-associated NAD glycohydrolase (NADase) can be solubilized by the treatment of phosphatidylinositol- specific phospholipase C (PIPLC) of *Bacillus cereus* [Kim,U.-H, et al. 1988. *Biochim. Biophys. Acta*, 965, 76-81]. We attempted to activate mouse peritoneal macrophages by endotoxin treatment following infection with *Mycobacterium tuberculosis*. NADase was released from endotoxin- treated mouse macrophages which were primed with *M. tuberculosis*, but not from normal macrophages. The release of NADase from mouse macrophages was observed in the presence of plasma which contains phosphatidylinositol-specific phospholipase D (PIPLD). These results suggest that endotoxin treatment on the primed macrophages increases PIPLC or PIPLD activities which are responsible for the cleavage of PI linkage of the ecto-NADase.

1211

Evidence for a Novel Type of ATP-Diphosphohydrolase (Type III) in Bovine Lungs. M. Picher, Y. P. Côté, and A.R. Beaudoin, Centre de recherche sur les mécanismes de sécrétion, Département de biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1K 2R1.

In the past few years, we have reported two distinct ATP-diphosphohydrolase (ATPDases) in pancreas (type I) and in bovine aorta (type II). The purpose of this work was to describe ATPase and ADPase activities in bovine lungs. Our results demonstrate both ATPase and ADPase activities sensitive to Ca^{2+} and Mg^{2+}. Analysis of substrate specificity show that the enzyme preparation hydrolyses both diphospho- and triphosphonucleosides. A series of inhibitors, including NaN_3, $HgCl_2$ and Gossypol, were tested and showed a comparable level of inhibition of ADPase and ATPase activities, suggesting the presence of an ATPDase. Analysis of the physico-chemical properties of the enzyme: optimum pH, heat denaturation curves, estimation of molecular weight by cobalt (^{60}Co) irradiation-inactivation and polyacrylamide gel electrophoresis under non-denaturing conditions demonstrate, on the one hand, that the same enzyme hydrolyses both ATP and ADP, and on the other hand, that the ATPDase described in bovine lungs is different from those reported in pancreas and bovine aorta. Our results thereby support the view that there is an hitherto undescribed type of ATPDase in bovine lungs. Supported by Quebec Heart Foundation and FCAR Quebec.

1212

Monoclonal Antibody Affinity Chromatography of Chicken Ecto-ATP-Diphosphohydrolase. R.S. Strobel and M.D. Rosenberg. Dept. of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108.

Ecto-ATP-Diphospholase is an integral membrane ectoenzyme which hydrolyzes extracellular nucleoside tri- and di- phosphates. Using a novel ATPase capture screening assay, six monoclonal antibodies have been produced to the chicken oviductal fluid enzyme. One antibody (MC22) coupled to hydrazide activated Sephacryl S-1000 has proven optimal for immunoaffinty chromatography of the enzyme. Using this column, the oviductal enzyme has been purified to a single band of 80K and 860 units\mg protein. The same column has been used to purifiy the chicken liver enzyme to a single band of 85K and 1230 units\mg protein. Studies are in progress to assess the crosreactivity of these antibodies in other species.

1213

Purification of Fungal β(1,3)-Glucan Synthase from *Neurospora crassa* by Product Entrapment. P.D. Awald, M. Zugel, D. Frost, and C.P. Selitrennikoff. Dept. Cellular and Structural Biology, University of Colorado Health Science Center, Denver, CO 80262.

The synthesis and assembly of carbohydrate polymers into cell-wall is essential for normal morphological development of fungi. *In vivo* inhibition of the synthesis of β(1,3)-glucan, an abundant carbohydrate polymer in the cell wall, by drug treatment or mutation, results in abnormal morphology and poor viability of *N. crassa*, a filamentous Ascomycete. Thus, purification and characterization of β(1,3)-glucan synthase will yield valuable information with regard to morphogenetic events in fungi. *N. crassa* hyphae were disrupted, and membrane-enriched fractions obtained by high-speed centrifugation. Glucan synthase activity was solubilized from membranes by treatment with octyl-β-D-glucoside and (3-[(Cholamidopropyl)dimethyl-ammonio]-propanesulfonate). Solubilized fractions were incubated with substrate (UDP-glucose), and glucan synthase, with its associated glucan chain, were purified by centrifugation through a 10% sucrose cushion (product entrapment). Partially purified enzyme had a mean specific activity of 3,000 nmoles glucose incorporated/min/mg protein; recovery was greater than 80%. This represents an 1,100 fold purification of enzyme activity when compared to crude lysates, and is over 100 fold greater than any reported purification on fungal glucan synthase to date. Separation of proteins by SDS-PAGE revealed a number of proteins copurifying with enzyme activity. Proteins of 105 kDa, 70 kDa, 55 kDa, 50 kDa, 42 kDa, 41 kDa, 35 kDa, 32 kDa, 31 kDa, and 28 kDa were especially prominent. These data reinforce the current view that fungal β(1,3)-glucan synthase is a multimeric enzyme complex.

1214

Localization of Human Placental Phospholipase A_2 in Cultured Placental Trophoblast Cells Using Immunocytochemical Techniques, G. Bowden, C. McCain, P. Hatch and L.M. Jordan

Phospholipase A_2 is an ubiquitous enzyme that is found in every cell that it has been sought. This enzyme catalyzes the hydrolysis of phospholipids in the sn-2-acyl position of phospholipids. Phospholipase A_2 is involved in various cellular abnormalities including asthma, arthritis, gastrointestinal disorders and septic shock.

We describe herein a series of experiments designed to determine the location of phospholipase A_2 (PLA_2) in cultured human placental trophoblast cells. Trophoblast cells were cultured from fresh term placenta on gold labelled coverslips. The cells were fixed using gluteraldehyde Gey's buffer solution. Cells which were cultured from t=0 to t=5 days were analyzed using a scanning transmission electron microscope. Phospholipase A_2 was localized with the aid of a polyclonal antibody.

1215

Characterization of Two-Dimensional Crystals of Photosystem II by Electron and Atomic Force Microscopy. P.S.Furcinitti, K.M.Marr, M.K.Lyon, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347.

Photosystem II (PS II) is different from all other reaction centers in that it splits water to evolve oxygen and hydrogen ions. This unique ability to evolve oxygen is due partly to three peripheral membrane polypeptides associated with the PS II complex. We have been able to induce the PS II complexes in spinach thylakoids to form two-dimensional arrays. The purified two-dimensional crystals have been characterized by room temperature absorption spectroscopy, immunoblots and gel electrophoresis. We have examined the topography of these arrays, as well as the surface of membrane fragments, with the atomic force microscope (AFM). The AFM provides a relatively high resolution, three dimensional view of individual PS II complexes, examined under hydrated buffered conditions, with no need for image averaging techniques. In addition, using low dose electron microscopy, we have produced a projection map of negatively stained PS II arrays at a resolution of 17 Å with a signal-to-noise ratio of 5.32. The projection map shows four distinct areas of density for each PS II complex. One area is much larger than the others and is likely to include the reaction center core. A second area extends out from the mass of the complex, where it may be involved in interaction with other membrane complexes. (This work supported by NIH GM 40735 and NIH Biotechnology Resources RR00592).

1216

Characterization of the Proteolytic Activity Responsible for Cleaving the Prenylated CAAX Box of Yeast Ras2 Protein. L. Farh, D.A. Mitchell and R.J. Deschenes, Department of Biochemistry, University of Iowa, Iowa City, IA 52242.

Ras proteins are initially synthesized as soluble precursors, but subsequently undergo posttranslational modifications that result in membrane localization. The signal lies in the CAAX box, where C is a cysteine, A is generally an aliphatic residue and X is the carboxyl terminal amino acid. Processing begins with farnesylation of cysteine, followed by proteolytic removal of the -AAX tail. In yeast, the protein farnesyltransferase is a dimer encoded by the *RAM1* and *RAM2* genes. The protease that cleaves the -AAX tail of the CAAX box has not been identified by genetic screens. We have established biochemical assays based on Ras2 protein synthesized in reticulocyte lysates. The assay detects farnesylation by incorporation of ^{3}H-farnesyl and proteolysis by a change in mobility by SDS PAGE. Attempts to inhibit the proteolysis with standard protease inhibitors have not been successful. However, we find that the activity is sensitive to EDTA and o-phenathroline, but not EGTA. Dialysis experiments show that the activity requires Zn^{2+} and Mg^{2+}. In this respect, the properties of the protease and farnesyltransferase are indistinguishable. We synthesized CIIS and CIIS-FAR, which are the unfarnesylated and farnesylated versions of the peptide Tyr-Arg-Ser-Gly-Gly-Cys-Ile-Ile-Ser, respectively. Only unfarnesylated CIIS showed appreciable inhibition of the Ras2 mobility shift. The failure of CIIS-FAR to inhibit suggests that the prenylation and proteolysis are tightly coupled *in vivo*.

1217

CaaX Box Sequence Requirements for Yeast RAS2 Protein Processing and Activity. D.A. Mitchell, T.K. Marshall and R.J. Deschenes Department of Biochemistry,University of Iowa, Iowa City, IA 52242

Saccharomyces cerevisiae contain two *ras* gene homologs at least one of which is required for vegetative growth. *Ras* proteins are synthesized as soluble precursors, but then undergo a series of posttranslational modifications resulting in localization to the cytoplasmic surface of the plasma membrane. These modifications are mediated through the CaaX box, the terminal four amino acids of the immature protein. The CaaX-box posttranslational modifications include: 1) farnesylation of the conserved cysteine residue (C319) four amino acids from the protein's carboxyl terminus; 2) cleavage of the C-terminal three amino acids; 3) α-carboxyl methylation; and 4) palmitoylation of the other cysteine (C318) located immediately upstream of the farnesylated cysteine. We have created a series of CaaX box mutants to probe the sequence requirement for processing and membrane localization. Mutations which prevent farnesylation abolish *RAS* activity, whereas palmitoylation and methylation do not appear to be essential. In the course of mutagenizing the yeast *ras2* CaaX box, we have found mutants which are not prenylated but still possess *RAS* activity. These mutants contain serine for cysteine substitutions at amino acid positions 318 and 319 followed by a basic amino acid extension of the sequence IIKLIKRK (CC-ext, SC-ext, and SS-ext). While loss of *Ras* activity is lethal to the cell, the extension mutants, at low copy number, are necessary and sufficient for growth in a *ras1ras2* background. Direct assays of prenylation reveal that none of the extension mutants becomes farnesylated. In addition, the extension mutants migrate more slowly than wild-type *RAS2* when subjected to SDS-PAGE indicating they are not proteolytically processed. In a *ras1ras2* background, these strains grow more slowly than wild-type. *RAS* produced from the CC-ext construct partitions to both the membrane and cytosolic fractions while *RAS* produced from SC-ext or SS-ext constructs is found predominantly in the cytosolic fraction. These observations suggest that alternate mechanisms exist which promote *RAS* function.

1218

Biogenesis of the Yeast Mating Pheromone a-Factor. P. Chen, S. Sapperstein, A. Kistler, R. Clyne, and S. Michaelis, Dept. of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

We are studying the biogenesis of the yeast mating pheromone a-factor. Mature a-factor is an isoprenylated, carboxymethylated dodecapeptide that exits the cell through a novel membrane pump, STE6, rather than via the classical secretory pathway (ER->Golgi->secretory vesicles). a-Factor is initially synthesized as a 36 amino acid precursor with a C-terminal CAAX box and an N-terminal extension. Through characterization of biosynthetic intermediates, we have previously shown that the first step in a-factor biogenesis is C-terminal modification, consisting of prenylation, cleavage of AAX, and methylation of the Cys residue of the CAAX box. a-Factor then becomes membrane-associated, and subsequently undergoes two N-terminal proteolytic cleavage steps to generate mature a-factor, which is exported. The first N-terminal proteolytic cleavage step, which was unanticipated, involves a clip within the first 7 amino acids of N-terminal extension of the a-factor precursor. The second cleavage step occurs, as expected, at the junction between mature a-factor and the remaining portion of N-terminal extension.

To identify signals within the a-factor precursor that are recognized by the proteolytic processing machinery, we have examined the effects of alterations (including substitutions and deletions) in the N-terminal extension of a-factor. We found that mutations which prevent the first proteolytic clip within the N-terminal extension have no detectable impact on production of mature a-factor. In contrast, alterations in amino acids flanking the junction between the N-terminal extension and mature a-factor, as well as deletions elsewhere in the N-terminal extension, can profoundly affect the production and secretion of mature a-factor. Taken together, these results indicate that this second proteolytic processing step is essential for a-factor biogenesis and export.

1219

CONSEQUENCES OF ALTERED ISOPRENYLATION TARGETS IN a-FACTOR EXPORT AND BIOACTIVITY

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Two distinct isoprenoids have been identified in the post-translational modification of proteins, the 15-carbon farnesyl moiety and the 20-carbon geranyl, geranyl group. Specific cysteine-containing amino acid sequences (CAAX, CC, CXC) are targets for attachment of these lipids by prenyl transferases and have been identified in various proteins including the oncogene product RAS, nuclear lamins, and rab vesicular trafficking proteins. The functional consequences of differential isoprenylation of proteins with either a C_{15} or C_{20} isoprenoid have not been elucidated. The primary gene product of the *S. cerevisiae* MFa1 gene is a 36 a.a. precursor that is processed to yield the farnesylated mating pheromone a-factor, YIIKGVFWDPAC[Farnesyl]-OCH_3.The farnesylation of a-factor is required for biological activity and export via the yeast STE6 protein, a homolog of the mammalian multiple drug-resistance P-glycoprotein. We have employed site-directed mutagenesis to alter the isoprenylation targeting sequence of a-factor from its native CVIA to both CVIL and SVCC, putative sequence targets for geranyl, geranylation. Yeast expressing these modified a-factor genes under the control of an inducible promoter secrete biologically active pheromones. In contrast, under similar conditions, *ste6* mutant cells containing these constructs do not exhibit a-factor activity. These results suggest that a-factor precursors containing a C-terminal target sequence for geranyl, geranylation are biosynthetically processed to pheromones which can be exported via STE6 and are also recognized by their receptor on the opposite mating-type cell.

1220

Maturation of Isoprenylated Proteins in *Saccharomyces cerevisiae*.

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In eukaryotic cells, there are a number of proteins and polypeptides whose initial translation product contains a -Cys-Xaa-Xaa-Xaa sequence at the COOH terminus, where -Xaa represents a variety of amino acids. This sequence can target these polypeptides for a series of posttranslational modifications that include sequential lipidation with either a farnesyl or geranylgeranyl isoprene group on the cysteine residue, proteolysis of the three terminal amino acids, and methyl esterification of the newly exposed α-carboxyl group of the cysteine residue. We have been interested in elucidating this pathway in the yeast *Saccharomyces cerevisiae* concentrating our efforts on the study of the methylation and proteolytic reactions. The enzyme involved in the final methylation step has been well characterized. We have shown that a membrane-associated COOH-terminal methyltransferase, the product of the *STE14* gene in *S. cerevisiae*, can catalyze the the methylation of both farnesylated and geranylgeranylated substrates including the RAS proteins and the a-factor mating pheromone. We have also determined that at least one of the small G proteins,YPT1, appears not to be methylated *in vivo* and are currently investigating the methylation state of other G proteins including the *RHO, RSR1*, and *CDC42* gene products. Another as yet unidentified membrane-associated 29kDa protein in *S. cerevisiae* appears to be methylated by the STE14 methyltransferase. In contrast, not much is known about the proteolytic processing enzymology either in yeast or other cell types. Through the development of an indirect coupled assay, we have identified three *in vitro* activities in *Saccharomyces cerevisiae* that can catalyze the proteolytic cleavage of the three COOH-terminal amino acids from the synthetic peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM. One of these is the vacuolar protease carboxypeptidase Y. Using a strain deficient in this enzyme, we find evidence for an additional soluble activity as well as for a membrane-associated activity that are both candidates for the roles in the physiological processing of isoprenylated protein precursors. Further characterization of the soluble activity revealed a novel carboxypeptidase of approximately 110kDa that catalyzes a processive removal of amino acids from the COOH terminus of both the farnesylated and non-farnesylated substrate, but not from three other unrelated peptides. We are presently purifying this activity from the cytosolic fraction.

1221

Essential target proteins of the *CAL1/CDC43* geranylgeranyl-protein transferase in *Saccharomyces cerevisiae*. Y. Ohya[1,2], H. Qadota[1], Y.Anraku[1], M.Tibbetts[3], J.R.Pringle[4], and D.Botstein[2], [1]Department of Biology, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, [2]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, [3]Department of Biology, The University of Michigan, Ann Arbor, MI 48109, [4]Department of Biology, The University of North Carolina, Chapel Hill, NC 27599.

Protein prenylation, including farnesylation (C15) and geranylgeranylation (C20), plays an important role in helping to anchor many proteins in the membrane. Proteins containing a C-terminal Cys-Ali-Ali-Xaa sequence can be modified by farnesyl-protein transferase and geranylgeranyl-protein transferase I (GGPTaseI). The Xaa position defines whether a protein is preferentially farnesylated or geranylgeranylated.

The *CAL1/CDC43* gene product in yeast has been shown to be essential for GGPTaseI activity. *CAL1/CDC43* is essential for growth. Mutations in the *CAL1/CDC43* gene were isolated using two different screens. The temperature sensitive *cal1* allele requires high levels of $CaCl_2$ in the media. The temperature sensitive *cdc43* alleles were isolated as cell division cycle mutants. *cal1-1* allele containing a ^{328}Gly to Ser change caused cell cycle arrest with a small bud and G2/M nucleus, whereas six *cdc43* mutants had missense mutations at 4 different positions; each of these alleles arrested cell cycle with unbudded multinucleate cells. The fact that there exist two classes of the *cal1/cdc43* mutant alleles in terms of terminal phenotype suggests that there are at least two different *CAL1/CDC43* target proteins which are essential for cell cycle progression.

Four yeast proteins contain the consensus sequence for GGPTaseI. Two of these, *CDC42* and *RHO1* are essential proteins. We have shown that simultaneous overproduction of *CDC42* and *RHO1* suppresses *cal1/cdc43* mutations, including the lethality of the null mutant. This implies that overproduction of two known essential targets of *CAL1/CDC43* suffices to suppress the *cal1/cdc43* defects.

1222

The Yeast Cell Adhesion Protein α-Agglutinin is Synthesized with a Glycosylphosphatidyl-Inositol Anchor. C.-F. Lu[1], J. Kurjan[2], and P.N. Lipke[1] Department of Biological Sciences, Hunter College, CUNY, New York, NY 10021; [2]Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, VT 05405 (Spon. by R. Rudner)

Saccharomyces cerevisiae α-agglutinin is a cell adhesion glycoprotein expressed on haploid cells of α mating type. The predicted amino acid sequence of α-agglutinin contains a C-terminal hydrophobic region that is characteristic of signal sequences for the addition of glycosylphosphatidyl-inositol (GPI) membrane anchors of numerous proteins. We therefore investigated whether α-agglutinin has a GPI anchor. Using Triton X-114 hydrophobic/hydrophilic phase separation, we identified a 140 kD membrane protein that reacted with α-agglutinin-specific antisera. This 140 kD membrane protein was a form of α-agglutinin, because 1) like α-agglutinin, this protein was expressed only on α cells and its production was pheromone inducible; 2) transformation of *MATα* cells with a multicopy plasmid containing the α-agglutinin structural gene increased the expression of the 140 kD protein by about 10-fold; and 3) this protein had α-agglutinin activity (specific binding to mating type **a** cells). This form of α-agglutinin was released from the detergent phase into the aqueous phase by the action of a bacterial phosphotidylinositol-specific phospholipase C. This protein could be metabolically labeled with myo-inositol and palmitic acid, indicating a covalent attachment of an inositol-containing phospholipid to this protein. This form of α-agglutinin contained N-linked and O-linked carbohydrate.

Supported by grants from NIGMS.

1223

Expression and Characterization of a Nonmyristylated Mutant Human Erythrocyte Band 4.2. M.A. Risinger, C. Korsgren, and C.M. Cohen, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA 02135 and Departments of Medicine and Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA 02111.

Band 4.2 is an abundant protein of the erythrocyte membrane which binds to band 3 and is apparently essential for normal red blood cell function since deficiencies in the protein are characterized by fragile and abnormally shaped red blood cells, resulting in hemolytic anemias. However, a specific function has not been determined for band 4.2. We have recently demonstrated that band 4.2 is an N-myristylated protein. In order to elucidate the function of the covalenty bound myristate, we used oligonucleotide directed mutagenesis to produce a glycine to alanine (G→A) mutation at the N-terminal myristylation site of band 4.2 and expressed the mutant protein in a Baculovirus expression system. ^{3}H-myristic acid labeling studies confirmed that the mutant protein is not myristylated. Immunofluorescence experiments revealed that nonmutated band 4.2 expressed in Sf9 cells is plasma membrane associated. In contrast, the G→A mutated band 4.2 demonstrated a punctate distribution at multiple focal levels, consistent with retention of the mutated protein within an intracellular compartment. Preliminary subcellular fractionation studies indicated that the nonmyristylated mutant protein may be associated with the rough microsomal fraction. These observations suggest that myristylation may be important for the targetting of band 4.2 to the plasma membrane in Sf9 cells. Both nonmutated band 4.2 and the G→A mutated protein were also expressed in a rabbit reticulocyte lysate system and the ^{35}S-labeled expressed proteins were tested for binding to erythrocyte alkaline stripped inside out vesicles. Comparable amounts of the nonmutated and G→A mutated proteins bound to the membranes and the binding appeared specific in that it was inhibited by excess cold band 4.2. These findings suggest that myristylation may not be required for the binding of band 4.2 to erythrocyte membranes. Supported by NIH grant HL37462.

1224

Cholesterol Regulates Cell Surface Expression of Glycophospholipid-Anchored (GPI-linked) CD14 Antigen. R.D. Bigler, J.L. Alfieri, S. Lund-Katz, and M. Esfahani, Hahnemann University, Philadelphia, PA 19102 and Medical College of PA, Philadelphia, PA 19129

Clustering of the GPI-linked receptors for 5-methyltetrahydrofolate into uncoated pits of MA104 cells is inhibited by sterol binding compounds or by partial depletion of cellular cholesterol. (Rothberg, K.G., Y.-S. Ying, B.A. Kamen and R.G.W. Anderson. 1990. J. Cell Biol. 111:2931-2938). Another GPI-linked surface protein, the CD14 antigen, is expressed on human monocytes and macrophages. Adherent human monocytes were cultured for 4 days in media containing delipidated fetal calf serum (DFCS) which depleted cellular cholesterol by 56%. Immunofluorescence analysis showed a decreased percentage of CD14^{+} cells from 93 ± 1% in FCS to 31 ± 6% in DFCS medium. The decrease in the fraction of cells expressing CD14 was parallel to a decrease in the average number of molecules per cell as indicated by a decrease in the mean value fluorescence intensity from 883 ± 17 to 172 ± 18. Expression of high affinity Fc receptors (FcRI) also was reduced under these conditions. The inhibition of CD14 expression was overcome by addition to the culture medium of cholesterol, low density lipoprotein, or very low density lipoprotein which replenish cellular cholesterol. Expression of FcRI was not restored by cholesterol. Taken together, these observations indicate that cholesterol regulates the lateral diffusion and surface expression of some GPI-linked glycoproteins. (Supported by Merck Research Laboratories, AR-40404, and HL-23633).

Membrane Fusion (1225-1226)

1225

CD4-Induced Activation of HIV-I gp120-gp41 Complex is pH and Temperature Sensitive. Y.-K. Fu, T. K. Hart, and P. J. Bugelski, SmithKline Beecham Pharm., King of Prussia, PA 19406.

Membrane fusion is mediated by the activation of an HIV envelope glycoprotein gp41 (fusogenic peptide) following binding of gp120 to CD4. Recombinant soluble CD4 (sCD4) induces shedding of gp120 from gp41 thereby blocking the fusion potential. BJAB cells stably expressing HIV-I envelope glycoproteins were used to study the interaction of CD4 and gp120-gp41 with regards to shedding of gp120 and fusion events. Both sCD4-induced shedding of gp120 and cellular CD4-mediated fusion with SupT1 cells were sensitive to pH and temperature. sCD4-induced shedding of gp120 was optimal at pH 4.5 to 5.5. Fusion was nonpermissive in that range, but favored at pH 7.5 to 8.5. The acidic pH effect on fusion was irreversible; shifting the pH from 5.0 to 7.5 resulted in slow (>16 h) recovery of fusion capability. At neutral pH, sCD4 did not induce shedding of gp120 at 4°C or 16°C, as it did at 22°C or 37°C. However, fusion occurred at 37°C but did not occur at or below 22°C. Incubation of co-cultures at 4°C, 16°C, or 22°C for 3 h, then shifting to 37°C for 1.5 h, resulted in similar, increased, or decreased numbers of fused cells compared to controls, respectively. These results show that cellular CD4 can inactivate the HIV-I gp120-gp41 fusion complex in a process analogous to sCD4-induced shedding of gp120. Furthermore, the physicochemical dissection of shedding and fusion implies that induced release of gp120 is not an integral step in fusion.

1226

Structure and Function of Glycosylphosphatidyl Inositol-Linked Viral Membrane Fusion Glycoproteins. J.M. Gilbert, G.W. Kemble, C.D. Weiss, L. Hernandez, and J.M. White, Departments. of Biochemistry and Pharmacology, University of California, San Francisco, San Francisco, CA 94143. (Spon. by J.M. White.)

We have generated stable cell lines expressing three GPI-linked viral membrane fusion glycoproteins: GPI-linked influenza hemagglutinin (HAPI), GPI-linked Rous sarcoma virus envelope (RenvPI), and GPI-linked human immunodeficiency virus envelope (HenvPI). Immunofluorescence and cell surface labeling demonstrated transport of each of these GPI anchored chimeras to the cell surface. Biochemical studies suggested that these molecules are properly folded. HAPI and RenvPI migrated similar to their wild type (wt) molecules on sucrose gradients. HAPI crosslinked as a trimer. Both HAPI and HenvPI interacted with reagents specific for the native oligomers.

Each of these GPI anchored glycoproteins was released from the cell surface by digestion with phosphatidyl inositol-phospholipase C (PI-PLC). Chemical crosslinking and sedimentation analysis demonstrated that HAPI and RenvPI remain oligomeric following release. PI-PLC released HAPI underwent the low pH induced conformational changes characteristic of wt-HA. Nonetheless, none of the GPI -anchored molecules mediated membrane fusion as evidenced by lipid mixing (HAPI) and syncytial assays (HenvPI). These results suggest an important and previously unrecognized role for the transmembrane domains in the fusion mechanism of viral glycoproteins.

1227

Fluorescence Dequenching of Octadecylrhodamine labeled Plasma Membrane Vesicles Fusing with Cells Expressing Vesicular Stomatitis Virus Glycoprotein: A New Approach to Measure Fusion Activity of Cloned Viral Envelope Proteins. A. Puri, M. Krumbiegel, D. Dimitrov, L. Prevec@ and R. Blumenthal
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We have studied fusion between fluorescently labeled plasma membrane vesicles (PMV), prepared from Vero cells by hypotonic lysis, with HeLa cells expressing vesicular stomatitis virus glycoprotein (G-protein). The G-protein was expressed on the cell surface either following infection with intact VSV, or with an adenovirus vector (AdG12) containing the gene for the VSV G-protein. The kinetics of fusion was monitored using a lipid mixing assay based on fluorescence dequenching of octadecyl rhodamine (R18). Fusion began immediately after lowering the pH below 6, and showed an approximately exponential time course. The pH dependence of PMV fusion paralleled that observed for VSV-cell fusion and VSV-induced syncytia formation. Fusion was very rapid, dependent on temperature and the density of G protein expressed on the cell surface. Fusion was inhibited in low ionic strength medium. Preincubation of G-protein expressing cells bound to R18PMV with G-protein antiserum resulted in inhibition of fusion. Lipid symmetric human erythrocyte ghosts which serve as targets for intact VSV did not efficiently bind or fuse with G-protein-expressing cells even in the presence of wheat germ agglutinin. The assay opens the possibilty to perform quantitative measurements of the fusion activity of mutant envelope proteins expressed in cells.

1228

Direct Measurement of Herpes Simplex Virus-Cell Fusion Kinetics

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Herpes Simplex Viruses (HSV) enter cells by fusion of the viral envelope with the cell plasma membrane. Among the ten identified envelope glycoproteins, three have essential roles in infectivity: gB, gD and gH. However, precise assignment of their functions in this multi - step process has been elusive. To determine which proteins are specifically involved in the membrane fusion step of viral entry, we have developed a direct assay. In this system HSV-1 envelopes were labeled with self-quenching concentrations of the fluorescence probe octadecylrhodamine. Labeled virus was incubated with mouse L-cells, in suspension, at 4° C to permit binding. After removal of unbound virus the cells were incubated at 37° C to monitor fusion. Increases in fluorescence, indicative of fusion - dependent relief of self - quenching were observed over 150 minutes. Over this time period at least 50% of bound virus had fused. These kinetics are similar to those derived from previous studies using electron microscopy or plaque assays. Fusion neutralization studies employing monoclonal antibodies against each of the relevant envelope glycoproteins are underway.

1229

Staining Methods for Characterization of Plasma Membrane Fusion Mutants in the Fission Yeast *Schizosaccharomyces pombe*.

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We intend to study plasma membrane fusion during conjugation of the fission yeast *S. pombe*. This organism is amenable genetically and biochemically to extensive characterization of the proteins involved. Using a genetic approach we have isolated several mutants which are blocked in a prezygotic stage, phenotypically defined by horse shoe shaped cell pairs. However, this general microscopic phenotype does not reveal whether blockage occurred at the stage of cell wall re-arrangement, of cytoplasmic membrane fusion or of some subsequent step.

To refine our definition of phenotypes, we have used stains and fluorophores which label the cell walls (PKH 26), the plasma membrane (octadecyl-Rhodamine (ODR), the cytoplasm (malachite-green) or the nuclei (DAPI). Visualization of these mutants with epifluorescence and confocal fluorescence microscopy will permit us to distinguish between mutants blocked at the cell-wall re-arrangement stage and those blocked at the membrane fusion stage. The latter will be further characterized.

1230

Intracellular Calcium Release is Necessary but Not Sufficient to Trigger Myoblast Fusion. J. D. David and A. Fitzpatrick, Division of Biological Sciences, University of Missouri, Columbia, MO 65211

The involvement of extracellular Ca^{2+}, prostaglandin E_1 (PGE_1), protein kinase C (PKC) and Ca^{2+} influx in chick skeletal muscle myoblast fusion has been well documented. The present study tests the hypothesis that release of intracellular Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores is an obligatory step in the regulatory signal cascade that leads from PGE_1 receptor occupancy to membrane union. We conclude that release of Ca^{2+} from IP_3-sensitive intracellular stores can trigger, and is necessary to trigger, membrane fusion based in part on the following evidence: (1) thapsigargin, which triggers release of Ca^{2+} from IP_3-sensitive intracellular stores, induces precocious fusion; (2) TMB-8, a drug which inhibits Ca^{2+} release from intracellular stores, inhibits fusion and; (3) thapsigargin attenuates inhibition by TMB-8. In contrast, release of Ca^{2+} from IP_3-insensitive intracellular stores can trigger fusion, but an inhibitor of that release does not block fusion. Ca^{2+} release from the IP_3-sensitive stores is not alone sufficient to trigger fusion since thapsigargin cannot attenuate inhibition by either the PKC inhibitor H-7 or the plasma membrane Ca^{2+} channel blocker D600. We have previously shown that D600 acts downstream of H-7 in the regulatory cascade, therefore the inability of thapsigargin to relieve inhibition by H-7 indicates that intracellular Ca^{2+} release occurs prior to PKC activation. We further conclude that intracellular Ca^{2+} release serves directly to activate PKC based on the following evidence: (1) thapsigargin acts synergistically with the PKC activators TPA and OAG to stimulate fusion; (2) thapsigargin stimulates Ca^{2+} influx through D600-sensitive Ca^{2+} channels with kinetics similar to stimulation by TPA; and (3) the TMB-8 sensitive step lies between PGE_1 receptor occupancy and PKC activation.

1231

Isolated Sea Urchin Cortical Granules can Fuse With Liposomes in Response to a Calcium Trigger. S.S. Vogel, L.V. Chernomordik & I.Zimmerberg
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Biological membrane fusion is mediated by proteins. While viral fusion-proteins have been identified, the identity and distribution of proteins that mediate exocytosis is unknown. Nevertheless, models for the mechanism of exocytosis have been proposed involving either cytoplasmic fusion proteins or pre-formed protein complexes, akin to gap-junctions, formed from hemiproteins in each of the two membranes. In opposition to these models, we present evidence here, using exocytotic granules from sea urchin eggs, that proteins in only one of the two membranes are required for fusion. We show using both light scattering and video microscopy that exocytotic granules treated with either trypsin or N-ethylmaleimide (NEM) can't fuse with other inactivated granules but do fuse with untreated granules. Moreover, we show using fluorescent microscopy that granules fuse with liposomes prepared from lipids extracted from sea urchin egg cortices, and with liposomes containing cholesterol and either DPPC or PI (1:1). Finally, we show that granule-liposome fusion requires no added cytoplasmic proteins, is inhibited by NEM, and is triggered with 59 μM calcium. Thus, our data suggests that calcium triggered exocytosis requires proteins in only one of the fusing membranes.

1232

Inhibition of sperm-egg fusion by peptides containing the TDE sequence in the disintegrin domain of the guinea pig sperm protein PH-30. D.G. Myles[1], L.H. Kimmel[1], C. Blobel[2], C. Turck[3], J. White[2], P. Primakoff[1].
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The PH-30 protein is a heterodimer which was initially identified as a potential sperm-egg fusion molecule by antibody inhibition studies (Primakoff et al, 1987, J Cell Biol 104:141). Determination of the cDNA sequence of PH-30 subunits showed that the α subunit contains a putative fusion peptide and the ß subunit contains an extracellular sequence homologous to the disintegrins, a class of snake venom peptides that bind to integrins (Blobel et al, 1992, Nature 356:248). The active site RGD tripeptide sequence found in most snake disintegrins is replaced by TDE in guinea pig PH-30 ß. To determine if the disintegrin region of PH-30 ß is required for sperm to bind to the egg plasma membrane via a mechanism that leads to membrane fusion, we tested the ability of PH-30 ß peptides containing the sequence TDE to inhibit guinea pig sperm-egg fusion. Fusion was assayed by scoring the appearance of swollen sperm heads in the egg cytoplasm. We found that PH-30 ß TDE containing peptides inhibited fusion in a dose-dependent manner with saturating inhibition of 80-100%. Irrelevant or scrambled control peptides, including GRGDTP, did not inhibit guinea pig sperm-egg fusion. These results are direct evidence that PH-30 functions in sperm-egg fusion and the TDE sequence in the disintegrin region is an active site. We propose the TDE binds to an egg integrin and that this adhesion leads to membrane fusion.

1233

The Pro Region of a Protein Active in Sperm-Egg Fusion Contains a Disintegrin Domain and a Metalloprotease Domain. T.G. Wolfsberg, C.P. Blobel, D.G. Myles, P. Primakoff, and J.M. White, Depts. of Pharmacology and Biochemistry and Biophysics, Univ. of California, San Francisco and Dept. of Physiology, Univ. of Conn. Health Center

PH-30 is a sperm surface protein shown to be involved in sperm-egg fusion (Primakoff et al. 1987. JCB 104:141). Sequence data presented here suggest that the protein also plays a role in sperm development in the testis. PH-30 is composed of two subunits, α and β, which are synthesized as precursors and processed, during sperm development, to yield the mature forms present on fertilization-competent sperm (Blobel et al. 1990. JCB 111:69). The complex of mature α and β resembles many viral fusion proteins in its membrane topology and its predicted binding and fusion functions (see Fig.) (Blobel et al. 1992. Nature 356:248). Furthermore, the mature α and β subunits are similar in amino acid sequence to each other as well as to a family of snake venom proteins which contain integrin-binding "disintegrin" domains. We report here the cDNA and deduced amino acid sequences of the α and β precursors (see Fig.). The pro-α region contains, from amino- to carboxy-terminus, (1) a domain of unknown function similar in sequence to the precursor domains of some snake venom disintegrins, (2) a metalloprotease domain similar to snake venom metalloproteases, and (3) a disintegrin domain. The pro-β subunit also contains the domain of unknown function and the metalloprotease domain; the mature β subunit contains a disintegrin domain. The putative metalloprotease catalytic residues are present in the α, but not the β, pro-region. We speculate that the metalloprotease and disintegrin domains of the PH-30 pro-α subunit are active in sperm development in the testis. Thus, PH-30 α is potentially a multifunctional protein that may enact metalloprotease, cell-cell adhesion, and membrane fusion functions. In addition, PH-30 α and β, as well as some snake venom proteins, likely stem from a common ancestor.

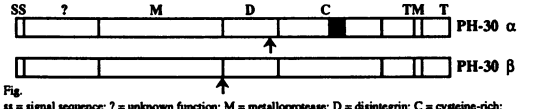

Fig.
ss = signal sequence; ? = unknown function; M = metalloprotease; D = disintegrin; C = cysteine-rich; TM = transmembrane; T = cytoplasmic tail ■ = fusion peptide ↑ = amino terminus of mature protein

1234

Microinjection of fodrin α10 peptides (SH3 homology region) and anti-fodrin antibodies induce migration and fusion of cultured epithelial MDBK cells. S.M. Eskelinen, J. Meriläinen, and V.-P. Lehto, Biocenter and Department of Pathology, University of Oulu, Kajaanintie 52 D, SF-90220 Oulu, Finland.

Fodrin is the main component of the membrane skeleton. It contains in its α-subunit the SH3 (src homology region 3) motif (α10 domain) which is characteristic of e.g. nonreceptor-type tyrosine kinases and phospholipase C. We have shown that manipulation of signal transduction pathway by PMA, TFP and pH leads to mobilization of the fodrin - actin network. This made us assume that fodrin may participate in signal transduction or actively respond to extracellular signals. We chose microinjection as a tool to manipulate the membrane skeleton in order to elucidate its function in cultured cells. We prepared a bacterially expressed fusion protein encompassing the α10 domain of fodrin linked to glutathione-S-transferase (α10-GST peptide). Microinjection of this peptide into cultured epithelial Madin-Darby bovine kidney (MDBK) cells led to their migration and fusion, resulting in heterokaryons with up to 6 - 8 nuclei. Fusion began one hour after injection and continued for four to five hours leading to an average fusion index of 14 %. Some of the fused cells were stable for at least 20 hours. Injection of antibodies against the α10-GST peptide or entire α-chain of fodrin induced rapid fusion (< one hour) of MDBK cells, but the fused cells were unstable and died within 4 - 5 hours. Fusion process was not dependent on intracellular calcium, since injection of EGTA did not prevent the fusion process. This is the first direct evidence that the membrane skeleton participates in the fusion process of nucleated mammalian cells. The results suggest that there is no absolute requirement for newly synthetized fusogenic proteins, but fusion can be induced by a rearrangement of preexisting membrane components.

1235

Characterization of Sequential Intermediates during Endosome Fusion. James M. Lenhard and Philip D. Stahl, Washington University School of Medicine, St. Louis, MO 63110

Studies with cell-free systems have allowed for identification of intermediates that form during vesicle-mediated protein transport. We have used an in vitro assay to characterize sequential events during early endosome fusion. GTPγS and 50 mM KCl were required for formation of a dilution-resistant intermediate in low and high concentrations of cytosol, respectively. KCl was also required for fusion of this intermediate. Formation of the dilution-resistant intermediate was blocked by addition of ARF plus GTPγS while fusion of the intermediate required NSF. Several lines of evidence suggest the sequential requirement for heterotrimeric and small GTP-binding proteins (smgs) before fusion. (I) Aluminum fluoride, an activator of heterotrimeric G proteins but not smgs, antagonized the effects of GTPγS on fusion. (II) Mastoparan, a peptide that activates nucleotide exchange on heterotrimeric G proteins, and a peptide from the NH_2-terminal domain of ARF1 reversed inhibition of fusion by GTPγS but not aluminum fluoride. (III) Preincubation experiments showed that endosomes formed aluminum fluoride (ie. heterotrimeric G protein)- and GTPγS (ie. smgs and heterotrimeric)-resistant intermediates with different kinetics. Taken together, the results indicate a cascade of distinct biochemical events occurs that are required for transport between early endosomes.

1236

In Vitro Reconstitution of a Late, Microtubule-Dependent Endocytic Fusion Event in Non-Polarized Cells. F. Aniento, N. Emans and J. Gruenberg. Cell Biology program. European Molecular Biology Laboratory. Heidelberg. Germany

We have investigated two aspects of membrane traffic in endocytosis: membrane fusion and microtubule-dependent transport. Previous studies have established that early endosomes (EE) exhibit a striking tendency to undergo fusion in vitro. However, it has been difficult until now to reconstitute fusion events between endosomes that may occur at later stages of the pathway. In vivo, markers destined to lysosomes appear sequentially in EE, late endosomes (LE) and then lysosomes. Previous studies of the group have suggested that the passage between EE and LE is microtubule-dependent and is mediated by multivesicular endosomes, which we termed endosomal carrier vesicles (ECV). Here we report a cell-free assay that measures the occurrence of fusion between ECV and LE. Fusion was measured by the formation of a product between two substrates (avidin & biotinHRP), which had been selectively internalized in two cell populations. We show that this in vitro process is cytosol and ATP-dependent and sensitive to low concentrations of GTP-γ-S. Electron microscopic studies using two electron-dense tracers established that the vesicular partners of the fusion reaction were ECV and LE, respectively and that both tracers colocalized in LE at the end of the reaction. In vitro fusion is stimulated several fold by the presence of taxol-stabilized microtubules (MT) and depends on microtubule-associated proteins, including cytoplasmic dynein. Direct binding of ECV with MT could be observed at the EM level. We are now using this assay to investigate the mechanisms that control that late stage of endocytic membrane traffic.

1237

Putative Lysosome-Fusion-Blocking Membrane Components. K.J. Kim, Y.E. Na, and K.W. Jeon, Department of Zoology, University of Tennessee, Knoxville, TN 37996.

Some intracellular vesicles such as symbiosomes containing endosymbionts avoid fusing with lysosomes, suggesting the possible presence of a fusion-blocking component(s) on their membranes. In the course of searching for such a component in the symbiont-bearing xD strain of Amoeba proteus, we found a 96-kDa protein and lipopolysaccharides (LPS) unique to symbiosome membranes. In an attempt to characterize these components further and to learn of their possible fusion-blocking activities, we obtained monoclonal antibodies (mAbs) and used them to study the origin of the molecules, their distribution on intracellular membranes, and the time course of their appearance in newly infected amoebae. We found that these molecules were produced by symbiotic bacteria and inserted/attached to the symbiosome membrane, which was apparently an amoeba membrane. When symbiont-free amoebae (D strain) were newly infected with bacteria isolated from xD amoebae, they digested most of the infecting bacteria and only a small portion survived. The vesicle membranes that enclosed surviving bacteria were initially devoid of 96-kDa protein or LPS, but they acquired the 2 types of molecules within 7 days as detected by indirect immunofluorescence. The intensity of fluorescence increased for up to 2 weeks after infection, when the labeling pattern was the same as in established xD amoebae. When we injected an anti-LPS mAb into symbiont-bearing amoebae to mask LPS, some of the symbiosomes that usually do not fuse with lysosomes fused, as determined by using a mAb specific to an amoeba lysosomal-membrane protein as a probe. Thus, it appeared that LPS was actively involved in preventing the lysosomal fusion with symbiosomes. [The work was supported by a grant from the National Science Foundation.]

1238

Effects of GTP on Lipid Metabolism and Membrane Structure in Derivatives of Rough Endoplasmic Reticulum from Rat Liver. J. Paiement, C. Lavoie*, M. Jolicoeur*, L. Roy*, A. Guénette*, Département d'anatomie, Université de Montréal, Montréal, H3C 3J7.

The accumulation of polyunsaturated free fatty acids (PUFAs) occurs coincident with GTP-dependent membrane fusion (Lavoie et al., B.B.A.1070:274,1991). We now report that cytidine nucleotides (5'-CMP>5'-CDP>5'-CTP) have a potentiating effect on PUFA accumulation and GTP-dependent membrane fusion and lead to the formation of multilamellar membrane structures (MMS). The analogues 3'-CMP, 2'-CMP, 5'-GMP and 5'-AMP are ineffective. In contrast, in the presence of 5'-GTP and 5'-CMP (500 μM) 10 μM coenzyme A, (CoA), plus 2 mM ATP cause a reduction in the accumulation of PUFAs as well as an inhibition in membrane fusion. Coenzyme A and ATP also stimulate the synthesis of triglycerides and the appearance of lipid droplets amongst the rough microsomes. Incubation of microsomes containing MMS in the presence of increasing amounts of CoA (1-5 μM) provokes the transformation of the MMS into lipid droplets. The use of an EM peroxidase cytochemical technique based on the generation of H_2O_2 using acyl-CoA oxidase indicated the accumulation of free fatty acids in the MMS. Thus GTP may permit the formation of membrane lipid microdomains which can lead to dramatic changes in membrane structure and influence membrane fusion. Supported by MRC Canada.

1239

Low molecular weight GTP-binding proteins in purified derivatives of rough endoplasmic reticulum membranes from rat liver and rat hepatocellular carcinomas. J. Lanoix and J. Paiement, Département d'anatomie, Université de Montréal, Montréal, Canada, H3C 3J7.

We have studied GTP-binding proteins in membrane preparations enriched in derivatives of rough endoplasmic reticulum (RER) and determined the effects of a variety of post-translational modifications of GTP-binding proteins on GTP-dependent membrane fusion. Analysis of binding of [α-^{32}P]GTP to proteins on nitrocellulose blots after separation by two-dimensional gel electrophoresis has revealed the presence of as many as 15 such proteins in the fraction containing rough microsomes (RM) (Lanoix, J. et al., Biochem. J. 262 : 497-503, 1989). These proteins were assessed for ras antigenicity with anti-ras monoclonal antibodies using both immunochemistry and immunocytochemistry. By immunochemistry, antibodies were observed to precipitate a 23.5 kDa [α-^{32}P]GTP-binding protein. Electron microscopic immunocytochemistry revealed ras antigenicity associated with smooth membrane contaminants of the RM. We carried out a variety of post-translational modifications including phosphorylation and ADP-ribosylation. A number of low molecular weight GTP-binding proteins were observed modified. However, quantitation of membrane fusion indicated that these post-translational modifications did not influence GTP-dependent fusion of rough microsomal membranes. We also studied RM isolated from rat hepatocellular carcinomas. These microsomes exhibited a greater capacity for GTP-dependent membrane fusion when compared with controls and biochemical studies revealed differences in the content and the isoelectric charge properties of associated low molecular weight GTP-binding proteins. The changes observed could, in part, account for differences observed in GTP-dependent membrane fusion. Supported by MRC Canada and CRSI Quebec.

Cell-to-Cell Interactions I (1240-1243)

1240

Calcium-Dependent Cell-Cell and Cell-Substratum Adhesion Mechanisms are Not Required for Expression of Differentiated Properties by LLC-PK$_1$ Cells. K. Amsler, J.-L. Chen, and L. Gatti. Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854 (Spon. by D.P. Beck).

Development of the mature, polarized epithelial cell requires calcium-dependent cell-cell contact events, including those mediated by E-cadherin (uvomorulin). The role of calcium-dependent cell-cell adhesion events in expression of apical membrane differentiated properties has not been examined. The pig renal epithelial cell line, LLC-PK$_1$, acquires progressively many properties characteristic of the mature renal proximal tubular cell, including Na-glucose symport activity, gamma-glutamyl transpeptidase activity, and villin protein, upon attaining a confluent density. Expression of these properties was compared in populations maintained in normal calcium medium supplemented with dialyzed serum and in calcium-free medium supplemented with dialyzed serum. Normal calcium cells exhibited a typical epithelial morphology and normal cell-cell interactions. In contrast, low calcium cells were elongated and adhered to one another very poorly. This demonstrated that the calcium-mediated cell-cell contacts had been disrupted. Despite these differences, expression of all three differentiated parameters was identical in the two cell populations. These results demonstrate that expression of differentiated properties by the LLC-PK$_1$ cell is not dependent on calcium-dependent cell-cell contacts, including those mediated by E-cadherin/uvomorulin, and thus does not require a completely polarized epithelial cell.

1241

Disruption of E-cadherin activity perturbs calcium-induced reorganization of the cytoskeleton in cultured human epidermal keratinocytes. J. E. Lewis*, P. J. Jensen§ and M. J. Wheelock.* *Department of Biology, University of Toledo, Toledo, OH 43606 and §Department of Dermatology, University of Pennsylvania, Philadelphia, PA 19104.

Human keratinocytes grown in 30 μM calcium display minimal cell-cell interactions. However, elevation of the calcium concentration to 1 mM induces the formation of adherens junctions and desmosomes. Formation of these intercellular junctions is accompanied by reorganization of actin and keratin filaments, which insert into the adherens junctions and desmosomes respectively. Function blocking antibodies to E-cadherin, an adhesion molecule localized to adherens junctions, delay the calcium induced formation of both adherens junctions and desmosomes. In the present study, we show that anti-E-cadherin antibody also delays the calcium-induced reorganization of the cytoskeletal elements, including not only actin and keratin filaments, but also microtubules. These studies indicate that E-cadherin function and intercellular junction formation are required for the cytoskeletal reorganization that occurs upon calcium elevation in human keratinocyte culture. (Supported by NIH CA44464 and AR39674)

1242

Regulation of E-Cadherin Binding Activity by Muscarinic Acetylcholine Receptors in Small Cell Lung Carcinoma. C.L. Williams and A.M. Hummel. Departments of Immunology, and Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN 55905.

We previously reported that cell-cell adhesion rapidly increases upon activation of M_3 muscarinic acetylcholine receptors (mAChR) in the SCC-9 small cell lung carcinoma cell line (*J. Cell Biol.* 115:71a, 1991). This response may be due to protein kinase C activation, since phorbol 12-myristate 13-acetate (PMA) similarly increases cell-cell adhesion of SCC-9 cells. We have identified E-cadherin as the molecule mediating increased adhesion, since antibodies to E-cadherin completely inhibit cell-cell adhesion induced by mAChR agonists or PMA. Immunoprecipitation of ^{35}S-methionine-labelled SCC-9 cell lysates with E-cadherin antibodies yields a protein with the MW of E-cadherin (140 kDa), as well as other proteins (109 kDa and 105 kDa) which may correspond to catenins. The amount and type of proteins that are immunoprecipitated are not altered by treating the cells with mAChR agonists or PMA. Using immunofluorescent techniques, we do not detect any changes in cell surface expression or localization of E-cadherin in response to PMA or mAChR agonists. These results indicate that increased binding activity of E-cadherin, rather than increased expression, causes cell-cell adhesion upon exposure of SCC-9 cells to PMA or mAChR agonists. These findings provide the first evidence that the binding activity of E-cadherin can be regulated by a cell surface receptor. Additionally, this is the first report that a neurotransmitter receptor can increase binding activity of an adhesion molecule. We are currently investigating the effects of mAChR agonists and PMA on the phosphorylation state of E-cadherin and associated proteins to determine the role of phosphorylation in E-cadherin binding activity. (Supported by NIH grant CA52471 to CLW).

1243

Characterization of Transfected E-cadherin in Human Breast Cancer Cell Lines. C.L. Sommers, E.P. Gelmann and S.W. Byers, Department of Anatomy and Cell Biology and the Lombardi Cancer Research Center, Georgetown University, Washington, D.C. 20007.

We and others have described loss of expression of E-cadherin in some human breast carcinoma cell lines (bccl's). All bccl's that were highly invasive when tested in modified Boyden chamber or Matrigel outgrowth assays had complete loss of E-cadherin expression. These cells also have a fibroblastoid phenotype, express vimentin and have down regulated keratins. To examine the effect of E-cadherin expression on invasiveness in these bccl's, we transfected the mouse E-cadherin cDNA into the invasive bccl's HS578T and BT549. For comparison we also transfected weakly invasive, cadherin-positive MCF-7 breast cancer cells and cadherin-negative mouse L929 fibroblasts. E-cadherin protein was detected in the transfected clones by immunofluorescence microscopy and immunoprecipitation. E-cadherin-transfected invasive cells did not assume a more epithelioid morphology and still exhibited an invasive phenotype in Matrigel. Triton treatment of transfected HS578T and BT549 cells prior to immunofluorescence microscopy resulted in loss of E-cadherin immunostaining. In contrast, Triton-insoluble E-cadherin was present at cell-cell borders in the transfected MCF-7 and L929 cells. These results indicate that in the invasive bccl's HS578T and BT549, the transfected E-cadherin is not appropriately linked to the actin cytoskeleton.

1244

Spatiotemporal Dissection of Early Molecular Events During Cadherin-Mediated Epithelial Cell Adhesion. Helen McNeill, Timothy A. Ryan, and W. J. Nelson. Department of Molecular and Cellular Physiology, Standford University School of Medicine, Stanford, CA 94305

Using high resolution differential interference contrast microscopy we have viewed MDCK epithelial cells as they initiate cell-cell contacts. Cells were processed for indirect immunofluorescence to determine the distribution of adhesion molecules and membrane cytoskeletal components at these early contacts. Using retrospective histochemical analysis, we correlated the stages in the formation of a cell contact with the distribution of E-cadherin, its association with the cytoskeleton, and the distribution of the cytoskeletal proteins actin and fodrin. Our data show that MDCK cells initially go through a stage during which cell contacts are often insubstantial and transient. Upon the formation of a stable contact, there is no significant change in the amount or in the organization of E-cadherin at the contact for ~ 10 minutes. After this lag period, there is a dramatic reorganization of the cadherin specifically at the contact point, indicated by a rapid acquisition of Triton insolubility, however the net amount of E-cadherin at the contact remains unchanged during this time. Only at much later time points (~ 1hr) is there an increase in the concentration of this adhesion molecule at the contact point. The distribution of the cytoskeletal proteins actin and fodrin was examined at these times.

1245

Expression in L-Cells of the Cell Surface N-Acetylgalactos-Aminylphosphotransferase which Modulates Cadherin Mediated Cell Adhesion. F. Evraert, J. Balsamo, and J. Lilien, Department of Biological Sciences, Clemson University, Clemson, South Carolina, 29634-1903

A cell surface N-Acetylgalactosaminylphosphotransferase (GalNAcPTase) has been shown to be associated with the cell adhesion molecule N-cadherin in embryonic chick neural retina and rat pancreatic islet cells. Anti-GalNAcPTase antibodies inhibit homophilic N-cadherin mediated adhesion and neurite outgrowth. We have isolated cDNA's that encode the GalNAcPtase. L-cells transfected with a single cDNA clone express protein that cross-reacts with anti-GalNAcPTase antibodies, transfers GalNAc from UDP GalNAc to an exogenous acceptor and localizes to the cell surface. The GalNAcPTase shares very high sequence similarity with chicken brain spectrin. We conclude that a close relative of spectrin is present at the outer cell surface, exhibits glycosyl transferase activity and modulates cell adhesion.

1246

Preparation and Characterization of Hybridomas Secreting Monoclonal Antibodies Directed Against the Cell Adhesion Recognition Sequence of Cadherins. O.W. Blaschuk, Division of Urology, Department of Surgery, McGill University, Montreal, Quebec, H3A 1A1.

The cadherins are a family of calcium-dependent cell adhesion molecules (CAMs). The molecular mechanisms by which these CAMs promote cell adhesion remain poorly understood. We have previously demonstrated that the tripeptide HAV is a component of a cadherin cell adhesion recognition (CAR) sequence (Blaschuk *et al.*, Dev. Biol. 139, 227). Synthetic peptides containing the HAV sequence are capable of inhibiting various cadherin-mediated processes, such as rat neurite outgrowth on astrocytes. In order to analyze the properties of the cadherin CAR sequence in greater detail, we have prepared a panel of mouse hybridomas that secrete monoclonal antibodies (Mabs) directed against the mouse N-cadherin (N-cad) CAR sequence. Four hybridomas (designated Hyb3, Hyb6, Hyb14 and HybW20, respectively) have been characterized. They all secrete Mabs of the IgM subclass. These Mabs bind to the synthetic peptide FHLRAHAVDINGNQV-amide, which was used as the immunogen. This peptide harbors the mouse N-cad CAR sequence. Three of the four hybridomas (Hyb3, Hyb6 and HybW20) also bind to the peptide LRAHAVDVNG-amide. The amino acid sequence of this peptide encompasses the chicken N-cad CAR sequence. Finally, two of the four hybridomas (Hyb3 and HybW20) secrete Mabs that bind to the synthetic peptide LDRERIATYTLFSHAVSSNG-amide, which harbors the human E-cadherin CAR sequence. Studies are now in progress to determine the ability of the Mabs to disrupt cadherin-mediated processes.

(Supported by the Medical Research Council of Canada)

1247

Two distinct but related proteins, β catenin and plakoglobin, are associated with E-cadherin in Madin-Darby canine kidney cells. Peter A. Piepenhagen and W. James Nelson.
Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305.

The cadherins represent a family of calcium dependent cell adhesion molecules. The catenins are a set of proteins found constitutively associated with the cadherin cytoplasmic domain. Their designations are α(102Kd), β(92Kd), and γcatenin(82Kd). Although their functions are unknown, the catenins appear to be important for cadherin function. A desmosomal protein, plakoglobin, has been shown to bear sequence similarity to β catenin, but the relationship between β catenin and plakoglobin was unclear from previous reports. In this study, we have used metabolic labeling, immunoprecipitation, western blotting, two dimensional gel electrophoresis, and immunofluorescence techniques to characterize the relationships among plakoglobin, β catenin, γ catenin, and E-cadherin in Madin-Darby canine kidney(MDCK) cells. Our results show that plakoglobin, β catenin, and γ catenin are all distinct proteins based upon their having different apparent molecular weights, detergent extractabilities, and isoelectric points. Despite these differences, all of them associate with E-cadherin as revealed by coimmunoprecipitation and western blotting. Plakoglobin association with E-cadherin is consistent with our observation that plakoblobin and E-cadherin immunostaining accumulate at lateral cell borders in a detergent insoluble form with the same kinetics. Our results also demonstrate that there are multiple isoelectric variants of plakoglobin present within MDCK cells but that only a subset of these associate with E-cadherin. These data more clearly define the composition of the cadherin associated proteins and suggest a mechanism for regulating the association of plakoglobin with E-cadherin.

1248

Plakoglobin, or an 83 kD homologue distinct from beta-catenin, interacts with E- and N-cadherin. K.A. Knudsen+ and M.J. Wheelock++, +The Lankenau Medical Research Center, Wynnewood, PA 19096 and ++Department of Biology, University of Toledo, Toledo, OH 43606.

E- and N-cadherin are members of a family of calcium-dependent, cell surface glycoproteins involved in cell-cell adhesion. Extracellularly, the transmembrane cadherins self-associate, while, intracellularly, they interact with the actin cytoskeleton. Several intracellular proteins, collectively termed catenins, co-immunoprecipitate with E- and N-cadherin and are thought to link the cadherins to the cytoskeleton. Two catenins have been identified: 102 kD vinculin-like alpha-catenin and 92 kD armadillo/plakoglobin-like beta-catenin. We observe that plakoglobin (or 83 kD plakoglobin-like protein) also co-immunoprecipitates and co-localizes with E- and N-cadherin. The 83 kD catenin is immunologically distinct from the 92 kD catenin and, because of its molecular weight, likely represents gamma-catenin. Thus, two different members of a plakoglobin family associate with N- and E-cadherin and, together with 102 kD alpha-catenin, appear to participate in linking these cadherins to the actin-based cytoskeleton.

1249

Peritubular Myoid Cells Modulate the Secretion of α2-Macroglobulin and Clusterin by Sertoli Cells. Ismail H. Zwain[1], Josephine Grima[1], Guido Verhoeven[2], C. Wayne Bardin[1], and C. Yan Cheng[1]. [1]The Population Council, 1230 York Avenue, New York, NY 10021; and [2]Laboratory for Experimental Medicine and Endocrinology, Onderwijs en Navorsing, Catholic University of Leuven, B-3000 Leuven, Belgium. (Spon. by A. Moo-Young)

Sertoli cells (SC) are the major secretory component in the seminiferous epithelium whose function is regulated predominantly by follicle-stimulating hormone (FSH) and testosterone (T). α2-Macroglobulin (MG) and clusterin (CL) are two putative SC secretory proteins that were purified from SC-enriched culture media. More recent studies from this laboratory have shown that the testicular MG, unlike its hepatic homologue, is not an acute-phase protein since its levels in the testis did not increase during acute-inflammation whereas the serum MG level increased by more than a 100-fold. Since the modulator that affects their secretion by SC is unknown, we sought to determine if FSH, testosterone, and other biomolecules as well as peritubular myoid cells (PMC) would affect their secretion. It was noted that SC cultured *in vitro* secreted increasing amounts of MG and CL as a function of time. FSH (0-1000 ng/ml) and testosterone ($0-10^{-5}$ M) had no apparent effect on the secretion of MG and CL by SC. Addition of interleukin-6 to SC in doses known to stimulate MG secretion by hepatocytes did not affect its secretion. Dexamethasone stimulated the MG secretion by SC dose-dependently but the addition of interleukin-6 had no synergistic effect on the dexamethasone stimulation of MG secretion. These findings coupled with the fact that the testicular MG is not an acute-phase protein suggest that the synthesis and/or secretion of MG by SC is regulated by a mechanism distinct from that of the liver. PMC cultured alone did not produce any measurable amount of either MG or CL; however, co-cultures of PMC with SC induced a significant increase in the MG and CL secretion by SC. Addition of T to these co-culture did not affect the levels MG and CL suggesting that the known PMC modulator of SC functions, P-Mod-S, is not involved in the secretion of MG and CL by SC. In summary, FSH and T at doses known to affect SC secretory function did not affect the secretion of MG and CL; however, PMC modulate the secretion of both MG and CL by SC via a T-independent factor.

1250

Inhibitin, A Novel Biological Factor Secreted By the Rat Seminiferous Tubules that Inhibits Leydig Cell Steroidogenesis. Ismail H. Zwain and C. Yan Cheng. The Population Council, 1230 York Ave. New York, NY 10021.

Seminiferous tubules (ST) secrete multiple biological factors that affect Leydig cell (LC) steroidogenic functions. Interleukin-1 (IL-1), a potent inhibitor of LC function, has been detected in the culture media of rat ST. These findings suggested that IL-1 plays an important role in the LC function. We sought to identify, purify, and elucidate the mechanism of action of a novel paracrine factor secreted by ST that affects the LC function. Using Sephadex G-100 gel filtration chromatography, it was noted that ST culture medium contained three distinctive peaks of LC inhibitory activity designated Peaks I, II, and III. The inhibitory activity under Peak III was subsequently purified to apparent homogeneity using five reversed-phase HPLC steps and was designated inhibitin. Inhibitin inhibited the basal testosterone (T) and hCG-stimulated T and cAMP production by Percoll purified LC. It also inhibited the forskolin (10^{-5} M; FK)- and cholera toxin (5 μg/ml; CT)-stimulated T and cAMP productions but had no apparent effect to compete the binding of [^{125}I]hCG to the LH receptors indicating that it exerts its inhibitory action at steps beyond the LH receptors but before the cAMP formation. IL-1β, like inhibitin, inhibited hCG-, FK-, and CT-stimulated T and cAMP production by LC; however, IL-1β, unlike inhibitin, stimulated the basal T production and inhibited the binding of [^{125}I]hCG to the LH receptors; whereas, IL-1α caused a significant increase in the basal T production but had no apparent inhibitory action on the hCG-stimulated testosterone and cAMP production by LC. Inhibitin also inhibited the 8-bromo-cAMP-stimulated T production, and the conversion of exogenously added 22R-hydroxy-cholesterol, pregnenolone, progesterone, or 17α-hydroxyprogesterone to T and had no effect on the conversion of androstenedione or dehydroepiandro-stenedione to T indicating that this factor exerts its effect on cytochrome $P450_{scc}$ and cytochrome P450 17α-hydroylase/17,20-lyase but not 3β-HSD and 17β-HSD. In summary, ST produces a unique biological factor that modulates LC function possibly mediated through the adenylate cyclase and steroidogenic enzymes. This factor is distinct from other known LC inhibitors such as IL-1, TGF-β, and activin.

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Expression of Neural Cell Adhesion Molecule and Retinoic Acid Receptor in Normal Brain and in Glial and Non-Glial Brain Tumors. D.R. Conway, B.K. Kleinschmidt-DeMasters, W.A. Franklin, M.P. Gaub*, C. Rochette-Egly*, C.A. Kruse. Depts. of Surgery and Pathology, Univ. of Colorado Health Sci. Ctr., Denver, CO 80262 and *INSERM, 67085 Strasbourg, France.

The expression of neural cell adhesion molecule (NCAM) and of retinoic acid receptor alpha (RARα) was assessed by immunohistochemistry in normal brain and in glial and nonglial brain neoplasms. Monoclonal antibodies NKH-1 and 9α(F), which react with NCAM and RARα respectively, were used in a sensitive alkaline phosphatase-antialkaline phosphatase technique. Both NCAM and RARα were strongly expressed by normal and neoplastic brain tissue. Anti-NCAM monoclonal antibody stained normal neuropil and frequently stained the cell membranes of glial tumors including glioblastoma multiforme, anaplastic astrocytoma and oligodendroglioma. Several glioma cell lines, derived from primary tumors which stained intensely for NCAM, had negligible NCAM expression. The nonglial neoplasms, pituitary adenoma, meningioma and metastatic melanoma were also labeled with anti-NCAM but metastatic adenocarcinoma and sarcoma were not. Anti-RARα labeled the nuclei of nonglial tissues with variable intensity but strongly labeled the cytoplasmic processes of astrocytic cells and tumors. The coexpression of NCAM and RARα and the unique pattern of RARα expression in the cytoplasm of astrocytic cells and tumors may have biological significance as well as diagnostic utility.

1252

Substrate bound GP130/F11 stimulates neurite outgrowth from DRG neurons. D.J. Moss, G.A. Clarke & C.A. White Department of Human Anatomy and Cell Biology, Liverpool University, P.O. Box 147, Liverpool L69 3BX, U.K.

GP130/F11 is a neuronal cell adhesion molecule belonging to the Ig superfamily. Its role during the development of the chick nervous system remains to be established. Although GP130/F11 is linked to the membrane by a glycosylphosphatidyl inositol group, our evidence suggests that GP130/F11 is exclusively located on the neuronal cell surface and is not, under normal conditions, released into the extracellular environment. It has been isolated under mild conditions by PI-PLC release and chromatography on HPLC. GP130/F11 bound to nitrocellulose stimulates neurite outgrowth from DRG neurons maximally at 270ng cm^{-2} compared to laminin at ~ 35ng cm^{-2}. Denaturing GP130/F11 reduces this ability which is not entirely abolished until the reduction of the disulphide bonds. Preliminary results suggest that neurons not expressing GP130/F11 will also adher and extend neurites on this molecule. This privides evidence that GP130/F11 can be a heterotypic cell adhesion molecule.

1253

Structural Characteristics of a Homophilic Binding Site in the Neural Cell Adhesion Molecule NCAM. Y. Rao, X. F. Wu, P. Yip, J. Gariepy*, and C.-H. Siu. Banting and Best Department of Medical Research and *Department of Biophysics, University of Toronto, Toronto, Ontario, M5G 1L6, Canada.

We have identified a homophilic binding site in the third immunoglobulin-like domain of the neural adhesion molecule NCAM. This site is located at a region corresponding to the C' strand of this domain. Further analysis using peptide analogues of different sizes in competition studies indicated that the decapeptide sequence KYSFNYDGSE is the shortest peptide sequence that still retained biological activity. Studies using peptide analogues with amino acid substitutions suggested a role for the charged residues Lys-243 and Asp-249. Substitution of the aromatic residues with Ala also rendered the peptide inactive. The role of this decapeptide sequence in NCAM homophilic binding was further confirmed by mutational analysis. Deletion of the decapeptide sequence or randomization of the first five amino acid residues of the decapeptide sequence totally abolished the binding activity of NCAM. Site-directed mutagenesis was also employed to alter individual amino acids. The homophilic binding activity of mutant NCAMs transiently expressed in COS-1 cells was assayed. Results suggest that hydrophobic interactions contributed by the three aromatic residues, Tyr-244, Phe-246 and Tyr-248, may play an important role in NCAM-NCAM binding and that the interaction may also be dependent on the b-structure backbone.

(Supported by MRC of Canada.)

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Identification of a Peptide Sequence Involved in Homophilic Binding in the Neural Cell Adhesion Molecule NCAM. Y. Rao, X. F. Wu, J. Gariepy*, Urs Rutishauser†, and C.-H. Siu. Banting and Best Department of Medical Research and *Department of Biophysics, University of Toronto, Toronto, Ontario, Canada; and †Department of Genetics, Case Western Reserve University, Cleveland, Ohio.

The neural cell adhesion molecule NCAM is capable of mediating cell-cell adhesion via homophilic interactions. In this study, three strategies have been combined to identify regions of NCAM that participate directly in NCAM-NCAM binding: analysis of domain deletion mutations, mapping of epitopes of monoclonal antibodies, and use of synthetic peptides to inhibit NCAM activity. Studies on L cells transfected with NCAM mutant cDNAs using cell aggregation and NCAM-Covasphere binding assays indicate that the third immunoglobulin-like domain is involved in homophilic binding. The epitopes of four monoclonal antibodies which have been previously shown to affect cell-cell adhesion mediated by NCAM were also mapped to domain 3. Overlapping hexapeptides were synthesized on plastic pins and assayed for binding with these monoclonal antibodies. One of them (PP) reacted specifically with the sequence KYSFNY. Synthetic oligopeptides containing the PP-epitope were potent and specific inhibitors of NCAM binding activity. A substratum with immobilized peptide-conjugates also exhibited adhesiveness for retinal cells. Cell attachment was specifically inhibited by peptides that contained the PP-epitope and by anti-NCAM univalent antibodies. Two active peptides were identified and they both contained the sequence KYSFNYDGSE, suggesting that this site is directly involved in NCAM homophilic interaction.

1255

Altered Distribution of Bile Canalicular Antigens in Chick Liver Aggregates Treated With Anti L-CAM Fab' Fragment and Whole IgG T.L. Terry and W.J. Gallin, Department of Zoology, University of Alberta, Edmonton, Alberta.

Monolayer cultures of embryonic chicken hepatocytes do not form structurally discernible bile canaliculi (BC), and only express bile canaliculus-specific antigens for 1 to 3 days in culture. In contrast, aggregates of hepatocytes formed in liquid rotation culture form BC within 24 hours of initiation of the culture and express canaliculus-specific antigens for at least 7 days. Cell shape and the extent of cell-cell contact differ in monolayer and aggregate cultured hepatocytes and one or both of these differences may be responsible for the observed differences in BC formation and antigen expression. We reasoned that it is possible to differentiate between effects caused by cell shape and cell-cell contact by blocking cell-cell adhesion without disrupting the overall structure of an aggregate of hepatocytes. To test this idea, we perturbed normal cell-cell adhesion in preformed aggregates without dissociating them, using anti-liver cell adhesion molecule (anti-L-CAM) Fab' fragments and whole IgG. Treatment of aggregates of 14-day hepatocytes for 1 to 7 days with anti-L-CAM Fab' fragment or whole IgG led to disruption of normal hepatocyte polarity. Specifically, treatment with the anti L-CAM antibodies led to disassociation of the BC and dispersion BC specific antigens over the hepatocyte cell surface, but not to a decrease in expression of these antigens. These results suggest that cell-cell contact mediated by L-CAM is necessary for the formation of BC, and that cell shape may play an important role in the maintenance of bile canalicular antigen expression.

1256

ZO-1 isoform distribution reveals two classes of tight junctions. M.S. Balda and J.M.Anderson. Dept. of Internal Medicine and Cell Biology, Yale School of Medicine, New Haven CT 06510

Tight junctions form intercellular barriers separating tissue compartments. Characteristics of these barriers are remarkably diverse among different epithelia and endothelia and are not explained by our limited knowledge of their molecular composition. Two isoforms of the tight junction protein ZO-1 differ by an internal 80 amino acid domain and result from alternative RNA splicing (Am.J.Physiol: C1119,1992). In order to correlate ZO-1 isoform distribution with junctional properties we performed double-labeled immunofluorescence microscopic localization with isoform-specific antibodies on rat tissues. We observed the ZO-1 1α- isoform is restricted to junctions of endothelial cells and highly specialized epithelial cells of both seminiferous tubules (Sertoli cells) and, as previously reported (Kurihara et al. PNAS in press) renal glomeruli (podocytes). This pattern was confirmed by immunoblotting and RNAse protection assays. Based on the known properties of these junctions, this dichotomy suggests the distinction between two classes of tight junctions; those expressing ZO-1α- are capable of readily opening the intercellular space and moving on the cell surface, those expressing ZO-1α+ are relatively less dynamic. In addition, correlation of isoforms distribution with previously reported freeze-fracture EM data, shows the fibrils of ZO-1α- junctions fraction with the outer membrane leaflet while those of ZO-1α+ junctions fraction with the inner membrane leaflet. We hypothesize the α-domain is responsible for an interaction which stabilizes the junction on the cytoplasmic surface. This interaction is not with ZO-2, because this protein was immunoprecipitated with both isoforms. We conclude expression pattern of ZO-1 isoforms reveals a fundamental molecular distinction between two classes of tight junctions and is a potential biochemical basis for variability in junctional properties.

1257

Isolation and Preliminary Characterization of ZO-2. L.A. Jesaitis and D.A. Goodenough. Program in Cell and Developmental Biology and the Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115

The tight junction regulates the permeability of the spaces between epithelial cells and plays a role in preventing the mixing of apical and basolateral plasma membrane components. We previously identified ZO-1, a 210 to 225 kD peripheral membrane phosphoprotein which localizes exclusively to the tight junction in a variety of epithelia (Stevenson, et al., 1986; Anderson, et al., 1988). ZO-2, a 160 kD protein coimmunoprecipitates with ZO-1 from MDCK cell extracts (Gumbiner, 1991). In this study, we found that anti-ZO-1 antibodies failed to react with ZO-2 as assayed either by immunoprecipitation of dissociated complexes or by immunoblotting, suggesting that ZO-2 is not a proteolytic fragment of ZO-1. Further, we isolated ZO-2 by bulk immunoprecipitation of extracts prepared from MDCK monolayers and subjected gel-purified protein to trypsin digestion and amino acid sequencing. No significant homology was found to previously identified proteins contained within the genbank database. Degenerate oligonucleotide primers derived from N-terminal sequence of a single tryptic polypeptide were used to PCR amplify a 75 bp DNA fragment from MDCK cDNA. The PCR fragment encoded an amino acid sequence identical to that obtained by Edman degradation and thus was used as a probe to isolate a 3.5 kb cDNA from an MDCK library in λZAP. Preliminary sequencing results suggest a small portion of the carboxy terminus of ZO-2 is encoded by this cDNA. We are also in the process of raising polyclonal and monoclonal antibodies to synthetic ZO-2 peptides.

1258

Phospholipase A_2-Gold Cytochemistry Reveals the Lipidic Nature of Tight Junction Strands. F.W.K. Kan, Department of Anatomy, Université de Montréal, Montreal, Quebec, Canada H3C 3J7. (Spon. by I. Hüttner)

Previous freeze-fracture experiments using either glutaraldehyde-fixed and cryoprotected specimens or unfixed, rapidly-frozen samples led to the proposal that cylindrical strands of tight junction (TJ) observed in freeze-fracture preparations are inverted cylindrical micelles made up of membrane lipids and possibly, membrane proteins. However, no one has yet been able to label directly the structural fibrils of the TJ. To test the hypothesis that TJ strands observed on freeze-fracture preparations are composed at least partially of lipids, we have combined the phospholipase A_2-gold (Eur J Cell Biol 46:564, 1988) and the fracture-label (Science 213:231, 1981) techniques for the localization of phospholipids. Phospholipase A_2, purified from bee venom, was adsorbed on gold particles and used for the specific labeling of its substrate. Phospholipase A_2-colloidal gold (PLA_2-CG) complex was applied to freeze-fractured preparations of rat exocrine pancreatic cells and testicular Sertoli cells, both of which are known to have extensive TJ complexes on their plasma membranes. Fracture-label replicas of exocrine pancreatic cells revealed specific association of gold particles with TJ fibrils on the protoplasmic fracture-face of the plasma membrane. A majority of these gold particles were observed either directly on the top of the TJ fibrils or adjacent to these cylindrical structures. High density of PLA_2-CG labeling was also observed over the complementary exoplasmic fracture-face of the TJ complex. This intimate association of PLA_2-CG labeling with the TJ is particularly evident in the Sertoli cell plasma membrane where rows of gold particles were observed to be superimposed on arrays of cylindrical strands of the TJ complex. The present findings provide direct cytochemical evidence, to support the hypothesis that cylindrical TJ strands observed in freeze-fracture preparations contain phospholipids, thus confirming the previously proposed lipidic nature of tight junctions. (Supported by MRC of Canada).

1259

Uvomorulin (UM) Expression is Neither Sufficient or Necessary to Confer Adherens Junction Formation in CHO Cells or Epithelial Morphology in L-929 Cells. H.Chopra and S.W. Craig. Department of Biological Chemistry, The Johns Hopkins School of Medicine, Baltimore, MD 21205.

During experiments to assess the ability of UM to recruit vinculin to the plasma membrane, we have been disappointed to uncover evidence that is inconsistent with the proposed function of UM in triggering junction formation and epithelioid morphology. We found that 4 clones of CHO cells that express UM at levels equivalent to MDCK cells did not exhibit morphology different from wild type (wt), did not form adherens junctions, and did not show clear differences in localization of vinculin and actin compared to wt cells. However, they did acquire the ability to co-aggregate under conditions where wt CHO cells remain as single cells. The UM-positive clones expressed α and β catenins to a similar level as MDCK cells, but only a trace of γ catenin. To determine if this unexpected failure of UM to induce junction formation was caused by expression in a non-competent cell line, we transfected L 929 cells with UM. At low density, wt cells grow isolated from one another and show several morphologies including round, spindle-shaped, and a small number of flat, polygonal cells. Four lines expressing levels of UM similar to MDCK cells were analyzed for morphology and three have been analyzed for catenin expression. All lines analyzed expressed α and β catenins at levels similar to MDCK, but only a trace of γ catenin. At low density, two of the lines show round cells and elongated chains of 3-4 cells attached at their ends, but become round and detached from one another at confluence. The other two lines exhibit epithelioid shape and morphology even at low density and remain epithelioid at high density. One of these lines SA-44, was not clonal and had about 40% UM-negative cells. At low density, islands of UM+ and UM-epithelial nests were observed. These results show that UM expression is neither necessary nor sufficient for expression of epithelial morphology. Supported by AHA Postdoctoral Fellowship (H.C.); and the W.W. Smith Foundation.

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Calcium Interacts at the Extracellular Face of the Plasma Membrane of Epithelial (MDCK) Cells to Trigger Tight Junctions Formation. Contreras, R.G., Miller*, I.H., Zamora, M., González-Mariscal, L., García-Villegas, M.R. and Cereijido, M. Department of Physiology, Biophysics and Neurosciences, Center for Research & Advanced Studies, Apartado Postal 14-740, México, D.F. 07000, México. (Spon. by Martinez-Palomo, A.).

Ca^{2+} plays a crucial role in the formation of tight junctions (TJs) between the epithelial cells. When confluent monolayers of Madin-Darby canine kidney (MDCK) cells are transferred from low (1-5 μM) to normal (1.8 Mm) Ca^{2+} medium, cytosolic Ca^{2+} increases and TJs assemble and seal. This increase does not seem to be necessary for junction formation because when cytosolic Ca^{2+} is kept low by adding La^{3+} to the normal Ca^{2+} medium, TJs form. This suggests that Ca^{2+} acts on the extracellular face of the plasma membrane. Here we analyzed this hypothesis by measuring unidirectional Ca^{2+} fluxes across the plasma membrane of MDCK cells to find suitable inhibitors and test their effects on the ability of Ca^{2+} to seal the TJs. We observed (1) that Ca^{2+} triggering of junction formation does not depend on its entry to the cell; (2) that cations like La^{3+} may impair the influx of Ca^{2+} without affecting the sealing of TJs; (3) that only Cd^{2+} is able to block both Ca^{2+} penetration and junction formation; yet (4) Cd^{2+} itself cannot trigger junction formation. We interpret that Ca^{2+} triggers junction formation by acting mainly on an extracellular membrane site, and that this site has a higher Ca^{2+} selectivity than the mechanisms for Ca^{2+} translocation across the membrane.

1261

Phosphorylation and Calcium Requirements for Glucocorticoid Induction of Tight Junctions in 31EG4 Mouse Mammary Cells.

K. S. Zettl and G. L. Firestone, Department of Molecular and Cell Biology, Univ. of California at Berkeley, Berkeley, CA 94720.

We have previously demonstrated that the synthetic glucocorticoid, dexamethasone, induces high resistance tight junctions (2000 $\Omega \cdot cm^2$) in the untransformed mouse mammary cell line, 31EG4. The hormonal induction of tight junctions in a single cell line is advantageous for studying molecular mechanisms responsible for changes in transepithelial electrical resistance (TER). Exposure of dexamethasone treated 31EG4 cell cultures to the phosphatase inhibitor okadaic acid (100 nM) resulted in a decrease in TER from 1097 $\Omega \cdot cm^2$ to background levels over 48 hours. This result, along with many others in the field, suggests that phosphorylation events and/or the direct phosphorylation of tight junction proteins may play a role in the regulation of tight junctions. Furthermore, consistent with studies using MDCK cells, switching to low Ca^{++} media (6 μM) abolished high TER and cell contact of 31EG4 mammary cells. In contrast to MDCK cells, reestablishment of TER and/or cell contact in normal Ca^{++} (1.8 mM) was sensitive to cyclohexamide, suggesting a dependency on de novo protein synthesis.

1262

The role of phosphorylation in the assembly and disassembly of epithelial junctions. Sandra Citi, Natasha Denisenko & Mauro Rabino. Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.

We have used protein kinase and phosphatase inhibitors to investigate the role of phosphorylation in the assembly and disassembly of intercellular junctions in MDCK cells. Upon removal of extracellular calcium (LC), all junctions are disassembled, as determined by immunofluorescent analysis with antibodies against cingulin, E-cadherin and desmoplakin, and by measurement of transepithelial resistance (TER). Pre-treatment with the serine protein kinase inhibitors H-7, H-8, and staurosporine and the tyrosine kinase inhibitor genistein prevent completely or partially the cell-cell dissociation, cytoskeletal reorganization and fall in TER induced by LC, suggesting a role for protein kinases in the events induced by LC. For example, LC may induce a cadherin-mediated activation of kinase(s) or, alternatively, phosphorylation of specific proteins may be required in order for LC to produce its effects (Citi, JCB 117, 169, 1992). Treatment with the tyrosine phosphatase inhibitor pervanadate mimics the effects of LC, is cumulative with LC, and can be partially prevented by H-7, suggesting the possibility that a cascade of phosphorylation events may modulate the organization of junctions and the cytoskeleton. Results of experiments to assay the level of phosphorylation of specific proteins following calcium removal and treatment with kinase and phosphatase inhibitors will be presented.

In the rapid assembly of junctions induced by the calcium switch, and in the slower assembly of trypsinized cells at normal calcium concentrations, serine and tyrosine kinase inhibitors prevent selectively the assembly of tight junction proteins and the establishment of TER, however E-cadherin, desmoplakin and actin appear to accumulate normally at sites of cell-cell contact. These results suggest that the assembly of adherens junctions and junctional actin is not sufficient for tight junction assembly, and that distinct pathways are used by MDCK cells to sort and/or target proteins of tight and adherens junctions.

1263

Increased Tyrosine Phosphorylation Accompanies Modification of Tight Junctions between Foot Processes of Glomerular Epithelial Cells (GEC). H. Kurihara, J.M. Anderson*, and M.G. Farquhar. Division of Cellular and Molecular Medicine and Center for Molecular Genetics, University of California San Diego, La Jolla, CA 92093, and *Dept. of Medicine, Yale University School of Medicine, New Haven, CT 06510.

We have previously shown that the tight junction protein ZO-1 rapidly redistributes from the attachment sites of slit diaphragms to the newly formed occluding-type junctions within 15 min after neutralization of the GEC cell surface charge by perfusion with a polycation (protamine sulfate (PS)) [Kurihara et al., 1992, Amer. J. Pathol., "in press"]. To investigate the role of transmembrane signaling in this process, tyrosine phosphorylation was analysed. In the normal rat kidney staining with anti-phosphotyrosine (P-tyr) antibody, was observed in the cytoplasm of mesangial cells by immunofluorescence. In PS-treated rats, however, it was concentrated along the foot process layer of GEC as well as in mesangial cells. In controls, little or no gold was seen in GEC after immunogold labeling with anti-P-tyr antibody. In PS-treated glomeruli numerous gold particles were localized along the altered GEC junctions and the basal membrane of podocytes. Some gold particles were also associated with the residual slit diaphragms after PS-treatment. Immunoblot analysis with anti-P-tyr antibody on glomerular lysates from control kidney demonstrates prominent 200, 140, 116, and 80 kD bands. In PS-treated glomerular lysates new bands were detected at 225, 180, and 100 kD. The 225 kD tyrosyl phosphorylated protein seen only in PS-treated glomerular fractions was identified as ZO-1 by immunoprecipitation followed by immunoblotting with anti-P-tyr. These findings suggest that tyrosine phosphorylation plays a key role in the formation of tight junctions between foot processes of glomerular epithelial cells upon neutralization of their cell surface charge. Supported by NIH grant DK 17724.

1264

Effect of polycations on permeability of glomerular epithelial cell (GEC) monolayers. M. Hammes and A. Singh, Department of Medicine, Loyola University Medical Center, Maywood, IL 60153

Polycations can interact with the surface negative charges of the GEC in the kidney and give rise to morphological changes usually associated with proteinuric states. The mechanism of charge neutralization-induced permeability loss is still unclear. We examined this question by studying the effect of polycations on the permeability property of GEC monolayers. Cells were grown to confluency on filter lined cups (millicell). They were treated apically with cationic BGG or protamine (100 ug/ml) for 2 hrs. at 37°. After washing the cells, the monolayers were tested for leakage of albumin by adding radioactive bovine serum albumin on the apical side and determining the time course of its appearance on the basal side. Polycation treatment caused significant leakage of albumin in absence of any toxic effect on viability or LDH release. The leakage was shown to be through the tight junctions of the monolayer. Permeability alterations were compared at 4°C and 37°C to determine whether impairment was due to the membrane ruffling effect of charge neutralization or due to intracellular metabolic changes. Significant leak occurred only at 37°C suggesting role of active processes in the maintenance of permselectivity. This was further substantiated by studying the protective effect of removing bound polycation with heparin. Removal of polycation after initial binding failed to protect the monolayer from albumin leakage.

It was concluded that neutralization of GEC cell surface charges results in subtle impairment of tight junction control of permeability to macromolecules across the monolayer. The functionality of the tight junctions is coupled to the negative charges via active processes in the cell.

1265

A Soluble, Thermolabile Factor Secreted into the Apical Medium of Epithelial Cells Monolayers Promotes Increase of Transepithelial Electrical Resistance. M. Jaeger, Y. Dodane, and B. Kachar. Laboratory of Cellular Biology, NIDCD, N.I.H., Bethesda, MD, 20892, U.S.A. (Spon. by. A. Sesso).

Tight junctions provide a selectively permeable occlusion along the paracellular pathway of epithelia. These structures limit the diffusion of molecules that are selectively concentrated on each side of the cell layer by transepithelial transport or secretory processes. We report evidence indicating the existence of a thermolabile factor(s) that is secreted by the apical membrane of various epithelial cell lines. This factor seems to influence transepithelial permeability when applied to the basolateral membrane of these cell lines. Cells from the A6 amphibian kidney cell line were grown on Costar chambers until they reached confluence and transepithelial electrical resistance (TER) was stabilized. When the basolateral surface of a confluent monolayer was exposed to apical medium, the TER increased progressively 16.5 ± 1% within 5-20 minutes. After 40-60 minutes, the TER returned slowly to baseline values. An increase of 21.5 ± 3.2 was obtained when the apical medium was concentrated 10 times by ultrafiltration through a 30,000 MW cutoff membrane. This increase in TER was not observed when the apical medium was preheated at 65 °C for 10 minutes or when using the 30,000 MW filtrate. All experiments were repeated a minimum of 10 times at constant temperature, pH and volume. The ability of the apical medium to promote the increase of TER was only observed when it was collected 4-7 days after confluence of the donor culture. This increase of TER was observed both in the presence and absence of the serum used to supplement the fresh medium. This suggests that the component which promotes the increase of TER is secreted and slowly accumulated in the apical medium. Similar results were obtained with MDCK and Caco-2 cell lines. Furthermore, apical medium collected from one cell line promoted increase of TER when applied to the basal side of another cell line. Apical medium collected from MDCK monolayers increased the TER of Caco-2 monolayers by 22.3 ± 4.7 %. We were not able to detect changes in the freeze-fracture morphology of the tight junction strands following the observed increase of TER. These preliminary results indicate that a component of the apical medium promotes an increase in TER, restricting the passage of ions through tight junctions. Leakage of this apical component to the basolateral domain could be involved in a feedback mechanism controlling TER.

1266

Regulation of Transepithelial Electrical Resistance: An Unexpected Cytokine Function. S. Myrdal and T. Bailey, Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, WA

We reported earlier that the novel cytokine Oncostatin M (OSM) produced polarized, bidirectional changes in the transepithelial electrical resistances (TER) of our MDCK clone BMS.1. That is, treating these epithelia with OSM at the apical surface caused an increase in TER, and at the basolateral surface, a decrease. We found no difference in size, numbers, or affinities of OSM receptors at the two surfaces. Both responses required tyrosine phosphorylation (Myrdal and Bailey, 1991, JCB 115:237a). We now report that several other cytokines exhibited TER regulatory effects. Among the unexpected findings was that TGF-β and TNF-α (both wholly unrelated to each other or to OSM regarding structural homology or receptor cross-talk) also induced polarized bidirectional TER changes in these epithelia. Unlike OSM, TGF-β neither induced tyrosine phosphorylation nor required it for TER regulation. Conversely, leukemia inhibitory factor (LIF) and IL-6 (which share structural homology and a receptor subunit (gp130) with each other and OSM) do not mimic the OSM bidirectional regulation. IL-6 neither alters TER nor binds to these epithelia. LIF causes TER increases from both apical and basolateral surfaces. Thus, LIF mimics and augments OSM on one side, and opposes OSM on the other. We do not know the molecular mechanisms underlying directionally opposite responses to a single cytokine, the redundancy of OSM with TGF-β and TNF-α, or the sided differences between OSM and LIF.

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Identification of a factor in urine that increases transepithelial electrical resistance in MDCK cells. J. M. Gallardo Montoya, M. Hernandez, R. G. Contreras, L. Gonzalez-Mariscal and M. Cereijido. Department of Physiology, Center for Research and Advanced Studies. Ap. Postal 14-740, México D.F., 07000

Epithelial cells of the mammalian kidney tubules develop an increasing transepithelial electrical resistance (TER) as well as more complex tight junctions as they proceed from the glomerulus to the distal convoluted tubule. We have searched for the presence of a factor(s) in urine that might regulate TER of kidney epithelia. Aseptically obtained urine from healthy male dogs was dialyzed against water and liophilized (DLU, dialyzed and liophilized urine). Confluent one day old MDCK cells, treated with DLU exhibit a significant increase in TER above control values, specially if DLU is applied to the basolateral surface (10% DLU produces an increment in TER of 14% when applied apically, and of 266% when added to the basolateral surface). Half maximal increments in TER were obtained 4 hs. after addition of DLU to the basolateral surface. The effect seems to be reversible, for once DLU is withdrawn TER returns to control values. The factor(s) in DLU is thermoresistant, as a 30 min incubation at 95 °C does not abolish the effect on TER.

1268

Structure of rat brain capillary endothelial cell tight junctions after cell isolation and short-term culture.
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Rat brain microvessel fragments were isolated by mechanical and enzymatic dissociation, and the endothelial cells cultured (Abbott *et al*, J. Cell Sci 1992, in press). The fine structural features of the endothelia were examined in thin sections and freeze-fracture replicas, with particular attention to the tight junctions. During isolation the cells rounded up, and immediately after isolation, few tight junctions could be seen in thin sections. Replicas showed one or two incomplete rows of particles forming discrete strands in the junctional zone. After 24hrs in culture, when cells were proliferating and growing out from the fragments, the complexity and number of junctional strands increased. The cell membranes also showed omega profiles in thin sections, and craters and volcanoes in replicas. Experiments with the exogenous tracer, cationized ferritin, are being made to test the hypothesis that the omega profiles are involved in endocytosis. Further studies are underway looking at the maturation and modifications of the junctions during development to confluence.

1269

Bacterial elastase facilitates Pseudomonas aeruginosa passage between epithelial cells.
A.O. Azghani and I.F. Williams, Department of Biochemistry, University of Texas Health Science Center. Tyler, TX 75710.

We have shown that elastase from Pseudomonas aeruginosa decreases transcellular electrical resistance of Madine-Darby canine kidney (MDCK) epithelial monolayers. The phenomenon coincides with a decreased fluorescent intensity of the ZO-1 protein of the tight junction. We reasoned that P. aeruginosa might use this mechanism to breach the epithelial barrier and invade the underlying tissue. Confluent MDCK monolayers on culture inserts (Costar) were pretreated with Pseudomonas elastase (PE) 30 min prior to the addition of ^{35}S-labeled bacteria (2-4 X 10^7) in phosphate buffered saline containing cycloserin. We measured transcellular electrical resistance (TER) before PE treatment and at the end of a 2.5 hr experiment. Monolayers were detached after three washes to determine the number of adherent bacteria to apical surfaces of the cells. Samples from basal media were taken to determine the number of bacteria that traverse the monolayers during the 2 hr incubation. While P. elastase (6 u/ml) increased bacterial adherence to MDCK more than 2 fold, it enhanced bacterial entry across epithelial monolayers significantly (0.06% vs 0.38%, $p<0.05$). This phenomenon coincided with decreased TER suggesting that bacteria probably traversed the paracellular space by opening the junctional complex. Thus, elastase may have an important role in pathogenesis of Pseudomonas infection.
Supported by grants from NHLBI (HL44473), AHA and ALA of Texas.

1270

Extracellular ATP regulates paracellular transport in human uterine cervical cells.
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The objective of the present study was to determine whether extracellular ATP (ATP_e) can regulate transepithelial paracellular transport. As a model we used Caski epithelial cell cultures, originally derived from squamous carcinoma of the human uterine cervix, grown on permeable support. The methodology included measurements of 1. transepithelial conductance (TC) and 2. transepithelial fluxes of inert, nonmetabolizable molecules with MW's varying from 182 to 70K. The latter included mannitol, sucrose and polydextrans. Results: Day 5-12 cultures of $Caski_2$ in FCS-enriched medium have a consistent TC of 35±7 $mS \cdot cm^{-2}$ and permeability(P) of 9.6±0.4 $cm \cdot sec^{-1}(\cdot 10^6)$ to pyranine (MW-500)$_f$. In serum-free medium both TC and P increase to 61±11 $mS \cdot cm^{-2}$ and 12.5±0.8 $cm \cdot sec^{-1}[\cdot 10^6]$ respectively. In the latter condition, compared to the former, small fluxes of 40K molecules can be measured (P of 2.2±0.3 $cm \cdot sec^{-1}[\cdot 10^7]$). ATP_e resulted in an acute and reversible dose-related decrease both in TC and P to molecules of MW 182-10K and the magnitude of the effect was 10-33% of baseline values. The effect began at 0.5μM added ATP and reached a plateau at 50μM, with EC_{50} of 3μM. Maximal ATP effect was obtained in experiments done in serum-free conditions with 33% reduction in both TC and P to molecules of MW 182-40K. Summary: ATP_e, in low, physiological concentrations, decreases acutely and reversibly transepithelial transport in Caski cells via, probably, a receptor mediated effect. We speculate that ATP_e may be an important factor in the regulation of paracellular transport in the human cervix, in vivo. Support: NIH AR-39750-04 and 1K08HD00977-01 to G.I.G.

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The Effect of Azide on Cell to Cell Diffusion of Large Molecules.
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The function of cytoplasmic streaming remains unknown but it may be required to mix the contents of plant cells and aid cell to cell diffusion of some molecules. Previously, we found that when cytoplasmic streaming in staminal hairs of Setcreasea purpurea was stopped with the metabolic inhibitor azide, the intercellular diffusion of microinjected carboxyfluorescein (CF) was not inhibited. Similar studies have now been done using fluorescent molecular probes that are larger and not as charged as CF. The molecular probes used: FITC-Gln, FITC-$(Gln)_2$, FITC-$(Gln)_3$, FITC-$(Gln)_4$ have molecular weight 535, 665, 796 and 926, respectively. They were found to have similar solubility and charge characteristics on chromatography and electrophoresis. Coefficients of diffusion between cells (D) and coefficients of loss into the vacuole (L) were determined for each probe using the kinetic analysis technique [Tucker et al. Plant Physiol. 90:1143-1147 (1989)]. Average Ds for FITC-Gln, $(Gln)_2$, $(Gln)_3$, and $(Gln)_4$ in untreated cells were 2.1, 3.2, 1.9 and 1.8 x 10^{-8} cm^2/sec, respectively and in azide treated cells were 2.5, 3.9, 3.8 and 4.1 x 10^{-8} cm^2/sec, respectively. Average Ls in untreated cells were 8.5, 3.4, 2.7, and 3.9 x 10^{-7}/um^2/sec, respectively and in azide treated cells were 2.9, 2.3, 2.7, and 3.1 x 10^{-7}/um^2/sec respectively. Azide treatments stopped cytoplasmic streaming, inhibited loss and enhanced cell to cell diffusion.

We concluded that cytoplasmic streaming does not drive the cell to cell diffusion of large, hydrophilic molecules. Furthermore, it appears that azide labile components of the cytoplasm or plasmodesmata, affect the rate of cell to cell diffusion.

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Intracellular Regulation of the Movements of E-cadherin on the Cell Surface.
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The movements of E-cadherin on the surface of living cells in culture were studied to elucidate the intracellular structural mechanism for regulation of cadherin assembly at the site of cell-cell contact. E-cadherin was labeled with 40 nm colloidal gold particles, whose movements were traced by analyzing the video-enhanced images at the temporal resolution of 33 msec (1.3 nm precision for determination of the spatial coordinates of the particles). The movements of normal and mutated E-cadherin polypeptides that contain various deletions in the cytoplasmic domain were examined by using five lines of transfected L-cells expressing these proteins. The analysis of the observed trajectories of normal E-cadherin showed that 1/3 of the molecules undergo transport-like movements or no motion, indicating binding to cytoskeletal network; 2/3 undergo fast movements (the microscopic diffusion coefficient = 1.0 x 10^{-10} cm^2/sec) and 1/2 of these molecules are confined within areas of ≈0.12 μm^2 (350 nm square) in the time scale of 30 sec. Deletion mutants that lost binding site(s) for catenins (E-cadherin-associated proteins) showed decrease in the cytoskeleton-bound fraction (10%) and increase in the diffusion rate by a factor of ≈2 for the mobile fraction (90%). One half of the mobile E-cadherin showed confined movements with the area size similar to normal cadherin. The size of the confined area increased by a factor of 4 for the mutant protein with a small cytoplasmic domain (24 a.a.). These results suggest regulation of E-cadherin movements by catenin-dependent cytoskeletal binding and by enclosing mechanism of the meshwork of the membrane skeleton (Tsuji et al., Biochemistry 27, 7447-7452, 1988).

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Microfilamentous Nets In the Region of the Golgi Stack May Serve as Structural Links and Organizers of Vesicular Trafficking. R.D. Allen and A.K. Fok, Pacific Biomedical Research Center and Department of Microbiology, University of Hawaii, Honolulu, HI 96822.

Recent studies on the Golgi apparatus have centered around vesicular trafficking, but the question of what links the Golgi stack to the transition element of the ER remains unexplained. By using physical fixation of Paramecium and deep-etch, rotary-shadow replicas at different phases in a culture-age study we have looked for an answer to this question. The Golgi stack in Paramecium is always small but the ER transition elements are very active in early-log-phase cells. Vesicles budding from the ER are coated with an 11 nm layer of subunits, presumably coatamer complexes. Linking these buds and coated vesicles together and linking the ER to the cis-cisterna of the Golgi stack is a non-etchable network of thin filaments. In late-log-phase cells this transition zone is filled with an extensive network of these microfilaments. Globular elements are bound to the network. Another microfilamentous array is associated with the trans-Golgi network. Thus we believe we have identified the structural specialization of the ER transitional element in Paramecium which holds the ER and Golgi stack together and which may serve to organize anterograde vesicular movement from the ER to the Golgi stack. Though poorly preserved by chemical fixatives, examples of such a network can be found in published accounts of both plant and higher animal cells. This network may represent a common feature of most cells. (Supported by NSF grants MCB 92-06097 and MCB 90-17455.)

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Microtubules and ER/Golgi trafficking: localization of membrane-bound kinesin on ER to Golgi transport intermediates. J. Lippincott-Schwartz and G. Bloom*. NICHD, NIH, Bethesda, MD. 20892, *Dept Cell Biol., UT Southwestern Med. Ctr, Dallas, TX, 75235.

In this study we show that delivery of membrane from the ER into the Golgi apparatus in fibroblast cell lines occurs through distinct transport intermediates which arise from multiple peripheral exit sites within the ER and then move toward the centrally located Golgi apparatus in a microtubule-facilitated process. The transport intermediates exist as tubular-vesicular structures which are largely devoid of resident ER and Golgi components but are enriched in the integral membrane protein, p53, the peripheral "coat" protein, βCOP, and the microtubule motor protein, kinesin based on immunofluorescence localization experiments. Since manipulations which alter the steady-state distribution of p53, including incubation at 16ºC, and nocodazole and brefeldin A treatment, cause a similiar shift in distribution of membrane-bound kinesin, we suggest that both of these molecules move constitutively within the ER/Golgi system. At 16ºC, p53, βCOP and kinesin show extensive, overlapping distributions in pre-Golgi intermediate structures. These structures move into the Golgi region upon 5 min of warming to 37ºC, but not when microtubules are depolymerized with nocodazole, suggesting a role for microtubules in facilitating ER to Golgi transport. Consistent with this, newly synthesized albumin was retarded in pre-Golgi/Golgi structures instead of being rapidly secreted in cells treated with nocodazole. The predominate localization of kinesin on pre-Golgi structures raises the question of what role it plays in the movement of these structures into the Golgi region. Since kinesin is thought to act as a plus-end directed microtubule motor protein, membrane-bound kinesin could function in the recycling of membrane from the Golgi to the ER and in intra-ER membrane movements (both of which involve microtubule plus end directed movements). The presence of kinesin on membrane structures moving toward the minus ends of microtubules (i.e. toward the Golgi) suggests its motor activity is either inhibited or altered on these structures. Since the cytoplasmic protein, βCOP, preferentially binds to ER-derived membrane moving toward the Golgi apparatus and is dissociated from membrane recycling back into the ER , we discuss the possibility that membrane binding of βCOP regulates kinesin motor acitivity on membrane that cycles between the ER and Golgi apparatus.

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Evidence for Lipid Sorting in the Cell-Free Vesicular Lipid Transfer from Endoplasmic Reticulum to Cis Golgi Apparatus. D.J. Morré, P. Moreau, C. Cassagne, D.M. Morré and T.W. Keenan, Purdue University, West Lafayette, IN, CNRS, Bordeaux, France and Virginia Polytechnic Institute and State University, Blacksburg, VA

Cell-free transfer of lipids from endoplasmic reticulum (ER) to Golgi apparatus was investigated using a system from rat liver. All major lipids were transferred except triglycerides and ceramides. Ceramides were present in both ER and Golgi apparatus but were absent from transition vesicles. Transition vesicles were much more efficient in transfer of phosphatidylcholine (PC) than the transitional ER from which they were derived. Transfer was temperature- and ATP-dependent and inhibited by N-ethylmaleimide (NEM). In contrast, there was little or no transfer of [^{3}H]-ceramide via transition vesicles and that transfer which occurred was ATP- and NEM- independent. Transfer of ceramide from ER to Golgi apparatus did occur in the cell-free system but via a non-vesicular mechanism that was temperature-dependent but not dependent on ATP or cytosol, nor inhibited by NEM. A component of PC transfer also was found that exhibited similar characteristics. Previous work showed triglycerides were excluded from transition vesicles (J. Biol. Chem. 266, 4322, 1991). The results provide evidence not only for lipid sorting for two mechanism of cell-free transfer of lipids from ER to Golgi apparatus. The first is vesicular and ATP- and temperature-dependent. The second is non-vesicular, temperature-dependent and neither ATP- nor cytosol-dependent. Supported by NIH GM44675 and 312 44.

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Antibody to α Subunits of Trimeric G-Proteins Recognizes 38 kDa Protein Resident to Cis-Golgi Apparatus. M. Paulik, A.O. Brightman, J.B. Lawrence, T. Reust, C.C. Geilen, K. Spicher, G. Schultz, W. Reutter, D. M. Morré and D.J. Morré, Purdue University, West Lafayette, IN 47907 and Free University, Berlin, Germany

Both low molecular weight, monomeric GTP-binding proteins [1] and trimeric G-proteins [2] have been implicated in the regulation of intracellular vesicle traffic. Included is evidence for their involvement in vesicular transport between the endoplasmic reticulum (ER) and the cis-Golgi apparatus. The transfer of vesicles between the ER and cis-Golgi occurs via small (50-70 nm) transition vesicles which bud from elements of transitional ER and fuse with cis cisternae of the Golgi apparatus. We have evidence for a 38 kDa protein (p38) that is concentrated in cis-Golgi membranes. Using a cell-free ER/cis-Golgi transfer system [3,4], vesicular transfer between ER and cis-Golgi was inhibited with polyclonal antisera directed against p38, but only when the antisera was preincubated with cis-Golgi. New findings using peptide specific antibodies directed against the α subunit nucleotide binding domain of trimeric G-proteins suggest that p38 may also be a GTP binding protein and a potential candidate for a targeting and/or docking receptor to help explain the GTPγS inhibition of specific binding of transition vesicles to the cis-Golgi apparatus. Supported by NIH GM44675.
[1] B. Goud and M. McCaffrey (1991) Curr. Opinion Cell Biol. 3, 626; [2] R.D. Burgoyne (1992) Trends Biol. Sci. 17, 87; [3] M. Paulik et al. (1988) J. Biol. Chem. 263, 177728; [4] P. Moreau and D.J. Moreau (1991) J. Biol. Chem. 266, 4329.

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Role of Endoplasmic Reticular Calcium in Oligosaccharide Processing of α_1-Antitrypsin. G. Kuznetsov, C.O. Brostrom, and M.A. Brostrom, Department of Pharmacology, UMDNJ-R.W. Johnson Medical School, Piscataway, NJ 08854.

Mobilization of sequestered Ca^{2+} from the endoplasmic reticulum (ER) inhibits post-translational processing and export of α_1-antitrypsin (α_1-AT) by HepG2 cells (Kuznetsov *et al.*, 1992, J. Biol. Chem. 267: 3932-3939). The present study explores the mechanism whereby ER Ca^{2+} depletion by such agents as thapsigargin (Tg), an ER Ca^{2+}-ATPase inhibitor, or ionomycin, a Ca^{2+} ionophore, results in the intracellular retention of glycosylated α_1-AT bearing high mannose, Endo H-sensitive oligosaccharide side chains. Arrest occurred at the removal of mannose residues such that intermediates with $Man_{8-7}GlcNAc_2$ side chains accumulated with the $Man_8GlcNAc_2$ structure predominating. Maturation of presynthesized α_1-AT bearing the $Man_{6-5}GlcNAc$ structure was unaffected by the drugs. In the presence of brefeldin A (BFA), which causes redistribution of Golgi components to the ER, α_1-AT was retained intracellularly but was resistant to Endo H. In ionomycin and Tg treated preparations, however, Golgi enzymes relocated to the ER by BFA failed to further process retained α_1-AT. A form of α_1-AT identical to that retained by ionomycin and Tg was observed upon treatment with 1-deoxymannojirimycin (1-DMJ), an inhibitor of ER/Golgi α1,2-mannosidases. However, treatment with 1-DMJ did not prevent secretion of α_1-AT bearing high mannose type, Endo H-sensitive oligosaccharide side chains. We conclude (1) that treatment of HepG2 cells with ER Ca^{2+} mobilizing agents interrupts ER processing of α_1-AT at the mannose side chain trimming steps and (2) that Ca^{2+} depletion produces misfolding in the ER such that normal mannose trimming does not occur. Dissociation of α_1-AT from α1,2 mannosidase may require Ca^{2+} or, alternatively, processing and folding of α_1-AT in the ER may require a Ca^{2+}-binding chaperonin. (Supported by a PMA Predoctoral Fellowship to G.K. and by NIH DK 35393)

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Myristoylation of an ADP-ribosylation Factor, a ~20-kDa Guanine Nucleotide-binding Protein, is Essential for its Binding to Golgi. R.S. Haun, S.-C. Tsai, R. Adamik, I. Moss, and M. Vaughan, Laboratory of Cellular Metabolism, NHLBI, NIH, Bethesda, MD 20892.

ADP-ribosylation factors (ARFs) are a family of ~20-kDa guanine nucleotide-binding proteins that were initially identified by their ability to enhance the *in vitro* ADP-ribosyltransferase activity of cholera toxin. ARFs, thought to be involved in protein trafficking, have been localized to the Golgi apparatus in mammalian cells and are associated with non-clathrin-coated transport vesicles and coat proteins. ARFs are N-terminally myristoylated and, although they are predominately cytosolic proteins, associate with membranes or phospholipids in a GTP-dependent manner. It appears that members of the ARF family may be preferentially associated with different membranes. We, therefore, used recombinant human ARF 5 to evaluate the role of myristoylation in membrane binding. Recombinant myristoylated ARF 5 was obtained from bacteria by co-expression with yeast myristoyl-CoA:protein *N*-myristoyltransferase. Bacterially expressed myristoylated and non-myristoylated ARF are functionally similar, as determined by their ability to enhance cholera-toxin catalyzed ADP-ribosylation, and display similar affinities for GTP in a nucleotide displacement assay. We show that only myristoylated ARF exhibits temperature- and GTP-dependent binding to Golgi, indicating that myristoylation is necessary, though not sufficient, for membrane association. Also, we demonstrate that ARF binding to Golgi is saturable, suggesting that it interacts with a specific membrane-associated protein.

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Dissociated β-COP Accumulates in Large Sedimentable Aggregates in BFA- or N_2-treated Pancreatic Exocrine Cells (PEC). L. C. Hendricks, M. McCaffery, G. E. Palade and M. G. Farquhar. Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093

In many cell types BFA blocks protein export from the ER and causes rapid disorganization of the Golgi complex with redistribution of Golgi proteins to the ER following dissociation of β-COP from Golgi membranes. In PEC BFA blocks early steps in secretory protein transport and stacked Golgi cisternae disappear, but vesicles and tubules containing Golgi markers persist and relocation of Golgi proteins to the ER is not detected. We have now examined the redistribution of β-COP in PEC of lobules incubated *in vitro* in the presence of BFA or under N_2, two agents known to inhibit ER to Golgi transport. In controls, β-COP was concentrated in the Golgi region by immunofluorescence and by immunogold was associated with Golgi cisternae and transport vesicles. In BFA-treated (7.2 μM) PEC, β-COP was concentrated in globular masses in the Golgi region by immunofluorescence. Large (>1 μm) electron-dense aggregates containing β-COP were seen in close proximity to Golgi remnants by immunogold. In N_2-treated (30 min) PEC, β-COP was concentrated in similar dense aggregates located predominantly on the cis side of the Golgi stacks which remained intact. Diffuse cytoplasmic staining was not seen after either BFA or N_2 treatment. To determine what proportion of the dissociated β-COP was sedimentable, we homogenized lobules in 0.3 M sucrose, removed nuclei (600g, 10 min) and centrifuged the postnuclear supernatants (100,000g, 60 min) yielding membrane and cytosol fractions. The proteins of the fractions were separated by SDS-PAGE and immunoblotted with anti-β-COP. In control, BFA- or N_2-treated lobules, β-COP was detected exclusively in the 100,000g pellet. These data indicate that: 1) upon arrest of ER to Golgi transport by either BFA or energy depletion β-COP dissociates from Golgi membranes and accumulates as large, sedimentable aggregates, 2) stacked cisternae persist under N_2 and Golgi remnants are present in BFA-treated specimens notwithstanding dissociation of β-COP from Golgi membranes, and 3) factors other than β-COP must be required for maintenance of Golgi integrity. Supported by NIH grants CA46128 and DK17780.

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Differential accessibility of the cytoplasmic domain of the H-2L^d class I MHC molecule to immunological and biochemical probes during intracellular transport. G.G. Capps and M.C. Zúñiga, Department of Biology, The University of California, Santa Cruz, CA 95064.

A rabbit antiserum specific for the cytoplasmic domains of H-2L^d and H-2D^b MHC class I molecules was generated against a synthetic peptide (GSQSSDMSLPDCK) corresponding to a portion of the cytoplasmic domain of the H-2L^d/D^b class I MHC molecules. This antibody preparation (R4) specifically immunoprecipitates H-2L^d and H-2D^b molecules bearing this sequence in their cytoplasmic domains. R4 binding of the H-2L^d molecule is unaffected by phosphorylation of the cytoplasmic domain. However, R4 binds only H-2L^d/D^b class I molecules which are not associated with β_2-m or with antigenic peptide. In β_2-m$^+$ cells, R4 binds only Endo H-resistant H-2L^d/D^b class I molecules, but in β_2-m$^-$ cells R4 binds H-2L^d molecules immediately after their synthesis. To explore further the molecular basis of the differential availability of the H-2L^d/D^b cytoplasmic tail to R4 we examined the susceptibility of the sole cytoplasmic domain tyrosine to radioiodination, using gently decavitated cell preparations. Density gradient fractionation of the radioiodinated organelles and subsequent immunoprecipitation analysis of gradient fractions showed that the cytoplasmic tyrosine of Endo H-resistant, but not of Endo H-sensitive, H-2L^d/D^b molecules is accessible to radioiodination. These results suggest that the failure of Endo H-sensitive H-2L^d/D^b molecules to react with R4 is due to association with specific factor(s) which shields the cytoplasmic domains until the H-2 molecules have traversed the medial Golgi, and that this factor(s) is sensitive to the state of assembly of the class I-β_2-m-antigenic peptide trimolecular complex. (Supported by NSF grants DCB-9196051 and DCB-9096241)

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The Sec13p-containing complex is required for vesicle formation in vitro. Nancy K. Pryer, Nina R. Salama and R. Schekman. HHMI and Molecular Cell Biology, U. C. Berkeley, Berkeley, CA 94720.

To begin to understand the mechanism of vesicle formation from the ER, we have characterized and purified the product of the SEC13 gene of S. cerevisiae. *Sec13* mutants are defective in ER to Golgi transport at a point prior to the formation of transport vesicles. The SEC13 gene product is a 34kD protein that partitions between membrane-bound and soluble fractions. Immuno-fluorescence localization of Sec13p reveals punctate staining concentrated around the nucleus. We have developed a Sec13p-dependent in vitro assay to reconstitute vesicle formation. For this assay, Sec13p is extracted from an ER-enriched microsome fraction by urea treatment. Cytosol depleted of Sec13p is produced using a SEC13-dihydrofolate reductase (DHFR) gene fusion expressed in a strain carrying a SEC13 deletion. A high speed supernatant prepared from this strain was applied to a methotrexate (MTX)-agarose column, which binds DHFR and retains the Sec13p-DHFR fusion protein. The flow through from this column is inactive in the vesicle formation assay, while wild type cytosol chromatographed over MTX-agarose retains activity. Activity can be restored to the Sec13p-depleted flow through by addition of small amounts of wild type cytosol or a Sec13p-enriched $(NH_4)_2SO_4$ precipitate. The majority of Sec13p in wild type cells is incorporated into a large complex that elutes from a gel filtration column at 700kD. Column fractions containing this complex are able to restore activity to the Sec13p-depleted assay. A small amount of Sec13p elutes from a gel filtration column as expected for a 34kD protein, but this fraction is inactive in the assay. Overexpression of SEC13 results in overproduction of the inactive monomeric form alone. We are using the Sec13p-dependent budding assay and Sec13p immunoreactivity to monitor the purification of the Sec13p complex.

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Yeast ER to Golgi Transport is Dependent on Sec7p Function. A. Franzusoff, E. Lauzé, and E. van Tuinen, Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, Denver, CO 80262.

The transport of proteins destined for post-endoplasmic reticulum (ER) locations in the secretory pathway is mediated by small vesicular carriers. Cytosolic components are required in the budding of transport vesicles, in a manner analogous to the formation of clathrin-coated vesicles. In the yeast cell-free ER to Golgi transport assay, the introduction of antibodies to the Sec7 protein (Sec7p) prevents α-factor transport to the Golgi, and results in the accumulation of vesicle intermediates, including those that have budded from the ER. These transport intermediates exhibit Sec7p on the vesicle surface, as determined by both biochemical and morphological criteria (Franzusoff *et al.*, (1992) *Nature* 355, 173-175). These results prompted a re-investigation of the cellular requirement for Sec7p *in vivo*. We report that yeast depleted of Sec7p by regulation of *SEC7* gene expression exhibit alterations in protein traffic from the ER as well as through the Golgi apparatus. Furthermore, antibodies to the yeast HDEL (ER retrieval) sequence highlight a novel budding intermediate on the ER membrane by immuno-EM of *sec7* mutants at the restrictive temperature. These results suggest that Sec7p participates in vesicle budding from the ER as well as from different compartments of the Golgi apparatus.

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Characterization of Proteins Associated with Sec7p in Extracts of *Saccharomyces cerevisiae*. J. Wolf and A. Franzusoff, Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, Denver, CO 80262.

Sec7p, encoded by the yeast SEC7 gene, is a 230 kDa acidic phosphoprotein that is required for vesicular transport from the ER and through the different compartments of the yeast Golgi apparatus (Nature 355: 173, 1992). The protein is equally distributed in cell lysates between a cytosolic form and associated with multiple intracellular structures. The mechanism by which this protein functions, however, is not understood. Sec7p apparently interacts with a number of other proteins. Non-denaturing immuno-precipitation of yeast cytosol followed by polyacrylamide gel electrophoresis and Coomassie blue staining show that proteins of 230 (Sec7p), 83, 60, and 32 kDa are specifically precipitated with antibody to Sec7p (Sec7-Ab). A 25 kDa protein is also associated with Sec7p, as demonstrated by GTP-ligand binding blots. A similar collection of proteins can be shown to be associated with Sec7p by experiments in which lysates of radiolabeled spheroplasts are covalently attached with a cleavable bifunctional crosslinking reagent and immunoprecipitated with Sec7-Ab. These results demonstrate that Sec7p is associated with a number of proteins in yeast and suggest that these proteins may function with Sec7p in vesicular trafficking at either unique or multiple stages of the yeast secretory pathway.

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New Gene Products that Interact with Bet1, a Membrane Protein Required for ER to Golgi Transport in Yeast. P. Mancini, K. Kolstad, V. Zarate and S. Ferro-Novick, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510. (Spon. by P. De Camilli.)

BET1 and *SEC22* genetically interact with each other and with a third gene, *BOS1*, to execute a common aspect of ER to Golgi transport (Newman, et al., 1990, MCB 10: 3405-3414; Shim, et al., 1991, JCB 113: 55-66) The *bet1* and *sec22* mutants are phenotypically similar in that they accumulate ER and small vesicles when they are shifted to their restrictive growth temperature. While screening a yeast genomic library for genes that suppress *bet1* and *sec22*, we identified three high copy suppressors (*BOS2*, *BOS3* and *BOS4*) of these mutants. The *BOS2* gene (bet one suppressor) is the best suppressor of this group of genes and it also suppresses several other mutants that block transport from the ER to the Golgi apparatus. DNA sequence analysis of *BOS2* has revealed that this 68KD hydrophilic protein is not a previously identified gene required for secretion in yeast. Currently, we are raising antibody to Bos2 and sequencing the other high copy suppressors. We are also determining the phenotypic consequences of depleting yeast cells of the *BOS2* gene product.

Additionally, we are utilizing a synthetic lethal screen to identify other proteins that interact with Bet1. Synthetic lethality results when two non-lethal mutations are combined in the same haploid strain and the resulting double mutant fails to grow. Thus far, our studies have led to the isolation of several new temperature-sensitive secretory mutants that we are presently analyzing.

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GTP hydrolase purified from endoplasmic reticulum is inhibited by retinol. J. Zhao[1], D. J. Morré[2] and D.M. Morré[1]. [1]Department of Foods and Nutrition and [2]Department of Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907.

The formation of transition vesicles from transitional endoplasmic reticulum is stimulated by all-trans retinol (Nowack et al., Biochim. Biophys. Acta, 1051, 250, 1990). The retinol stimulation is favored under conditions of low GTP levels suggesting an interaction between retinol and a GTP-requiring step in the vesicle formation process. A GTP hydrolase was purified from transitional endoplasmic reticulum by DEAE-cellulose column chromatography, gel filtration chromatography, HPLC and native gel electrophoresis that was inhibited by all-trans retinol with a K_i of 0.03 mM. Binding of all-trans retinol to the purified GTP hydrolase, as determined by equilibrium dialysis, was with a k_d of ca. 0.1 nM and a stoichiometry of ca. 1 mole retinol bound per mole of purified protein. The specific activity of the 300- to 400-fold GTP hydrolase was 275 µmole/h/mg protein. Two peptides of differing mobility on non-denaturing gels were identified using a GTPase activity stain as being inhibited by retinol. On SDS-PAGE, the two peptides yielded similar molecular weights of 55 kD. Cyanogen bromide fragments of the two peptides exhibited no obvious sequence homology with any previously described retinoid binding protein. Supported in part by NIH GM 44675 and Phi Beta Psi Sorority.

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Isolation, Characterization, and Partial Sequencing of the Principal ATPase Activity of Transitional Endoplasmic Reticulum from Rat Liver. L. Zhang and D.J. Morré, Department of Medicinal Chemistry, Purdue University, West Lafayette 47907

Transition vesicle formation from transitional endoplasmic reticulum in a cell-free system from rat liver is an ATP-dependent process. In order to begin to characterize some of the functional molecules involved in the transitional vesicle formation, the principal ATPase activity of transitional endoplasmic reticulum has been characterized. Unique inhibitor specificities and ion requirements distinguish the ATPase activity from that of other ATPases present in liver homogenates. The ATPase activity was purified as a single band of 100 kDa on SDS-PAGE. A 25 amino acid sequence obtained by digestion of the 100 kDa with cyanogen bromide exhibited 80% identity to p97-ATPase. This 25 amino acid sequence shared 55% identity with cell division control protein (CDC 48). Both p97-ATPase and CDC 48 display homology to the mammalian N-ethylmaleimide sensitive fusion protein (NSF) and yeast Sec 18p which are essential for fusion in secretory process. Supported by NIH GM44675.

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A Highly Conserved Sequence near the Carboxyl Terminus of Serpins Effects The Transport of A1Pi From The Endoplasmic Reticulum To The Golgi Apparatus. **R.M. Brodbeck and J.L. Brown, Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262.**

In the human disease α1-proteinase inhibitor deficiency, some variants of human α1-proteinase inhibitor (A1Pi) are poorly secreted, and others are not secreted at all. To examine possible causes for defective secretion we altered the A1Pi cDNA to encode a series of C-terminally truncated forms of this protein. Examination of the fates of these shortened proteins in transiently transfected Cos 1 cells via pulse-chase followed by immunoprecipitation, SDS PAGE and phosphor-imagery, shows that truncation prior to Pro^{391} (total length is 394 amino acids) prevents movement from the endoplasmic reticulum (ER) to the Golgi apparatus and therefore secretion. Comparison of the primary sequences of the C-terminal regions of all serine protease inhibitors (serpins) reveals a highly conserved region from residues 383 through 391 (A1Pi numbering). Saturation mutagenesis within this conserved region, and subsequent analysis as indicated above, show that amino acids of specific classes are required at specific positions of A1Pi. Our results from the expression of a series of Pro^{391} point mutants in Cos 1 cells suggests that a hydrophobic residue is required at amino acid 391 for movement from ER to Golgi. Analysis of a series of variants resulting from mutations of the codon specifying Lys^{387} show that the putative salt bridge between Lys^{387} and Glu^{264} is not required for efficient secretion. Replacement of Lys^{387} with other polar residues, including Arg and Glu, severely depressed A1Pi's transport from ER to Golgi. These data are consistent with a model which has the conserved C-termini of serpins playing a key role in ER to Golgi transport. Studies are underway to determine if this conserved region plays a similar role for other members of the serpin superfamily of proteins.

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Nanomelic Chondrocytes Synthesize, but Fail to Translocate, a Truncated Aggrecan Precursor. B.M. Verzel, L.M. Walters, B.L. Grier, N. Maine and P.F. Goetink.* Department of Cell Biology and Anatomy, The Chicago Medical School, No. Chicago, IL. 60064 and *Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129.

Cartilage extracellular matrix (ECM) is composed primarily of type II collagen and chondroitin sulfate proteoglycan (aggrecan). Maturation and function of these complex macromolecules are dependent upon sequential processing events which occur during their movements through specific subcellular compartments in the secretory pathway. Failure to complete these events results in assembly of a defective ECM and may produce skeletal abnormalities. *Nanomelia* is a lethal genetic mutation of chickens characterized by shortened and malformed limbs. Biochemical studies show that cultured nanomelic chondrocytes synthesize a truncated aggrecan core protein precursor that disappears with time; however, the protein does not appear to be processed by the Golgi or secreted. More recent studies investigate the intracellular trafficking of the defective aggrecan precursor using immunofluorescence, immunoelectron microscopy and several inhibitors. Results indicate that nanomelic chondrocytes assemble an ECM that contains type II collagen, but lacks aggrecan. Instead, aggrecan precursor is localized intracellularly, within small cytoplasmic structures corresponding to extensions of the endoplasmic reticulum (ER). At no time are precursor molecules observed in the Golgi. In contrast, normal and nanomelic chondrocytes exhibit no difference in the intracellular or extracellular distribution of type II procollagen. Therefore, retention of the aggrecan precursor appears to be selective. Incubation of chondrocytes at 15°C results in the retention and accumulation of product in the ER. After a return to 37°C, translocation of the product to the Golgi is observed for normal, but not for nanomelic chondrocytes, although the precursors disappear with time. Ammonium chloride, an inhibitor of lysosomal function, has no effect on protein loss, suggesting that the precursor is removed by a non-lysosomal mechanism, possibly by ER-associated degradation. Based on these studies, we suggest that the nanomelic chondrocyte is a useful model for examining cellular trafficking and sorting events and the processes by which abnormal products are targeted for retention or degradation. Further investigations should provide insights into the mechanisms underlying chondrodystrophies and other related diseases. (Supported by NIH grants AM 28433, HD 22016.)

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A Novel Germ Cell Associated Domain of the Sertoli Cell Central Vacuolar System May Regulate Protein Secretion. K. Johnson, and K. Boekelheide. Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912.

Germ cell regulation of Sertoli cell protein secretion has been widely observed although little is known of its mechanism. Using the fungal metabolite Brefeldin A (BFA), we have revealed a unique compartment within the Sertoli cell central vacuolar system. Following 45 min BFA exposure, the Sertoli cell Golgi complex (GC) is specifically resorbed to areas immediately surrounding elongate spermatid heads using the GC antibody marker Mannosidase II on testis frozen sections. This compartment is likely endoplasmic reticulum associated with complex Sertoli cell/germ cell adhesion junctions termed ectoplasmic specializations. Colchicine treatment prior to BFA exposure results in Mannosidase II immunostaining throughout the Sertoli cell indicating that GC transport to this compartment is microtubule dependent. Following washout of BFA, the Sertoli cell GC returns to its normal intracellular location within the basal cytoplasm. We propose that this novel Sertoli cell compartment is involved in germ cell paracrine regulation of Sertoli cell protein secretion.

1290

GAF: Factors Which Potentiate ARF Function Within the Golgi Complex. T. C. Taylor and P. Melançon. Dept. of Chemistry and Biochemistry; University of Colorado; Boulder, CO 80309-0215.

Protein transport through the Golgi complex *in vitro* is regulated by GTP-dependent Golgi Binding Factors (GGBFs) which are members of the ARF family of GTP-binding proteins. Two soluble proteins of 20 kDa were purified by their ability to mediate a GTPγS-dependent inhibition of transport ("GGBF-activity"; Taylor et al., (1992) *Cell*, **70**, 69). Poor yields of GGBF-activity during purification prompted us to investigate the existence of additional factors involved in regulating intra-Golgi transport. Here we describe the partial characterization of GAF, or GGBF-Associated Factor, which potentiates the activity of GGBF. GAF-GGBF interaction may be monitored by performing GGBF assays in the presence of GGBF at a concentration below the sensitivity of the GGBF assay. Upon the addition of fractions containing soluble proteins including GAF, a strong GTPγS-dependent inhibition is observed. Using the soluble fraction (cytosol) from bovine brain, GAF can be localized exclusively to a high-salt-eluting fraction by ion-exchange chromatography. Furthermore, cytosolic GGBFs and GAF can be completely separated by this technique. By combining a fraction containing GGBFs with one containing GAF, GGBF-activity equivalent to that measured in whole cytosol can be reconstituted. By size-exclusion chromatography GAF elutes at an apparent M_r of > 200 kDa and is most likely a complex of distinct proteins. The possibility that unique GAFs interact preferentially with different ARF proteins is being investigated.

1291

Binding of the p200 coat protein to Golgi membranes is regulated by pertussis toxin sensitive G proteins.

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We have previously described a 200kd, brefeldin sensitive, phosphoprotein (p200) which is found predominantly in the cytosol and associated with Golgi membranes. The role of heterotrimeric G proteins in binding p200 to Golgi membranes was investigated by in vitro incubation of highly purified rat liver Golgi membranes with cytosol in the presence of AlFn, mastoparan and GTPγS to activate G proteins. Activation of heterotrimeric G proteins with AlFn and mastoparan induced binding of 30-40% of the cytosolic p200 onto Golgi membranes; in the presence of GTPγS 100% of p200 was bound to the membranes. Intact cells treated with AlFn showed altered immunostaining of p200, seen as depletion of cytoplasmic staining with more intense staining of the Golgi complex. Pertussis toxin specifically ADP-ribosylates Gαi subunits, such as the $G\alpha_{i-3}$ which is on Golgi membranes. Pertussis toxin pretreatment partially blocked the AlFn-induced binding of p200 to membranes, both in the in vitro assay and in intact cells. These results show that pertussis toxin sensitive heterotrimeric G proteins on Golgi membranes are involved in the binding of p200 protein onto Golgi membranes. This provides further evidence that p200 is similar to other coat proteins, β-COP and γ-adaptin, suggesting that p200 may also be involved in Golgi vesicle trafficking.

1292

Staphylococcal ADP-ribosyltransferase-sensitive small G protein is involved in brefeldin A action. C.-H. Chen, M. Sugai, and H.C. Wu.

Department of Microbiology, Uniformed Services University of the Health Sciences, MD 20814-4799.

Brefeldin A (BFA) is a fungal antibiotic which inhibits protein secretion and disintegrates the Golgi apparatus, resulting in the redistribution of the Golgi proteins into the ER in mammalian cells. An early event in the action of brefeldin A is the dissociation of the 110-kD protein from the Golgi membrane. We recently found that staphylococcal ADP-ribosyltransferase (EDIN) with substrate specificity of a member of small G protein, *rho*, disassembles the Golgi apparatus and dissociates the 110-kD protein from the Golgi apparatus in Vero cells. Three independent BFA-resistant cell lines (BER-40 from Vero cells, PtK_1, and MDCK) showed cross resistance to EDIN regarding the release of the 110-kD protein and other Golgi markers from the Golgi membrane by EDIN or BFA. It has been shown that ADP-ribosylation of *rho* protein induces the disassembly of actin microfilaments. BFA as well as EDIN induced the disassembly of the actin microfilaments in Vero cells, and they both failed to induce the disassembly of actin microfilaments in BER-40, PtK_1, and MDCK cells. EDIN differed from BFA in its effect on protein secretion in Vero cells; BFA inhibited protein secretion in Vero cells but not in BFA-resistant cell lines, whereas EDIN did not inhibit protein secretion in either Vero or other cell lines. AlF_4^- which has been shown to activate a trimeric G protein but not small G proteins of the *ras* superfamily, inhibited the effect of EDIN on the distribution of the 110-kD protein. Taken together, these results suggest that an EDIN-sensitive *rho* protein is one of the biochemical targets of BFA in Vero cells, and this small G protein together with a trimeric G protein is involved in the regulation of the assembly of coated vesicles and vesicular transport in the Golgi apparatus.

Organization of Extracellular Matrix (1293-1296)

1293

The α5β1 integrin fibronectin receptor, but not the α5 cytoplasmic domain, is required for fibronectin matrix assembly. C. Wu[1], J. Bauer[2], R. L. Juliano[2], and J.A. McDonald[1], [1]Mayo Clinic Scottsdale, AZ 85259, [2]Dept. Pharmacology, Univ.North Carolina, Chapel Hill, N.C.

The α5β1 integrin mediates cell adhesion and migration on fibronectin (FN) matrices. We used a molecular genetic approach to definitively examine a more controversial role of α5β1 integrins, that of FN matrix assembly. Mutant CHO cells deficient in α5 integrin expression could not assemble a FN matrix. Reconstituting α5β1 integrin expression by transfecting them with a full length cDNA encoding the human α5 chain restored FN matrix assembly ability. Thus, α5β1 integrins are required for an initial, critical step in matrix assembly. Cells expressing an α5 chain lacking the cytoplasmic domain also assembled a FN matrix. To form a FN matrix, cells must bind FN's 29 kDa domain, a site distinct from the RGD containing integrin binding domain. The α5 deficient cells were also deficient in 29 kDa binding. We conclude that α5β1 integrins not only mediate cell adhesion to FN, but also play an essential role in the assembly of a FN matrix. This role includes direct binding to FN, and modulating a distinct binding event involving the interaction of FN's aminoterminal matrix assembly domain with the cell surface. In this novel system, a low avidity integrin receptor appears to facilitate the activity of a distinct cell-matrix recognition system of much higher avidity that leads to matrix assembly on the cell surface.

1294

Collagen Fibril Assembly And Deposition During Extracellular Matrix Morphogenesis. D.E. Birk and E.I. Zycband, Department of Pathology, Robert Wood Johnson Medical School, Piscataway, NJ.

Collagen fibril organization is directly related to tissue function. Discrete fibril segments are assembled and deposited into developing connective tissues. A lateral and/or linear fusion of segments may be responsible for the formation of mature, continuous fibrils. This would produce a change in fibril diameter and/or length with development. Fibril segments were isolated from 12 to 17-day chick embryo metatarsal tendons. Tendons were gently homogenized in PBS with protease inhibitors, which almost completely disrupted the 12 to 15-day tendons. Transmission electron microscopy demonstrated intact segments of discrete length. Between days 12-15 of development, mean segment length increased from ~22 to 33 μm. The incremental increase in length with development indicates a limited linear fusion of segments. Morphological examination of extracted segments support this suggestion. At 16 days of development there was a significant decrease in segment extratability and by 17 days intact segments were nearly unextractable. Mean segment lengths were 37 and >70 μm for 16 and 17 day tendons respectively. During this period fibril diameter also increased. These data demonstrate both lateral and linear fusion of segments. This also was supported by morphological data. In all cases, the segment ends were asymmetric as described in situ. These observations are consistent with in situ data, indicating an increase in fibril length between 14 and 18 days of development. Similar increases in segment length were observed in chick embryo cornea and dermis. These data indicate that segments are precursors in fibril formation in a variety of soft connective tissues. The post-depositional lateral and/or linear fusion of fibril segments are important processes allowing for orderly development and growth.

1295

Regulation of Collagen Fibrillogenesis in vitro by Cartilage Matrix Protein. M. M. Tondravi, D. Haudenschild, and P. F. Goetinck. Cutaneous Biology Research Center, MGH/Harvard Medical, Charlestown, Ma. 02129.

The extracellular matrix of cartilage is a highly specialized tissue deposited by chondrocytes with the principal property of being highly compressible and having high tensile strength. The compressibility is mediated by the ternary complex of aggrecan, link protein and hyaluronic acid while tensile strength of the tissue is imparted by the collagenous fibers. In an effort to understand the molecular interactions that occur in the extracellular matrix of cartilage, we are investigating the role of cartilage matrix protein (CMP) in the organization of the collagen fibrils. CMP is a disulfide bonded multimeric protein. The monomer (54 kDa) consists of two homologous 190-amino acid domains (CMP domains) that are separated by a central ~40 amino acid region with homology to an EGF-precursor motif. In a previous publication, we demonstrated by ELISA that CMP binds to collagen (Winterbottom et al. 1992. Dev. Dyn., 193:266.) and that CMP binds to collagen in a periodic manner which is consistent with its binding to ends of the collagen molecules that make up the fibril. Experiments on the effects of CMP on collagen fibrillogenesis in vitro demonstrate that the addition of purified CMP to type II collagen molecules increases the extent of collagen fibril formation as measured by change in absorbance at 400 nm. The effect of CMP on in vitro collagen fibrillogenesis is concentration dependent, and the fibrils formed in the presence of CMP appear thinner than those formed in the absence of CMP as visualized by EM. The effects of the addition of bacterially expressed full-length CMP as well as various deletions of CMP on in vitro collagen fibrillogenesis will also be presented. Immunoelectron microscopy is being used to determine if the CMP in the in vitro fibrils is organized in a periodic manner analogous to the in vivo pattern established by primary chondrocytes. (Supported by NIH grant HD 22016 and the Arthritis Foundation)

1296

Coordinated Regulation of Alternative Splicing of Fibronectin Pre-mRNA at ED-A and ED-B Regions. H.Hirano, T.Tachikawa, F.Oyama[1], K.Titani[1], and K.Sekiguchi[2]

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Molecular diversity of fibronectin (FN) results from alternative RNA splicing at two regions, termed ED-A and ED-B. We have shown that the alternative splicing at these regions is regulated in an oncodevelopmental and organ-specific manner. The expression of the ED-A$^+$ and/or ED-B$^+$ isoforms is significantly increased in fetal and tumor tissues when compared with normal adult tissues. It is not clear, however, whether the splicing at the ED regions within a single FN pre-mRNA is regulated independently or coordinately. In order to address this question, we have prepared monoclonal antibodies against these ED regions and developed ELISA systems specific for ED-A$^+$ and ED-B$^+$ isoforms. By combination of immunoaffinity chromatography using anti-ED-A monoclonal antibody and these ELISA, we have fractionated placental FN into ED-A$^+$ and ED-A$^-$ isoforms and examined whether the ED-B$^+$ isoform was cosegregated with the ED-A$^+$ isoform. We found that the ED-B$^+$ isoform was three fold enriched in the bound ED-A$^+$ fraction while it was completely depleted from the unbound ED-A$^-$ fraction. Similarly, the ED-B$^+$ isoform was cosegregated with the ED-A$^+$ isoform, when the "cellular FN" isolated from the spent medium of human fibroblast was fractionated. These results strongly suggest that the alternative splicing of FN pre-mRNA at two ED regions is coordinately regulated within a single pre-mRNA, thus the ED-B exon is preferably included in the mRNA with the ED-A exon than in the mRNA without it.

1297

Thrombospondin 1 and Thrombospondin 2 are Expressed as Both Homo and Heterotrimers. K. M. O'Rourke, C.D. Laherty, and V.M. Dixit, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109.

There exist two distinct thrombospondin molecules (designated TSP1 and TSP2) which are encoded by separate genes. TSP1 is a *trimeric* cell surface and extracellular matrix molecule. Sequence comparison reveals that the two cysteines involved in interchain disulfide linkage and trimer assembly in TSP1 are conserved in TSP2 (Laherty, C.D., O'Rourke, K.M., Wolf, F.W., Katz, R.M., Seldin, M.F., and Dixit, V.M. (1992) *J. Biol. Chem.* 267:3274-3281). Swiss 3T3 fibroblasts express both TSP1 and TSP2, and, therefore, an important question is whether TSP in such cells is expressed as homotrimers or as heterotrimers. We find that Swiss 3T3 cells and epithelial cells transfected with TSP expression vectors express both homo and heterotrimeric forms of TSP. In addition, homotrimeric TSP2 has a lower heparin affinity for heparin than homotrimeric TSP1. Thus, the heparin affinity of TSP can be modulated by the expression of TSP as homo or heterotrimers.

1298

A PCR Search for Tenascin in Fish and Invertebrates. B.K. Maddox and H.P. Erickson, Department of Cell Biology, Duke University Medical Center, Durham, NC 27710. (Sponsored by H.P. Erickson.)

Tenascin is a prominent extracellular component of developing embryonic tissues, healing wounds, and tumors. The biological functions of tenascin remain elusive, although the pattern of expression suggests a role in the cell-matrix interactions of growing and restructuring tissues. While tenascin has been observed in most vertebrates, a more manipulable genetic system with which to investigate function would be highly desirable. Towards this goal, we have screened for the gene in an assortment of organisms using the polymerase chain reaction (PCR). Degenerate oligonucleotide primers were designed for each of the four types of structural domains of tenascin from published sequences of human and chick cDNA clones. Paired segments of conserved amino acids were identified that corresponded to ~20 base pairs, and were separated by 150-500 base pairs. The four different pairs of primers were used for PCR amplification of genomic and cDNA templates isolated from fish, tunicate, octopus, sea urchin, *Drosophila*, and *C. elegans*. This technique demonstrated the expression of tenascin in goldfish brain using a primer pair within TNfn4 (fibronectin type III, repeat #4) and another pair from the EGF-like repeats. PCR primers that recognized TNfn12, an alternatively spliced domain, and the central crosslinking region, were unsuccessful in amplifying template from fish, in contrast to control mouse brain tissue and cultured chick fibroblasts. Although several primers amplified bands of the expected size from the invertebrate species screened, no sequence related to tenascin has been identified. One possible conclusion is that these invertebrates may not have the gene for tenascin.

1299

Link Protein is a ubiquitous structural component of non-cartilaginous tissues. F. Binette, J. Cravens and P. F. Goetinck. Cutaneous Biology Research Center, MGH-Harvard Medical School, Charlestown, MA 02129

Link Protein (LP) is a member of the hyaladherins, a category of proteins defined by their ability to bind to hyaluronic acid (Toole,B., Curr. Op. Cell Biol. 2:839; 1990). Hyaladherins share structural homologies to the tandem repeats of LP, the region responsible for hyaluronic acid binding. In the ECM of cartilage, LP stabilizes the interaction of the proteoglycan aggrecan with hyaluronic acid. Although cartilage contains large amounts of LP, the protein has also been reported in the aorta (Gardell et al., BBRC 95:1823; 1980), the mesonephros (Stirpe et al., Dev. Biol. 137:419; 1990), eyes (Poole et al., JCB 93:910; 1982) and brain (Ripellino et al., JCB 108:1899; 1989). In the present study, using the RT-PCR technique, we report on the wide distribution of LP mRNA in a large number of embryonic tissues. We also identified on western blots the LP translation product in these tissues using a new monoclonal antibody (4B6/A5) that recognizes LP. LP mRNA and protein were found in dorsal skin, kidney and aorta from 10 day old embryos, in brain, heart, proventriculus, gizzard, intestine, liver from 10 and 15 day old embryos and in pectoral muscle, ceca, calvaria and sterna from 15 day old embryos. Using the new monoclonal antibodies 4B6/A5 and 3H8/C3 in indirect immunofluorescence, we localized LP on frozen sections of chicken embryos. In addition to cartilage, aorta, kidney and eyes, reactive material was found in the connective tissue along the entire digestive tract from the esophagus to the large intestine. The finding of LP mRNA and its translation product in a wide variety of tissues suggests that this protein may also play an important structural role in the stabilization of extracellular matrix of non-cartilaginous tissues. The interaction of LP with molecules in a non-cartilaginous environment is currently under investigation. (Supported by NIH grants HD 22016 and HD 22050.)

1300

Isolation and Partial Characterization of a new Fibrillin-like Protein M.A. Gibson, E. Davis, M. Filiaggi, and R.P. Mecham, Department of Pathology, University of Adelaide, Adelaide, Australia, and Departments of Cell Biology & Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.

While screening nuchal ligament cDNA libraries with antibodies to MP340 (Fibrillin-15), a partial cDNA for a fibrillin-like protein (clone cL9) was identified, isolated and sequenced. Northern blotting of nuchal ligament RNA demonstrated hybridization of the isolated clone to two large mRNAs of 9-10 kb and 8 kb. DNA sequencing revealed in cL9 an open reading frame coding for a protein that contained multiple EGF-like repeats and several motifs containing 8-cysteine repeats that contain the sequence CCC. On comparison with other fibrillin sequences, the isolated clone demonstrated the same motif structure as Fib15 and Fib5 but differed in individual sequence within the motifs. Using the cL9 clone for additional library screening resulted in the isolation of several clones that exhibited complete homology with the 5' region of cL9 but their sequences diverged at the same point to give a different 3' region which was over 1kb longer than that of cL9. These results suggest alternative splicing of the cL9 transcript, which would also explain the existence of two sizes of cL9 mRNA detected by northern analysis. Evidence supporting the identification of the cL9 protein as a fibrillin includes similarity in domain structure, immunogold localization of peptide antibodies specific for cL9 to elastic fiber microfibrils and a strong correlation between cL9 expression and expression of the elastin phenotype in developing tissues.

1301

Fibulin is a Component of Connective Tissue Microfibrils. E. F. Roark, S. Godina, M. Mattei, H. Tran, and W.S. Argraves. Biochemistry Laboratory, American Red Cross, Rockville, MD 20855.

Fibulin is an extracellular matrix and blood glycoprotein. As part of our efforts to determine the function of this protein we have examined its pattern of expression in various tissues by immunohistochemical analysis using monoclonal antibodies. Fibulin was found to be widely distributed in connective tissues such as in the dermal layer of skin, the embryonic connective tissues of the umbilical cord and basement membranes such as that underlying the amnion epithelium. Fibulin staining was particularly evident in fibrous structures such as those found in the dermis of skin and the smooth muscle layers of blood vessel walls. The fibers in skin reactive with fibulin antibodies also stained with elastin antibodies and elastic Van Giesen stain indicating that they contained elastin. These fibers were also positive using fibrillin antibodies. The results suggest that fibulin is a component of connective tissue microfibrils and highlight the potential for interactions between fibulin and other microfibril glycoproteins including elastin, fibrillin, gp115 and MAGP. Supported by NIH grant GM42912 (WSA) and NRSA grant HD07515 (EFR).

1302

Domain Interactions in Elastin Crosslinking and Fiber Assembly P. L. Brown, T. Broekelmann, C. Sloan, and R.P. Mecham, Departments of Cell Biology and Medicine, Jewish Hospital at Washington University School of Medicine, St. Louis, MO 63110.

Assembly of tropoelastin into polymeric elastin requires oxidative deamination of lysyl ε-amino groups yielding active aldehydes that condense to form covalent crosslinkages between tropoelastin molecules. To investigate which domains of tropoelastin align during crosslink formation, we isolated and sequenced crosslink-containing peptides from insoluble elastin obtained from copper-deficient pig aorta (a gift from Dr. Larry Sandberg) with the hypothesis that the crosslinks formed in insoluble elastin would be those primary in tropoelastin polymerization. Purified insoluble elastin was treated with NaB^3H_4 to radiolabel reducible crosslinks and was then solubilized by successive treatments with trypsin and chymotrypsin. Solubilized peptides were fractionated by reverse phase HPLC. Cross-link containing peptides were rechromatographed by reverse phase HPLC and sequenced by automated Edman degradation . Preliminary data suggests that most of the crosslinks that form in copper deficient elastin are in the amino terminal half of the tropoelastin molecule. One desmosine-containing peptide contained sequences from exon 6 and exon 10. Exon 6 contains the only alanine-rich crosslinking domain in the amino terminal end of the molecule while exon 10 contains two lysines separated by a single proline. Although it was not possible to determine whether the two sequences derive from the crosslinking of different tropoelastin molecules or from crosslinking between two sites within a single molecule, these results imply that the amino terminal half of tropoelastin may contain important nucleation sites for tropoelastin crosslinking.

1303

Modification of Tropoelastin Cross-link Domains during Elastic Fiber Formation. E.C. Davis, P.L. Brown and R.P. Mecham. Departments of Cell Biology and Medicine, Jewish Hospital at Washington University School of Medicine, St. Louis, MO 63110.

The formation of cross-links in mature elastin is critical for the characteristic properties of elastogenic tissues. Although numerous alanine- and lysine-rich cross-link domains have been identified in the tropoelastin molecule, the inter- and intramolecular organization of these cross-links remains to be established. In an attempt to study the formation of elastin cross-links in developing elastic fibers, synthetic peptides corresponding to putative cross-link and non-cross-link domains of bovine tropoelastin were made and used to generate polyclonal antisera. All antisera raised to the synthetic peptides demonstrated positive immunoreactivity to tropoelastin by Western blot analysis. To investigate the modification of the tropoelastin cross-link domains in developing elastic fibers, the antisera were localized by immunoelectron microscopy in fetal bovine aorta and ligamentum nuchae. Antisera raised against non-cross-link domains of tropoelastin showed strong positive immunoreactivity over the insoluble elastin of elastic laminae and fibers. Similar immunoreactivity was observed with an antiserum raised to a cross-link domain at the carboxy terminal end of the tropoelastin molecule. In contrast, antisera raised to several different cross-link domains in the amino terminal end of the molecule showed little or no immunoreactivity over elastic laminae and elastic fibers. Although the extracellular localization of the antisera to the cross-link domains showed contrasting results, all antisera showed similar positive intracellular labeling. These results suggest that upon secretion of the tropoelastin the antigenicity of the amino terminal cross-link domains is rapidly alterated by the oxidation of lysyl residues during cross-link formation.

1304

Elastase Activity of Lysostaphin and Its Homology to Metalloenzymes P.W. Park, G.L. Griffin, M.S. Mudd, R.M. Senior, and R.P. Mecham. Departments of Cell Biology and Medicine, Jewish Hospital at Washington University School of Medicine, St. Louis, MO 63110.

Lysostaphin is a bacterial zinc metalloenzyme that degrades bacterial cell wall peptidoglycans through proteolysis of the glycyl-glycyl cross-link. In using lysostaphin to solubilize staphylococcal cell wall components for isolation of elastin-binding proteins, we found that lysostaphin both binds to and degrades purified insoluble elastin. Specific elastin binding was demonstrated through elastin affinity chromatography, and the elastase activity of lysostaphin was determined using a [^{3}H]elastin release assay. Similar to the staphylolytic activity, the elastolytic property of lyso-staphin is pH-dependent and is inhibited by zinc and EDTA. Interestingly, elastolytic activity was not inhibited by trypsin treatement, which has been shown to inactivate the enzyme's staphylolytic properties. Although the mechanism of zinc inhibition is unknown, exogenously added zinc does not inhibit the ability of the enzyme to bind to elastin. This result suggests distinct substrate recognition and catalytic domains on lysostaphin. The first five amino acids, AATHE, of mature lysostaphin have perfect homology with an internal sequence of matrilysin (PUMP-1, a metalloenzyme with elastase activity). This sequence has been hypothesized to play a role in zinc binding and to define a portion of the enzymatic active site. Similar sequences are also present in other metalloenzymes such as stromelysin, collagenase and Pseudomonas elastase. Whether the amino terminal sequence of lyso-staphin contains the zinc binding and elastolytic domains remains to be established. However, based on enzymatic properties and sequence homology, lysostaphin may belong to the growing family of matrix metalloproteinases with elastase activity.

1305

Developmental Expression of Microfibrillar Components in Fetal Bovine Aorta and Nuchal Ligament. M.C. Filiaggi, M.A. Gibson, J. Rosenbloom, and R.P. Mecham. Department of Cell Biology & Medicine, Jewish Hospital at Washington University Medical Center; Department of Pathology, University of Adelaide; and Department of Anatomy-Histology, University of Pennsylvania School of Dental Medicine.

The expression of four putative microfibrillar components - fibrillin 15 (Fib 15), MAGP, MP70, and a unique fibrillin-like protein (clone cL9) - was assessed during fetal development (120 to 270 days, gestation = 270 days) in bovine aorta and nuchal ligament and compared to the pattern of tropoelastin expression over the same period. Northern analysis of total tissue RNA using cDNA probes for each component revealed that in both tissues the patterns of Fib 15 and cL9 expression are similar. In ligament, message levels for both proteins increased 3 to 5 fold over the last two trimesters to a peak at 270 days, preceding the rise in tropoelastin expression by 20 days. In the aorta, where elastin synthesis begins earlier than in ligament, Fib 15 and cL9 levels peaked by mid gestation and decreased through the third trimester. As was observed in the ligament, this increased expression occurred before changes in tropoelastin expression. The message for MAGP was constant at a high level in both tissues during this period, while the that for MP70 was constant at low levels. The similar developmental pattern of Fib 15 and cL9 expression suggest that both may contribute to the structure of elastin-associated microfibrils; the temporal relationship between the expression of these components and tropoelastin during fibrillogenesis supports the hypothesis that microfibrils may nucleate the formation of elastin fibers.

1306

Fibronectin and Laminin Synthesis by Type II Pulmonary Epithelial Cells. S. E. Dunsmore and D. E. Rannels, Dept. of Cellular & Molecular Physiology, Penn State Univ. College of Medicine, Hershey PA 17033.

Bidirectional interactions of type II pulmonary epithelial cells (T2Ps) with the extracellular matrix (ECM) affect both cell differentiation and ECM composition. The following studies focus on the relative synthesis of fibronectin (FN) and laminin (LAM) in matrix fractions from T2P primary cultures. Relative synthesis rates were examined between *day 1* and *day 3* of culture. During this interval, T2Ps undergo significant changes in differentiation and the proportion of total cell protein synthesis devoted to the matrix fraction is regulated, reaching a peak at *day 2*. Matrix fractions were obtained by treatment of T2Ps with 0.25M NH_4OH. [^{35}S]methionine-labeled proteins in the matrix fraction were analyzed by immunoprecipitation, SDS-PAGE, and fluorography. Fluorographic analysis of the matrix fraction on *day 1* shows a predominant band at 50 kD. On *day 3*, the matrix fraction contains predominant bands at 220 kD and 50 kD with several less predominant bands between 220 kD and 50 kD. These intermediate molecular weight bands are more prevalent in continuous labeling protocols than in pulse labeling protocols. Fluorographic analysis of matrix fractions immunoprecipitated with anti-FN (Collaborative Biomedical) reveals a predominant band at 220 kD on *day 1* and predominant bands at 220 kD and 46 kD on *day 3*. When immunoprecipitated with anti-LAM (gift of Dr. D. J. Carey), predominant bands at 200 kD and 46 kD are observed on both *day 1* and *day 3*. The relative synthesis of both FN and LAM is greater on *day 3* than on *day 1*. These results indicate that the relative synthesis of FN and LAM is regulated as a function of time in primary culture and suggest that bidirectional T2P-ECM interactions play a role in the regulation of FN and LAM synthesis.
Supported by HL-31560 and HL-07223.

1307

The Substrate Can Affect the Net Synthesis/Retention and Secretion of Laminin in NHEK Cells J. R. Cook and R. G. Van Buskirk. Department of Biological Sciences, State University of New York at Binghamton. Binghamton, New York 13902-6000.

The factors needed to elicit the synthesis and secretion of basement membrane components are not known. Using SDS PAGE, immunoprecipitation and autoradiography, we explored the possibilities that the type of underlying substrate coupled with the arrangement of the cytoskeleton can affect the synthesis/retention and secretion of laminin. When Normal Human Epidermal Keratinocytes (NHEK) cells are grown on cross-linked Type I collagen gels and Type I collagen-coated Millipore Millicell CM microporous cell culture inserts, they exhibit a marked increase in beta laminin synthesis/retention but not alpha laminin synthesis as compared to conventional plastic-grown NHEK cells. These substrates also regulated the apical and basal release of beta laminin. This increase in the synthesis/retention of the beta laminin may be related to the substratum-directed arrangement of the cytoskeleton because the microtubule-inhibiting drug, nocodazole, further changes the amount of beta laminin detected. This observation is not apparent when NHEK cells are grown on conventional plastic substrata. We conclude that the substrate can influence laminin synthesis/retention and secretion and this may be, in part, regulated by the arrangement of the cytoskeleton.

1308

Apparent Value for the Viscosity of the Pericellular Matrix. G.M. Lee, F. Zhang, A. Ishihara, and K.A. Jacobson. Dept. of Cell Biology and Anatomy, Univ. of North Carolina, Chapel Hill, NC 27599.

At the interface of the plasma membrane and the extracellular matrix is the pericellular matrix where the extracellular domains of plasma membrane molecules intermingle with extracellular matrix molecules. The structure and physical properties of the pericellular matrix were probed with Nanovid microscopy (video- enhanced microscopy of 30 nm colloidal gold particles) and gold- labeled lipids. Gold labeled lipids diffuse freely in artificial planar membranes (Lee et al., PNAS 88:6274, 1991). With cells, the gold must move through the pericellular matrix as the attached lipid diffuses in the plasma membrane. The diffusion of the gold labeled lipids will be restricted if there are filamentous barriers or extensive cross-linking within the pericellular matrix. Fluorescein-phosphatidylethanolamine, incorporated into the plasma membranes of cultured fibroblasts, epithelial cells and keratocytes, was labeled with 30 nm colloidal gold conjugated to anti-fluorescein. The trajectories of the gold-labeled lipids were used to compute diffusion coefficients (D_G) and to test for restricted motion. On the cell lamella, the gold-labeled lipids diffused freely in the plasma membrane with no evident restriction. This result suggests that any extensive filamentous barriers in the pericellular matrix are at least 40 nm from the plasma membrane surface. The average diffusion coefficients ranged from 1.1 to 1.7 x 10^{-9} cm^2/sec. These values were lower than the average diffusion coefficients (D_F) (5.4 to 9.5 x 10^{-9} cm^2/sec) obtained by fluorescence recovery after photobleaching. The lower D_G is partially due to the pericellular matrix as demonstrated by the result that heparinase treatment of keratocytes significantly increased D_G to 2.8 x 10^{-9} cm^2/sec, but did not affect D_F. Pericellular matrix viscosity was estimated from the frictional coefficients computed from D_G and D_F and ranged from 0.5 to 0.9 poise for untreated cells. Heparinase treatment of keratocytes decreased the apparent viscosity to approximately 0.1 poise.

1309

Removal of Soluble Growth Factors from Matrigel™ Basement Membrane Matrix and the Demonstration and Quantitation of Insoluble, Matrix-Bound TGF-Beta. F.J. Mannuzza, Collaborative Biomedical Products, Inc., Becton Dickinson Labware, Bedford, MA 01730.

Basement membranes are thin extracellular matrices (ECM) underlying epithelial cells, separating them from connective tissues in vivo. Matrigel™ Basement Membrane Matrix (Matrigel™ BMM) extracted from the Englebreth-Holm-Swarm mouse tumor consists predominantly of laminin, type IV collagen, heparan sulfate proteoglycan and entactin which polymerize under physiologic conditions to produce reconstituted biologically active extracellular matrix for use as a tissue culture substrate. Growth factors, including EGF, IGF1, bFGF, PDGF, and TGF-Beta, are also present in Matrigel™ BMM and may contribute to the proliferation and differentiation of cells grown on the matrix. These growth factors, with the exception of TGF-Beta, can be reduced to biologically inactive levels by repeated precipitation using 20% $(NH_4)_2SO_4$. We have quantitated these growth factors and the major structural components before and after repeated salt precipitations. The growth factor-reduced Matrigel™ BMM retains the gelation properties of the starting matrix material and is useful for cell studies under growth factor-defined conditions.

Collagen and Basement Membranes (1310-1313)

1310

Genomic Organization of Human Surfactant Protein D - SP-D is Encoded at 10q22.2-23.1. E.Crouch, K.Rust, R.Veile, H.Donis-Keller. Depts. of Path. and Genet., Wash.Univ.Schl.Med., St.Louis, MO. 63110.

Surfactant protein D (SP-D) is a member of a family of collagenous C-type lectins that includes surfactant protein A (SP-A), and the serum mannose binding proteins (MBP). We have previously described cDNAs specific for human SP-D (Arch.Biochem.Biophys. 290:116, 1991). We now describe the characterization of genomic sequences for hSP-D, and present evidence for an SP-D gene on the long arm of chromosome 10 - in the company of SP-A, MBP-C, XIII collagen, and bullous pemphigoid antigen-2. DNA sequencing of two overlapping clones demonstrated at least seven exons spanning ~ 7 kb. The collagen domain is encoded on five exons - including 4 sequential exons of 117 bp, which appear unique to SP-D. These exons begin and end with split glycine codons, and show marked conservation in the predicted distribution of hydrophilic amino acids, consistent with tandem replication of this collagen gene sequence during evolution. The sequence also predicts a 39 a.a. periodicity in polar residues within the triple helical domain similar to the D/6 periodicity observed for the interstitial collagens. The carbohydrate recognition domain (CRD) and the connecting region between the collagen domain and conserved region of the CRD are each encoded by a single exon. Previous segregation analysis of HindIII digests of genomic DNA using specific cDNA probes demonstrated selective hybridization of radiolabeled hSP-D cDNA to chromosome 10q-containing human/hamster hybrids - findings confirmed by DNA amplification using oligomers specific for intron and exon sequences within the collagen domain. Fluorescence *in situ* hybridization of metaphase chromosomes using genomic probes gave selective labeling of 10q22.2-23.1. We speculate that SP-D is encoded at a "locus" on 10q that includes the genes for SP-A.

1311

Collagen Gene Expression in Human Scar : Quantiation and Localization of Type L III and VI Collagen mRNA in Vitro and in Vivo, Lu-gian Zhang, Matti Laato and Juba Peltonen, Department of Medical Biochemistry and Surgery, University of Turku, Turku Finland

The studying on hypertrophic scar clearly indicated the presence of defferent pathogenic mechanisms in scars of defferent clinical diagnosis, as evaluated by the expression of the major collagen types at mRNA level. The lesions diagnosed as hypertrophic scars demonstrated a clear elevation mRNA for al(I) and al(III),but much less of typeVI collagen. This is in striking contrast to keloidal lesions, which have been demonstrated to contain elevated steady-state levels of type I and VI collagen mRNA, but not much of that of type III collagen. Other hand In vitro the collagen gene expression profiles of hypertrophic scar cells were markedly different from that observed in Vivo. In essence, The stoichiometric ratios of MRNAs for collagen types studied were the same in cultures from hypertrophic scars compared to age matched controls.. these findings clearly suggest that factors operative in individuals developing excessive scar formation relate to an in vovi situation only. Furthermore, fibroblasts in hypertrophic scars may not display a auto regulatory mechanism detectable in vitro.

1312

Basic Fibroblast Growth Factor in the Extracellular Matrix Decreases Collagen Synthesis and α1(III) Procollagen mRNA Levels in Arterial Smooth Muscle Cells. AK Majors and LA Ehrhart. Cleveland Clinic Research Institute, Cleveland, Ohio 44195.

Previously we showed that arterial smooth muscle cells (SMC) plated on a preformed extracellular matrix (ECM) synthesized only 67% as much collagen as cells plated on plastic. Levels of α1(III) procollagen mRNA were also reduced in cells on the SMC-derived ECM. Total protein synthesis was unchanged. Furthermore, we demonstrated that recombinant basic fibroblast growth factor (bFGF) could elicit the same effects. In the present study we have plated SMC sparsely on an untreated, SMC-derived ECM, on an ECM pretreated with heparitinase to release bFGF, or into plastic dishes. In a separate experiment, SMC were plated onto an ECM in the presence of either a neutralizing polyclonal antibody directed against bFGF or nonimmune goat IgG. Control SMC cultures were plated directly onto plastic. After 19 h on the various substrata, the cultures were incubated for 5 h with ^3H-proline and sodium ascorbate. TCA-precipitable protein was hydrolyzed and collagen synthesis was determined from the radioactivity in hydroxyproline and proline which were resolved by ion exchange chromatography. Both heparitinase treatment of the matrix and addition of the anti-bFGF antibody abolished the inhibitory activity of the matrix on collagen synthesis. Together, these results indicate bFGF is the active component of the ECM responsible for its suppression of SMC collagen synthesis.

1313

Increased Collagen type III in the Neurogenic Non-compliant Bladder. D.H. Ewalt, S. Constantinescu, G. Bellon, J.P. Borel, H.M. Snyder, J.W. Duckett and E.J. Macarak. Children's Hospital of Philadelphia and Connective Tissue Research Institute, University of Pennsylvania, Philadelphia, PA 19104.

Collagen distribution in neuropathic bladder tissue is extensively altered. In such tissues, a fibrotic response occurs which is characterized by the altered accumulation of type III collagen. The increase in type III collagen is associated with the muscle bundles of the bladder wall and may be a product of the smooth muscle cells. In patients with non-compliant bladders, total hydroxyproline concentration increased from 31.2±4.1 mg/g to 37.5 ±mg/g, p= 0.15. Fibrillar collagen concentration estimated from 4-hydroxyproline concentration increased in the non-compliant bladders from 231±31 mg/g to 278± 14mg/g, p=.15. Quantitation of type I and III collagens showed the percentage of type III collagen increased from 24%± 2.5 in the normal bladder to 32%± 2.4, p=0.04 in the noncompliant bladder (33% increase) while the percentage of type I collagen decreased from 76% ± 2.5 to 68% ±2.4, p=0.04 in the non-compliant bladder (10% decrease). When combined with the slight increase in total collagen concentration, type III collagen actually increased 61% in the noncompliant bladder while type I increased by only 7%. Increased type III collagen accumulation within the smooth muscle bundles of the noncompliant bladder wall may contribute to altered mechanical properties of this tissue. Supported by NIH Grant HL34005.

1314

Isolation of Chondrons and Two Distinct Collagen Fiber Populations from Articular Cartilage. G.N. Smith, Jr., E.A. Mickler, and A.M. Lovelace. Arthritis Center, Indiana University School of Medicine, Indianapolis, IN 46202-5103

Isolation of intact elements of cartilage after homogenization allows direct determination of the distribution of specific collagen types within morphologically distinct cartilage matrix structures. The chondron--the cell, the pericellular matrix, and the capsule--can be isolated as a unit from articular cartilage by homogenization and filtration. To increase the yield and purity of chondrons for biochemical studies, we fractionated homogenates of canine articular cartilage on gradients of Percoll. The homogenates were suspended in PBS containing 24% Percoll and centrifuged for one hour at 12,000 x g. The resultant gradient contained three zones of collagen-rich fragments. The chondrons formed a sharp zone near the top of the gradient, while collagenous fibers formed two distinct zones within the gradient. More than 90% of the chondrons were in the zone at 1.016 g/ml. The bulk of the fibers migrated at density greater than 1.07 g/ml, while a second population of collagen fibers migrated at a density of 1.018 g/ml. The biochemical difference between low density and high density fibers is under investigation. The bulk of the proteoglycan in the gradient, as measured by DMMB binding, co-migrated with the chondrons, but more rigorous studies of the fiber populations are in progress to determine if proteoglycan content is the basis for the difference in density. The collagen extracted from the chondrons and the low and high density fibers by pepsin digestion and analyzed on SDS-polyacrylamide gel electrophoresis was predominantly type II, but type XI also was present in each of the fractions. When these collagen types were quantified by HPLC chromatography on TSK-heparin, there was more type XI relative to type II in the chondrons than in either the low-density fibers or high-density fibers. These data are consistent with the observation in studies of chick cartilage that all cartilage collagen fibers contain type XI collagen, but suggest that the pericellular matrix is enriched in this collagen type, as suggested by immunofluorescence staining.

1315

Force Microscopy of Collagen at 143 K. M.B. Shattuck[1], M.G.L. Gustafsson[2], K.C. Yanagimoto[3], R.S. Bhatnagar[1], J. Clarke[2], and K.A. Fisher[3]* [1]Group in Bioengineering, Univ. Calif., San Francisco, CA 94143; [2]Dept. Physics, Univ. Calif., and Center for Advanced Materials, Materials Science Division, Lawrence Berkeley Laboratory, Berkeley, CA 94720; [3]Depts. Anatomy, Biochemistry & Biophysics, and F.I. Proctor Foundation, Univ. Calif., San Francisco, CA 94143.

Imaging biological molecules by scanned probe microscopy is often limited by interactions between the probe and the soft sample. It has been suggested that such limitations may be diminished by scanning frozen samples at cryogenic temperatures. Reproducible images of individual molecules of type I collagen have been obtained using a newly designed low temperature scanning force microscope (cryo-SFM) operating at 143 K in isopentane. Characterization of identically prepared samples, by transmission electron microscopy (TEM) and gel electrophoresis, supported the conclusion that the structures imaged were indeed collagen. Cryo-SFM images revealed intramolecular features of single collagen molecules that have not been visualized by conventional TEM techniques. This represents the first time frozen single biomolecules and sub-molecular structure have been imaged by a low temperature force microscope. Supported by NIDA Grant No. DA-05043 and NEI Grant No. EY-02162 (K.A.F.) and by the Director, Office of Energy Research, Office of Basic Energy Science, Materials Science Division of the U.S. Dept. of Energy, under contract No. DE-AC03-76F00098 (J.C. and M.B.L.G.), NIH Grant No. DE-09859 (M.B.S. and R.S.B.), and NIH Training Grant No. GM-08155 (M.B.S.).

1316

Contact and Non-contact Atomic Force Microscopy of Type I Collagen. E.A.G. Chernoff, D.A. Chernoff, and K. Kjoller, Department of Biology, Indiana University-Purdue University at Indianapolis (IUPUI), Indianapolis, IN 46202; Advanced Surface Microscopy, Inc., Indianapolis, IN 46220; Digital Instruments, Inc., Santa Barbara, CA, 93117

Type I collagen was examined using two types of atomic force microscopes (AFM) in a continuing effort to refine the process of obtaining molecular information from biological materials using scanning probe microscopy. Operating in air, a contact mode (Nanoscope II) and a non-contact mode AFM (Nanoscope III) were used to image collagen fibrils polymerized from pepsin-extracted type I bovine skin collagen adsorbed onto mica substrates. Fibrillar collagen samples were polymerized from phosphate-buffered saline at pH 7.4 at 37°C, monomer samples were adsorbed from 0.012N HCl at 21°C. The contact AFM images show well-defined D-banded fibrils with a banding period of 70nm. Well-formed fibrils with tapered ends lie on a meshwork of finer fibrillar material, probably oligomeric collagen. Adsorption of unpolymerized collagen at acidic pH produced a meshwork of monomeric collagen with an occasional studcture of oligomeric size. Using the non-contact AFM we obtained further, higher resolution information from the fibrillar samples: sub-bands at intervals as small as 30 nm are found in both the regions of subunit overlap and in the gap region. In the region of overlap two principal bands show the characteristic asymmetry seen in negative stained material visualized using transmission electron microscopy. This assymetry of the sub-bands in region of overlap indicates the direction of the carboxy-termini of the polymerized monomers within the fibril.

1317

Morphometric Analyses of Collagen Types II and X mRNA Expression in the Growth Plate Cartilage of Normal and Rachitic Chicken Vertebrae and Tibiae
M. Hayashi, K. Hayashi, R. Adox Dept. of Pathology, Robert Wood Johnson Medical School, Piscataway, NJ

Rachitic chickens were produced by raising 1-day-old male chickens on a vitamin-D-free diet for 4 wk. Vertebral bodies (C9-C11) and proximal tibiae were examined. Serial sections from paraffin-embedded tissues were processed for in situ hybridization to localize type II and type X collagen mRNAs using 3H-labeled cDNA probes, and the autoradiographic silver grains were quantified by computer-based image analysis. **Histograms** of the distribution of silver grains clearly demonstrated two separate peaks for type II and type X collagen mRNA zones in the growth plate cartilage of normal vertebrae; and the altered distribution of the two collagen mRNAs in the enlarged and disorganized growth plate of rachitic vertebrae. **The areal density** of silver grains for type II collagen mRNA in the growth plate cartilage of rachitic chickens was almost double that of normal chickens in both vertebrae and tibiae. In the rickets, the areal density for type X collagen mRNA was reduced by 50% in the tibial growth plate but it was increased by 40% in the vertebral growth plate. In contrast, in the epiphyseal cap cartilage, areal densities for types II and X collagen mRNAs showed no significant changes in rachitic chickens. (Supported by a NIH grant AG09199)

1318

Basement Membrane Gene Expression in Rat Sertoli and Peritubular Myoid Cells *in vitro*. L.L. Richardson, [1]H.K. Kleinman, and M. Dym. Department of Anatomy and Cell Biology, Georgetown University Medical Center, Washington, DC 20007 and [1]Laboratory of Developmental Biology, National Institute of Dental Research, Bethesda, MD 20892

As demonstrated by Skinner et al (JCB 100:1941, 1985), Sertoli cells (SC) and peritubular myoid cells (MC) in monoculture synthesize and secrete basement membrane components, but only deposit highly complex matrix fibrils in coculture. In order to determine the mechanism by which these cell types cooperate in the production of basement membrane, we sought to determine how the expression of the genes for basement membrane components are regulated. Total RNA obtained over 5 days from monocultures of SC, MC, or SC:MC cocultures was analyzed by Northern blot for laminin A, B1, and B2 chains, type IV collagen (IV), and fibronectin (FN). Surprisingly, the genes for the laminin chains were not coordinately expressed. SC produced mRNA for the laminin A and B2 chains which did not change with time in culture. Low yet increasing amounts of mRNA for the B1 chains and IV were observed only on days 3-5 of culture. No FN mRNA was seen in SC. In contrast, MC produced mRNA for the laminin B1 and B2 chains, but had no detectable A chain transcripts. Collagen IV and FN mRNAs were also present in MC and there was no change in the levels of any of these mRNAs with time in culture. In SC:MC cocultures, the levels of expression of the A and B2 chains were similar to those in the SC monocultures. B1 chain, IV, and FN transcripts were lower in the SC:MC cocultures than in MC monocultures and decreased with time in coculture. The data suggest that these basement membrane genes may be regulated by paracrine factor(s).

1319

Generation of Glycoprotein Fragments by Proteolytic Clipping of the C-Terminal Regions of Perlecan. P. Valente[1,2], D.M. Noonan[2], and J.R. Hassell[1], [1]The Eye & Ear Institute and University of Pittsburgh, PA 15213 and [2]Ist. Naz. per la Ricerca sul Cancro, 16132 Genova, Italy.

The core protein of perlecan, a proteoglycan present in most basement membranes, undergos proteolytic modifications (J. Cell Biol. 106:963-970, 1988). The nature and positions of these modifications are not yet known. cDNA clones encoding for portions of Domain III and Domain IV of perlecan were expressed in E. coli using the p-mal expression vector. The recombinant proteins were partially purified by Q Sepharose and Mono S chromatography and subsequently linked to activated Affi-gel columns. Polyclonal antiserum to perlecan were affinity purified over these columns, resulting in antibodies specific to domain III or to domain IV. The extracellular matrix produced by M1536-B3 cells was extracted and separated into glycoprotein and proteoglycan fractions by DEAE chromatography. Antibodies to both recombinant proteins recognized the 400 kDa core protein isolated after heparatinase digestion of the proteoglycan fraction and specifically immunoprecipitated the 400 kDa precursor protein to perlecan. Antibodies to domain III did not react with glycoprotein fraction. Antibodies to the domain IV, however, recognized an 130 kDa protein in the glycoprotein fraction in western blots. The deduced sequence of domain IV encodes for a peptide of 139 kDa. Predicted disulfide bonding within domain IV suggests it forms a single globule. These results suggest that proteolytic clips occur between domains III and IV as well as between domain IV and V to release a possibly intact domain IV. (Supported by NIH Grant E R01 GM45389 (JH); CNR 91.02482.CT14 (DN); and AIRC (PV)).

1320

A lamina lucida does not appear in the basement membranes of cryofixed or paraformaldehyde-fixed tissues processed by freeze substitution F.L.Chan, S.Inoue, and C.P.Leblond, Department of anatomy, McGill University, Montreal, Quebec, Canada H3A 2B2

Ultrastructurally, the basement membrane is generally divided into two main layers: the electron lucent lamina lucida and the electron dense lamina densa. However, after cryofixation by slam freezing followed by freeze substitution in osmium-acetone, the basement membranes of rat seminiferous tubules, epididymis and ciliary body show a lamina densa making contact with the plasmalemma of the associated cells without an intervening lamina lucida. It thus appears that the lamina lucida is an artefact arising in the course of conventional tissue processing. To find out whether the formation of a lamina lucida is produced by aldehyde fixation or by dehydration, mice have been perfused with glutaraldehyde and the fixed tissues have been frozen in Freon 22 followed by freeze substitution to insure a slow gentle dehydration. Under these conditions, the basement membranes of seminiferous tubules, epididymis, ciliary body and kidney show no lamina lucida. Hence, the slow dehydration has resulted in the absence of lamina lucida. Nevertheless, two tissues, plantar skin and trachea still show a lamina lucida. The experiment has then been repeated using fixation by paraformaldehyde perfusion instead of glutaraldehyde. No distinct lamina lucida is then observed in skin and trachea. It is concluded that the lamina lucida is formed by disruption of the fragile attachment of the lamina densa to the associated cells, mainly during conventional dehydration but facilitated in some tissues by glutaraldehyde fixation.

1321

Axonal Behavior on Enzymatic Treated Inner Limiting Membrane from Chicken Embryonic Retina. L. Chai, and J.E. Morris, Department of Zoology, Oregon State University, Corvallis, OR 97331.

Our previous study has shown that heparan sulfate proteoglycan (HSPG) is a major component of the inner limiting membrane (ILM) from chicken embryonic retinas (Chai et al., J. Cell Biol. 150a, 1990). In the present study, we explored the possible functions of this proteoglycan on growth of ganglion cell axons (GCA) on the ILM, the natural substrate for their migration. We isolated the ILM according to Halfter et al. (J. Neurosci. 7:3712, 1987) by attaching the retina, with ganglion cell side facing down, to a polylysine-coated glass cover slip. The attached ILM was treated with 2% Triton-X 100 to remove Müller cell endfeed. The coverslip with attached ILM was then cut in half. One half was treated either with enzymes or with antibodies and the other was left untreated as a control. After treatment, the two pieces were then combined and a 300 μm-wide strip of retina was placed across the two pieces of coverslip. We found that density of GCA growing from the explant was greatly reduced by heparitinase, whereas the growth rate of GCA was only slightly less than the control. Chondoitinase had no effect on density or growth rate. Heparitinase greatly inhibits outgrowth of GCA from 6- and 8-day embryos but not from 13-day embryos. We also found that axonal density and growth rate were inhibited by exogenous heparin, but less by chondroitin sulfate. These results using the natural substrate for GCA outgrowth support the conclusion of others using plastic or glass substrates that HSPG promotes retina cell adhesion. We conclude that these molecules have a key role in normal axonal migration. (supported by NIH grant HD 19530)

1322

Cloning and Biological Function of a Primitive Laminin in Hydra. M.P.Sarras Jr., L.Yan, X.Zhang, A.Grens, P.L. St.John, and D.R. Abrahamson. Department of Anatomy & Cell Biology, University of Kansas Medical Center, Kansas City, KS; Developmental Biology Center, University of California at Irvine, Irvine, CA; Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL.

Hydra is characterized by having a simple body structure composed of a gastric tube with a foot process at the basal pole and a mouth and adjacent tentacles at the apical pole. Its entire body wall consists of a epithelial bilayer with an intervening extracellular matrix (ECM) termed the mesoglea which contains Type IV collagen, fibronectin, laminin, and heparan sulfate proteoglycan. Mesoglea components have been shown to be important in developmental processes such as head regeneration and hydra cell aggregation. The latter process involves complete morphogenesis of the adult hydra structure within 96 hrs from pellets formed from dissociated cells. In the present study, monoclonal antibodies raised to isolated mesoglea were used to further determine the role of ECM components in hydra development and to characterize these components using rDNA techniques. One of these antibodies (mAb52) binds to a single protein with a mass of about 300kDa under nonreducing conditions and ultrastructurally localizes to the subepithelial zone of the mesoglea on both the ectoderm and endoderm side. mAb52 was found to block development of morphogenesis as assayed in hydra cell aggregate experiments and to block in vivo cell migration as assayed in grafting experiments. Using mAb52 we have screened a Lambda Zap II cDNA library made from Poly(A) RNA from Hydra vulgaris and have isolated several clones with insert sizes of from 2.5 to 3.0 kb. Partial sequencing of these clones and analysis of these sequences using Genbank indicates that they code for a protein with high homology to laminin B1 chain. This homology crosses species lines from drosophila to human. Given that hydra developed some 500 million years ago, these experiments indicate the highly conserved nature of laminin structure and function. The studies also support the use of hydra as an experimental system to study cell/matrix interactions during development. Supported by funds from NIH grants RR06500 (MPS), DK34972 (DRA), and the Juvenile Diabetes Foundation (MPS).

1323

Quantitative Analysis of Extracellular Matrix Formation In Vivo and In Vitro. E. Papadimitriou[*], B.R. Unsworth[*], M.E. Maragoudakis[#] and P.I. Lelkes[*], (*)Marquette University, Milwaukee, WI, (a) University of Patras, Greece, (#) University of Wisconsin - Medical School, Milwaukee, WI

Angiogenesis, i.e. the formation of new blood vessels from pre-existing ones, is associated with the formation of a sub-endothelial basement membrane (BM). The sequential deposition of extracellular matrix (ECM) proteins, such as fibronectin (FN), laminin (LM), collagen IV (C-IV) and collagen-I (C-I) have been studied both in vivo in the chick chorioallantoic membrane (CAM) and in vitro in cultured rate adrenal medullary endothelial (RAME) cells using indirect immunofluorescence, enzyme-linked immunoadsorption (ELISA) and Western blotting techniques. In the CAM, FN levels increased transiently with the peak at day seven of development, after which it decreased gradually. By contrast, the levels of LM and collagen-I increased steadily during development. Quantitation at later stages of CAM development showed the predominance of C-I, whereas LM comprised only a minor component of the ECM proteins. A similar temporal sequence of ECM protein deposition was observed with RAME cells *in vitro*. FN formed an increasing extracellular network immediately after plating and until the cells became confluent. After confluence, the amounts of FN remained constant. By contrast, although LM and C-IV were synthesized by the cells from the onset of culture, they were deposited into the ECM only after the cells became confluent. The temporal sequence of the gene expression for the various BM proteins is currently being assessed using molecular biological approaches.

1324

Diffusion Rates of Solutes in Extracellular Matrix Measured by Fluorescence Recovery after Photobleaching S.-C. Chen, J. Hoying & S. Williams, Department of Surgery, University of Arizona, Tucson, AZ 85724

Transglomerular basement membrane protein exchange illustrates the degree of selectivity which can occur during the diffusion of macromolecules through matrices. We have developed an in vitro model of molecular diffusion using reconstituted gels of extracellular matrix proteins saturated with FITC labelled solutes. The diffusion of these labelled molecules was assessed by fluorescence recovery after photobleaching (FRAP). Fluorescence intensity profiles were subsequently analyzed and diffusion coefficients were derived using a PC based curve fitting program. Initial studies have focussed on collagen gels reconstituted from rat tail collagen. The diffusion coefficient for FITC-labelled native bovine serum albumin (Alb)in collagen gels was determined to be 1.63×10^{-7} ($\pm$ 0.22) cm^2/s. Glycated albumin exhibited a diffusion coefficient of 1.42×10^{-7} ($\pm$ 0.21) cm^2/s. Finally, immunoglobulin G diffusion coefficient was determined to be 0.38×10^{-7} ($\pm$ 0.15) cm^2/s. The mean diffusion rates of these proteins were therefore Alb > g-Alb > IgG. We suggest that matrix gels can be used to evaluate the diffusion characteristics of molecules and the diffusion barrier that matrix components create with regard to molecular transport processes. (supported by grant DK 43620).

1325

Cartilage differentiation and calcification during skeletal development in organ culture. B.J. Klement and B.S. Spooner. Biology, Kansas State University, Manhattan, KS 66506-4901.

Bone formation is a process that includes cellular differentiation and concomitant tissue morphogenesis. We have developed an organ culture system, using embryonic mouse pre-metatarsals, where these events occur in a pattern like that observed *in vivo*. Pre-metatarsals are isolated at 13 days of gestation, and cultured in serum-free, defined BGJ_b medium, on Nucleopore supports. The explants are initially composed of mesenchyme. In culture, the mesenchyme differentiates into cartilage that is morphogenetically organized into discreet rods. With further culture, a sub-population of the chondrocytes undergo terminal differentiation into hypertrophied chondrocytes, surrounded by a calcifying ECM. When the medium is supplemented with ITS, mesenchyme differentiation to cartilage occurs, but the time required for terminal cartilage differentiation is accelerated, i.e. hypertrophy begins in less than half the control time. Supplementation with 10% fetal bovine serum also results in normal cartilage rod formation and accelerated terminal differentiation. However, continued culture in FBS results in the formation of osteoid-like tissue similar to that seen in the metatarsals of newborn mice. This *in vitro* organ culture system is advantageous for the study of embryonic bone formation since it models pre-metatarsal tissue development *in vivo*, while being isolated from additional embryonic and maternal tissues and accessible for manipulation of the system. Supported by NASA NAGW 2328.

1326

Modulation of Chondrocyte Differentiation from Mesenchymal Stem Cells. D. McK. Ciombor and R.K. Aaron. Department of Orthopaedics, Brown University and the Orthopaedic Research Laboratory, Department of Surgery, Roger Williams Medical Center, Providence, RI.

The expression of the differentiation of chondrocytes from stem cells has been studied mainly in chick limb bud micromass cultures. These cultures are arduous and provide limited cell number. We have worked with another model system for the study and modulation of chondrocytic differentiation, in which stem cells are recruited with decalcified bone matrix (DBM).

The process of endochondral ossification can be induced by the introduction of DBM into the subcutaneous space of an adolescent rat. The inducing agent (osteogenin) causes a recruitment of mesenchymal stem cells to the site of the implant within the first day following implantation. On day 2, the newly formed plaques are harvested and the cells are enzymatically released and placed in culture on a membrane system. The expression of the chondrocyte phenotype is determined by morphological assessment, incorporation of radiolabelled sulfate into proteoglycan, and production of mRNA to type II collagen and proteoglycan core protein. ^{3}H TdR incorporation is used to assess proliferation.

The differentiation of stem cells is modulated in this system by several factors. The composition of the culture surface can be shown to be permissive for either proliferation or differentiation. Collagen coated membranes provide a surface which permits differentiation. Addition of EGF to the cultures induces proliferation. bFGF induces a large differentiative response by day 2 in culture. The time course of differentiation varies with the exogenous factors used.

1327

In Vitro Chondrogenic Potential of Embryonic Chick Calvarial Cell Subpopulations. M. Wong, and R.S. Tuan, Developmental Biology/Teratology Program and Dept. of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA 19107.

Intramembranous ossification in embryonic chick calvaria (CV) occurs through direct differentiation of mesenchyme into bone. However, under certain conditions, chondrocyte-like cells have been observed in the developing CV [Dev. Biol. 115: 215; 133: 221]. To analyze their chondrogenic potential, primary CV cells from day 14 embryos were Percoll-density fractionated. After 14 days in culture, one subfraction of CV cells (fraction F, sp. gr. =1.055-1.060) showed a rounded phenotype, with abundant refractile extracellular matrix. The chondrogenic potential of CV cells was characterized by co-culturing the CV cell fractions, as side-by-side micromasses, with an equal number of chondrogenic, stage 23-24 embryonic chick limb bud (LB) mesenchymal cells. Chondrogenesis was assayed after 4 days by the number of Alcian Blue staining nodules and by [^{35}S]-sulfate incorporation. None of the CV cell fractions cultured alone formed nodules. However, when co-cultured with LB cells, all CV fractions had a 3-fold increase in [^{35}S]-sulfate incorporation. In the presence of fraction F CV cells, LB cells had 1.5-fold more [^{35}S]-sulfate incorporation ($p<0.05$). Interestingly, conditioned media from fraction F CV cells stimulated [^{35}S]-sulfate incorporation (1.4-fold, $p<0.05$) by LB cells cultured alone, whereas conditioned media from other fractions had no effect. In summary, embryonic chick CV cells (1) may be stimulated to incorporate more [^{35}S]-sulfate by co-cultured LB cells, and (2) contain a fraction of cells (fraction F) which can differentiate into a chondrocyte-like phenotype in vitro. Furthermore, (3) this chondrogenic CV cell fraction enhanced sulfate incorporation by co-cultured LB cells, possibly through a secreted factor in the medium. These findings suggest that although chondrogenesis is normally inhibited in CV cell differentiation, a subpopulation of CV cells are responsive to chondro-stimulatory influences and are involved in aberrant CV development. Support: NIH HD 15822.

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In vitro Proteoglycan Synthesis in Response to Extracts of Bone Matrix. M.A. Nathanson, Dept. of Anatomy, Cell Biology & Injury Science, New Jersey Medical School, Newark, NJ 07103.

Two extracts of bovine bone, termed BMP (Urist) and OFE (Celtrix Labs), were tested for the ability to elicit formation of cartilage from embryonic skeletal muscle. Skeletal muscle from 20-day rat fetuses was placed into organ culture on substrata of type I collagen and fed on alternate days with medium CMRL-1066 containing 15% fetal calf serum and either 50μg/ml BMP or 20μg/ml OFE. Controls on authentic bone matrix consistently produce hyaline cartilage within 6-12 days *in vitro*. Experimental data indicated that only BMP was capable of eliciting a similar morphologic transformation, even though both extracts supported GAG and Sepharose CL2B elution profiles that were consistent with the expected appearance of cartilage-type proteoglycan, Aggrecan. Unexpectedly, the cell layer of cultures treated with BMP contained little or none of the newly-synthesized proteoglycan; the latter was lost to the culture medium. Proteoglycan produced in response to OFE turned out not to be Aggrecan, but a proteoglycan with short GAG chains that had only limited ability to aggregate with hyaluronic acid. These results demonstrate that BMP is effective in promoting chondrogenesis by virtue of its ability to upregulate both the core protein and the GAG of an Aggrecan-like molecule. However, BMP may be only one of several factors required during the induction of cartilage, as "accessory" matrix components appear to be required to anchor Aggrecan within the matrix of the cell layer. BMP, then, may be the sum of several activities that collectively upregulate chondrogenesis. Supported by NIH grant AR37940.

1329

Alternative Splicing of Fibronectin During Chondrogenesis *in Vitro*. A.L. Gehris and V.D. Bennett. Department of Orthopaedic Research, Thomas Jefferson University, Philadelphia, PA 19107.

Fibronectin, a large extracellular glycoprotein that mediates interaction of cells with the extracellular matrix, appears to be present in cartilage matrix. Previous studies demonstrated that the splicing patterns of fibronectin RNA change during chondrogenesis (cartilage development) in the chick limb. Prechondrogenic limb mesenchyme contains both exon IIIB and exon IIIA (B+, A+) while the fibronectin RNA from cartilage contains exon IIIB, but no exon IIIA (B+, A-). Therefore, the structure and function of cartilage fibronectin may differ from fibronectin in prechondrogenic mesenchymal cells, possibly due to interaction with the matrix. To determine if the splicing patterns of fibronectin RNA play a role in the chondrogenic process, we have begun to examine the structure of fibronectin RNA during chondrogenic differentiation of chick limb mesenchymal cells *in vitro*. Cultures were assessed for chondrogenesis by [^{35}S]-sulfate incorporation and enhancement of accumulation of alcian blue stainable extracellular matrix. Poly $(A)^+$ RNA was prepared from the cultures at various times *in vitro* and the splicing patterns of the fibronectin RNAs were examined by RNase protection assays. Preliminary results suggest that the amount of fibronectin RNA containing exon IIIA decreases as chondrogenesis increases in the cultures. The result is consistent with the loss of exon IIIA in fibronectin RNA that occurs during chondrogenesis *in vivo*.

1330

Characterization of Fibronectin in Cartilage: Generation of an Antibody to the Region of Fibronectin Encoded by the Alternatively Spliced Exon IIIB. D.W. Brandli and V.D. Bennett. Department of Orthopaedic Research, Thomas Jefferson University, Philadelphia, PA 19107.

Fibronectin (FN), a large extracellular glycoprotein, is found in a variety of tissues and participates in many critical cellular processes including differentiation, adhesion, and migration. Heterogeneity in the structure of fibronectin is largely due to the alternative splicing of at least three exons during processing of the FN primary transcript. Previously, we determined that chick cartilage FN mRNA differs from the mRNA in mesenchyme as a result of these differential splicing events. Cartilage FN mRNA contains exon IIIB but not exon IIIA (B+A-), while the mRNA in prechondrogenic mesenchyme contains exon IIIB and exon IIIA (B+A+). In order to characterize the FN protein in cartilage, we have generated a polyclonal antibody (Ab) which recognizes the region of fibronectin encoded by exon IIIB. Immunoblot experiments with the exon IIIB Ab and an exon IIIA FN Ab indicate that both antibodies react with the FN present in prechondrogenic mesenchyme. In contrast, only the exon IIIB Ab reacts with the FN present in chick cartilages. Neither Ab reacts with chick plasma FN, known to lack both of the regions corresponding to exons IIIB and IIIA. Thus the exon IIIB Ab appears to react with FN in a tissue-specific manner indicating that the Ab is specific for FN isoforms containing the region corresponding to exon IIIB. Furthermore, characterization of cartilage FN with the exon-specific Abs confirms our previous characterization of the FN mRNA in cartilage.

1331

In Situ Hybridization of Alternatively Spliced Type II Procollagen N-propeptides in Human Fetal Laryngeal and Tracheal Cartilages. R.A. Reife, A.M. Nalin and L.J. Sandell. Departments of Biochemistry and Orthopaedics, University of Washington and Veterans Administration Medical Center, Seattle, WA, 98195.

Two isoforms of type II procollagen exist. The difference between them resides in the presence or absence of exon 2, encoding the 69 amino acid cysteine-rich domain of the NH_2-propeptide. Type IIA procollagen is defined as containing exon 2 while type IIB procollagen does not contain exon 2. We examined the distribution of IIA in cartilages of the hypopharynx and trachea; cartilages which are not ultimately replaced by bone thus avoiding the additional complexity of osteogenesis. Human fetal larynx and trachea tissues from 59 - 89 days of gestation were probed for mRNAs of types IIB, IIA and I by *in situ* hybridization. Hybridization of the oligonucleotide probe for type IIB mRNA localized to chondrocytes of the cricoid, thyroid and arytenoid cartilages of the larynx and cartilage rings of the trachea while type IIA message was observed in cells of the perichondrium. In addition, the probe for type I mRNA localized to cells of the mesenchyme external to the perichondrium and did not appear to be synthesized by cells expressing type IIA. Upon closer morphological examination, the type IIA-expressing cells appeared to be fibroblastic in shape compared to the polygonal appearance of the cells synthesizing type IIB. This study demonstrates that mRNAs for the type II procollagen isoforms are expressed in spatially and phenotypically distinct cell populations during chondrogenesis. It seems that as chondroprogenitor cells mature to chondrocytes, the pattern of collagen expression switches from type IIA mRNA to type IIB and is accompanied by an overt accumulation of a cartilage matrix. To further examine the temporal expression of both isoforms as an indicator of their potential roles in chondrogenesis, we employed an *in vitro* system where isolated periosteal cells cultured at high density differentiate into chondrocytes and form cartilage. Cryosections of cells after 8 days of culture were examined for procollagen mRNAs by *in situ* hybridization and revealed that type IIA was more abundant than IIB. In contrast, after 16 days of culture, the ratios between the two isoforms reversed where IIB appeared to predominate. Collectively, these results support our hypothesis that there is a switch from type IIA to type IIB prior to chondrogenesis and that IIA may be the molecular marker for chondrogenic potential.

1332

Enhancement of cytoskeletal organization by metallic substrates. R.K. Sinha and R.S. Tuan. Dept. of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA.

The composition and topography of a biomaterial substrate exert substantial effects on cell attachment and morphology (J. Cell Biol, 115:448a, 1991. We describe here differences among various orthopaedically relevant materials in actin filament reorganization in cultured normal human osteoblasts. The substrates used (Biomet, Inc.) were of two chemical compositions--Ti6Al4V (Ti) and CoCrMo (CC); and two different topographies--polished (Pol) and rough (R; 4.62X the surface area of Pol). Tissue culture polystyrene (PS) was the control substrate. The cells were plated at 10,000/cm^2 and at 3, 6, 12 or 24 hours were stained with rhodamine-phalloidin. Morphology was analyzed based on 100 nonoverlapping high power fields on two separate sample preparations for each surface and time point. Upon PS, the proportions of the three distinct morphological arrangements changed as a function of time: Type I cells-- faint staining, no discernible actin filaments, most abundant at 3h but decreased over time; Type II cells-- cortical filaments below the membrane and a few radially oriented filaments, percentage increased from 3 to 6 h but decreased thereafter; Type III cells- distinct actin filaments aligned parallel to the long axis of the cells, percentage increased steadily with time. The temporal sequence of each cell type suggests that Type I cells represent little cytoskeletal organization, Type II cells are intermediate, and Type III cells have the greatest degree of cytoskeletal organization. For the Ti surfaces, at 3h, there were a greater percentage of Type III cells on R Ti (55.04%) than on Pol Ti (22.73%). This difference remained at 6h (71.15% vs 44.84%), equalized at 12 h (79.36% vs 74.39%), but became reversed at 24h (73.88% vs 89.06%). Upon CC surfaces, a similar relationship was observed. At 3h, the percentage of Type III cells was higher on R CC (34.61%) than on Pol CC (15.59%). Again, this difference was sustained at 6h (52.21% vs 37.95%), but was equalized at 12h (54.02% vs 58.11%) and 24h (79.11% vs 80.18%). Comparing Ti to CC, there was a higher percentage of Type III cells on Pol Ti vs Pol CC at all time points, and a higher percentage on R Ti vs R CC at all time points except 24h. Thus, the initial rate of cytoskeletal organization in human osteoblasts is influenced by surface chemistry (Ti faster than CC) and topography (R faster than P). These differences in early cell-substratum interaction may result from the surface chemistry and surface area. (Supported by NIH HD15822 and AR01877)

1333

Poly-L-Lysine Stimulation of Limb Mesenchyme Chondrogenesis Involves Interaction with Membrane Proteoglycan. R.S. Tuan and M. Yagami. Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA 19107.

Polyionic compounds, such as poly-L-lysine (PL) and heparin-like glycosaminoglycans (GAG), stimulate chondrogenesis by limb mesenchyme micromass cultures *in vitro* (*Devel. Biol.* 115: 313, '86; 123: 17, '87). Based on our recent findings (*Devel. Biol.*, in press), which suggest a strong interaction between PL and GAG during modulation of chondrogenesis, we have examined the possibility of a cell surface "PL receptor"-like component(s) on limb mesenchymal cells. Whole chick embryonic limb buds (HH stage 23/24) were metabolically labeled with either ^{35}S-Met or ^{35}S-sulfate, and homogenized for membrane isolation. The PL-binding assay is as follows: solubilized membranes were incubated with PL(240 KD)-impregnated nitrocellulose disks, and then washed sequentially with buffer (to remove unbound materials), PL(4KD), lysine (0.25M and 0.5M), and pH 10 borate buffer. The amount of radioactivity eluted with each wash and remaining on the disk were determined. Radiolabeled materials were found to bind to PL-disk with high specificity, and consisted primarily of high-Mr (~180 KD) components as analyzed by SDS-PAGE. The proteoglycan nature of the bound components is demonstrated by: 1) labeling by both ^{35}S-sulfate and ^{35}S-Met, and 2) chymotrypsin removal of bound Met radiolabel, but not sulfate label (with reduction in Mr). Compositional analyses, including nitrous acid sensitivity and glycohydrolase treatments, indicate that the bound materials consist of heparan sulfate GAG; heparin also effectively competes with and displaces the limb mesenchyme material in PL-binding. Finally, the amount of PL-binding materials increase as a function of embryonic development *in vivo* and culture time of the mesenchyme *in vitro*. Taken together, these results suggest that a heparan sulfate proteoglycan is present in pre-cartilage limb bud and may be involved in PL-mediated stimulation of chondrogenesis. (Supported in part by NIH HD 15822)

1334

Tenascin plays a role in early cartilage formation. J.D. Brooks and M.L. Tanzer, Department of BioStructure and Function, University of Connecticut Health Center, Farmington, CT 06030.

The extracellular matrix glycoprotein, tenascin, shows a unique pattern of temporal and spatial distribution in the developing avian limb bud. Micromass culture conditions which allow chondrogenesis of chick limb bud mesenchyme simulate in vivo developmental events, and such cultures have been used in the present studies. The purpose of this investigation was to demonstrate the effect on chondrogenesis of blocking the function of tenascin through addition of anti-tenascin antibody. The monoclonal antibody, M1-B4, against tenascin was raised in ascitic fluid, then purified by affinity chromatography through Protein-G Sepharose. When purified antibody IgG was added to micromass culture media at a concentration of 10 µg/ml, chondrogenesis was inhibited. Alcian blue stain bound to cartilage matrix was extracted with 8M guanidinium chloride and quantified by spectrophotometry. Micromass cultures of stage 23/24 whole wing bud mesenchyme treated with anti-tenascin antibody during the five-day culture period exhibit one-half the amount of stained matrix in comparison to untreated cultures or those exposed to 10 µg/ml non-specific IgG. To determine whether cultures in the three groups contained equivalent numbers of cells, cell counts were performed with a hemocytometer after digestion of cultures with 0.25 % trypsin and 0.1 % collagenase. Cell counts were not affected by antibody treatment. These data suggest that tenascin promotes chondrogenesis in micromass culture of avian limb mesenchymal cells.

1335

Immunogold Transmission Electron Microscopic Demonstration of Vimentin in Osteoblast and Osteocyte Cell Processes in Rat Bone. F. Shapiro, M.D., C. Cahill and R.C. Nayak, Ph.D., Laboratory for Skeletal Disorders, Children's Hospital and Joslin Diabetes Center, Boston, MA 02115.

Although the intermediate filament protein vimentin has been shown to be expressed in mesenchymal cells, its specific localization at the ultrastructural level in bone cells has not been reported. We have examined the expression of vimentin in rat bone by immunogold staining and TEM. After perfusion fixation with 4% paraformaldehyde plus 0.5% or 1.0% glutaraldehyde and decalcification in 7.5% EDTA, tissues were embedded in several resins. Araldite 502 and Lowicryl HM20 gave poor results whereas Lowicryl K4M proved to be the resin of choice. Sixty nanometer sections were placed on nickel formvar grids and floated on 3% ovalbumin overnight followed by flotation on anti-vimentin antibody (monoclonal, VIM 13.2, IgM) for two hours at room temperature. Antibody binding was detected with gold conjugated goat anti-mouse IgM (μ). Control staining was performed by omission of primary antibody and also with a control antibody (MOPC 104 E, IgM myeloma protein). Results. We demonstrated very good binding on the cell processes and cell body intracellular filaments in both osteoblasts and osteocytes with minimal matrix binding at preferential concentrations of 1:25. In the control studies there was no uptake in cells or matrices. The discernable uptake was present over the intracellular filaments at the periphery of the cell body cytoplasm near the point of exit of the cell processes and in the cell processes of both osteoblasts and osteocytes. Preferential uptake adjacent to the gap junctions linking cell processes in the canaliculi was not seen. Although dense uptake in the cell processes was seen on occasion, most filaments show no uptake. It is a point of current investigation whether this is antibody-concentration related or indicative of the existence of other filamentous processes.

1336

Expression and Functional Involvement of N-Cadherin during Cartilage Development in the Chick Embryonic Limb Bud. S.A. Oberlender and R.S. Tuan. Departments of Orthopaedic Surgery and Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107

Prior to chondrogenesis, the pre-cartilage mesenchyme of the developing limb bud undergoes a crucial cellular condensation phase. Because condensation is most likely dependent on cell-cell interactions, we have examined the involvement of N-cadherin, a neural Ca^{++}-dependent cell adhesion molecule. Using NCD-2, a monoclonal antibody directed against the binding region of N-cadherin, our immunohistochemical studies demonstrate that N-cadherin is expressed on limb mesenchymal cell surface in a development-specific manner. High levels of staining occur around H-H stage 24/25, corresponding to the period of cellular condensation *in vivo*. At later stages, when cartilage growth is apparent, N-cadherin is localized to the surrounding mesenchyme and perichondrium, but not in the growing cartilage core. In addition, immunohistochemistry of limb mesenchyme cultured as micromass *in vitro*, reveals N-cadherin protein expression in distinct cellular aggregates. Immunoblot analysis of limb bud proteins using NCD-2 reveals a single protein band around 130 kD, corresponding to N-cadherin, which increases as a function of limb development. N-Cadherin is detected as early as H-H stages 17/18. *In situ* hybridization using N-cadherin cDNA reveals N-cadherin mRNA expression as early as H-H stage 17/18, and continued expression through limb bud development and chondrogenesis. To examine the functional importance of N-cadherin, NCD-2 antibodies were added to micromass cultures of limb mesenchyme, which inhibited chondrogenesis in a concentration-dependent manner, perhaps by interfering with active recruitment of uncommitted mesenchymal cells into growing cartilage nodules. These results demonstrate that N-cadherin is expressed in a development specific manner in the limb bud, and implicates a specific role for N-cadherin in chondrogenesis, such as mediating cell-cell interactions during mesenchymal condensation. (Support: NIH HD15822)

1337

Inhibition by Estrogens of Differentiation of Cultured Hemopoietic Stem Cells into Osteoclast-like Cells.

T. Tsutsui[1], N. Iwasaki[2] and H. Ishikawa[3], Department of Pharmacology[1], Periodontology[2] and Endodontology[3], The Nippon Dental University, Tokyo 102, Japan.

To examine a possible involvement of estrogens in the differentiation of hemopoietic stem cells (HSC) into osteoclasts, HSC were treated with estradiol or diethylstilbestrol (DES) and the incidence of multinucleated cells with a tartrate-resistant acid phosphatase (TRAP) activity was scored. HSC from a mouse spleen were cultured for 7 days with methylcellulose medium containing interleukin-3 (IL-3) to form colonies consisting of blast cells. The blast cells were replated on 24-multiwell plates at $1.5X10^4$ cells per well with IL-3 medium (α-MEM + 5% fetal bovine serum + 10% IL-3 supplement). Following 3 to 10 days of incubation, the cells were exposed for 3 to 10 days to 10nM 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and stained to detect TRAP activity. The incidence of TRAP-positive multinucleated (TRAP) cells were increased by the exposure to 1α,25$(OH)_2D_3$. The highest incidence was induced when the blast cells were cultured with IL-3 medium for 5 days and subsequently exposed to 1α,25-$(OH)_2D_3$ for 3 days. Treatment with estradiol or DES for 3 days at 1pM to 1nM resulted in decrease in the incidence of TRAP cells in a dose-dependent manner. The results indicate that treatment of hemopoietic stem cells from a mouse spleen with estrogens inhibited the differentiation of the cells into osteoclast-like cells. This suggests a direct response of estrogens to cells of the osteoclast lineage, and a regulation by estrogens of differentiation of the cells.

1338

OSTEOBLASTS AND OSTEOCLASTS SECRETION AND ACTIVATION OF LATENT TRANSFORMING GROWTH FACTOR-β. M.J. Oursler, B.L. Riggs, and T.C. Spelsberg. Mayo Clinic and Foundation, Rochester, MN 55905.

Normal bone metabolism involves a finely-tuned coupling between osteoblast (OB)-mediated bone formation and osteoclasts (OC)-mediated bone resorption. This coupling suggests that autocrine and paracrine factors are produced within the bone environment. Transforming growth factor β (TGF-β) is a potent cytokine which influences bone metabolism by modulating the activities of both OBs and OCs. Osteoblasts synthesize TGF-β as a latent complex which must be activated in order to influence cellular activity, and OCs are known to activate this latent complex. Therefore, a likely candidate for a paracrine coupling factor may be TGF-β. Glucocorticoids have diverse effects on bone metabolism including involvement in glucocorticoid-induced osteoporosis. We have investigated the influence of dexamethasone (DEX) on the secretion and activation of latent TGF-β by normal human OB-like cells (hOB). We have also examined highly purified avian OCs for synthesis of TGF-β, and investigated the mechanism by which avian OCs activate the latent TGF-β complex. TGF-β protein production was measured in conditioned media using growth factor inhibition of CCL-64 cells, and verified by blocking the effects with anti-TGF-β antibodies and northern blotting. Both hOBs and OCs secreted measurable TGF-β within 4 hours of culture. Nearly all the secreted OC TGF-β and hOB TGF-β from Dex treated cells were activated. When presented with exogenous latent TGF-β, both OCs and DEX treated hOBs activated latent TGF-β from various sources. Furthermore, both hOBs and OC conditioned media retained the ability to activate latent TGF-β during cell-free incubations at 37°C. Activation was inhibited by protease inhibitors and weak base treatment of the cells. DEX treatment of hOBs resulted in a dose-dependent increase in lysosomal protein mRNAs. Taken together, these data suggest that DEX treatment of hOBs induces production and secretion of lysosomal proteases which could activate the TGF-β present within the bone environment. Both OCs and OBs may therefore activate TGF-β which then might be involved in autocrine and paracrine effects on bone cells.

1339

Effects of Recombinant Human Osteogenic Protein (hOP-1) on the Differentiation of Osteoclast-like Cells and Bone Resorption. T.A. Hentunen, P.T. Lakkakorpi, J. Tuukkanen, T.K. Sampath and H.K. Väänänen, Department of Anatomy, University of Oulu, Oulu, Finland and Creative BioMolecules, Inc., Hopkinton, MA 01748.

Both purified bovine bone osteogenic protein and recombinant hOP-1 have previously been shown to be equally potent in bone induction in vivo. Here we show that recombinant hOP-1 significantly and in a dose-dependent manner stimulates the formation of tartrate-resistant acid phosphatase-positive multinucleated cells in rat bone marrow culture. The effect of hOP-1 on osteoclast-like cell formation was further enhanced (8-fold) when 1,25$(OH)_2D_3$ was present in the culture. In addition to hOP-1, the presence of 1,25$(OH)_2D_3$ was needed to induce multinucleated cells capable of bone resorption. hOP-1 at the concentration of 20 ng/ml also stimulated bone resorption by measuring ^{45}Ca-release in neonatal mouse calvaria-assay and by counting the resorption pits on bovine bone slices made by isolated rat osteoclasts. Interestingly, concentrations below 20 ng/ml and above 40 ng/ml did not have any effect on resorption. These and previous studies indicate that OP-1 could be one of the coupling factors in bone remodellation.

1340

Non-genomic Regulation of Osteopontin (OPN, Spp1) Charge State by 1,25-Dihydroxyvitamin D_3. R. E. Devoll, R. Khoury, G. C. Wright, W. T. Butler, and M.C. Farach-Carson, Department of Biological Chemistry, University of Texas Dental Branch, Houston, TX 77030

Previous studies have shown that in the presence of low nM levels of 1,25$(OH)_2$ vitamin D_3, both the mRNA and protein levels of OPN produced by osteoblasts are increased. This increase is mediated through a cis-element upstream of the OPN gene (VDRE) which is recognized by the vitamin D receptor (VDR). Using osteoblast-like ROS 17/2.8 cells as a model, we found that this up-regulation of OPN assessed by Northern blotting is relatively slow and is maximal after 24-48 hours. In contrast, we have found that in the presence of 2.5 nM 1,25$(OH)_2D_3$, the isoelectric point of OPN measured following 2-D gel electrophoresis rapidly changes from 4.6 to 5.1 without a shift in apparent molecular weight. This change in charge state is apparent within 3 hours of treatment with 1,25$(OH)_2D_3$, far too quick to be accounted for by a genomic mechanism. We have isolated OPN from the conditioned medium of ROS 17/2.8 cells either with or without exposure to 2.5 nM 1,25$(OH)_2D_3$. Experiments are underway with the purified proteins to identify the molecular mechanism for the shift in charge state, which we believe to occur through a change in post-translational modification. (Supported by RO1 AR39273)

Extracellular Matrix and Cell Signaling (1341-1342)

1341

The Organization of Extracellular Matrix Regulates PDGF-Induced DNA Synthesis and Autophosphorylation of PDGF Receptors. Y.-C. Lin and F. Grinnell, Department of Cell Biology and Neuroscience, University of Texas, Southwestern Med Ctr, Dallas, Tx 75235.

Collagen matrices contracted *in vitro* by fibroblasts acquire morphologic and biosynthetic features similar to wound tissue or dermis respectively depending upon whether or not stress is allowed to develop in the matrices during contraction. Previous studies have shown that fibroblasts in stressed but not relaxed collagen matrices proliferate in response to serum stimulation. To learn more about the role of extracellular matrix organization in regulation of fibroblast proliferation, we studied DNA synthesis and intracellular signaling pathways for DNA synthesis induced by PDGF treatment of fibroblasts in stressed and relaxed collagen matrix cultures. Fibroblasts in relaxed collagen matrices showed reduced PDGF-induced DNA synthesis and tyrosine phosphorylation of PDGF receptors compared to fibroblasts in stressed collagen matrices. On the other hand, the number of PDGF receptors and their clustering, internalization, and down-regulation were similar for fibroblasts in relaxed and stressed collagen matrices. Therefore, the loss of fibroblast proliferative capacity in relaxed collagen matrices can be explained by a reduction in the ability of PDGF receptors to undergo autophosphorylation.

1342

Structural Features of Fibronectin (Fn) Required for Regulation of Matrix Metalloproteinase (MMP) Expression and Focal Contact Formation. G. Santos, P. Tremble, *J. Schwarzbauer, Z. Werb and C. Damsky. University of California San Francisco, CA 94341-0512, and *Dept. Biol. Princeton Univ., Princeton, NJ.

Rabbit synovial fibroblasts (RSF) express low levels of MMP when plated on intact Fn and enhanced levels when plated on the 120kD cell binding domain, or immobilized antibody against the α5/β1 Fn receptor (Werb, et al 1989 JCB 109). This suggests that the ability of RSF to regulate MMP expression depends on their ability to recognize sites within the cell binding domain, via α5/β1. and outside this domain via as yet unknown receptor(s). To determine which domains of Fn are required to control MMP expression, dimeric constructs with specific Fn domain composition have been used as substrate ligands for RSF, and expression of interstitial collagenase and the 92 kD gelatinase was monitored. RSF plated on dimeric recombinant cellular Fn.expressed basal levels of these MMP. RSF plated on a dimeric Fn construct containing type III repeats 3-15, but lacking the N-terminal assembly and collagen binding domains expressed elevated levels of MMP as did RSF plated on a construct lacking only type III repeats 1-7. RSF plated on intact Fn displayed well organized focal contacts. In contrast, conditions that supported elevated MMP expression resulted in poor localiztion of both α5/β1 and cytoskeletal proteins to focal contacts. This approach should allow us to identify specific regions of Fn outside the cell binding domain that control MMP, as well as receptor(s) that cooperate with α5/β1 in communicating this information to the signaling machinery of the cell. Supported by CA42032, DE-AC03-76F01012.

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Ectopic expression of integrin $\alpha_5\beta_1$ suppresses in vitro growth and tumorigenicity of human colon carcinoma cells. J. A. Varner, M. H. Fisher, and R. L. Juliano, Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599 (spon. by R.L. Juliano)

The integrin superfamily of $\alpha\beta$ heterodimers mediates the adhesion of most mammalian cells to a variety of extracellular matrix ligands and to some recently described cell surface immunoglobulin superfamily molecules. Although several integrin receptors for vitronectin, collagen, or laminin ($\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_3\beta_1$, $\alpha_2\beta_1$) impart tumorigenic or metastatic capabilities to the transformed cell, overexpression of the fibronectin receptor ($\alpha_5\beta_1$) in Chinese hamster ovary fibroblasts (CHO) has been associated with loss of tumorigenicity. We have expressed the fibronectin receptor alpha subunit (α_5) in HT29 colon carcinoma cells, which, like most adult epithelial cells and many carcinoma lines, do not normally express alpha 5 and thus exhibit minimal adhesion to fibronectin. In addition to imparting significant cellular adhesion to fibronectin, focal contact formation and signal transduction events commonly associated with $\alpha_5\beta_1$, the classical fibronectin receptor completely suppresses the tumorigenicity of human colon carcinoma cells in nude mice as well as depresses the growth of transfected cells in vitro. We are studying the growth inhibitory signals transduced by the fibronectin receptor.

1344

Tyrosine Kinase Inhibitor Effects on Cytoskeletal Organization and Cell Motility. L.H. Romer[1], C.E. Turner[2], and K. Burridge[3]. Pediatric Critical Care[1], and Cell Biology and Anatomy[3], University of North Carolina at Chapel Hill, 27599-7220, and [2]Dept of Anatomy and Cell Biology, SUNY Health Sciences Center, Syracuse, New York

Integrin-mediated cell adhesion is accompanied by an increased level of tyrosine phosphorylation in the proteins paxillin (70kD) and pp125^{FAK}. The tyrosine kinase inhibitor herbimycin A blocked the adhesion-associated tyrosine phosphorylation in paxillin and pp125^{FAK}, as measured by Western blotting and immunoprecipitation. To investigate the importance of tyrosine phosphorylation in cytoskeletal assembly during adhesion, REF52 were plated on fibronectin-coated surfaces in the presence or absence of 875nM herbimycin A. Many of the cells treated with herbimycin A had a marked decrease in the size and number of focal adhesions when stained with antibodies against phosphotyrosine, paxillin, pp125^{FAK}, and talin. In addition, a paucity of stress fibers was noted in the herbimycin A-treated cells. Cell spreading was also diminished in the REF52 treated with herbimycin A. We then investigated the role of tyrosine phosphorylation in cell migration. Human umbilical vein endothelial cells grown to confluence on gelatin-coated coverslips and wounded with a pipette tip were stained for phosphotyrosine. Increased tyrosine phosphorylation was seen in the focal adhesions of cells at the wound margins. Western blotting of cell extracts identified increased phosphotyrosine in a band at 125 kD in the samples from multiply wounded monolayers as compared with intact controls. The addition of several tyrphostins to endothelial monolayers delayed the time to wound closure from 48 hours for untreated and DMSO-treated controls to 96 hours for tyrphostin-treated monolayers. Tyrphostin-treated cells demonstrated a decrease in focal adhesions and stress fibers. We conclude that tyrosine phosphorylation serves an important role in the cytoskeletal reorganization events that enable cell spreading and motility. Supported by NIH grants GM 29860, and HL 44918.

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Signalling by Integrins: The P125^{FAK} Tyrosine Kinase Connection. LJ Kornberg[1], HS Earp[2], JT Parsons[3], RL Juliano[1], Departments of Pharmacology[1] and Medicine[2], University of North Carolina, Chapel Hill, NC 27599 and Department of Microbiology[3], University of Virginia, Charlottesville, VA.

The interaction of cell surface integrins with the ECM leads to profound changes in cell behavior. This suggests that integrins transmit biochemical signals. We have recently shown that changes in tyrosine phosphorylation of a 130 kD protein(s) (pp130) may be involved in integrin-signalling (Kornberg, et al. Proc. Natl. Acad Sci, 88: 8392). We now demonstrate that one component of the pp130 protein complex reacts with an antibody generated against p125^{fak} which is a focal adhesion-associated tyrosine kinase (Schaller, et al., Proc. Natl. Acad Sci., 89:5192). Both antibody mediated integrin-clustering and adhesion of KB cells to fibronectin leads to increased tyrosine phosphorylation of p125^{fak}. The phosphorylation of p125^{fak} is coincident with adhesion of cells to fibronectin and is maximal prior to cell spreading. Tyrosine phosphorylation of p125^{fak} is induced when KB cells are allowed to adhere to fibronectin, collagen, or laminin, but is not induced on polylysine. When KB cells are subjected to immunofluorescence microscopy, p125 co-localizes with talin in focal adhesions. These data lend further evidence that tyrosine kinases are involved in integrin signalling.

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Integrin β1 Transmits Mechanical Forces Across the Cell Surface and to the Cytoskeleton. N. Wang, J.P. Butler, and D.E. Ingber, Respiratory Biology Program, Harvard School Public Health; Depts. of Surgery & Pathology, Children's Hospital & Harvard Medical School, Boston, MA 02115

Studies were carried out to identify molecules that mediate mechanical force transmission across the cell surface. A cell magnetometry system (Valberg & Butler, Biophys. J. 52:537, 1987) was adapted in order to apply mechanical loads directly to specific cell surface receptors, independent of any global cell shape change. Adherent cells were allowed to bind to ferromagnetic beads (5.5 μm diam.; approx. 5/cell) coated with specific ligands for transmembrane integrin receptors (RGD-containing peptides, anti-integrin β1 antibodies) or with non-specific ligands (e.g., acetylated-LDL which binds to scavenger receptors). The magnetic moments of all beads were aligned and then magnetically twisted in a perpendicular direction using applied magnetic fields. Stress (applied force), strain (angular rotation of beads in the direction of the twisting field), and elastic recoil (angular rotation in the opposite direction when the twisting field is turned off) were all quantitated. In cells bound to RGD-beads, we found that increasing the stress applied to cell surface receptors from 7 to 40 dynes/cm^2 resulted in a increase in both cytoskeletal stiffness (30 to 90 dynes/cm^2) and elastic recoil (4 to 11°). This alteration of cytoskeletal mechanics was prevented when soluble GRGDSP (1 mg/ml) was included in the medium; GRGESP was ineffective. Importantly, beads coated with anti-β1 antibodies mediated similar transfer of mechanical forces to the cytoskeleton whereas acetylated-LDL beads had no effect. These data demonstrate that a single type of transmembrane molecule, integrin β1, can mediate mechanical force transfer across the cell surface and thereby produce a cytoskeletal response. This finding is consistent with the concept that integrins act as mechanoreceptors and provide a molecular mechanism for mechanochemical transduction across the cell surface (Ingber, Curr. Opin. Cell. Biol. 3:841, 1991). [Supported by NIH grants HL33009 & CA45548 and by NASA grant NAG-9-430.]

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Oligosaccharide(s) in the Extracellular Coat of Sea Urchin Eggs (the Egg Jelly Coat) Induce the Acrosome Reaction. S.H. Keller and V.D. Vacquier, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093.

The major signal transduction event in sea urchin fertilization is the induction of the acrosome reaction (AR) when sperm contact the extracellular egg jelly coat. A glycoprotein fraction induces the AR not the fucose-sulfate polymer (Keller and Vacquier, unpub. data). Here we summarize evidence that oligosaccharide components are sufficient to induce the AR. (a) A strong alkaline hydrolysis with sodium borohydride (1M NaOH, 4M $NaBH_4$, 60°C, 48 hr) should cleave peptide bonds but leave oligosaccharides relatively intact; proteinase-K treatment should produce the same result. Our results demonstrate that AR inducing activity remains (although lowered by one-half) after jelly samples are treated separately by the above treatments. (b) Strong alkaline hydrolysis without sodium borohydride should destroy proteins and alter oligosaccharides by a peeling reaction. We found that AR activity is inhibited with this treatment. (c) After whole egg jelly is treated with PNGase-F, the fucose-sulfate-protein glycoconjugate absorbed on SM-2 resin (which should remove proteins) and the flow-through fractionated on a Sephacryl-200 column, molecules included within the column volume have AR activity. This active fraction is not included without PNGase treatment, it strongly reacts with the phenol-sulfuric assay indicating the presence of neutral sugars.

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Hyaluronic Acid Induces Lymphocyte Signal Transduction and GP85/CD44-Cytoskeleton Interaction. Lilly Y.W. Bourguignon, Jian Zhang and W. Glenn L. Kerrick. Departments of Cell Biology & Anatomy and Physiology and Biophysics, University of Miami Medical School, Miami, FL 33101 (Spon. by Robert Warren.)

Hyaluronic acid (HA) is a major extracellular glycosaminoglycan found in many types of extracellular matrix in mammalian systems. Recently, the hyaluronan receptor (the HA binding site) has been shown to be identical to GP85 (CD44) which has been implicated in lymphocyte homing-related cellular adhesion. In this study we have examined various signal transducing events occurring after HA binding to its receptor on a mouse T-lymphoma cells. Our results indicate that the concentration of intracellular Ca^{2+} (as measured by Fura-2 fluorescence) increases as early as 2-5 min after the addition of HA to the cells. Calcium channel blockers, such as nifedipine and bepridil, readily inhibit HA-induced Ca^{2+} mobilization. Following the increase of intracellular Ca^{2+}, hyaluronic acid receptors (GP85/CD44) form surface patched/capped structures. Cytoskeletal proteins such as actomyosin, fodrin and ankyrin, are preferentially accumulated underneath the HA-induced GP85/CD44 patched/capped structures. The Ca^{2+} ionophore, ionomycin stimulates HA-induced receptor patching and capping. Various agents, such as EGTA (a Ca^{2+} chelator), nefedipine/bepridil (Ca^{2+} channel blockers), W-7 (a calmodulin antagonist) and cytochalasin D (a microfilament inhibitor), inhibit HA-induced receptor redistribution. These findings suggest that the sub-plasma membrane actomyosin contractile system, which is regulated by Ca^{2+} and calmodulin, is involved in hyaluronic acid-mediated lymphocyte adhesion function.