IN SEARCH OF:
A MEMBRANE SIALOGLYCOPROTEIN CELL GROWTH INHIBITOR

by

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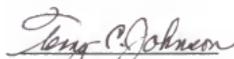
Major Professor

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SECTION II

HISTORICAL REVIEW

Density-dependent Cell Growth

Cell-cell interactions are involved in the regulation of cell growth. The study of such cellular interactions and cell growth control was simplified by the development of *in vitro* cultivation. Eukaryotic cells were first successfully grown in culture in the early 1900s by Harrison (1907) and Carrel (1912), and these earliest studies of cultured cells included simple observations of cellular behavior in this unnatural environment. Abercrombie and Heaysman (1954) described the formation of "monolayers" of cells grown in hanging drop slides and demonstrated that the cellular arrangement into monolayers resulted from "contact inhibition." Contact inhibition referred to the phenomenon whereby cellular movement was restricted through cell-cell contact. Since that initial observation, numerous researchers have explored the causes of such cell behavior.

Further investigations suggested that not only was cell motility diminished, but also cell proliferation was reduced upon cell-cell contact. Later, these two events

were seen to be separate; Stoker and Rubin (1967) proposed terminology to distinguish between contact inhibition, meaning restricted movement of cells, and density-dependent inhibition, meaning restricted growth of cells. Subsequently, however, these terms have been used inter-changeably in reference to cell growth control.

A loss of contact or density-dependent inhibition of cell growth was correlated to tumorigenicity of cell populations. Aaronson and Todaro (1968) found that cell lines which grew efficiently at high saturation densities *in vitro* produced tumors *in vivo* more frequently than cell lines which demonstrated contact inhibition in culture. About the same time, Holley and Kiernan (1968) demonstrated that contact inhibition of cells in culture could be overcome by serum factors. They found that the final cell density was directly proportional to the concentration of serum in the medium. In other words, the more serum (thus, serum factors) present, the greater the number of cells produced in a given time and space. Such growth-promoting molecules have been the object of many investigative pursuits.

Positive Growth Regulators

Enhancement of cellular proliferation has been attributed to many agents including known transforming substances (such as viruses and phorbol esters), serum factors and cell-associated molecules. The numerous transforming substances are beyond the scope of this review and the serum components have not yet been completely identified; therefore, only those agents isolated from cells will be discussed here.

The majority of cell-derived molecules have been described as polypeptide growth factors. These polypeptide growth factors are similar to hormones in that they are relatively small polypeptides and that they are released and received by cells. Several such molecules have been isolated, purified and characterized in detail; while others have been purified and/or characterized to a lesser extent.

One of the first of these molecules to be isolated and carefully studied was epidermal growth factor (EGF). EGF activity was initially discovered about thirty years ago in an extract of mouse submaxillary glands by Cohen (1962), while he was studying nerve growth factor (NGF). Later, Cohen also isolated EGF from human urine (Cohen

and Carpenter, 1975), where originally it was described as urogastrone and subsequently determined to be equivalent to EGF. The physical properties of EGF include a low molecular weight of approximately 6,000 Daltons and an isoelectric point of 4.6. Its amino acid sequence has been determined, revealing a single polypeptide chain of 53 residues with three intramolecular disulfide bonds (Taylor *et al.*, 1972). Generally, EGF elicits a mitogenic response in cultured cells, and the response has been characterized experimentally by increased cell density and increased macromolecular synthesis, including DNA, RNA and protein (Cohen and Stastny, 1968; Hoober and Cohen, 1967a; 1967b; Westermark, 1976). Additionally, biological responses to EGF, which have been measured in various cells, involve ion transport, glycolysis, membrane alterations, viral growth and production of specific molecules (i.e. human chorionic gonadotropin, prostaglandin, etc.) (Carpenter and Cohen, 1979).

NGF activity was described by Bueker in 1948 in a mouse sarcoma. NGF was subsequently discovered in mouse submaxillary gland, snake venom, chick embryo, rat granuloma and other tissues and cells (reviewed in Gospodarowicz and Moran, 1976). The purified polypeptide

was determined to be a dimer consisting of chains of 118 amino acids bonded by three disulfide linkages (Angeletti and Bradshaw, 1971). Structurally and functionally, NGF is similar to both insulin and EGF, although it does not exhibit a mitogenic effect on cultured cells. NGF must be present in order to stabilize embryonic nerve cells *in vitro*, without NGF most nerve cells would disintegrate in culture (Bradshaw, 1978). Levi-Montalcini and Booker (1960a) demonstrated that NGF was necessary for cultured neurons to extend neurites. They also showed that the sympathetic nervous system of neonatal animals injected with anti-NGF antibodies was destroyed (1960b).

Another growth factor, which proved to be extremely interesting, was isolated from virally-transformed cells in culture and was, therefore, called transforming growth factor-β (TGF-β). TGF-β was shown to be a polypeptide of 25,000 Dalton molecular weight composed of two 12,500 Dalton subunits linked by disulfide bonds. TGF-β, although larger than EGF, was categorized with EGF due to similarites in structure (James and Bradshaw, 1984). TGF-β elicited opposing biological responses from various cells *in vitro*. Generally, fibroblasts were stimulated mitogenically, while epithelial cell lines appeared to be inhibited in proliferation. This contradictory activity

has not been fully explained through experimentation to date, but the resolution of this paradox may uncover some important information related to the positive and/or negative regulation of cell growth.

These various growth factors have been proposed to exert their influence by one of three modes of transmission (James and Bradshaw, 1984). The first model was related to normal hormone activity, or endocrine secretion. Endocrine secretion described the process whereby a molecule was produced by a cell or tissue, released into a transport system (i.e. the bloodstream), and subsequently, received by another cell far-removed from the original secreting cell. Alternatively, the released molecule might not have traversed such distances, but merely migrated to a neighboring, non-secreting cell where it was received. Factors confined to a limited area illustrated the paracrine secretion model. Independent of the reaction of distant or neighboring cells, an actively secreting cell could contain receptors for the released molecules, thereby causing an effect in the manufacturing cell itself. This process has been referred to as autocrine secretion. Most of the growth factors have been demonstrated to operate in a paracrine fashion, but some that utilized

endocrine (insulin-like growth factors) or autocrine (TGFs) signaling have also been identified.

Regardless of the route of transport, these positive growth regulators induce mitogenesis by binding to specific receptors on the cell surface. The growth factors do not necessarily need to be internalized into the cell in order to initiate a response. Once a receptor-ligand complex is formed, a cascade of events proceeds within the cell. Among the intracellular changes that occur following receptor binding are influx of sodium ions, efflux of hydrogen ions, release of stored calcium ions, increased turnover of phosphatidyl inositols, and activation of protein kinase C (Deuel, 1987).

The multitude of changes that occur within the cell prior to division seem to be intricately connected. For example, the hydrolysis of phosphatidyl inositols produces diacylglycerol and inositol phosphates. Inositol triphosphate may be a second messenger which couples membrane receptor binding to the release of intracellular calcium. At the same time, diacylglycerol triggers the activation of protein kinase C.

Molecules which may induce the hydrolysis of phosphitidyl inositols by acitivating phospholipase C are

what are termed G proteins. G proteins are membrane molecules composed of three polypeptide subunits which dissociate upon activation. The α-subunit of G proteins binds GTP, and in this state, is active. The active subunit of various G proteins not only may activate phosopholipase C, but also may stimulate or inhibit adenylate cyclase, which alters cellular cyclic AMP levels. Cyclic AMP triggers intracellular responses to hormones and possibly other regulatory factors.

The various positive growth regulators cause cell division via different pathways. Because the processes involved in mitosis are intertwined, it is plausible that a single inhibitory element could block the action of more than one stimulatory factor.

Negative Growth Regulators

I. Soluble Factors Responsible for Inhibition

The discovery of growth stimulatory factors in serum
and various tissues, and the demonstration that cells in
culture were density-dependent growth-arrested led some
investigators to believe that antagonistic, inhibitory
factors might also exist. Some researchers believed that
such inhibitory factors might be released from cells in
response to cell-cell contact and be present in
conditioned culture media.

In 1974, the first cell-elaborated inhibitory factor
was isolated by Lipkin and Knecht (1974). This
diffusible factor which was isolated from culture media
of a contact-inhibited, amelanotic melanoma cell line was
capable of restoring contact inhibition to highly
malignant melanocytes *in vitro*. The factor was
demonstrated to contain protein and be of high molecular
weight, but was not purified to homogeneity. Similarly,
Holley *et al*. (1978) have shown that the density-
dependent regulation of growth of BSC-1 cells (African
green monkey kidney) resulted, in part, from the
accumulation of inhibitors in the medium. In 1980,
Holley *et al*. (1980) partially purified two high

molecular weight growth inhibitors from culture medium of BSC-1 cells. These factors were shown to inhibit thymidine incorporation in BSC-1 cells by 85%. The actual number of cells in culture was also limited by the inhibitory proteins. The action of these inhibitors could be counteracted by the addition of EGF. One of the BSC-1 cell inhibitors was later shown to be structurally similar to TGF-β (Tucker *et al.*, 1984). In the last decade, several other groups have isolated and/or partially purified inhibitors from various sources. The hepatic proliferation inhibitor (HPI) was separated from a growth stimulatory fraction of rat liver homogenates by McMahon, Farrelly, and Iype (1982). HPI was found to have a molecular weight of 26,000 Daltons and an isoelectric point of 4.65; its inhibitory activity was demonstrated by significantly reducing the number of colonies formed by Fischer nonmalignant rat liver cells.

Harel, Blat, and Chatelain (1985) isolated both inhibitory and stimulatory factors from 3T3 cell conditioned medium. The separated factors were IDF45, a 45,000 Dalton inhibitory polypeptide, and SDF10, a 10,000 Dalton stimulatory protein. IDF45 was demonstrated to inhibit the stimulation, by SDF10, of nucleic acid synthesis in 3T3 cells. Also from 3T3 cell conditioned

medium, Steck *et al.* (1982) isolated a polypeptide
enriched fraction which they dubbed FGR-s (Fibroblast
Growth Regulator-secreted). Partially purified, FGR-s
contained two major polypeptides of molecular weights of
10,000 and 13,000 Daltons. This extract was shown to
reduce the number of 3T3 cells per colony by two-thirds.
Later, Hsu, Barry, and Wang (1984) produced a monoclonal
antibody to FGR-s that bound the 13,000 Dalton protein
and successfully neutralized the inhibitory activity.

In 1984, Boehmer *et al.* (1984) isolated and
subsequently purified MDGI (Mammary Derived Growth
Inhibitor) from normal bovine mammary glands. This
protein had a molecular weight of 13,000 Daltons and
inhibited the number of Ehrlich ascites mammary carcinoma
cells *in vitro* by up to 40%. The MDGI inhibitory effect
was not only abolished in culture by both EGF and
insulin, but also neutralized by anti-inhibitor antiserum
raised in mice. Interestingly, in 1987, Boehmer *et al.*
(1987) discovered that antibodies prepared against MDGI
cross-reacted with FGR-s (13 kD). There may be
additional revelations such as that of the MDGI and FGR-s
investigators. Such released inhibitors may function in
an autocrine, paracrine and/or endocrine fashion, and
presumably, these molecules usually act in an autocrine

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or paracrine manner. As discussed above, a number of the
inhibitors exert their effects on cells identical to
those from which they were isolated. Logically,
autocrine or paracrine secretion would explain density-
dependent behavior of cells in culture, because cells
within a confined space seem to signal themselves and/or
each other to cease proliferation. Additionally,
although positive growth regulators (growth stimulatory
factors) have been shown to be present in serum, none of
the inhibitors mentioned here have been isolated from
serum.

II. Cell-associated Factors Responsible for Inhibition

Dulbecco and Stoker (1970) proposed that the cell
growth inhibition of normal cells *in vitro* might be a
result of cell-to-cell contact either directly or
indirectly. As cell membranes approach one another, the
receptors for various growth factors could be blocked,
thus indirectly stopping cell proliferation. Later,
Dulbecco (1970) showed that most likely "topoinhibition"
was not caused by occlusion of receptors, but resulted
directly from cellular interactions.

When Dulbecco showed that cell growth inhibition
seemed to be a result of direct contact, other groups

(Burger, 1970; Sefton and Rubin, 1970) demonstrated that cells in culture lost contact inhibition when treated with proteolytic enzymes. Shortly thereafter, Pardee (1971) noted that during the process of cell division, the cell surface changed. Taken together, these observations suggested that some cell surface protein(s) which was(were) responsible for conferring density-dependent inhibition of growth to cells might indeed exist.

Following this lead, several groups attempted to isolate such a molecule. In 1976, Fisher and Koch (1976) isolated inhibitory peptides by treating HeLa cells with dilute pronase. HeLa cell surface peptides (HSP) were shown to inhibit protein synthesis of HeLa cells in culture and also in cell-free translation systems. HSP were incorporated into HeLa cells rapidly and appeared to associate with monosomes and polysomes. Using a similar method, Yaoi (1984) isolated inhibitory glycopeptides from primary chick embryo fibroblasts. These molecules reduced cell growth rate and thymidine uptake of chick embryo fibroblasts *in vitro*, but this inhibitory effect was partially destroyed by neuraminidase treatment and completely destroyed by periodate oxidation of the

sialoglycopeptides, suggesting that carbohydrates were responsible for the inhibition.

Finally, the Johnson laboratory isolated and purified glycopeptides from both mouse and bovine brain (see table for comparisons). The first glycopeptide inhibitors were proteolytically removed from mouse cerebral cortex cells in 1979 (Kinders, Johnson, and Rachmeler, 1979). These molecules, called BCSG (brain cell surface glycopeptides), had molecular weights of 10,000-25,000 Daltons and were shown to inhibit protein synthesis of various cells in culture. Subsequently, Kinders and Johnson (1982) isolated similar glycopeptides from bovine cerebral cortices. The first bovine derivative, which bound to *Ulex europaeus* agglutinin, thereby suggesting the presence of fucose, had an apparent molecular weight of 18,000 Daltons. The glycopeptide was demonstrated to inhibit cell proliferation and protein synthesis of BHK-21 (baby hamster kidney) cells *in vitro*. More recently, another bovine cerebral cortex glycopeptide has been purified (Sharifi *et al.*, 1986) which also had a molecular weight of approximately 18,000 Daltons, but bound to *Limulus polyphemus* agglutinin and not to *Ulex europaeus* agglutinin, suggesting the presence of sialic acid

instead of fucose. The sialoglycopeptide (SGP) inhibitor

reduced macromolecular synthesis and cellular mitosis in

a wide spectrum of cell types (Fattaey, Johnson, and

Chou, submitted). Binding of the SGP to a specific

receptor (Bascom, Sharifi, and Johnson, 1986) was

sufficient to produce the effects (Sharifi, Bascom, and

Johnson, 1986), which were determined to be reversible.

The inhibitor was capable of antagonizing the stimulatory

effects of bombesin (Sharifi, unpublished), TPA (12-*O*-

tetradecanoylphorbol-13-acetate) (Chou *et al.*, 1987), and

EGF (Bascom, Sharifi, and Johnson, 1987).

      If these molecules were indeed cell surface

proteins, then the groups highlighted above only isolated

a portion of the inhibitory proteins by limited

proteolysis. Concurrently, other investigators were

working with cell membranes. Natraj and Datta (1978)

extracted FGFR (Fibroblast Growth Regulatory Factor) from

3T3 cell membranes with urea. Previously, Whittenberger

and Glaser (1977) had demonstrated that isolated 3T3 cell

membranes were capable of inhibiting growth of cells in

culture. Also in 1978, Whittenberger *et al.* (1978)

proceeded to extract the inhibitory molecule from

membrane fractions with the detergent, octylglucoside.

Proteins were then partially purified from this

octylglucoside extract by Raben, Lieberman, and Glaser
(1981).

TABLE. The information illustrated was collated from the Johnson laboratory. In each case, the cerebral cortices were prepared similarly for isolation of the various inhibitors. Briefly, cerebral cortex cells were treated with dilute protease, the elaborated proteins were ethanol precipitated and then extracted with chloroform-methanol.

Abbreviations are as follows: UEA-1=*Ulex europaeus* agglutinin, WGA=Wheat germ agglutinin, S-E HPLC=size-exclusion HPLC (high performance liquid chromatography), HI HPLC=hydrophobic interaction HPLC, HA HPLC= hydroxy-apatite HPLC, LPA=*Limulus polyphemus* agglutinin, ND=not determined, Met=methionine, Thy=thymidine, and Urd=uridine. Molecular weights in parentheses were determined using BioGel, others were determined by electrophoresis.

Comparison of inhibitors from cerebral cortices.

Factor	Murine[1]	I. Bovine[2]	II. Bovine[3]
Purification Steps	BiogelP-100[1] UEA-1[1]	BioGelP-100[2] UEA-1[2] CM agarose[7]	DEAE agarose[3] WGA[3] S-E HPLC[3] HI HPLC[3] HA HPLC[9] LPA[3]
Molecular Weight	(26,000)[1] (10,000)[1]	(45,000)[2] (10,000)[2] 18,000[2]	18,000[3]
Isoelectric Point	ND	8.1[8]	3.0[3]
Inhibition:			
Met incorporation	+[1]	+[2]	+[3]
Thy incorporation	ND	ND	+[10]
Urd incorporation	−[4]	ND	+[11]
Cell division	+[4]	+[2]	+[10,12]
Cell cycle block	G2[5]	ND	G1[12]
Cell Specificity	A	B	C

A Primary cell lines most sensitive, transformed cell
  lines least sensitive, tumor cells sensitized by ganglio-
  sides.[1,6]
B Established cell lines and cells treated with
  gangliosides sensitive, transformed cells insensitive.[2,7]
C All cells tested to date sensitive, including primary,
  established, transformed, avian, and insect cell lines.[12]
1 Kinders, Johnson, and Rachmeler, 1979.
2 Kinders and Johnson, 1982.
3 Sharifi et al., 1986.
4 Kinders et al., 1980.
5 Kinders and Johnson, 1981.
6 Kinders, Rintoul, and Johnson, 1982.
7 Bascom et al., 1985.
8 Johnson, Kinders, and Sharifi, 1985.
9 Bascom et al., 1986.
10 Chou et al., 1986.
11 Bascom et al., in preparation.
12 Fattaey, Johnson, and Chou, submitted.

SECTION III

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SECTION IV

MANUSCRIPT: IN SEARCH OF--A MEMBRANE SIALOGLYCOPROTEIN
CELL GROWTH INHIBITOR

INTRODUCTION

The regulation of cell growth has been the subject
of many studies and several investigators have isolated
molecules from cells which inhibit cellular metabolism.
A few of these inhibitory factors have been demonstrated
to be membrane components, but none have been completely
purified as membrane proteins. The possible residence of
such molecules on the cell surface suggests that they may
play an important role in cell-cell recognition, cell-
cell communication and thus, the density-dependent growth
behavior of cells in culture. The function of such
growth inhibitors has been studied *in vitro*, using
purified or partially purified molecules.

Sharifi *et al.* (1986) have isolated and purified, to
near homogeneity, a glycopeptide inhibitor from intact
bovine cerebral cortex cells that had an apparent
molecular weight of 18,000 Daltons, an isoelectric point
of 3.0 and has been shown to contain sialic acid by
selectively binding to a *Limulus polyphemus* lectin
affinity column.

The sialoglycopeptide (SGP) was purified by
treating bovine cerebral cortex cells in suspension with
dilute pronase, followed by ethanol precipitation,

chloroform/methanol extraction, DEAE-agarose
chromatography, wheat germ agglutinin chromatography,
size exclusion HPLC, and hydroxyapatite HPLC (Sharifi *et
al.*, 1986).

The SGP inhibited the incorporation of radiolabeled
methionine into cellular proteins and radiolabeled
thymidine and uridine into cellular nucleic acids
(Sharifi *et al.*, 1986; Bascom *et al.*, in preparation;
Fattaey, Johnson, and Chou, submitted). The lack of
incorporation of such precursors into cellular products
suggested an inhibition of cell division. It has also
been shown that the SGP reduced the proliferation of
cells, as determined by direct cell count (Chou *et al.*,
1987; Fattaey, Johnson, and Chou, submitted). The
inhibitor appeared to synchronize 3T3 cells in the G1
phase of the cell cycle and upon removal of the SGP, the
cells resumed proliferation.

In order to supress cell proliferation, the SGP did
not need to be internalized by the cells. Binding to a
specific cell surface receptor was sufficient for the SGP
to mediate its inhibition (Bascom, Sharifi, and Johnson,
1986; Sharifi, Bascom, and Johnson, 1986). Additionally,
the inhibitor was shown to be a potent antagonist of the
mitogenic activity of growth factors and a tumor

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promoter. The SGP successfully inhibited the stimulation of cells by bombesin (Sharifi, unpublished), epidermal growth factor (Bascom, Sharifi, and Johnson, 1987), and TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Chou *et al.*, 1987).

As stated previously, the SGP was isolated by proteolytic cleavage from the surface of bovine cerebral cortex cells, thereby presumably generating a fragment of a resident membrane glycoprotein, or "parental" glycoprotein. The inhibitory fragment was soluble in water and therefore, unlike the typical hydrophobic proteins found in membranes, was convenient to purify by routine biochemical procedures that employ aqueous solvents.

Prior to this study, rabbit polyclonal anti-SGP antibody was produced and the availability of this antibody provided an opportunity to assess a "one-step" purification scheme. Theoretically, the cell growth inhibitor (SGP) and/or its "parental" membrane protein could be isolated by applying an extract from cerebral cortex cells to a matrix coupled with anti-SGP polyclonal antibody. Such a strategy was employed in the following attempt to purify the "parental" sialoglycoprotein in its native state, as a membrane molecule.

MATERIALS AND METHODS

Isolation

Slices of bovine cerebral cortex tissue were removed
from fresh bovine brain, placed in ice-cold DMEM
(Dulbecco's Modified Eagle Medium, GIBCO Laboratories
Life Technologies, Inc., Grand Island, NY) containing
25 mM HEPES buffer (pH 7.1) and weighed. A cell suspen-
sion was prepared by repeated pipetting. The cells were
pelleted by centrifugation at 3,000 rpm for 5 minutes and
resuspended in 1-2 ml ice-cold solubilization buffer per
gram of wet tissue. Solubilization buffer contained 1%
w/v octyl-β-D-glucopyranoside (Behring Diagnostics, La
Jolla, CA), 10 nM phenylmethylsulfonylfluoride (Serva
Fine Biochemicals, Inc., Westbury, NY), 5 nM
phosphoramidon (Sigma Chemical Company, St. Louis, MO),
10 nM leupeptin (Serva Fine Biochemicals, Inc., Westbury,
NY), 10 nM pepstatin A (Sigma Chemical Company, St.
Louis, MO), in phosphate buffered saline (PBS), where PBS
was composed of 0.9% w/v sodium chloride, 10 mM sodium
phosphate, pH 7.2. After incubation on ice for 30
minutes with periodic mixing, the suspension was
centrifuged at 5,000 rpm for 5 minutes. The supernatant

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fluid was collected and re-centrifuged at 100,000 x g for
one hour. The supernatant liquid was then concentrated
to a final volume of 10 ml by ultrafiltration using a
Diaflo YM100 membrane (Amicon Corporation, Danvers, MA).

Protein Determination

Protein content was determined by the BCA
(bicinchoninic acid) protein assay (Pierce Chemical
Company, Rockland, IL) using bovine serum albumin in PBS
containing 1% (w/v) octyl-β-D-glucopyranoside
(octylglucoside) as a standard.

Polyclonal Antibody Affinity Column

Typically, 1.5-2 ml activated agarose slurry, Affi-
gel 10 (BioRad Laboratories, Richmond, CA), were washed
with at least 5 column volumes of ice-cold distilled
water followed by approximately 5 column volumes of PBS.
Approximately 200 μg of polyclonal antibody, adjusted to
a final volume of 1 ml, were added to the column matrix
and rotated on a Fisher Hematology/Chemistry Mixer
(Fisher Scientific, Pittsburgh, PA) continuously, over-
night, at 4°C. The Affi-gel 10 was then washed with at

least 10 column volumes of PBS and fractions were saved
to determine the amount of uncoupled antibody. Unreacted
Affi-gel 10 was then blocked by washing successively with
several volumes of 3 M ammonium thiocyanate, 50 mM
ethanolamine (pH 11.0) and/or 2 M glycine (pH 2.5).
Excess blocking agents were then removed by extensive
washing with 100-500 column volumes of PBS, followed by
equilibration with 10 column volumes of PBS containing 1%
(w/v) octylglucoside.

Polyclonal Antibody Affinity Chromatography

Concentrated, solubilized bovine cerebral cortex
proteins were applied to the polyclonal antibody affinity
column and incubated at 4°C, overnight. The column was
washed with PBS containing 1% (w/v) octylglucoside until
no absorbance at 280 nm was detectable. Elution buffer
(1% w/v octylglucoside in either 50 mM ethanolamine or 2
M glycine) was added to elute antibody-bound proteins.
Samples were collected until absorbance was zero. A
single protein peak was eluted and the collected
fractions were pooled, dialyzed against dilute PBS
containing 1% (w/v) octylglucoside, dried in a Speed Vac

Concentrator (Savant Instruments, Inc., Hicksville, NY)
and stored at -20°C.

SDS-PAGE

Proteins were diluted 1:2 (v/v) in sample buffer (36
mM sodium phosphate, pH 7.2 with 35% glycerol, 4% sodium
dodecyl sulfate, 0.1% bromphenol blue with or without 18%
β-mercaptoethanol). Samples were incubated at 100°C on a
heating block for 5-10 minutes and then were electro-
phoresed in a 5%-20% polyacrylamide gradient, essentially
by the method of Laemmli (1970). Molecular weight
standards (prestained, Bethesda Research Laboratories
Life Technologies, Inc., Gaithersburg, MD) were myosin H-
chain (200,000), phosphorylase B (97,400), bovine serum
albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen
(25,700), β-lactoglobulin (18,400) and lysozyme (14,300).

Isoelectric Focusing

Isoelectric focusing was performed by a technique
similar to the method of Vesterberg (1972). The gel was
poured between a Gel-Bond (FMC Corporation, Rockland, ME)
and glass plate sandwich, using the following solution:

5% (v/v) acrylamide stock, 10% (v/v) glycerol, 0.9% (v/v)
Triton-X 100 (CalBiochem, La Jolla, CA), 0.3% (v/v)
Ampholine pH 4-6 (LKB Wallac, Finland), 0.3% (v/v)
Ampholine pH 5-7, 0.6% (v/v) Ampholine 9-11, 4.6% (v/v)
Ampholine pH 3.5-10, and 0.05% (w/v) ammonium persulfate.
The electrodes were saturated with 0.5 N sodium hydroxide
and 0.5 N acetic acid. After focusing for 90 minutes at
25 Watts, the gel was removed. The pH gradient was
measured using a flat-bed electrode, unless isoelectric
focusing standards were used. Broad Range IEF Standards
from BioRad included phycocyanin-pI 4.65, β-lactoglobulin
B-pI 5.10, bovine carbonic anhydrase-pI 6.00, human
carbonic anhydrase-pI 6.50, equine myoglobin-pI 7.00,
whale myoglobin-pI 8.05, chymotrypsin-pI 8.80, and
cytochrome c-pI 9.60.

Size Exclusion HPLC

A molecular sieving column (TSK G3000, Phenomenex,
Rancho Palos Verdes, CA) was equilibrated with PBS
containing 1% (w/v) octylglucoside, samples were applied,
and 1 ml aliquots collected, at a flow rate of 1
ml/minute. Absorbance was monitored at 280 nm.

Limulus polyphemus Agglutinin (LPA) Chromatography

About 2 ml (final column volume) of LPA (E-Y
Laboratories, San Mateo, CA) were equilibrated with at
least 10 column volumes of 10 mM Tris buffer, pH 8.0,
containing 10 mM calcium chloride and 1% (w/v)
octylglucoside. Bovine cerebral cortex solubilized
proteins at various stages of purification were added to
the matrix and rotated on a Fisher Hematology/Chemistry
Mixer (Fisher Scientific, Pittsburgh, PA) continuously,
at 4°C, overnight. The LPA was washed until no
absorbance at 280 nm was detectable. Subsequently,
lectin-bound molecules were eluted with 100 mM sialic
acid, pH 8.0 with 0.1% EDTA and 1% (w/v) octylglucoside.
Eluted fractions were dialyzed against dilute PBS
containing 1% (w/v) octylglucoside and dried in a Speed
Vac Concentrator (Savant Instruments, Inc., Hicksville,
NY).

Reduction and Blocking

Bovine cerebral cortex protein extracts were reduced
by adding β-mercaptoethanol to a final concentration of
9% (v/v) and incubating at room temperature for 2 hours.

The sulfhydryl groups were alkylated by dialysis against 100 mM iodoacetamide for 4 hours followed by dilute (0.1 mM) iodoacetamide at 4°C, overnight.

Cell Culture

Swiss 3T3 cells were maintained as monolayer cultures in humidified, 37°C incubators containing 95% air and 5% carbon dioxide, as described by Kinders *et al.*, (1979). Cells were grown in Dulbecco's Modified Eagle Medium (GIBCO Laboratories life Technologies, Inc., Grand Island, NY), supplemented with 10% calf serum (Hazelton Dutchland, Inc., Denver, PA) and were harvested for assay by scraping culture flasks with sterile rubber policemen.

Biological Inhibitory Activity

The method utilized to determine the capability of a molecule to inhibit protein synthesis of cells has been described by Kinders *et al.*, (1979). Basically, Swiss 3T3 cells were harvested from subconfluent monolayers after washing briefly with methionine-free Minimal Essential Medium containing 25 mM HEPES buffer (MEM-

HEPES). The cell suspension was then centrifuged; the pelleted cells were resuspended and counted in a hemacytometer, and diluted to provide 2-5×10^5 cells in 100 µl of warm (37°C), methionine-free MEM-HEPES. Potential inhibitory substances were pipetted into 13 X 100 mm test tubes at different concentrations, each in a final volume of 40 µl in PBS. Cell aliquots were then added and the tubes were incubated, at 37°C, for 30 minutes. At the end of this incubation, 2.0 µCi of ^{35}S-methionine in 10 µl of methionine-free MEM were added and the tubes immediately reincubated, at 37°C, for 10 minutes. Incorporation was stopped by the addition of 2 ml ice-cold HKM buffer (10 mM HEPES, 120 mM potassium chloride, and 5 mM magnesium chloride, pH 7.1) and centrifugation of the cells, at 2,000 rpm, for 3 minutes. After removal of supernatant liquid, the cells were lysed with distilled, deionized water and 1 N sodium hydroxide, followed by precipitation of proteins with an equal volume of ice-cold 20% trichloroacetic acid. Precipitated molecules were collected by centrifugation and the previous steps repeated. On the third repetition, trichloroacetic acid was omitted and a 100 µl aliquot was removed for liquid scintillation counting.

Immunoblotting

Samples were either directly spotted onto pre-wet nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) using a Minifold (Schleicher and Schuell, Inc., Keene, NH) or electrophoresed and electroblotted using a Transphor Electrophoresis Cell (Hoefer Scientific Instruments, San Francisco, CA) by the method of Towbin, *et al.*, (1979). Following transfer, the nitrocellulose membrane was dried and then blocked overnight, at room temperature, in blocking buffer (0.1 mM EDTA, 0.01% w/v sodium iodide, 5 mM sodium chloride, 1 mM Tris, pH 7.0 and 0.3% Tween 20). The membrane was subsequently washed 5 times, for 5 minutes each, with wash buffer (1% w/v sodium chloride, 100 mM Tris, pH 7.5 and 0.3% Tween 20). Antibody was added, in blocking buffer, and the membranes were incubated overnight, at room temperature. The blot was washed again, followed by the addition of ^{125}I-radiolabeled staphylococcal Protein A at 100,000 CPM per ml of blocking buffer. Finally, the membrane was washed, dried and used to expose an X-ray film (Agfa-Gevaert, Belgium).

Isolation

The strategy designed to purify the parental sialoglycoprotein inhibitor is illustrated in Figure 1. Fifteen independent isolates from fresh bovine cerebral cortex tissue cell suspensions were studied. The cells were pelleted by low speed centrifugation and extracted with 1% (w/v) octylglucoside. Only 4.0% of the total cellular protein was extracted by this method. Following solubilization, the cells were again centrifuged; the supernatant fluid, containing detergent-extracted proteins, was retained and concentrated by ultrafiltration. The concentrated extract contained 3.0% of the total cerebral cortex cell protein, as measured by BCA protein assay (Table 1).

Polyclonal Antibody Affinity Purification

The detergent-extracted proteins were applied to a polyclonal antibody affinity column and subsequently eluted with either ethanolamine or glycine, containing 1% (w/v) octylglucoside (Fig. 2). In either case, the

collected fractions were immediately neutralized, dialyzed against dilute PBS, and analyzed by electrophoresis. In parallel experiments with the 18,000 Dalton SGP inhibitor, it was determined that the sialoglycopeptide was not efficiently released from a polyclonal antibody affinity column by 50 mM ethanol-amine. Subsequently, biologically active SGP was successfully eluted from the antibody column with less than ten column volumes of 2M glycine, pH 2.5.

Because of the previous observation, the majority of "parent" isolations were conducted using glycine as elution buffer. The amount of protein recovered by elution with 50 mM ethanolamine was 0.002% of the total cerebral cortex cell protein compared to 0.003% of the total cerebral cortex cell protein eluted with 2 M glycine, pH 2.5 (Table 1).

Molecular Analysis of Polyclonal Antibody-Eluted Material

Proteins bound to the polyclonal antibody affinity column were subjected to SDS-polyacrylamide gel electrophoresis. Non-reduced preparations failed to enter the separating gel, whereas, reduced preparations demonstrated the presence of four distinct protein bands

of apparent molecular weights of 175 kD, 135 kD, 66 kD, and 56 kD (Fig. 3). This indicated that the protein (or proteins) might be a large complex that included four subunits connected by disulfide bonds.

Although the non-reduced proteins failed to enter polyacrylamide gels, it was possible that these'high molecular weight species might be discernable by size-exclusion HPLC (high performance liquid chromatography). Molecular sieving HPLC could have provided a convenient means of separation of the antibody eluted molecules, but the chromatographs from several preparations were inconsistent (Fig. 4). The hydrophobicity of these membrane proteins may have caused them to aggregate randomly into various conformations in different isolates. These complexes of diverse molecular size could have produced different elution times and peak areas in size-exclusion HPLC. In any event, it was impossible to assess the molecular weight species by HPLC.

Interestingly, the four reduced bands present in SDS gels were no longer obvious upon isoelectric focusing. Isoelectric focusing of the polyclonal antibody affinity purified material, revealed only a slight difference between non-reduced and reduced samples. Focusing did

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not produce distinct protein bands, but did show that the molecules present were acidic and heterogeneous with regard to their isoelectric points (Fig. 5). This heterogeneity suggested that the proteins were glycosylated and/or sialylated to varying degrees.

In order to determine if the antibody-bound molecule(s) contained N-acetylneuraminic acid (NANA), binding to a NANA-specific lectin, *Limulus polyphemus* agglutinin (LPA), was examined. It appeared that at least a portion of the isolated material contained sialic acid because, on average, forty percent of the protein applied to the LPA column bound, and in an early experiment neuraminidase-treated proteins were shown not to bind to the LPA. However, the amount of glycoprotein, in separate preparations, eluted from LPA by competition with sialic acid varied from three to seventy-six percent bound of the total protein applied. Polyacrylamide gels revealed similar staining patterns in both the LPA-bound and LPA-unbound fractions.

Reduction and Blocking of Polyclonal Antibody-Eluted Material

Due to the problem of aggregation of the molecules when under non-reducing conditions, it was thought that reduction followed by alkylation to prevent reformation of disulfide bonds might aid in the elucidation of the specific subunit directly related to the sialoglyco-peptide inhibitor. By decreasing the probability for subunits to bind together in disulfide linkages, it seemed possible that the molecules might remain separate during analysis or that, perhaps, the subunit most similar to the SGP might be less hydrophobic than the other subunits.

Reduction was accomplished using a final concen-tration of 9% (v/v) β-mercaptoethanol and subsequent alkylation was performed with 100 mM iodoacetamide. Sufficient blocking was demonstrated to have occurred by subjecting the alkylated molecules to SDS-PAGE in the absence of reducing agents (Fig. 6). The proteins stained by Coomassie Blue were analogous to those seen previously under reduced conditions (Fig. 3).

Reduction and alkylation did not appear to facilitate separation and identification of the subunit directly related to the SGP. For example, a reduced and blocked preparation was applied to an LPA column, producing results which were similar to those seen with non-reduced, non-alkylated protein. Approximately fifty percent of the sample applied to the LPA column was bound and released. Which of the four bands seen in SDS-PAGE actually had NANA residues was not well-defined by electrophoretic analysis of the LPA-bound and unbound fractions. Based on SDS gels, all of the proposed subunits recovered from the lectin column contained sialic acid. Nearly identical staining patterns were observed in both bound and unbound samples.

Biological Activity of Polyclonal Antibody-Eluted Material

Although the molecular characterization was ambiguous, auspicious results were obtained from a biological activity assay. One of the initial criteria demanded that the "parent" to the sialoglycopeptide cell growth inhibitor should be capable of inhibiting cell

growth and/or protein metabolism. Polyclonal antibody purified material was dialyzed to remove detergent and tested for its ability to inhibit Swiss 3T3 cells from incorporating radiolabeled methionine.

In one such experiment, protein synthesis was effectively inhibited by the putative "parent" molecule. A dose-responsive inhibition of protein synthesis was observed in Swiss 3T3 cells treated with various concentrations of antibody-purified proteins (Fig. 7). Additional trials of the biological activity assay did not reproduce the inhibition described here; therefore, the conclusion that the isolated protein was the "parent" of the SGP inhibitor was not experimentally confirmed.

The amount of potential "parent" protein used in biological activity assays was based on experiments performed with the inhibitory peptide. In preliminary isolations of the "parent", the protein was demonstrated to have an apparent molecular weight of 180,000 Daltons, which was ten times as large as the peptide inhibitor. At the time this assay was performed, 2 µg of SGP (peptide) was equivalent to one unit of biological activity, which was defined as that amount of protein that inhibited methionine incorporation by 25%.

Consequently, 20 µg of the suspected "parent" protein was
the minimum used in cell protein synthesis experiments.

After heating the antibody-isolated fraction to
100°C for 30 minutes, no inhibition of methionine
incorporation was evident in Swiss 3T3 cells, suggesting
that the inhibition described above resulted from the
presence of a non-denatured protein.

The inhibition was not due to the presence of
detergent in the protein samples, because the dialysate
from the octylglucoside removal step was used as a
control. Additionally, dilutions of detergent were
assayed in a separate experiment which demonstrated that
up to 0.02% octylglucoside would not significantly
inhibit protein synthesis in Swiss 3T3 cells (Table 2).

Immunoblotting of Polyclonal Antibody-Eluted Material

Since at least one experiment indicated that a
protein in the polyclonal antibody purified fraction was
inhibitory, it seemed logical that the molecule
responsible for inhibition could be identified by
immunoblot analysis. Polyclonal antibody-isolated
material, LPA-eluted material, and crude octylglucoside
solubilized material were subjected to immunoblot

analysis. Blots were performed by direct application of
samples to nitrocellulose (dot blots) and by SDS-PAGE
followed by electrophoretic transfer to nitrocellulose
(Western blots). Efficient transfer of proteins was
demonstrated by negative gel staining with Coomassie Blue
and positive membrane staining with Ponceau S. The
18,000 Dalton SGP inhibitor, which had been used as the
immunogen, successfully bound polyclonal antibody in an
immunoblot. During the course of this study, no reactive
species from either affinity purified samples or crude
octylglucoside extracted samples were detected in
immunoblots. However, more recently generated antiserum
does react with detergent solubilized and immunoaffinity
column purified proteins in a dot blot.

Non-specific Binding to the Polyclonal Antibody Column

Based on the lack of evidence that the isolated
proteins consistently reacted with polyclonal antibody,
it was suspected that the immunoaffinity column may not
have been binding specifically to SGP-related molecules.
Non-specific binding was tested experimentally by
applying solubilized material to an uncoupled, blocked
agarose column. In this manner, it was shown that

detergent-extracted proteins actually bound to the uncoupled agarose to nearly the same extent that they bound to antibody-coupled agarose (Table 3). This suggested that the concentration of antibody ligated to the matrix was too low or that the isolated proteins had some affinity for the matrix itself.

In order to reduce such non-specific binding, a new immunoaffinity column was prepared using a 10-100 fold greater concentration of antibody and an uncoupled agarose column was inserted into the isolation strategy as a pre-column. Neither of these procedures appeared to facilitate the purification of an immunoreactive protein, as measured by blotting.

DISCUSSION

The ultimate goal of this project was to purify the native form, or "parent" of the bovine cerebral cortex cell surface sialoglycopeptide growth inhibitor. During the process of attempting to purify the membrane sialoglycoprotein, valuable information was discovered.

The purification of the native membrane sialo-glycoprotein cell growth inhibitor proved to be a more formidable exercise than originally perceived. Purification was difficult for several reasons. First, the "parent" sialoglycoprotein probably constituted a mere fraction of the total cellular proteins, because its derivative was a minor bovine brain protein. Only microgram quantities of the SGP cell growth inhibitor were separated from over 100 grams of cerebral cortex tissue (Sharifi *et al.*, 1986).

The isolation of some protein which was relatively abundant would have been a less arduous task. For example, the isolation of comparatively large quantities of hemoglobin would have been facilitated by its known high concentration in blood: 14-16 grams of hemoglobin per 100 ml of whole blood (from New England Journal of Medicine, 1980).

While hemoglobin is relatively abundant in blood, growth factors are present in much lower concentrations in serum and growth factor receptors are comparatively scarce on cell surfaces. One group of researchers was fortunate that a unique cell line was discovered. The recognition that an epidermoid carcinoma cell line, A431, specifically bound approximately ten-fold more EGF than other human cell lines (Fabricant, DeLarco, and Todaro, 1977) enhanced the purification of the EGF receptor (Cohen, Carpenter, and King, 1980). In the present study, an abnormally rich source of the desired molecule might have been utilized, if such a source were available or identifiable.

As in the isolation of the SGP, it was speculated that brain tissue, because of its growth-arrested state *in vivo*, was a propitious source of potential cell growth inhibitory elements. It was known, as stated above, that the 18,000 Dalton SGP inhibitor was present on the surface of bovine cerebral cortex tissue in limited quantities, but it was uncertain whether or not the number of parental molecules would correlate with the number of SGP molecules.

Isolating a minute portion of the total cellular protein was difficult enough, but the fact that the

extracted membrane proteins were hydrophobic and predisposed to aggregation added another dimension to the dilemma. Further increasing the magnitude of the purification problem was the finding that at least some of the solubilized proteins tended to bind non-specifically to the immunoaffinity column.

Some of these difficulties must have been encountered by others in the pursuit of membrane inhibitory molecules. Several researchers have claimed to purify such negative growth regulators, but have not demonstrated conclusively that their preparations were indeed pure.

The "purification" of a growth inhibitor from Ehrlich ascites mammary carcinoma cells by Boehmer *et al.*, (1984) demonstrated the presence of an inhibitory protein of approximate molecular weight between 12,000 and 14,000 Daltons. The molecular weight of the major protein isolated by Grosse's group was later described as 13,000 Daltons (Boehmer *et al.*, 1985), but the ^{125}I-labeled inhibitory fraction appears as a single protein band only after fixing and washing gels as if preparing for silver staining. When gels were not prepared for silver staining, multiple bands were present. Biological activity was tested in samples which were not treated for

silver staining, therefore, inhibitory properties cannot be unconditonally attributed to the 13,000 Dalton protein. Because it was not clear that the major protein discussed above was responsible for cellular inhibition and, in an earlier publication (Boehmer *et al.*, 1984), the greatest proportion of inhibitory activity was attributed to a fraction which displayed an almost invisible protein band, the possibility exists that a minor protein actually affected cell growth.

Another group that happened upon difficulties in working with inhibitory molecules was Luis Glaser's lab. Glaser and coworkers isolated 3T3 cell membranes which were shown to inhibit cell growth (Whittenberger and Glaser, 1977). The published work was criticized because Glaser *et al.* had not purified the specific inhibitory element from the cell membrane. The arguments against Glaser included the fact that the membrane fragments could, in essence, be "smothering" the cells by not allowing interaction of growth factors in the medium with receptors on the cell surface. To refute the contention that cells treated with membrane fragments were inhibited due to lack of stimulation by resident growth factors, Whittenberger and Glaser (1978) submitted an elegant

experiment which displayed that limited diffusion was not responsible for interruption of cell growth in culture.

Whittenberger *et al.*, (1978) subsequently solubilized the membrane fragments and demonstrated that the extracted proteins could also inhibit cell metabolism. Three years later, the growth inhibitory proteins remained "partially purified" (Raben, Lieberman, and Glaser, 1981), suggesting that the researchers had not been successful at separating a homogeneous protein.

One of the biggest obstacles in the present study was the tendency of the extracted molecules to aggregate. There is a precedent for such difficulties: Patt and Houck (1980) reported that "chalones", endogenous mitotic inhibitors, had an "aggregative avidity...for various macromolecules."

The propensity for the isolated proteins to aggregate may have been the cause of some of the inconsistent experimental results. The inconsistencies may have resulted from variations in the arrangement of the aggregates. For instance, one of five biological activity assays was successful in demonstrating inhibition of cellular protein synthesis. This may have been because, in that particular trial, the protein complex was arranged such that the inhibitory portion of

the "parent" molecule was available for binding to the
surface of 3T3 cells, thereby signaling the cells to
cease proliferation.

Aside from its relatively low abundance on cerebral
cortex cells and the aggregative behavior of the native
sialoglycoprotein, a substantial inconvenience was the
preparation and utilization of an immunoaffinity column.
This project was based on the supposition that a "parent"
molecule actually existed on the surface of bovine
cerebral cortex cells. Furthermore, it was speculated
that the membrane glycoprotein in its native conformation
contained an epitope which would bind to and was
available to polyclonal antibodies raised against the SGP
inhibitor.

Consequently, the preparation of an immunoaffinity
column using the previously isolated polyclonal
antibodies was presumed to be of vital significance.
While utilizing the antibody affinity column, non-
specific binding of the solubilized proteins to the
column became apparent. The interpretation of this
result was that either the extracted molecules had a high
affinity for the column (matrix, etc.) or the antibodies
had a low (or no) affinity for the "parent" glycoprotein.
The antibody may not have bound the "parent" molecule

because the membrane glycoprotein complex conformation was such that the epitope(s) was (were) hidden. More than likely, a combination of the former was responsible for these results.

The polyclonal antibody used in the present study had been passed through an affinity column coupled with purified SGP (Sharifi, unpublished). The affinity-purified antibody was shown to have high affinity for the peptide inhibitor in radioimmunoassays (Fleenor, unpublished), but assays with tissue culture cells and bovine cerebral cortex cells failed to display immunofluorescence (Fleenor, unpublished). The inability to demonstrate fluorescence was attributed to a cell surface concentration of SGP that was under the limits of detection of the assay. Alternatively, the epitope(s) might have been masked by other cell surface proteins or the native protein itself.

A potential solution to the dilemma of non-specific binding to the immunoaffinity column would be to increase the quantity of antibody coupled to the column matrix, provided that the non-specific binding described here was due to proteins binding to the matrix and/or the plastic column. The evidence presented by the binding of molecules to the uncoupled agarose strongly suggests that

this could be the case. It is plausible that the
purification of the parental sialoglycoprotein could be
enhanced by the availability of a greater supply of
antibody and/or by a higher affinity antibody.

The affinity columns prepared in this study were not
coupled to maximum capacity, which may have provided
extra matrix surface area to which the proteins might
adhere. Logically, if the agarose beads were coupled to
capacity, then there would be fewer potential binding
sites on the matrix itself. Other laboratories have
encountered difficulties in protein purification on
activated agarose columns which were coupled at low
densities (J. Hughes, personal communication).

Additionally, antibody with a greater affinity or
alternative binding site might alleviate some of the
problems mentioned. The study described here may have
resulted in the isolation of the native membrane
sialoglycoprotein, but because of the difficulties
encountered, the "parent" molecule has not been
conclusively identified. Newly generated antibody could
conceivably react positively in an immunoblot and,
thereby, remove some of the ambiguity and doubt described
above. Such an antibody appears to have been produced
recently. This novel antiserum has not only bound crude

bovine cerebral cortex protein, detergent-extracted protein, and several of the original brain isolates in a dot blot, but also bound potential "parent" molecule to a freshly prepared immunoaffinity column. Thereafter, an immunoreactive species was identified in the eluate from the new column. These preliminary results indicate that an antigenic determinant is actually present and accessible in the octylglucoside solubilized material. Hopefully, this antibody will assist in the search for the native membrane sialoglycoprotein, provided the other obstacles described here are also conquered.

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TABLE 1. Purification of the parental sialoglycoprotein.

Purification Step	Protein (mg/g wet tissue)	Total (%)
Cerebral Cortex Cells	125.0	100
Octylglucoside Extract	5.5	4
Concentrated Extract	3.9	3
Polyclonal Antibody Bound (released with glycine)	0.004	0.003

Protein concentrations were determined by the BCA protein assay as described in Materials and Methods. Data shown are from a typical bovine cerebral cortex preparation.

TABLE 2. Inhibition of methionine incorporation by octylglucoside.

Octylglucoside Concentration (% w/v)	Inhibition (% of control)
0 (control)	0
0.012	0
0.025	6
0.05	12
0.1	40
0.25	99
0.5	99
1.0	99

Aliquots of 100 µl methionine-free MEM-HEPES containing 2-5 x 10^5 Swiss 3T3 cells were incubated with 40 µl samples of various concentrations of octylglucoside in PBS for 30 minutes, at 37°C. At the end of this incubation, the cells were radiolabeled with 2.0 µCi of ^{35}S-methionine, at 37°C, for 10 minutes and processed as described in Materials and Methods. These data represent the averages of duplicate samples.

TABLE 3. Non-specific binding of proteins to uncoupled agarose.

Column Type	Protein Bound (μg)	Total (%)
Polyclonal Antibody Coupled, Blocked Affi-gel 10	20	1.9
Uncoupled, Blocked Affi-gel 10	18	1.7
Agarose	31	2.9

Aliquots of 1 mg of solubilized bovine cerebral cortex proteins were applied to the columns, incubated at 4°C, overnight, and subsequently eluted with 2 M glycine, pH 2.5. Protein concentrations were determined by BCA protein assay. These data represent the averages of three experiments.

Figure 1. General purification procedure for the
isolation of the bovine cerebral cortex cell
membrane sialoglycoprotein.

```
          Bovine Cerebral Cortex Cells
          (suspended in DMEM-HEPES)
                      |
                      |
          Centrifuge at 1500 rpm
                      |
          +-----------+-----------+
          |                       |
        Pellet            Supernatant fluid
          |                    (discard)
          |
      Solubilization with
  1% Octyl-beta-D-glucopyranoside
          |
          |
      Centrifuge at 1500 rpm
          |
      +---+-----------+
      |               |
  Supernatant fluid  Pellet
      |            (discard)
      |
      |
  Concentration by ultrafiltration
      |
  +---+---------------+
  |                   |
Polyclonal antibody  PAGE
affinity column
  |
  +---+
  |   |
  | Unbound
  |
Bound
```

Figure 2.   Polyclonal antibody affinity column elution of
            the potential "parent" sialoglycoprotein.   The
            octylglucoside extracted fraction containing
            approximately 5 mg protein was loaded onto
            Affi-gel 10 previously coupled with polyclonal
            antibody.   The single eluted peak was
            collected and analyzed.
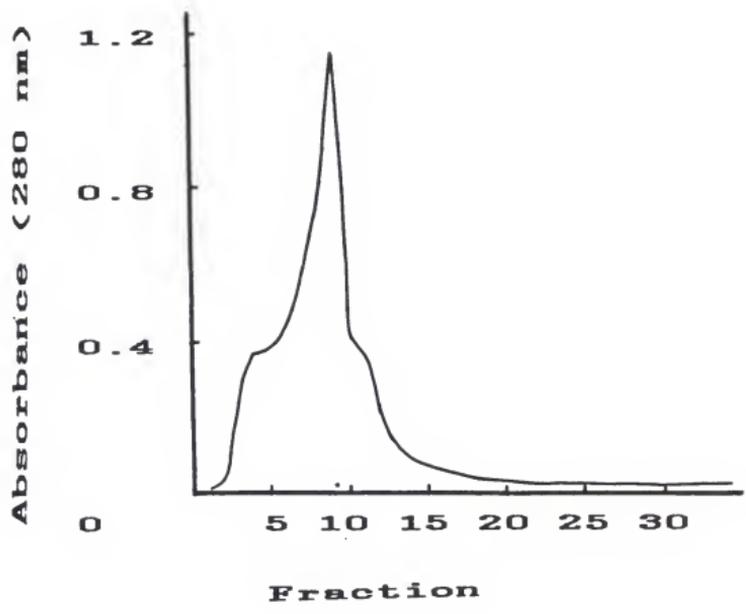
Figure 3. SDS-PAGE of fractions purified by
immunoaffinity chromatography. Three separate
preparations were electrophoresed in a gel
with a linear gradient of 5-20%
polyacrylamide. The preparations were
analyzed under non-reducing (Lanes 1-3) and
reducing (Lanes 5-7) conditions. Each lane
was loaded with 5 µg protein. Lanes 1 and 5
contain samples from the same brain
preparation, as do Lanes 2 and 6, and Lanes 3
and 7. Lane 4 contains molecular weight
standards.
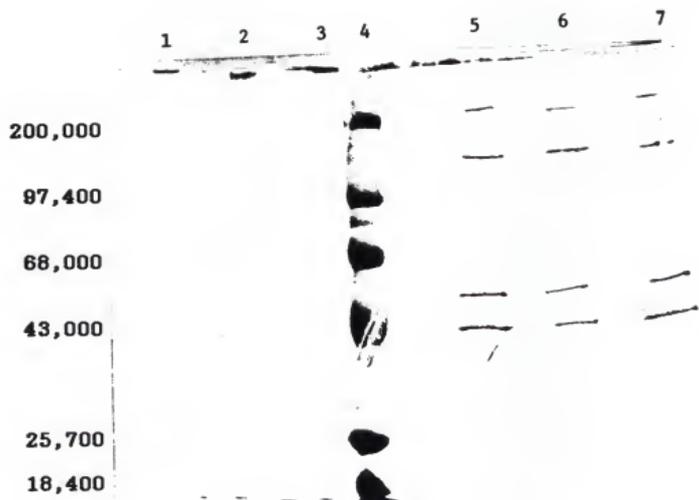
Figure 4. Size-exclusion HPLC analysis of immunoaffinity
eluted fraction. Samples containing 10 µg of
protein were loaded on a TSK G3000 HPLC column
previously equilibrated with phosphate-
buffered saline, pH 7.2. The attenuation was
set at 16 and the flowrate was 1 ml per
minute. Panels A-D illustrate HPLC profiles
from four separate brain isolates. Elution
times are as indicated.

A

8.90
10.94
11.74
12.76
13.44
.25

B

8.95

C

.18
3.33
4.68
8.75
10.17
12.11
13.27

D

.22
3.46
8.85
11.83
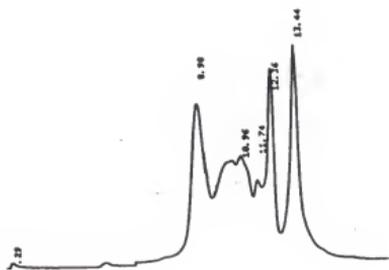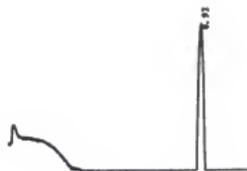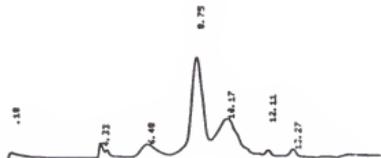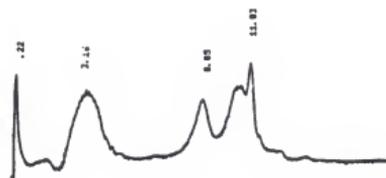
Figure 5.   Isoelectric focusing of immunoaffinity eluted
             fraction. The purified protein was radio-
             iodinated and isoelectric focused for 1.5
             hours at 25 Watts. Samples of 100,000 CPM
             were loaded into each lane. Sample A was
   reduced while Sample B was not reduced.
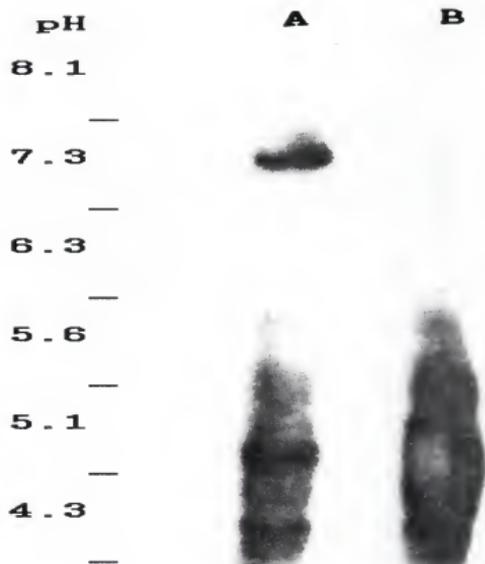
Figure 6. SDS-PAGE of reduced and alkylated
immunoaffinity eluted fraction. A sample of
5 μg of blocked protein was electrophoresed in
a gel with a linear gradient of 5-20%
polyacrylamide in the absence of reducing
agents. Lane 1 is the blocked sample and Lane
2 contains molecular weight standards.
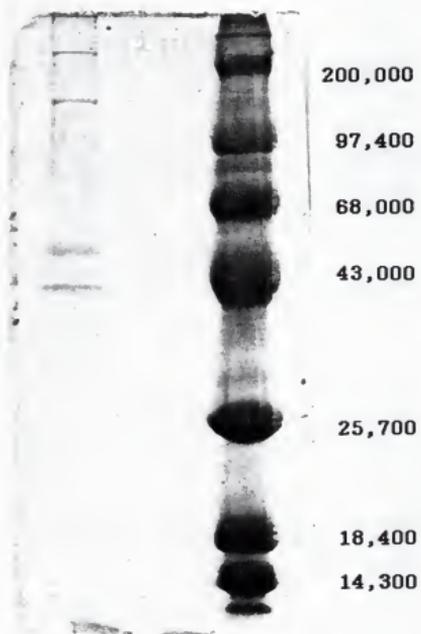
Figure 7.   Inhibition of 3T3 cell protein synthesis by an
immunoaffinity eluted fraction.   Cells in
100 µl of DMEM-HEPES were incubated at 37°C in
the presence of 20 µl of the isolated protein
in PBS, as described in Materials and Methods.
Assay was performed in duplicate and results
are expressed as percentage of inhibition as
compared with control cultures, which received
only PBS.   The vertical bars depict the range
of duplicate samples and the * indicates a
sample which was heated to 100°C for 30
minutes prior to addition to cells.
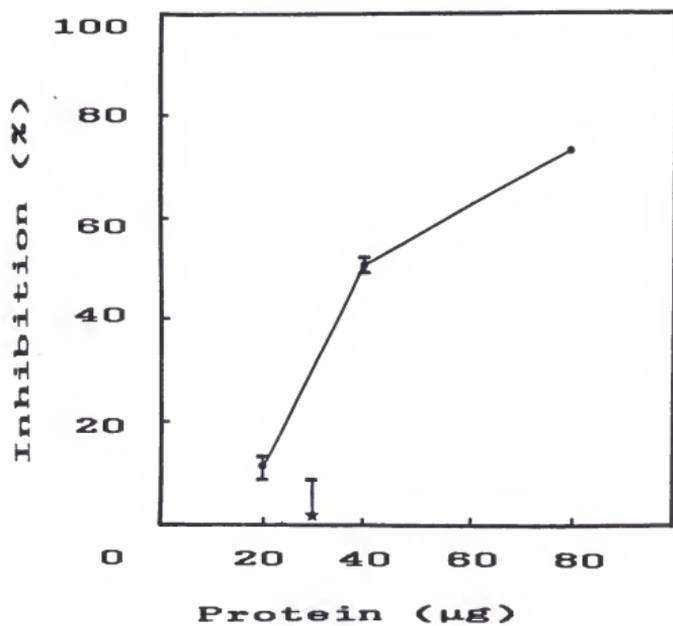
IN SEARCH OF:
A MEMBRANE SIALOGLYCOPROTEIN CELL GROWTH INHIBITOR

by

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AN ABSTRACT OF A THESIS

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ABSTRACT

Density-dependent control of cell growth in culture
may result from specific inhibitory molecules in cell
membranes interacting with specific receptors in
neighboring cell membranes. A molecule with such
potential has been proteolytically cleaved from the
surface of bovine cerebral cortex cells and has been
shown to inhibit cell division, protein synthesis, DNA,
and RNA synthesis of several cultured cell lines. The
present study utilized polyclonal antibody against the
sialoglycopeptide inhibitor in an attempt to isolate the
intact, native membrane protein from which the inhibitor
was originally derived. Bovine cerebral cortex cells
were solubilized with octyl-β-D-glucopyranoside
(octylglucoside) and the solubilized molecules subjected
to immunoaffinity chromatography. The polyclonal
antibody bound fraction was examined by SDS-PAGE, IEF,
HPLC, lectin affinity chromatography and immunoblot
analysis. The isolated material was also tested for
biological activity in a protein synthesis inhibition
assay. Upon reduction in an SDS polyacrylamide gel, the
antibody-bound fraction displayed four distinct molecular
weight bands. The extracted protein inhibited protein
synthesis in Swiss 3T3 cells; however, this inhibition

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was not predictably repeatable. Immunoblots failed to reveal antibody binding activity in either the affinity purified material or in crude detergent solubilized fractions. Based on the evidence presented, it cannot be concluded that the fraction isolated was indeed the native membrane sialoglycoprotein. However, at the time of this writing, preliminary results indicate that octylglucoside extracted molecules react with more recently generated antiserum.