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In Vivo Analysis of Protein Crowding in the Nuclear Pore Complex during Interphase and Mitosis
Hide A Konishi[1], Suguru Asai[1], Tomonobu M Watanabe[2], Shige H Yoshimura[1].
[1]Kyoto university, Kyoto, Japan, [2]RIKEN Quantitative Biology Center (QBiC), Suita, Osaka, Japan.
The nuclear pore complex (NPC) of eukaryotic cells regulates macromolecular traffic between the cytoplasm and the nucleoplasm. The central channel of the pore is thought to form a crowded hydrophobic environment, due to the high content of Phe-Gly (FG) motifs in pore-forming subunits (Nups), which determines the selectivity of the pore. Here, we analyzed the spatiotemporal formation of crowded environments within the NPC by utilizing a crowding-sensitive fluorescent protein probe (GimRET), which was constructed by fusing ECFP with YFP carrying a single amino acid insertion (YFP-G1). The fluorescent properties of GimRET exhibited sensitivity to high concentrations of protein (> ~100 mg/mL). When GimRET-fused Nups were expressed in HeLa cells. The FRET signal in the nuclear envelop revealed that the extent of crowding is different among Nups. Nups located in the outer rim of the pore (Nups50, 153, 214 and 358) exist in a highly crowded environment, whereas Nups located in the middle channel (Nups54, 58 and 62) exhibited a minimal crowding, suggesting that a large entropic barrier exits at both ends of the pore. We also investigated the formation of a crowded environment during post-mitotic reassembly of the NPC. Quantitative analysis of the probe signals from metaphase to G1 phase indicated that some Nups are in a less crowded environment when dispersed in the cytoplasm at metaphase than when assembled in the NPC during interphase. However, some Nups exhibit a similar amount of crowding in both cases, suggesting that some Nups remain in subcomplexes, but others dissociate during mitosis. In addition, a lag period between the localization of Nups around the chromatin surface and the detection of crowding suggests that there is a dynamic rearrangement of Nups after assembly on the chromatin surface.

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Microinjection of fl-tRNA for the Study of tRNA Subcellular Dynamics
Sean E. Anderson[1], Anna Kashina[2], Haim H. Bau[1], Barry S. Cooperman[3].
[1]Mechanical Engineering and Applied Mechanics, University of Pennsylvania, Philadelphia, PA, USA, [2]Animal Biology/Biochemistry, University of Pennsylvania, Philadelphia, PA, USA, [3]Chemistry, University of Pennsylvania, Philadelphia, PA, USA.
Transfer-RNA (tRNA) is the key adaptor molecule in protein synthesis, but recent studies demonstrate behavior and interactions that suggest a far greater role in cellular function. This includes priming viral protein synthesis, retrograde nuclear trafficking, binding to cytochrome C, and post-translational arginylation of proteins. Defects in tRNA modifications are also linked to a number of human diseases with relatively unknown mechanisms. Here we describe the use of live-cell imaging techniques to monitor tRNA nuclear-cytoplasmic trafficking and localization in real time via microinjection of fluorescently-labeled tRNA (fl-tRNA) into adherent mammalian cells. These results represent an important step toward our eventual goal of developing assays for the full characterization of the subcellular dynamics of specific isoacceptor tRNAs.

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Involvement of Water Molecules in the Formation of Hydrophobic Barrier in the Nuclear Pore Complex
Suguru Asai, Hide A. Konishi, Shige H. Yoshimura.
Graduate School of Biostudies, Kyoto University, Kyoto, Japan.
The Nuclear pore complex (NPC) is a protein complex existing in the nuclear envelope which functions as a selective channel between the cytoplasm and the nucleoplasm. The central channel of the pore, which is composed of Phe/Gly motif-rich subunits (FG-nucleoporins), is thought to form a hydrophobic barrier and regulate the passage of soluble proteins and nucleotides. In this study, we combined biochemical approaches and molecular simulations to reveal the structural dynamics of nucleoporin interactions with transport receptor proteins. i) Structural analyses of purified transport receptor proteins, such as importin β, revealed that amphiphilic helices undergo conformational changes in their tertiary structure upon exposure to hydrophobic environments, indicating that the structural flexibility of importin β plays a critical role in passage through the crowded hydrophobic environment of the NPC. ii) Molecular dynamics simulations of FG-containing peptides (STFGST and AVFGAV) at a high concentration (200 mg/mL) were performed to reveal the dynamics of water molecules around the peptides. The result suggested that Ser/Thr residues around Phe are involved in the formation of hydrogen bond networks between the peptides and water and contribute to exposing Phe to surrounding water molecules. iii) The addition of importin β to the crowded FG-peptide environment resulted in a conformational change of importin β mediated mainly by the Phe residue in the peptide. Comparison of two different peptides revealed the important role of Ser/Thr residues in the conformational change of importin β. These results suggested that the hydrogen bond network among water and hydroxyl groups of nucleoporins plays an important role in the formation of the barrier within the NPC and also in the interaction with transport receptors.

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Monitoring and Modeling Effects of IGF1, Insulin and Green Tea Compound EGCG on Nuclear-Cytoplasmic Distribution of Foxo1-GFP in Skeletal Muscle Fibers
Robert Wimmer[1], **Sarah Russell**[1], Bradford Peercy[2], Martin Schneider[1].
[1]Department of Biochemistry and Molecular Biology, University of Maryland, Baltimore, MD, USA, [2]Department of Mathematics and Statistics, University of Maryland Baltimore County, Baltimore, MD, USA.
The transcription factor Foxo1 promotes transcription of the ubiquitin ligases MuRF1 and MAFbx/atrogin1, which contribute to muscle fiber protein breakdown, leading to muscle atrophy and wasting. Foxo1 phosphorylated at its protein kinase B (Akt) sites is transported out of nuclei, and Foxo1 dephosphorylated at these sites is transported into nuclei. Foxo1 net nuclear efflux decreases Foxo1 transcriptional activity, thus opposing muscle protein breakdown and atrophy. Here we use quantitative fluorescence confocal imaging of the time course of Foxo1-GFP net nuclear fluxes together with mathematical modeling of Foxo1-GFP nuclear movements to examine the effects of upstream signaling on nuclear Foxo1. IGF1 and insulin , which activate Akt via the PI3k/PDK1/Akt signaling pathway, caused concentration dependent decreases of nuclear Foxo1-GFP. High IGF1 or insulin caused similar rapid and marked Foxo1-GFP net nuclear efflux. ECGC caused a more delayed net loss of nuclear Foxo1-GFP, possibly indicative of need for EGCG to enter the fiber to initiate its effect, whereas IGF1 and insulin work at cell surface receptors. Excluding the delay, the final rate and extent of decay of nuclear Foxo1-GFP was similar for EGCG, IGF1 and insulin. Analysis using our recently presented reduced (2 state)model for Foxo nuclear fluxes (Wimmer et al, 2014) showed that IGF1, insulin and EGCG all markedly and similarly increased the apparent unidirectional rate constant for Foxo1-GFP nuclear efflux, as well as markedly decreasing the apparent unidirectional rate constant for nuclear influx, indicative of increased Foxo1 phosphorylation in both cytoplasm and nuclei, both of which contribute to the observed decrease in nuclear Foxo1-GFP. Thus, IGF1, insulin and EGCG all activate Akt, both in the cytoplasm and within the nuclei. Supported by NIH R01AR056477 and by UMB-UMBC Seed Grant

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The Autophagosome Marker LC3 Undergoes Regulated Targeting to the Nucleus and Nucleolus
Lewis J. Kraft[1], Jacob Dowler[2], Anne K. Kenworthy[2].
[1]Chemistry and Chemical Biology, Harvard University, Cambridge, MA, USA, [2]Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN, USA.
Autophagy is a lysosomal degradation pathway that is important for the maintenance of cellular homeostasis. Although the formation of autophagosomes occurs in the cytoplasm, it is increasingly evident that several key proteins in the autophagy pathway shuttle in and out of the nucleus. We previously reported that LC3, a protein that participates in autophagosome formation and cargo selection, is enriched in the nucleus in a slowly diffusing form. However, the mechanisms that retain LC3 in the nucleus and control its nuclear dynamics remain poorly understood. To address this, we used a combination of fluorescence microscopy and FRAP to study Venus-LC3 in HeLa cells. We find that mutating residues involved in binding of LC3 to other proteins and RNA moderately decreases its nucleocytoplasmic ratio, but disrupting LC3 lipid modification does not change its nucleocytoplasmic ratio. This suggests that the majority of LC3 in the nucleus is soluble, and it is retained in the nucleus by interactions with either proteins or RNA. Consistent with this, soluble nuclear LC3 diffuses more slowly than predicted for a monomer. Perturbing the autophagy pathway with rapamycin or chloroquine has little effect on LC3's nuclear localization, but does change the apparent size of LC3-associated complexes. This implies that soluble nuclear LC3 associates with complexes with functions possibly related to autophagy. Unexpectedly, we also find that nuclear LC3 is enriched within the nucleolus. We show that the triple arginine motif of LC3, a region previously shown to bind to both proteins and RNA, shares similarities with a consensus nucleolar detention sequence. Furthermore, perturbation of LC3's triple arginine motif completely abolishes its localization to the nucleolus. Together, these findings reveal a

potential role for both protein-protein and protein-RNA interactions in targeting LC3 to the nucleus and nucleolus.

Voltage-gated Na Channels

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Resting State of S4 Identified for Each Domain of Nav1.2 using Omega Current Technique

Claudia Lehmann, Hansjakob Heldstab, Nikolaus G. Greeff.
Biophysics Institute Greeff, Uetikon am See, Switzerland.

During gating transitions the voltage sensor S4 slides through the narrow so-called gating pore. The positively charged amino acids of S4, arginine (R) or lysine (K) sense the transmembranal electric field and promote S4 in or out. If one or more long R or K is replaced by the short neutral glutamine (Q), a leak (omega-) pore is created through which a leak current, the omega current will flow when S4 is at the appropriate position. The occupancy of this leaky position can then be electrically monitored. Rat brain sodium channels Nav1.2 were studied at high expression in X. laevis oocytes with two-electrode voltage clamping at strong hyperpolarization to force S4 into the resting state. Mutant channels with single gaps R1Q, R2Q or R3Q as well as with double gaps RRn,n+1QQ were tested for the presence of omega leaks. We found unambiguous omega currents for double gaps in domain DI (RR12QQ), DII (RR12QQ), DIII (RR23QQ) and DIV (RR12QQ), indicating the resting position of S4 in each domain. These findings are in contrast to skeletal muscle sodium channels Nav1.4 where single gap omega leaks are reported for DII and DIII. However, our single gap mutants in Nav1.2 produced only very small leak currents similar to artifacts sometimes also occurring at wild-type channels at very strong hyperpolarizing pulses and at least ten times smaller than those with double gaps. Based on this study, we currently use our double gap channel constructs as a tool to selectively investigate which S4 of the four domains I-IV are immobilized by inactivation.

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Selective Immobilization of S4 in Domain III and IV of Rat Brain Nav1.2 Shown by Omega Currents

Nikolaus Greeff, Hansjakob Heldstab, Claudia Lehmann.
Biophysics Institute Greeff, Uetikon am See, Switzerland.

Recently, we have identified the minimally necessary number of mutations of the outer positively charged arginine (R) or lysine (K) to glutamine (Q) in the voltage sensor S4 of each domain, producing an inward omega leak current in the resting state. In all four domains a double gap (RRn,n+1QQ) gave distinct omega currents. In this study we use these double gap channel constructs as a tool to investigate which S4 of the four domains I-IV is immobilized by inactivation. The recovery time constant of sodium current after inactivation was measured with a classical double pulse protocol for a wide range of recovery potentials from −100 to −240 mV. In addition, the onset of the omega current at the same recovery potentials was measured twofold: without and with an inactivating prepulse. We found that the onset of omega current was fast and not affected by inactivation in domain I and II; however, in domain III and IV the onset was fast without prepulse but was slowed after the inactivating prepulse. The return to the resting state seems to be hindered due to immobilization. The time constant of the recovery of omega current matches well the recovery of sodium current over the wide potential range studied. We corroborated our results by using the mutation R4H in S4DIV, which slows the sodium current recovery about twentyfold (Kühn and Greeff, 1999). Adding the mutation R4H in S4DIV to our double gap constructs, we found that the omega current was also slowed by the same factor. This suggests that the same mechanism which keeps the alpha pore closed for ionic current in inactivated channels would also hinder the return of S4III and S4IV to the resting position.

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Voltage Sensor Domains and Closed-State Inactivation in Sodium Channels

James R. Groome.
Biological Sciences, Idaho State University, Pocatello, ID, USA.

Sodium channels enter into a state of fast inactivation after opening, or directly from closed states. We examined the roles of the four voltage sensor domains in hNaV1.4 in closed-state fast inactivation using a mutagenesis approach. Charge reversing mutations of outer arginine residues in domains I, III and IV depolarized the steady-state fast inactivation curve and accelerated entry. Similar effects on closed-state fast inactivation were observed for charge-reversing mutations of inner negative charges in domains I and IV, suggesting that electrostatic interaction of these residues limits S4 translocation in response to sub-threshold depolarization. This work was supported by NIH 2P20GM103408 to ISU.

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Gating Pore Currents are Common Defects of Two Nav1.5 Mutations in Patients with Mixed Arrhythmias and Dilated Cardiomyopathy

Adrien Moreau[1], Pascal Gosselin-Badaroudine[1], Lucie Delemotte[2], Michael L. Klein[2], Mohamed Chahine[1,3].
[1]Electrophysiology, CRIUSMQ, Quebec, QC, Canada, [2]Institute of Computational Molecular Science, Philadelphia, PA, USA, [3]Department of Medicine, Université Laval, Quebec, QC, Canada.

The gating pore current, also called omega current, consists of a cation leak through the typically non-conductive voltage sensor domain (VSD) of voltage gated ion channels (VGIC). While the study of gating pore current refined the knowledge of the structure and the function of VGIC, their implication in cardiac disorders has not been established. Two Nav1.5 mutations (R222Q and R225W) localized in the VSD are associated with complex arrhythmias and dilated cardiomyopathy. Using the patch clamp technique, in-silico mutagenesis and molecular dynamic simulations, we tested the hypothesis that these two mutations may generate gating pore currents potentially accounting for their atypical clinical phenotypes. Our findings suggest that the gating pore current generated by the R222Q and R225W mutations could constitute the yet unrevealed pathological mechanism linking Nav1.5 VSD mutations with cardiac arrhythmias and dilatation of cardiac chambers in humans.

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Interaction of the Cardiac Sodium Channel Alpha-Subunits Leads to Coupled Gating Properties

Jérôme Clatot, Haiyan Liu, Eckard Ficker, Isabelle Deschênes.
Case Western Reserve University, Case Western Reserve University, OH, USA.

Objective: The cardiac sodium channel has been linked to cardiac arrhythmias. We have shown the existence of dominant-negative mutations in Brugada Syndrome due to interactions between alpha-subunits. Here we investigated the stoichiometry of the interaction and whether the interaction leads to coupled gating properties.

Methods: Single-molecule pull-down experiments and blue native gels have been performed to study the stoichiometry of the interaction, while FRET/TIRF experiments were performed to investigate the interaction at the cell surface. Biophysical properties were studied by patch-clamp analysis in the whole-cell configuration.

Results: Biochemistry results support the dimerization of alpha-subunits. FRET/TIRF experiments showed interacting channels at the plasma membrane. Also, we investigated if the dimerization of the channel leads to biophysical consequences. To do so, we used different mutants leading to specific biophysical. R1860X and R1629Q mutants were used for inactivation and R878C for gating deficiency. When cells expressed WT and R1629Q mutant channels, inactivation properties behaved more closely to the WT contrarily to what would be expected of 2 channels working independently. In addition, when R1629Q was coepxressed with the gating deficient mutant R878C we showed a significant improvement of the R1629Q inactivation properties, even though R878C is not conducting. We then used a truncated channel R1860X and once again the coexpression with R878C significantly improved the inactivation defect, which was not the case with the expression of a C-terminus fragment alone. This, strongly suggest that the presence of the full length channel could rescue the inactivation defect of the delta-Cter channel.

Conclusions: Our data indicate that the alpha-subunits of the cardiac sodium channel present coupled gating properties due to the formation of dimers.

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Superresolution Microscopy Reveals Sodium Channel Localization within Intercalated Disk Microdomains: Implications for Ephaptic Coupling

Rengasayee Veeraraghavan[1], Joyce Lin[2], James P. Keener[3], Steven Poelzing[1], Robert G. Gourdie[1].
[1]Virginia Tech Carilion Research Institute, Virginia Polytechnic University, Roanoke, VA, USA, [2]California Polytechnic State University, San Luis Obispo, CA, USA, [3]Dept. of Mathematics, University of Utah, Salt Lake City, UT, USA.

Pore-forming (Nav1.5) and auxiliary (β1; SCN1b) subunits of cardiac sodium channels are enriched at the cardiomyocyte intercalated disk (ID). Mathematical models suggest that this may facilitate conduction via ephaptic mechanisms. We previously demonstrated anisotropic conduction slowing during acute interstitial edema (AIE), possibly due to weakened ephaptic coupling. Here we assessed Nav1.5 and β1 localization to ID microdomains using