between proteins and sugars, peptides or nucleic acids, which may be useful to study catalysis by enzymes such as amylases, cellulases, proteases or nucleases. This new generation of substrates holds the promise of becoming a key tool to study single-bond chemistry under force.

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Single-Molecule Analysis of the Recognition Forces Underlying Nucleo-Cytoplasmic Transport

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Macromolecular exchange between the nucleus and cytoplasm of cells is gated at nuclear pores by a family of intrinsically-disordered nucleoporins (nups). These feature phenylalanine-glycine repeats in a cohesive domain (FD domain) that interact to form a physical barrier. Through unknown mechanisms, karyopherins (importins, exportins, transportins, NTRs) penetrate this barrier to facilitate the movement of large proteins and RNPs across without paying an external energetic cost, simply by interacting with FG domains. To address the molecular binding and dissociation mechanisms involved in this coupled gating-translocation process, single molecule force spectroscopy was used here to measure the interaction force between nup FG repeats, and between importin beta and nup FG repeats. As predicted, cohesive FG domains bound each other through multiple FG repeat interactions. In contrast importin bound only relaxed coil multiple FG repeats simultaneously, whereas just one FG binding site was assessed in collapsed coil multiple FG domains. Most importantly, the interaction forces and fast dissociation rate constants measured between two FG repeats, and between importin and one FG repeat, were almost identical. This suggests that the force needed to separate interactions between FG repeats of nups at the NPC (i.e., for kins to penetrate the gate and translocate across) could be provided in full by the enthalpy gained through the formation of karyopherin-FG repeat interactions.

60-Plat

Direct Observation of Catch-Bonds in Focal Adhesions of Living Cells

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Single molecule force spectroscopy data have demonstrated that the chemical bonds between extracellular matrix proteins, integrins and several proteins of the focal adhesion complex show catch-bond behavior: the binding strength increases under mechanical load. It remains unknown, however, whether catch-bond mechanisms are of any relevance for stabilizing matrix adhesions in living cells. To measure adhesion strength, we bind RGD-coated magnetic beads to integrin adhesion receptors of living cells and apply forces of up to 80 nN with a magnetic tweezer. Under mechanical load, the beads detach stochastically from the cell surface, and the characteristic force at which 50% of the beads detach is a measure of the adhesion strength. In the case of a pulling force that increases linearly with time, the characteristic bead detachment force is expected to increase logarithmically with the loading rate for thermally activated Bell-type molecular bonds. We find that the detachment force tends to increase faster than logarithmically, demonstrating that the adhesion bonds strengthen under force. This may be indicative of catch bonds, but could also arise from a complex binding energy landscape that, as it is tilted under a mechanical load, presents different energy barriers against detachment. To distinguish between these two possibilities, we applied a staircase-like mechanical load with the same average loading rate but with forces that at all times exceeded those of the linear ramp protocol. We find significantly increased detachment forces under a staircase-like loading protocol compared to a linear force ramp, which rules out other mechanisms except catch-bond behavior.

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Single-Molecule Measurements of Catch Bond Formation in Cadherin Cell-Adhesion Proteins

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We report on mechanical interactions by which cadherin cell adhesion proteins tune the mechanical properties of cell-cell junctions. Cells in multicellular organisms undergo constant rearrangements; during tissue formation and wound healing, cells divide, change positions and tug on their neighbors. While the genes that orchestrate these processes have been studied extensively, we do not know nearly enough about how cell-cell contacts resist physical force. At the molecular level, cells are held together by adhesion proteins, most commonly by the cadherin family of proteins. Cadherins are essential for tissue formation and for maintaining tissue integrity. Using single molecule force measurements, we demonstrate that cadherin bonds become stronger as cells are pulled apart. This counter-intuitive data is the first demonstration that cadherin’s form catch bonds; the proteins grip strongly in the presence of an external force but detach when force is removed. Our results suggest a molecular mechanism for regulating cell-cell adhesion under conditions of variable mechanical stress.

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Direct Measurement of the Diffusion Dynamics of an Extended Poly-Ubiquitin Under Constant Force

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Diffusion is a passive transport process that results from thermal fluctuations. It is commonly measured at zero force. However, the force dependence of the diffusion process is not well understood. Force spectroscopy is a technique in which single proteins are probed under mechanical perturbation. The force-length course measured in force spectroscopy is commonly described in terms of a diffusive process over a one dimensional potential of mean force, which reflects the end to end motion of the protein. Accordingly, the diffusion coefficient of a protein or polypeptide over its potential of mean force, D, is a basic property that relates the detected kinetics to the applied load. So far, D had been calculated from measurements taken in bulk, where the molecule is free to diffuse without any applied mechanical loads. The reported values indicate rapid dynamics that cannot explain the substantially slower timescales observed by a collapse trajectory of a single molecule under force. To this end we built a fast AFM apparatus with an improved characteristic time response of ~100 μs. Using this novel setup we pulled on poly-ubiquitin, which has a distinctive footprint, and then applied force quenching between 250 and 100 pN to probe its recoiling dynamics. We fitted this data with a high force approximation analytical expression, which was verified using Brownian Dynamics, to measure the value of D for the recoiling traces. We report here for the first time an averaged value of D = 1374 ± 222 nm^2/s, which is interestingly about five orders of magnitude smaller than the ones measured in bulk. The value of D measured here accounts for the observed slow recoiling timescales (~1 ms) of a single poly-ubiquitin under mechanical load. Moreover, this value is significant when describing elastic systems where proteins are bound on both sides and still undergo conformational changes.

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Filamin’s Force Sensing Mechanism Revealed by Optical Tweezers

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1Technische Universität München, Garching, Germany, 2Department of Biological and Environmental Science, Jyväskylä, Finland. Cell adhesion, spreading and migration, as it occurs during embryogenesis or wound healing and hemostasis, involve a complex interplay between the extracellular matrix, transmembrane adhesion receptors and the cytoskeleton. A prominent role plays human filamin A, since it is a widely expressed actin cross-linker which additionally binds a multitude of transmembrane receptors such as beta integrins or the von Willebrand Factor receptor GP Ib/IX. Although it has been shown that beta integrins or GP Ib/IX bind to the Ig-like domain 21 of filamin A, the molecular-level mechanism for filamin binding, especially its regulation, is still unclear. Filamin can auto-inhibit the interaction by binding the binding site through intra-molecular interactions and it has been suggested that the binding site can be exposed by a force-induced conformational change. To address the question whether filamin’s binding site in domain 21 was activated by force, we adapted a highly sensitive optical tweezers setup to pull at single domain pairs of human filamin A containing the receptor binding site and the auto-inhibiting region. We were able to apply and detect low forces in the physiological relevant regime around 3 pN and monitor the conformational change by measuring the length increase of the protein on a sub-millisecond timescale. This allowed us to distinguish in real-time the inhibited closed conformation and the active open one. Additionally, we were able to detect the binding of different ligands added in solution and how the applied force increased the binding rate. Therefore, filamin A can be regarded as a force sensor.

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Studying the Bacterial Flagellar Motor using an Optical Torque Wrench

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At the biomolecular level, the physical quantity torque manifests itself in a number of ways e.g. in the conformational changes of biopolymers such as DNA and in actions of both linear and rotary molecular motors. The optical torque wrench, an optical tweezers setup with the capability of applying and
measuring both force and torque, has been developed as a versatile setup in tackling biologically relevant issues at high spatial and temporal resolution. Torque sensors for slow inactivation, the latter on the membrane level and readout of the polarization state of light used to trap nano-fabricated birefringent cylinders. The flagellar motor of *Escherichia coli* is a well-known rotary motor of only about 45 nm embedded in the cellular membrane, but besides its protein content the exact functioning of this intriguing motor remains unknown. The rotary motor consists of a rotor attached to a flagellum and of stators ‘pushing’ the rotor around. Stators diffuse in the cytoplasmic membrane upon engaging in the motor complex. The temporal resolution of our setup allows to investigate fast stator dynamics. We are studying the response of the motor at stall torque, forward rotation and backward rotation by optically adjusting the load torque on the motor, on which we present preliminary results. Deploying our optical setup we are trying to unravel the mechanism by which this molecular motor works to propel bacteria.

**Platform: Voltage-gated K Channels: Gating**

65-Plat

**The Role of the 2nd Threonine Within the TTVGYGD Sequence of Kv-Channels in C-Type Inactivation Gating, Ion Selectivity and Permeation**

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An allosteric communication between the activation gate (AG) in K-channels and their selectivity filter (SF) promotes the collapsing of the former, prompting the channel to enter the C-type inactivated state (OI). The 2nd Threonine within the TTVGYGD sequence of Kv-channels (2T) could be a key component for this allosteric coupling[1]. Here we report that an Alanine substitution of 2T prevents all human delayed rectifier Kv-channel subtypes (Kv1.5, Kv 2.1 and Kv3.1), the Shaker as well as the KcsA channels, from entering the (OI). Prolonged depolarization or pH activation causes Kv and KcsA channels respectively to enter (OI). However, the T to A substitution yielded non-inactivating channels that instead displayed a second (slow) activation process, which suggested the conversion of the (OI) into a second conductive state (O2).

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**Molecular Determinants of Slow Inactivation in Voltage-Gated Potassium Channels**

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Potassium channels respond to prolonged depolarizations with structural rearrangements that result in inactivated, nonconducting channels through a process termed slow inactivation. Significant efforts to understand this process have been made using structural, theoretical and experimental approaches yet the molecular details of slow inactivation in eukaryotic voltage-gated potassium (Kv) channels remain poorly understood. Data gleaned from prokaryotic (KcsA) and eukaryotic (Kv1.2/2.1) channels have implicated two adjacent residues and highly conserved aromatic side chains near the selectivity filter as critical for slow inactivation. In the indole nitrogen through fluorination (and therefore strengthening its hydro-bond donor ability), the rate of slow inactivation was substantially decreased (4-fold slower than WT). Conversely, the novel unnatural amino acid side chain, Ind, which lacks the indole nitrogen but is otherwise isosteric to Trp, increased the rate of inactivation more than 10-fold when Ind-containing channels were co-expressed with WT channels, and Ind-containing channels alone did not produce ionic current. In contrast to Trp434, the indole nitrogen of Trp435 does not contribute to slow inactivation as even relatively nonconservative mutations to Phe or Tyr at this site do not affect slow inactivation. Taken together, these results directly demonstrate the functional importance of the H-bonding ability of Trp434 in open pore stability in Kv channels.

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**Phe233 in the Voltage-Sensor is Rate Limiting for Channel Closure but not for the Opening**

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During activation, the charged S4 segment in the voltage sensor domain (VSD) of voltage-gated ion channels is required to translate across a hydrophobic zone. This constitutes a thin aperture clearly separating the open/relaxed and closed/resting states. The nature of this barrier is critical for channel function, and how it changes during activation has been the focus of many studies. We determined the single preserved residue F233 (F290 in Shaker) as a structural barrier for the gating charges, that uniquely determines intermediate state and substitutions modulate the deactivation barrier. A fundamental understanding of the S4 activation/deactivation barrier as well as kinetics is an important remaining challenge to decipher gating. Here, we study the free barrier and kinetics of the VSD through converting in-vitro and in-silico experiments. We used site-directed mutagenesis and measured the voltage-dependence in-vitro to study the effect of F290L compared to the wild-type (WT). In parallel, molecular dynamics simulations allowed us to identify residues interacting with the phenyl ring. Through in-silico mutations we show their impact on the barrier, as well as the structural and kinetics effects for the phenyl ring orientation during the first step of deactivation and the (reverse) last step of the activation. Strikingly, the channel closing transition shows a huge speedup from the F290L (1ms, WT 10ms), in contrast to the opening that is completely unaffected. This indicates that F233 is only rate-limiting for the channel closure, but not opening and suggests different kinetics for the activation/deactivation barriers. Additionally the ring is clearly stabilized through vdW interactions with surrounding hydrophilic residues, and appears to always open by upward rotation both for activation/deactivation. This upward rotation suggests a model where the closing is possibly a mostly entropic process, while opening would be largely enthalpic.

**Movement of the S4-S5 Linker of KvAP during Gating**

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Voltage-gated potassium channels are formed by four voltage sensor domains (VSD) and a central pore domain. The voltage sensors are activated during membrane depolarization, leading to pore opening through a process called electromechanical coupling. Functional and structural evidence suggests that the covalent link between the VSD and the pore, the ß-helical S4-S5 linker, plays a crucial role in the electromechanical coupling. However, no data on the rearrangements at the cytosolic face of the channel is presently available leaving the molecular mechanism of coupling subject for speculation. In this study, we determined the position of the S4-S5 linker in the open and closed state of the bacterial KvAP channel. To this end, we utilized LRET (Lanthanide Resonance Energy Transfer) to measure the cross pore distances of various positions in the S4-S5 linker. A KvAP dimer with a single cysteine has been constructed resulting in a tetramer with two symmetric cysteines, which we label with a Tb-echlate and an organic fluorophore, respectively. The labeled protein is reconstituted in lipid vesicles. We have measured distances in both closed and open state for 8 positions along the 10 amino acid linker. Based on the results we aim to create a 3D model of the movement of the S4-S5 linker during the opening of the pore.

**Structural Insights into Calmodulation of Neuronal KCNQ Channels**

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Calmodulin (CaM) is a ubiquitous intracellular calcium sensor for many potassium channels. For the voltage-gated KCNQ family of potassium channels, CaM binds to the intracellular C-terminus to mediate channel assembly, yet the direct demonstration of this notion is missing in Kv channels. Here we used a combination of in-vitro and in-silico experiments. We used site-directed mutagenesis and labeled protein is reconstituted in lipid vesicles. We have measured distances in both closed and open state for 8 positions along the 10 amino acid linker. Based on the results we aim to create a 3D model of the movement of the S4-S5 linker during the opening of the pore.