Cellular Adhesion

DOI: 10.1002/ange.200803552

A Bond for a Lifetime: Employing Membrane Nanotubes from Living Cells to Determine Receptor–Ligand Kinetics**

*Michael Krieg, Jonne Helenius, Carl-Philipp Heisenberg, and Daniel J. Muller**

Interactions of cell surface receptors with their ligands determine how cells respond to their environment. Receptors bind both soluble ligands, such as growth factors or neurotransmitters, and immobile ligands, such as extracellular matrix components or proteins on the surface of neighboring cells. While many interactions initiate outside–in signaling pathways, interactions with immobile ligands are the means by which cells adhere to their environment. The ability of these adhesive interactions to withstand mechanical forces is crucial to their function. The rate at which receptors and ligands dissociate depends on the force pulling them apart,[1] with increasing force the off-rate is expected to rise exponentially. The Bell model[1] describes the off-rate, k, of a bond as a function of the applied force, F [Eq. (1)]:

$$k(F) = k_{off} \exp\left(\frac{-x_u F}{k_B T}\right) \quad (1)$$

where k_{off} (s^{-1}) is the dissociation rate in the absence of an applied force, x_u is the distance the binding partners must be separated to force dissociation, and $k_B T$ is the thermal energy.

Single-molecule force spectroscopy (SMFS) approaches can probe these adhesive forces at different loading rates (applied force versus time) to provide insights into the kinetics of isolated receptor–ligand pairs.[2] Such in vitro measurements have limitations. Ligands and receptors are commonly purified and removed from their cellular environment, so one cannot be confident of their functional state. This is of particular concern for cell adhesion molecules (CAMs) which are functionally modulated in vivo by the cell.[3] While many adhesive molecular interactions follow the Bell model, and can be studied using dynamic force methods, there are exceptions. The most striking exception are catch bonds, such as P-selectin and its ligand, whose lifetime increases with small force loads.[4] Therefore, constant-force methods are needed because these do not presume a dissociation model.

The relation between unbinding rate and force for various CAMs has been studied by different SMFS methods including atomic force microscopy (AFM), laser traps, biological membrane force probes, and flow assays.[5–8] Herein we implement a SMFS method that uses membrane nanotubes (tethers) formed by living cells to exert a constant force on a specific biological bond. The benefit of using a live cell is that it allows the strength and lifetime of fully functional receptor–ligand interactions to be studied at forces that are determined by plasma membrane properties.

To validate this method we set out to study the binding of Concanavalin A (Con A) to N-linked oligosaccharides attached to extracellular domains of membrane receptors.[9] To measure specific binding forces in the piconewton (pN) range, AFM cantilevers were functionalized with Con A. In repeated cycles, AFM cantilevers were pressed onto single mesendoderm zebrafish embryo cells for short contact times (less than 0.2 s) with a force of 100 pN (Figure 1a,b). Upon separating the Con A functionalized AFM stylus, adhesive interactions with the cell surface bend the cantilever. As these interactions rupture, the cantilever deflection relaxes to zero force. This force drop is seen as a step in the force–distance curve (indicated by an arrow in Figure 1c). The contact time and force were adjusted so that single connective bonds formed in about 40%, separating the stylus. To demonstrate binding specificity, the interaction between Con A and N-linked oligosaccharides was inhibited (Figure 1d). Substitution of Con A on the cantilever with bovine serum albumin (BSA) and the incubation of Con A coated cantilevers in heat-inactivated fetal calf serum (HiFCS) nearly eliminated binding between the cantilever and the cell. Cantilever incubation with 4-nitrophenyl-α-D-mannopyranoside (NPM), a low-affinity competitive inhibitor of Con A binding, also reduced the binding rate. We conclude that greater than 80% of the interactions were between Con A and cell-surface-exposed oligosaccharides.

By retracting the cantilever, an outward force was applied to bonds formed between Con A and the cell. In this situation, either the bond breaks or the protein to which the oligosaccharide is covalently attached is pulled away from the cell surface at the tip of a membrane nanotube (Figure 1b). Once the membrane nanotube is initiated, the force required to extend the nanotube stays essentially constant. Upon unbinding of Con A, the force on the cantilever is instantly reduced. Representative force–distance curves shown in Figure 1c show constant force plateaus. The physical model of lipid membranes predicts the extraction force required to pull the nanotube, F_t [Eq.(2)]:

[*] M. Krieg, Dr. J. Helenius, Prof. D. J. Muller
Dept. of Cellular Machines, Biotechnology Center,
University of Technology, 01307 Dresden (Germany)
Fax: (+49) 351-463-40330
E-mail: mueller@biotec.tu-dresden.de
Dr. C.-P. Heisenberg
Max Planck Institute of Molecular Cell Biology and Genetics
01307 Dresden (Germany)

[**] This project was supported by the European Union, the BMBF, the Free State of Saxony, and the Boehringer Ingelheim Fonds (M.K.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803552.

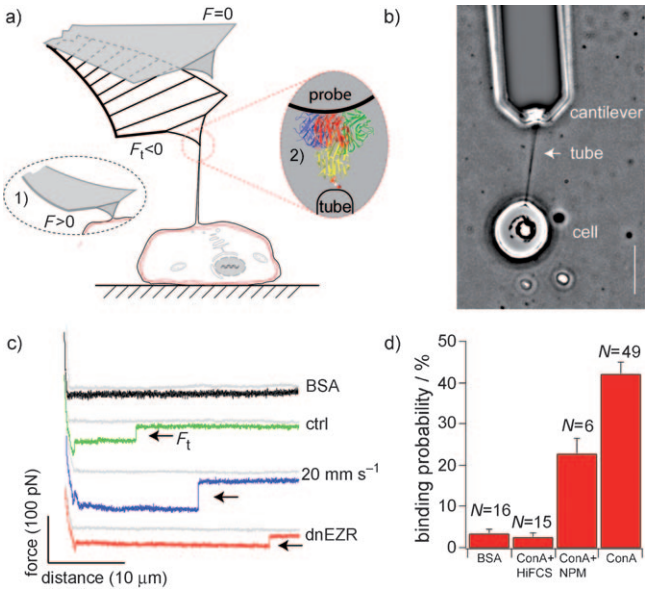

Figure 1. a) Depiction of the cell-based SMFS assay. An AFM cantilever coated with Con A (inset 2, PDB code 1VAM) is pressed onto a living cell resting on a glass slide ($F>0$, inset 1). If an interaction between Con A and N-linked oligosaccharides on the cell surface is established, a membrane nanotube may be formed upon retraction of the cantilever. The extraction force of the nanotube deflects the cantilever ($F_t<0$). b) Phase contrast image of a membrane nanotube extruded between cantilever and cell (scale bar = 20 µm). c) Force–distance curves acquired using a BSA-coated cantilever (no bond failure is observed; black), and Con A coated cantilevers at retraction velocities of 10 µm s^{-1} (green) and 20 µm s^{-1} (blue). A force curve (red) obtained with a cell expressing dominant negative version of ezrin (dnEzr) at a retraction speed of 10 µm s^{-1} is shown. The rupture of the bond between Con A and its ligand results in a force step (indicated by arrows) equal to the nanotube extraction force (F_t). Approach traces are shown in light gray. The bond lifetime equals the tether length at bond rupture divided by the velocity of cantilever retraction. d) Probability of pulling a nanotube using cantilevers coated with BSA and Con A. Preincubating Con A coated cantilevers in heat-inactivated fetal calf serum (HiFCS) as well as in 20 mM 4-nitrophenyl-α-D-mannopyranoside (NPM) suppresses specific binding. *N* is the number of cells tested for each condition. Error bars represent the standard error of the mean.

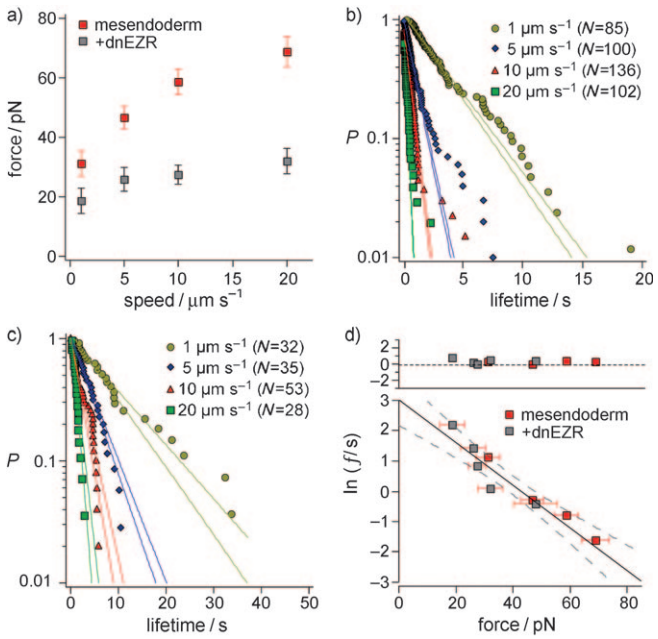

Figure 2. a) Median extraction force of nanotubes with respect to pulling speed. Error bars denote standard error of the median. At least 35 cells were tested for wild-type cells and at least 14 cells for mesendoderm cells expressing dnEzr. b) Lifetimes of Con A–N-linked oligosaccharide bonds plotted for different pulling velocities. *P* is the number of nanotubes having a lifetime greater than or equal to the lifetime of the data point, divided by the total number of nanotubes analyzed for the condition (*N*). The mean bond lifetime, *f*, was fitted at each velocity by using Equation (3). Paired lines represent the 99% confidence interval of the fit. Deviations at very long lifetimes may result from nanotubes bound by more than one interaction. *N* is the number of nanotubes analyzed. c) Data from dnEzr cells represented in the same manner as in (b). d) Mean lifetimes of the Con A–oligosaccharide bond plotted with respect to the median tether force. Data points stem from (b) and (c). The solid line represents the fit calculated by using the Bell model [Eq. (1)], dashed lines represent the 99% confidence interval of the fit. For fitting, data points were weighted by the inverse of the error in the lifetime. Horizontal bars denote standard error of the median force while the errors of the lifetimes were smaller than the data points. The residuals of the fit are shown in the upper section.

$$F_t = 2\pi\sqrt{\sigma\kappa} + \frac{2\pi\eta V}{C} \qquad (2)$$

where σ is the far field isotropic membrane tension, κ is the membrane bending rigidity, η is the membrane viscosity, V is the nanotube extension velocity, and C is a correction factor (1.6).[10,11] This relation implies that, without changes in plasma membrane properties, the nanotube extraction force depends on the extension velocity, as shown in Figure 1c. Thus, adjusting the extension velocities of the membrane nanotube allowed tuning the constant force applied to the receptor–ligand bond. However, in disagreement with Equation (2), the velocity–force dependence was not linear (Figure 2a). This is likely because the cell plasma membranes were not ideal lipid membranes but a mixture of lipids and proteins. Nevertheless, by changing the extension velocity the extraction force could be controlled.

In addition to normal mesendoderm cells, cells expressing a dominant negative form of ezrin (dnEzr) were used.[12] It is likely that the disruption of ezrin activity decreased membrane–cortex adhesion, thereby lowering the membrane tension, σ.[10,13] As predicted, the expression of dnEzr reduced the extraction force at all extension velocities (Figure 2a). The use of two cell lines and varying the extension velocities from 1 to 20 µm s^{-1} allowed force clamps from 15 to 75 pN to be applied (Figure 2a).

To characterize interactions between Con A and N-linked oligosaccharides, the nanotube length at the time of unbinding and the pulling force were determined for nanotubes longer than 0.25 µm. At given nanotube extension velocities the extraction forces were normally distributed (Figure S1 in the Supporting Information). Extraction forces were independent of the length of the membrane nanotube (Figure S2 in the Supporting Information). Therefore, it can be con-

cluded that the nanotube length divided by the cantilever speed represents the lifetime of the anchoring bond at a given extraction force.

Figure 2b,c shows the lifetime of the Con A–N-linked oligosaccharide bond measured for different extension velocities of nanotubes. Indicative of the stochastic unbinding of a common ligand–receptor interaction, simple exponential decay curves characterize the nanotube lifetime distribution at all pulling forces [Eq. (3)]:

$$P(t) = A \exp\left(-\frac{t}{f}\right) \quad (3)$$

with f being the mean bond lifetime and $P(t)$ the probability of bond survival after a given time t.

As expected, the mean Con A bond lifetime decreased with increasing extraction force (Figure 2d). In the assayed range of forces clamped by membrane nanotubes of living cells, the Bell model with a k_{off} value of 0.049 s^{-1} and a x_u value of 2.9 Å describes the observed rates well. The bond between Con A and single mannose residues has been examined using dynamic force spectroscopy and was found to have a considerably higher k_{off} value of 0.17 s^{-1} and an x_u value of 2.7 Å.[14] However, because N-linked oligosaccharides of the cell surface bind Con A with approximately 50 times higher affinity (k_a) than single mannose residues,[15] a significantly higher dissociation rate (k_{off}) is expected in the latter case.

Other methods that use membranes to measure molecular interactions include biological membrane force probes and flow assays. However, AFM allows the most accurate spatial control and has the least complicated means to measure the nanotube extraction force. Our use of nanotubes extracted from living cells has several advantages. This method examines unbinding at forces that are innate to cells. Moreover, the binding of in situ cell surface proteins that are neither manipulated, modified, purified, nor artificially immobilized can be studied. This simple assay should allow the cellular regulation of CAMs to be characterized. The method is not limited to the study of CAMs because soluble signaling molecules, such as growth factors, can be bound to AFM cantilevers that are used to probe their binding kinetics with receptors on live cells. In summary, this method is an uncomplicated approach to the study of fully native receptor–ligand interactions at the single-molecule level.

Experimental Section

Embryo injections and primary cell culture: Freshly fertilized zebrafish eggs were injected at the one-cell stage with *cyclops* RNA (100 pg) to transform all cells into mesendodermal fate.[16] To produce cells with reduced membrane tension, embryos were co-injected with dominant negative ezrin RNA (300 pg). Embryos were grown until 5 hr post fertilization at 31 °C. Cells were isolated after removal of the chorion in Pronase (2 mg mL^{-1} in E2 medium for 8 min, Roche) by mixing in culture medium (DMEM/F12 supplemented with 15 mM HEPES, Invitrogen, and Pen/Strep, Sigma). Yolk proteins were removed by centrifugation at 300 *g* for 30 s.

AFM nanotube extrusion: AFM cantilevers (Olympus Biolever, 6 mN m^{-1}) were plasma-cleaned for 5 min and incubated in either 2.5 mg mL^{-1} Concanavalin A (Sigma) or 5 mg mL^{-1} BSA (Sigma) in PBS buffer for 2 hr. Prior to use, cantilevers were rinsed in PBS. Cantilevers were then mounted in an AFM (NanoWizard, JPK) that is integrated into an inverted light microscope (Axiovert 200 M, Zeiss).[16] Cantilever spring constants were determined using the thermal noise method.[17] Cells were seeded in culture medium (1 mL) onto glass surfaces (GoldSeal) in a home-built fluid chamber. Nanotubes were extracted at room temperature (ca. 25 °C). To optimize the number of bonds between the cantilever and the cell, the contact force was 100 pN while the contact time was varied between 0.0 and 0.6 s. Force–distance curves showing more than one nanotubes or nanotubes shorter than 0.25 μm were omitted from the analysis. The approach velocity was 5 μm s^{-1} for all conditions. To control the nanotube extrusion force, the cantilever retraction velocity was varied between 1 and 20 μm s^{-1} (or up to 50 μm s^{-1} when using dnEzr cells). Specificity was tested using BSA-incubated cantilevers. Alternatively, cantilevers were incubated with Con A over night at 4 °C and subsequently immersed in 4-nitrophenyl-α-D-mannopyranoside (20 mM; Sigma) or pure heat-inactivated fetal calf serum (Invitrogen) for 2 h.

Data processing: Nanotube extraction forces and lifetimes were determined from force–distance curves displaying a single unbinding event using an in-house Igor Pro (WaveMetrics) algorithm. The median and standard error of the median for the force at each velocity was computed using Igor Pro. Exponential decay curves to cumulated lifetimes were fitted using Igor Pro. A linear fit of ln(lifetime) versus median force was used to calculate the potential width and unbinding rate at zero force according to the Bell model.[1]

Received: July 21, 2008

Keywords: biomolecular bonds · nanotubes · scanning probe microscopy · single-molecule studies · transition states

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