Published in final edited form as:

IEEE Trans Nanobioscience. 2005 December ; 4(4): 269–276.

Molecular Handles for the Mechanical Manipulation of Single-Membrane Proteins in Living Cells

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Abstract

We have developed a procedure to selectively biotinylate a specific membrane protein, enabling its attachment to external force probes and thus allowing its mechanical manipulation within its native environment. Using potassium channels as model membrane proteins in oocytes, we have found that Maleimide-PEG3400-biotin is the crosslinker with highest conjugation selectivity and accessibility to external probes. Neutravidin-coated beads provide for directed attachment while avoiding nonspecific interactions with the cell. The technology was successfully tested by mechanical manipulation of biotinylated extracellular residues of channels in oocytes using an atomic force microscope under conditions which preserve function of the channels. Binding forces of ~80 pN at 100 nN/s were measured.

Keywords

Atomic force spectroscopy; living cells; patch clamp; selective molecular attachment; single-molecule force measurements

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This work was supported in part by the National Institutes of Health under Grant RO1 MH60711-02, in part by the Gaspar de Portolà Program (Generalitat de Catalunya), and in part by the Molecular Foundry (Lawrence Berkeley National Laboratory) under Project 13-2004. The work of P. Gorostiza was supported by postdoctoral fellowships from the Generalitat de Catalunya (Nanotechnology Program), the Ministerio de Educación y Ciencia (Spain), and the Human Frontier Science Program.

I. INTRODUCTION

THE mechanical manipulation of single biomolecules is revolutionizing the way we understand biological processes [1]. Single-molecule studies have been possible thanks to the development of new *in vitro* experimental assays that make it possible to manipulate a desired molecule while preserving its structural integrity and function. This kind of work has been successful in purified preparations. However, achieving the same manipulation accuracy when a molecule of interest is located in the complex context of a living cell, without disrupting its native conformation or modifying its environment, is still a great challenge. There has been a number of successes in mechanical studies of single molecules in their native environment: DNA packaging into bacteriophage viruses [2], DNA translocation into bacteria [3], and the rotation of flagella in bacteria [4].

Here we present a procedure for selectively biotinylating extracellular residues of membrane proteins expressed in living cells, and for attaching them to external force probes. Our motivation is to develop functional assays for mechanical studies of membrane proteins. A direct interaction between the protein and a microscopic force probe is required. It is essential to specifically attach force probes to the desired protein, while minimizing nonspecific interactions. An additional challenge is to physically access the protein without disrupting the cell membrane, thus maintaining the electrochemical gradients across the membrane which constitute the driving force for transmembrane electrical signaling and transport.

As the expression system we chose oocytes, which are widely used for eukaryotic membrane proteins in studies ranging from functional characterization of new proteins to the screening of drugs on known therapeutical targets. The developed methods can thus be applied to other cloned proteins without the need of purification and reconstitution and can be directly combined with existing electrophysiological methods and chemical modification techniques available for oocytes. Ion channels were chosen as the model proteins, since a protocol for fluorescent labeling and functional characterization in oocytes was previously developed [5]. Our approach was to selectively biotinylate the protein under study and interface the modified protein with known single-molecule manipulators such as force microscopy probes and magnetic beads.

Some of the problems that had to be solved are outlined in Fig. 1. Target proteins in the membrane (black) have extracellular cysteines introduced by mutagenesis and can be chemically modified with thiol-selective reagents. Other proteins (white) present in the plasma membrane (PM) have native cysteines that must be blocked prior to expression of the target protein. The vitelline membrane (VM) is required for cell survival, but it is permeable to small molecules like biotinylation reagents and fluorescent labels. However, cells must be devitellineized to allow the attachment of micrometer-sized beads, force microscopy probes, or to seal patch pipettes for electrophysiology. The follicular layer (FL) is not vital and is enzymatically removed from most cells in the preparation, but if present it poses a barrier to the diffusion of molecules. In defolliculated and devitellineized cells, the PM is exposed to the external solution, but a carbohydrate-rich extracellular matrix (ECM) still hinders physical access of micrometer-sized objects to the membrane and is a source of electrostatic charge. As will be seen, the ECM gives rise to nonspecific interactions that can also be minimized by removing it enzymatically and using biotin-binding proteins with low charge at neutral pH (neutravidin).

II. MATERIALS AND METHODS

A. Channel Expression

All labeling experiments were performed on Shaker channels ShH4 Δ (6–46) with the mutations 359C (to target the thiol-reactive handles to the extracellular loop between S3 and S4 helices) and 434F (to render the channel nonconducting, which also increases expression levels). m-RNA was transcribed *in vitro*, injected at 1- μ g/ μ l concentration into *Xenopus laevis* oocytes that were first defolliculated by treatment with 2 mg/ml collagenase. Cells were incubated for 4–8 days after injection. Native cysteines were blocked on the injection day by conjugation with the charged reagent tetraglycine maleimide (TGM), which was synthesized as previously described [5]. Channel expression was verified by labeling with tetramethylrhodamine maleimide (TMRM T-6027, Molecular Probes, Invitrogen Corp., Carlsbad, CA), and voltage clamp fluorometry was used to record the voltage-dependent fluorescent changes ΔF and the total fluorescence F from the cells at a fixed gain of the photomultiplier tube.

B. Labeling

Injected cells and uninjected controls (both blocked with TGM on the injection day) were labeled with TMRM or biotinylated, together with positive and negative controls prepared from the same oocyte batch the day of the experiments. Negative controls (con $-$) were cells that were blocked with TGM immediately before labeling with TMRM or biotinylation reagents. Positive controls (con $+$) were treated with 1 mM β -mercaptoethanol (β ME) to break disulfide bonds and expose a greater number of reduced cysteines. Unless otherwise specified, incubations and washouts were carried out with ND-96 (NaCl 96 mM, KCl 2 mM, CaCl₂ 1.8 mM, MgCl₂ 1 mM, HEPES 10 mM, pH7.6).

Biotinylation reactions were carried out at a concentration of 10–100 μ M for 30–90 min in depolarizing solution (“high K $^{+}$ ”: KCl 92 mM, CaCl₂ 0.75 mM, MgCl₂ 1 mM, HEPES 10 mM, pH7.5), gently stirring at room temperature and in the dark. This solution provides maximum exposure to the extracellular medium of the residue 359 [6] and thus allows optimal labeling. Unless otherwise specified, the ECM was routinely digested prior to biotinylation with a mixture of 1 mg/ml hyaluronidase (H-2126, Sigma, St. Louis, MO) and 1 unit/ml neuraminidase (N-2876, Sigma) for 30 min at room temperature.

Several biotinylating reagents were tested: maleimide-PEG3400-biotin (0H2D0F12, Nektar Therapeutics, San Carlos, CA), PEO-iodoacetyl biotin (Pierce 21 334, Rockford, IL), MTS-PEG3400-biotin (Toronto Research Chemicals B396 950, North York, ON, Canada). Biotinylation reagents with hydrophobic chains were also tested (N α -(3-maleimidylpropionyl) biocytin, Molecular Probes M-1602, and N-iodoacetyl-N-biotinylhexylenediamine, Pierce 21333) and yielded qualitatively similar results. Occasionally, the amine-selective biotinylating agent sulfo-NHS-LC-biotin was used (Pierce 21 335, Rockford, IL).

Commercially available crosslinkers are convenient because the reagent can be used directly, but they have the disadvantages of short lengths (well below 10 nm, e.g., 2.5 nm for PEO-iodoacetyl biotin). This limits the separation between the protein and force probe (e.g., the bead), which should be large to minimize nonspecific interactions with the cell surface and to allow the characteristic stretching of the polymer. On the other hand, polyethylene glycol (PEG) linkers are longer but have a wide distribution of chain lengths [7]. For the PEG polymer of molecular weight 3400 included in the crosslinkers cited above, the length can be calculated as 25 ± 3 nm. In order to have a linker of greater, well-controlled length and mechanical properties, we also tested double-stranded DNA molecules that were biotinylated at one end and maleimide-activated in the other end. Modified oligonucleotides (Sigma Genosys, St.

Louis) had complementary sequences $\text{H}_2\text{N}(\text{CH}_2)_{12}\text{-5' -CGCTCTCGTCGGACCTTG-3'}$ and biotin – TEG – $5' - \text{CAAGGTCCGACGAGAGCG} - 3'$. The length of this heterobifunctional DNA molecule is approximately 6 nm, but greater lengths can be achieved by ligating a long vector to two double-stranded DNA fragments having compatible overhangs in one end and biotin or amino modifications at the other end [8]. Oligonucleotides were resuspended in 50 mM NaCl, 10 mM phosphate pH7.2 to a final concentration of 2 mM, mixed, and annealed by warming up to 95 °C for 1 min and allowed to cool to room temperature. Maleimide activation of the amine group was carried out immediately before labeling, in a solution 50 μM DNA, 500 μM NaCl, 2 mM sulfo-SMCC (Pierce 22 322, Rockford, IL), incubating for 1 h at 37 °C. Unreacted sulfo-SMCC, which contains maleimide that can interfere with labeling, was eliminated by three successive centrifugations in chromatography columns (Micro Biospin P6 in SSC buffer, BioRad 732-6200, Hercules, CA). Prior to use, buffer in the columns was exchanged three times with 20 mM phosphate, pH7.1. Maleimide activation was verified using the thiol and sulfide quantitation kit (T-6060, Molecular Probes, Invitrogen Corp.) and yielded efficiencies of around 30%. Presence of the biotin was confirmed by retention of the DNA in a polyacrylamide gel after mixing with neutravidin beads (Spherotech NVP-20-50, Libertyville, IL, washed three times as described below, and resuspended at the initial dilution of 0.5% w/v). The resulting maleimide-activated DNA was added to a PCR tube containing devitellineized oocytes in 0.2 ml high K^+ solution to give a final concentration of $\sim 1 \mu\text{M}$. Concentrations in the μM range (similar to those successfully used for TMRM labeling) can thus be achieved using medium scale oligo amounts and keeping incubation volumes in the 0.1-ml range. Longer DNA tethers (e.g., after ligation to a linearized vector) yield lower final concentrations, which may decrease the overall coupling efficiency.

Biotinylation reactions were usually carried out in oocytes with the VM intact with the assumption that small, partially hydrophilic molecules can effectively cross the vitelline layer and reach the PM as shown for TMRM [5]. In the case of maleimide-activated DNA, cells were devitellineized prior to incubation. Images for bead quantification were always recorded in clean, uniform regions of each cell. In atomic force microscope (AFM) and patch experiments, biotinylated oocytes were kept at 12 °C and devitellineized just before the recordings, without further incubation.

C. Voltage-Dependent Blocking

In order to maximize the efficiency of selective channel labeling, a strategy was used which took advantage of the known voltage dependence of S4 accessibility [6], [9]. 359C, at the outer end of S4, is not accessible to external thiol-reactive reagents at negative voltage. Thus, native cysteines were blocked while protecting the 359C introduced in the channel. Oocytes were carefully poked and clamped at -110 mV for 2 min while perfusing 20 μM TGM in ND-96. Cells were kept at -110 mV during 5-min washout in ND-96, then removed from the chamber and sit at 12 °C for recovery. Labeling was carried out 1–2 h after blocking.

D. Biotin Quantification

Oocytes were incubated in neutravidin-coated beads to quantify the extent of biotinylation and to evaluate the accessibility of the channel-attached biotin to the external force probes. Two-micrometer polystyrene beads (neutravidin-coated NVP-20-5, or streptavidin-coated SVP-20-5, Spherotech) were washed three times by removing the supernatant after centrifugation in ND96 solution, and resuspended in fresh solution. Cells were then devitellineized to expose biotin, washed three times on agar-coated cell culture wells (to protect the exposed PM) and incubated for 30 min at 12 °C with beads at 1 : 10 dilution. The solution was mixed to keep beads from sitting at the bottom. Cells were carefully washed again three times and imaged without staining on an upright confocal microscope Zeiss LSM 510 META, Oberkochen, Germany, using an Achro-plan 20x/0.5W water immersion objective (WD = 1.9

mm). Polystyrene bead autofluorescence was visible under 488-nm laser excitation. Confocal optical slices of the top of the oocyte were acquired and projected on a single image. The number of beads was counted using the software NIH Image [10].

E. Force Spectroscopy

Force curves were recorded in a 3D-MFP system (Molecular Force Probe, Asylum Research, Santa Barbara, CA). AFM force probes were microfabricated silicon nitride cantilevers (spring constant 10–30 pN/nm). They had a 4.5- μ m-diameter polystyrene bead glued to the tip and were coated with streptavidin or neutravidin (Novascan PT.PS.SA.SN and PT.PS.NA.SN, respectively, Ames, IA). In order to preserve the protein coating, probes were kept under argon atmosphere in a glove box until the day of the experiments. Cantilevers directly coated with neutravidin (without polystyrene bead, NovaScan CT.AU.SA.SN) were found to produce noisy signals when electrodes were introduced in the bath, possibly as a result of gold coating used for neutravidin attachment. Tips were mounted in the microscope head and their elastic constant was measured using the thermal method in air [11]. They were then immersed in the recording solution (ND-96). The laser and detector were aligned and allowed to drift for 1–2 h prior to the start of the experiment. Deflection calibration was carried out on a glass surface in solution after the end of the experiment. Cells were biotinylated as described above and kept at 12 °C. Biotinylated and devitellinized oocytes were washed three times in ND-96 on agar-coated cell culture wells and transferred to the microscope.

F. Patch Clamp

Pipettes were pulled from borosilicate glass tubing (Warner G-150-TF4, Hamden, CT) and outside-out patches were obtained by the standard procedures using an Axopatch 200A amplifier. Cells were biotinylated, devitellinized, and washed as described above. Neutravidin-coated paramagnetic beads were 5 μ m in diameter (Spherotech NVM-50-5). Before use, they were washed thrice by centrifugation in ND96, and 0.05% BSA was added to prevent adhesion of the beads to the pipette glass.

III. RESULTS

A. Biotinylation of Membrane Proteins

The biotinylation procedure was quantified and compared to the amount of cysteines on the membrane, using the following assay. We measured the surface density of neutravidin beads attached after biotinylating the cells with maleimide-PEG3400-biotin and compared it to the total TMRM fluorescence in oocytes from the same frog [5]. The measurements are shown in Fig. 2 for the negative control (con[−]), uninjected cells (uninj), cells expressing ShH4 Δ 359C, and the positive control (con⁺). A large difference was seen between con[−] blocked with TGM immediately before biotin labeling and con⁺, where native cysteines were all available for conjugation. TMRM labeling of con⁺ was sixfold greater than con[−], and the density of neutravidin beads on con⁺ was 30-fold greater than con[−]. The correlation between TMRM and bead–biotin labeling shows that this method should be able to detect specific bead conjugation to channels. Indeed, oocytes blocked by TGM on the day of RNA injection had significantly greater fluorescence and bead density than uninjected controls. It must be noted that bead density is lower than the density of conjugated TMRM molecules, since most likely a great number of biotin molecules are attached to each neutravidin-coated bead.

B. Factors Affecting Nonspecific Binding

In order to reduce background and data dispersion, we explored crosslinkers with different cysteine-reactive moieties, lengths and polarity of the spacer chain. We compared the number of beads attached to each group of oocytes after biotinylating them with MTS-PEG3400-biotin,

PEO-iodoacetyl biotin and maleimide-activated DNA. The results are shown in Fig. 3. MTS-PEG3400-biotin produces a large number of beads even in negative controls, with a large variability. Both PEO-iodoacetyl biotin and maleimide-activated DNA had lower background in the con⁻, a larger difference between con⁻ and con⁺, and greater labeling in 359C than in uninjected oocytes. Cells labeled with PEO-iodoacetyl biotin display essentially the same behavior as maleimide-activated DNA, although with a somewhat reduced reactivity for the positive control.

In an attempt to reduce nonspecific interactions between the polar tethers and the negatively charged cell surface, we repeated incubations in the presence of 10 mM EDTA and 10 mM EGTA, but no significant differences were found (not shown). The effect of pH was also evaluated, in order to reduce the net charge of the streptavidin-coated beads (the isoelectric point of streptavidin is at pH5). However, ND-96 solutions of pH5, 6.3, and 7.6 did not show significant differences in nonspecific attachment of beads to biotinylated and nonbiotinylated cells (not shown). In contrast, using neutravidin-coated beads (isoelectric point pH6.3) almost completely abolished nonspecific attachment of beads to nonbiotinylated cells even at the regular pH7.6 of ND-96 (Fig. 4). The number of beads in biotinylated cells was also reduced modestly. In these experiments, cells were biotinylated using sulfo-NHS-LC-biotin (amine-specific) and compared to untreated cells so that we could study the interaction between beads and biotin, without taking into account the efficiency of cysteine blocking.

Another potential source of nonspecific interaction is the ECM of the oocyte PM, a 1- μ m-thick carbohydrate-rich mesh [12] that contains polar and charged groups, and that might physically hinder the specific interactions between biotinylated channels and streptavidin-coated beads. Enzymatically removing the ECM increases the specificity of bead attachments, as is shown in Fig. 5. The number of beads attached to the TGM blocked cells is reduced substantially, and there is a smaller reduction in labeling of the con⁺. Oocytes with the FL intact despite treatment with collagenase also have lower specificity of labeling.

A major source of background labeling comes from the native cysteines present in oocyte membrane proteins. These were routinely blocked on the injection day, but natural recycling of membrane proteins proceeds steadily as the exogenous protein is overexpressed following mRNA injection. In the best cases, injected cells displayed a fourfold larger total fluorescence compared to uninjected cells, but a factor of two or lower was more common. This problem is intrinsic to the expression level of a particular protein and usually cannot be avoided. However, in the channel that we used in these experiments, the residue 359C displays state-dependent accessibility [6], [9], i.e., it is exposed to the extracellular medium under depolarizing conditions (high K⁺ solution, or voltage-clamping the cell near 0 mV), and it is not exposed under hyperpolarizing conditions (ND-96 or low K⁺ solutions, or clamping the cell equal or more negative than -80 mV). We took advantage of this fact to selectively block native and recycled cysteines while protecting 359C. Fig. 6 compares bead coverage of cells injected and uninjected (expressed for three days), and after blocking the cells for 2 min at -110 mV, 20 μ M TGM in ND96. As can be seen, in oocytes where there was little or no difference with block on the injection day (day 0), block on day 3 at -110 mV followed by labeling in high K⁺ showed a substantial difference between injected and noninjected.

C. Force Measurements in Membrane Proteins

There are several ways to access these molecular handles with microscopic probes in order to sense and apply forces. We tested the application of our methodology by measuring the binding forces between biotinylated membrane proteins on the cell, and neutravidin-coated AFM probes. The approach-retract force curve on a biotinylated cell (con⁺, 50 μ M maleimide-PEG3400-biotin) using a neutravidin-coated AFM probe, is shown in Fig. 7(a). Biotinylated cells expressing the channel mutant 359C yield similar results with a lower number of

unbinding events (not shown), in agreement with the higher density of labeling in Fig. 2. The force-extension plots show compression as positive (up) and tension as negative (down), as indicated in the insets. On the approach trace, irregular jumps were found in some curves, which may be related to the complex morphology of the oocyte surface [12]. On the retract trace, discrete deadhesions of 50–150 pN can be observed. These display a Gaussian distribution [Fig. 7(b)]. The center force of the distribution, as well as its full width at half maximum (FWHM), are dependent on the pulling rate as is shown in Fig. 7(a), inset. At 10^5 pN/s, the center force is 84 ± 1 pN and $\text{FWHM} = 42$ pN. The slope of the retract curve is decreased with each deadhesion event, as the total number of tethers between probe and cell (and hence the effective elastic constant) is reduced. Nonbiotinylated cells (Fig. 7(a), gray trace) display an adhesion region that is about four-fold lower with neutravidin coated tips (56 ± 7 pN, $N = 44$) than with streptavidin tips (209 ± 22 pN, $N = 25$). This is in agreement with bulk measurements shown in Fig. 3, in which nonspecific attachment of neutravidin-coated beads to nonbiotinylated cells is practically nonexistent.

We have also explored the combination of patch clamp and magnetic tweezers for simultaneous current, voltage, and force recordings in outside-out patches of membrane. Outside-out patches of biotinylated cells, with seal resistances above $1 \text{ G}\Omega$, were obtained, and carefully dipped in neutravidin-coated paramagnetic bead mounds placed at the bottom of the patch perfusion chamber. In more than 70% of the attempts, this procedure resulted in beads clearly attaching to the biotinylated patch with no decrease in the seal resistance (see Fig. 8). Addition of BSA 0.05% to the bead solution helped to keep beads at the tip, by preventing bead attachment to the pipette glass. Forces were applied by approaching a fragment of fixed magnet glued to the tip of a pipette and immersed in the recording solution. The beads can occasionally be seen to move in the magnetic field, but not detach from the membrane. Comparing to the adhesion forces between a biotinylated cell and a neutravidin-coated AFM probe having a bead of similar dimensions (see above), we estimate that the forces applied to the patch are ~ 100 pN.

IV. DISCUSSION

Biotin moieties attached to introduced cysteines in the external domain of membrane proteins, are physically accessible to microscopic probes outside the cell, and the extent of biotinylation is proportional to the amount of cysteines on the cell surface (see Fig. 2). There are, however, several sources of background and variability. The high background of the MTS-PEG reagent compared to the maleimide-PEG (Figs. 2 and 3) could be due to the higher reactivity and lower specificity of MTS. The background is much lower for the iodoacetamide, which is similar in reactivity to maleimide. However, the short length of available iodoacetamide-biotin crosslinkers (~ 3 nm) made them less attractive for pulling experiments, because the stretched length is more difficult to resolve in force-extension curves. Also, iodoacetamides are very sensitive to moisture and light, which tend to decrease reactivity over time. In principle, using maleimide-activated DNA is the best method for having well-controlled length and mechanical properties, compared to the chemical synthesis of the longest PEG linkers which give a relatively wide distribution of lengths [7]. However, when using oocytes as an expression system, DNA linker conjugation requires devitellinization, making the cell sensitive to physical perturbations and limiting practicality. Mammalian cells, which are smaller and have a more accessible PM, may be better suitable for these DNA tethers. However, depending on the desired DNA length, the final concentration of activated DNA may be well below the μM range, which will reduce the final biotinylation efficiency. Overall, the best crosslinker for this application was maleimide-PEG3400-biotin because it yields the lowest background labeling in (con-), it is simple to prepare, and its long PEG linker allows better accessibility and stretch in single-molecule recordings.

The extremely low nonspecific bead attachment of neutravidin compared to streptavidin (Fig. 4) is in agreement with their reported properties [13]. Neutravidin is an avidin derivative that has been modified to remove universal cell surface recognition sequences (present in streptavidin), glycosylation, and a number of charged residues, raising the isoelectric point to 6.3 as compared to 5 for streptavidin. In this way electrostatic interactions that contribute to nonspecific binding are reduced. Neutravidin is therefore very useful to minimize nonspecific interactions. Many streptavidin-coated surfaces are often available with neutravidin as well, and the protein coating procedure is very similar (the major difference being the resuspension of free neutravidin, which must be done in pure water due to the low charge of the modified protein).

Digestion of the ECM (Fig. 5) is advantageous to reduce nonspecific interactions and it was adopted routinely as part of the biotinylation protocol. It is also important to verify oocyte defolliculation. Removal of the FL increases the efficiency of enzymatic removal of the ECM. Another possible explanation for the high background in the negative control is that some TGM may be taken up by the FL, thus reducing blocking efficiency, but most of the high background in folliculated cells can be accounted for by the effect of the FL alone.

State-dependent accessibility of certain residues is sometimes known for membrane proteins, e.g., in the presence of a ligand or under certain electrochemical gradients. Voltage-dependent blocking experiments (Fig. 6) show that further improvement of labeling selectivity can be achieved by taking advantage of the state-dependence of accessibility.

Force experiments with the AFM (Fig. 7) make it possible to sense and manipulate these molecular handles, and to design mechanical assays on membrane proteins at the single-molecule level on living cells. The unbinding forces measured between biotin and neutravidin are lower than the forces between biotin and streptavidin at comparable rates on solid substrates [14]. These differences may be due to a lower binding affinity between biotin and neutravidin, and/or to differences in the loading rate due to the elasticity of the cell. In the patch clamp–magnetic tweezers setup, the forces applied on the membrane are estimated about 100 pN. These forces are enough to promote biologically relevant conformational changes, and could be detected externally with very high sensitivity if linked to changes in membrane conductance.

In summary, we have presented a method to selectively biotinylate extracellular residues of membrane proteins expressed in oocytes. The biotin moiety can be seen as a versatile and reversible “handle” that can be grabbed specifically with a variety of biotin-binding probes. Several sources of nonspecific interactions have been identified and addressed. Maleimide-PEG3400-biotin is the best behaved crosslinker, combining ease of use and high specificity with a relatively long hydrophilic linker. Neutravidin-coated beads were found to effectively minimize nonspecific interactions with the cell surface, especially when combined with the digestion of the oocyte ECM. This procedure is generally applicable to any cloned membrane protein that can be expressed in oocytes. Membrane proteins presenting state-dependent accessibility allow further labeling specificity by selective blocking of native cysteines.

Biotinylation for single-molecule experiments should use lower crosslinker concentrations and shorter expression times after block, which in turn provides a lower nonspecific background [12]. On the other hand, a common strategy in single-molecule studies is to chemically modify the probe surface with a variety of functional groups [15], [16] and ligands [17] specific for each application. This approach has been successful in force spectroscopy studies of single bonds, and in ligand-receptor recognition microscopy [18]. Here we propose to functionalize the membrane protein with a “universal handle” directed to cysteines that consists of a biotinylated PEG. Probes can then be standardized, and hence the strategies and conditions to reduce their nonspecific adhesion can be employed generally. This setup is also suitable to

study mechanosensitive channels, tip-link channels of hair cells, DNA pores, the translocation of (biotinylated) substrates across the PM, or that of isolated nuclei or mitochondria. The method could also be applied to bind primary antibodies against a membrane protein, and then use the neutravidin-coated AFM tip as a standardized secondary probe of antibody location.

A great number of single-molecule studies have dealt with prokaryotic proteins in vitro, yielding a wealth of information about their inner workings under isolated conditions. As our understanding of the operation of proteins reaches the single-molecule level, it becomes more important to put this knowledge back in the context of the living cell. Studies in the native environment of the cell will allow to “put the cell back together” but involve highly demanding experimental conditions regarding specificity and accessibility. We have addressed some of these issues by using standard probes and expression systems for eukaryotic membrane proteins. Further developments include functional assays of membrane proteins by combining force and electrophysiological recordings.

Acknowledgment

The authors would like to thank L. Fang and M. Salmeron (Molecular Foundry, Lawrence Berkeley National Laboratory) for the use of the molecular force probe, V. F. Puentes and A. P. Alivisatos (University of California, Berkeley) for the use of the argon glove box, and members of the Isacoff, Bustamante, and Kramer laboratories for help and advice. We also thank B. Onoa and D. Pareto for comments on the manuscript.

REFERENCES

1. Bustamante C, Chemla YR, Forde NR, Izhaky D. Mechanical processes in biochemistry. *Annu. Rev. Biochem* 2004;73:705–748. [PubMed: 15189157]
2. Smith DE, Tans SJ, Smith SB, Grimes S, Anderson DL, Bustamante C. The bacteriophage 29 portal motor can package DNA against a large internal force. *Nature* 2001;314:748–752. [PubMed: 11607035]
3. Maier B, Chen I, Dubnau D, Sheetz MP. DNA transport into *Bacillus subtilis* requires proton motive force to generate large molecular forces. *Nat. Struct. Mol. Biol* 2004;11:643–649. [PubMed: 15184891]
4. Ryu WS, Berry RM, Berg HC. Torque generating units of the flagellar motor of *Escherichia coli* have a high duty ratio. *Nature* 2000;403:444–447. [PubMed: 10667798]
5. Mannuzzu LM, Moronne MM, Isacoff EY. Direct physical measure of conformational rearrangement underlying potassium channel gating. *Science* 1996;271:213–216. [PubMed: 8539623]
6. Baker OS, Larsson HP, Mannuzzu LM, Isacoff EY. Three transmembrane states and sequence-dependent displacement of the S4 domain in Shaker K⁺ channel gating. *Neuron* 1998;20:1283–1294. [PubMed: 9655514]
7. Ratto TV, Langry KC, Rudd RE, Balhorn RL, Allen MJ, McElfresh MW. Force spectroscopy of the double-tethered concanavalin-A mannose bond. *Biophys. J* 2004;86:2430–2437. [PubMed: 15041680]
8. Hegner M. DNA handles for single molecule experiments. *Single Mol* 2000;1:139–144.
9. Larsson HP, Baker OS, Dhillon DS, Isacoff EY. Transmembrane movement of the Shaker K⁺ channel S4. *Neuron* 1996;16:387–397. [PubMed: 8789953]
10. NIH Image. <http://rsb.info.nih.gov/nih-image>Online
11. Hutter JL, Bechhoeffer J. Calibration of atomic-force microscope tips. *Rev. Sci. Instrum* 1993;64:1868–1873.
12. Sonleitner A, Mannuzzu LM, Terakawa S, Isacoff EY. Structural rearrangements in single ion channels detected optically in living cells. *Proc. Nat. Acad. Sci. USA* 2002;99:12759–12764. [PubMed: 12228726]
13. Hiller Y, Gershoni JM, Bayer EA, Wilchek M. Biotin binding to avidin. Oligosaccharide side chain not required for ligand association. *Biochem. J* 1987;248:167–171. [PubMed: 3435435]
14. Chen A, Molloy VT. Single-molecule force measurements. *Methods Cell Biol* 2002;68:301–309. [PubMed: 12053735]

15. Friedsam C, Del Campo Bécáres A, Jonas U, Gaub HE, Seitz M. Polymer functionalized AFM tips for long-term measurements in single-molecule force spectroscopy. *Chem. Phys. Chem* 2004;5:338–393.
16. Afrin R, Yamada T, Ikai A. Analysis of force curves obtained on the live cell membrane using chemically modified AFM probes. *Ultramicroscopy* 2004;100:187–195. [PubMed: 15231309]
17. Schültz GJ, Pastushenko V, Ph. Gruber HJ, Knaus H-G, Pragl B, Schindler H. 3D imaging of individual ion channels in live cells at 40nm resolution. *Single Mol* 2000;1:25–31.
18. Raab A, Han W, Badt D, Smith-Gill SJ, Lindsay SM, Schindler H, Hinterdorfer P. Antibody recognition imaging by force microscopy. *Nat. Biotechnol* 1999;17:902–905.

Fig. 1. Sketch of the structure of the oocyte surface and all the hurdles for the biotinylation of target membrane proteins (black) in the PM. Native membrane proteins are depicted white. The VM is shown with dashed lines and the FL as a black stripe. The ECM is indicated in gray.

Fig. 2.

Assay for quantification of biotinylated cysteines on the cell surface. In each set of cells, the average density of neutravidin beads was measured after biotinylation with maleimide-PEG3400-biotin 10 μ M for 90 min. These measurements were compared to the average fluorescence after TMRM labeling (which is proportional to the amount of cysteines on the membrane [5]). Measurements were repeated in the negative control (con-), uninjected cells (uninj), cells expressing Sh Δ 359C during six days, and the positive control (con+). Each data point shows the average $\pm$ s.e.m. [fluorescence: $N = 14$ (con-), 13 (uninj), 8 (inj), 14 (con+); beads: $N = 11$ (con-), 13 (uninj), 8 (inj), 12 (con+)]. Inset: confocal projection images showing representative coverages of control cells con- and con+.

Fig. 3.

Comparison of the extent of biotinylation for three biotinylation reagents: MTS-PEG3400-biotin 10 μ M [$N = 6$ (con-), 5 (con+)], iodoacetyl biotin 100 μ M [$N = 8$ (con-), 9 (uninj), 8 (inj), 9 (con+)] and maleimide-activated DNA 1 μ M [$N = 9$ (con-), 10 (uninj), 3 (inj), 9 (con+)]. Injected cells were allowed to express for five days.

Fig. 4. Nonspecific interactions between beads and cell due to protein coating on the beads: comparison of streptavidin and neutravidin bead attachment to cells untreated, and biotinylated with sulfo-NHS-LC-biotin 1 mM for 30 min [streptavidin $N = 7$ (biot), 5 (untreat); neutravidin $N = 8$ (biot), 8 (untreat)].

Fig. 5.

Nonspecific interactions between beads and cell due to the ECM and the presence of FL during blocking and biotinylation with iodoacetyl biotin 100 μ M, 60 min. Bead attachment to defolliculated cells after enzymatic removal of the ECM using hyaluronidase and neuraminidase [$N = 11$ (con-), 10 (con+)]; defolliculated cells after ECM digestion [$N = 9$ (con-), 5 (con+)]; and folliculated cells after incubation in hyaluronidase and neuraminidase [$N = 11$ (con-), 10 (con+)].

Fig. 6.

Surface density of beads in cells blocked with TGM on the injection day and allowed to express for three days before biotinylation. Recycling of native (blocked) cysteines increases background labeling [$N = 10$ (uninj), 6 (inj)]. Native cysteines can be selectively blocked using TGM 20 μM perfusion under cell voltage clamp at -110 mV for 2 min, immediately prior to biotinylation [$N = 11$ (uninj), 9 (inj)].

Fig. 7.

(a) AFM force-extension curves using neutravidin-coated AFM bead probes on the surface of (con+) cells biotinylated with maleimide-PEG3400-biotin 50 μM (black trace) and untreated cells (gray trace). (b) Histogram of the unbinding forces in biotinylated cells as a function of the pulling rate. Each histogram was fitted to a Gaussian function and in (a, inset) the mean unbinding force is plotted together with the error (black error bars) and the FWHM in each case (gray bars).

Fig. 8. Outside-out patch of (con+) cell biotinylated with 50 μ M maleimide-PEG3400-biotin (left). Neutravidin-coated magnetic beads were attached to the membrane patch by carefully dipping it in bead mounds at the bottom of the recording chamber.