Measurement of Membrane Binding between Recoverin, a Calcium-Myristoyl Switch Protein, and Lipid Bilayers by AFM-Based Force Spectroscopy

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ABSTRACT Myristoyl switch is a feature of several peripheral membrane proteins involved in signal transduction pathways. This unique molecular property is best illustrated by the "Ca^{2+}-myristoyl switch" of recoverin, which is a Ca^{2+}-binding protein present in retinal rod cells of vertebrates. In this transduction pathway, the Ca^{2+}-myristoyl switch acts as a calcium sensor involved in cell recovery from photoactivation. Ca^{2+} binding by recoverin induces the extrusion of its myristoyl group to the solvent, which leads to its translocation from cytosol to rod disk membranes. Force spectroscopy, based on atomic force microscope (AFM) technology, was used to determine the extent of membrane binding of recoverin in the absence and presence of calcium, and to quantify this force of binding. An adhesion force of 48 $\pm$ 5 pN was measured between recoverin and supported phospholipid bilayers in the presence of Ca^{2+}. However, no binding was observed in the absence of Ca^{2+}. Experiments with nonmyristoylated recoverin confirmed these observations. Our results are consistent with previously measured extraction forces of lipids from membranes.

INTRODUCTION

It has been widely demonstrated that many viral and cellular proteins are N-terminally acylated by myristic acid (C14:0) and other fatty acids (i.e., C12:0, C14:1, C14:2, C16:0) (for reviews see Dunphy and Linder, 1998; Resh, 1999). Generally, N-myristoylation consists of a covalent attachment of a myristic acid to an N-terminal glycine residue of a protein via an amide linkage (Duronio et al., 1993). It takes place during protein synthesis and is catalyzed by N-myristoyl transferase. This modification has been shown to play a key role in protein-protein interaction and/or in binding of proteins to plasma membranes. Interestingly, in some cases, ligands like GTP, phosphate, or Ca^{2+} are involved in the modulation of membrane binding by controlling the orientation of the myristoyl moiety relative to the protein (for a review see McLaughlin and Aderem, 1995). In these cases, myristoyl groups and ligands constitute a molecular switch, the so-called myristoyl switch. So far, the most studied is the Ca^{2+}-myristoyl switch of recoverin.

Recoverin is a 23-kDa calcium-binding protein originally purified from retinal rod outer segments (ROS) of vertebrates (Dizhoor et al., 1991). This protein is a member of the EF-hand superfamily, which contains proteins that bind Ca^{2+} via the EF-hand motif, a helix-loop-helix of 12 residues arranged to coordinate Ca^{2+} with pentagonal bipyramidal symmetry (for reviews see Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001). Of the four EF-hand present in recoverin, only two (EF-2 and EF-3) bind Ca^{2+} (Fig. 1 *A*) (Ames et al., 1995; Flaherty et al., 1993). Recoverin contains an amino-terminal myristoyl group (Dizhoor et al., 1992, 1993; Zozulya and Stryer, 1992) and acts as a calcium sensor by regulating the rod cell response to the change in intracellular Ca^{2+} upon photoactivation. Recoverin prevents the phosphorylation of rhodopsin by inhibiting rhodopsin kinase at a high concentration of Ca^{2+} (Chen et al., 1995; Gray-Keller et al., 1993; Kawamura et al., 1993; Klenchin et al., 1995; Senin et al., 1995) Indeed, in the dark, the binding of two Ca^{2+} ions to recoverin induces the extrusion of its myristoyl group (calcium-myristoyl switch) (Fig. 1 *B*), which enables it to bind ROS disk membranes and to inactivate peripheral protein rhodopsin kinase (Ames et al., 1995, 1997; Hughes et al., 1995). In contrast, light induces lowering of intracellular Ca^{2+}, which results in a conformational change of recoverin and sequestration of the myristoyl group in a hydrophobic cleft (Fig. 1 *A*) (Ames et al., 1994; Flaherty et al., 1993, Tanaka et al., 1995). Consequently, recoverin loses its affinity for membranes and moves to the cytosol, which allows rhodopsin kinase to phosphorylate light-activated rhodopsin. In addition, patch clamp studies of truncated ROS have shown that myristoylated recoverin is 12-fold more active than nonmyristoylated recoverin to prolong the lifetime of light-activated rhodopsin, indicating that the myristoyl group is essential for the proper transduction of the calcium signal in rod cells (Erickson et al., 1998).

Few studies have been done on the partitioning of acylated proteins in bilayers. Free-energy data for small myristoylated peptides (Peitzsch and McLaughlin, 1993) and for an acylated protein (Pool and Thompson, 1998) have

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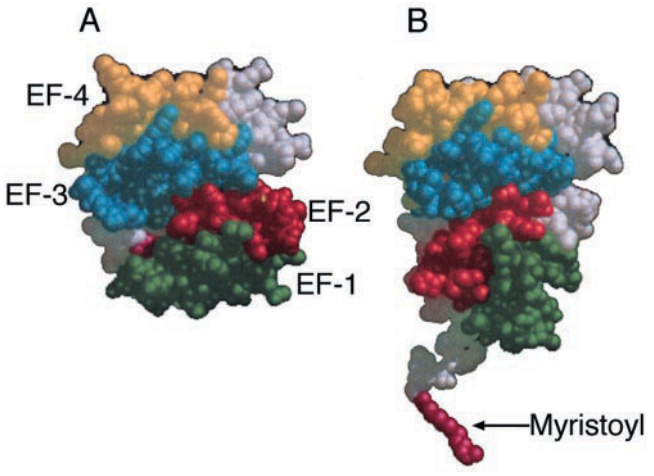

FIGURE 1 Space-filling model of (*A*) Ca^{2+}-free state of myristoylated recoverin (Tanaka et al., 1995; liku.pdb) and (*B*) Ca^{2+}-bound state of myristoylated recoverin (Ames et al., 1997; 1jsa.pdb). This figure has been modified from Fig. 2 *A* published by Ames et al. (1997).

shown that a myristoyl moiety is barely enough (or not sufficient) to anchor a protein to membranes. Indeed, other contributions by the protein arising from charge residues (Kim et al., 1994; Sigal., 1994), conformational and mass-dependent entropy (Silvius and Zuckerman, 1993; Finkelstein and Janin, 1989), and steric effects and hydrophobic residues (Grenier et al., 1998) can be involved in binding of acylated proteins to membranes. However, surface plasmon resonance spectroscopy studies have revealed that binding of recoverin to membranes was strictly dependent on Ca^{2+} and its myristoyl group, indicating that electrostatic contribution is minimal or negligible in this case (Lange and Koch, 1997). Considering all these studies, it is not clear whether a single myristoyl group is sufficient to fully anchor a protein to membranes.

The atomic force microscope (AFM) (Binnig et al., 1986) has become a powerful tool for observing biological structures (for reviews see Hansma and Hoh, 1994; Morris, 1994) and studying intramolecular and intermolecular interactions at the single molecular level. In fact, the ability of applying and measuring minute forces between the AFM tip and the sample and the development of functionalization methods of AFM tips (Florin et al., 1994; Lee et al., 1994; Moy et al., 1994, Grandbois et al., 1999, 2000) have allowed the emergence of AFM-based force spectroscopy. This technique has opened the door to a new structural parameter within and between molecules that is the measurement of force (for reviews see Engel et al., 1999; Fisher et al., 1999; Leckband, 2000; Rief et al., 1999). Force spectroscopy by AFM has been used to measure many types of forces, such as intermolecular forces between various ligands and receptors (Florin et al., 1994; Lee et al., 1994; Moy et al., 1994; Grandbois et al., 2000), rupture force of covalent bond (Grandbois et al., 1999), unfolding forces of individual proteins (Müller et al., 1999; Oberhauser et al., 1998; Oesterhelt et al., 2000; Rief et al., 1997), and cell-cell interaction forces (Benoit et al., 2000).

We have extended the use of AFM-based force spectroscopy to further understand the effect of conformational changes of recoverin on its membrane binding ability and to obtain important data on the strength of binding of myristoyl groups with membranes. In this study we report the statistical distribution of adhesion forces between recoverin and a supported dipalmitoylphosphatidylcholine (DPPC) bilayer in absence and presence of calcium for myristoylated and nonmyristoylated recoverin.

MATERIALS AND METHODS

Expression and purification of recombinant recoverin

Nonmyristoylated and myristoylated recoverins were expressed and purified by phenyl-Sepharose chromatography essentially as described by Ray et al. (1992). Briefly, 0.5 l of Luria-Bertoni medium containing both kanamycin and ampicillin (50 μg/ml) were inoculated by 1 ml of overnight culture of *Escherichia coli* strain BL21(DE3) pLysS containing plasmids encoding for recoverin (pET11a-mr21) and N-myristoyl-transferase (pBB131), which was kindly provided by Anthony J. Scotti and James B. Hurley. The culture was grown at 37°C with shaking. At $A_{600\ nm} = 0.3$, protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside and cells were incubated for an additional 3 h at room temperature. Then, cells were harvested by centrifugation at 7000 $\times$ *g* for 10 min. In the case of myristoylated recoverin, 200 μg/ml of myristic acid was then added to the growth medium 15–20 min before inducing protein expression (Duronio et al., 1990). The pellet was resuspended in 20 ml of buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM DTT, 1 mM $CaCl_2$, 1 mM PMSF) and the cells were disrupted by sonication and centrifuged to remove insoluble materials (20,000 $\times$ *g* for 30 min). The cleared lysate was filtrated through a 0.45-mm filter and loaded on a 5 ml phenyl-Sepharose column equilibrated with buffer A. After washing, recoverin was eluted by decreasing Ca^{2+} concentration with elution buffer B containing EGTA (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM DTT, and 5 mM EGTA). The eluted fractions containing recoverin were frozen at -70°C until use. The purity was at least 99% as judged by gel electrophoresis and Coomassie blue staining. Myristoylation of recoverin has been verified by fluorescence spectroscopy as described by Ray et al. (1992). Our measurements strongly suggest that close to 100% of our samples of recombinant myristoylated recoverin are myristoylated.

Functionalization of AFM tips

Myristoylated and nonmyristoylated recoverin were tethered to Si_3N_4 tips (Microlever, Park Scientific Instruments, Sunnyvale, CA) using carboxymethylated amylose spacers. This protocol, based on carbodiimide chemistry (Hermanson, 1996), was developed by Grandbois et al. (1999) for AFM tip functionalization. First, the Si-OH layer of the Si_3N_4 cantilever was silanized by immersion in *N*′-(3-(trimethoxysilyl)-propyl)-diethylentriamin (Aldrich, Milwaukee, WI) at 90°C for 15 min. After silanization, the amino-functionalized cantilever was rinsed in ethanol and then water-cured for 1 h at 90°C. A PBS (pH 7.4) solution of 10 mg/ml carboxymethylamylose (Sigma, St Louis, MO) was prepared and activated with 50 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) and 10 mg/ml of *N*-hydroxysuccinimide (NHS) (Aldrich) to introduce NHS ester groups along the amylose chain. The amino-functionalized tip was incubated with this NHS-activated amylose for 2 min and rinsed in PBS. The

tip was then coated with recoverin by immersing it in a solution containing 1 mg/ml of myristoylated or nonmyristoylated recoverin in buffer C (50 mM HEPES, pH 7.4, 100 mM NaCl, 2.5 μM EGTA) for 1 h and rinsed with buffer C to remove unbound recoverins. During this last step, an amide bond linkage is formed between the free amino groups of recoverin and the NHS ester groups of amylose. Functionalized recoverin tips were kept hydrated in buffer C and immediately used for force measurements. Tips functionalized only with carboxyamylose were also prepared as described above for control experiments.

Substrate preparation

Supported 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, Alabaster, AL) bilayers were prepared by Langmuir-Blodgett-Schaefer transfers onto freshly cleaved mica according to a procedure reported by Tamm and McConnell (1985). Briefly, the mica was submerged into the water subphase of a home-built Langmuir trough. A DPPC solution (2 mg/ml in chloroform) was then spread at the air-water interface at 25°C. After evaporation of the solvent, the monolayer of DPPC was compressed to 35 mN/m. The Langmuir-Blodgett (LB) film was then prepared by a single upstroke of the mica at 50 μm/s through the air-water interface. This first monolayer was allowed to dry for 15 min before the transfer of the second layer by a single downstroke of the substrate by the Langmuir-Schaefer (LS) method through the air-water interface. The substrate was then placed in a petri dish at the bottom of the trough, which was then removed from the subphase and immediately transferred to the AFM-based force measuring device.

AFM imaging

Before the force spectroscopy experiments, the phospholipid-supported bilayers were imaged in buffer C at high resolution with an atomic force microscope (Digital Instruments Nanoscope III, Santa Barbara, CA). The mica-supported bilayer was imaged in the constant force contact mode (loading force $<$0.5 nN) at a scan rate of 5 Hz (lines per second). A Park Scientific Instruments microlever with a nominal spring constant of 10 mN/m was used. The piezo scanner was calibrated for *x-y-z* against a grid of a known dimension.

Force spectroscopy

The softest triangular microlever of a silicon-nitride cantilever (Microlever, tip C, Park Scientific Instruments) was used for all measurements. Each microlever was calibrated after a given experiment using the thermal noise amplitude (Florin et al., 1995; Butt and Jaschke, 1995; Hutter and Bechhoefer, 1994). The measured spring constants were between 8 and 11 mN/m, which were in good agreement with the nominal spring constant of 10 mN/m provided by the supplier (Park Scientific Instruments). All force measurements were performed using a home-made force spectrometer based on the design and operation of an AFM. This apparatus is optimized for vertical approach-retract cycles (perpendicular to the substrate plane) and *x-y* translation can be done manually with micrometer screws. A piezo transducer equipped with a strain-gauge position sensor was used to set the position of the cantilever relative to the substrate. The fluid cell was a petri dish that contained the substrate in buffer C as described above. Ca^{2+}, when present, was added directly to buffer C from a 2.0 M $CaCl_2$ stock solution to reach a final concentration of 5 mM (buffer D). The cantilever deflection was monitored optically by a laser beam focused onto the end of the cantilever and reflected onto a split photodiode. A typical experiment was performed as follows: the recoverin functionalized tip was approached until a contact with the DPPC bilayer occurred (Fig. 3 *A*) and the adhesive force was calculated from vertical excursion of the last rupture peak of the retract force curve (Fig. 3 *C*). Moreover, force curves were recorded in the absence and presence of Ca^{2+} with the same functionalized tip and several experiments were performed with different functionalized tips to verify reproducibility of the data. Analysis of force curves were done off-line using routines written in IgorPro 3.11.

RESULTS AND DISCUSSION

We have used AFM-based force spectroscopy to perform force measurements of the interaction between recoverin and a DPPC bilayer in the absence and presence of Ca^{2+}. To carry out these experiments, we have tethered recoverin to an AFM tip through an amylose spacer using carbodiimide chemistry. The use of amylose spacers provides sufficient distance between recoverin and the tip to maximize the spatial accessibility of recoverin to the substrate. Moreover, the amylose polysaccharide coating of the tip minimizes undesirable nonspecific adhesions. Substrates were prepared by LB and LS transfers of DPPC on mica and characterized by AFM imaging (Fig. 2). The bright region in Fig. 2 *A* corresponds to the phospholipid bilayer in the gel phase, whereas the dark regions are defects (holes) in the film that are 6 nm deep, a thickness value consistent with a bilayer organization of the film (Fig. 2 *B*). As can be seen in Fig. 2 *A*, the surface coverage of the mica was found to be very uniform over several micrometers, which makes the force experiments with recoverin very reliable. In a typical adhesion force measurement experiment, recoverin is brought in contact with the DPPC bilayer and then retracted from the bilayer until detachment occurs (Fig. 3). Many hundreds of approach-retract cycles were performed at several locations of the DPPC bilayer and force versus tip-sample distance curves were recorded. The adhesion force between recoverin and the membrane was obtained by measuring the last "pull-off" event on a force curve (Fig. 3 *C*). This sharp "pull-off" of the recoverin from the membrane occurs when the restoring force of the cantilever equals or exceeds the adhesion force between recoverin and the membrane.

Binding was rarely observed between myristoylated recoverin and the DPPC bilayer in the absence of Ca^{2+}. However, in the presence of Ca^{2+}, binding events occurred between recoverin and the DPPC bilayer. Fig. 4 shows typical retract force-distance curves recorded in the presence of Ca^{2+}. Because several recoverins are tethered to the AFM tip through amylose spacers of different length, rupture can occur at different distances from the phospholipid bilayer surface, as can be seen in Fig. 4. The rupture forces for a series of 190 force-distance curves were quantified and plotted in a force histogram shown in Fig. 5. This histogram shows a distribution of the rupture forces between 10 and 130 pN, with the most frequent rupture force centered at 48 $\pm$ 5 pN.

As presented in Fig. 6, the addition of Ca^{2+} dramatically increases the adhesion probability of the myristoylated recoverin. However, the approach-retract cycle did not always

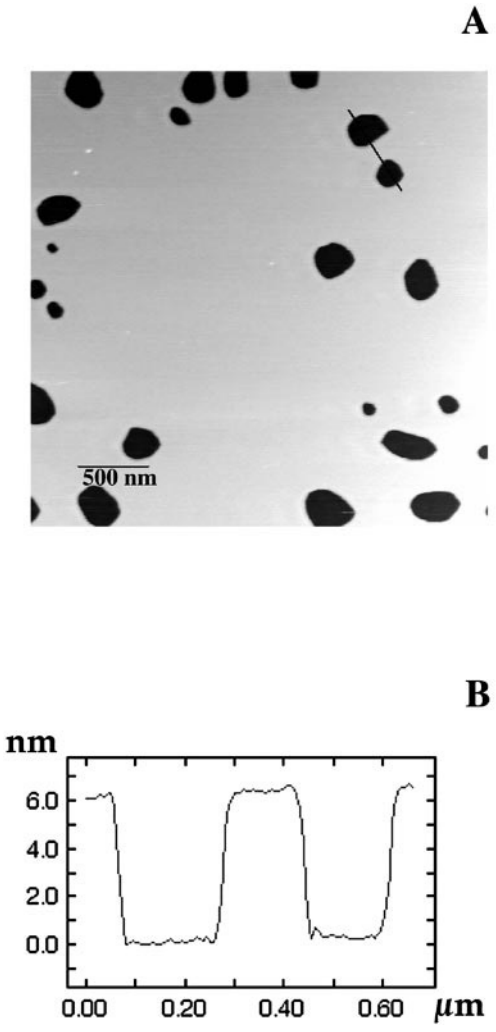

FIGURE 2 (*A*) AFM topographic image of a mica-supported DPPC bilayer in buffer C (50 mM HEPES, pH 7.4, 100 mM NaCl, 2.5 μM EGTA). (*B*) Profile plot along the line drawn in *A*. The height difference between the mica and the DPPC surface (6 $\pm$ 0.3 nm) corresponds to the thickness of the DPPC bilayer.

result in an adhesion event between recoverin and the bilayer in the presence of Ca^{2+}, which makes it more likely to observe single adhesion events between the recoverin-coated tip and the bilayer. In addition, to further demonstrate the specificity of the force measurements for myristoylated recoverin and to rule out the role of Ca^{2+}, measurements with nonmyristoylated recoverin were performed. Ca^{2+} binding to recoverin leads to the unclamping and extrusion of the myristoyl group and to a 45° rotation of the N-terminal domain relative to the carboxy-terminal domain, which exposes hydrophobic residues (Ames et al., 1997; Hughes et al., 1995). Previous reports have shown that the structure of the nonmyristoylated recoverin is essentially similar, in terms of surface hydrophobicity, to the Ca^{2+}-bound state of the myristoylated recoverin (Ames et al., 1997; Hughes et al., 1995). Nevertheless, no binding

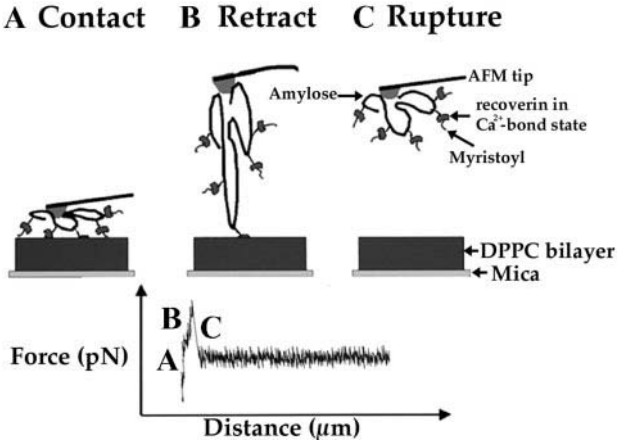

FIGURE 3 Schematic diagram of the experimental setup. Myristoylated recoverin was covalently tethered via a carboxymethylated amylose spacer to the AFM tip. A DPPC bilayer has been prepared on a mica substrate by LB transfer. In the presence of Ca^{2+}, adhesion between the myristoylated recoverin tip and the DPPC bilayer was observed. This adhesion causes an extra force signal during tip retraction until the rupture between recoverin and the bilayer occurs. (*A*) The tip is brought into contact with the DPPC bilayer. (*B*) The tip is retracted from the bilayer. (*C*) The bridge between recoverin and the bilayer is ruptured.

was observed between nonmyristoylated recoverin and the bilayer in the presence of Ca^{2+}. As shown in Fig. 6, the adhesion probability of the nonmyristoylated recoverin was near zero in the absence of Ca^{2+} and in the presence of Ca^{2+}. These results suggest that the myristoyl group is solely responsible for the membrane adhesion of the Ca^{2+}-bound myristoylated recoverin and that the contribution of the hydrophobic cluster is minimal in this process. This is in agreement with the observation of Lange and Koch (1997) by surface plasmon resonance spectroscopy, where no binding was measured between nonmyristoylated recoverin and phospholipid bilayers in the absence and presence of Ca^{2+}. Consequently, contributions from hydrophobic residues and basic residues in the N-terminal or from the basic residues in the C-terminal region (six lysines and two glutamates are located between K_{192} and L_{202}) are not involved in the membrane binding of recoverin. However, we cannot entirely exclude a possible contribution from surface residues within the signal noise (10 pN), but one can assume that this effect is minimal when compared to the strength of binding of the myristoyl group or to the contribution of basic clusters of other proteins. For example, it has been shown that the presence of six basic residues in the N-terminal region following the myristoyl group (G_2-R_{15}, net charge = +5, myristoyl electrostatic switch) of the Src, the product of the *v-Src* oncogene of Rous sarcoma virus, enhances its binding to membranes containing acidic lipids by nearly 3000-fold compared to nonmyristoylated Src (Buser et al., 1994; Sigal et al., 1994). The same region of recoverin has a net charge of -1, which is consistent with the conclusion that the myristoyl group is much more important than electrostatic

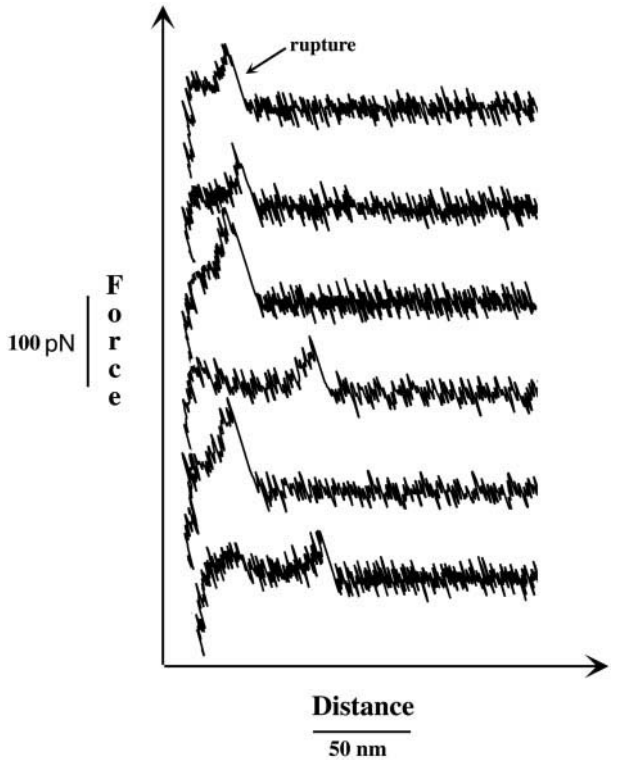

FIGURE 4 Typical retract force curves recorded on the DPPC bilayer with a myristoylated recoverin-functionalized tip in the presence of Ca^{2+}.

interactions for membrane binding of recoverin. In short, our results revealed that only the Ca^{2+}-myristoyl switch of recoverin is involved in membrane binding with no electrostatic and hydrophobic contributions by residues at the surface of the protein.

In addition, several control experiments were performed to exclude possible contributions from the amylose grafting material or Ca^{2+} ions to the rupture force measured. Indeed,

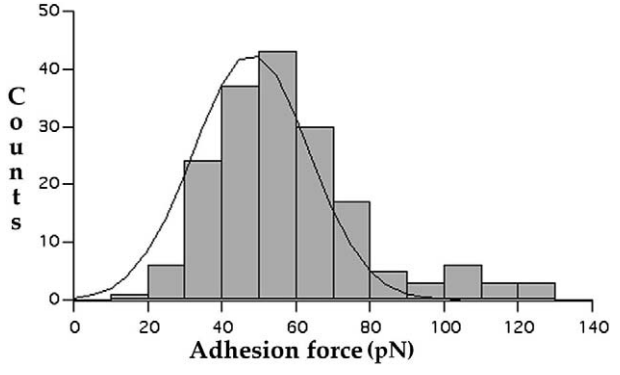

FIGURE 5 Histogram of the rupture forces between a myristoylated recoverin-functionalized tip and a DPPC bilayer in the presence of calcium at a loading rate of 500 pN/s. The line is a Gaussian fit used to determine the mean rupture force (±SD). All data were from force-distance curves recorded in buffer D and at room temperature. For clarity, zero force adhesion events were omitted from the histogram.

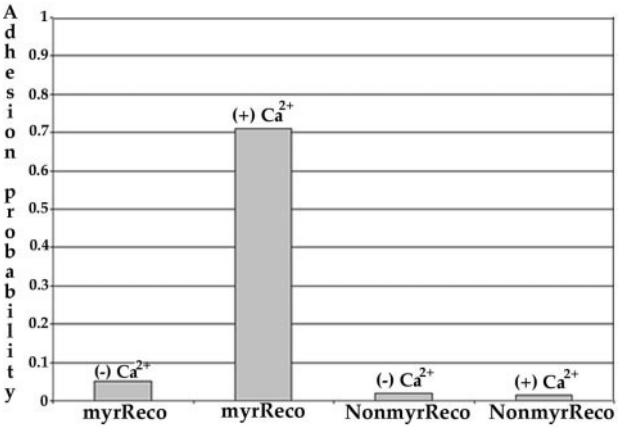

FIGURE 6 Effect of Ca^{2+} on the adhesion probability of myristoylated and nonmyristoylated recoverin-functionalized tips with a DPPC bilayer. The adhesion probability is the probability for observing an adhesion event in an approach-retract cycle. MyrReco, myristoylated recoverin; NonmyrReco, nonmyristoylated recoverin.

no binding was observed between an AFM tip functionalized only with amylose spacers and a DPPC bilayer in the absence and presence of Ca^{2+}. These results indicate that the amylose grafting material does not contribute to the rupture force measured for myristoylated recoverin in the presence of Ca^{2+}.

The control experiments and the Ca^{2+}-dependence of the adhesion between myristoylated recoverin and lipid bilayers have confirmed the specificity of our measurements. Consequently, it is clear that the rupture force measured in the presence of Ca^{2+} is due to the interaction between the myristoyl moiety of recoverin and the phospholipid bilayer. In other words, our results suggest that the rupture forces recorded are a direct measurement of hydrophobic interactions. This conclusion is further supported by previous reports, which have shown by electrophoretic mobility and equilibrium dialysis measurements that the binding energy of fatty acids and acylated peptides to phospholipid vesicles increases linearly with the number of carbons of the acyl chain (Peitzsch and McLaughlin, 1993). These previous results are in good agreement with the data of Tanford (1980) for the partitioning of hydrocarbons between water and a bulk alcane phase, indicating that the membrane-binding energy measured by Peitzsch and McLaughlin (1993) is due to the classical hydrophobic effect. In addition, Pool and Thompson (1998) have shown for an acylated protein (bovine pancreatic trypsin inhibitor) that its membrane-binding energy increases linearly with the acyl chain length.

The mean rupture force we have measured (48 ± 5 pN, Fig. 5) from the last jump of the adhesion peak is thus a direct measure of the hydrophobic interaction of individual or multiple myristoyls with the bilayer. Few studies have reported values of adhesion forces or extraction forces of

lipids measured using biomembrane force probe (BFP) and surface force apparatus (SFA). First, a BFP decorated with streptavidin was used by Evans and Ludwig (2000) to attach, and then to extract, biotin-PEG-distearoylphosphatidylethanolamine (biotin-PEG-DSPE) present at extremely low concentration in mixed giant vesicles with DSPE. They obtained values of rupture forces between 10 and 60 pN that varied linearly over a range of loading rates from 2 pN/s to 25,000 pN/s. At the loading rate that we have used (500 pN/s), they have measured a rupture force of ~23 pN. In a similar experiment they measured a force of 19 pN at 500 pN/s for the extraction of a biotin-PEG-dimyristoylphosphatidylethanolamine (biotin-PEG-DMPE) from mixed giant vesicles containing stearoyloleoylphosphatidylcholine and cholesterol (Ludwig and Evans, 2000). Those results suggest that the mean rupture force measured in our experiments with myristoylated recoverin is due to the interaction of no more than a few myristoyl groups with the DPPC bilayer. Leckband et al. (1995) using SFA have calculated, from adhesion energy measurements, a much larger rupture force of 70 pN for a DPPC molecule, assuming a bond length of 2.0 nm and a constant adhesion force along the unbinding path. Marrink et al. (1998) have shown by nonequilibrium molecular dynamic (MD) simulations that the adhesion force is not constant along the unbinding path. They recalculated a rupture force assuming a linear increase of the rupture force up to the rupture point and obtained a value of 140 pN for the SFA experiment of Leckband et al. (1995). Moreover, their MD simulations of the extraction of a DPPC molecule revealed a force stronger than 200 pN even under the slowest pull rate used. According to Marrink et al. (1998), at the lowest pull rates, the lipid has enough time to find an energetically favorable conformation during the extraction process that would reduce friction forces. Taking into account the results of Leckband et al. (1995) and Marrink et al. (1998), it is likely that a binding interaction between a single myristoyl group and the phospholipid bilayer was probed in our experiments. In this regard, it is interesting to compare the force we have measured with energy estimates for the binding of a myristoyl to a phospholipid membrane. Because energy = force × distance, we can estimate the distance over which the myristoyl group is pulled before its extraction from the phospholipid membrane. Using a $\Delta G = -8 \times 10^{-20}$ J/molecule (Peitzsch and McLaughlin, 1993; Tanford, 1980) and the mean rupture force we have measured (48 pN), we calculated a pulling distance of 1.7 nm, which is very consistent with the length of a myristoyl tail (1.8 nm) calculated from x-ray data (Petrov et al., 1999; Kjaer et al., 1989).

Our results obtained with recoverin and phospholipid-supported membranes clearly establish AFM-based force spectroscopy as a prime tool that can be used for the characterization of myristoyl switches and acylated proteins. Recently, Ames et al. (2000) have shown that Frq1, a novel calcium sensor protein in the yeast *Saccharomyces cerevisiae*, has an overall structure very similar to recoverin. However, its myristoyl group becomes solvent-exposed in a Ca^{2+}-free state in contrast to the Ca^{2+}-free state of recoverin, where its myristoyl group is buried in the protein. Moreover, they found that the nonmyristoylated Frq1 associates with the membrane in the presence, but not in the absence, of Ca^{2+}, thus indicating a contribution of nonpolar side chains in the Ca^{2+}-bound state. In addition, many other acylated proteins could be studied by force spectroscopy in the same way as performed for recoverin. Very important data on hydrophobic contributions from both the non-polar residues and the acyl group of these proteins could be obtained by force spectroscopy using an AFM-functionalized tip with covalently bound proteins via a spacer, and a substrate with a good surface coverage, such as a DPPC bilayer on mica, as performed in the present study.

CONCLUSIONS

In this study we have extended the use of AFM-based force spectroscopy to quantify the strength of binding of a myristoyl group with membranes and to further understand the effect of conformational changes of recoverin on membrane binding. We have measured the adhesion of myristoylated recoverin and a phospholipid bilayer in the absence and presence of Ca^{2+}. Control experiments with the amylose spacer and the Ca^{2+}-dependence of the adhesion observed have demonstrated the specificity of our measurements. In addition, no adhesion was observed for nonmyristoylated recoverin in the absence and presence of Ca^{2+}. These results indicate that the myristoyl group alone is responsible for the membrane binding of recoverin. Moreover, the adhesion force measured for the myristoyl moiety of recoverin is consistent with previously measured extraction forces of lipids with membranes. Our work shows that force spectroscopy can be used to quantify the contribution of protein acylation to membrane binding of other proteins and to study the effect of length and unsaturation of acyl chains on hydrophobic interactions by overexpressing recoverin or other proteins with acyl chains of different lengths and unsaturation. The effect of unsaturation of the acyl chain and of the physical state of the phospholipid bilayer are currently under investigation.

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