Single Molecule Recognition of Protein Binding Epitopes in Brush Border Membranes by Force Microscopy

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ABSTRACT Sidedness and accessibility of protein epitopes in intact brush border membrane vesicles were analyzed by detecting single molecule interaction forces using molecular recognition force microscopy in aqueous physiological solutions. Frequent antibody-antigen recognition events were observed with a force microscopy tip carrying an antibody directed against the periplasmically located γ-glutamyltrans- peptidase, suggesting a right side out orientation of the vesicles. Phlorizin attached to the tips bound to NA^+/D-glucose cotransporter molecules present in the vesicles. The recognition was sodium dependent and inhibited by free phlorizin and D-glucose, and revealed an apparent K_D of 0.2 μM. Binding events were also observed with an antibody directed against the epitope aa603-aa630 close to the C terminus of the transporter. In the presence of phlorizin the probability of antibody binding was reduced but the most probable unbinding force f_u = 100 pN remained unchanged. In the presence of D-glucose and sodium, however, both the binding probability and the most probable binding force (f_u = 50 pN) were lower than in its absence. These studies demonstrate that molecular recognition force microscopy is a versatile tool to probe orientation and conformational changes of epitopes of membrane components during binding and *trans*-membrane transport.

INTRODUCTION

Sodium-solute symport systems represent one of the prototypes of secondary active transport systems for organic and inorganic solutes that are used by cells to accumulate nutrients. Despite their general biological importance and the cloning from various organisms and tissues, our understanding of the structural events accompanying the *trans*-membrane movement of the transportates is rather limited. This is predominantly due to the fact that thus far only a few membrane transport systems have been amenable to protein crystallography in their phospholipid environment. In addition, the transport systems can be expected to assume different conformations during their catalytic cycles, thus making the crystallization and analysis of single states very difficult. The same situation prevails with the sodium glucose cotransporter (SGLT) family where topological assignment of epitopes are still a matter of controversy, and conformational changes have either been deduced intuitively or demonstrated after chemical modification of the molecule (Koepsell and Spangenberg, 1994; Lin et al., 1999; Turk et al., 1996).

In the present study we applied atomic force microscopy (AFM) (Binnig et al., 1986) as a dynamic molecular approach in aqueous physiological solutions (Drake et al., 1989; Müller et al., 1995; Shao and Yang, 1995) to detect molecular recognition of binding epitopes at different environmental conditions. Specific receptor-ligand interaction forces (Lee et al., 1994; Florin et al., 1994; Hinterdorfer et al., 1996) on cell membranes (Lehenkari and Horton, 1999; Chen and Moy, 2000) were measured on the single molecule level. These forces are dependent on the loading rate of the experiment (Evans and Ritchie, 1997; Grubmüller et al., 1996; Fritz et al., 1998; Merkel et al., 1999; Kienberger et al., 2000; Baumgartner et al., 2000a; Schwesinger et al., 2000) and give insight into the molecular dynamics of the recognition process. An antibody or a ligand was coupled to an AFM tip via a flexible cross-linker (Hinterdorfer et al., 1996, 1998; Willemsen et al. 1998; Raab et al., 1999; Haselgrübler et al., 1995) for the molecular recognition of enzymes and transporters embedded in brush border membrane vesicles. Unbinding forces, i.e., pulling forces required to disrupt single receptor-ligand bonds in dynamic experiments, were measured with anti-γ-glutamyltranspeptidase antibody, phlorizin and anti-SGLT antibody, respectively, bound to the atomic force microscopy tip. Their values were compared at one fixed loading rate. Kinetic rates k_{on}, k_{off}, and the dissociation constant K_D were estimated from these data. The results obtained in this study provide information about membrane sidedness of epitopes of the SGLT and about structural changes of the binding pocket during interaction with the substrate D-glucose and the inhibitor phlorizin.

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This paper is dedicated to the memory of Hansgeorg Schindler (died 28 August 2001).

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MATERIALS AND METHODS

Production of antibodies

Epitope specific antibodies (AB) against SGLT (anti-SGLT-AB) were produced as described previously (Lin et al., 1999), anti-γ-glutamyl-

transpeptidase antibodies (anti-γ-GT-AB) were made by using γ-glutamyltranspeptidase isolated from bovine kidney as antigen (Biotrend, Köln, Germany).

Conjugation of antibodies and ligands to tips

Conjugation of AB, anti-SGLT-AB, and anti-γ-GT-AB, both immunopurified antibodies directed against membrane proteins of the brush border membrane, to AFM-tips via a flexible cross-linker was done as described before (Hinterdorfer et al., 1996, 1998). Phlorizin as competitive inhibitor of the SGLT protein was coupled to the cross-linker on AFM tips following the same protocol. For this, 3-iodoacetamido-phlorizin was prepared similar to 3-bromoacetamido-phlorizin (Lin et al., 1982) starting from phlorizin (Sigma, Deisenhofen, Germany). Cross-linker conjugated AFM tips were incubated with 1 mM dithiothreitol in pH 4.5 buffer to reduce the disulfide of the 2-pyridyldithiopropionyl residue-group. Immediately after washing with buffer, tips were immersed in 500 μL of a 4 mM 3-iodoacetamido-phlorizin solution in 0.1 M NaH_2PO_4 buffer (pH 7.4) for 1 h at room temperature. After subsequent washing in buffer, tips were stored at 4°C.

Preparation of samples

Brush border membrane vesicles (BBMV) were prepared from rabbit kidney cortex using the Ca^{2+} precipitation method described previously (Lin et al., 1981), resulting in tightly sealed right side out oriented membrane fragments (Haase et al., 1978). The membrane protein was determined according to Lowry et al. (1951). BBMV were deglycosylated by overnight incubation with N-glycosidase F (Boehringer, Mannheim, Germany) at 20°C. For atomic force microscopy studies 10 μL of a 0.1 mg/mL BBMV suspension in 100 μL hypotonic buffer (10 mM HEPES/Tris, 5 mM EGTA, 0.01% thimerosal, pH 7.4), KCl-buffer (120 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM $KHCO_3$, pH 7.4) or NaCl-buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 7.4) was incubated on freshly cleaned gold surfaces for 30 min. Then gold plates were washed before the probe was immediately inserted into a fluid cell and imaged with the atomic force microscope.

Atomic force microscopy

We used a Macmode PicoSPM magnetically driven dynamic force microscope (Molecular Imaging, Phoenix, AZ) with a Molecular Imaging Macmode interface. Topography images (Fig. 1) were recorded using the magnetic AC mode (Han et al., 1996, 1997a,b; Raab et al., 1999) with antibody- or ligand-conjugated MacLevers (Molecular Imaging) of 0.1 N/m nominal spring constant. Measurements were performed with 5-nm free tip oscillation amplitude at 5 kHz driving frequency and 20% amplitude reduction. The small free cantilever oscillation amplitude of 5 nm provides a most gentle tip-surface interaction that minimizes the compression of the soft vesicle structures on the surface induced by the force applied from the scanning tip. Single vesicular structures (see Fig. 1) of 150 to 300 nm in diameter and 30 to 100 nm in height were clearly resolved. No deterioration or disassembling of the structures during the imaging process was observed, which indicates that the vesicles were tightly bound to the surface.

For the detection of antibody-antigen or ligand-antigen recognition, force distance cycles were performed with MacLevers of 0.1 N/m nominal spring constant in the conventional contact force calibration plot mode. At lateral positions where brush border membrane vesicles were identified in the topography imaging mode (Fig. 1), the *x-y* scan was stopped, and force distance cycles were recorded. An AFM tip carrying a ligand or antibody was first approached (trace) and then retracted (retrace) from a BBMV, and the deflection angle of the cantilever was measured as a function of the *z* position. The deflection is directly proportional to the interaction force according to $f = k\Delta z$ (k is the spring constant of the cantilever; Δz is the deflection up (+) or down (−) of the cantilever). The sweep-amplitude was 60 nm at a sweep duration of 1s. For quantification of forces, spring constants of cantilevers were determined with the thermal noise method. Analysis of force distance cycles was performed using the Matlab Version 4.2c.1 (Math Works Inc., Natick, MA) as described previously (Baumgartner et al., 2000b).

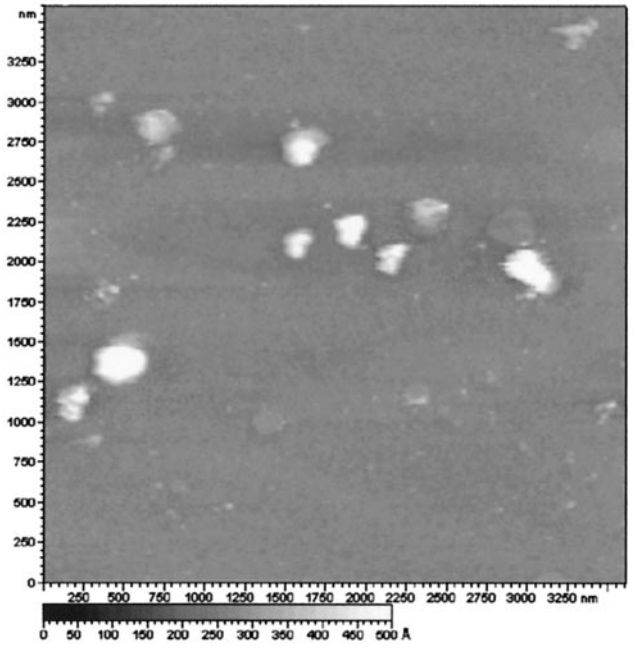

FIGURE 1 Topography image of brush border membrane vesicles. BBMV were adsorbed a to a gold surface and imaged in the Macmode at 3.5-μm scan size. Singly distributed vesicular structures are clearly resolved. They appear 150 to 300 nm in diameter and up to 100 nm in height.

RESULTS AND DISCUSSION

Recognition of γ-GT on the brush border membrane surface by antibodies

The high sensitivity of the AFM in force detection was used to measure the interaction force of a single receptor-ligand bond (Lee et al., 1994; Florin et al., 1994; Hinterdorfer et al., 1996). Force distance cycles with an AFM tip carrying an anti-γ-glutamyltrans-peptidase antibody directed against the γ-glutamyltranspeptidase on BBMV surfaces were recorded. Upon tip-surface approach (Fig. 2 *A*), the deflection angle remains zero (*z* position 50−0 nm) until the tip contacts the surface (at *z* position 0) from where a repulsive force develops that increases with further down movement of the tip (0 to −10 nm). Subsequent retraction of the tip from the surface (retrace) leads to relaxation of the repulsive force in the contact region (−10 to 0 nm). If the antibody on the tip has bound to an antigen on the surface an attractive force develops during further retraction (0–18 nm) because the tip is physically connected to the surface by the antibody-antigen bond. The attractive force develops in a delayed

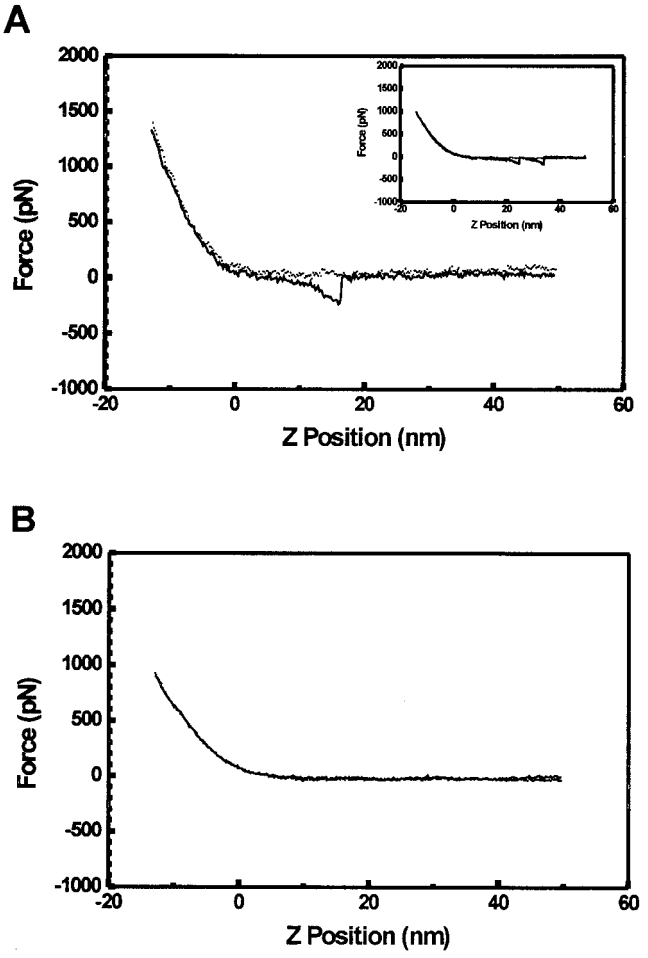

FIGURE 2 Force-distance cycle with an antibody containing tip on a brush border membrane vesicle membrane. (*A*) Single antibody-antigen recognition event. An anti-γ-GT antibody conjugated tip was moved toward a brush border membrane vesicle surface (trace, *dotted line*) and subsequently retracted (retrace, *solid line*) at a constant lateral position. During tip approach the antibody on the tip binds to an antigen embedded in the vesicle membrane surface. This leads to formation of a single antibody-antigen complex. Upon contact ($z = 0$ nm), a repulsive force develops ($z = 0$ to -10 nm), which reversibly relaxes ($z = -10$ to 0 nm) during subsequent tip retraction (retrace). The attractive force that develops in the retrace ($z = 0$ to 18 nm) arises from the antibody-antigen bond connecting tip and surface (recognition event). Further retraction finally leads to antibody-antigen dissociation ($z = 18$ nm) and the tip jumps back into the resting position. The *z* amplitude of the shown force-distance cycle was 60 nm at a sweep duration of 1 s. (*Inset*) Force-distance cycle consisting of two recognition events. They are clearly separated in space and time. (*B*) Control. In the presence of 1 μM anti-γ-GT antibody in solution the recognition signal disappears and retrace looks like trace.

and nonlinear fashion, reflecting the stretching and the nonlinear spring constant of the flexible cross-linker by which the antibody is covalently coupled to the AFM tip. This signal is of particular characteristic shape and reflects the signature of the recognition event of a single antibody-antigen pair (Hinterdorfer et al., 1996, 1998). At a distinct critical force, termed as unbinding force f_u (*z* position of ~18 nm), the antibody on the tip dissociates from the antigen on the surface and the cantilever jumps back to zero deflection. The signal shown (Fig. 2 *A*) has $f_u = 235 \pm 10$ pN. The accuracy of force quantification of a few pN (Hinterdorfer et al., 1996) is thereby determined by the thermal noise of the cantilever (Baumgartner et al., 2000b). Force distance cycles containing two or more recognition events were very rarely (<1%) observed (inset Fig. 2 *A*) because of the low density of antibodies on the AFM tip. These multiple events are always well separated in space and time, according to the different positions of the antibodies on the tip (Hinterdorfer et al., 1998). For data analysis, only curves containing single recognition events were considered.

At the conditions used, approximately every second force distance cycle showed an unbinding event. That these events arise from the specific interaction between the anti-γ-GT antibody on the tip and the γ-GT in the brush border membrane vesicles was concluded from the strong inhibition (>90%) of recognition events (see Fig. 2 *B* as a typical example) after addition of free anti-γ-GT antibody in solution. Recognition events disappear because free anti-γ-GT antibodies bind to the γ-GT on the membrane surface and block recognition by the anti-γ-GT antibody on the tip. The specificity of the events was further supported by the block wash-out experiment shown in Fig. 3 *A*, obtained from hundreds of force distance cycles. The binding probability, which is the probability to detect a recognition event in a force-distance cycle or not, was determined at different experimental situations. In pure buffer, the anti-γ-glutamyltranspeptidase antibody on the tip bound to γ-glutamyltranspeptidase in brush border membranes with a probability of ~0.5 (Fig. 3 *A*, 0–15 min), which is a typical value also observed for other receptor-ligand combinations (Hinterdorfer et al., 1996, 1998; Willemsen et al. 1998; Baumgartner et al., 2000b; Kienberger et al., 2000). Addition of free anti-γ-GT antibody in solution almost completely abolished binding (Fig. 3 *A*, 15–50 min). Apparently, anti-γ-GT antibodies blocked the γ-GT binding sites on the BBMV and, thus, prevented recognition by anti-γ-GT on the AFM tip. When anti-γ-GT antibodies were subsequently washed out with buffer the binding probability increased again back to the initial value of 0.5 (Fig. 3 *A*, 50–120 min) because the anti-γ-GT antibodies dissociated from γ-GT on BBMV. This clearly demonstrates the specificity and reversibility of the antibody-antigen reaction. The interaction strength of γ-GT/anti-γ-GT was quantified by constructing an empirical probability density function of the unbinding force distribution (Hinterdorfer et al., 1996; Baumgartner et al., 2000b) (Fig. 3 *B*). The maximum of the probability density function, $f_u = 131 \pm 44$ pN, reflects the most probable unbinding force, i.e., the force required to dissociate a single receptor bond. That this value is approximately one-half of the pulling force reported to extract single helix pairs out of cell membranes (Oesterhelt et al., 2000) additionally supports the specificity of the observed antibody-antigen unbinding events.

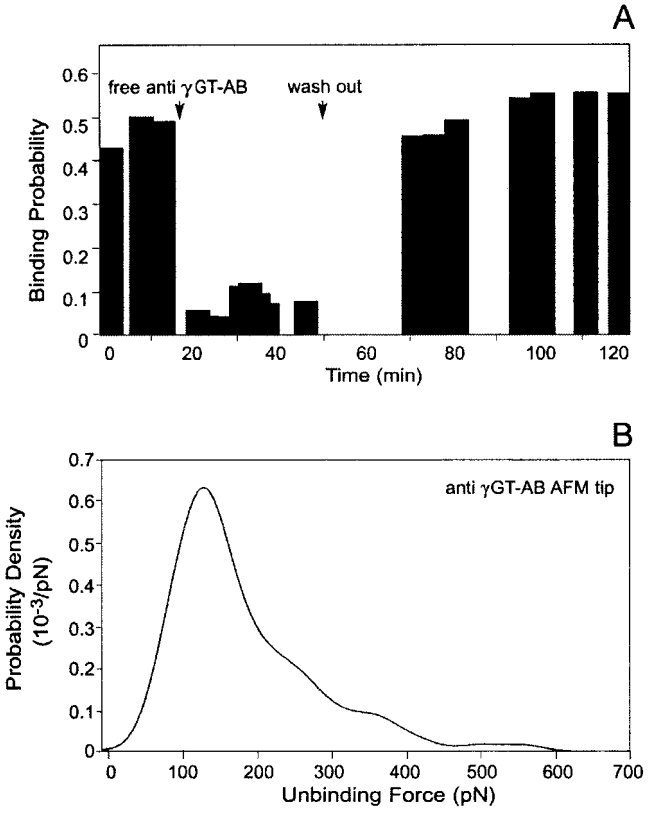

FIGURE 3 Recognition of γ-glutamyl transferase (GT) in BBMV by anti-γ-GT antibody. (*A*) Binding probability. Binding of anti-γ-GT-Ab on the AFM tip to γ-GT in BBMV probed in force-distance cycles occurred with a probability of 0.47 (0–15 min). Injection of 1 μM anti-γ-GT-AB in solution resulted in a dramatic decrease of the binding probability. Upon anti-γ-GT-AB wash out with buffer binding is fully recovered after 30 min. (*B*) Distribution of unbinding forces. The empirical probability density function of unbinding forces detected in force-distance cycles shows a maximum at 130 pN and a width σ of 44 pN.

Apparently, specific recognition of the peripheral membrane protein γ-glutamyltranspeptidase can be probed by an antibody on the AFM tip on the single molecule level. Because γ-glutamyltranspeptidase is known to be exclusively located on the luminal surface of the brush border membrane (Kenny and Booth, 1976) the orientation of the BBMV on the gold surface must be such that the former luminal side faces the aqueous phase and the AFM tip. The right side out orientation observed for the membrane preparation used in our studies had already been proposed from freeze fracture studies (Haase et al., 1978). The binding events observed show that a periplasmatically localized enzyme can be reached by the antibodies on the AFM tip.

Recognition of Na^+/D-glucose cotransporters by phlorizin

The question whether also membrane embedded transporters can be analyzed by molecular recognition atomic force microscopy was tested using a cantilever, which carried a phlorizin molecule attached to it. Phlorhizin is known to bind to the sodium-D-glucose transporter with a high affinity and to competitively inhibit D-glucose transport (Diedrich, 1963; Kinne, 1976). To ensure maximal interaction between the phlorizin ligand and the transport molecule 3-iodoacetamidophlorhizin was used, which carries the reactive group at the B ring of the molecule, which according to recent pharmacophore investigations is only of minor importance for the interaction with the transporter (Wielert-Badt et al., 2000).

AFM tips containing phlorizin showed recognition events in force-distance cycles on brush border membrane vesicles similar to the ones shown in Fig. 2 *A*. Apparently, the phlorizin on the tip recognizes SGLT, which is embedded as *trans*-membrane protein in BBMV. The specificity of binding is demonstrated in the block experiment of Fig. 4 *A*. The initial binding probability of 0.6 in pure NaCl buffer (Fig. 4 *A*, 0–25 min) is effectively reduced upon addition of phlorizin in solution (Fig. 4 *A*, 25–65 min) and fully recovered after wash out with buffer (Fig. 4 *A*, 65–120). Therefore, the phlorizin binding epitope on SGLT is freely accessible to the phlorizin on the AFM tip and consequently located on the luminal side of the brush border membrane as well.

Binding of phlorizin to SGLT in BBMV is also strongly diminished in the presence of D-glucose (Fig. 4 *B*). As expected (Frasch et al., 1970), D-glucose competes with phlorizin for the binding site at the SGLT and blocks therefore recognition by phlorizin on the tip. This result provides evidence that the Na^+/D-glucose cotransporters in the surface of adsorbed brush border membrane vesicles are still functionally active. Recognition events detected in pure buffer were analyzed, and their unbinding forces were determined. The experimental probability density function constructed from these measurements (Fig. 4 *C*) reveals a most probable unbinding force f_u of 120 ± 44 pN for phlorizin/Na^+-D-glucose cotransporter unbinding.

Kinetic rates and equilibrium constant of phlorizin binding

The wash-out recovery time of phlorizin (Fig. 4 *A*) reflects the lifetime τ_0 of the phlorizin-SGLT bond at zero force and reveals the kinetic off rate k_{off} of receptor-ligand binding, according to $k_{off} = \tau_0^{-1}$. A measured value of ~20 min (Fig. 3 *A*) yields $k_{off} \sim 10^{-5}\ s^{-1}$. The kinetic on rates of receptor-ligand binding k_{on} can be estimated from the time $t_{0.5}$ the ligand on the tip has a chance to bind to the receptor on the surface with a binding probability of 0.5 during a force distance cycle and from the effective concentration c of the ligand on the tip, using $k_{on} = c^{-1}\, t_{0.5}^{-1}$ (Hinterdorfer et al., 1996, 1998). For our experimental conditions, $k_{on} \sim 5 \times 10^3\ M^{-1}\ s^{-1}$. Values for the equilibrium dissociation constants, estimated from k_{on} and k_{off}, yield $K_D \sim 0.2$ μM

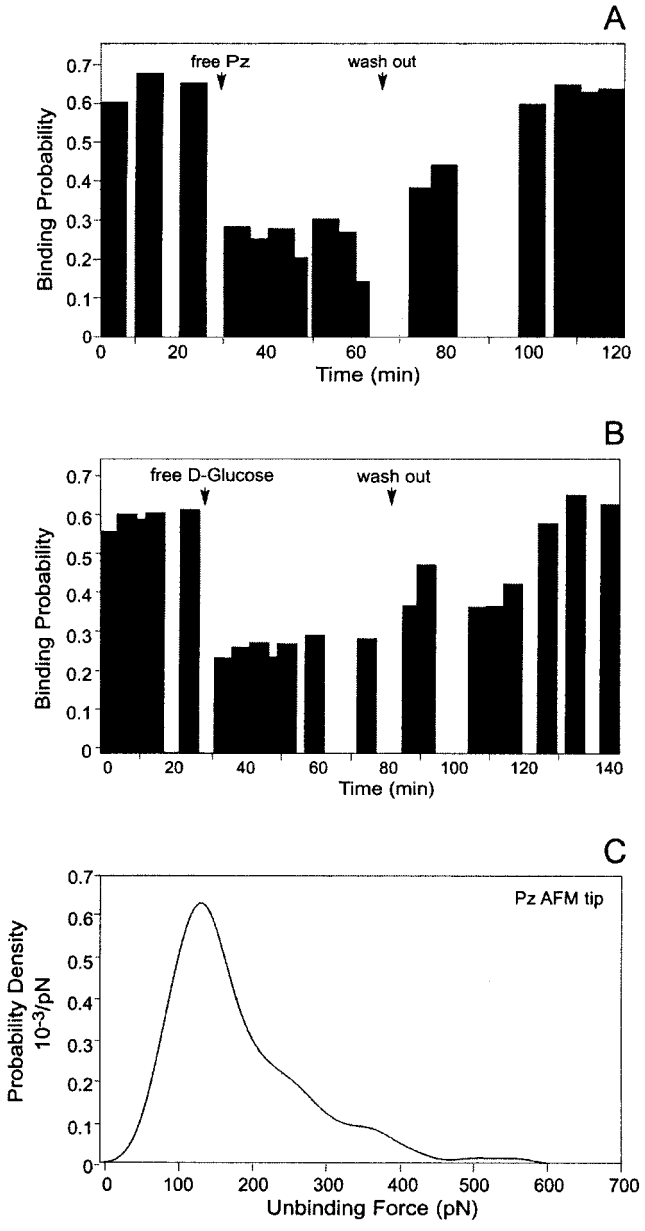

FIGURE 4 Ligand binding to Na^+/D-glucose cotransporters SGLT1 in BBMV. Recognition events detected in force-distance cycles on BBMV using AFM tips to which phlorizin is coupled yield binding probability and unbinding force of phlorizin-SGLT1 binding. The measurements were carried out in hypotonic buffer. (*A*) Block. The binding probability of 0.6 (0–25 min) decreases significantly after injection of 1 mM phlorizin in NaCl buffer (30–70 min). Wash out (min 70) results in full recovery of binding after an incubation time 30 of min. (*B*) Competitive inhibition. Addition of glucose in NaCl buffer (30–80 min) results in a reversible decrease of the binding probability compared with pure buffer min (0–30 min and from min 130). (*C*) Unbinding force. The probability density function of forces reveals a most probable unbinding force of 120 pN and a width σ of 44 pN.

for phlorizin/SGLT binding. The apparent affinity of the binding derived from the molecular recognition atomic force microscopy data is in the range observed for isolated plasma membranes in suspension (Kinne, 1976; Frasch et al., 1970).

A length of the binding pocket l_p can be calculated from the reduction of the receptor-ligand bond under an applied force τ_f (Hinterdorfer et al., 1996), using Bell's formula (Bell, 1978): $\tau_f = \tau_0 \exp(-l_p f/k_B T)$, in which τ_f and τ_0 denote the bond lifetime at force f and 0, respectively, k_B Boltzmann's constant, and T the absolute temperature. τ_f, estimated from the width of the force window (Hinterdorfer et al., 1996) yields $\tau_f \sim 5$ ms, which results in $l_p \sim 0.5$ nm. The size of the binding pocket calculated from our data is in agreement with other publications (Wielert-Badt et al., 2000).

Recognition of Na^+/D-glucose cotransporter by antibodies

Binding of antibodies to SGLT in BBMV was probed with antibody doped AFM tips. The antibody, PAN3, was raised against a peptide construct (A606) from the sequence aa606-aa630 of SGLT 1, which is part of a supposedly extra-membrane loop (Lin et al., 1999). Force-distance cycles contained frequent recognition events. Their probability of 0.5 (Fig. 5 *B*) decreased to almost 0 (not shown) upon injection of anti-SGLT antibodies, because the antibody binding epitopes of SGLT are occupied by the antibodies ligated from the solution. Reversibility of binding was demonstrated by the full recovery of the binding probability after antibody wash out. Recognition of SGLT in BBMV by the epitope specific antibody was also prevented by the peptide A606 in solution because of blockage of the antibody on the AFM tip. These results unequivocally demonstrate the specificity of PAN3 recognition.

The most probable unbinding force f_u and its width σ_f for SGLT-PAN3 antibody obtained from the empirical probability density function of Fig. 5 *B*, were 100 ± 47 pN. The width of the force distribution and the recovery time allowed for a first estimate (Hinterdorfer et al., 1996) of the affinity of SGLT-PAN3 antibody binding and yielded $K_D \sim 0.1$ μM. These results demonstrate that the loop of the SGLT against which the antibody is directed is oriented toward the outside of the membrane (as suggested by previous studies using the same antibody on CHO cells expressing SGTL 1 (Lin et al., 1999)) and that this region of the molecule was detected in a functionally active state by the anti-SGLT antibody.

Structural changes of the PAN3-antibody binding epitope of SGLT during function

The study with the epitope-specific PAN3-antibody was further expanded for the investigation of conformational changes of the binding epitope of SGLT during D-glucose translocation. For this, binding of the antibody on the AFM

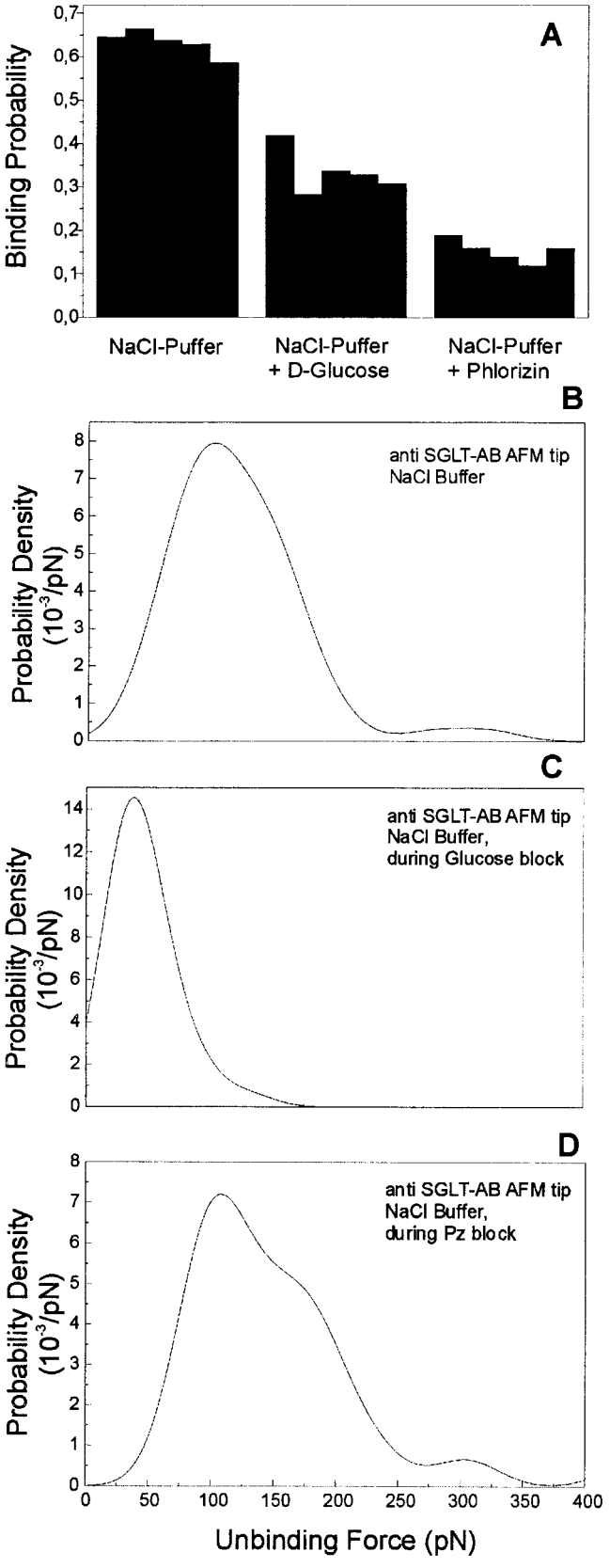

FIGURE 5 Influence of phlorizin and glucose on the binding of PAN-3 antibody to Na^+/D-glucose cotransporter SGLT1. (*A*) Binding probability. The probability for binding of PAN-3 antibody on the AFM tip to SGLT1 in BBMV is shown for three different situations. The value of 0.62 in pure NaCl buffer (see Materials and Methods) reduces to ~0.3 in the presence of 250 mM D-glucose and 0.15 in the presence 1 mM phlorizin, respectively. Each bar corresponds to 240 force distance cycles. (*B–D*) Unbinding force. The maximum of the probability density function of unbinding forces is at 98 pN in pure NaCl buffer (*B*), at 47 pN in NaCL buffer containing 250 mM D-glucose (*C*), and at 102 pN in NaCl buffer containing 1 mM phlorizin (*D*), respectively.

tip to Na^+/D-glucose cotransporters in brush border membrane vesicles was followed for three different conditions (Fig. 5). In pure NaCl buffer, SGLT is in the 1) nontransporting state, changing to the 2) transporting state in the presence of D-glucose in NaCl-buffer, and to the 3) fully blocked state upon addition of phlorizin.

The probability of SGLT-anti-SGLT antibody binding of 0.6 decreases to 0.3 when D-glucose is present in NaCl buffer (Fig. 5). In contrast, the binding probability remains unchanged (0.6) in a buffer containing D-glucose but lacking Na^+ (not shown). This confirms former findings that D-glucose requires Na^+ for the binding to the external surface of SGLT (Kinne, 1976). In addition, the presence of D-glucose in NaCl buffer does not only decrease the binding probability (Fig. 5 *A*), it also has an influence on the unbinding force (Fig. 5, *B* and *C*). Whereas the most probable unbinding force in pure NaCl buffer is ~100 pN (Fig. 5 *B*), this value changes to ~50 pN when D-glucose is added (Fig. 5 *C*). Apparently, D-glucose binding to SGLT influences antibody recognition in two ways; it either 1) completely blocks recognition (reduction of binding probability) or alternatively 2) decreases the interaction force of the remaining binding events from 100 to 50 pN. Because the binding probability in the absence of glucose is approximately twice as high as its presence the latter two observed phenomena occur with approximately the same frequency.

The presence of phlorizin (a nontransported β-glucoside that occupies the glucose binding site of the transporter) blocks recognition of SGLT by anti-SGLT antibody in a different way. The binding probability is even more decreased (0.15, compare Fig. 5 *A*), whereas the force distribution of the few remaining recognition events (Fig. 5 *D*) is similar to the force distribution obtained in pure NaCl-buffer (Fig. 5 *B*) with its maximum at ~100 pN. Thus, the residual antibody binding activity arises from an incomplete block, most likely caused by the dynamics of phlorizin binding and release from SGLT.

These results altogether strongly suggest that the PAN3 binding epitope of SGLT was caught in at least three different configurations. 1) Binding of anti-SGLT antibody to SGLT in pure NaCl buffer with the observed interaction force of 100 pN is considered as undisturbed SGLT-anti-SGLT antibody recognition. 2) The almost complete block by phlorizin indicates that the binding epitope of SGLT is not accessible for the antibody when phlorizin is bound. The

decrease in binding probability without any change in the unbinding force of the remaining recognition events probably indicates an all or none change in the accessibility of the PAN3-epitope when the carrier is occupied by phlorizin. As shown in several studies including the current one phlorizin only interacts with high affinity with the outside of the transporter (Koepsell and Madrala, 1987; Firnges, 2000; Panayotova-Heiermann et al., 1997). Thus, the inaccessibility of the epitope at the C terminus by PAN3 antibody might be the hallmark of the state in which the D-glucose-binding site facing the extracellular medium is occupied.

A similar performance of the epitope was also observed as one of the two effects during D-glucose binding by SGLT. However, 3) alternatively to complete block, the presence of D-glucose can also lead to a reduction of the SGLT/anti-SGLT antibody interaction force from 100 to 50 pN. This reduction indicates a structural or conformational change in the PAN3-epitope itself and can be most easily attributed to the fact that D-glucose is translocated across the membrane and thus is able to interact with the transporter both at the periplasmic and the cytoplasmic face. The blocked state compares with the situation where the D-glucose binding site is occupied from outside, whereas the conformation with the lower interaction force to the antibody might reflect a state where D-glucose is associated with the transporter at the inside of the vesicle. Further studies taking into account the different sodium affinities and substrate specificity at this side of the membrane are (Firnges, 2000; Panayotova-Heiermann et al., 1997), however, necessary to further substantiate the latter assumption.

CONCLUSION

The current studies extend molecular recognition force microscopy to its application to transport-capable membrane vesicles. Such vesicles have been very useful in determining the driving forces and kinetics of membrane transport systems under conditions that closely mimic the events occurring in the intact cell with a proper control of the *cis* and *trans* compartment of the plasma membrane (Kinne, 1991). To allow conclusions on the topology of the events studied in vesicles the knowledge of their orientation is a prerequisite. Initial studies using a cantilever carrying an antibody against the γ-glutamyltranspeptidase, an enzyme known to be located on the surface of the cell and thus the periplasmic side of the brush border membrane (Kenny and Booth, 1976), clearly showed specific binding events on BBMVs, indicating the right side out orientation of the membrane preparation.

Binding epitopes of the *trans*-membrane Na^+/D-glucose cotransporter SGLT1 were mapped using PAN3, an antibody specifically directed against a peptide region close to the C terminus, and phlorizin, a competitive high affinity inhibitor of D-glucose, as ligands on the AFM tip, respectively. It is noteworthy in this context that mutagenesis studies locate the site of the transporter responsible for D-glucose transport and or binding also close to the C terminus (Sauer et al., 2000). In the presence of D-glucose, a reduction of the binding probability for both PAN3 and phlorizin was found, suggesting that this reduction is due to the occupancy of the D-glucose binding site. In addition, a decrease in the unbinding force was observed for PAN3, indicating that the PAN3 binding epitope of SGLT undergoes conformational changes during D-glucose translocation. Molecular recognition atomic force microscopy appears to be very suitable to distinguish between various states of transporters in their natural lipid environment when performing *trans*-membrane transport.

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REFERENCES

Baumgartner, W., P. Hinterdorfer, W. Ness, A. Raab, D. Vestweber, H. Schindler, and D. Drenckhahn. 2000a. Cadherin interaction probed by atomic force microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 97:4005–4010.

Baumgartner, W., P. Hinterdorfer, and H. Schindler. 2000b. Data analysis of interaction forces measured with the atomic force microscope. *Ultramicroscopy.* 82:85–95.

Bell, G. I. 1978. Models for the specific adhesion of cells to cells. *Science.* 200:618–627.

Binnig, G., C. F. Quate, and Ch. Gerber. 1986. Atomic force microscope. *Phys. Rev. Lett.* 56:930–933.

Chen, A., and V. T. Moy. 2000. Cross-linking of cell surface receptors enhances cooperativity of molecular adhesion. *Biophys. J.* 78: 2814–2820.

Diedrich, D. F. 1963. The comparative effects of some phlorizin analogs on the renal reabsorption of glucose. *Biochim. Biophys. Acta.* 71:688–700.

Drake, B., C. B. Prater, A. L. Weisenhorn, S. A. C. Gould, T. R. Albrecht, C. F. Quate, D. S. Cannell, H. G. Hansma, and P. K. Hansma. 1989. Imaging crystals, polymers, and processes in water with the atomic force microscope. *Science.* 243:1586–1589.

Evans, E., and K. Ritchie. 1997. Dynamic strength of molecular adhesion bonds. *Biophys. J.* 72:1541–1555.

Firnges, M. A. 2000. Inaugural-Dissertation zur Erlangung des Doktorgrades im Fachbereich Chemie der Mathematisch-Naturwissenschaftlichen Fakultät der Westfälischen. Wilhelms-Universität, Münster. 1–99.

Florin, E. L., V. T. Moy, and H. E. Gaub. 1994. Adhesion forces between individual ligand receptor pairs. *Science.* 264:415–417.

Frasch, W., P. P. Frohnert, F. Bode, K. Baumann, and R. Kinne. 1970. Competitive inhibition of phlorizin binding by D-glucose and the influence of sodium: a study on isolated brush border membrane of rat kidney. *Pflügers Arch.* 320:265–284.

Fritz, J., A. G. Katopidis, F. Kolbinger, and D. Anselmetti. 1998. Force-mediated kinetics of single P-selectin/ligand complexes observed by atomic force microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 95:12283–12288.

Grubmüller, H., B. Heymann, and B. Tavan. 1996. Ligand binding and molecular mechanics calculation of the streptavidin-biotin rupture force. *Science.* 271:997–999.

Haase, W., A. Schäfer, H. Murer, and R. Kinne. 1978. Studies on the orientation of brush-border membrane vesicles. *Biochem. J.* 172:57–62.

Han, W., M. Dlakic, Y. J. Zhu, S. M. Lindsay, and R. E. Harrington. 1997b. Strained DNA is kinked by low concentrations of Zn^{2+}. *Proc. Natl. Acad. Sci. U.S.A.* 94:10565–10570.

Han, W., S. M. Lindsay, M. Dlakic, and R. E. Harrington. 1997a. Kinked DNA. *Nature.* 386:563.

Han, W., S. M. Lindsay, and T. Jing. 1996. A magnetically driven oscillating probe microscope for operation in liquid. *Appl. Phys. Lett.* 69:1–3.

Haselgrübler, Th., A. Amerstorfer, H. Schindler, and H. J. Gruber. 1995. Synthesis and applications of a new poly(ethylene glycol) derivative for the crosslinking of amines with thiols. *Bioconjugate Chem.* 6:242–248.

Hinterdorfer, P., W. Baumgartner, H. J. Gruber, K. Schilcher, and H. Schindler. 1996. Detection and localization of individual antibody-antigen recognition events by atomic force microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 93:3477–3481.

Hinterdorfer, P., K. Schilcher, W. Baumgartner, H. J. Gruber, and H. Schindler. 1998. A mechanistic study of the dissociation of individual antibody-antigen pairs by atomic force microscopy. *Nanobiology.* 4:39–50.

Kenny, A. J., and A. G. Booth. 1976. Organization of the kidney proximal-tubule plasma membrane. *Biochem. Soc. Trans.* 4:1011–1017.

Kienberger, F., G. Kada, H. J. Gruber, V. Ph. Pastushenko, C. Riener, M. Trieb, H.-G. Knaus, H. Schindler, and P. Hinterdorfer. 2000. Recognition force spectroscopy: studies of the NTA-His_6 bond. *Single Molecules.* 1:25–31.

Kinne, R. 1976. Properties of the glucose transport system in the renal brush border membrane. *Curr. Top. Membr. Trans.* 8:209–267.

Kinne, R. 1991. Selectivity and direction: plasma membranes in renal transport. *Am J. Physiol.* 260:153–162.

Koepsell, H., and A. Madrala. 1987. Interaction of phlorizin with the Na^+-D-glucose cotransporter from intestine and kidney. *Top. Mol. Pharmacol.* 4:169–202.

Koepsell, H., and J. Spangenberg. 1994. Function and presumed molecular structure of Na^+-D-glucose transport measurements. *J. Membr. Biol.* 138:1–11.

Lee, G. U., D. A. Kidwell, and R. J. Colton. 1994. Sensing discrete streptavidin-biotin interactions with atomic force microscopy. *Langmuir.* 10:354–357.

Lehenkari, P. P., and M. A. Horton. 1999. Single integrin molecule adhesion forces in intact cells measured by atomic force microscopy. *Biochem. Biophys. Res. Commun.* 259:645–650.

Lin, J.-T., M. E. M. DaCruz, S. Riedel, and R. Kinne. 1981. Partial purification of hog kidney sodium-D-glucose cotransport system by affinity chromatography on a phlorizin polymer. *Biochim. Biophys. Acta.* 640:43–54.

Lin, J.-T., K.-D. Hahn, and R. Kinne. 1982. Synthesis of phlorizin derivatives and their inhibitory effect on the renal sodium/D-glucose cotransport system. *Biochim. Biophys. Acta.* 693:379–388.

Lin, J.-T., J. Kormanec, D. Homerová, and R. Kinne. 1999. Probing transmembrane topology of the high-affinity sodium/glucose cotransporter (SGLT1) with histidine-tagged mutants. *J. Membr. Biol.* 170: 243–252.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Merkel, R., P. Nassoy, A. Leung, K. Ritchie, and E. Evans. 1999. Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy. *Nature.* 397:50–53.

Müller, D. J., F. A. Schabert, G. Büldt, and A. Engel. 1995. Imaging purple membranes in aqueous solutions at sub-nanometer resolution by atomic force microscopy. *Biophys. J.* 68:1681–1686.

Oesterhelt, F., D. Oesterhelt, M. Pfeiffer, A. Engel, H. E. Gaub, and D. J. Müller. 2000. Unfolding pathways of individual bacteriorhodopsins. *Science.* 288:143–146.

Panayotova-Heiermann, M., S. Eskandari, E. Turk, G. A. Zanpighi, and E. M. Wright. 1997. Five transmembrane helices form the sugar pathway through the Na^+/glucose cotransporter. *J. Biol. Chem.* 272: 20324–20327.

Raab, A., W. Han, D. Badt, S. J. Smith-Gill, S. M. Lindsay, H. Schindler, and P. Hinterdorfer. 1999. Antibody recognition imaging by force microscopy. *Nat. Biotech.* 17:902–905.

Sauer, G. A., G. Nagel, H. Koepsell, E. Bamberg, and K. Hartung. 2000. Voltage and substrate dependence of the inverse transport mode of the rabbit Na^+/glucose cotransporter (SGLT1). *FEBS Lett.* 469:98–100.

Schwesinger, F., R. Ros, T. Strunz, D. Anselmetti, H.-J. Güntherodt, A. Honegger, L. Jermutus, L. Tiefenauer, and A. Plückthun. 2000. Unbinding forces of single antibody-antigen complexes correlate with their thermal dissociation rates. *Proc. Natl. Acad. Sci. U.S.A.* 97:9972–9977.

Shao, Z., and J. Yang. 1995. Progress in high resolution atomic force microscopy. *Q. Rev. Biophys.* 28:195–251.

Turk, E., C. J. Kerner, M. P. Lostao, and E. M. Wright. 1996. Membrane topology of the human Na^+/glucose cotransporter SGLT1. *J. Biol. Chem.* 271:1925–1934.

Wielert-Badt, S., J.-T. Lin, M. Lorenz, S. Fritz, and R. K.-H. Kinne. 2000. Probing the conformation of the sugar transport inhibitor phlorizin by 2D-NMR, molecular dynamics studies, and pharmacophore analysis. *J. Med. Chem.* 43:1692–1698.

Willemsen, O. H. E., M. M. Snel, K. O. van der Werf, B. G. De Groth, J. Greeve, P. Hinterdorfer, H. J. Gruber, H. Schindler, Y. Van Kooyk, and C. G. Figdor. 1998. Simultaneous height and adhesion imaging of antibody antigen interactions by atomic force microscopy. *Biophys. J.* 57:2220–2228.