Ligands on the string: single-molecule AFM studies on the interaction of antibodies and substrates with the Na^+-glucose co-transporter SGLT1 in living cells

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Summary

Atomic force microscopy (AFM) was used to probe topology, conformational changes and initial substrate-carrier interactions of Na^+-glucose co-transporter (SGLT1) in living cells on a single-molecule level. By scanning *SGLT1*-transfected Chinese hamster ovary (CHO) cells with AFM tips carrying an epitope-specific antibody directed against the extramembranous C-terminal loop 13, significant recognition events could be detected. Specificity was confirmed by the absence of events in nontransfected CHO cells and by the use of free antigen and free antibody superfusion. Thus, contrary to computer predictions on SGLT1 topology, loop 13 seems to be part of the extracellular surface of the transporter. Binding probability of the antibody decreased upon addition of phlorizin, a specific inhibitor of SGLT1, suggesting a considerable conformational change of loop 13 when the inhibitor occludes the sugar translocation pathway. Using an AFM tip carrying 1-thio-D-glucose, direct evidence could be obtained that in the presence of Na^+ a sugar-binding site appears on the transporter surface. The binding site accepts the sugar residue of the glucoside phlorizin, free D-glucose, and D-galactose, but not free L-glucose and probably represents the first of several selectivity filters of the transporter. This work demonstrates the potential of AFM to study the presence and dynamics of plasma membrane transporters in intact cells on the single molecule level.

Key words: SGLT1, Na^+-D-glucose co-transporter, Phlorizin, AFM, Force spectroscopy

Introduction

The Na^+-D-glucose co-transporter SGLT1 present in the apical membrane of epithelial cells in the small intestine and in the kidney plays an important role in intestinal glucose absorption and in renal glucose reabsorption in many organisms (Crane, 1977). The transporter facilitates the translocation of glucose into cells driven by the Na^+ electrochemical potential difference across the membrane (Crane, 1977) and is a prime example for the large family of biologically important ion-gradient-driven co-transport systems.

Several methods were used to investigate the topology of the SGLT1, indicating that the SGLT1 contains 14 transmembrane α-helices with both the N-terminus and C-terminus facing the extracellular compartment (Lin et al., 1999; Turk et al., 1996; Turk and Wright, 1997). The N-terminal half of the protein contains the Na^+-binding sites, whereas the sugar pathway is located in the C-terminal domain, particularly helices 10-13 of the protein (Panayotova-Heiermann et al., 1997; Panayotova-Heiermann et al., 1996; Vayro et al., 1998). A six-state kinetic model has been proposed that Na^+ binding causes long-range structural alterations in the protein, which increases the affinity of the carrier for sugar (Hirayama et al., 1997; Loo et al., 2005; Loo et al., 1998; Meinild et al., 2002; Wright, 2001). Phlorizin, an aromatic β-glucoside, is a well-known potent competitive inhibitor of the SGLT1 transporter (Diedrich, 1966). It is postulated that phlorizin is supposed to bind with a two-step mechanism to both the sugar-binding site of the transporter (mainly through hydrogen bonds) and to a binding site for the aglucone moiety (phloretin) mainly through hydrophobic interactions (Hirayama et al., 2001; Oulianova and Berteloot, 1996; Oulianova et al., 2001). Site-directed mutagenesis studies and studies using reconstituted peptide in vitro have shown that the C-terminal loop 13 is involved in the binding of phlorizin (Novakova et al., 2001; Raja et al., 2003; Xia et al., 2003). However, direct information on the surface of SGLT1, in particular on the single-molecule level is missing. This is a general problem in cell biology that such information can be obtained elegantly and successfully for channels with single-molecule analysis by patch clamp techniques (Neher and Sakmann, 1976) but this method is not applicable to transporters with a much lower translocation velocity.

In the present study we applied atomic force microscopy (AFM) (Binnig et al., 1986) as a pioneering approach to probe the topology and ligand-induced conformational changes of the SGLT1 on the single-molecule level on native cells. The principle and operation of AFM have been described extensively (Binnig et al., 1986; Hoh and Hansma, 1992; Lal and John, 1994). With the potential of AFM to image surface structures at the molecular scale and to measure ultra-low (a few pN) forces at high lateral resolution, the AFM offers a

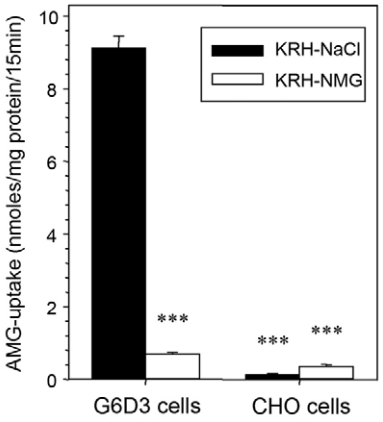

Fig. 1. Representative experiment of [^{14}C]AMG uptake by non-transfected CHO cells and SGLT1 stably transfected G6D3 cells. Uptake was measured by incubation with 0.1 mM [^{14}C]AMG for 15 minutes at 37°C in the presence of Na^+ (KRH-NaCl, black bar), or in the absence of Na^+ (KRH-NMG, white bar). Values are mean ± s.e.m., n=9; ***P<0.005 compared with levels in Na^+-treated G6D3 cells.

unique opportunity to detect molecular recognition of binding epitopes at different environmental conditions (Almqvist et al., 2004; Chen and Moy, 2000; Dupres et al., 2005; Florin et al., 1994; Hinterdorfer et al., 1996; Lee et al., 1994; Lehenkari and Horton, 1999; Pfister et al., 2005; Wielert-Badt et al., 2002). Biochemical modification of the cantilever tips (Hinterdorfer et al., 1996; Raab et al., 1999; Willemsen et al., 1998) has paved the way for detecting ligand-membrane protein interactions at the single molecular level which gives insight into the molecular dynamics of the recognition process.

Here we investigated the molecular recognition of SGLT1 on the cell membrane surfaces of live cells in different buffer conditions by an individual antibody or D-glucose molecule coupled to AFM tips. The results obtained in this study reveal different states of the SGLT1 molecule in the absence and presence of Na^+ and of the inhibitor phlorizin. The D-glucose-binding site of the transporter was investigated, which opens new possibilities for investigating the glucose-transport pathway. Furthermore we successfully probed the transmembrane topology of the wild-type transporter in intact cells and could show that under these experimental conditions the late part of loop 13 is oriented to the outside of the cells – in contrast to computer modeling predictions and results obtained with mutated SGLT1. These studies demonstrate that AFM is a useful method to explore the structural and functional dynamics of plasma membrane transport proteins in live cells on a single-molecule level.

Results

SGLT1 expression in G6D3 cells

In order to verify the presence of functional SGLT1 in the G6D3 cells (CHO cells stably transfected with *rbSGLT1*) used in this study, Na^+-D-glucose co-transport activity was determined using an α-methylglucoside (AMG) uptake assay. As shown in Fig. 1, in non-transfected CHO cells no Na^+-dependent uptake was observed. By contrast, AMG uptake by the G6D3 cells was 9.46±0.34 nmoles/mg cell protein/15 minutes (n=9) in the presence of Na^+ (KRH-NaCl) and 0.69±0.03 nmoles/mg cell protein/15 minutes (n=9) in the absence of Na^+ (KRH-NMG). These data demonstrate that the G6D3 cells used in these studies express SGLT1 as a functionally active transporter on their membrane surface. In addition, previous investigations with G6D3 cells have shown that phlorizin, a high-affinity inhibitor of SGLT1, inhibits AMG uptake with a K_i of 2.4 μM (Lin et al., 1998). Moreover, immunoprecipitation assays with a specific antibody have confirmed the high-level expression of SGLT1 in these cells (Lin et al., 1998). The transporter expressed in the transfected-CHO cells (G6D3 cells) shows thus far all the kinetic properties know from studies in its native environment of the rabbit kidney brush border membrane.

Surface morphology of G6D3 cells in culture

AFM offers the potential for imaging morphology of living cells in a non-destructive manner. Monolayers of live G6D3 cells grown on poly-L-lysine-coated glass coverslips were imaged in the KRH-Na^+ medium used in the uptake studies by contact mode AFM at forces of less than 100 pN. Images recorded using dynamic force microscopy (MAC mode) (Pfister et al., 2005) looked very similar to contact mode images (data not shown). Large-scale topography and deflection images of the cells are shown in Fig. 2a,b. Typical cells were about 30-70 μm in diameter with a characteristic bright zone corresponding to the nuclei region appearing about 1-3 μm in height. The region surrounding the nucleus area appeared flat which assisted adhesion of the cells to the glass plate. Another prominent feature observed in the AFM images

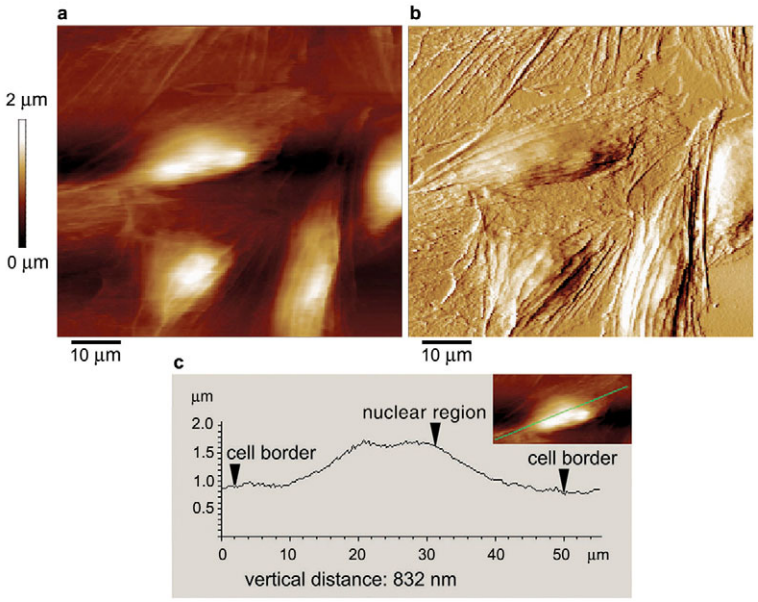

Fig. 2. Cell images of living G6D3 cells. (a) Topographical image and (b) deflection image of living cells imaged at a scan size of 70 μm. The bright regions of the image correspond to the nuclei region. The dominant features observed in both images are the cytoskeleton. (c) Cross-section analysis along the flattened cell showing the height of 832 nm. These images did not change during the course of the AFM experiments.

were structures of the cytoskeleton (Fig. 2a,b). An ~80% confluent cell monolayer, which contained cells with the maximum height of about 1 μm, were chosen for AFM force measurements (see cross-section analysis, Fig. 2c).

Recognition of SGLT1 with an AFM tip carrying an epitope-specific antibody

A scheme of AFM force-distance cycles with a cantilever carrying an epitope-specific antibody performed on living G6D3 cells is shown in Fig. 3a. The specific binding of antibody to a cell surface protein was observed with a unique characteristic of a particular unbinding force (f_u) (see Materials and Methods). Fig. 3b shows a typical force curve (retraction) for a single molecule antibody-antigen recognition event on the surface of a G6D3 cell with a PAN3-2-conjugated tip. For confirmation of the specificity of recognition of SGLT1 by PAN3-2, blocking experiments by injecting free antibodies were performed. As illustrated in Fig. 3b (inset), in the presence of free antibodies no binding events were observed because no free epitopes were present on the cell surface.

In addition, binding probabilities from several experiments were quantified (Fig. 3c). A binding probability (probability of finding an unbinding event in force-distance cycles) of 10.6±0.7% was observed in Na^+-containing KRH medium for G6D3 cells. As another indicator of specificity, a very low binding probability was found for the parent CHO cells, which contained no functional SGLT1. When free PAN3-2 antibodies were present in solution, the binding probability decreased significantly to 3.6±0.9%. After PAN3-2 antibodies were subsequently washed out, the binding probability increased again (6.6±2.3%). By constructing an empirical probability density function (pdf) of the unbinding forces (Fig. 3d), the maximum of the distribution was found to be 100±30 pN. These results demonstrate that SGLT1 on the surface of living cells can be specifically detected at the molecular level by using epitope-specific antibodies. In addition, one has to conclude that the epitope, which is part of the loop 13 of SGLT1, is localized extracellularly in living cells.

Effects on antibody binding induced by ligands

Phlorizin is a strong inhibitor for Na^+-D-glucose co-transport that interacts with SGLT1 in a Na^+-dependent manner (Frasch et al., 1970; Glossmann and Neville, Jr, 1972). Mutagenesis studies and in vitro investigations have suggested that loop 13 is one of the binding domains for the inhibitor (Raja et al., 2004; Raja et al., 2003; Wielert-Badt et al., 2002; Xia et al., 2003). In order to determine whether this assumption also holds for the 'native' wild-type carrier in living cells, the effect of Na^+ and phlorizin on binding of PAN3-2 antibody to the SGLT1 was investigated. As illustrated in Fig. 4, before the addition of phlorizin, SGLT1 could be recognized by the PAN3-2 antibody on the AFM tip in both Na^+-containing (KRH-NaCl) and Na^+-free (KRH-NMG) buffers with ~8% binding probability (from several experiments the binding probabilities of about 8-12% were observed for both conditions). Upon addition of phlorizin, the recognition probability dramatically decreased in the presence of Na^+ (Fig. 4a, 2.5±0.4%), whereas the binding probability remained unchanged in the absence of Na^+ (Fig. 4b, 9.1±1.3%). To confirm the specificity of the PAN3-2 recognition in KRH-NMG buffer, we applied the peptide A606 (antigen used for PAN3-2 antibody production) in solution to saturate the PAN3-

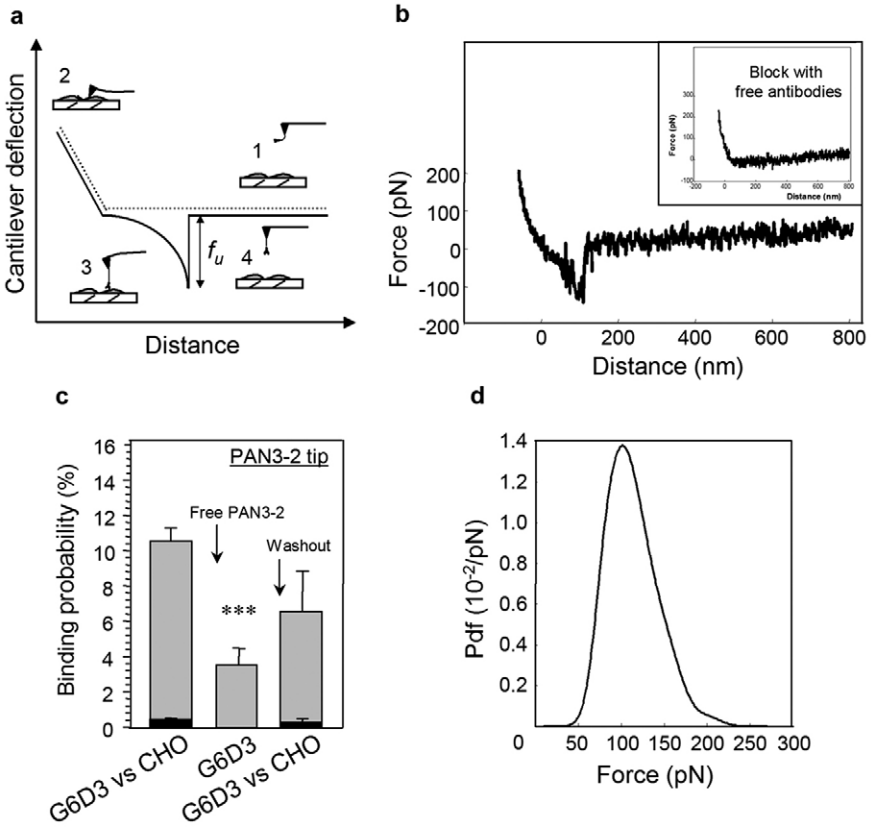

Fig. 3. Recognition of SGLT1 on the surface of intact cells by an AFM tip carrying an epitope-specific antibody (PAN3-2). (a) Schematic representation of a force-distance cycle. The tip was moved toward the cell surface (dotted line, 1-2) and subsequently retracted (solid line) at a constant lateral position. During tip approach, the antibody forms a complex with an antigen that leads to a force signal with a distinct shape (3) during tip retraction. The force increases until dissociation occurs (4) at an unbinding force (f_u). (b) Force curve showing specific interaction between the antibody and SGLT1 upon tip-surface retraction. The specific interaction is blocked by injecting free PAN3-2 antibodies in the solution (inset). (c) Quantitative comparison of binding probabilities of PAN3-2-coated tips on G6D3 (gray) and CHO cells (black) in the absence or presence of free PAN 3-2 in the medium. Values are mean ± s.e.m., n=2000-4000; P<0.005 compared with levels in the control group (unblocked). (d) Probability density function (pdf) giving the distribution of the unbinding force (f_u) of PAN3-2 to SGLT1 (n=1000).

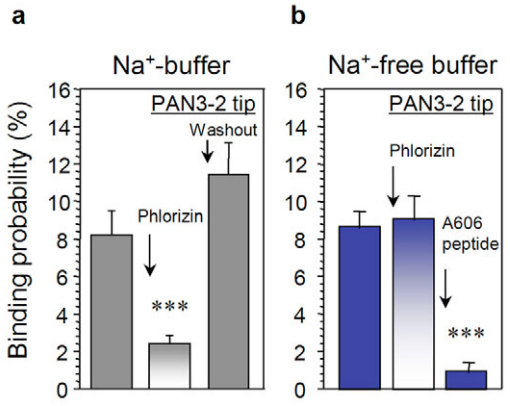

Fig. 4. Effect of Na^+ and phlorizin on the recognition probability of SGLT1 by PAN 3-2. (a) The binding probabilities of PAN3-2 tip on G6D3 cells in the presence of Na^+ (KRH-NaCl buffer, gray) and (b) in the absence of Na^+ (KRH-NMG buffer, blue) in the absence (dark columns) or in the presence (light columns) of phlorizin, a specific inhibitor of SGLT1. Values are mean ± s.e.m., n=2000-4000; and P<0.005 compared with levels in the control group (unblocked).

2 antibody on the AFM tip with antigen. This resulted in an almost complete loss of binding (Fig. 4b, 1.0±0.5%). PAN3-2 binding to the cell surface recovered after removal of phlorizin with buffer (Fig. 4a). These results indicate that in the presence of phlorizin and Na^+, the antigenic sites, aa 606-630, on loop 13 of the transporter become inaccessible to the antibody, suggesting either strong conformational changes of the transporter in this region or a direct obstruction of the site by phlorizin (see Discussion).

Interaction of SGLT1 with a D-glucose carrying AFM tip

To study the initial event in D-glucose transport, i.e. the binding of the sugar to SGLT1 on the surface of intact cells, 1-thio-β-D-glucose was coupled to the flexible crosslinker at the AFM tip by a thio glycosidic bond at the C1 position (creating the 1-thio-D-glucose tip). Using this cantilever tip, force-distance cycles were performed on either live G6D3 cells or the control CHO cells in Na^+-containing buffer. Distinct recognition events were only observed in G6D3 cells, in CHO cells the recognitions were almost absent. Binding probabilities of 9.0±0.3% and 1.4±0.2% were measured on G6D3 and CHO cells, respectively (Fig. 5a). Under the same experimental condition we examined the effect of phlorizin on glucose binding. Upon injection of phlorizin in the Na^+-buffer, the binding probability of D-glucose decreased significantly from 9.0±0.3% to 3.2±1.3%, and subsequently recovered during washout to 7.8±0.6% (Fig. 5a). The specificity of the interaction was confirmed by experiments with free antibodies against SGLT1. The binding probabilities of D-glucose to the receptor decreased significantly after injecting the antibody PAN3-2 (Fig. 5a) The constructed pdf of the unbinding force distribution revealed a maximum of probability of f_u=51.4±12 pN (n=1000) (Fig. 5b). These results demonstrate that the D-glucose-binding site of SGLT1 is accessible on live cells for AFM force spectroscopy investigations.

Several mechanisms have been proposed how Na^+ effects the interaction of SGLT1 with D-glucose (Wright, 2001). Kinetic models propose that initially there are ordered binding events where Na^+ interacts first with the carrier and creates a state where then D-glucose can bind. In order to test whether such an event can be demonstrated directly, we performed recognition studies with 1-thio-D-glucose tip either in the presence or in the absence of Na^+. The results in Fig. 5c show that binding probability drops from 9.0±0.3% in Na^+-containing buffer to almost zero (0.9±0.5%) when no Na^+ is present (KRH-NMG buffer). These results strongly suggest that the site where the initial binding of sugar to SGLT1 occurs is detectable from the outside only in the presence of Na^+ but not in the absence of Na^+.

Stereospecificity of the initial D-glucose-binding site

The availability of the 1-thio-D-glucose tip also allowed us to investigate the stereospecificity of the initial binding events. To this end the effect of various sugars on the binding probability of the 1-thio-D-glucose tip to the receptor was investigated (Fig. 6). In the absence of sugar the binding probabilities of the tip to SGLT1 in Na^+ buffer were similar to those found earlier, i.e. about 8-12%. Upon addition of 10 mM free D-glucose to the medium, the binding probability was reduced significantly with ~63% inhibition as shown in Fig. 6a (compared with the initial condition without D-glucose a drop from 9.1±1.3% to 3.3±0.9%). By contrast, L-glucose inhibited only by 15% as indicated by the change from 7.8±2.4% to 6.6±1.2% (Fig. 6b). In the case of D-galactose, which is a natural substrate for SGLT1, the binding probability decreased from 11.7±0.1% to 5.5±0.9% resulting in a 53% inhibition (Fig. 6c). Phlorizin, as we stated in the previous sections, was

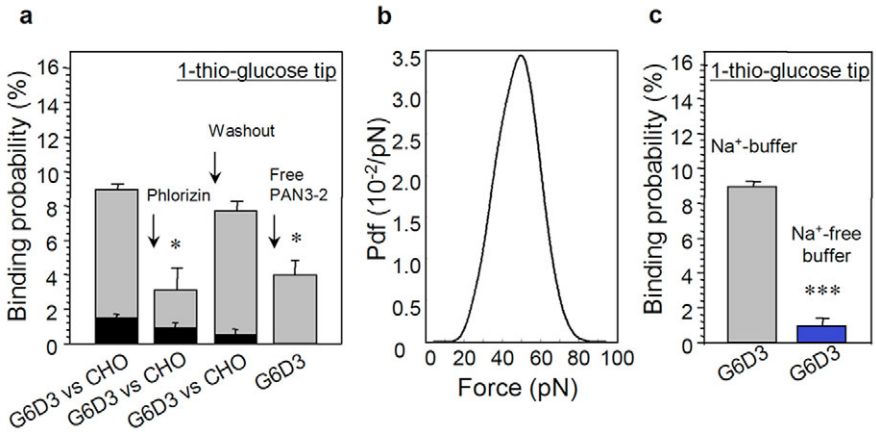

Fig. 5. Recognition of SGLT1 on the surface of intact cells by a D-glucose primed AFM tip. (a) The effect of phlorizin and the antibody PAN3-2 in the presence of Na^+ (KRH-NaCl) on the binding probabilities of D-glucose tip on G6D3 (gray) and CHO cells (black). (b) Probability density function (pdf) giving the distribution of the unbinding force f_u required to disrupt single glucose-receptor interaction (n=1000). (c) Binding probabilities of D-glucose tip on G6D3 cells in the presence and in the absence of Na^+ (KRH-NaCl) (gray) and KRH-NMG (sodium replaced by N-glucosamine) (blue), respectively. Values are mean ± s.e.m., n=2000-4000. *P<0.05 and ***P<0.005 compared with levels in the control group (unblocked).

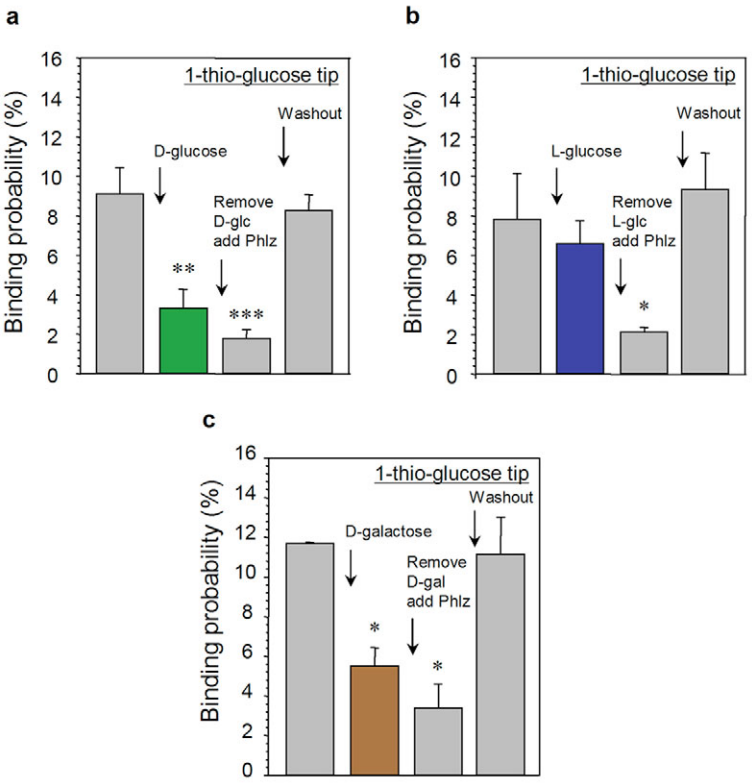

Fig. 6. Inhibition of D-glucose binding with the stereospecific monosaccharide. Binding probabilities of D-glucose tip and the effect of D-glucose (a, green), L-glucose (b, blue), and D-galactose (c, brown). Values are mean ± s.e.m., n=2000-4000. D-glc, L-glc and D-gal are D-glucose, L-glucose and D-galactose, respectively. Phlz, Phlorizin. *P<0.05, **P<0.01 and ***P<0.005 compared with levels in the relevant controls (absence of carbohydrates in solution).

observed to have the strongest inhibitory effect (Fig. 6). All recognition probabilities increased again after washout of phlorizin.

Discussion

In this study, we showed that AFM single-molecule-recognition spectroscopy is a suitable method to obtain information on the structure and function of single transporters on the surface of living cells. One prerequisite for such investigations is that the cells are attached firmly to their support. AFM deflection images of the cell cultures reveal that this is the case for G6D3 cells, although owing to the high flexibility and softness of the cell membrane of living cells no transporters on the membrane were directly visualized. For the detection of SGLT1 we used interaction-force spectroscopy. This method uses single-molecule tips, which contain a very low surface density of ligands (about 400 molecules per μm^2) on the tip surface to allow single detection of single molecular events. It has been used in the past in other, mainly in vitro, systems (Florin et al., 1994; Hinterdorfer et al., 1996; Lee et al., 1994), but was applied in the current study to living cells in their near-physiological surrounding. G6D3 and CHO cells are easily grown and maintain vitality at room temperature for at least 6 hours. Therefore they seem to be a very suitable cell model for AFM force spectroscopic investigations.

Surface topology of SGLT1 using force spectroscopy

Several approaches have been used previously to determine the topology of the transmembrane protein SGLT1, for example, computational algorithms, scanning mutagenesis and immunostaining methods (Lin et al., 1999; Turk et al., 1996; Turk et al., 1994). Here we introduce an alternative way to probe the surface topology by using AFM force spectroscopy. The advantages of this approach are that we could probe unaltered cells, i.e. unfixed (living cells) under near-physiological conditions in terms of ion composition of the intra- and extracellular medium, membrane potential, and membrane fluidity. It is important to note that the transporters were not modified by mutagenesis, e.g. addition of glycosylation sites or tags, because the latter can influence the protein orientation in the membrane. In addition, association of the transporter with other proteins inside the membrane, the cytoskeleton, and/or the cytoplasm are not likely to be disturbed.

The structural intramembrane topology of the SGLT1, especially of the large C-terminal loop 13, is still under discussion (Lin et al., 1999; Turk et al., 1996; Turk et al., 1994). The current work demonstrates that the PAN3-2 antibody coupled to the AFM tip and directed against an epitope of the late loop 13 could interact specifically with the antigenic-binding epitope of functional SGLT1. These results confirm an extracellular orientation of the late part of loop 13, which supports our assumptions derived from mutation studies and in vitro analysis of functional domains of the transporter (Lin et al., 1999; Lin et al., 1998; Raja et al., 2004; Raja et al., 2003; Xia et al., 2003). Interestingly they contradict computer models and results obtained with strongly modified SGLT1 mutants, demonstrating the need to investigate the wild-type transporter under near-physiological conditions.

The observed unbinding force (f_u) required to dissociate PAN3-2 antibody from SGLT1 is in agreement with a previous investigation investigating PAN3-2–SGLT1 interaction on isolated brush border membrane vesicles (BBMVs) (Wielert-Badt et al., 2002), suggesting that the topology of the transporter in this preparation was quite close to that occurring in vitro. The obtained single molecule receptor-ligand force was also in the range of 50-250 pN observed for other biological systems (Florin et al., 1994; Hinterdorfer et al., 1996; Pfister et al., 2005). Unlike kinetic rate constants, affinities and energies, single-molecular-recognition forces do not resemble unitary values, because they depend on the dynamics of the experiment. More precisely, the forces increase with the loading rate in a logarithmic fashion (Evans and Ritchie, 1997). The loading rate can be directly obtained from the slope before unbinding in the force-distance cycles, and is given by the product of retraction velocity and effective spring constant, of which the latter assembles from a series of springs from the cantilever, the cell membrane, the crosslinker and the ligand molecule. Therefore, the experiments with all substrates and ligands were performed at the same loading rate, so that the forces obtained in all experiments can be compared as a measure of the binding strength.

Changes of epitope accessibility by phlorizin

We observed that phlorizin significantly reduced the binding of PAN3-2 to SGLT1, but only in the presence of Na^+. This observation is in agreement with phlorizin-binding studies on isolated BBMVs, which showed that the affinity of the membranes for phlorizin binding decreased when the Na^+ concentration is lowered (Glossmann and Neville, Jr, 1972). After phlorizin binding to the transporter, the loop 13 epitope (aa 606-630) is no longer accessible to the antibody PAN3-2. This change could be due to an obstruction of the antibody binding sites by phlorizin itself or due to a conformational change in the transporter. Support for the former assumption is rather weak because the epitope is much larger than the region between amino acids 601 and 611 of loop 13, which our group has proposed act as a phlorizin-binding domain (Raja et al., 2004; Raja et al., 2003; Wielert-Badt et al., 2002; Xia et al., 2003). Since it has been shown that the C-terminal part (region between transmembrane helices 10-13) is responsible for sugar translocation (Panayotova-Heiermann et al., 1997; Panayotova-Heiermann et al., 1996; Vayro et al., 1998), we rather assume that phlorizin induces a movement of loop 13 which brings it close to or even into the plasma membrane plane, thereby making the interaction with the antibody impossible. There is evidence for such behavior of extramembranous loops in several other transporter proteins, e.g. the glutamate transporter (GLT) and the citrate and/or malate transporter (CimH) (Grunewald and Kanner, 2000; Krom and Lolkema, 2003; Slotboom et al., 1999).

Recognition of SGLT1 by the 1-thio-D-glucose tip

In the current study we observed distinct recognition events between the D-glucose-loaded cantilever and the G6D3 cells. This interaction was only observed in the cells expressing functional SGLT on their surface but not with the parent CHO cells showing no Na^+-D-glucose co-transport. In addition, the recognition events were inhibited by phlorizin and sugars that are translocated by the transporter. Also, the binding probability and the recognition of SGLT1 by PAN3-2 were quite similar. This evidence strongly supports our assumption that the tip recognizes the SGLT1 transporter. The sugar is linked to the tip in such a way that the major recognition sites of the molecule (C2, C3 and C4) remained unaltered and therefore close interaction with the carrier can occur. It is relatively certain that superficial binding of the sugar to the transporter is observed because the bulky vinylsulfon group present at the end of the flexible linker most probably prevents a translocation. Thus, this study demonstrates that single-molecule recognition AFM can also be applied to study the interaction of substrates with their transporters in the membrane of living cells.

Effect of Na^+ on glucose binding

Our studies with the 1-thio-D-glucose tip show that an interaction between the sugar and the transporter only occurs when Na^+ is present. Thus Na^+ in addition to increasing the affinity of sugar binding (Hirayama et al., 1997; Loo et al., 1998) as postulated previously, plays an essential role in making the initial sugar-binding site accessible to the extracellular medium, which is shown for the first time directly and on the native transporter.

These conclusions can also explain the observation that phlorizin alters the binding of the antibody PAN3-2 to SGLT1 only in the presence of Na^+. Several authors have proposed a two-step mechanism for phlorizin binding to SGLT1, involving a low-affinity (aglucone) binding site and a high-affinity (glucose) binding site. Opening of the sugar-binding site would make the interaction of the glucoside with high affinity possible.

Stereospecificity of D-glucose-binding site

It is known that SGLT1 strongly discriminates among its natural substrates, D-glucose, D-galactose and other hexoses (Diez-Sampedro et al., 2001; Hirayama et al., 1996; Kanai et al., 1994; You et al., 1995). It is hypothesized that there exist at least two sites of interaction of the transporter with the sugars, one involved in the initial binding of the sugar and the other in the translocation. However these results are based upon transport kinetics rather than direct measurements of the binding affinity itself. In the current study a direct preliminary analysis of the properties of the initial D-glucose-binding step without a subsequent translocation was possible. The binding probability of the D-glucose-coupled AFM tip was reduced significantly after applying free D-glucose, whereas only a slight effect was observed in the presence of L-glucose. This indicates that the discrimination between D- and L-glucose occurs at the initial binding event. A significant change in binding probability could, however, be detected upon injection of D-galactose, whereby the effect of D-galactose was only slightly smaller than that of D-glucose. Transport studies with rabbit kidney outer medullary BBMVs (containing SGLT1) showed that D-galactose is about four times less effective in inhibiting D-glucose transport than D-glucose (Turner and Moran, 1982) itself. Thus a second discrimination between the two sugars probably occurs at the translocation site that leads to the different affinity of the two sugars for the transport cycle.

Although several questions on the molecular level still remain unanswered, we have demonstrated in this study that AFM force spectroscopy is a very sensitive and suitable method for probing the surface topology and for characterizing conformational states of membrane proteins in intact cells in near-physiological media. In the specific case of SGLT1, this study has further elucidated the action of Na^+ on the transporter as well as defined the initial binding events and the stereospecificity of the SGLT1 in sugar transport. Furthermore, one interaction site with the inhibitor phlorizin and the conformational changes associated with this interaction have been identified. These investigations were performed at the single-molecule level, a resolution thus far not achieved for membrane transporters with a slower transport rate than channels. The method used in this study has great potential for the investigation and localization of membrane proteins on the surface of living cells with several pN force resolution and a few nanometers positional accuracy.

Materials and Methods

Materials

Immunopurified polyclonal antibodies PAN3-2 were raised against a peptide construct from the sequence aa 606-630 (C-terminal loop13) of SGLT1 as described previously (Lin et al., 1999; Lin et al., 1998). 1-thio-β-D-glucose, D-glucose, L-glucose, D-galactose, phlorizin and poly-L-lysine were obtained from Sigma (Schnelldorf, Germany). All other reagents were of the highest purity available.

Cell cultures

Parental CHO cells were obtained from the American Type Culture Collection

(Rockville, MD). CHO cells and *rbSGLT1*-expressing G6D3 cells, a CHO cell line stably transfected with rabbit SGLT1 generated in our laboratory (Lin et al., 1998), were grown in 25-cm^2 flasks (Falcon, Heidelberg, Germany) under 5% CO_2 at 37°C. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing high glucose (25 mM) supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× minimal essential medium (MEM), and 25 μM β-mercaptoethanol. To maintain selection of transfected cells, culture medium for G6D3 cells contained 400 μg/ml paneticin G420 (PAN Biotech, Aidenbach, Germany). Culture medium was renewed three times a week and the cells were subcultured at 80% confluence. Cells were used below passage 15 for all experiments. For cellular uptake studies, confluent monolayers of CHO cells and G6D3 cells were grown on six-well plates (Falcon) for 3 days. For AFM investigation, the cells were seeded on 22-mm^2 poly-L-lysine-coated glass coverslips and the experiments were performed within 1-4 days of seeding the coverslips.

Transport studies

Na^+-D-glucose co-transport activity of G6D3 cells was determined by examining [α-^{14}C]methylglucoside (AMG) uptake. Before the transport assays, CHO and G6D3 cells were incubated in a D-glucose-free medium for 1 hour at 37°C to remove the extracellular D-glucose and to reduce the intracellular D-glucose concentration. Na^+-dependent uptake of AMG into the cells was performed at 37°C in Na^+-containing KRH (Krebs-Ringer-HEPES) medium or Na^+-free KRH-NMG medium at a concentration of 0.1 mM AMG (containing 0.5 μCi ^{14}C-labeled AMG) as described previously (Lin et al., 1998). The KRH-Na^+ medium contained in 120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 10 mM HEPES; the pH was adjusted to 7.4 with Tris base. NaCl was substituted by N-methylglucamine (NMG) for the KRH-NMG medium. Three independent experiments were performed with different cell passages and the triplicate samples were examined in each experiment. Results were expressed in mean values ± s.e.m. The statistical significance was tested using a Student's *t*-test.

Conjugation of antibody and ligand to AFM tips

Conjugation of immunopurified antibody PAN3-2 to AFM-tip by a flexible PEG [poly(ethylene glycol)]-crosslinker was done as described previously (Hinterdorfer et al., 1996). In brief, antibody PAN3-2 was modified with SATP [N-succinimidyl-3-(S-acetylthio)propionate] in order to generate free SH groups for coupling to the PDP [2-(pyridyldithio)propionyl]-PEG-modified tip. In separate experiments, the free SH group of 1-thio-β-D-glucose was reacted with VS (vinylsulfon)-PEG-conjugated AFM tips. The corresponding crosslinker (termed VS-PEG-NHS) was synthesized by coupling succinimidyl-(4-vinylsulfonyl)benzoate (Molecular Biosciences) to the amine function of NH2-PEG-COOH and activating the terminal carboxyl as described before (Riener et al., 2003), except that a new differential extraction method was used to isolate pure VS-PEG-COOH before converting the terminal carboxyl into the NHS (N-hydroxysuccinimide) ester (A. S. M. Kamruzzahan, L.W., A. Ebner, C. Hahn, P.H. and H.J.G., unpublished observations). Tips were finally washed in the AFM working buffer and stored in the cold room. This method provides tips which are primed with single molecules of antibodies or ligands (Hinterdorfer et al., 1996).

Atomic force microscopy

All AFM experiments were performed using a magnetically driven dynamic force microscope (PicoSPM II, Molecular Imaging, Tempe, AZ). Topographical and deflection images of cells were acquired with contact mode AFM in the Na^+-containing KRH medium at room temperature with non-functionalized AFM cantilevers (cantilever from Veeco, Mannheim, Germany) of 0.03-0.5 N/m nominal spring constants.

For the detection of antibody-SGLT1 and glucose-SGLT1 recognitions, force-distance cycles were performed at room temperature using ligand-coated cantilevers (rectangular cantilever, Veeco Instruments) with 0.02 N/m nominal spring constants in the conventional contact force calibration plot mode. Force distance cycles were recorded on cell surfaces with the assistance of a CCD camera for the localization of the AFM cantilever on isolated cells or cell monolayers. At a fixed lateral position an AFM tip carrying a glucose or antibody was first approached (trace) and then retracted (retrace) from the cell surface. Upon approach (trace line), no bending of the cantilever occurs, thus, the cantilever deflection (Δz) from which the force (f) is calculated according to Hook's law ($f=k\Delta z$, k is the spring constant of the cantilever). As the cantilever reaches the cell surface it is deflected upwards due to a repulsive tip-sample interaction. Subsequently the tip is retracted. When antibody binding to a cell surface protein has occurred, an attractive force develops upon retraction causing the cantilever to bend downwards. The cross-linker with which the ligand is tethered to the tip will be stretched more and more with increasing tip-surface separation, leading to a force signal of distinct shape. The force increases until the antibody detaches from the protein at a particular unbinding force (f_u) and the cantilever jumps back to zero deflection. The sweep-amplitude of the force-distance cycle was 1000 nm at 1 Hz sweep rate. Up to 500-1000 force-distance cycles were performed for each location on the surface of cells and up to four locations (different cells) for each condition, i.e. initial condition, ligand addition and washout condition. For ligand addition of PAN3-2-coupled AFM tip experiments, 0.3 μM free PAN3-2 antibodies, 10 μg/ml A606-antigen peptide or 0.5-1 mM phlorizin were separately applied for the different conditions. 10 mM of each D-glucose, L-glucose, D-galactose or 0.5 mM phlorizin as well as 0.25 μM free PAN3-2 was separately added for competition assays. The effect of Na^+ ions was studied by substituting sodium with N-methylglucamine. The incubation and washing periods for each condition were about 30 minutes to 1 hour, except for the sugar addition where the force distance cycles were recorded after 10 minutes of incubation. The binding probability for each condition was derived and expressed as mean value ± s.e.m., n=2000-4000. Several experiments were performed and one typical experiment for each condition is shown in this study.

For the quantification of the forces, spring constants of cantilevers were determined in air using the thermal noise method (Butt and Jaschke, 1995; Hutter and Bechhoefer, 1993). The deflection sensitivity of the photo-detector was determined from the slope of the force-distance curves taken on the bare surface of glass coverslips. Analysis of force-distance cycles was performed using the Matlab Version 6.5 (Math Works, Natick, MA) as previously described (Baumgartner et al., 2000; Hinterdorfer et al., 1996).

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References

Almqvist, N., Bhatia, R., Primbs, G., Desai, N., Banerjee, S. and Lal, R. (2004). Elasticity and adhesion force mapping reveals real-time clustering of growth factor receptors and associated changes in local cellular rheological properties. *Biophys. J.* **86**, 1753-1762.

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

Binnig, G., Quate, C. F. and Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Lett.* **56**, 930-933.

Butt, H.-J. and Jaschke, M. (1995). Calculation of thermal noise in atomic force microscopy. *Nanotechnology* **6**, 1-7.

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

Crane, R. K. (1977). The gradient hypothesis and other models of carrier-mediated active transport. *Rev. Physiol. Biochem. Pharmacol.* **78**, 99-159.

Diedrich, D. F. (1966). Competitive inhibition of intestinal glucose transport by phlorizin analogs. *Arch. Biochem. Biophys.* **117**, 248-256.

Diez-Sampedro, A., Wright, E. M. and Hirayama, B. A. (2001). Residue 457 controls sugar binding and transport in the Na(+)/glucose cotransporter. *J. Biol. Chem.* **276**, 49188-49194.

Dupres, V., Menozzi, F. D., Locht, C., Clare, B. H., Abbott, N. L., Cuenot, S., Bompard, C., Raze, D. and Dufrene, Y. F. (2005). Nanoscale mapping and functional analysis of individual adhesins on living bacteria. *Nat. Methods* **2**, 515-520.

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

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

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

Glossmann, H. and Neville, D. M., Jr (1972). Phlorizin receptors in isolated kidney brush border membranes. *J. Biol. Chem.* **247**, 7779-7789.

Grunewald, M. and Kanner, B. I. (2000). The accessibility of a novel reentrant loop of the glutamate transporter GLT-1 is restricted by its substrate. *J. Biol. Chem.* **275**, 9684-9689.

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

Hirayama, B. A., Lostao, M. P., Panayotova-Heiermann, M., Loo, D. D., Turk, E. and Wright, E. M. (1996). Kinetic and specificity differences between rat, human, and rabbit Na+-glucose cotransporters (SGLT-1). *Am. J. Physiol.* **270**, G919-G926.

Hirayama, B. A., Loo, D. D. and Wright, E. M. (1997). Cation effects on protein conformation and transport in the Na+/glucose cotransporter. *J. Biol. Chem.* **272**, 2110-2115.

Hirayama, B. A., Diez-Sampedro, A. and Wright, E. M. (2001). Common mechanisms of inhibition for the Na+/glucose (hSGLT1) and Na+/Cl–/GABA (hGAT1) cotransporters. *Br. J. Pharmacol.* **134**, 484-495.

Hoh, J. H. and Hansma, P. K. (1992). Atomic force microscopy for high-resolution imaging in cell biology. *Trends Cell Biol.* **2**, 208-213.

Hutter, J. L. and Bechhoefer, J. (1993). Calibration of atomic-force microscope tips. *Rev. Sci. Instrum.* **64**, 1868-1873.

Kanai, Y., Lee, W. S., You, G., Brown, D. and Hediger, M. A. (1994). The human kidney low affinity Na+/glucose cotransporter SGLT2. Delineation of the major renal reabsorptive mechanism for D-glucose. *J. Clin. Invest.* **93**, 397-404.

Krom, B. P. and Lolkema, J. S. (2003). Conserved residues R420 and Q428 in a cytoplasmic loop of the citrate/malate transporter CimH of Bacillus subtilis are accessible from the external face of the membrane. *Biochemistry* **42**, 467-474.

Lal, R. and John, S. A. (1994). Biological applications of atomic force microscopy. *Am. J. Physiol.* **266**, C1-C21.

Lee, G. U., Chrisey, L. A. and Colton, R. J. (1994). Direct measurement of the forces between complementary strands of DNA. *Science* **266**, 771-773.

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

Lin, J. T., Kormanec, J., Wehner, F., Wielert-Badt, S. and Kinne, R. K. (1998). High-level expression of Na+/D-glucose cotransporter (SGLT1) in a stably transfected Chinese hamster ovary cell line. *Biochim. Biophys. Acta* **1373**, 309-320.

Lin, J., Kormanec, J., Homerova, D. and Kinne, R. K. (1999). Probing transmembrane topology of the high-affinity Sodium/Glucose cotransporter (SGLT1) with histidine-tagged mutants. *J. Membr. Biol.* **170**, 243-252.

Loo, D. D., Hirayama, B. A., Gallardo, E. M., Lam, J. T., Turk, E. and Wright, E. M. (1998). Conformational changes couple Na+ and glucose transport. *Proc. Natl. Acad. Sci. USA* **95**, 7789-7794.

Loo, D. D., Hirayama, B. A., Cha, A., Bezanilla, F. and Wright, E. M. (2005). Perturbation analysis of the voltage-sensitive conformational changes of the Na+/glucose cotransporter. *J. Gen. Physiol.* **125**, 13-36.

Meinild, A. K., Hirayama, B. A., Wright, E. M. and Loo, D. D. (2002). Fluorescence studies of ligand-induced conformational changes of the Na(+)/glucose cotransporter. *Biochemistry* **41**, 1250-1258.

Neher, E. and Sakmann, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260**, 799-802.

Novakova, R., Homerova, D., Kinne, R. K., Kinne-Saffran, E. and Lin, J. T. (2001). Identification of a region critically involved in the interaction of phlorizin with the rabbit sodium-D-glucose cotransporter SGLT1. *J. Membr. Biol.* **184**, 55-60.

Oulianova, N. and Berteloot, A. (1996). Sugar transport heterogeneity in the kidney: two independent transporters or different transport modes through an oligomeric Protein? 1. Glucose transport studies. *J. Membr. Biol.* **153**, 181-194.

Oulianova, N., Falk, S. and Berteloot, A. (2001). Two-step mechanism of phlorizin binding to the SGLT1 protein in the kidney. *J. Membr. Biol.* **179**, 223-242.

Panayotova-Heiermann, M., Loo, D. D., Kong, C. T., Lever, J. E. and Wright, E. M. (1996). Sugar binding to Na+/glucose cotransporters is determined by the carboxyl-terminal half of the protein. *J. Biol. Chem.* **271**, 10029-10034.

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

Pfister, G., Stroh, C. M., Perschinka, H., Kind, M., Knoflach, M., Hinterdorfer, P. and Wick, G. (2005). Detection of HSP60 on the membrane surface of stressed human endothelial cells by atomic force and confocal microscopy. *J. Cell Sci.* **118**, 1587-1594.

Raab, A., Han, W., Badt, D., Smith-Gill, S. J., Lindsay, S. M., Schindler, H. and Hinterdorfer, P. (1999). Antibody recognition imaging by force microscopy. *Nat. Biotechnol.* **17**, 901-905.

Raja, M. M., Tyagi, N. K. and Kinne, R. K. (2003). Phlorizin recognition in a C-terminal fragment of SGLT1 studied by tryptophan scanning and affinity labeling. *J. Biol. Chem.* **278**, 49154-49163.

Raja, M. M., Kipp, H. and Kinne, R. K. (2004). C-terminus loop 13 of Na+ glucose cotransporter SGLT1 contains a binding site for alkyl glucosides. *Biochemistry* **43**, 10944-10951.

Riener, C. K., Stroh, C. M., Ebner, A., Klampfl, C., Gall, A. A., Romanin, C., Lyubchenko, Y. L., Hinterdorfer, P. and Gruber, H. J. (2003). Simple test system for single molecule recognition force microscopy. *Anal. Chim. Acta* **479**, 59-75.

Slotboom, D. J., Sobczak, I., Konings, W. N. and Lolkema, J. S. (1999). A conserved serine-rich stretch in the glutamate transporter family forms a substrate-sensitive reentrant loop. *Proc. Natl. Acad. Sci. USA* **96**, 14282-14287.

Turk, E. and Wright, E. M. (1997). Membrane topology motifs in the SGLT cotransporter family. *J. Membr. Biol.* **159**, 1-20.

Turk, E., Martin, M. G. and Wright, E. M. (1994). Structure of the human Na+/glucose cotransporter gene SGLT1. *J. Biol. Chem.* **269**, 15204-15209.

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

Turner, R. J. and Moran, A. (1982). Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Membr. Biol.* **67**, 73-80.

Vayro, S., Lo, B. and Silverman, M. (1998). Functional studies of the rabbit intestinal Na+/glucose carrier (SGLT1) expressed in COS-7 cells: evaluation of the mutant A166C indicates this region is important for Na+-activation of the carrier. *Biochem. J.* **332**, 119-125.

Wielert-Badt, S., Hinterdorfer, P., Gruber, H. J., Lin, J. T., Badt, D., Wimmer, B., Schindler, H. and Kinne, R. K. (2002). Single molecule recognition of protein binding epitopes in brush border membranes by force microscopy. *Biophys. J.* **82**, 2767-2774.

Willemsen, O. H., Snel, M. M., van der Werf, K. O., de Grooth, B. G., Greve, J., Hinterdorfer, P., Gruber, H. J., Schindler, H., van Kooyk, Y. and Figdor, C. G. (1998). Simultaneous height and adhesion imaging of antibody-antigen interactions by atomic force microscopy. *Biophys. J.* **75**, 2220-2228.

Wright, E. M. (2001). Renal Na(+)-glucose cotransporters. *Am. J. Physiol. Renal Physiol.* **280**, F10-F18.

Xia, X., Lin, J. T. and Kinne, R. K. (2003). Binding of phlorizin to the isolated C-terminal extramembranous loop of the Na+/glucose cotransporter assessed by intrinsic tryptophan fluorescence. *Biochemistry* **42**, 6115-6120.

You, G., Lee, W. S., Barros, E. J., Kanai, Y., Huo, T. L., Khawaja, S., Wells, R. G., Nigam, S. K. and Hediger, M. A. (1995). Molecular characteristics of Na(+)-coupled glucose transporters in adult and embryonic rat kidney. *J. Biol. Chem.* **270**, 29365-29371.