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Conformational changes in the intestinal brush border sodium–glucose cotransporter labeled with fluorescein isothiocyanate

(fluorescence/brush border vesicles/membrane proteins)

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ABSTRACT **Fluorescein isothiocyanate (FITC) was used to label the rabbit intestinal brush border Na^+–glucose carrier, identify the carrier protein on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and monitor the effect of ions and substrates on fluorescence quenching. Enriched brush border preparations were employed to study both glucose transport and FITC binding. FITC and a nonfluorescent analog (phenyl isothiocyanate, PITC) both inhibited Na^+-dependent D-glucose transport irreversibly. Inhibition was blocked completely by the presence of Na^+ and D-glucose during labeling. PITC was used to label nonspecific amino groups in the presence of glucose and Na^+, and then the glucose carrier was labeled with FITC in the absence of substrates. Fluorescence of FITC bound to the carrier was quenched specifically with Na^+ in a saturable fashion, and this indicates a Na^+-dependent conformational change in the carrier. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of FITC-labeled membranes revealed specific labeling of a 71,000-dalton peptide. We conclude that Na^+ induces a conformational shift in the 71,000-dalton glucose carrier, and this is quite consistent with the kinetics of Na^+-dependent glucose transport in these membranes.**

Sodium-dependent organic solute transport across the brush border membrane of the small intestine plays a primary role in the absorption of sugars and amino acids (1). In these systems Na^+ gradients provide the driving force for uphill solute uptake, and kinetic experiments suggest that Na^+ increases the affinity of the carrier for the solute through conformational changes. However, little direct information is available about either the nature of the conformation changes or even the identity of the carrier. Since site-specific reagents with fluorescent labels have been used successfully to probe conformation changes in other transport proteins (2–7), we have explored this approach with intestinal brush borders. Fluorescein isothiocyanate (FITC), an irreversible group-specific reagent for amino groups, has been used to label the Na^+–glucose carrier, identify the carrier on $NaDodSO_4$ gels, and monitor conformation changes. Nonspecific FITC binding was minimized by using enriched membrane preparations and labeling nonspecific sites with nonfluorescent isothiocyanates in the presence of excess Na^+ and glucose. We find that Na^+ produces a specific, saturable decrease in FITC fluorescence that is consistent with a Na^+-dependent conformational change in the glucose carrier.

METHODS

Brush border membrane vesicles (BBMVs) were isolated from rabbit small intestine by the Ca^{2+} differential centrifugation method (8) and further purified by an adaptation of Hopfer's KSCN treatment (9). Briefly, the crude brush borders were resuspended by homogenization in 0.6 M KSCN and 10 mM Hepes/Tris buffer at pH 7.5, incubated at 4°C, and then diluted 1:10 with 10 mM Hepes/Tris, pH 7.5. This suspension was centrifuged at 6000 × *g* for 10 min, and the resultant supernatant was centrifuged at 38,000 × *g* for 30 min. This high-force pellet was resuspended in 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5, and washed twice. The final pellet was suspended in 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5, at a protein concentration of 10–15 mg/ml, and stored in liquid nitrogen.

Glucose transport was measured by using D-[^{3}H]glucose and a rapid mixing and filtration procedure as described elsewhere (10). All transport experiments were carried out at 22–23°C. Uptakes are expressed as nmol/mg of protein.

Binding of FITC and phenyl isothiocyanate (PITC) to BBMVs was performed by incubating membranes (250–500 μg of protein) at 22–23°C in the dark with FITC or PITC in solutions containing 2 mM EDTA and 50 mM Tris·HCl, pH 9.2. Under alkaline conditions, FITC and PITC are thought to react with lysine amino groups. Stock solutions of FITC and PITC were prepared in dimethylformamide. The reaction was stopped by dilution with isothiocyanate-free buffer and centrifugation at 38,000 × *g* for 30 min. The membrane pellet was resuspended in either 50 mM Tris·HCl, pH 9.2, for fluorescence studies or 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5, for transport studies.

Fluorescence experiments were performed on an Amin-co/SLM SPF 500 spectrofluorometer at 22–23°C with an excitation wavelength of 495 nm and an emission wavelength of 525 nm in the ratio mode. Slit widths were set at 4 nm. In all these experiments, 100 μg of FITC-treated membranes was added to 2 ml of 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA. Ions were added from 1 M stock solutions and the level of fluorescence was corrected for the dilution of the membranes. All fluorescence data refer to uncorrected emission spectra.

$NaDodSO_4$/polyacrylamide gel electrophoresis of 50–200 μg of membrane protein was performed on 7.5% gels according to the Laemmli method (11). The distribution of protein was determined on tracks stained with Coomassie blue, and FITC binding was determined on unstained parallel tracks. The gels were cut into 3-mm slices, and these were incubated overnight in 2% $NaDodSO_4$ and 50 mM Hepes/Tris, pH 7.5, at room temperature to remove the protein from the gel. The acrylamide was sedimented by centrifugation, and the protein and FITC in the supernatant were measured by absorbance at 550 and 490 nm, respectively. Sucrase and alkaline phosphatase were measured as described (12), and protein was assayed by using the Bio-Rad protein reagent. FITC binding to membranes was obtained from absorbance measurements at 490 nm, using the extinction coefficient measured for FITC bound to 10 mM lysine.

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Abbreviations: FITC, fluorescein isothiocyanate; PITC, phenyl isothiocyanate; BBMV, brush border membrane vesicle.

RESULTS

Our study of FITC binding to intestinal brush border Na^+/glucose carriers was facilitated by the use of enriched brush border membranes. As described by Hopfer *et al.* (9), KSCN treatment increased the specific activities of the brush border marker enzymes sucrase and alkaline phosphatase 3- to 5-fold above the Ca^{2+}-precipitated brush border membranes—i.e., to 129 ± 3 and 70 ± 5 fold higher than the initial homogenate (mean ± SEM for three measurements). As shown in Fig. 1, KSCN treatment also enriched glucose uptake as judged by the increase in initial rate, the peak overshoot, and the J_{max} of the Na^+-dependent uptake. The J_{max} was almost an order of magnitude higher than that obtained in our laboratory with "crude" BBMVs (13). Fig. 2 shows that PITC and FITC both inhibit Na^+-dependent glucose uptake. In this experiment, preincubation of membrane with either 2 mM PITC for 30 min or 50 μM FITC for 15 min at pH 9.2 inhibited glucose uptake ≈50%. The maximal inhibition observed at pH 9.2 ranged from 45% to 70% and, as judged by extensive dilution and washing, this was irreversible. However, preincubation with PITC or FITC in the presence of 100 mM NaCl and 10 mM glucose completely blocked the action of these inhibitors (see Fig. 2). Protection against isothiocyanates is substrate specific, because substitution of neither K^+ for Na^+ nor L-glucose for D-glucose affords protection of glucose uptake. This suggests that the isothiocyanates bind irreversibly to the glucose transporter in the absence of substrates.

Specific binding of FITC to the Na^+/glucose carrier can be monitored by labeling the nonspecific sites with PITC in the presence of Na^+ and glucose, washing the membranes free of glucose, Na^+, and unbound PITC, and then exposing to FITC. As shown in Fig. 2, this reduced FITC binding 85%, although FITC still inhibited glucose uptake to the full extent. In control experiments not shown, inclusion of 100 mM NaCl together with 10 mM D-glucose further reduced FITC binding under these circumstances to 5% of the total. This suggests that only 10% of the total FITC binding sites on the membrane are located on the carrier.

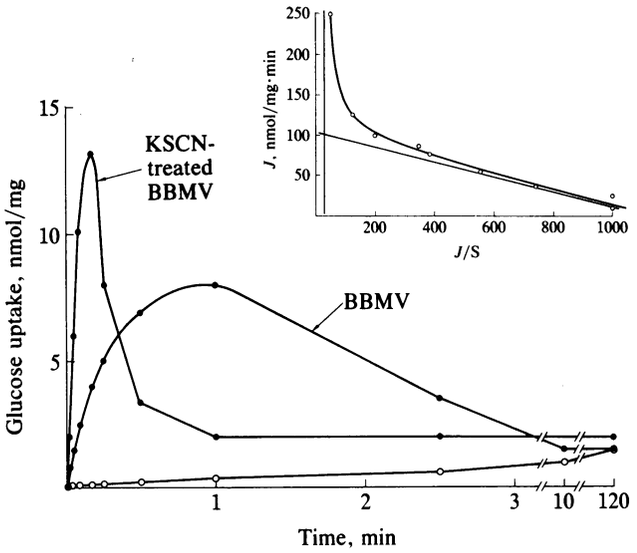

FIG. 1. Time course of D-glucose uptake. Vesicles were equilibrated in 290 mM mannitol, 10 mM NaCl or KCl, and 10 mM Hepes/Tris, pH 7.5. Uptake buffers contained 100 mM NaCl or KCl, 100 mM mannitol, 10 mM Hepes/Tris, pH 7.5, and 50 μM D-[6-^{3}H(N)]-glucose. Each data point is the mean of two estimates. Uptakes (nmol/mg of protein) were determined in BBMVs and KSCN-treated BBMVs in the presence (●) and absence (○) of Na^+ gradients. The glucose uptakes in the absence of Na^+ were essentially the same for treated and untreated vesicles. (*Inset*) Kinetics of D-glucose uptake into KSCN-treated BBMVs. Uptakes were measured at 2 sec for D-glucose concentrations of 10 μM to 10 mM, and the rates of uptake, J (nmol/mg·min), are plotted against J/S, in which S is the D-glucose concentration in mM. The plot was resolved into two components: one saturable and one diffusional. The J_{max} was 104 ± 16 nmol/mg·min, the K_m was 85 ± 7 μM, and the permeability was 30 ± 8 μl/mg·min. The error is expressed as SEM. The correlation coefficient for the line describing the fit to the data points, the diffusional and the saturable components, was 0.993.

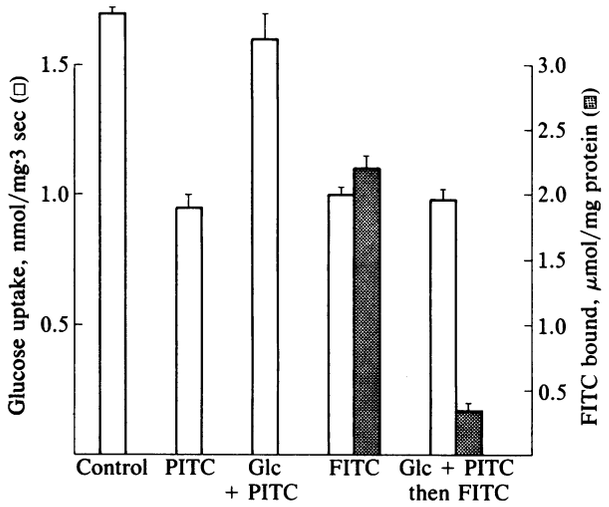

FIG. 2. Effect of PITC pretreatment on glucose transport and FITC binding. Vesicles (500 μg) treated with 50 μM FITC or 2 mM PITC in the presence or absence of 10 mM glucose (Glc) and 100 mM NaCl in 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA were then assayed for glucose transport. A sample of the PITC-treated membranes was also exposed to 50 μM FITC in 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA for 15 min in the dark at room temperature. Unreacted FITC and PITC were removed by centrifugation and the pellets were resuspended in 290 mM mannitol, 10 mM NaCl, and 10 mM Hepes/Tris, pH 7.5. Na^+-dependent glucose transport was assayed at room temperature, using 3-sec uptakes as in Fig. 1. FITC was determined spectrophotometrically at 490 nm in 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA. Results are presented as mean ± SEM.

The fluorescence response of FITC bound to the specific sites on the membrane to various cations is shown in Fig. 3. Addition of Na^+, but not K^+, results in a quenching of the fluorescence signal. The concentration dependence of the Na^+ quench response is given in Fig. 3*B*. Maximal quenching saturated at 20% with a $K_{0.5}$ of 10 mM and a Hill coefficient of 0.92. No quenching was observed with the addition of 100 mM D-glucose or 50 mM K^+, Cs^+, Li^+, Rb^+, or choline chlorides. Furthermore, FITC bound to nonspecific sites—i.e., bound in the presence of NaCl and D-glucose—exhibited no Na^+-dependent quenching. These results demonstrate that Na^+ produces a specific, saturable conformational change at one class of FITC binding sites.

FITC was also used to locate the Na^+-dependent D-glucose carrier on $NaDodSO_4$ gels. KSCN-treated brush border membranes were run on $NaDodSO_4$/7.5% polyacrylamide slab gels according to the method of Laemmli (11). The results shown in Fig. 4*A* indicate an FITC peak corresponding to 71,000 ± 3000 daltons (n = 3) for the major FITC binding protein in brush borders preincubated with 2 mM PITC and substrates followed by FITC. Elimination of the PITC pretreatment step (Fig. 4*B*) or removal of substrates during PITC pretreatment results in a reduction in the FITC bound at slice 19 and increases FITC binding to other membrane proteins. Pretreatment of membranes with the nonfluorescent FITC analog increases specific binding to the 71,000-dalton peptide 50% while reducing background binding an

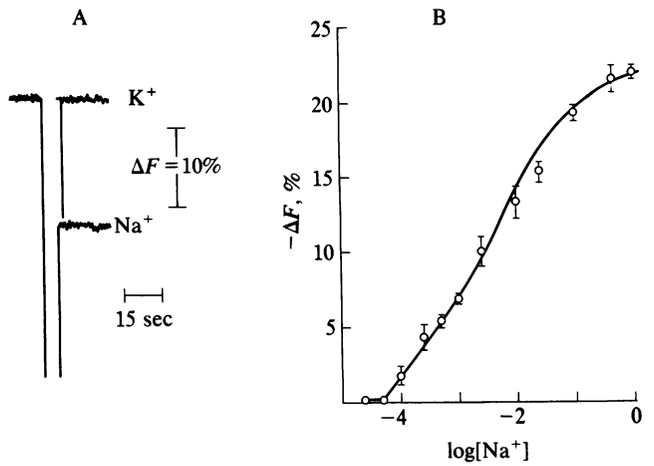

FIG. 3. Quenching of FITC fluorescence by Na^+. Membranes were pretreated with PITC in the presence of 100 mM NaCl and 10 mM D-glucose and then were treated with FITC in 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA. Fluorescence of 100 μg of FITC-treated membranes was recorded in 2 ml of 50 mM Tris·HCl, pH 9.2, and 2 mM EDTA at 525 nm upon excitation at 495 nm with slit widths of 4 nm. (*A*) Tracings of the fluorescence recording in the absence of salt and upon addition of 50 mM NaCl or KCl. Similar experiments showed that Cs^+, Rb^+, Li^+, choline, and K^+ all failed to quench the FITC fluorescence. (*B*) Dose–response curve of the Na^+-dependent quench in FITC fluorescence. The data points at each Na^+ concentration are the mean ± SD of five estimates on three different membrane preparations. Na^+ concentrations are molar.

additional 47%. Protection is substrate specific and limited to the glucose carrier, since neither proline nor phenylalanine (data not shown) cross-protect. These results suggest that the 71,000-dalton peptide is the Na^+-dependent glucose carrier.

DISCUSSION

Site-specific reagents coupled to fluorescent probes provide a useful tool in the study of protein conformational changes resulting from ligand binding. Previously these studies have been limited to homogeneous systems in which the protein of interest contributes 50–100% of the total protein. With regard to vesicle systems heterogeneous with respect to membrane protein, special strategies are required to ensure specificity to probe response. Reduction of probe nonspecific binding through the use of nonfluorescent probe analogs is an example of such a strategy and may be employed, provided the analog binds irreversibly and the protein of interest may be reversibly protected against the analog.

PITC is a useful analog that mimics the behavior of FITC in intestinal BBMVs. Both isothiocyanates bind irreversibly to BBMVs and the glucose carrier in the absence of substrates. Inhibition is eliminated by the addition of substrates, while nonspecific binding appears unaffected. Previous studies with isothiocyanates (14) have suggested that they react with a lysine group at or near the glucose site. Our results with FITC are consistent with this interpretation. Protection of the transporter against FITC and PITC requires both Na^+ and glucose and has the same ion and sugar specificity as transport. The ion sensitivity of the FITC bound to the glucose-protectable amino groups on the membrane further suggests probe coupling to the glucose carrier. Na^+, but not K^+, Cs^+, Rb^+, Li^+, or choline, quenches the FITC fluorescence. This fluorescence quench indicates a change in the local environment of the probe that is consistent with a change in protein conformation. The Na^+ quench is saturable with a single class of Na^+ binding sites and a $K_{0.5}$ of 10 mM. Transport of glucose by the high capacity carrier in brush borders is activated by *cis* Na^+, and the apparent glucose affinity increases with increasing Na^+ (13). It is, therefore, not unreasonable to conclude that the Na^+-dependent conformation change of the FITC binding protein is related to Na^+ activation of glucose transport. The FITC binding protein (71,000 daltons) is most likely the glucose carrier since it is very similar to the one purified to homogeneity by Semenza's group, using monoclonal antibodies (15), and identified through 4-azidophlorizin photoaffinity labeling (16). In agreement with Semenza's group, we estimate from specific FITC binding that this protein represents no more than 1% of the protein in the enriched BBMVs.

FITC offers a unique tool for probing the mechanism of the Na^+–glucose symporter, and it should allow us to define

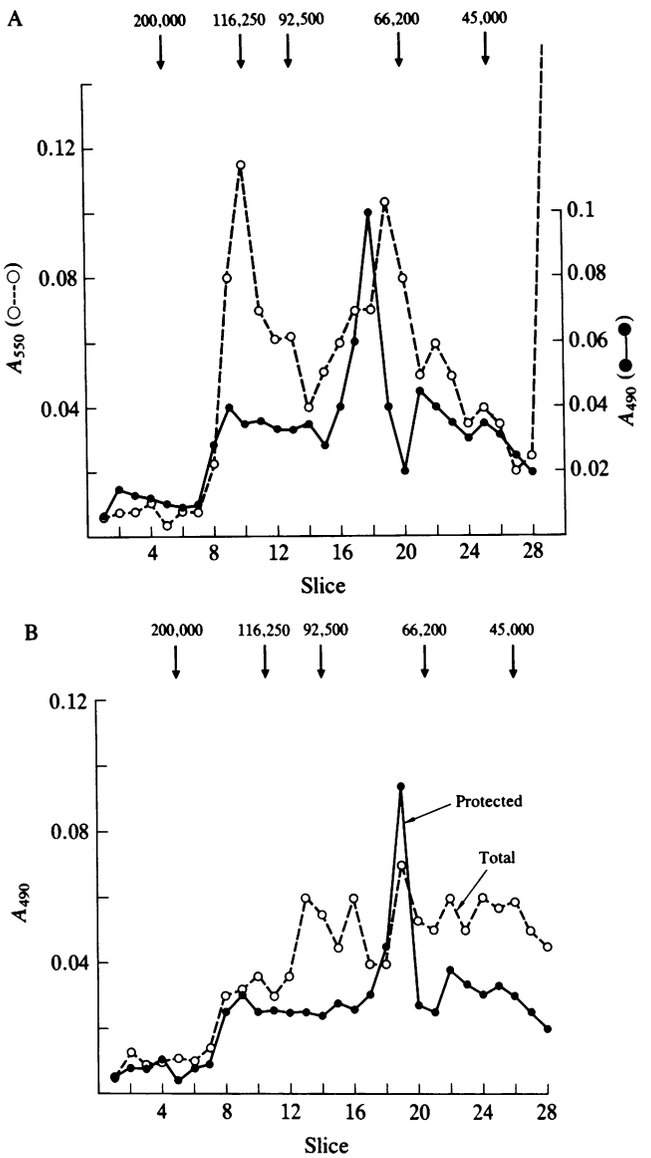

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of FITC-treated brush border membranes. Electrophoresis was performed with 100 μg (Coomassie blue staining) or 200 μg (FITC binding) of membrane protein on 7.5% slab gels. Molecular weight standards (Bio-Rad) were run in parallel and were stained with Coomassie blue and are indicated by the arrows. (*A*) Protein (broken line) and FITC (solid line) distributions were determined by measuring the absorbance of Coomassie blue (A_{550}) and FITC (A_{490}) of protein extracted from 0.5-cm gel slices. FITC-treated membranes were pretreated with PITC in the presence of 100 mM NaCl and 10 mM D-glucose. (*B*) FITC binding in the absence (broken line) and presence (solid line) of PITC pretreatment. A_{550} and A_{490} are plotted against slice number.

the identity of the FITC binding sites and measure the kinetics of the conformational changes. The use of coupled FITC derivatives should aid in the isolation of the symporter.

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