Lever, J. E. (1984) *Biochemistry 23*, 4697-4702.
Lever, J. E., Yoneyama, Y., Wu., J. S. R., Wygant, J., & Kennedy, B. G. (1986) in *Ion Gradient Coupled Transport* (Alvarado, F., & Van Os, C. H., Eds.) INSERM Symp. No. 26, pp 355-361, Elsevier, New York.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Malathi, P., & Preiser, H. (1983) *Biochim. Biophys. Acta 735*, 314-324.
Malathi, P., Preiser, H., Fairclough, P., Mallet, P. Z., & Crane, R. K. (1979) *Biochim. Biophys. Acta 554*, 259-263.
Malathi, P., Preiser, H., & Crane, R. K. (1980) *Ann. N.Y. Acad. Sci. 358*, 253-266.
Peerce, B. E., & Wright, E. M. (1984) *J. Biol. Chem. 259*, 14105-14112.
Peerce, B. E., & Wright, E. M. (1985) *J. Biol. Chem. 260*, 6026-6031.
Pretzman, C. I., Ralph, D., Mishler, L., & Bodine, J. (1985) *J. Immunol. Methods 83*, 301-307.
Schenk, D. B., & Leffert, H. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5281-5285.
Schmidt, U., Eddy, B., Frazer, C. M., Venter, J. C., & Semenza, G. (1983) *FEBS Lett. 161*, 279-283.
Semenza, G., Kessler, M., Hosang, M., Weber, J., & Schmidt, U. (1984) *Biochim. Biophys. Acta 779*, 343-379.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Turner, R. J., & Kempner, E. S. (1982) *J. Biol. Chem. 257*, 10794-10797.
Turner, R. J., & Moran, A. (1982) *J. Membr. Biol. 70*, 37-45.
Weber, J., & Semenza, G. (1983) *Biochim. Biophys. Acta 731*, 437-447.
Wu, J.-S. R., & Lever, J. E. (1987) *Biochemistry* (following paper in this issue).
Yoneyama, Y., & Lever, J. E. (1987) *J. Cell. Physiol. 131*, 330-341.

Monoclonal Antibodies That Bind the Renal Na^+/Glucose Symport System. 2. Stabilization of an Active Conformation†

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ABSTRACT: Conformation-dependent fluorescein isothiocyanate (FITC) labeling of the pig renal Na^+/glucose symporter was investigated with specific monoclonal antibodies (MAb's). When renal brush border membranes were pretreated with phenyl isothiocyanate (PITC), washed, and then treated at neutral pH with FITC in the presence of transporter substrates Na^+ and glucose, most of the incorporated fluorescence was associated with a single peak after resolution by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The apparent molecular mass of the FITC-labeled species ranged from 79 to 92 kDa. Labeling of this peak was specifically reduced by 70% if Na^+ and glucose were omitted. Na^+ could not be replaced by K^+, Rb^+, or Li^+. FITC labeling of this peak was also stimulated after incubation of membranes with MAb's known to influence high-affinity phlorizin binding, and stimulation was synergistically increased when MAb's were added in the presence of Na^+ and glucose. Substrate-induced or MAb-induced labeling correlated with inactivation of Na^+-dependent phlorizin binding. MAb's recognized an antigen of 75 kDa in the native membranes whereas substrate-induced FITC labeling was accompanied by loss of antigen recognition and protection from proteolysis. These findings are consistent with a model in which MAb's stabilize a Na^+-induced active conformer of the Na^+/glucose symport system.

Secondary active transport of glucose in renal and intestinal cells is mediated by a symport mechanism in which sugar movement across the plasma membrane is coupled to movement of Na^+ down its electrochemical potential gradient (Crane, 1977). Kinetic studies (Kaunitz & Wright, 1984) and inactivation studies (Weber & Semenza, 1983) of substrate translocation in intestinal membranes strongly support a coupling mechanism in which conformational changes in the symporter induced by Na^+ binding induce increased carrier affinity for glucose. Na^+-induced conformational changes in an intestinal brush border membrane protein identified as a good candidate for the glucose symporter were detected by using a substrate-protectable FITC[1] label as a reporter group (Peerce & Wright, 1984, 1985). However, due to difficult progress in the unequivocal identification of the protein(s) that catalyze Na^+/hexose symport, very little is known about the role of conformational changes in transporter function at the molecular level.

As an approach to develop highly specific probes to investigate the structure and function of the renal Na^+/glucose

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[1] Abbreviations: FITC, fluorescein isothiocyanate; PITC, phenyl isothiocyanate; MAb, monoclonal antibody; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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symporters, we have isolated and preliminarily characterized several monoclonal antibodies (MAb's) that recognize a 75-kDa antigen identified as a component of the renal Na^+/glucose symporter [preceding paper in this issue (Wu & Lever, 1987)]. In the present study, we have utilized two of these MAb's to investigate conformational changes in the Na^+/glucose symporter of pig renal brush border membranes. Results from this analysis can be interpreted in terms of a model in which MAb's stabilize an active conformation of the transporter generated in the presence of Na^+.

Materials and Methods

Materials. FITC and PITC were from Sigma. The sources of all other materials and the monoclonal antibodies used in this study are described in the preceding paper (Wu & Lever, 1987).

FITC Labeling of Renal Brush Border Membranes. Renal brush border membranes were prepared from pig kidney cortex by the method of Malathi et al. (1979) as modified by Wu and Lever (1987). Membranes, 0.3 mg, were incubated at room temperature in 250-μL volumes with 2.0 mM phenyl isothiocyanate (PITC) in 50 mM Tris-HCl, pH 7.0, and 2 mM EDTA (buffer A). After 40 min, the incubation was terminated by addition of 1 mL of buffer A, and samples were centrifuged at 38000g for 30 min. All subsequent steps were carried out in the dark. Membranes were resuspended in 0.2 mL of buffer A in the presence or absence of the indicated additions and incubated for 30 min with the indicated concentration of fluorescein isothiocyanate (FITC). Where indicated, membranes were incubated with 50 μL of 10 mM K^+-HEPES, pH 7.2, and 5 mM $MgCl_2$ either with or without MAb for 30 min at room temperature before FITC treatment. The incubation was terminated by addition of 1 mL of buffer B-1 (0.05 M sucrose, 2 mM $MgCl_2$, 4 mM K^+-HEPES, pH 7.2) and centrifugation at 38000g for 30 min. Membranes were washed again with 1 mL of the same buffer and then either resuspended in buffer B for assay of [^{3}H]phlorizin binding or solubilized in SDS–PAGE sample buffer and resolved by 7.5% acrylamide SDS–PAGE. Gels were photographed by using Polaroid type 55 film under UV light in order to visualize fluorescent bands.

Fluorescence eluted from 2-mm gel slices by the procedure of Peerce and Wright (1984) was measured at room temperature on a Perkin-Elmer LS-5 fluorescence spectrophotometer at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. Slit widths were set at 5 nm. The fixed scale was set at 100 or as otherwise indicated.

Assay of Phlorizin Binding. Phlorizin binding to membranes was assayed as described in the preceding paper (Wu & Lever, 1987).

Results

Inactivation of Phlorizin Binding by PITC Treatment: Effect of pH and Substrates. The covalent reaction of PITC and FITC with the ϵ-amino residue of lysine, which has a pK_a near 10, is optimal above pH 9.2 (Sen et al., 1981). Peerce and Wright (1984) have shown that, at pH 9.2, inactivation of phlorizin binding activity of intestinal brush border membranes was protected by inclusion of the substrates Na^+ and glucose during the PITC treatment. In contrast to the intestinal Na^+/glucose symporter, which is much less sensitive to pH changes in this range (Toggenberger et al., 1978), renal Na^+/glucose symporter activity is markedly sensitive to pH in the range 6–8, with an optimum at pH 7. At pH 9.2, Na^+-dependent hexose transport activity and Na^+-dependent phlorizin binding in renal membranes are inactive (Lever, 1984). Thus, it would be expected that substrate protection would not be observed during PITC treament of renal membranes at alkaline pH. Incubation of renal brush border membranes at pH 9.2 for 30 min with various concentrations of PITC resulted in a dose-dependent inactivation of phlorizin binding activity, with an IC_{50} of 0.3 mM (concentration required for 50% inhibition) (Figure 1A). In this experiment, membranes were washed after PITC treatment and resuspended in phlorizin binding assay mixture at pH 7.2 for binding assay. Protection by Na^+ and glucose was not observed even at greatly increased substrate levels in the presence of suboptimal PITC concentrations: 50 mM glucose, 20 μM phlorizin, 100 mM NaCl, and 0.2 mM PITC (not shown).

At pH 7.0, phlorizin binding required higher PITC concentrations (IC_{50} > 4 mM) for inactivation than at pH 9.2, as expected on the basis of the lower reactivity of isothiocyanates at neutral pH (Figure 1A). Inactivation reached a

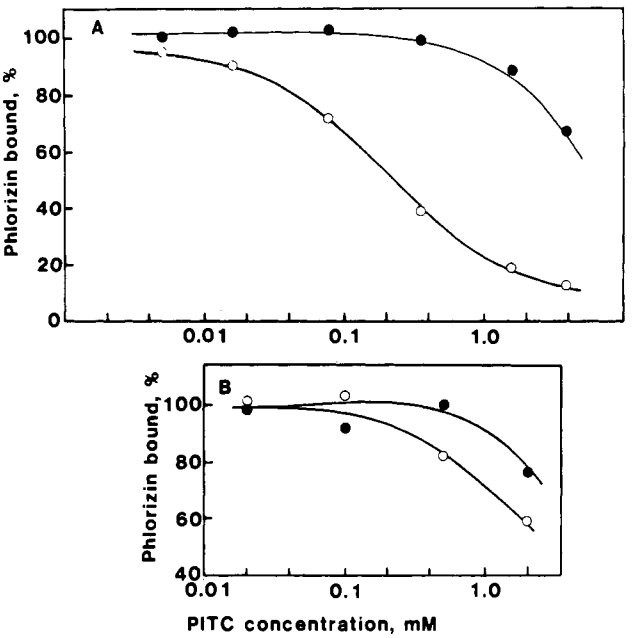

FIGURE 1: Inactivation of phlorizin binding to renal membranes after PITC pretreatment: effect of pH and transport substrates. (A) Membranes were incubated with the indicated concentration of PITC for 30 min at room temperature at either (○) pH 9.2 or (●) pH 7.0, washed, and assayed for [^{3}H]phlorizin binding as described under Materials and Methods. (B) Membranes were pretreated 40 min with 2 mM PITC at pH 7.0 in the absence of substrates and then treated with the indicated concentration of PITC in the presence (○) or absence (●) of 10 mM glucose and 100 mM NaCl. Total phlorizin binding activity is shown, without subtraction of Na^+-free binding, which was 14.5% of total binding. In (A), 100% refers to 10.41 pmol/mg. In (B) 100% refers to 7.81 pmol/mg, the activity of membranes after the first PITC pretreatment. Membranes preincubated with substrates alone showed activity identical with that of control membranes.

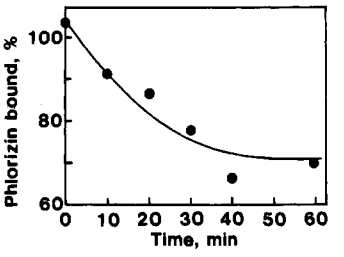

FIGURE 2: Time dependence of PITC inactivation of phlorizin binding. Membranes were incubated for the indicated time with 1.5 mM PITC at pH 7.0. One hundred percent refers to 18.9 pmol/mg, the phlorizin binding activity of membranes incubated without PITC, without subtraction of Na^+-free binding (14% of total).

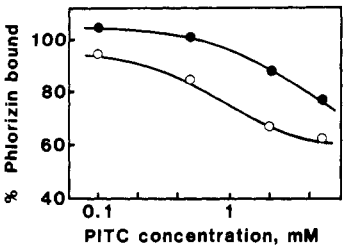

FIGURE 3: Inactivation of phlorizin binding by PITC is increased in the presence of MAb 11A3D6. Membranes were incubated with 15 μg of purified 11A3D6 (○) or buffer (●) for 30 min at room temperature, followed by 30-min incubation with the indicated concentration of PITC at pH 7.0. Samples were washed and assayed for phlorizin binding activity as described under Materials and Methods. Total phlorizin binding activity is shown. One hundred percent refers to 15.7 pmol/mg in the case of MAb-treated samples and 13.4 pmol/mg for controls.

plateau value after 40-min incubation with PITC (Figure 2).

Inclusion of glucose and NaCl during PITC treatment at neutral pH increased the extent of inactivation of phlorizin binding. At 1.5 mM PITC, phlorizin binding was inactivated by 10% in the absence of substrates and by 50% in the presence of 10 mM glucose and 100 mM NaCl (not shown). In order to examine this substrate-dependent component in more detail, membranes were first incubated with 2.0 mM PITC for 40 min and then washed and incubated in the presence of various concentrations of PITC from 0.01 to 2 mM in the presence or absence of 10 mM glucose and 100 mM NaCl (Figure 1B). After resuspension in assay buffer, phlorizin binding activity of each sample was measured. These data indicate that, at a given PITC concentration in this range, greater inactivation of phlorizin binding was observed in the presence of cotransporter substrates. Membranes preincubated with substrates alone showed identical phlorizin binding activity to control untreated membranes (not shown).

These observations are compatible with the hypothesis that, at neutral pH, the phlorizin binding site undergoes a conformational change in the presence of Na^+ and glucose. Reaction of previously buried residues with PITC results in inactivation of phlorizin binding. At alkaline pH, where the transporter is inactive, this conformational change does not occur and therefore the increased inactivation in the presence of substrate is not observed.

Effect of MAb's on Inactivation by PITC. In the experiment shown in Figure 3, membranes were preincubated with or without MAb 11A3D6, shown in the preceding paper to bind to a 75-kDa polypeptide identified as a component of the Na^+/glucose symporter (Wu & Lever, 1987). Membranes were then treated at pH 7 with the indicated concentrations of PITC and assayed for phlorizin binding activity. An increased inactivation of phlorizin binding after treatment with a given PITC concentration was observed in the case of membranes exposed to the transporter-specific MAb compared with controls. This effect was not observed when Sigma mouse IgG_{2b} was used (data not shown). Thus, binding of specific MAb to the transporter induced a similar increase in sensitivity to inactivation by PITC to that observed in the presence of Na^+ and glucose.

Substrate-Dependent Labeling by FITC. These observations suggested a two-step approach for specific labeling of the phlorizin binding site. In the first step, samples were treated with 2 mM PITC at neutral pH in order to block nonspecific residues. Then, in the second step, washed membranes were exposed to FITC in the presence of glucose and NaCl in order to put a fluorescent tag on residues newly exposed in the presence of substrates.

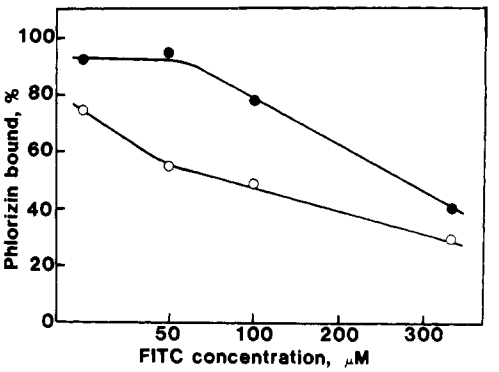

FIGURE 4: Substrate-inducible inactivation of phlorizin binding by FITC. Membranes were pretreated with 2 mM PITC, washed, and then incubated with the indicated concentrations of FITC in the presence (○) and absence (●) of 50 mM glucose and 500 mM NaCl as described under Materials and Methods before assay for total phlorizin binding activity. Results are shown without subtraction of Na^+-free binding, which was 15% of total. One hundred percent refers to 8.28 pmol/mg, the activity observed after the PITC pretreatment without addition of FITC.

Table I: Na^+-Specific Stimulation of FITC Incorporation into a Membrane Protein[a]

glucose (mM)	salt (100 mM)	n	fluorescence (%)
0		9	100
50	NaCl	9	167 ± 21.9
50	KCl	3	88.3 ± 2.70
50	RbCl	1	77.5
50	LiCl	1	90.1
50		4	124 ± 9.51
0	NaCl	3	175 ± 32.3

[a] Incorporation was carried out as described in the legend to Figure 7, with substitution of salts and glucose as indicated. Fluorescence values are mean ± SE, where n indicates the number of experiments.

We first demonstrated that FITC produced a similar substrate-dependent inactivation of Na^+-dependent phlorizin binding to that observed after PITC treatment. Figure 4 shows that when membranes were pretreated with 2 mM PITC and then exposed to various FITC concentrations in the presence and absence of 50 mM glucose and 500 mM NaCl, inactivation of Na^+-dependent phlorizin binding was significantly augmented in the presence of substrates.

Membranes that had been pretreated with 2 mM PITC and then labeled with 500 μM FITC in the presence and absence of NaCl and glucose were analyzed by SDS–PAGE. When the gel was visualized under UV light, one intense fluorescent band was visible at 79 kDa with a much less intense diffuse band at 39 kDa (Figure 5A). Analysis of Coomassie Blue staining patterns of two parallel samples after resolution by SDS–PAGE revealed a marked shift in mobility of a large number of membrane proteins after treatment with PITC and FITC (Figure 5A, lanes C and D). Such changes in apparent molecular mass would be expected after covalent incorporation of isothiocyanate residues into proteins. Thus, apparent mobility on SDS–PAGE is not a sufficient criterion to identify the 79-kDa FITC-labeled species as the glucose cotransporter.

Quantitation of fluorescence eluted from gel slices revealed that fluorescence associated with the 79-kDa FITC-labeled species was significantly increased when Na^+ and glucose were included during FITC treatment (Figure 5A). In different experiments, the molecular mass of this band varied from 79 to 92 kDa.

Figure 6 shows that substrate-stimulated incorporation of fluorescence into the major band increased as a function of FITC concentration, reaching saturation at 1 mM FITC. The inset shows a saturation curve of the net incorporation induced

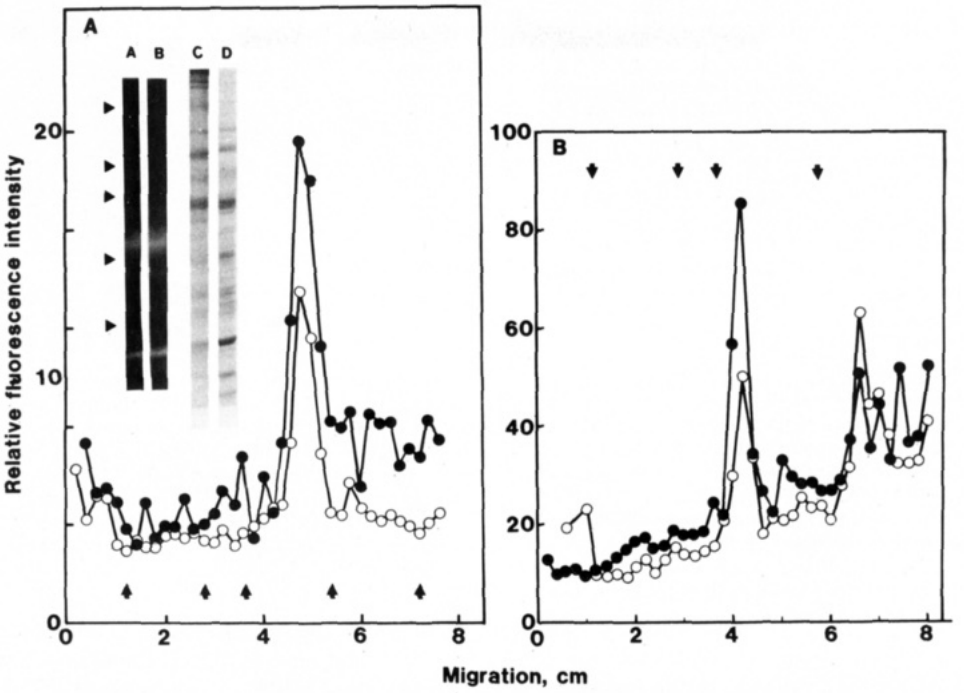

FIGURE 5: (A) Analysis of FITC-labeled membranes by SDS–PAGE. Membranes were pretreated with 2 mM PITC and then labeled with 500 μM FITC plus (●) (lanes B and C) or minus (○) (lane A) 10 mM glucose and 100 mM NaCl as described under Materials and Methods. Samples were resolved by SDS–PAGE (7.5% acrylamide), and the gel was photographed under UV light (lanes A and B). Fluorescence eluted from 2-mm slices is plotted vs. distance of migration. The fixed scale of the spectrophotometer was set at 10. The arrows indicate the positions of molecular mass markers at 45, 66, 92.5, 130, and 200 kDa, respectively. The position of the Na^+- and glucose-stimulated FITC-labeled peak corresponds to an apparent molecular mass of 79 kDa. In other experiments the position of this peak varied in the range 83–93 kDa. Coomassie Blue staining patterns of FITC-labeled (lane C) and untreated (lane D) membranes are shown. (B) Stimulation of FITC labeling of the major peak by a MAb that inhibits Na^+-dependent phlorizin binding. Membranes were pretreated with 2 mM PITC, washed, and then incubated for 30 min at room temperature with (●) or without (○) the IgM MAb 18H10B12 which had been fractionated on a PD-10 column. Membranes were then labeled with 50 μM FITC plus 50 mM glucose and 100 mM NaCl and resolved by SDS–PAGE, and fluorescence eluted from 2-mm gel slices was analyzed as a function of distance of migration. Arrows indicate the positions of molecular mass markers. The major peak corresponds to an apparent molecular mass of 88 kDa.

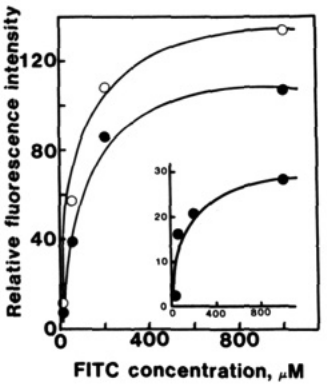

FIGURE 6: Na^+- and glucose-stimulated fluorescence labeling of the major peak as a function of FITC concentration. Membranes were pretreated with 2 mM PITC and then incubated with the indicated concentration of FITC in the presence (○) or absence (●) of 10 mM glucose and 100 mM NaCl. Samples were washed as described, solubilized in SDS–PAGE sample buffer, and resolved by 7.5% acrylamide SDS–PAGE. Fluorescence eluted from 2-mm gel slices was measured. The total fluorescence associated with the major FITC-labeled peak is shown after subtraction of background, basal fluorescence. The inset shows net substrate-stimulated increase in fluorescence after subtraction of incorporation observed in the absence of Na^+ and glucose.

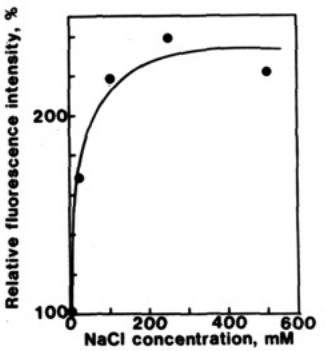

FIGURE 7: Stimulation of FITC labeling as a function of Na^+ concentration. Membranes were pretreated with 2 mM PITC and then exposed to 50 μM FITC in the presence of the indicated concentration of NaCl plus 50 mM glucose. Fluorescence content of the major peak is shown after analysis of membranes by SDS–PAGE and elution of fluorescence from gel slices as described in the legend to Figure 5. One hundred percent refers to samples labeled with FITC in the absence of NaCl and glucose.

by cotransporter substrates after subtraction of values observed in the absence of Na^+ and glucose. Incorporation of fluorescence into this band increased as a function of NaCl concentration, reaching saturation at 100 mM NaCl (Figure 7). NaCl could not be replaced by either RbCl, LiCl, or KCl (Table I). A small but reproducible stimulation was observed in the presence of glucose alone, but stimulation by glucose was blocked in the presence of Rb^+, Li^+, or K^+ (Table I). The significant and reproducible stimulation by Na^+ and glucose of FITC labeling strongly implicated this FITC-labeled species as the cotransporter.

In order to obtain additional support for this interpretation, we examined the interaction of the FITC-labeled species with MAb's shown in the preceding paper (Wu & Lever, 1987) to influence high-affinity, Na^+-dependent phlorizin binding. Membranes were pretreated with PITC and then exposed to FITC ± substrates, as described in the legend to Figure 3. Then samples were analyzed by Western blot analysis. Examination of the nitrocellulose sheet under UV light after

Table II: MAb's Stimulate FITC Incorporation into a Membrane Protein[a]

addition	rel fluoresence of major FITC-labeled peak (%)	
	500 μM FITC	50 μM FITC
none	100	100
Na^+, glucose	140	152
11A3D6 minus Na^+, glucose	157	150
11A3D6 plus Na^+, glucose	171	244
18H10B12 minus Na^+, glucose	proteolysis	proteolysis
18H10B12 plus Na^+, glucose	303	290
11F7B2 minus Na^+, glucose	110	ND[b]
11F7B2 plus Na^+, glucose	151	ND[b]
nonspecific mouse IgG_{2b}		110

[a] Membranes, pretreated with 2 mM PITC and washed as described under Materials and Methods, were incubated with the indicated MAb or buffer control for 30 min at room temperature and then treated for 30 min with the indicated concentration of FITC in the presence or absence of 50 mM glucose and 100 mM NaCl. MAb 11A3D6, 20 μg, was purified by protein A affinity chromatography; 18H10B12 and 11F7B2, 0.3 mg each, were fractionated on PD-10 columns; affinity-purified nonspecific mouse MAb IgG_{2b}, 25 μg, was from Sigma. At each FITC concentration, a representative experiment from three determinations is shown. [b] ND, not determined.

electrotransfer confirmed the cotransfer of the fluorescence-labeled major FITC-labeled species. MAb 11A3D6 detected a 75-kDa band in control membrane samples without PITC or FITC treatment, but this band was not visible in samples exposed to FITC in the presence of substrates (not shown). In a different approach, immunoprecipitation of solubilized FITC-labeled membranes was attempted with a different transporter-specific MAb, 18H10B12. A 75-kDa antigen was immunoprecipitated from untreated membranes but not from FITC-labeled membranes (not shown). These findings indicate that the substrate-induced FITC labeling destroys the antigenic site recognized by MAb's that bind the glucose cotransporter. Treatment of the immunoprecipitate with FITC resulted in an increase in apparent molecular mass on SDS–PAGE from 75 to 82.6 kDa (not shown), suggesting that this covalent modification could account for the increased apparent size of the FITC-labeled species compared with the 75-kDa antigen.

The fluorescence of FITC-labeled membranes prepared in the presence of substrates, and then washed free of substrates, was not quenched by addition of Na^+ and glucose (not shown).

Stimulation of FITC Labeling by MAb's Specific for the Symporter. Independent, compelling evidence that the major FITC-labeled band represented the Na^+/glucose symporter was obtained by using our panel of MAb's that interact with the high-affinity Na^+-dependent phlorizin binding site. We investigated the possibility that these MAb's would either stimulate or block substrate-induced FITC labeling. Figure 5B shows an experiment in which membranes were pretreated with PITC, washed, and then incubated with MAb's. After 30-min incubation with antibody, membranes were then treated with either 50 or 500 μM FITC in the presence of 50 mM glucose and 100 mM NaCl. Samples were resolved by SDS–PAGE, and incorporation of fluorescence was quantitated in gel slices. Membranes that had been preincubated with MAb 18H10B12, an IgM known to strongly inhibit Na^+-dependent phlorizin binding, exhibited an additional 150% augmentation of incorporation of fluorescence into the major FITC-labeled species compared with samples incubated with FITC and substrates alone.

Table II summarizes the effects of a number of control MAb's and MAb's known to inhibit phlorizin binding on the incorporation of fluorescence into the major FITC-labeled species. Values are obtained by analysis of fluorescence of

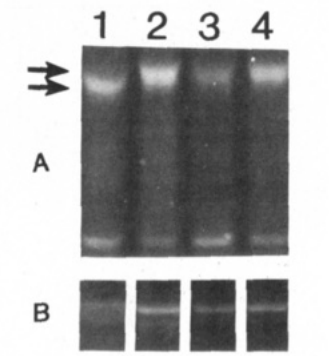

FIGURE 8: Substrate-protectable proteolysis of the major FITC-labeled species after MAb binding. Membranes were pretreated with 2 mM PITC, washed, incubated in the presence or absence of substrates (10 mM glucose and 100 mM NaCl) with the indicated MAb, and then treated with either (A) 50 or (B) 500 μM FITC ± substrates. After resolution by SDS–PAGE, the gel was photographed under UV light. Lane 1, 18H10B12 minus substrates; lane 2, 18H10B12 plus substrates; lane 3, 11F7B2 minus substrates; lane 4, 11F7B2 plus substrates.

gel slices after resolution of labeled membranes by SDS–PAGE and represent total fluorescence associated with the major peak. Stimulation was specific for this band, and samples did not differ significantly in other regions of the gel. MAb 11A3D6, which interacts with the phlorizin receptor, stimulated the labeling of the peak by 50% in the absence of substrates, the same stimulation as produced by NaCl and glucose in the absence of antibody. Addition of 11A3D6 in the presence of Na^+ and glucose had a synergistic effect, producing a greater stimulation than that observed by either antibody alone or substrates alone. This was most noticeable at 50 μM FITC. Control MAb 11F7B2, which does not affect phlorizin binding but recognizes another antigenic site in the same membranes, did not stimulate FITC labeling of the peak, nor did it affect the stimulation produced by substrates. Similarly, a commercial preparation of mouse MAb IgG_{2_b} did not stimulate FITC labeling.

Substrate-Protectable Proteolysis. Extensive proteolysis of the major FITC-labeled species was observed in the case of membranes incubated with MAb 18H10B12 (treated by gel filtration on PD-10 columns); therefore, it was not possible to evaluate FITC incorporation into this band (Table II). Proteolysis was visible as a decrease in apparent molecular mass (shown by arrows, Figure 8A, lane 1) or as a broadening of the band and decrease in intensity (Figure 8B, lane 1). By contrast, no proteolysis was observed after incubation of membranes with a control MAb ascites fluid, 11F7B2 (Figure 8; Table II). However, when substrates Na^+ and glucose were added together with MAb 18H10B12, the major FITC-labeled species was protected from proteolysis (Figure 8, lane 2; Table II). Under these conditions, labeling of the major FITC-labeled species was 2.9 times higher than that of corresponding control samples (Table II). Taken together, these observations strongly implicate the major FITC-labeled band as the glucose cotransporter.

By contrast, incubation of untreated membranes with MAb's 18H10B12 and 11F7B2 ascites fluid resulted in proteolysis of the 75-kDa antigen detected by Western blot analysis which was not protectable by substrates [preceding paper (Wu & Lever, 1987)]. Possibly covalent modifications resulting from PITC treatment protect nonspecific sites from proteolysis. Binding of the specific MAb 18H10B12 to isothiocyanate-treated membranes would induce a conformational change, exposing a new site for cleavage by proteases contained in the antibody preparation, while this effect is not seen with a closely

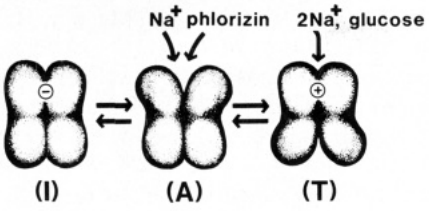

FIGURE 9: Conformation-dependent FITC labeling of the renal glucose symporter: a hypothesis. Assignment of a net negative charge to the inactive form (I), electrical neutrality to the activated, nontranslocating, phlorizin-binding form (A), and a net positive charge to the active translocating complex (T) is based on previous studies of Na^+ stoichiometry and response to membrane potential (Lever, 1984).

matched control MAb, 11F7B2, which does not bind this antigen or induce this conformational change. Binding of substrates would mask this new specific cleavage site from proteolysis in isothiocyanate-treated membranes, but this protective effect would not be visible against the background of nonspecific cleavage site available in membranes not pretreated with isothiocyanates.

Discussion

Several lines of evidence support the identification of a major FITC-labeled peptide of renal brush border membranes as the Na^+/glucose symporter. Incorporation of FITC into this band was significantly stimulated in the presence of symporter substrates Na^+ and, to a lesser extent, glucose. Furthermore, two different MAb's, shown in the preceding paper (Wu & Lever, 1987) to interact with the high-affinity, Na^+-dependent phlorizin binding site of the symporter, mimicked the effects of Na^+ and glucose in stimulating FITC labeling of this species. A synergistic further stimulation was observed when substrates were added together with specific MAb's. Stimulation was specific for Na^+ among alkali cations tested, and the Na^+ concentration dependence of FITC labeling paralleled that for activation of Na^+/glucose symport. This conclusion was further reinforced by the observation that Na^+ and glucose protected the FITC-labeled peptide from proteolysis.

Considerable evidence indicates that renal and intestinal membranes may each contain two distinct Na^+/glucose symporters [reviewed in Semenza et al. (1984)]. Furthermore, while renal and intestinal Na^+/glucose symporters broadly resemble each other in general features of the symport mechanism, they differ sharply in specific functional parameters. Intestinal glucose symport is largely insensitive to pH in the range of 6.5–9.5 (Toggenberger et al., 1978) while renal glucose symport activity declines sharply above pH 7.5 (Lever, 1982). These systems also differ 10-fold in affinity for phlorizin (Semenza et al., 1984). A striking example of functional differences between the two systems is revealed by conformation-dependent FITC labeling. Thus, while the intestinal glucose symporter exhibited Na^+- and glucose-*protectable* FITC labeling at pH 9.2 (Peerce & Wright, 1984, 1985), this could not be demonstrated in the case of renal symport activity, presumable due to the known differences in pH sensitivity between the two systems. Rather, we observed substrate-*inducible* FITC labeling of a polypeptide at neutral pH. These differences may also reflect the possibility that different functional groups are labeled with FITC at each pH (Sen et al., 1981).

A working hypothesis to explain these observations is represented schematically in Figure 9. In this view, the renal symporter exists in an equilibrium between an inactive conformation (I), an activated, nontranslocating form that can bind phlorizin (A), and an active translocating complex conformation (T). In the presence of Na^+, the accumulation of active conformers is favored. The conformational change upon activation is accompanied by exposure of previously buried residues which can be selectively labeled by FITC. MAb's to the symporter stabilize an active conformation. A possible mechanism for activation by MAb's is preferential binding to an active conformation, forming a tight complex that pulls the equilibrium in favor of accumulation of an active conformer. Alternately, Mab's may first bind the inactive form, triggering a conformational charge leading to activation, or Mab's may stabilize by binding a transition-state conformation of the transporter. Thus, MAb binding is accompanied by stimulation of FITC labeling and enhancement of PITC inactivation of phlorizin binding, mimicking the effects of Na^+ and glucose. This would also explain the stimulation of Na^+-dependent phlorizin binding in the case of MAb 11A3D6 (preceding paper). While MAb 18H10B12 stimulated FITC labeling yet inhibited [^{3}H]phlorizin binding (preceding paper), these observations are still consistent with stabilization of an active conformer, since this antibody is an IgM (molecular mass 900 kDa) which may sterically hinder the phlorizin binding site. Preliminary evidence that binding of ^{3}H-labeled MAb 11A3D6 to membranes is stimulated by Na^+ suggests that this antibody recognizes an epitope revealed after Na^+ activation (Wu and Lever, unpublished results).

We observed that binding of MAb 11A3D6 significantly decreased the sensitivity of symport to inhibition by phlorizin, expressed as a 3-fold increase in the K_i for transport inhibition by phlorizin (preceding paper). This MAb did not protect transport by blocking access of phlorizin to its binding site; in fact, phlorizin binding was stimulated in the presence of MAb 11A3D6. Furthermore, in the absence of phlorizin, this MAb had no detectable effect on glucose symport. One explanation for these findings is that MAb 11A3D6 uncouples glucose symport from inhibition by bound phlorizin by stabilizing a conformer associated with the active, translocating complex. The mechanism of phlorizin inhibition of symport is not fully understood and cannot be completely accounted for by its competitive interactions with sugar substrates. Semenza et al. (1984) and Lever (1984) have proposed that phlorizin binding locks the transporter in an uncharged, nontranslocating complex.

While MAb's imitated conformational effects of substrates on carrier activation monitored by exposure of residues to isothiocyanates, antibodies could not replace the Na^+ requirement for activation of symport activity. Thus, MAb's had no measurable effect on transport in the presence or absence of Na^+ and did not affect Na^+-independent phlorizin binding. These observations suggest that the obligatory role of Na^+ in the symport mechanism as cosubstrate and determinant of the net charge of the translocating complex cannot be replaced by MAb stabilization of an active conformation.

Studies using apical membrane vesicles from LLC-PK_1 cells indicated that Na^+/hexose symport exhibited a stoichiometry of 2 Na^+:1 glucose, whereas the Na^+ stoichiometry of phlorizin binding was 1:1 (Lever, 1984). Imposition of an interior-negative membrane potential in vesicles increased the number of Na^+-dependent phlorizin binding sites, consistent with a model in which the unloaded transporter is negatively charged and its orientation influenced by membrane potential (Lever, 1984). Since a similar stimulation of phlorizin binding and FITC labeling by MAb's was observed in both sealed and leaky membrane preparations, it is unlikely that Mab effects are due to immobilization of the carrier in an outwardly facing orientation. The lack of effect of antibody binding per se on transport rates suggests that symport does not occur by a

rotating carrier mechanism.

This model is based on the generally accepted but not rigorously proven assumption that high-affinity, Na^+-dependent phlorizin binding sites are localized in the same polypeptide that mediates Na^+/glucose symport. Previous attempts at solubilization and reconstitution of this transport system have failed to recover comparable activities of both Na^+-dependent sugar uptake and Na^+-dependent phlorizin binding in the same preparation (Koepsell et al., 1983). These MAb's will provide useful probes to investigate the possible role of homologous and heterologous subunit interactions in the function of this system.

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REFERENCES

Crane, R. K. (1977) *Rev. Physiol., Biochem. Pharmacol. 78*, 99-159.

Gibbs, E. M., Hosang, M., Reber, B. F. X., Semenza, G., & Diedrich, D. F. (1982) *Biochim. Biophys. Acta 688*, 547-556.

Kaunitz, J. D., & Wright, E. M. (1984) *J. Membr. Biol. 79*, 41-51.

Koepsell, H., Menuhr, H., Ducis, I., & Wissmuller, T. F. (1983) *J. Biol. Chem. 258*, 1888-1894.

Koepsell, H., Korn, K., Ferguson, D., Menuhr, H., Ollig, D., & Haase, W. (1984) *J. Biol. Chem. 259*, 6548-6558.

Koepsell, H., Neeb, M., & Madrala, A. (1986) in *Ion Gradient Coupled Transport* (Alvarado, F., & Van Os, C. H., Eds.) INSERM Symp. No. 26, pp 117-125, Elsevier, New York.

Lever, J. E. (1982) *J. Biol. Chem. 257*, 8680-8686.

Lever, J. E. (1984) *Biochemistry 23*, 4697-4702.

Malathi, P., Preiser, H., Fairclough, P., Mallet, P. Z., & Crane, R. K. (1979) *Biochim. Biophys. Acta 554*, 259-263.

Peerce, B. E., & Wright, E. M. (1984) *J. Biol. Chem. 259*, 14105-14112.

Peerce, B. E., & Wright, E. M. (1985) *J. Biol. Chem. 260*, 6026-6031.

Pretzman, C. I., Ralph, D., Mishler, L., & Bodine, J. (1985) *J. Immunol. Methods 83*, 301-307.

Schmidt, U. M., Eddy, B., Frazer, C. M., Venter, J. C., & Semenza, G. (1983) *FEBS Lett. 161*, 279-283.

Semenza, G., Kessler, M., Hosang, M., Weber, J., & Schmidt, U. (1984) *Biochim. Biophys. Acta 779*, 343-379.

Sen, P. C., Kapakos, J. G., & Steinberg, M. (1981) *Arch. Biochem. Biophys. 211*, 652-661.

Toggenberger, G. Kessler, M., Rothstein, A., Semenza, G., & Tannenbaum, C. (1978) *J. Membr. Biol. 40*, 269-290.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.

Turner, R. J., & Moran, A. (1982) *J. Membr. Biol. 70*, 37-45.

Weber, J., & Semenza, G. (1983) *Biochim. Biophys. Acta 731*, 437-447.

Wu, J.-S. R., & Lever, J. E. (1987) *Biochemistry* (preceding paper in this issue).

Enzyme II of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Protein–Protein and Protein–Phospholipid Interactions†

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ABSTRACT: The mannitol-specific enzyme II (EII), purified free of phospholipid, exhibits a concentration dependence in its specific activity with P-HPr and mannitol as the donor and acceptor substrates, respectively. This concentration dependence, previously observed only in the case of the mannitol ↔ mannitol phosphate exchange reaction, indicates that an oligomeric form of the enzyme is responsible for catalyzing the phosphorylation reaction (P-HPr + mannitol → mannitol-P + HPr) as well as the exchange reaction. Kinetic analysis revealed that the monomeric enzyme has a much lower specific activity than the associated species. The specific activity can be increased by raising the steady-state level of phosphorylation of EII and also by adding phospholipid, demonstrating that phosphorylation and the binding of phospholipid facilitate the association process. Kinetic measurements and fluorescence energy transfer measurements demonstrate a strong preference of EII for phospholipids with specific head group and fatty acid composition.

Enzymes II (EII) of the bacterial phosphoenolpyruvate-dependent phosphotransferase systems are responsible for phosphorylating and transporting their specific sugar substrates across the cytoplasmic membrane. Four species of this enzyme have been purified from different microorganisms, *Escherichia coli* EII^{Mtl} (Jacobson et al., 1979, 1983a), *Staphylococcus aureus* EII^{Lac} (Schafer et al., 1981), *Salmonella typhimurium* EII^{Glc} (Erni et al., 1982), and *E. coli* EII^{Man} (Erni & Zanolari, 1985).

Two of these EII species have been observed to occur as oligomers. Erni et al. (1982) suggested that purified EII^{Glc} occurred as a dimer or trimer. The enzyme electrophoresed

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