in a NOESY spectrum obtained at a longer mixing time. The interdependence of β-sheet MCD patterns will also be useful. β-Sheets on average consist of 6.45 residues per strand and 4.7 strands per sheet (Sternberg & Thornton, 1977). Thus the loss of fidelity in the parallel sheet patterns obtained at long mixing times will be compensated by the appearance of other MCD patterns in the sheet structure formed by one (or both) of the strands with other strands in the sheet. This highly cooperative and confirmatory behavior was observed in the MCD analysis of human ubiquitin based upon a single NOESY spectrum (Di Stefano & Wand, 1987).

MCD Algorithm

These observations lead us to propose the following algorithm for applying the main-chain-directed assignment strategy to the analysis of 2D ^{1}H NMR spectra of proteins.

(1) NH–C_αH–C_βH units: define all apparent NH–C_αH–C_βH J-coupled sets through analysis of J-correlated spectra.

(2) Helix: (a) search for all groups of two consecutive interlocking helical MCD patterns; (b) extend each of these groups in both directions; (c) remove from further consideration all NOEs exclusively involving the main-chain protons of these residues.

(3) Antiparallel sheet: (a) search for the full antiparallel sheet pattern; (b) extend each full pattern by application of the hybrid and, when present, additional full antiparallel sheet patterns; (c) remove from further consideration all NOEs exclusively involving the main-chain protons of these residues.

(4) Parallel sheet: (a) search for the double parallel sheet pattern; (b) extend each double parallel sheet pattern by application of the single parallel sheet pattern; (c) remove from further consideration all NOEs exclusively involving the main-chain protons of these residues.

(5) Reconcile the results of (3) and (4). Construct the MCD-defined sheets found and incorporate turns revealed by (2) and (3).

(6) Extended chain: using only the remaining NOEs and main-chain units, search for unambiguous (i.e., no degeneracy) main-chain NOEs to define random coil regions.

(7) Structure alignment: identify and align the elements of secondary structure in the primary sequence by identification of amino acid side-chain spin systems that can be easily and reliably defined in J-correlated spectra.

(8) If desired, use the constraints imposed by (7) to help define the more complex amino acid side-chain J-correlated spin systems.

One appreciates that certain aspects of this approach have been used by other workers. However, we expect that when the entire approach is used as a general strategy, in conjunction with the tactical application of the closed loop patterns, a considerable gain in efficiency can be achieved. In addition, the MCD approach lends itself to computer-assisted analysis. This will be reported in a future communication.

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Purification and Reconstitution of a 75-Kilodalton Protein Identified as a Component of the Renal Na^+/Glucose Symporter†

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ABSTRACT: A 75-kilodalton (kDa) protein was purified from solubilized renal brush border membranes by using high-pressure liquid chromatography (HPLC) and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Functional and immunological properties identified the 75-kDa protein as a component of the Na^+/glucose symport system. The purified protein was specifically recognized by a monoclonal antibody that functionally interacts with the Na^+/glucose symporter. Na^+-dependent phlorizin binding activity was associated with fractions containing the 75-kDa protein during HPLC fractionation on the anion exchanger Mono-Q and was greatly increased after reconstitution into egg yolk phosphatidylcholine vesicles. The final purified preparation contained glucosamine and a blocked N-terminus.

Transporters localized in the apical membrane of renal proximal tubule and intestinal epithelial cells catalyze the coupled translocation of glucose and Na^+ in a symport (cotransport) mechanism (Crane, 1977). Na^+-dependent binding of phlorizin, a specific high-affinity inhibitor of this transport mechanism, has been established as an indication of the transport activity (Frasch et al., 1970; Lever, 1984). Attempts

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to identify and purify the protein components of the symport system using different approaches such as solubilization and reconstitution (Crane et al., 1976; Ferguson & Burton, 1977; Im et al., 1982; Koepsell et al., 1983; Malathi & Preiser, 1983; Kano-Kameyama & Hoshi, 1983; Silverman & Speight, 1986), negative purification and selective thiol labeling (Klip et al., 1979), photoaffinity labeling (Hosang et al., 1981; Gibbs et al., 1982), and alkylation (Arita & Kawanami, 1980) have proven difficult and inconclusive. Recently, Peerce and Wright (1984) observed substrate-protectable labeling of a 75-kDa[1] protein in intestinal brush border membranes. A preliminary report by Schmidt et al. (1983) described a monoclonal antibody (MAb) that recognized a 75-kDa polypeptide in intestinal brush border membranes, but more direct evidence for the identification of this polypeptide as the glucose transporter was not obtained.

Previously we have isolated and preliminarily characterized a panel of MAb's that recognized a 75-kDa protein in renal brush border membranes identified as a component of the renal Na^+/glucose symporter (Wu & Lever, 1987a,b). The present study describes the purification of this 75-kDa protein from solubilized renal brush border membranes and the reconstitution of Na^+-dependent phlorizin binding activity of this preparation. This represents the first purification to homogeneity of a subunit of a mammalian Na/organic solute cotransport system.

Materials and Methods

Materials. [*phenyl*-3,3′,5,5′-^{3}H,*propanone*-3-^{3}H]Phlorizin, 58 Ci/mmol, was purchased from New England Nuclear Corp. Kidneys from domestic hogs were obtained through the generosity of Texas A&M University, College Station, TX. Protein A–Sepharose and SDS–PAGE reagents were from Bio-Rad. Nitrocellulose sheets were from Schleicher and Schuell, Keene, NH. Rabbit anti-mouse (IgG + IgM + IgA) antibody and its peroxidase conjugate were from Zymed. *n*-Octyl β-D-glucopyranoside (*n*-octyl glucoside) was from Boehringer Mannheim. All other reagents were of analytical grade.

Monoclonal antibodies (MAb's) were described previously (Wu & Lever, 1987a). MAb's 11A3D6, an IgG_{2b}, and 3F8E12, an IgG_1, were purified on protein A–Sepharose (Bio-Rad MAPS system).

Purification of the Na^+/Glucose Symport Protein. Brush border membranes were prepared from pig kidney cortex and then treated with 0.1 M EDTA at pH 11.2 to remove extrinsic membrane proteins (depleted membranes) as described previously (Wu & Lever, 1987a).

Proteins were then solubilized from 30–50 mg of depleted membranes by stirring the membranes at 0 °C for 20 min in 8–14 mL of buffer A containing 20 mM Tris-HCl, pH 7.7, 2 mM dithiothreitol, and 46 mM *n*-octyl glucoside. The unsolubilized materials were removed by centrifugation at 160000*g* for 1 h.

Solubilized samples were injected onto a Mono-Q HR 5/5 column (Pharmacia) through a Model 7125 syringe loading sample injector and eluted with concentration gradients of 0–1 M NaCl in 50 mL of buffer A at a flow rate of 1 mL/min. Absorbance at 280 nm was monitored with a LKB 2138 UVICORD S UV monitor. Fractions from the Mono-Q column were analyzed by SDS–PAGE to identify protein bands.

Fractions containing the 75-kDa protein were pooled and resolved by preparative SDS–PAGE using a 3 mm thick 7.5% acrylamide running gel and a 3% stacking gel. The gel was stained briefly with Coomassie Blue, and the 75-kDa protein was then eluted from the gel by using an ISCO electrophoresis concentrator with 40 mM Tris–acetate buffer, pH 8.6, 0.1% SDS in the electrode and inner buffer compartments and 10 mM Tris–acetate buffer, pH 8.6, 0.1% SDS in the sample cup.

Assay of Phlorizin Binding by Filtration. Phlorizin binding activities of renal brush border membranes and depleted membranes were assayed by incubating 0.12 mg of membranes for 5 min at 21 °C in 100-μL volumes with the indicated concentration of [^{3}H]phlorizin, 1.25 μCi, in buffer B (0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K^+-HEPES, pH 7.2) containing either 100 mM NaCl or 100 mM choline chloride. The reaction was terminated by addition of wash buffer (0.8 M NaCl, 10 mM Tris-HCl, pH 7.5) and rapid filtration through a nitrocellulose filter as described previously (Lever, 1984).

Assay of Phlorizin Binding by Gel Filtration. Since solubilized membranes and column fractions could not be retained by nitrocellulose filters, an alternative assay method using gel filtration was developed. Columns containing 1 mL of preswollen Sephadex G-50 (fine) in buffer B with either 100 mM NaCl or 100 mM KCl were centrifuged at 100*g* for 2 min. Before assay, all solubilized samples and column fractions were dialyzed against buffer B′ (5 mM $MgCl_2$, 10 mM K^+-HEPES, pH 7.2) overnight in order to remove *n*-octyl glucoside. Samples were then incubated with the indicated concentration of [^{3}H]phlorizin in buffer B as described above, and 100 μL of the mixture was applied to each column and centrifuged at 100*g* for 2 min. One portion of the eluent was used for radioactivity determination, and the other portion was used for protein determination using the method described by either Lowry et al. (1951) or Bradford (1976). Similar values of K_d and B_{max} were observed for phlorizin binding to unsolubilized depleted membranes when determined either by nitrocellulose filtration or by gel filtration.

Reconstitution of Na^+-Dependent Phlorizin Binding Activity. Egg yolk phosphatidylcholine (Sigma) was dissolved in chloroform, dried under a stream of N_2 gas, and then sonicated at 2 mg/mL in 20 mM Tris-HCl, pH 7.7, until clear. Solubilized membranes or fractions from HPLC in buffer A were mixed with equal volumes of sonicated lipids and dialyzed against 2 L of buffer B′ for 24 h with one change of dialysis buffer. Samples were then assayed for Na^+-dependent phlorizin binding activity by the gel filtration method.

Western Blot Analysis. Samples were resolved by SDS–PAGE using a 7.5% acrylamide running gel and a 3% stacking gel. Proteins were electrophoretically transferred to nitrocellulose sheets for Western blot analysis by the method of Towbin et al. (1976) as modified by Yoneyama and Lever (1987). Molecular weight markers were localized on the filter by staining with Amido Black. Filters were incubated with the indicated monoclonal antibody at 4 °C for 24 h, washed, and then incubated with peroxidase-conjugated rabbit anti-mouse (IgG + IgA + IgM) antibody at a 1:200 dilution at room temperature for 2 h. Filters were washed and then peroxidase activity was visualized by the intensification protocol described by Adams (1981).

Results and Discussion

Enrichment of Phlorizin Binding Activity during Purification of a 75-kDa Membrane Protein. Pig kidney brush

[1] Abbreviations: MAb, monoclonal antibody; M_r, molecular weight; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton; HPLC, high-pressure liquid chromatography; Ig, immunoglobulin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

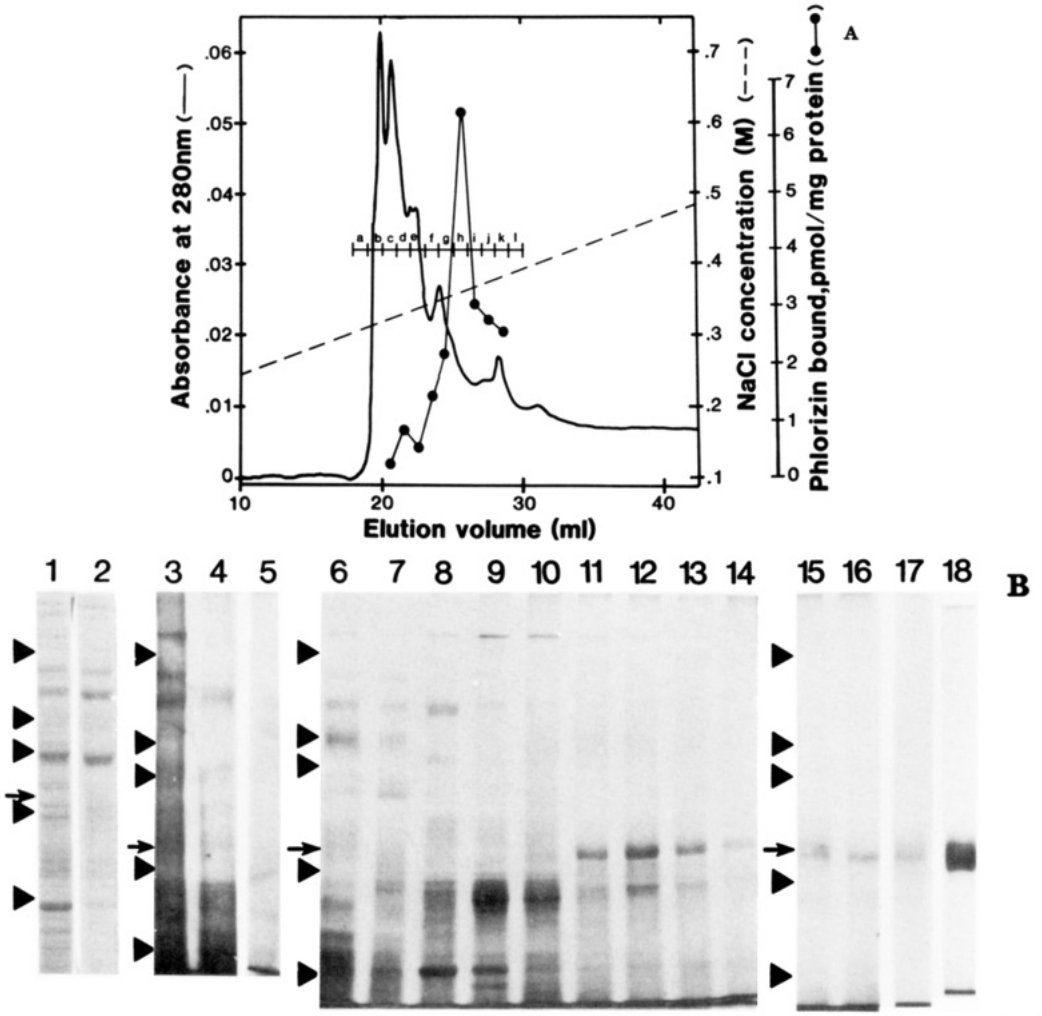

FIGURE 1: (A) Ion-exchange HPLC on a Mono-Q column. Samples solubilized from 50 mg of depleted pig kidney brush border membranes were applied to the column and eluted by using a NaCl gradient indicated by the dashed line. Fractions c–k were dialyzed and assayed for Na^+-dependent phlorizin binding activities with 0.3 μM [^{3}H]phlorizin, 1.25 μCi, as described. Activity shown is the Na^+-dependent phlorizin binding activity after subtraction of Na^+-free binding activity. (B) SDS–PAGE analysis: lane 1, pig kidney brush border membranes (250 μg); lane 2, depleted membranes (250 μg); lane 3, solubilized membrane fraction (30 μg); lane 4, flow-through (30 μg); lanes 5–16, fractions from Mono-Q chromatography (5–15 μg), which correspond to fractions a–l in part A; lane 17, purified 75-kDa protein (1.5 μg); lane 18, purified 75-kDa protein (7 μg). After detergent solubilization, all samples were dialyzed against buffer B′ before SDS–PAGE analysis. Lanes 1 and 2 were stained by Coomassie Blue, and the remainder were visualized by silver stain.

border membranes were depleted of extrinsic proteins by EDTA treatment at high pH, resulting in a 2-fold enrichment in Na^+-dependent phlorizin binding specific activity (Wu & Lever, 1987a). Depleted membranes were then solubilized by using 46 mM *n*-octyl glucoside and fractionated by HPLC and preparative SDS–PAGE (Figure 1). Fractions were analyzed by assay of Na^+-dependent phlorizin binding activity, protein pattern by SDS–PAGE, and Western blot reactivity with MAb 11A3D6, shown previously to interact with the Na^+/glucose symporter (Wu & Lever, 1987a).

Na^+-dependent phlorizin binding activity was completely abolished at *n*-octyl glucoside concentrations above 8 mM, whereas Na^+-independent binding was not affected (not shown). However, Na^+-dependent phlorizin binding activity of detergent-treated membranes was partially restored (K_d = 0.57 μM; B_{max} = 50.66 pmol/mg) after detergent was removed by dialysis (not shown). These values are approximately 8-fold lower than those observed in intact depleted membranes (Wu & Lever, 1987a). If the detergent-treated membranes were centrifuged for 1 h at 160000*g* and then the supernatant was dialyzed as above, K_d and B_{max} values for Na^+-dependent phlorizin binding were reduced to 0.37 μM and 4.08 pmol/mg, respectively (not shown). This decrease presumably represented removal of phospholipids rather than loss of the symporter since most proteins including a 75-kDa protein were enriched in the supernatant (Figure 1B, lane 3).

Na^+-dependent phlorizin binding activity was greatly increased after addition of phospholipids during reconstitution. The K_d and B_{max} were restored to 0.77 μM and 120.2 pmol/mg, respectively, after the solubilized fraction was reconstituted into egg yolk phosphatidylcholine vesicles.

Figure 1A illustrates the resolution of solubilized membranes by high-pressure liquid chromatography (HPLC) using a Mono-Q column and elution by a NaCl gradient. Nonabsorbed proteins appearing in the flow-through were of low molecular weight ($M_r \leq 62\,000$) (Figure 1B, lane 4), and no proteins were eluted at NaCl concentrations below 0.3 M. Between 0.3 and 0.35 M NaCl (fractions b–f), several high molecular weight proteins were eluted (Figure 1B, lanes 6–10). Beginning at 0.35 M NaCl (fractions g–l), fractions were enriched in a 75-kDa protein (Figure 1B, lanes 11–16). This 75-kDa protein was barely visible in the starting brush border membranes and depleted membrane preparations (Figure 1B, lanes 1 and 2). No detectable proteins were eluted from 0.39 to 0.7 M NaCl, and a few proteins were eluted above this salt concentration (not shown).

Table I: Reconstitution of Na^+-Dependent Phlorizin Binding Activity in Phospholipid Vesicles Accompanying Purification of a 75-kDa Protein

		Na^+-dependent phlorizin binding activity				
fraction	total protein (mg)	K_d (μM)	B_{max} (pmol/mg)	total act. (pmol)	recovery	rel sp act.
octyl glucoside treated membranes	43.8	0.57[a]	50.7	2220	100	1
octyl glucoside supernatant	16.8	0.77[b]	120[b]	2020	91	2.37
pooled HPLC fractions (0.30–0.35 M NaCl)	4.52	ND[c]	ND[c]	ND[c]	0	0
pooled HPLC fractions (0.35–0.39 M NaCl)	0.672	1.2[b]	3820[b]	2560	115	75.4

[a] The starting material (depleted membranes), before detergent treatment, exhibited K_d = 3.7 μM and B_{max} = 426 pmol/mg. [b] Samples reconstituted in the absence of phospholipids exhibited K_d = 0.37 μM and B_{max} = 4.08 pmol/mg in the case of the octyl glucoside supernatant and K_d = 0.63 μM and B_{max} = 8.83 pmol/mg for the pooled HPLC fractions eluting between 0.35 and 0.39 M NaCl. [c] ND, activity not detectable.

Most of the Na^+-dependent phlorizin binding activity (ranging from 2 to 6.5 pmol/mg, assayed at a subsaturating phlorizin concentration of 0.3 μM) was associated with fractions eluted between 0.35 and 0.39 M NaCl (Figure 1A) corresponding to the fractions containing the 75-kDa protein. Fractions not containing the 75-kDa protein exhibited greatly decreased binding activity (Figure 1A). Fractions g–l (Figure 1), which contained the 75-kDa protein and other minor proteins, were pooled, dialyzed, and analyzed for phlorizin binding activity. Binding was Na^+-dependent and saturable; K_d and B_{max} values were determined as 0.63 μM and 8.82 pmol/mg, respectively (not shown). This B_{max} value determined in the absence of phospholipids represents a 2-fold enrichment in specific activity compared with that of the solubilized membrane preparation applied to the column. K_d and B_{max} of Na^+-dependent phlorizin binding activity were increased to 1.66 ± 0.40 μM and 4588 ± 716 pmol/mg (n = 3), respectively, after reconstitution into phosphatidylcholine vesicles. A representative experiment is shown in Figure 2. These values represent a 520-fold activation when compared with samples reconstituted in the absence of phospholipids. When fractions a–f (Figure 1A), which did not contain the 75-kKa protein, were similarly pooled and assayed, no Na^+-dependent phlorizin binding activity was detected either before or after phospholipid reconstitution (not shown). Table I summarizes the results from a typical purification experiment. The relative specific activity of Na^+-dependent phlorizin binding activity in the pooled 75-kDa-protein-enriched fractions was increased by 32-fold when compared with that of the detergent-solubilized fraction (octyl glucoside supernatant) and was increased by 75-fold when compared with that of the detergent-treated depleted membranes. It should be noted that this comparison may not be an accurate indication of the actual increase in specific activity since samples at different stages of purity may differ in endogenous lipid content.

In the final purification step, fractions g–l containing the 75-kDa protein from the Mono-Q step were pooled and fractionated by preparative SDS–PAGE, and the 75-kDa protein was eluted from the gel by using an ISCO electrophoresis concentrator. Analysis of this preparation by SDS–PAGE followed by silver stain revealed a band with an apparent M_r of 75 000 (Figure 1B, lane 17). In an overloaded sample, the preparation appeared as a broad band with a M_r ranging from 72 000 to 79 000 (Figure 1B, lane 18). This apparent heterogeneity is typical of glycoproteins.

Analysis indicated that the purified protein contained approximately 2 mol of glucosamine/mol and contained a blocked N-terminus.

The final 75-kDa preparation after preparative SDS–PAGE was denaturated in the presence of SDS, and phlorizin binding activity could not be restored.

The 75-kDa Protein Binds MAb's That Functionally Interact with the Na^+/Glucose Symporter. We have recently isolated a panel of Mab's that bind a renal Na^+/glucose

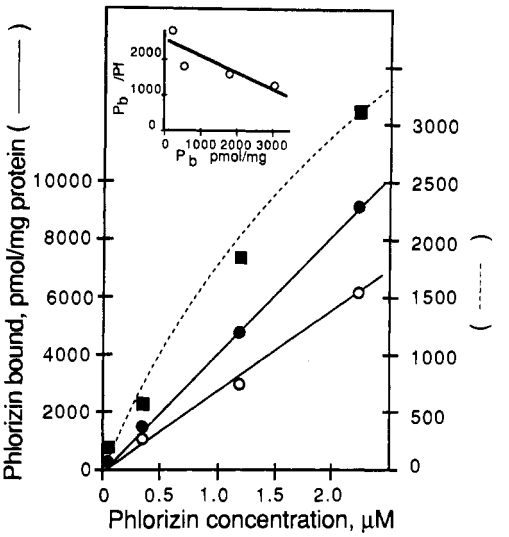

FIGURE 2: Characterization of Na^+-dependent phlorizin binding activity of fractions containing the 75-kDa protein. Samples were incubated with the indicated concentration of [^{3}H]phlorizin, 1.25 μCi, in the presence of either (●) 100 mM NaCl or (○) 100 mM KCl and then spun through gel filtration columns as described under Materials and Methods. The Na^+-dependent component is shown (■) after subtraction of the Na^+-free binding. Apparent K_d and B_{max} values of 2.5 μM and 5543 pmol/mg, respectively, were calculated after subtraction of Na^+-free binding activity as shown in the Scatchard plot in the inset.

symport system (Wu & Lever, 1987a). All MAb's in this panel, including MAb 11A3D6, recognized a 75-kDa protein in pig kidney brush border membranes as detected by Western blot analysis. The purified 75-kDa protein after the preparative SDS–PAGE step was recognized by MAb 11A3D6 by Western blot analysis (Figure 3, lane b). Membrane fractions after resolution by a Mono-Q column were also screened by Western blot analysis using MAb 11A3D6. Figure 3 shows that MAb 11A3D6 recognized a 75-kDa protein in total brush border membranes (lane a) and in fractions eluted between 0.35 and 0.39 M NaCl (lanes h–k), whereas control MAb 3F8E12 did not react with any of the fractions. The remaining fractions, including the flow-through fraction, which lacked the 75-kDa protein, did not show reactivity with MAb 11A3D6 (lanes c–f). MAb 11A3D6 also recognized a 91-kDa antigen in brush border membranes (lane a), which was also enriched in a fraction (lane f) eluting prior to elution of the 75-kDa protein. This band may represent either a precursor form of the 75-kDa protein or cross-reactivity with an unrelated protein and was not recognized by other preparations of MAb 11A3D6 (Wu & Lever, 1987a). The 200-kDa band visible in total brush border membranes (lane a) is presumably an artifact as noted previously (Wu & Lever, 1987a) since it appeared before peroxidase color development and was not present in fractions after HPLC purification.

These results strongly suggest that the 75-kDa glycoprotein purified from brush border membranes in this simple two-step

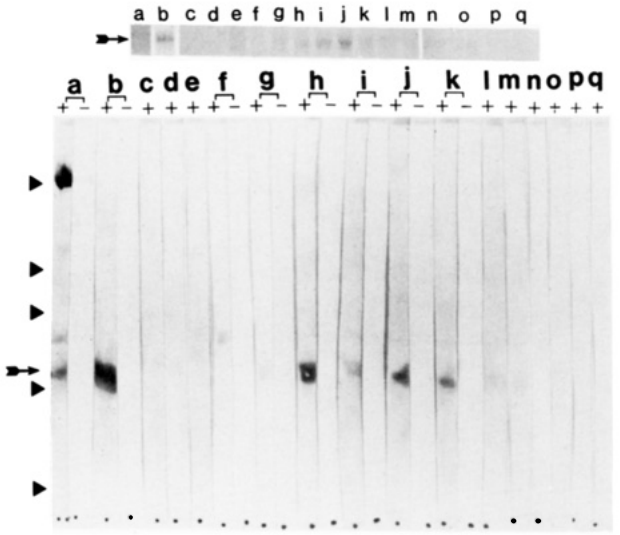

FIGURE 3: Purification of the 75-kDa antigen recognized by MAb 11A3D6. Samples were resolved by SDS–PAGE and transferred to nitrocellulose sheets for Western blot analysis. Either (+) purified 11A3D6 or (−) 3F8E12 (control antibody) was used as the first antibody (25 μg/mL). Lane a, renal brush border membranes (825 μg); lane b, purified symport protein (100 μg); lane c, flow-through fraction after Mono-Q (80 μg); lanes d–q, fractions from Mono-Q that correspond approximately with fractions b–l in Figure 1A. Amount of protein used: lanes c and d, 75 μg; lanes e and f, 200 μg; lanes g and h, 150 μg; lanes i and j, 90 μg; lanes k–m, 60 μg; lanes n–q, 40 μg. M_r markers of 200 000, 130 000, 92 500, 66 000, and 45 000, respectively, are indicated by the filled triangles. Arrows indicate the position of the 75-kDa protein. (Top) Coomassie Blue stained pattern of the 75-kDa region in the corresponding fractions. Amount of protein used: lane a, 250 μg; lane b, 1.5 μg; lanes c, d, and h–k, 15 μg; lanes e–g, 35 μg; lanes l–q, 10 μg.

procedure is a component of the Na^+/glucose symport system. This conclusion rests on (1) the strong correlation between Na^+-dependent phlorizin binding activity and enrichment of the 75-kDa protein after resolution by HPLC and (2) the interaction of the purified 75-kDa protein with a MAb known to functionally interact with the symporter.

Activity of the solubilized symporter is strongly dependent on the presence of phospholipids, as noted in previous studies (Ducis & Koepsell, 1983; Poiree et al., 1986). Reconstitution of activity of solubilized membrane proteins by phosphatidylcholine addition during removal of detergent has been observed in a number of different systems including the erythrocyte anion transporter (band III protein) (Gerritsen et al., 1978) and cytochrome P-450 (Taniguchi et al., 1979).

On the basis of estimates of the target size of the Na^+/glucose symporter by radiation inactivation, Lin et al. (1984) have obtained convincing evidence that multisubunit interactions are important in symport function. Also, the large conformational changes postulated after Na^+ activation of the symporter (Peerce & Wright, 1985) are energetically more favorable in a multisubunit interaction model than if mediated by refolding of a single polypeptide. Although not ruled out, our present results suggest that it is unlikely that the symporter complex also contains an unidentified subunit which is nonidentical with the one we have purified. No other polypeptide was immunoprecipitated from brush border membranes by our antibody to the 75-kDa protein (Wu & Lever, 1987a), and no other detectable polypeptide copurified with the 75-kDa subunit under nonionic detergent conditions known to preserve oligomeric structure in other systems.

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