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Immunological identification of the human erythrocyte glucose transporter

(membrane protein/cytochalasin B/HeLa cells)

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ABSTRACT **A rabbit antibody against the human erythrocyte glucose transporter was purified by affinity chromatography and used to determine the distribution of transporter on polyacrylamide gels after electrophoresis in sodium dodecyl sulfate. Fresh erythrocyte ghosts showed transporter only at the broad 55,000 M_r band, as did the isolated transporter. HeLa cell plasma membranes showed a similar band of crossreacting material at M_r 55,000. The amount of crossreacting material in human erythrocyte ghosts and in plasma membranes from human HeLa cells and mouse L-1210 cells was determined in an enzyme-linked immunosorbent assay which gave results consistent with the extent of glucose-reversible binding of cytochalasin B.**

The glucose transporter from human erythrocytes has been purified by using as an assay glucose transport activity reconstituted in liposomes (1, 2). As determined by $NaDodSO_4$/polyacrylamide gel electrophoresis, the transporter has a M_r of 55,000, but it has been claimed that the isolated protein is a proteolytic fragment of the native transporter (3). Antibody against transporter can be prepared in rabbits and used to identify its molecular weight distribution on gels of fresh erythrocyte membrane proteins. Because glucose transport in cells is affected by hormones (4), withdrawal of carbon sources (5), and transformation (6, 7), an accurate estimate of the amount of transporter would be useful. We report here that the molecular weight of the glucose transporter stained with antibody upon $NaDodSO_4$/polyacrylamide gel electrophoresis of fresh erythrocyte ghosts is 55,000, the same as that of the isolated transporter. In addition, the amount of transporter in other cells is determined by immunological methods.

MATERIALS AND METHODS

Materials. Human glucose transporter was prepared as described (2, 8). Tween 20, 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), protein A-Sepharose CL-4B, and horseradish peroxidase were purchased from Sigma; microtiter plates were from Cooke (Alexandria, VA); peroxidase-conjugated goat anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA); Freund's adjuvant was from Difco; rabbits (Flemish giants) were from Cornell's rabbit colony; and $NaDodSO_4$ was from Bio-Rad.

Antisera Production. Rabbits were injected intramuscularly with purified glucose transporter (200 μg) with Freund's complete adjuvant followed by subsequent boosts of antigen (200 μg) with incomplete adjuvant. Three different rabbits all produced comparable titers of antibody against transporter as described below. Rabbits maintained high titers of antibody for over 6 months after boosting.

Enzyme-Linked Immunosorbent Assay (ELISA). Titer of antibody and amount of transporter were determined by ELISA (9, 10). For the first step of both assays, protein (either antigen or antibody) in 60 mM sodium carbonate (pH 9.6) was adsorbed to each well of a substrate microtiter plate (Cooke, no. 1-223-24) for 4–20 hr at 37°C. Excess protein that did not adsorb to the plastic was discarded. Plates were then washed three times with 20 mM sodium phosphate, pH 7.5/0.85% NaCl (P_i/NaCl) containing 0.05% Tween 20 after this and subsequent incubations with protein. The final step for both assays was the determination of the amount of peroxidase activity (11) adsorbed to each well by addition of 0.1 ml of 0.5 mM 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) and 2.5 mM H_2O_2 in 50 mM sodium citrate (pH 4.0) and incubation with mixing for 15 min at 25°C. The reaction was stopped by addition of 0.1 ml of 10 mM HF containing 1% $NaDodSO_4$, and the absorbance (to 2.0) at 414 nm was determined.

For antibody titers, alkaline-washed erythrocytes (8) were sonicated in 60 mM sodium carbonate (pH 9.5) at 100 μg of protein per ml in polypropylene tubes. The sample was diluted with carbonate buffer to 10 μg of protein per ml and 200 μl of the solution was added to each well of the microtitration plates. Solutions containing antibody in P_i/NaCl/Tween 20 were added to the first well, diluted 1:2 serially across the plate, and made up to 0.2 ml per well with P_i/NaCl/Tween 20. After 1 hr at 37°C, peroxidase-conjugated goat anti-rabbit IgG was added (0.2 ml) and the mixture was incubated at 37°C for 30 min. The wells were again washed with P_i/NaCl/Tween 20 and the amount of peroxidase present was assayed. The absorbance at 414 nm was related linearly to the amounts of antibody present in the serum. Titer is defined as the protein concentration required to produce an absorbance of 1.0 at 414 nm.

For quantitation of the amount of transporter, antibody (250 ng) purified by affinity chromatography was adsorbed in each well. Antigen sources were dissolved in $NaDodSO_4$ as described in *Results*, applied in P_i/NaCl/Tween 20 to one well, and diluted 1:2 serially as above. After 2 hr at 37°C, the protein not bound to the antibody was discarded and the plates were washed with P_i/NaCl/Tween 20. Purified antibody conjugated to horseradish peroxidase was added and the mixture was incubated for 2 hr at 37°C. Peroxidase activity was determined and a standard curve for each experiment was constructed by using the isolated protein as a standard.

Purification of Antibody by Affinity Chromatography.

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Abbreviations: P_i/NaCl, phosphate-buffered saline (20 mM sodium phosphate, pH 7.5/0.85% NaCl); ELISA, enzyme-linked immunosorbent assay.

Purified glucose transporter was attached to Bio-Rad Affi-Gel 102 by reductive alkylation by oxidized carbohydrate of the glycoprotein. Protein (2 mg) was delipidated (8) and oxidized with 40 mM $NaIO_4$ for 40 min at pH 8 in 0.5% $NaDodSO_4$. Ethylene glycol (25 μl) was added and after 6 hr the reaction mixture was dialyzed against 10 mM sodium carbonate, pH 9.5/0.1% $NaDodSO_4$ at 0°C with three changes of buffer (1 liter each). The oxidized transporter in about 5 ml was then added to 5 ml of Affi-Gel that had been washed with carbonate buffer. After 2 hr at 0°C, 20 mg of $NaBH_4$ was added and the mixture was allowed to react overnight. Excess $NaBH_4$ was destroyed by lowering the pH to less than 7 with HCl. The gel was then washed and used for purification of antibody against the transporter. Most of the protein in the serum (1 ml containing 50 mg of protein, diluted to 3 ml) did not bind to the column (5 ml). The column was then washed with 1.4 M NaCl, which eluted less than 200 μg of protein. Purified antibody was then eluted by washing with 300 mM NaCl adjusted to pH 2.5 with HCl (12); 2-ml fractions were collected into tubes containing 50 μl of 1 M Na_2HPO_4. The salt was required for maximal recovery of antibody. The purified antibody had a titer 50 times that of serum and 20 times that obtained in the pellet after serum was treated with 35% saturated $(NH_4)_2SO_4$ at 25°C.

Plasma Membranes. The membranes were prepared by the procedure of Lever (13) from either HeLa or mouse L-1210 cells and stored frozen. Erythrocyte membranes were prepared as described by Dodge *et al.* (14).

Binding of Cytochalasin B. The binding was determined by equilibrium dialysis as described (15). Nonspecific binding to HeLa cell plasma membranes was determined by measuring the amount of cytochalasin B bound to membranes that had been heated at 70°C for 15 min. The nonspecific binding, probably due to lipid, at each free concentration of cytochalasin B was subtracted from the amount bound to the unheated samples to obtain a corrected Scatchard plot (16). All of the specific binding and none of the nonspecific binding were reversible with glucose.

Staining with Antibody after $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Protein samples (0.025–200 μg) were applied to gels and run as described by Fairbanks *et al.* (17) or by Laemmli (18). Each gel was fixed in 50 ml of acetic acid/isopropanol/H_2O, 10:25:65 by vol, with one change for 16 hr at 4°C and then in P_i/NaCl/Tween 20 with two changes for 2 hr each. Gels were sliced to 1–2 mm. The slices were homogenized with a Teflon pestle (7 mm in diameter) in culture tubes (100 × 75 mm) and incubated for 1 hr in 0.5 ml of purified anti-glucose transporter (10 μg/ml of P_i/NaCl/Tween 20) containing bovine serum albumin (10 mg/ml). The unreacted antibody was washed out with six changes of P_i/NaCl/Tween 20 over 1 hr at 25°C by centrifuging at 1200 × *g* and decanting the supernatants. The second antibody, peroxidase-conjugated goat anti-rabbit IgG, was added for 1 hr and washed out as above. Peroxidase activity was determined by adding the substrate solution (0.7 ml) used above and incubating for 30–120 min. The reaction was stopped as above and $A_{414\ nm}$ was determined.

Conjugation of Anti-Glucose Transporter. Purified anti-glucose transporter was conjugated to horseradish peroxidase by the method of Nakane and Kawaoi (19).

Removal of $NaDodSO_4$. In some instances $NaDodSO_4$ was used to solubilize antigen and the excess had to be removed. $NaDodSO_4$ will crystallize at 0°C at concentrations greater than 0.25%, and that remaining in solution is present as monomers (20). Protein solubilized with $NaDodSO_4$ remained in solution after the temperature was lowered and could be filtered through Sephadex G-25 to remove the monomers not bound to the protein.

RESULTS

Glucose Transporter Reacts Completely with Purified Antibody. Addition of antibody to the isolated glucose transporter inhibited cytochalasin B binding a maximum of only 50%. This is probably because the transporter is in vesicles of endogenous lipid and only half of the molecules are correctly oriented to react. Similarly, the maximal inhibition of glucose transport in reconstituted vesicles was 50% (T. J. Wheeler, personal communication), presumably for the same reason. To show that the antibody would react completely with the glucose transporter, we titrated a crude Triton extract of alkaline-washed ghosts with antibody before removing the Triton X-100, which would allow access to all of the molecules. Various amounts of purified antitransporter were incubated for 4 hr at 0°C with 100 μg of protein from the Triton extract in 0.5 ml of 10 mM Na morpholinepropanesulfonate/0.1% Triton. Protein A-Sepharose (20 mg) was then added and the mixture was incubated for 4 hr at 0°C to bind the antibody. The solution was then centrifuged, the Triton was removed from the supernatant with Biobeads SM-2 (1), and the binding of cytochalasin B was measured by equilibrium dialysis (15). The antibody at 10 μg, 40 μg, and 320 μg removed 27%, 43%, and 94%, respectively, of the cytochalasin B binding activity. The residual 6% of binding activity was nonspecific binding to lipids in the extract (15).

Identification of Glucose Transporter after $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. As shown in Fig. 1*A*, the isolated glucose transporter from human erythrocytes appears as a broad band both by absorbance at 600 nm due to staining with Coomassie blue G for protein (20 μg of protein applied to the gel) and by staining with the purified antibody (500 ng of protein). The antibody staining also showed a small shoulder at higher molecular weights and an extra peak (10% of total area) around M_r 20,000 although little absorbance from Coomassie blue was observed in these regions. Protein that was further purified by gel filtration in $NaDodSO_4$ and free of lipid and band 7 material (8) showed no extra peak, and the antibody response closely followed the stain due to Coomassie blue (Fig. 1*B*). Because the purified antitransporter was purified by affinity chromatography with isolated transporter that had been denatured in $NaDodSO_4$ and had had its carbohydrate oxidized with periodate, the antibody probably reacts with linear sequences of amino acids or stable conformations that form again after removal of $NaDodSO_4$ from the gels.

Erythrocytes. Proteolysis of proteins in erythrocyte membranes has been reported (21, 22). To determine if proteolysis of the transporter from M_r 95,000 to M_r 55,000 had occurred during detergent extraction of the transporter, as suggested by Mullins and Langdon (3), we prepared ghosts from freshly drawn blood and dissolved the membranes at 100°C in $NaDodSO_4$. The membrane proteins (20 μg) were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and the distribution of glucose transporter was determined by antibody binding. A parallel gel with 50 μg of protein was stained with Coomassie blue G. As seen from Fig. 1*C*, the transporter was labeled as a broad band with a peak at M_r 55,000. The band was so broad that some transporter migrated in the band 3 region (M_r 95,000 at 17 mm), but there was no indication that two components were reacting with the antibody. The same labeling pattern was also seen for intact erythrocytes (data not shown). By using ammonium sulfate-precipitated serum, a similar pattern was observed except that a small amount of band 7 material (≈5% of the total area) was also labeled. Antibody did not react with slices from the glycolipid region of the gels or with glycoproteins other than the transporter. The peak at M_r 20,000 seen in Fig. 1*A* is not present in erythrocytes and is

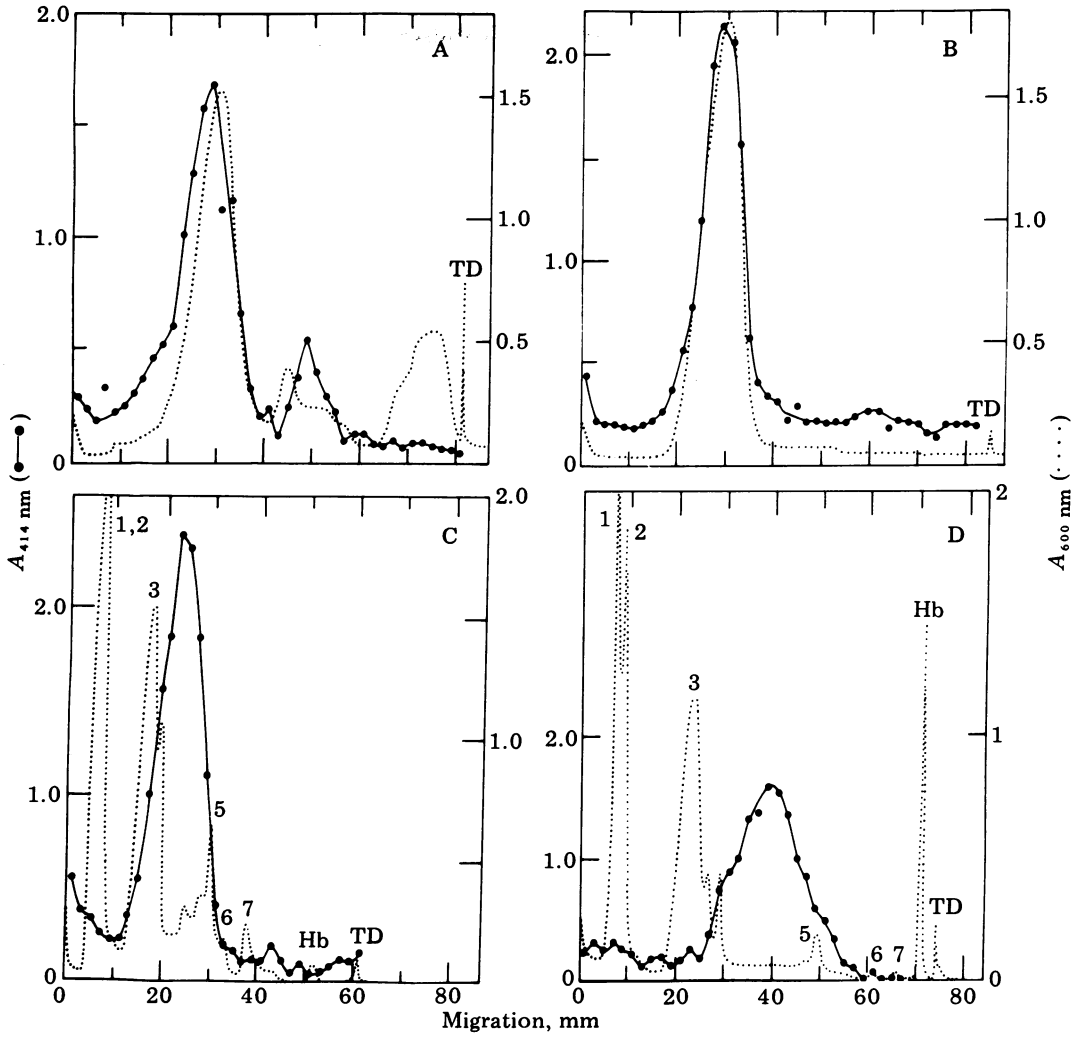

FIG. 1. Identification of transporter after NaDodSO$_4$/polyacrylamide gel electrophoresis. (*A*) Purified transporter. (*B*) Delipidated transporter. (*C*) Erythrocyte membranes; numbers label the positions of the major bands (17). *A–C* used the gel system of Fairbanks *et al.* (17). (*D*) Erythrocyte membranes; the Laemmli (18) procedure for electrophoresis was used. $A_{600\ nm}$ (·····) is due to Coomassie blue G stain on gels containing 25–50 μg of protein; $A_{414\ nm}$ (●—●) is due to antibody labeling. TD, tracking dye.

probably a minor proteolysis product. In control experiments, no labeling was observed with pre-immune serum. As shown in Fig. 1*D*, little staining with antibody was seen in the region of band 3 when fresh ghosts were analyzed by the discontinuous gel buffers of Laemmli (18). We previously observed that the isolated transporter formed dimers at $M_r \approx 100{,}000$ when analyzed on the Laemmli system (8), but we have not seen such dimers when fresh ghosts were used and stained with the antibody as in Fig. 1*D*. It is essential to use recrystallized NaDodSO$_4$ with the Laemmli gels to prevent precipitation of protein on top of the gel. It is possible that minor differences in the NaDodSO$_4$ or other factors may cause dimer formation, as occurs with glycophorin (23).

HeLa Cell Plasma Membranes. Proteins of plasma membranes from HeLa cells, a human cell line, were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis and stained as above. As shown in Fig. 2, a protein in the region of M_r 55,000 was also labeled with antibody as a broad band. Thus, HeLa cells appear to contain a similar protein that crossreacts with the antibody to the erythrocyte glucose transporter and is probably the same protein. An additional peak was observed at lower molecular weight (15,000) and may reflect some proteolysis.

Quantitation of Glucose Transporter. Fig. 3 shows that the amount of transporter can be quantitated after NaDodSO$_4$/polyacrylamide gel electrophoresis. However, the procedure is lengthy and accurate to only 25 ng of protein (improved sensitivity could be obtained if the protein migrated as a sharp band). To develop a more sensitive quantitative assay for the protein, we used the ELISA (9, 10). If antigen was applied directly to the microtiter plates, two problems were encountered. First, when purified protein was used as a standard, the amount of transporter estimated in ghosts was 6 times that which would be predicted from the extent of binding of cytochalasin B. Apparently the purified protein was not detected by the assay as efficiently as the protein in the original membranes. The poor reaction may reflect either incomplete adsorption of the protein to the plates (possibly due to the Triton X-100 still remaining in the sample) or the aggregation of the protein which then masks antigenic sites. Second, due to the limited surface in wells, we estimate that more than 0.2% of the total protein present must be the antigen of interest in order to obtain a significant response.

To avoid these problems we used the ELISA procedure outlined in *Materials and Methods*. In preparation for assay, isolated transporter (200 μg/ml), ghosts (1 mg of protein per

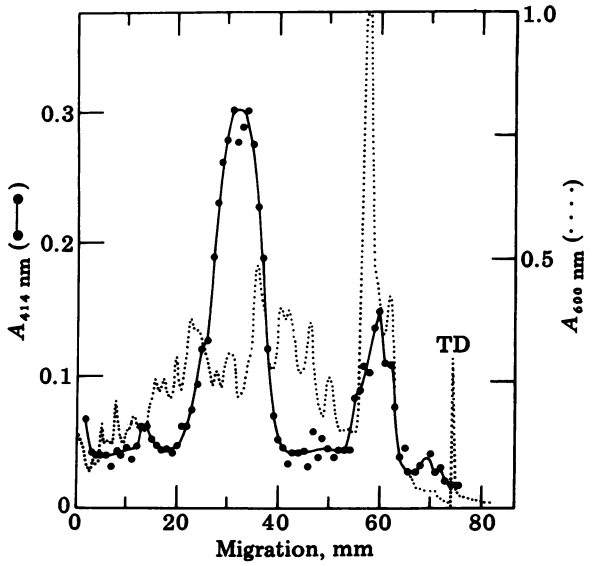

FIG. 2. Identification of transporter in HeLa cell plasma membranes after $NaDodSO_4$/polyacrylamide gel electrophoresis. Membrane protein (·····) was run as in Fig. 1 *A–C*. For antibody staining (●—●), 100 μg of protein was applied to the gel.

ml), and alkaline-washed ghosts (1 mg of protein per ml) were dissolved in 1% $NaDodSO_4$ and then cooled and centrifuged to decrease the detergent concentration to 0.25%. $NaDodSO_4$ at final concentrations greater than 0.002% interferes with the assay; however, for the highest concentration of protein used, these samples were diluted more than 500-fold with P_i/NaCl/Tween 20. HeLa and L-1210 cell plasma membranes required higher amounts of protein. For these, $NaDodSO_4$ was removed by crystallization followed by gel filtration (see *Materials and Methods*). As seen in Table 1, the amounts of antigen estimated immunologically and by binding of cytochalasin B agree. Fig. 4 shows that the ELISA was linear from 0.1 to 3 ng. Triton X-100 could elicit a maximal response for ghosts or alkaline-washed ghosts provided the Triton concentration was less than 0.15% in the sample containing the highest protein concentration; however, isolated transporter required solubilization with $NaDodSO_4$ to give a maximal and consistent response.

Also shown in Table 1 is the estimate of cytochalasin B

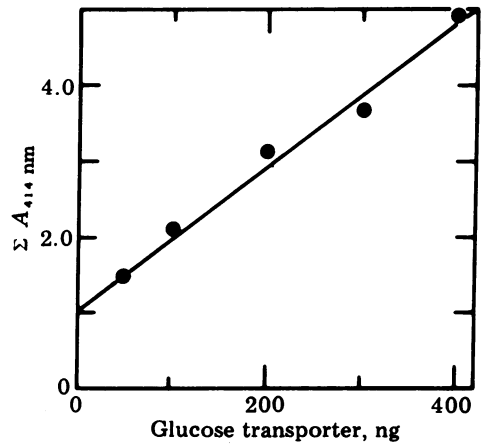

FIG. 3. Quantitation of glucose transporter from $NaDodSO_4$/polyacrylamide gel electrophoresis. Four gel slices (5 mm each) from the region of gels (containing 25–400 ng of protein) in which the transporter migrated were homogenized and assayed immunologically for transporter. The sum of the four absorbances for each gel was plotted.

Table 1. Amount of glucose transporter as determined by ELISA

Source of transporter	% by ELISA	% by binding of cytochalasin B
Purified protein	100	100
Ghosts	5	4.6*
Alkaline-washed ghosts	12	11.3†
HeLa cell plasma membrane	0.16	0.2–0.3
Mouse L-1210 cell plasma membrane	0.03	—

By use of ELISA, the fraction of total protein responding as transporter was determined for each sample. The isolated protein was used as a standard. Purified protein bound 11 nmol of cytochalasin B per mg of protein.
* From ref. 24.
† From ref. 15.

binding sites and the amount of transporter determined immunologically for HeLa cell membranes. These membranes have about 1/25th the transporter of erythrocytes, as determined by both the ELISA and the specific cytochalasin B binding.

Mouse L-1210 cell plasma membranes have a protein that crossreacts with the antibody (Table 1). The amount is lower than that for the HeLa cells but still significant. The amount of transporter in the mouse cells was too low to measure the binding of cytochalasin B by our equilibrium dialysis procedure.

In control experiments, detergent extracts of chloroplast membranes and rat erythrocyte ghosts [reported not to contain any glucose transport activity (25)] did not react in the ELISA at protein concentrations as high as 100 μg/ml. A reliable lower limit for the assay is 1 ng of transporter in 20 μg of total protein.

DISCUSSION

Kasahara and Hinkle (2) purified a glycoprotein of M_r 55,000 from erythrocyte ghosts based on its glucose transport activity after reconstitution in liposomes. Further work has indicated that this protein is responsible for glucose transport and is the site of binding of cytochalasin B (8, 15, 26). Among various differential labeling experiments, some have identified the transporter as a component of band 3 (3, 27, 28) whereas others have indicated that the protein migrates in the region known as 4.5 with a M_r of 55,000 (29, 30). Recently Mullins and Langdon (3) reported that maltosylisothiocyanate labels a protein at 100,000 M_r by $NaDodSO_4$/polyacrylamide gel electrophoresis in a reaction that is partly inhibited by D-glucose or cytochalasin B. In addition, they showed that after extraction

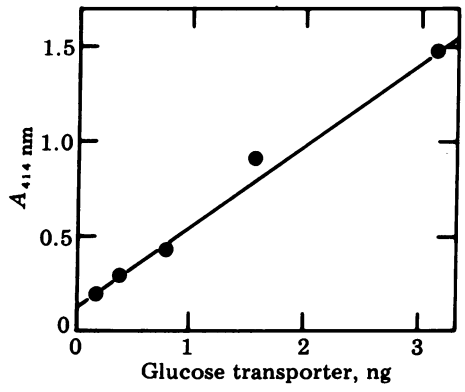

FIG. 4. Quantitation with ELISA. Protein was assayed as described; $NaDodSO_4$ was used to initially solubilize the isolated transporter.

with Triton X-100, the label appeared in the 50,000 M_r region, indicating that proteolysis occurred during extraction. However, our finding that the antibody made against the isolated transporter labels $NaDodSO_4$/polyacrylamide gels of fresh erythrocytes or erythrocyte ghosts at the 55,000 M_r region indicates that the native protein has a subunit M_r of 55,000, not 100,000. The antibody reacts with $NaDodSO_4$-denatured protein in this procedure and the antigenic sites cannot be hidden in a larger precursor form of the protein. One possible explanation for the results of Mullins and Langdon is that the transporter may form a dimer at the high protein loads used for the electrophoresis, as occurs with glycophorin (23). We previously observed variable amounts of dimer on $NaDodSO_4$/polyacrylamide gel electrophoresis of the isolated transporter by the Laemmli procedure, but did not observe dimers when erythrocyte ghosts were used (Fig. 1*D*). Another possible explanation for the discrepancy is that the maltosylisothiocyanate may label the anion transporter as the predominant product at 100,000 M_r. The labeled glucose transporter may have been such a broad band that it was missed.

When proteins from HeLa cell plasma membranes were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and labeled immunologically, a broad band was identified at the same molecular weight as the isolated erythrocyte transporter. The broadness of the band for protein from both sources may indicate that variable glycosylation is a common property of the glucose transporter.

We have found that the methods of antibody-stained $NaDodSO_4$/polyacrylamide gel electrophoresis and quantitative ELISA are superior to immunodiffusion or immunoelectrophoresis for analysis of the glucose transporter, and probably other membrane proteins, because of the tendency of the protein to aggregate in the nonionic detergent used in the latter techniques. Depending on the exact conditions, the protein ran as a single line or multiple lines in immunodiffusion experiments. Surprisingly, after immunoelectrophoresis in 1% agar containing 0.2% Triton X-100/75 mM sodium barbital, pH 8.6, the protein precipitated as an arc migrating toward the cathode. If the protein is positively charged at pH 8.6, many of the acidic residues must be present as amides because we found 27 basic and 63 acidic residues based on amino acid analysis (8).

Quantitation of the transporter by the ELISA procedure provides a sensitive (to 100 pg of protein) linear assay (Fig. 3). The binding of cytochalasin B provides an important control on the accuracy of the assay. The presence of crossreacting material in both HeLa and mouse L-1210 cells will permit analysis of the state of glycosylation, location and amount of transporter present in other mammalian sources, and changes in response to different growth conditions.

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