Monoclonal Antibody to the Human Glucose Transporter That Differentiates between the Glucose and Nucleoside Transporters†

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ABSTRACT: A monoclonal antibody to the glucose transporter has been prepared with band 4.5 (M_r 45 000–65 000) from human erythrocyte ghosts as antigen. This antibody, designated 7F7.5, is of the IgG_{2b} type. The antibody bound exclusively to proteins in the band 4.5 region of immunoblots of human erythrocyte ghosts separated on sodium dodecyl sulfate–polyacrylamide gels. Immobilized 7F7.5 antibody removed glucose transport activity from solubilized alkaline-treated ghosts. The material that was eluted from the immobilized antibody matrix migrated primarily in the band 4.5 region of electrophoretic gels and bound the antibody in immunoblots. To test the specificity of the antibody, glucose and nucleoside transporters in alkaline-treated human erythrocyte ghosts were affinity labeled with [^{3}H]cytochalasin B and [^{3}H]-*S*-(nitrobenzyl)thioinosine (NBMPR), respectively. Both of these transporters are band 4.5 proteins and "copurify" by DEAE-cellulose chromatography. A filter paper assay was developed to assess the presence of the labeled transporters. Immobilized 7F7.5 antibody bound 99% of the labeled glucose transporter. In contrast, only 3% of the specifically labeled nucleoside transporter bound to the immobilized antibody. Furthermore, the antibody did not remove nucleoside transport or NBMPR binding activities from detergent solution. The antibody recognized two tryptic fragments, M_r 23 000 and 18 000, which contain the cytochalasin B binding site of the glucose transporter. By immunoblot, the monoclonal antibody recognized the glucose transporter in cultured human IM9 lymphocytes, synovial cells, and HBL 100 mammary cells but not cells of murine or rat origin. These results indicate that the glucose and nucleoside transporters are distinct proteins which can be distinguished by monoclonal antibody 7F7.5. The method developed to quantitate covalently labeled glucose and nucleoside transporters should have broad applicability as a rapid and easy method for determining the recovery of affinity-labeled membrane proteins in detergent solution during purification. Because of the location of the epitope, the antibody itself should prove to be a valuable tool in establishing the molecular basis for the function and regulation of the glucose transporter.

The glucose transporter in human erythrocytes and most cell types catalyzes the facilitative diffusion of D-glucose across the cell membrane in a stereospecific manner. During the past 15 years, a number of different methods have been developed to identify and study the glucose transporter (Wheeler & Hinkle, 1985). In general, these methods have identified in human erythrocytes a band 4.5 protein [nomenclature of Steck (1974)] which migrates in sodium dodecyl sulfate (SDS)[1]–polyacrylamide gels as a broad band with M_r 45 000–65 000. Although the regulation of glucose transport by various hormones and factors has been under intensive study, the molecular mechanisms by which the transporter functions and is regulated are still not fully understood. The lack of specific and sensitive probes has presented a major difficulty in studying the structure and regulation of glucose transport at the molecular level, particularly in non-erythrocyte cells in which the transporter is a low abundance protein. Although [^{3}H]cytochalasin B binding (Baldwin et al., 1979, 1981), affinity-labeling (Carter-Su et al., 1982; Shanahan, 1982; Holman et al., 1986), and reconstitution (Kasahara & Hinkle, 1977; Sogin & Hinkle, 1978) assays have been used to identify glucose transporters in a variety of cell types (Wheeler & Hinkle, 1985), these techniques have rather low sensitivity, are difficult to perform, and are of limited use in dissecting the topology of the glucose transporter. It is also difficult to monitor the purity of transporter preparations with these assays, especially in non-erythrocyte membranes.

In this paper, we report the preparation and characterization of a monoclonal antibody to the glucose transporter that can be used to identify, quantify, purify, and study the structure of the glucose transporter. Because a monoclonal antibody is against a single epitope, it is much more likely to be against a single protein than a polyclonal antibody prepared against impure preparations of glucose transporters (Sogin & Hinkle, 1980; Baldwin & Lienhard, 1980). Although this is not the first monoclonal antibody to the glucose transporter described in the literature (Allard & Lienhard, 1985; Boyle et al., 1985), there are several unique features of this antibody and/or its characterization. To facilitate characterizing this antibody, we developed a rapid and easy assay to quantitate D-glucose-sensitive photoaffinity labeling of the transporter with cytochalasin B so that the amount of transporter in column fractions could be easily assessed. Using this technique in conjunction with reconstitution and binding assays, we show

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[1] Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; HAT, hypoxanthine, aminopterin, and thymidine; DEAE-cellulose, [(diethylamino)ethyl]cellulose; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LITC, lactosyl isothiocyanate; MITC, maltosyl isothiocyanate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; PMSF, phenylmethanesulfonyl fluoride; NBMPR, *S*-(nitrobenzyl)thioinosine; NBTGR, *S*-(nitrobenzyl)thioguanosine; ASA-BMPA, *N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)-2-propylamine.

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unequivocally that the glucose and nucleoside transporters which are both present in the substantially purified preparation used to prepare the antibody (Wu et al., 1983; Tse et al., 1985) are separate proteins and that the antibody is specific for the glucose transporter. We also show that the antibody binds to the M_r 18 000 tryptic peptide of the glucose transporter which contains the cytochalasin B binding site.

Experimental Procedures

Materials. *S*-(Nitrobenzyl)thioinosine (NBMPR), *S*-(nitrobenzyl)thioguanosine (NBTGR), protein A–Sepharose 4B, Sephadex G-25 (80 mesh), molecular weight protein standards, and cholesterol were purchased from Sigma. Cytochalasins B and D were purchased from Aldrich. Dimethyl pimelimidate and sodium lauryl sulfate (sequanal grade) came from Pierce and octyl glucoside from Calbiochem. Egg phosphatidylcholine, egg phosphatidic acid, and egg phosphatidylethanolamine came from Avanti. [4-^{3}H]Cytochalasin B (13–15 mCi/mmol), ^{125}I-protein A (9.8 μCi/μg), D-[^{14}C]-glucose (341 mCi/mmol), L-[^{3}H]glucose (10.7 Ci/mmol), tissue solubilizer (Protosol), Omnifluor, and Atomlight liquid scintillation fluid were obtained from New England Nuclear. [5,6-^{3}H]Uridine (49 Ci/mmol) came from ICN and [G-^{3}H]NBMPR (36 Ci/mmol) from Moravek Biochemicals. Rabbit anti-mouse IgG and alkaline phosphatase conjugated rabbit anti-mouse IgG were purchased from Hyclone and prestained molecular weight protein standards from Bio-Rad. Trypsin (TPCK) came from Worthington. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Hyclone, Irvine, or Grand Island Biological Co. Anti-band 3 monoclonal antibody (IV-F12) was a kind gift of Dr. M. Jennings, University of Texas at Galveston. Blood was obtained from the Detroit Chapter of the American Red Cross. Cultured human HBL100 mammary cells were obtained from Dr. B. Peters, murine 3T3-F442A cells from Dr. J. Schwartz, and human synovial cells from Dr. W. Castor (all from the University of Michigan).

Preparation of Antigen. Human erythrocyte ghosts were prepared according to the method of Dodge et al. (1963). Alkaline-treated ghosts were made by exposing the ghosts to pH 11.5 to remove cytoskeletal proteins (Gorga & Lienhard, 1981). The alkaline-treated ghosts were solubilized in 2% octyl glucoside, and band 4.5 proteins consisting primarily of glucose transporters were purified by DEAE-cellulose chromatography as described by Baldwin et al. (1982). In experiments described for Figures 1, 4, 7, and 8, protein was determined according to Lowry et al. (1951). In experiments in which octyl glucoside was used (Figures 2, 3, 5, and 6), protein was determined according to Bradford (1976). Bovine serum albumin (BSA) was used as a standard.

Preparation of the Monoclonal Antibody. Mice were injected with DEAE-cellulose-purified band 4.5 according to the injection schedule and fusion protocol developed by John Kearny (personal communication to Dr. L. Claflin, head of the Hybridoma Facility of the Michigan Diabetes Research and Training Center). The transporter (80 μg of protein/mouse) was emulsified with complete Freund's adjuvant and injected into the foot pads, axial lymph nodes, and inguinal lymph nodes of 4 BALB/c mice. Mice were boosted 9 times with 80 μg of protein per boost over a period of 5 months. One day after the final injection, cells from popliteal, axial, and inguinal lymph nodes were fused with cells of the NS-1 variant of the P3(MOPC21) myeloma parent cell line, in poly(ethylene glycol) 4000. Cells were subcloned in soft agar and resubcloned by limiting dilution. Mouse sera and hybridoma supernatants were screened for the presence of antibodies by ELISA using DEAE-cellulose-purified band 4.5 as the test substance and alkaline phosphatase conjugated rabbit anti-mouse IgG. Antibodies from the clone designated 7F7.5 gave the greatest response by ELISA and were chosen for further study.

SDS–PAGE and Immunoblotting. SDS–PAGE was performed according to the two-phase system of Laemmli (1970) with a 7.5, 9, or 11% polyacrylamide separating gel and a 3.5% polyacrylamide stacking gel [30:0.08 acrylamide:bis(acrylamide)]. Samples were diluted 1:1 with sample preparation buffer consisting of 167 mM Tris, 33% glycerol, and 3.3% SDS (w/v), pH 6.8. Proteins were visualized with Coomassie blue or transferred electrophoretically overnight at 1.8 A (4 °C) onto nitrocellulose (Towbin et al., 1979). The blots were incubated first with 2% milk proteins (Carnation instant nonfat milk) in PBS + 0.05% Tween-20 (blocking buffer) followed by purified antibody (1–2 μg/mL) in blocking buffer overnight at 24 °C. After being washed, the blots were incubated with ^{125}I-protein A (125 000 cpm/lane) in blocking buffer for 2 h at 24 °C. To detect the glucose transporter in non-erythrocyte cell types, the immunoblots were incubated with a second antibody (rabbit anti-mouse IgG) for 2–16 h at room temperature followed by ^{125}I-protein A. The antigen was visualized by autoradiography. Molecular weight standards included myosin (M_r 205 000), β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 400), BSA (M_r 66 000), catalase (M_r 58 000), ovalbumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), trypsinogen (M_r 24 000), soybean trypsin inhibitor (M_r 20 100), and α-lactoalbumin (M_r 14 200). In immunoblots, the standards were visualized by staining with 0.1% Amido Black in 50% methanol–7.5% acetic acid. In some experiments, prestained standards were used and included phosphorylase *b* (M_r 130 000), BSA (M_r 75 000), ovalbumin (M_r 50 000), carbonic anhydrase (M_r 39 000), soybean trypsin inhibitor (M_r 27 000), and lysozyme (M_r 17 000).

Production and Purification of Antibody. For routine maintenance, cells were grown in 90% DMEM + 10% fetal calf serum, supplemented with L-glutamine and antibiotics. For purification of monoclonal antibody, cells were transferred to serum-free medium. The supernatant was collected, and antibody was concentrated by $(NH_4)_2SO_4$ (50% saturation) precipitation. After dialysis against 20 mM Tris, pH 7.4, 20 mL of the concentrated supernatant was chromatographed on a column (2 mL) of protein A immobilized on Affi-Gel 10 (Bio-Rad). After extensive washing of this affinity resin with PBS, the monoclonal antibody was eluted with 0.1 M citric acid, pH 3.6. The pH of the eluate was adjusted immediately to 7–8 by addition of 1 M Tris, pH 11.

Immunoprecipitation of the Glucose Transporter. Purified monoclonal antibody (2–3 mg) was attached to protein A–Sepharose 4B (1 mL) with dimethyl pimelimidate according to Schneider et al. (1982). Un-cross-linked antibody was removed by washing the resin with 0.1 M citric acid, pH 3.6. The immobilized antibody was washed further with 0.1 M sodium borate, pH 8.2, and stored at 4 °C. Alkaline-treated ghost proteins (0.5–1 mg/mL) (Bradford, 1976) in 50 mM Tris, pH 7.4, were solubilized in 2% octyl glucoside on ice for 20 min. Dithiothreitol (2 mM) was added, and 10 min later, solubilized membrane proteins were cleared by centrifugation at 230000g_{max} for 1 h. The supernatant was diluted 4 times with 1% octyl glucoside in 50 mM Tris, pH 7.4, and combined with 0.5 mL of the immunomatrix (preequilibriated with 1% octyl glucoside in 50 mM Tris, pH 7.4). Following 2–4 h of gentle mixing using a 360° rotator at 4–8 °C, the proteins that

did not bind to the immunomatrix (designated as "unbound fraction") were collected, and the immunomatrix was washed extensively with buffer containing 1% octyl glucoside, 50 mM Tris, 1 mM EDTA, and 0.1 M NaCl, pH 7.4, until no protein was detected in the wash buffer. Antigen was eluted by successive washes with 0.1 M citric acid, pH 3.6, containing 1% octyl glucoside. The immunomatrix was regenerated by extensive washing with 0.1 M citric acid, pH 3.6, followed by 0.1 M sodium borate, pH 8.2.

Reconstitution of Membrane Proteins into Phospholipid Vesicles. Octyl glucoside solubilized proteins from alkaline-treated ghosts were incubated with immobilized 7F7.5 antibody. Portions of the starting material and the unbound fraction were reconstituted into phospholipid vesicles according to modifications of the procedures of Carter-Su and Czech (1980) and Baldwin et al. (1982). Briefly, solubilized proteins were added to octyl glucoside solubilized phosphatidylcholine (1.9 mM), phosphatidylethanolamine (0.5 mM), phosphatidic acid (0.5 mM), and cholesterol (0.5 mM). For the glucose transport assay, the protein and lipid mixture was dialyzed overnight against reconstitution buffer (100 mM NaCl, 10 mM Tris, 1.5 mM $MgSO_4$, pH 7.4). For the nucleoside transport assay, the mixture was dialyzed against 10 mM Tris, pH 7.4 (Tse et al., 1985). The resulting phospholipid vesicles were frozen in an acetone–dry ice bath and stored at −70 °C. Most were used within 3 days; all were used within 2 weeks.

Transport Assays. For the glucose transport assay, the frozen vesicles were thawed, sonicated briefly in a Bransonic bath sonicator, and incubated at room temperature for ~20 min. D-[^{14}C]glucose (0.75 μCi) and L-[^{3}H]glucose (1.5 μCi) in reconstitution buffer (10 μL) were added to 40-μL vesicles, and transport was assayed as described previously (Carter-Su et al., 1980). Nonspecific trapping and binding were accounted for by subtraction of a "zero" value determined by adding the radioactivity and ice-cold stopping buffer simultaneously and filtering immediately. The validity of this approach is supported by the finding that the zero value in picomoles of glucose was the same for both D- and L-glucose and vesicles containing proteins from the starting material and unbound fraction.

To assess the presence of nucleoside transport activity, uptake of [^{3}H]uridine by reconstituted vesicles was measured by a centrifugal gel filtration method according to Tse et al. (1985) with slight modification. The entire procedure was carried out in a 4 °C cold room. Briefly, 1-mL tuberculin syringes were filled with Sephadex G-25 (80 mesh) equilibrated in 10 mM Tris, pH 7.4, containing 20 μM NBMPR. The columns were centrifuged at 1000 rpm for 2 min in a Sorvall GLC centrifuge immediately before use. Vesicles (45 μL) were preincubated with or without 20 μM NBTGR for 5 min at 16 °C. Uptake was initiated by the addition of 45 μL of [^{3}H]uridine (10 μCi/mL, 49.7 μM final concentration) (16 °C). The reaction was terminated by addition of 20 μL of NBTGR (50 μM, ice cold); 100 μL of the reaction mixture was added to the column. Following centrifugation for 2 min, the eluate was collected and counted for radioactivity. [^{3}H]Uridine and 50 μM NBTGR were added simultaneously to vesicles to determine nonspecific trapping and binding.

NBMPR Binding Assay. Reconstituted vesicles were incubated with [^{3}H]NBMPR in the presence and absence of 20 μM NBTGR for 30 min. Bound and unbound [^{3}H]NBMPR were separated by centrifugation through small columns of Sephadex G-25 (80 mesh) as described above, except that no unlabeled NBMPR was present in the column buffer and the entire procedure was carried out at 24 °C. Specific binding was determined by subtracting nonspecific (in the presence of unlabeled 20 μM NBTGR) from total (in the absence of unlabeled NBTGR) binding.

Photoaffinity Labeling of Glucose and Nucleoside Transporters and Filter Paper Assay for the Presence of Transporters. To label the glucose transporter, alkaline-treated ghosts in 5 mM sodium phosphate, pH 8.0, were incubated with and without 500 mM D-glucose for 30 min at room temperature. In the absence of D-glucose, an equivalent concentration of D-sorbitol was included as an osmotic control. D-Sorbitol is not transported by the glucose transporter and does not inhibit binding of [^{3}H]cytochalasin B to the glucose transporter (Carter-Su et al., 1982). Cytochalasin D (10 μM) was added to block binding of cytochalasin B to actin (Carter-Su et al., 1982; Shanahan, 1982). [^{3}H]Cytochalasin B at different concentrations was then added. After 20–30 min, alkaline-treated ghosts were irradiated with UV light according to Carter-Su et al. (1982) with an American Ultraviolet Portacure lamp (1000 W). The nucleoside transporter was labeled by a modification of the method of Wu et al. (1983). Alkaline-treated ghosts were incubated with [^{3}H]NBMPR for 20 min in the presence and absence of 25 μM unlabeled NBMPR and then irradiated with UV light as described above. Portions (50 μL) of the UV-irradiated ghosts labeled with [^{3}H]cytochalasin B or [^{3}H]NBMPR were spotted on pieces of filter paper (2 × 2 cm, Whatman No. 3) which were then dropped into an ice-cold bath of 10% trichloroacetic acid (TCA). After 10 min, the filters were washed twice with 95% ethyl alcohol and once with acetone. The filters were dried and counted for radioactivity. Alternatively, the samples were subjected to SDS–PAGE. After staining and destaining, the gel was sliced into 1-mm segments, and every three slices were pooled. The samples were incubated overnight with Protosol (0.5 mL), and toluene–Omnifluor (3 mL) was added for scintillation counting. The amount of specifically labeled glucose transporter was expressed as the difference between the amount of covalently bound [^{3}H]cytochalasin B in the presence of 500 mM D-sorbitol and that in the presence of 500 mM D-glucose. The amount of specifically labeled nucleoside transporter was expressed as the difference between the amount of covalently bound [^{3}H]NBMPR in the absence of 25 μM unlabeled NBMPR and that in the presence of 25 μM unlabeled NBMPR.

Preparation of Membranes from Other Cell Types. Cultured IM9, HBL100, human synovial cells, and 3T3-F442A fibroblasts were washed 3 times with PBS, sonicated briefly in a Bransonic bath sonicator, and homogenized in 10 mM Tris, 1 mM EDTA, and 250 mM sucrose, pH 7.4, in a glass homogenizer fitted with a Teflon pestle. The homogenization buffer was supplemented with 1 mM PMSF, 100 μg/mL leupeptin, and 100 μg/mL aprotinin to avoid potential proteolysis. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C to remove unbroken cells and nuclei. The membrane proteins were pelleted by centrifugation at 230000*g* for 1 h at 4 °C. Rat erythrocyte membranes were prepared as described for human erythrocyte membranes. Rat adipocyte membranes were prepared as described previously (Carter-Su & Okamoto, 1985). Membranes were stored at −70 °C.

RESULTS

Binding of Antibody 7F7.5 to Band 4.5 Proteins in Human Erythrocyte Membranes. As a first test of the specificity of the 7F7.5 antibody, we determined whether the antibody bound to proteins migrating in SDS gels in the same region as the glucose transporter (band 4.5). Immunoblot analysis of proteins from human erythrocyte ghosts (Figure 1, lane A)

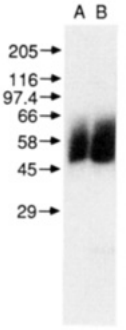

FIGURE 1: Immunoblot of membrane proteins from human erythrocytes with 7F7.5 monoclonal antibody. Human erythrocyte ghosts (30 μg, lane A) or alkaline-treated ghosts (30 μg, lane B) were electrophoresed on a 7.5% acrylamide gel. After electroblotting, the nitrocellulose was incubated with 7F7.5 antibody followed by ^{125}I-protein A and autoradiography. The migration of molecular weight standards ($\times 10^{-3}$) is indicated on the left side of the figure.

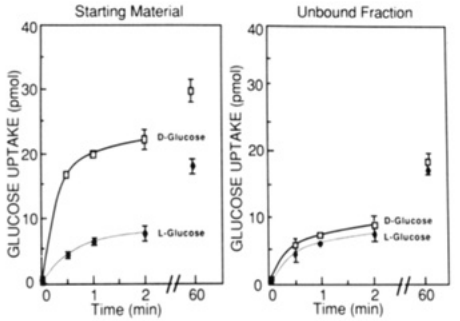

FIGURE 2: Removal of glucose transport activity by immobilized anti-glucose transporter monoclonal antibody. Solubilized alkaline-treated ghost proteins were incubated with immobilized antibody. The starting material (480 μg of protein/2 mL) (left panel) and unbound proteins (310 μg/2 mL) (right panel) were reconstituted into phospholipid vesicles as described under Materials and Methods. The uptake of D-[^{14}C]glucose (solid line) and L-[^{3}H]glucose (dotted line) was measured simultaneously and corrected for uptake at "0" time. Means ± SEM of triplicate samples are shown for a representative experiment. Where error bars are not visible, the SEM was smaller than the size of the symbol. This experiment was performed 3 times with similar results. In experiments not shown, equilibrium was found not to occur until 120 min, at which time intracellular concentrations of D- and L-glucose reached the same value.

and alkaline-treated ghosts (Figure 1, lane B) shows that the 7F7.5 antibody binds almost exclusively to band 4.5 proteins. A very small amount of antibody sometimes bound to a protein of M_r 23 000 in alkaline-treated ghosts, thought to be a proteolyzed form of the transporter (see below; Davies et al., 1987).

Removal of Glucose Transport Activity by Immobilized Antibody. To test whether the monoclonal antibody binds to the glucose transporter in band 4.5, we assessed the ability of immobilized antibody to remove glucose transport activity from octyl glucoside solubilized alkaline-treated ghosts. Portions of the starting material and the fractions containing proteins that did not bind to the antibody (unbound fraction) were reconstituted into phospholipid vesicles. Glucose transport activity was determined by monitoring simultaneously the uptake of D-[^{14}C]glucose and L-[^{3}H]glucose. L-Glucose is a poor substrate for the transporter (LeFevre, 1961). Its uptake was monitored to provide an estimate of the contribution of passive diffusion to the total uptake of D-glucose into these vesicles. The difference between the uptake of these two enantiomers of glucose was taken to indicate carrier-mediated glucose transport activity. When proteins in the starting material were reconstituted (Figure 2, left panel), the rate of D-[^{14}C]glucose uptake was significantly greater than the rate

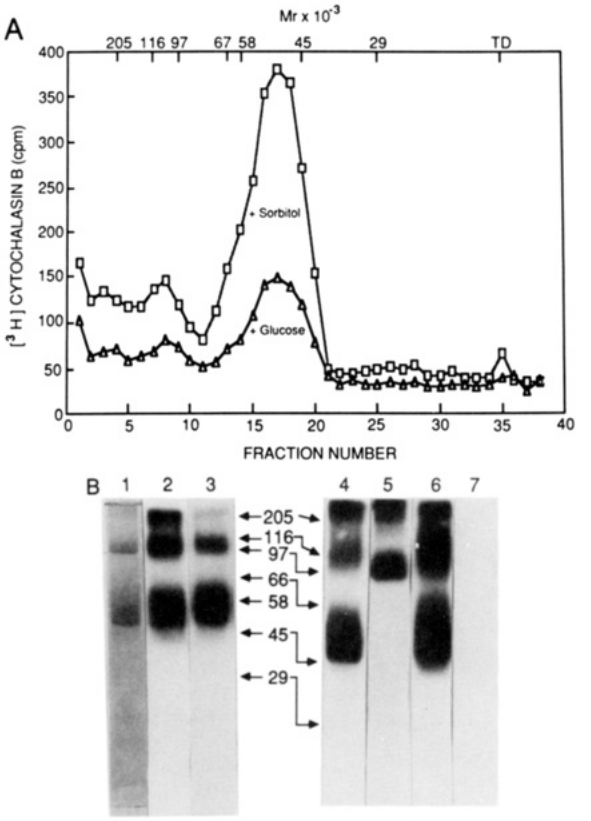

FIGURE 3: Immunoprecipitation of the glucose transporter. Alkaline-treated ghosts were labeled with [^{3}H]cytochalasin B, solubilized with octyl glucoside in the presence of D-glucose or D-sorbitol, and then subjected to immunoprecipitation. Bound proteins were eluted with 0.1 M citric acid, pH 3.6, and subjected to SDS–PAGE (9% acrylamide). The gel was either sliced and counted for radioactivity (10 μg of antigen) (panel A), stained with Coomassie blue (10 μg of antigen) (panel B, lane 1), or subjected to electroblotting followed by incubation with monoclonal antibody (1 μg of antigen) (panel B, lanes 2 and 3). In panel B, lane 3, the immunopurified antigen was dialyzed for 40 h to remove the detergent before freezing. In another experiment, 5 μg of immunopurified (panel B, lanes 4 and 5) or DEAE-cellulose-purified band 4.5 (dialyzed for 16 h) (panel B, lanes 6 and 7) was probed with either 7F7.5 antibody (panel B, lanes 4 and 6) or anti-anion transporter antibody (panel B, lanes 5 and 7). The migration of molecular weight standards is indicated at the top of panel A and in the center of panel B. TD indicates the migration of the tracking dye in panel A.

of L-[^{3}H]glucose uptake. In contrast, no specific transport of D-glucose was observed when proteins in the unbound fraction were reconstituted (Figure 2, right panel). These results suggest that the monoclonal antibody removed glucose transport activity from the starting material.

Ability of the Monoclonal Antibody To Immunoprecipitate Glucose Transporters Covalently Labeled with [^{3}H]Cytochalasin B. To provide additional evidence that the 7F7.5 monoclonal antibody bound to the glucose transporter, glucose transporters in alkaline-treated ghosts were labeled covalently with [^{3}H]cytochalasin B in the presence or absence of 500 mM D-glucose. Membrane proteins were solubilized in octyl glucoside and incubated with immobilized antibody. SDS–PAGE analysis of the proteins bound to and eluted from the antibody revealed the presence of a major peak of radioactivity in the band 4.5 region of the gel (Figure 3A) which was greatly diminished when D-glucose was present during the incubation with [^{3}H]cytochalasin B. This indicates that [^{3}H]cytochalasin B labeled transporter bound to and could be eluted from the antibody.

Coomassie blue staining of the immunopurified proteins revealed the presence of protein primarily in the band 4.5

region of the gel (lane 1, Figure 3B). Proteins of M_r 100 000 were also visible. In immunoblots of the immunoprecipitated protein (lanes 2–4, Figure 3B), the monoclonal antibody bound primarily to band 4.5 proteins. Some antibody also bound to proteins migrating with M_r 100 000, thought to be aggregates of band 4.5 proteins. The presence of band 4.5 aggregates is supported by several findings: (1) the antibody bound exclusively to band 4.5 proteins in ghosts and alkaline-treated ghosts not exposed to detergent (Figure 1); (2) the amount of M_r 100 000 protein and higher molecular weight aggregates was greatly diminished when the immunoprecipitates were dialyzed extensively in the presence of phospholipids (lane 3, Figure 3B); (3) proteins larger than band 4.5 were labeled with [^{3}H]cytochalasin B in a D-glucose-sensitive fashion in the immunoprecipitate (Figure 3A); (4) 7F7.5 antibody bound to proteins of M_r 45 000–65 000 and 100 000 in immunoblots of DEAE-cellulose-purified band 4.5 (lane 6, Figure 3B). This preparation contained no anion transporter (the major M_r 95 000–100 000 protein of human erythrocytes) as judged by its inability to bind anti-anion transporter monoclonal antibody (lane 7, Figure 3B). However, the Coomassie blue stained M_r 100 000 proteins precipitated by 7F7.5 were not exclusively aggregated glucose transporters, because anti-anion transporter antibodies also bound to M_r 100 000 proteins in the 7F7.5 immunoprecipitate (lane 5, Figure 3B).

Filter Paper Method To Assay for [^{3}H]Cytochalasin B Labeled Glucose Transporters and [^{3}H]NBMPR-Labeled Nucleoside Transporters. To establish firmly the specificity of the 7F7.5 monoclonal antibody for the glucose transporter, we tested whether the antibody could discriminate between the glucose transporter and nucleoside transporter, a membrane protein thought to be similar in structure. We tested the relative ability of the antibody to precipitate [^{3}H]cytochalasin B labeled glucose transporter and [^{3}H]NBMPR-labeled nucleoside transporter. To permit easy and rapid quantitation of the recovery of labeled glucose and nucleoside transporters in each fraction of an immunoaffinity preparation, we developed a filter paper assay to measure labeled transporters. Alkaline-treated ghosts were affinity-labeled with [^{3}H]cytochalasin B or [^{3}H]NBMPR. Aliquots of the membrane proteins were spotted onto filter paper and precipitated in 10% TCA. Free [^{3}H]cytochalasin B and [^{3}H]NBMPR were eliminated by washing the filters first with ethanol (95%) followed by acetone.

To verify that photoaffinity labeling in combination with the filter paper assay was adequate for assessing the presence of transporters, we tested the effect of different concentrations of ^{3}H-labeled ligand on the amount of labeled transporter subsequently recovered on the filter paper. The amount of covalently bound [^{3}H]cytochalasin B (Figure 4A) and [^{3}H]-NBMPR (Figure 4B) recovered on the filter paper increased with increasing concentrations of ^{3}H-labeled ligands. The amount of label on the filter paper was negligible when membrane proteins were incubated with [^{3}H]cytochalasin B but not exposed to UV light (shown as the "zero" value). For glucose transporter (Figure 4A), labeling with [^{3}H]cytochalasin B was inhibited 59% by 500 mM D-glucose. Average inhibition was 53 ± 2% (n = 7) at 0.5 μM cytochalasin B. D-Glucose at 100 mM was only slightly less effective (15% less, n = 2) than 500 mM D-glucose in preventing labeling of the transporter at 0.5 μM cytochalasin B. The amount of specifically labeled glucose transporter saturated at 0.3 μM [^{3}H]cytochalasin B, with half-maximal saturation at approximately 0.14 μM. For nucleoside transporter (Figure 4B), labeling with [^{3}H]NBMPR was inhibited substantially when

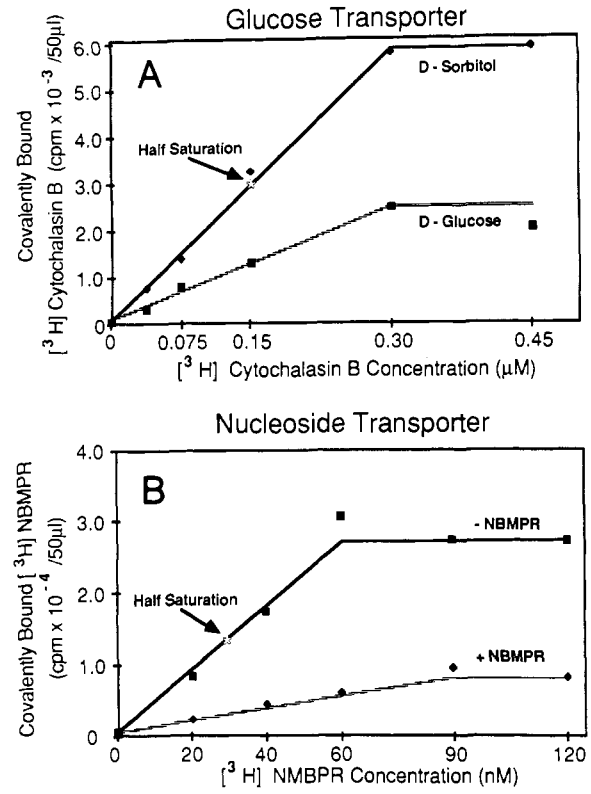

FIGURE 4: Filter paper method to quantitate the amount of photoaffinity-labeled glucose and nucleoside transporter. Alkaline-treated ghosts (1.0 mg/mL) were photoaffinity labeled with the designated concentration of [^{3}H]cytochalasin B in the presence of 500 mM D-sorbitol or D-glucose (panel A) or [^{3}H]NBMPR in the absence or presence of 25 μM unlabeled NBMPR (panel B). Labeled proteins (50 μL) were spotted on a filter, precipitated, washed, and counted as described under Materials and Methods. Each symbol shows the average of two filter papers from the same assay. The experiment was performed 3 times with similar results.

25 μM unlabeled NBMPR was present during the treatment with UV light. The amount of specifically labeled nucleoside transporter saturated at approximately 60 nM. Half-maximal label incorporation was observed at 31 nM.

Monoclonal Antibody Discriminates between the Glucose and Nucleoside Transporters. The filter paper method described above allowed us to assess whether monoclonal antibody 7F7.5 binds exclusively to the glucose transporter. Alkaline-treated ghosts were labeled with 0.5 μM [^{3}H]cytochalasin B in the presence and absence of 500 mM D-glucose or with 75 nM [^{3}H]NBMPR in the presence and absence of 25 μM unlabeled NBMPR. Membrane proteins were solubilized, and the antigen was immunopurified as described earlier. The amount of each transporter recovered in each fraction was determined by spotting 50 μL of the solubilized protein sample onto filter paper. Figure 5A (lower two panels) shows that 99% of the specifically [^{3}H]cytochalasin B labeled glucose transporter present in the starting material was found to bind to and could be eluted from the immunomatrix. No [^{3}H]cytochalasin B labeled transporter was detected in unbound fractions. In contrast, 92% of the [^{3}H]NBMPR-labeled nucleoside transporter added to the immunomatrix was recovered in the unbound fractions (Figure 5B, lower two panels). Only 3% of the specifically labeled nucleoside transporter bound to and was eluted from the immunomatrix. The amount of protein in the unbound, wash and eluted fractions is shown in the upper panels of Figure 5. The amount of protein recovered in each fraction was the same whether the proteins were labeled with [^{3}H]cytochalasin B ± D-glucose or [^{3}H]-NBMPR ± unlabeled NBMPR, indicating that the solubility

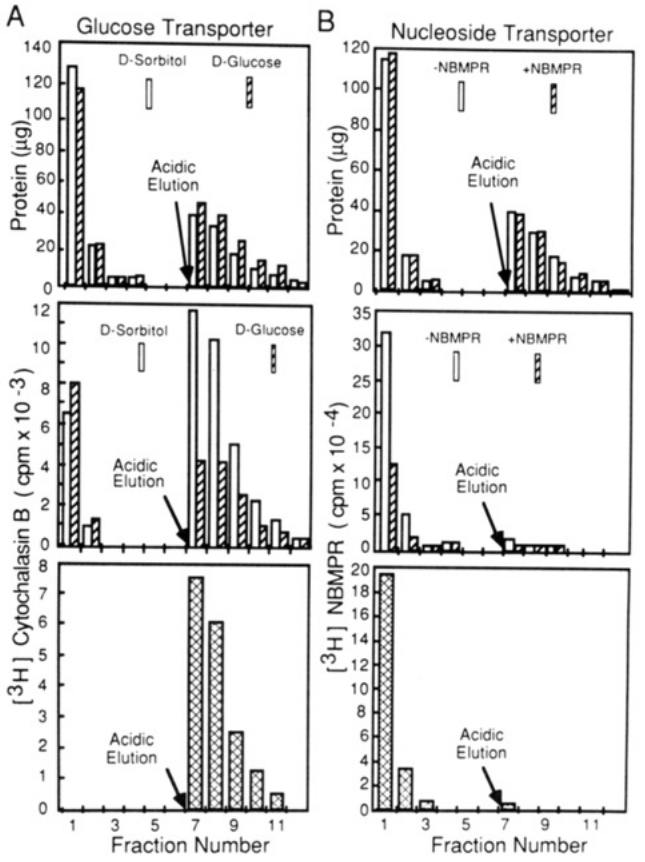

FIGURE 5: Antibody differentiates between the glucose and nucleoside transporters. Alkaline-treated ghosts (720 μg of protein/1 mL) were prelabeled with [^{3}H]cytochalasin B (panel A) or [^{3}H]NBMPR (panel B), solubilized with octyl glucoside, and then incubated with the immobilized monoclonal antibody. After extensive washing, the antigen was eluted with 0.1 M citric acid (pH 3.6). The amount of protein (upper panels) and the radioactivity of proteins labeled with ^{3}H-labeled ligands (middle panels) were determined for all unbound, wash, and eluted fractions. The amount of specifically labeled glucose and nucleoside transporter (lower panels) was determined as described under Materials and Methods. This experiment was repeated once with similar results.

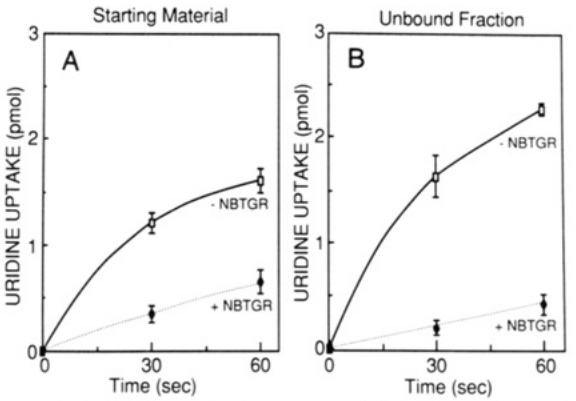

FIGURE 6: Antibody 7F7.5 does not precipitate NBTGR-sensitive uridine uptake from detergent solution. Octyl glucoside solubilized alkaline-treated ghosts (810 μg of protein) were incubated with immobilized antibody. Portions of the starting material (810 μg of protein/4.5 mL) (panel A) and unbound proteins (495 μg/4.5 mL) (panel B) were reconstituted into phospholipid vesicles and assayed for [^{3}H]uridine uptake in the absence (solid line) and presence (dashed line) of 20 μM NBTGR. Means ± SEM of triplicate samples are shown for a representative experiment. The experiment was performed 2 times with similar results.

of the antigen and the binding between the antigen and the antibody were not affected by the different conditions of photoaffinity labeling.

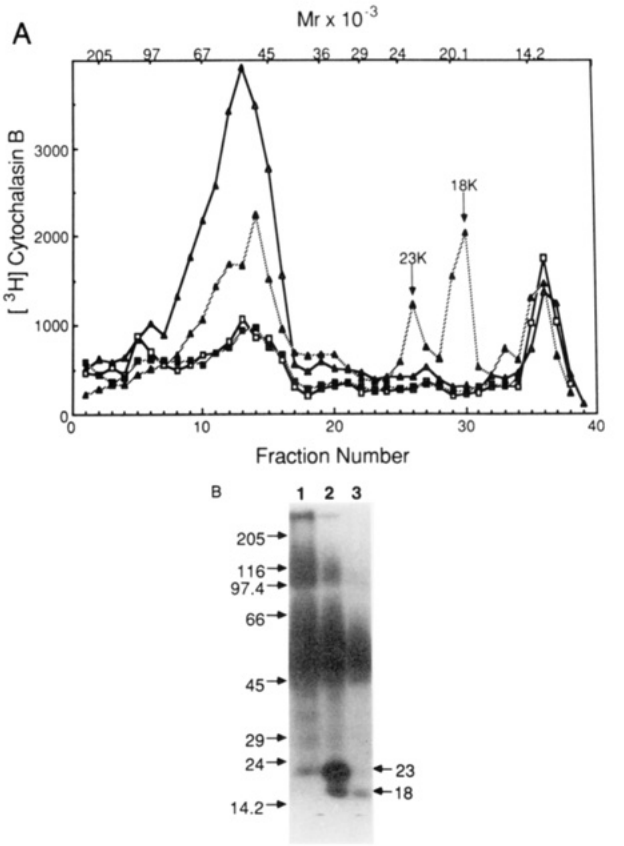

FIGURE 7: Immunodetection of the tryptic fragments of glucose transporter. (Panel A) Alkaline-treated ghosts (600 μg of protein) were covalently labeled with 0.5 μM [^{3}H]cytochalasin B in the presence of either 500 mM D-sorbitol (▲—▲, ▲---▲) or D-glucose (□—□, ■---■) and then treated with (▲---▲, ■---■) or without (▲—▲, □—□) trypsin (10 μg/mL) at room temperature for 1 h. Proteolysis was terminated by addition of PMSF at a final concentration of 20 μg/mL. The membrane proteins were then subjected to SDS–PAGE (11% acrylamide), and the amount of radioactivity (cpm) was determined for every 3-mm gel. (Panel B) DEAE-cellulose-purified band 4.5 (20 μg of protein) was treated with 0 (lane 1), 1 (lane 2), or 10 μg/mL (lane 3) trypsin and then subjected to SDS–PAGE (9% acrylamide) and immunoblot analysis using the 7F7.5 antibody. The migration of molecular weight standards is indicated at the top of panel A and to the left of panel B. The migrations of the M_r 23 000 and 18 000 fragments ($\times 10^{-3}$) are indicated at the right of panel B.

Since the [^{3}H]NBMPR-labeled nucleoside transporter represents only 20% of the total transporter, it was important to determine whether 7F7.5 antibody also recognized the native nucleoside transporter. Solubilized ghost proteins were incubated with the immunomatrix, and portions of the starting material and unbound fraction were reconstituted into phospholipid vesicles. Vesicles were assayed for NBTGR-sensitive uridine uptake and [^{3}H]NBMPR binding. NBTGR and NBMPR are both potent inhibitors of the nucleoside transporter. Figure 6 illustrates that NBTGR-sensitive uridine uptake activity was not removed by immobilized antibody. An average of 90% recovery of [^{3}H]NBMPR binding activity in the unbound fraction (Table I) is consistent with the value of 92% recovery of affinity-labeled NBMPR binding sites in the unbound fraction (Figure 5B) and indicates that the antibody recognizes neither the native nor the photoaffinity-labeled nucleoside transporter.

Monoclonal Antibody Binds to Tryptic Peptides That Contain the Cytochalasin B Binding Site of the Glucose Transporter. Limited digestion of the alkaline-treated ghosts labeled with [^{3}H]cytochalasin B with trypsin (10 μg/mL) resulted in decreased amounts of intact labeled transporter (M_r 55 000) and generation of two [^{3}H]cytochalasin B labeled

Table I: 7F7.5 Antibody Does Not Immunoprecipitate NBMPR Binding Proteins[a]

	NBMPR binding (pmol)			
	starting material		unbound fraction	
	expt 1	expt 2	expt 1	expt 2
−NBTGR[b]	85.90	72.93	83.24	76.87
+NBTGR[c]	21.91	22.89	23.75	33.23
specific[d]	63.99	50.04	59.49	43.64
recovery (%)	100	100	93.0	87.2

[a] Portions of the reconstituted vesicles (80 μL) used for Figure 6 were incubated with 60 nM [^{3}H]NBMPR in the absence or presence of 20 μM NBTGR for 30 min at room temperature. Bound and unbound [^{3}H]NBMPR were separated by centrifugation. [b] Total binding. [c] Nonspecific binding. [d] Total minus nonspecific binding.

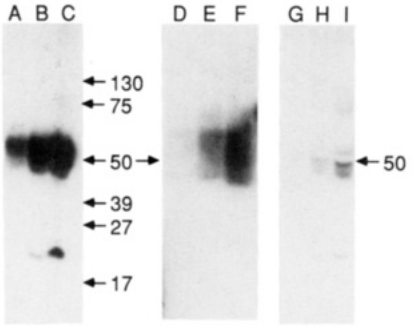

FIGURE 8: Immunoblot of membrane proteins from cultured human cells with the 7F7.5 antibody. Membrane proteins were isolated from cultured human HBL-100 mammary cells (lanes A–C), IM-9 lymphocytes (lanes D–F), and synovial cells (lanes G–I). Proteins (12.5, 25, and 50 μg) were separated by SDS–PAGE (9% acrylamide) and subjected to immunoblot analysis with the 7F7.5 antibody.

fragments of M_r 23 000 and 18 000 (Figure 7A). The labeling of all three proteins was partially or totally prevented by preincubating the membrane proteins with 500 mM D-glucose, indicating that the two proteolytic fragments are derived from the intact glucose transporter. Immunoblot analysis of tryptic-digested DEAE-cellulose-purified band 4.5 shows that the antibody binds to both tryptic fragments (Figure 7B). Before treatment with trypsin, the antibody bound primarily to the intact transporter, although a faint band at M_r 23 000 was also visible (Figure 7B, lane 1). Trypsin added at 1 μg/mL decreased the intensity of the M_r 55 000 band, increased that of the M_r 23 000 band, and introduced a new band of M_r 18 000 (Figure 7B, lane 2). When the trypsin concentration was increased to 10 μg/mL, the intensities of all three bands decreased (Figure 7B, lane 3). These results indicate that the 7F7.5 antibody recognizes tryptic fragments of M_r 23 000 and 18 000 that contain the cytochalasin B binding site.

Binding of Antibody to the Transporter in Other Cell Types. To determine the tissue and species specificity of the antibody, we assessed the ability of the monoclonal antibody to identify glucose transport proteins in immunoblots of membrane proteins prepared from three different human cell lines, two rat tissues, and one murine cell line. As shown in Figure 8, the monoclonal antibody recognized a protein migrating as a broad band of M_r 45 000–65 000 in human HBL100 mammary cells and human IM9 lymphocytes and M_r 45 000–50 000 in human synovial cells. The antibody also bound to a protein of M_r ~23 000, suggesting that endogenous proteolysis occurred in these membrane preparations despite the presence of protease inhibitors. In contrast to the cells of human origin, the monoclonal antibody did not recognize any protein in immunoblots of membrane proteins prepared from rat adipocytes, rat erythrocytes, or cultured murine 3T3-F442A preadipocyte fibroblasts (data not shown). These results suggest that the glucose transporter migrates as a broad band of M_r 45 000–65 000 in multiple cell types and that monoclonal antibody 7F7.5 recognizes the glucose transporter in different human cell types but not in cells of rodent origin.

Discussion

The 7F7.5 Monoclonal Antibody Recognizes the Human Glucose Transporter. Results from this work clearly demonstrate that the 7F7.5 antibody recognizes the human glucose transporter. First, it identifies band 4.5 proteins in immunoblots of human erythrocyte ghosts and alkaline-treated ghosts. Second, the immobilized antibody removes glucose transport activity from solubilized membrane preparations. Third, the immobilized antibody immunoprecipitates 99% of the [^{3}H]cytochalasin B labeled glucose transporter from detergent-solubilized alkaline-treated ghosts.

Filter Paper Assay for Covalently Labeled Glucose and Nucleoside Transporters. Since it is so difficult to quantitate transporters by activity in reconstituted vesicles, transport proteins are generally quantitated by the binding activity of specific inhibitors. Unfortunately, in most cases, ligand–protein binding is lost when the membrane proteins are exposed to the concentrations of detergent needed for solubilization. Although ligand binding can often be restored by removing the detergent by dialysis, the recovery is often low, and the procedure is usually tedious and time consuming, making the purification of integral membrane proteins by chromatographic techniques difficult. One must be able to estimate the amount of the membrane protein of interest collected in each fraction from a column before protein denaturation occurs. To overcome this problem, we developed a fast (less than 1 h) and easy filter paper assay to assess the presence of labeled proteins in column fractions. Loss of ligand binding activity in detergent is avoided because the photolabeling is performed with the intact membrane prior to detergent treatment. In the present study, this allowed us to show complete immunoprecipitation of the glucose transporter by 7F7.5 antibody and to demonstrate that the antibody can differentiate between the glucose and nucleoside transporters.

The adequacy of the filter paper method was assessed by the following criteria. First, the amount of covalently bound [^{3}H]cytochalasin B was decreased by glucose during the affinity-labeling step, as expected for the glucose transporter. Measured radioactivity was decreased by an average of 53% and 42% by preincubating the membrane proteins with 500 mM and 100 mM D-glucose, respectively, at 0.5 μM cytochalasin B. This agrees well with previous reports that 500 mM D-glucose inhibits by 60–70% the amount of [^{3}H]cytochalasin B labeled protein migrating in the band 4.5 region of electrophoretic gels (Carter-Su et al., 1982; Shanahan, 1982) and that 120 mM D-glucose is 90% as effective as 500 mM D-glucose (Carter-Su et al., 1982). Similarly, the amount of covalently bound [^{3}H]NBMPR was substantially inhibited by the presence of 25 μM unlabeled NBMPR during the labeling step. Second, the concentrations of ligand at which half-maximal labeling was observed agree well with values reported in the literature for half-maximal binding. For glucose transporters, a value of 0.14 μM [^{3}H]cytochalasin B for half-maximal labeling is consistent with K_d's of 0.1–0.2 μM reported for cytochalasin B binding to glucose transporters in human erythrocyte membranes (Lin & Spudich, 1974; Sogin & Hinkle, 1978; Baldwin et al., 1979). It is also comparable to the value of 0.7 μM for half-saturation of D-glucose-inhibitable [^{3}H]cytochalasin B incorporation into band 4.5 proteins reported by Carter-Su et al. (1982). Our esti-

mated value of 31 nM for half-maximal incorporation of [^{3}H]NBMPR into the nucleoside transporter is comparable to the half-saturation concentration (13 nM) of [^{3}H]NBMPR incorporation into band 4.5 proteins reported by Young et al. (1983). Both values are substantially higher than the K_d of 0.3 nM for reversible high-affinity [^{3}H]NBMPR binding to human ghosts reported by Wu et al. (1983).

Glucose and Nucleoside Transporters Are Distinct Proteins That Can Be Separated by Use of the 7F7.5 Monoclonal Antibody. To ensure the specificity of the 7F7.5 antibody, we tested whether the antibody cross-reacts with other membrane proteins. Because the antibody identified only band 4.5 proteins in immunoblots of erythrocyte ghosts or alkaline-treated ghosts, our primary concern was whether the antibody cross-reacted with proteins other than the glucose transporter which migrate in the band 4.5 region of the gel. The inability of the antibody to precipitate the nucleoside transporter, a band 4.5 protein with many molecular similarities to the glucose transporter, was felt to provide the most stringent test possible for the specificity of the antibody for several reasons. First, the two transporters cannot be separated by electrophoresis, DEAE-cellulose chromatography, or reverse-phase high-pressure liquid chromatography (Klip et al., 1986). Thus, the antigen used for immunization contained some nucleoside transporter (estimated 3%; Jarvis & Young, 1981). Second, Jarvis et al. (1985) prepared monoclonal antibodies from DEAE-cellulose-purified band 4.5 that immunoprecipitate both the glucose and nucleoside transporters. These investigators reported no antibodies that bound to either transporter exclusively. Kwong et al. (1986) reported almost identical migration patterns of the transporters (M_r 44 000 for the nucleoside transporter and M_r 45 000 for the glucose transporter) following deglycosylation with endoglycosidase F, although differential sensitivities of [^{3}H]cytochalasin B labeled and [^{3}H]NBMPR-labeled proteins to endoglycosidase F (Klip et al., 1986) and trypsin (Janmohamed et al., 1985) digestion have been reported. Most recently, an attempt by Jarvis et al. (1986) to photoaffinity label the nucleoside transporter with a covalent probe, 8-azidoadenosine, resulted in affinity labeling of the glucose transporter. The identity of the labeled substrate was based upon the finding that cytochalasin B and D-glucose, but not cytochalasin E or L-glucose, inhibited the labeling. The finding in the present work that the two transporters can be completely separated by the 7F7.5 monoclonal antibody provides strong evidence that the two transporters are different proteins and is consistent with the two transport systems having distinct inhibitors and substrate specificities. It also supports a similar conclusion reached by Boyle et al. (1985), who used a monoclonal antibody to remove cytochalasin B binding activity but not nucleoside binding activity from solubilized DEAE-cellulose purified band 4.5.

Identity and Size of the Glucose Transporter in Human Erythrocytes. Coomassie blue staining of the immunopurified protein (Figure 3B) showed that the antibody bound primarily band 4.5 proteins. It also bound proteins migrating with M_r 100 000, at least a portion of which are thought to be transporter dimers, formed after detergent exposuue, for reasons discussed under Results. A tendency of the glucose transporter to aggregate upon boiling and exposure to detergent has been discussed previously by Lienhard et al. (1982) and Haspel et al. (1985). The presence of relatively small amounts of anion transporter relative to glucose transporter in 7F7.5 immunoprecipitates suggests that band 3 proteins may also form aggregates with glucose transporters, since immunoblots of ghost proteins indicate that band 3 proteins by themselves do not bind to 7F7.5 antibody. A close association of band 3 proteins with the glucose transporter has also been suggested from studies using the sugar analogues MITC and LITC to study the glucose transporter. Mullins and Langdon (1980) found MITC interfered with glucose transport although [^{14}C]MITC preferentially labeled proteins in band 3, rather than in band 4.5. Since the incorporation of this compound was decreased by cytochalasin B, D-glucose, and other transported sugar analogues, and substantially purified band 3 protein supported hexose transport when reconstituted into liposomes (Shelton & Langdon, 1983), these investigators concluded that the glucose transporter is a band 3 protein. Rees et al. (1987) found that LITC not only inhibited glucose transport but also decreased both reversible binding and photoaffinity labeling of band 4.5 with [^{3}H]ycytochalasin B, even though the LITC analogue [^{14}C]MITC bound almost exclusively to band 3 proteins. These investigators hypothesized an interaction between a band 3 LITC/MITC binding component and the intracellular cytochalasin B binding site on the glucose transporter. Similarly, May (1987) found that MITC binding was decreased by the anion transport inhibitor dihydro-DIDS and that MITC inhibited both tritiated dihydro-DIDS labeling of band 3 and sulfate uptake in intact cells. He speculated that the glucose and anion transporters are separate proteins but may lie in close physical proximity in the membrane or exist as part of a larger oligomeric complex. This may explain why band 4.5 and some band 3 proteins appear to copurify in our immunoaffinity chromatography procedure. Thus, a one-step purification procedure using 7F7.5 antibody may not be ideal for the purification of glucose transporters from crude membranes. However, since DEAE-cellulose-purified band 4.5 contains no band 3, a two-step purification utilizing DEAE-cellulose in conjunction with immunoaffinity chromatography should produce homogeneous preparations of nucleoside and glucose transporters.

Epitope Recognized by the 7F7.5 Monoclonal Antibody Resides within the Cytochalasin B Binding Tryptic Fragment of the Transporter. Both alkaline-treated ghosts and DEAE-cellulose-purified band 4.5 were subjected to limited trypsin digestion to determine the epitope recognized by the anti-glucose transporter monoclonal antibody. Limited trypsin digestion has been reported to dissect the glucose transporter into two large fragments, with the N-terminal half migrating in SDS gels with M_r 23 000–42 000 and containing the carbohydrate moiety and the C-terminal half migrating with M_r 21 500–23 500 and retaining the cytochalasin B binding site. The latter fragment can be further digested into a M_r 18 000–19 000 form which lacks the C terminus (Cairns et al., 1984; Davies et al., 1987; Shanahan & D'Artel-Ellis, 1984). Using relatively mild conditions, we found that 10 μg/mL trypsin added to alkaline-treated ghosts generated two major fragments of M_r 23 000 and 18 000 which were labeled with [^{3}Hycytochalasin B. Immunoblot analysis of DEAE-cellulose-purified band 4.5 indicated that the antibody recognized both tryptic fragments and is consistent with the M_r 18 000 form being derived from the M_r 23 000 fragment. This is the only monoclonal antibody reported to bind to the M_r 18 000 and 23 000 fragments (Allard & Lienhard, 1985; Boyle et al., 1985; Jarvis et al., 1985). The M_r 18 000 fragment is of particular interest because of recent studies of Davies et al. (1987) and Holman and Rees (1987). Using a polyclonal antibody against a synthetic peptide corresponding to the C-terminal region (residues 477–492) of the deduced amino acid sequence of the Hep G2 glucose transporter (Mueckler et al., 1985), Davies et al. (1987) suggested that the M_r 23 000

tryptic fragment spans amino acid residues 256–492 and the M_r 18 000 fragment spans amino acid residues 256–458 of the intact transporter. The finding that the 7F7.5 antibody recognizes the M_r 18 000 cytochalasin B binding component and does not bind to the exofacial surface of the transporter (data not shown) suggests that the epitope recognized by the antibody lies between residues 256 and 458, either on the cytoplasmic face or in the membrane spanning domain of the intact transporter. It is unlikely that the epitope resides within the cytochalasin B binding site itself, because transporters labeled covalently with [^{3}H]cytochalasin B continue to bind to the antibody. Furthermore, in experiments not shown, preincubation of alkaline-treated ghosts with antibody did not decrease binding with cytochalasin B. Studying the fragmentation pattern of the glucose transporter affinity labeled on the internal side with [^{3}H]cytochalasin B or on the external side with [^{3}H]ASA-BMPA, Holman and Rees (1987) proposed that the core of the transporter is located in the membrane spanning segments H7–H10 (residues 272–402) of the glucose transporter. This segment is thought to include the exofacial active site, the internal cytochalasin B binding site, and a possible hydrogen-bonding channel through which sugar could move. Since this segment is located within the M_r 18 000 cytochalasin B binding tryptic fragment which is recognized by the 7F7.5 antibody, this antibody will provide a powerful tool to elucidate the topology and function of the glucose transporter.

Summary. We have produced a monoclonal antibody (designated 7F7.5) which is specific for the glucose transporter. It is the first antibody shown to immunoprecipitate the glucose transporter from a solubilized membrane preparation, rather than the already substantially pure DEAE-cellulose-purified band 4.5 used to prepare the antigen. Furthermore, the avidity is not so great as to preclude eluting the transporter from the antibody with citric acid rather than SDS and boiling. The antibody data in conjunction with the newly developed filter paper assay to assess the presence of covalently labeled membrane transporters suggest that the glucose and nucleoside transporters are two distinct proteins. Furthermore, the 7F7.5 antibody, which recognizes the cytochalasin B binding tryptic fragment, provides us with an excellent tool for purifying both the nucleoside and glucose transporters and elucidating the structure of the glucose transporter.

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Multiple Phosphorylation Sites in the 165-Kilodalton Peptide Associated with Dihydropyridine-Sensitive Calcium Channels†

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ABSTRACT: Evidence from electrophysiological and ion flux studies has established that dihydropyridine-sensitive calcium channels are subject to regulation by neurotransmitter-mediated phosphorylation and dephosphorylation reactions. In the present study, we have further characterized the phosphorylation by cAMP-dependent protein kinase and a multifunctional Ca/calmodulin-dependent protein kinase of the membrane-associated form of the 165-kDa polypeptide identified as the skeletal muscle dihydropyridine receptor. The initial rates of phosphorylation of the 165-kDa peptide by both protein kinases were found to be relatively good compared to the rates of phosphorylation of established substrates of the enzymes. Phosphorylation of the 165-kDa peptide by both protein kinases was additive. Prior phosphorylation by either one of the kinases alone did not preclude phosphorylation by the second kinase. The cAMP-dependent protein kinase phosphorylated the 165-kDa peptide preferentially at serine residues, although a small amount of phosphothreonine was also formed. In contrast, after phosphorylation of the 165-kDa peptide by the Ca/calmodulin-dependent protein kinase, slightly more phosphothreonine than phosphoserine was recovered. Phosphopeptide mapping indicated that the two kinases phosphorylated the peptide at distinct as well as similar sites. Notably, one major site phosphorylated by the cAMP-dependent protein kinase was not phosphorylated by the Ca/calmodulin-dependent protein kinase, while other sites were phosphorylated to a high degree by the Ca/calmodulin-dependent protein kinase, but to a much lesser degree by the cAMP-dependent protein kinase. The results show that the 165-kDa dihydropyridine receptor from skeletal muscle can be multiply phosphorylated at distinct sites by the cAMP- and Ca/calmodulin-dependent protein kinases. As the 165-kDa peptide may be the major functional unit of the dihydropyridine-sensitive Ca channel, the results suggest that the phosphorylation-dependent modulation of Ca channel activity by neurotransmitters may involve phosphorylation of the 165-kDa peptide at multiple sites.

Voltage-dependent calcium channels are believed to be regulated by neurotransmitter-mediated phosphorylation and dephosphorylation reactions. Evidence from electrophysiological and ^{45}Ca flux studies has shown that cAMP and cAMP-dependent protein kinase can influence the opening of Ca channels in cardiac and skeletal muscle (Reuter, 1983; Tsien et al., 1986; Schmid et al., 1985; Arreola et al., 1987), as well as in vertebrate (Fedulova et al., 1985) and invertebrate neurons (Chad & Eckert, 1986). Other studies have suggested that certain Ca channels also are subject to regulation by agents which activate protein kinase C (DeRiemer et al., 1985; Rane & Dunlap, 1986; Di Virgilio et al., 1986; Miller, 1987). Ca channels are also known to be regulated by intracellular calcium, which could conceivably involve phosphorylation mediated by Ca/calmodulin (CaM)[1]-dependent protein kinases. As dephosphorylation would be expected to reverse effects of phosphorylation, several studies have shown that Ca channel activity can be regulated by phosphoprotein phosphatases (Kameyama et al., 1986; Chad & Eckert, 1986; Hescheler et al., 1987). Despite the numerous observations concerning effects of protein kinases and phosphatases on Ca channel activity, very little is known about the molecular events underlying the regulation of Ca channels by phosphorylation and dephosphorylation. It remains to be determined if components of the channels, or regulatory proteins, are reversibly phosphorylated in intact cells in response to signaling by different neurotransmitter-activated pathways.

It is now recognized that there are muliple types of Ca channels which differ in their voltage dependencies, kinetics, and pharmacological sensitivities (Nowycky et al., 1985; Cognard et al., 1986; Miller, 1987). Only one type of Ca channel, known as the L type (Nowycky et al., 1985), slow or dihydropyridine (DHP)-sensitive Ca channel, has been well

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[1] Abbreviations: DHP, dihydropyridine; T-tubule, transverse tubule; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate; WGA, wheat germ agglutinin; $NaDodSO_4$, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CaM, calmodulin.

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