Evidence That Functional Erythrocyte-type Glucose Transporters Are Oligomers*

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In this study we tested the hypothesis that functional erythrocyte-type glucose transporters (GLUT1) exist as oligomeric complexes by expressing chimeric transporter proteins in Chinese hamster ovary cells harboring endogenous GLUT1 transporters. The chimeric transporters were GLUT1-4c, in which the 29 C-terminal residues of human GLUT1 were replaced by the 30 C-terminal residues of rat skeletal muscle glucose transporter (GLUT4), and GLUT1n-4, containing the N-terminal 199 residues of GLUT1 and the 294 C-terminal residues of GLUT4. Endogenous GLUT1 was quantitatively co-immunoprecipitated by using an anti-GLUT4 C-terminal peptide antibody from detergent extracts of Chinese hamster ovary cells expressing either of the chimeric proteins, as detected by immunoblotting the precipitates with an anti-GLUT1 C-terminal peptide antiserum. No co-immunoprecipitation of native GLUT1 with native GLUT4 from extracts of 3T3-L1 adipocytes, which contain both these transporters, was observed with the same antibody. These data are consistent with the hypothesis that GLUT1 transporters exist as homodimers or higher order oligomers and that a major determinant of oligomerization is located within the first 199 residues of GLUT1.

The facilitated diffusion of glucose in mammalian cells is mediated by a family of integral membrane glycoproteins composed of at least five isoforms identified by cDNA cloning (1-9). All glucose transporters share deduced primary structures that predict 12 transmembrane domains and cytoplasmic locations for the N-terminal and C-terminal segments as well as for a large hydrophilic loop connecting the sixth and seventh transmembrane regions (1-9). Little is known about the molecular mechanism by which D-glucose transport occurs, due in part to a lack of detailed information describing the three-dimensional structures of glucose transporter proteins. Previous studies on the size of the native human erythrocyte glucose transporter, measured by irradiation inactivation of either cytochalasin B binding (10, 12) or D-glucose flux (11) and by freeze-fracture electron microscopy (13), suggested molecular sizes compatible with either a dimer (12, 13) or a tetramer (10, 11). However, these findings were also consistent with the possibility that the glucose carrier is assembled with one or more heterologous proteins. Such physical associations with the erythroid glucose transporter have been recently proposed for hexokinase (14) and glyceraldehyde-3-phosphate dehydrogenase (15).

The aim of the present studies was to test directly whether multiple erythrocyte-type glucose transporters (GLUT1) are present in oligomeric complexes in cell membrane preparations. We engineered cDNA constructs encoding two chimeric transporter proteins which contain N-terminal regions of GLUT1 and C-terminal regions of the adipocyte-type transporter (GLUT4) and expressed these proteins in stable CHO cell lines. Immunoprecipitation of these chimeric transporters from membrane detergent extracts with an anti-GLUT4 C-terminal peptide antibody also precipitated native GLUT1 molecules, endogenously expressed in CHO fibroblasts. These data strongly indicate that GLUT1 transporter proteins exist as homooligomeric structures.

EXPERIMENTAL PROCEDURES

Production of the Chimeric Constructs—GLUT1-4c chimera is composed of the coding region for the first 463 codons of the human GLUT1 cDNA linked to the coding region for the last 30 codons of the rat GLUT4 cDNA. To obtain a compatible restriction fragment containing the first 463 codons of GLUT1, a non-unique Sau3A I site at position 1565 on the cDNA from the plasmid pGem3Z-GLUT1 (16) was mutated to a unique BglII site by site-directed mutagenesis. The chimeric cDNA was generated by ligating a 1565-bp (BamHI-BglII) fragment from GLUT1 cDNA to a 3' 763-bp (BglII-BamHI) fragment of the GLUT4 cDNA at the BglII site. GLUT1n-4 chimera has the first 215 codons of rat GLUT4 cDNA replaced by the first 199 codons of GLUT1 cDNA. The chimeric cDNA was assembled by ligating a 799-bp BamHI-PstI fragment from GLUT1 cDNA to a 1549-bp PstI-BamHI fragment spanning codons 216-509 of GLUT4 cDNA, including 670 bp of its 3' untranslated region. Both chimeras were initially assembled in the bacterial plasmid vector pUC18. The identities of the chimeric cDNAs were verified by restriction analysis and DNA sequencing.

Transfection of the Chimeric cDNAs into CHO-K1 Cells—The chimeric inserts were subcloned into the BamHI cloning site of the mammalian expression vector pLEN, and transfection of the resultant expression vectors into subconfluent CHO-K1 cells was performed as described previously (16). Two clones, CHO 1-4c and CHO

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1 The abbreviations used are: GLUT1, erythrocyte/HepG2-type glucose transporter; GLUT4, adipocyte/skeletal muscle-type glucose transporter; CHO, Chinese hamster ovary; [125I]HAPS, 3-iodo-4-azidophenylethylamido-7-O-succinyldeacetyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; bp, base pairs.
1n-4, each expressing the corresponding type of chimera, were chosen for this study. 

Cell Culture Conditions—CHO-K1 fibroblasts and 3T3-L1 preadipocytes were obtained from American Type Culture Collection. CHO-K1 control fibroblasts, and CHO 1-4c and CHO 1n-4 cells were cultured in Ham’s F-12 medium containing 10% fetal calf serum. CHO-3T3-L1-L1 cells were grown in DMEM containing 10% calf serum. Two days after confluence, CHO-3T3-L1 fibroblasts were incubated to differen-
tiate basically as described in Ref. 17. Briefly, cells were incubated in DMEM supplemented with 10% fetal calf serum, 5 μg/ml insulin, 0.25 mM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. After 2 days, the medium was replaced by the same lacking dexamethasone and 3-isobutyl-1-methylxanthine, and the incubation was prolonged for another 2 days. Cells were then cultured in DMEM containing 10% fetal calf serum and used from day 8 to day 15 after the beginning of the differentiation.

Immunochemical Studies—Total mem-
bones from CHO-K1, CHO 1-4c, and CHO 1n-4 cells were subjected to photoaffinity labeling with [3H]IAPS-forskolin, essentially as de-
scribed by Wardzinski et al. (18). Briefly, cells were grown to conflu-
ence in 150-mm dishes and then harvested, homogenized in 20 mM Hepes, pH 7.4, 250 mM sucrose, and 1 mM EDTA in the presence of 1 mM phenylmethylsulfonyl fluoride. Total membranes were prepared by ultracentrifugation of the homogenate at 200,000×g for 90 min at 4°C. Membranes (200 μg of total membrane proteins for each sample) were resuspended in 50 μl of 20 mM Hepes, pH 7.4, 1 mM EDTA (resuspension buffer). When the photoaffinity labeling was performed, 1-2 μl of a 50 nM solution of [3H]IAPS-forskolin was added to each sample (1-2 nM final concentration) in the absence or in the presence of 200 μM cytochalasin B, and the incubation was performed for 30 min in the dark. Samples were then diluted in resuspension buffer ≥ 200 μM cytochalasin B and irradiated for 5 s with a 1000-watt UV lamp. Subsequently, 2-mercaptoethanol was added to a final concentration of 1%. Membranes were then collected by ultracentrifugation at 200,000×g for 90 min, solubilized in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (10% resolving gel). Gels were dried under vacuum and autoradiographed by using Kodak X-OMAT AR films, and the intensity of the bands was determined by laser densitometric scanning.

Immunoblotting—Total cell membranes—Immunoblotting of total membranes from CHO 1-4c, CHO 1n-4, and parental cells to detect endogenous GLUT1 was performed by using a 1:1000 dilution of R-480 antibody, an anti-GLUT1 C-terminal antisera directed against residues 475-492 of human GLUT1 (17). Membrane proteins (100 μg for each sample) were resolved by SDS-polyacrylamide gel electrophoresis (10% resolving gel) and then electrothermally transferred to a nitro-
cellulose filter at 200 mA for 3 h. Filters were incubated with R-480 antiserum (2 h at 23°C), and antibody binding was detected by [125I] Protein A (Du Pont-New England Nuclear). Autoradiography of the filters and quantification of the intensity of the bands were performed as described previously.

Immunoprecipitation with Anti-GLUT4 C-terminal Peptide Anti-
sen—Total membranes from CHO 1-4c, CHO 1n-4 cells (100 μg for each sample), and 3T3-L1 adipocytes (200 μg for each sample) were solubilized in a buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 2% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (0.25 mM final concentration) for 30 min at either 0 or 23°C, and subjected to centrifugation at 100,000×g for 1 h at 4°C. The supernatant and the insoluble pellet were assayed for glucose trans-
porters in CHO-K1 cells. Parental cells (CHO-K1), CHO-K1 fibroblasts transfected with chimeric GLUT1-4c molecules (CHO 1-4c), or chimeric GLUT1n-4 molecules (CHO 1n-4) were assayed for 2-deoxyglucose uptake (clear bars), [3H]IAPS-forskolin binding to total cell membranes (hatched bars), and GLUT1-immunoreactive protein (dotted bars). A representative experiment of each assay is presented.
That stable CHO cell lines transfected with cDNAs encoding either of these chimeric proteins exhibit 5-6-fold increases in rates of 2-deoxyglucose uptake compared with the parental CHO-K1 cells. Similar increases in 2-deoxyglucose transport rates were observed in previous studies in which CHO cells were transfected with cDNA encoding native human GLUT1 and GLUT4 and GLUT4c proteins, respectively, was observed (16). A 10- and 13-fold increase in [125I]APS-forskolin photo-labeling of total membranes from CHO cell lines expressing the GLUT1n-4 and GLUT1-4c proteins, respectively, was observed (Fig. 1b). This reagent (18) effectively labels both endogenous GLUT1 and the expressed chimeric transporters, thereby providing an estimate of total transporter proteins present in these cells. The amounts of endogenous GLUT1 are similar in all transfected and parental cell lines, as assessed by immunoblotting total membranes with an anti-GLUT1 C-terminal peptide antibody (R-480), raised against amino acids 475-492 of GLUT1 (17) (Fig. 1b). It is interesting to note that stable CHO cell lines transfected with the rat GLUT4 cDNA expressed GLUT4 protein, as detected by immunoblot (not illustrated). However, no elevations of 2-deoxyglucose uptake rates or [125I]APS-forskolin labeling were observed, indicating very low GLUT4 expression in these transfected cells. The results described above indicate that both chimeric transporters can be expressed at high levels in CHO cells. Furthermore, the chimeric carriers are capable of catalyzing glucose transport when expressed in CHO cells.

In order to assay whether endogenous GLUT1 and the expressed chimeras in CHO cells are physically associated, chimeric transporters in Nonidet P-40 extracts of total membranes from both CHO 1-4c and CHO 1n-4 cells were immunoprecipitated with an anti-C-terminal peptide antibody (R-1288) directed against amino acids 498-509 of rat GLUT4 (19). The immunoprecipitates were resolved by electrophoresis, and transport proteins were detected by immunoblot analysis (Fig. 2) using the R-1288 antisera, which recognizes both GLUT1-4c (lanes 1-3) and GLUT1n-4 transporters (lanes 7-9), as well as using the anti-GLUT1 C-terminal peptide antibody R-480 (lanes 4-6 and 10-12). Both antibodies are isofrom-specific in their ability to immunoprecipitate and immunoblot the respective native GLUT1 and GLUT4 transporters. In particular, R-1288 antisera fails to either immunoprecipitate or immunoblot GLUT1 from total membrane extracts from parental CHO-K1 cells (not shown). Immunoprecipitation performed with R-1288 antisera provided a quantitative recovery of both GLUT1-4c and GLUT1n-4 transporter proteins (lanes 2 and 8, respectively), compared with the total amount of chimeric transporters present in the membrane extracts (lanes 1 and 7, respectively). Nonspecific immunoprecipitation was assessed as described in detail under "Experimental Procedures" (lanes 3, 6, 9, and 12). Importantly, analysis of these immunoprecipitates by immunoblotting with the anti-GLUT1 C-terminal antisera R-480 also showed quantitative recovery of endogenous GLUT1 (lanes 5 and 11 compared with lanes 4 and 10, respectively). Therefore, GLUT1 was entirely co-immunoprecipitated with the chimeric molecules. These data indicate that nearly all native GLUT1 from CHO 1-4c and CHO 1n-4 cells is physically associated with the chimeric transporters in the detergent extracts. Immunoprecipitations with the anti-GLUT1 C-terminal antisera R-480 were not performed due to low efficiency of GLUT1 recovery using this antibody.

In an effort to evaluate the specificity of the co-immunoprecipitation of GLUT1 with chimeric transporters, similar experimental protocols were performed with membrane extracts from 3T3-L1 adipocytes. These cells express both native GLUT1 and native GLUT4 transporter isoforms in a ratio of about 3:1 (20). GLUT4 was quantitatively immunoprecipitated from total membrane extracts of 3T3-L1 adipocytes using the R-1288 anti-GLUT4 C-terminal peptide antibody (Fig. 3, lane 2). However, no GLUT1 could be detected...
in the same immunoprecipitate when probed with the R-480 antiserum (Fig. 3, lane 5). Similar results were obtained when detergent extracts of either plasma membranes or low density microsomes from 3T3-L1 adipocytes were studied in this manner (not illustrated). These results, demonstrating that GLUT1 and GLUT4 fail to co-immunoprecipitate, are consistent with the previous findings of Calderhead et al. (20), using 3T3-L1 adipocytes, and Zorzano et al. (21), using primary rat fat cells. It is noteworthy that the latter cell type expresses at least 10-fold more GLUT4 transporter protein than GLUT1. If hetero-oligomeric complexes of GLUT1 and GLUT4 occurred in these extracts, quantitative precipitation of GLUT1 should have been observed. The weight of the results presented here and those previously published indicate that GLUT1 molecules are not physically associated with GLUT4 transporters under the conditions of these experiments. Furthermore, the absence of co-precipitation of GLUT1 and GLUT4 in rat adipocytes indicates that the 10-fold greater expression of GLUT4 alone is not sufficient to drive a physical association of this isoform with GLUT1 (21). Therefore, it is unlikely that the expression of chimeric transporters in CHO cells to a level severalfold greater than that of endogenous GLUT1 causes an artifactual oligomerization of the two molecules. Moreover, we observed co-precipitation of endogenous GLUT1 in cells where the expression of the chimeric molecule was only 3–4-fold over the parental level (not shown).

In order to better compare the results of Figs. 2 and 3, the relative amounts of GLUT1 and GLUT4 immunoreactivity in CHO 1-4c cells and in 3T3-L1 adipocytes were determined. The epitope recognized by R-1288 is conserved between mouse GLUT4 and the chimeric molecule. Since the hamster GLUT1 has not been cloned to date, we assumed that R-480 antibody would bind GLUT1 molecules from the two species with comparable affinity, given the high degree of sequence homology in this isoform from different species. Nevertheless, GLUT1 quantitation must be considered approximate. Based on these considerations, the amount of GLUT4 immunoreactivity in CHO 1-4c cells was found to be only 1.8-fold greater than in 3T3-L1 adipocytes, when normalized per cell number. On the other hand, GLUT1 immunoreactive protein is approximately 4.5-fold greater in 3T3-L1 adipocytes than in CHO 1-4c fibroblasts (not shown). These observations combined with the report that the former cell line expresses more GLUT1 than GLUT4 (20) imply that the total amount of glucose transporter proteins harbored by chimera-expressing CHO cells is significantly lower than that expressed by 3T3-L1 adipocytes. These results further argue against the possibility of artificial glucose transporter physical association driven by elevated expression of chimeric transporters.

The results in Figs. 2 and 5 suggest that co-immunoprecipitation of GLUT1 transporter with GLUT1-4c and GLUT1n-4 proteins is due to specific interaction and not to random association mediated by their hydrophobic properties. Moreover, it would be difficult to attribute the transporter protein association described here to an artifact due to solubilization. Nonidet P-40 is a nonionic detergent with a hydrophile-lipophile balance value in the lipophilic range. This type of detergent appears to provide a hydrophobic environment most closely matched to that of the membrane, thereby maintaining membrane proteins in their native conformation (22). Proteins that appear to exist as monomers in the membrane are found as monomers following detergent solubilization (see, for example, rhodopsin (23)). Membrane proteins existing as oligomers in situ are found as oligomers in detergents (see, for example, Ca²⁺-ATPase (24) and the erythrocyte anion transporter (25)). It is noteworthy that the co-immunoprecipitation of GLUT1 with GLUT1-4c chimera was still observed when CHO 1-4c total membranes were solubilized in radioimmune precipitation buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (not illustrated). Furthermore, we performed experiments where membranes from CHO 1-4c cells were mixed with membranes from CHO GT3 fibroblasts, a cell line overexpressing human GLUT1 (16) prior to solubilization at 0 °C in 2% Nonidet P-40 and immunoprecipitation with R-1288 antiserum. GLUT1 immunoreactivity expressed by CHO GT3 cells is 17–20-fold greater than that expressed by parental cells (16). If the physical association of GLUT1 and the chimeric transporters occurred in the detergent solution, the amount of GLUT1 immunoreactive protein co-precipitating with GLUT1-4c molecules should be much greater from the mixed membrane lysate than from a lysate containing CHO 1-4c membranes alone. We found that the same amount of GLUT1 immunoreactive protein coprecipitates with the chimeric molecules under both conditions, indicating that glucose transporter physical association is present prior to membrane solubilization (results not illustrated).

The finding that GLUT1 does not associate with GLUT4 in these experiments indicates that GLUT1 oligomerization involves sequences intrinsic to the GLUT1 primary structure not present in GLUT4. Since GLUT1 co-immunoprecipitates with the GLUT1n-4 chimera, the site of interaction between the two molecules (or between the two molecules and a binding protein) must be located within the first 199 residues of GLUT1. Interestingly, within this GLUT1 segment, a “leucine zipper-like” motif is present (26). This structure is clearly a candidate for driving the association between glucose transporter molecules. However, this sequence is partially conserved in GLUT4 and thus may not be the only sequence required for interaction. The potential involvement of this “leucine zipper-like” motif suggests the hypothesis of a dimeric transporter structure, since leucine repeats have been shown to play a major role in dimerization of several DNA-binding proteins (27).

The results in Fig. 3, showing that expressed chimeric glucose transporters exhibit catalytic activity, combined with the evidence that they are oligomeric (Fig. 2) indicate that transporter oligomers are functional. A multimeric assembly of glucose carrier proteins could provide a rational explanation for the observed catalytic properties of the human erythrocyte sugar transport system. The human erythrocyte sugar carrier exposes both influx and efflux sites to the substrate simultaneously (28, 29). When these sites are occupied by nontransported, competitive inhibitors of transport, they interact with negative cooperativity (28, 29). Cooperative interactions are frequently observed in multimeric enzymes and ligand-binding proteins, where binding of a ligand to one subunit of the complex affects the affinity of the remaining subunit(s) for substrate (30, 31). Sugar transport by most cells is consistent with a homodimer model in which sugar influx and efflux sites are arranged in an antiparallel fashion and localization of substrate by one subunit promotes the complementary translocation of sugar by the second subunit (32).
define whether other glucose transporter isoforms also exist as oligomers and whether control of the transporter oligomeric state may play a role in regulation of transporter function.

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