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Protein Interactions with the Glucose Transporter Binding Protein GLUT1CBP That Provide a Link between GLUT1 and the Cytoskeleton

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Subcellular targeting and the activity of facilitative glucose transporters are likely to be regulated by interactions with cellular proteins. This report describes the identification and characterization of a protein, GLUT1 C-terminal binding protein (GLUT1CBP), that binds via a PDZ domain to the C terminus of GLUT1. The interaction requires the C-terminal four amino acids of GLUT1 and is isoform specific because GLUT1CBP does not interact with the C terminus of GLUT3 or GLUT4. Most rat tissues examined contain both GLUT1CBP and GLUT1 mRNA, whereas only small intestine lacked detectable GLUT1CBP protein. GLUT1CBP is also expressed in primary cultures of neurons and astrocytes, as well as in Chinese hamster ovary, 3T3-L1, Madin–Darby canine kidney, Caco-2, and pheochromocytoma-12 cell lines. GLUT1CBP is able to bind to native GLUT1 extracted from cell membranes, self-associate, or interact with the cytoskeletal proteins myosin VI, α-actinin-1, and the kinesin superfamily protein KIF-1B. The presence of a PDZ domain places GLUT1CBP among a growing family of structural and regulatory proteins, many of which are localized to areas of membrane specialization. This and its ability to interact with GLUT1 and cytoskeletal proteins implicate GLUT1CBP in cellular mechanisms for targeting GLUT1 to specific subcellular sites either by tethering the transporter to cytoskeletal motor proteins or by anchoring the transporter to the actin cytoskeleton.

INTRODUCTION

Glucose transporters are required for efficient movement of glucose across the plasma membrane of mammalian cells. GLUT1–GLUT5 and GLUT7 are six functional isoforms that comprise the family of facilitative glucose transporters (Mueckler, 1994). Despite the high degree of sequence homology, each can be subject to distinct modes of regulation. GLUT1 is a ubiquitously expressed transporter, and like that of other members of this transporter family, its rate of synthesis, subcellular localization, and activity each contribute to the regulation of the rate of glucose uptake by cells expressing this isoform.

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As a mode of regulation, insulin-induced translocation of GLUT1 to the plasma membrane occurs but is less pronounced than is that for GLUT4. GLUT1 is more commonly regulated via alterations in the level of transporter expression or via translocation-independent changes in the activity of the transporter residing in the plasma membrane. For example, no changes in the plasma membrane concentration of GLUT1 are noted when glucose transport is stimulated by protein synthesis inhibitors in 3T3 fibroblasts (Clancy *et al.*, 1991), by anoxia in Clone 9 cells (Shetty *et al.*, 1993), or by glucose starvation in 3T3-L1 adipocytes (Fisher and Frost, 1996). Recently, it was shown that GLUT1 in erythrocyte ghosts could be activated by agents that disrupt the actin cytoskeleton (Zhang and Ismail-Beigi, 1998). Although no GLUT1-specific regulatory proteins have been identified to date, it is

likely that GLUT1 binding proteins exist that could either directly alter GLUT1 activity or mediate interactions with other GLUT1 regulatory factors.

Although a unique insulin-regulated pattern of membrane targeting exists for GLUT4 in muscle and fat tissues, specialized targeting of GLUT1 occurs in other cell types. For example, GLUT1 expression is polarized in cells comprising the blood–brain barrier (Pardridge *et al.*, 1990), the blood–placental barrier (Takata *et al.*, 1994), peripheral nerve cell sheaths (Muona *et al.*, 1992), kidney proximal tubules (Heilig *et al.*, 1995), mammary glands (Camps *et al.*, 1994), the rat oviduct (Tadokoro *et al.*, 1995), and the intestine (Boyer *et al.*, 1996). In Caco-2 and Madin–Darby canine kidney (MDCK) cells, which are model systems for intestinal and kidney epithelia, respectively, GLUT1 is localized to the basolateral plasma membrane (Harris *et al.*, 1992; Pascoe *et al.*, 1996). GLUT1 is localized to glial fibrillary acidic protein (GFAP)[1]-positive processes in primary astrocytes (Maher, 1995), and in the heart, GLUT1 is localized to the specialized membrane surfaces of the intercalated discs (Doria-Medina *et al.*, 1993). Thus, *in vivo*, GLUT1 subcellular localization serves an important and presumably regulated role to facilitate transcellular interchange of glucose between vascular and cellular compartments.

The mechanism(s) by which GLUT1 achieves a polarized distribution within certain cells is unknown. In the parasitic protozoan *Leishmania enriettii*, association of glucose transporters with the cytoskeleton is a critical determinant for their proper intracellular targeting (Snapp and Landfear, 1997), and a critical requirement of microtubules and motor proteins for targeting viral proteins in polarized cells has been demonstrated (Lafont *et al.*, 1994). Targeting motifs have been identified that direct some integral membrane proteins to either the apical or basolateral membrane in polarized epithelial cells (Mostov *et al.*, 1992; Brown and Stow, 1996). Although GLUT1 may contain such motifs, these signals have not been identified.

The cytosolic, C-terminal domain of GLUT1 is 42 amino acids in length and is functionally important because mutant GLUT1 molecules that lack the C terminus exhibit significantly reduced activity (Oka *et al.*, 1990; Muraoka *et al.*, 1995; Dauterive *et al.*, 1996). One source of seizures and delayed development in humans can be directly attributed to the loss of the GLUT1 C terminus and the resulting impaired sugar movement across the blood–brain barrier (De Vivo *et al.*, 1991). The C-terminal domain of GLUT1 is conserved between species but is dissimilar in sequence to that of other GLUT isoforms. Therefore, the C terminus is a potential binding site for isoform-specific regulatory and/or targeting proteins. The observations that antibodies against the GLUT1 C terminus stimulate GLUT1 transport activity (Tanti *et al.*, 1992) and that peptides derived from the GLUT1 C terminus block azide-induced regulation of GLUT1 activity in Clone 9 cells (Shi *et al.*, 1995) both provide indirect evidence supporting the existence of such regulatory proteins, because their normal interactions with the C terminus and subsequent alteration of GLUT1 activity would be blocked by these reagents. The unique sequence and functional importance of the C terminus of GLUT1 and indirect evidence that it serves as a regulatory target prompted a search for proteins capable of interacting with this unique domain.

Using the yeast two-hybrid system, we isolated a cDNA clone whose polypeptide product, GLUT1 C-terminal binding protein (GLUT1CBP), binds to the GLUT1 C terminus. GLUT1CBP is unique among previously reported GLUT1 binding proteins (Lachaal *et al.*, 1990; Lachaal and Jung, 1993; Liu *et al.*, 1995; Shi *et al.*, 1995) because of its demonstrated binding specificity and the presence of a novel PDZ domain. This study examines the characteristics of GLUT1CBP, which include the binding specificity of its PDZ domain, patterns of mRNA and protein expression, self-association, and interaction with cytoskeletal motor proteins. The data support a model in which GLUT1CBP links GLUT1 to cytoskeletal motor proteins, thereby facilitating targeting of GLUT1 to specific subcellular sites.

MATERIALS AND METHODS

The Yeast Two-Hybrid System

Coding sequences for the following transporter C-termini were PCR amplified and inserted downstream of the coding sequence for the Gal4 DNA binding domain (Gal4 DBD) in pGBT9 (Clontech, Palo Alto, CA): from mouse GLUT1, amino acids 451–492 (GT1), 451–468 (GT1Δ24), 451–488 (GT1Δ4), and 451–492 with a valine(492)-to-alanine mutation (GT1V492A); from human GLUT3, amino acids 475–522 (HGT3); or from mouse GLUT4, amino acids 464–509 (GT4). The PCR-amplified portions of all constructs were sequenced to ensure that no PCR-induced errors were present. The entire GLUT1CBP cDNA was excised from pACT with *Bgl*II and ligated into the *Bgl*II site of pGBT9 for two-hybrid screening. The coding sequence for amino acids 107–247 of GLUT1CBP (the PDZ domain) was PCR amplified and inserted into pGBT9 or pGAD10. Other domains of GLUT1CBP were subcloned using available restriction sites. Construction of the rat brain cDNA library has been described (Brondyk *et al.*, 1995). Two-hybrid screens were performed as detailed in the Clontech Matchmaker Library protocol PT1020-1 and the Clontech Matchmaker two-hybrid system protocol PT1265-1. To exclude false positives, we cotransformed DNA (in pACT) from positive clones into HF7c with either pGBT9 (Gal4 DBD alone) or pSE1112 (Snf-1 fused to Gal4 DBD). These cotransformants failed to

[1] Abbreviations used: CHO, Chinese hamster ovary; DBD, DNA binding domain; ECL, enhanced chemiluminescence; GFAP, glial fibrillary acidic protein; GLUT1CBP, GLUT1 C-terminal binding protein; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; HUAEC, human umbilical artery-endothelial cells; HUVEC, human umbilical vein-endothelial cells; KIF-1B, kinesin superfamily protein 1B; MDCK, Madin–Darby canine kidney; PC-12 cells, pheochromocytoma-12 cells; PSD, postsynaptic density; PVDF, polyvinylidene fluoride.

demonstrate LacZ activity or growth on minus-histidine plates above background levels.

Sequencing of GLUT1CBP

The *Bgl*II fragment of the GLUT1CBP cDNA (in pACT) was subcloned into pBSKII (Bluescript II SK+; Stratagene, La Jolla, CA), and the sequence was determined at the DNA sequencing and synthesis facility at Iowa State University (Ames, IA).

Northern Blot Analysis

RNA purification (guanidinium thiocyanate extraction and sedimentation through CsCl) and Northern blot analysis were performed as described (Sambrook *et al.*, 1989). Poly(A)+ RNA was purified by oligo(dT)-cellulose chromatography. Ethidium bromide staining confirmed that an equal amount of RNA was loaded in each lane.

Preparation of His_6–GLUT1CBP, Glutathione S-Transferase (GST)–GLUT1CBP(249–333), and GST–GLUT Fusion Proteins

GLUT1CBP was subcloned into the *Xho*I site of pET30a(+) (Novagen, Madison, WI). The resulting fusion protein contained His_6- and S-tag sequences attached to the N terminus of GLUT1CBP. This construct was transformed into *Escherichia coli* BL21 (DE3)pLysS. Cells were induced, and the fusion protein was purified by Ni affinity chromatography from inclusion bodies using guanidine HCl as described by the manufacturer, except that 1 M sodium chloride was included to maintain the protein in solution during the removal of guanidine HCl. The concentration of GLUT1CBP was estimated assuming $A_{280}^{0.1\%} = 1$. cDNA's encoding glucose transporter C-termini were subcloned from pGBT9 vectors into pGEX-4T-1 (Pharmacia, Piscataway, NJ). The coding sequence for amino acids 249–333 of GLUT1CBP was inserted into pGEX-4T-1. Bacterial cells were grown and GST fusion protein synthesis was induced as described by the manufacturer.

Protein Overlay Assay

Cell pellets from bacteria expressing GST fusion proteins were dissolved in gel-loading buffer containing 50 mM Tris, pH 6.8, 2 mM EDTA, 6 M urea, 2% SDS, and bromophenol blue. The proteins were separated by SDS-PAGE using 10% gels and transferred to nitrocellulose membranes. The overlay assay was performed as described (Li *et al.*, 1992). However, buffer A in their procedure was modified to contain 10 mM HEPES-NaOH, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 40 nM purified His_6–GLUT1CBP as a probe. Bound His_6–GLUT1CBP was detected by enhanced chemiluminescence (ECL) using horseradish peroxidase (HRP)-labeled S-protein that recognizes the S-tag sequence present in His_6–GLUT1CBP.

Production of Antibody against GLUT1CBP

For antibody LSU43, peptide I, Cys-Gln-Arg-Ser-Ser-Gly-Gly-His-Pro-Gly-Ser-Gly-Pro-Gln-Leu-(amide), corresponding to amino acids 223–236 of GLUT1CBP (Figure 1, dashed line), was coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described (Sambrook *et al.*, 1989), dialyzed against PBS (minus KCl), and injected into New Zealand White rabbits (Cocalico Biologicals, Reamstown, PA). The antibody (LSU43) was purified and concentrated by adsorption to peptide I coupled to Sulfolink Gel (Pierce, Rockford, IL) according to the manufacturer's instructions. Antibody was eluted from the washed gel with glycine buffer, pH 3.0. The eluate was immediately neutralized with 1 M Tris, pH 9.5. The antibody was precipitated in 50% ammonium sulfate, resuspended, and dialyzed against PBS.

Antibody GAb(249–333) against the purified GST fusion protein to amino acids 249–333 of GLUT1CBP was raised in goat (Department of Veterinary Science, Louisiana State University Agricultural Center, Baton Rouge, LA).

Western Blot Analysis of the Cellular and Tissue Distribution of GLUT1CBP

Tissues from one male Sprague Dawley rat (Harlan Sprague Dawley, Indianapolis, IN) were isolated, quick frozen in liquid nitrogen, and stored at −80°C overnight. Frozen tissues (0.6–3.0 g) were disrupted with a Polytron 10N homogenizer in a 50-ml conical tube containing 2–4 ml of homogenization buffer (15% glycerol, 100 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2 mM EDTA, 10 μg/ml leupeptin, 1 mM PMSF) (Kain *et al.*, 1994). Samples were transferred to a glass–teflon homogenizer and further homogenized 10 times (on ice). Aliquots of the homogenate were frozen at −80°C. Cell monolayers were scraped directly into gel-loading buffer containing 4% SDS and 10 mM DTT. Protein content was determined by bicinchoninic acid analysis (Pierce) using bovine serum albumin as a standard. One hundred micrograms of protein from tissue or cellular extracts were diluted into gel-loading buffer, heated at 80°C for 5 min, separated by SDS-PAGE in 10% gels, and then transferred to nitrocellulose membranes in Tris-glycine buffer containing 20% (vol/vol) methanol. Membranes were blocked overnight in buffer containing 5% dry milk and 0.1% Tween 20 and then incubated with a 1:750 dilution of GAb(249–333). Membranes were washed, and bound GAb(249–333) was detected with HRP-conjugated monoclonal anti-goat IgG (Sigma, St. Louis, MO) and ECL.

Cell Culture

Chinese hamster ovary (CHO)-K1-HIR cells were seeded into 6-cm Falcon dishes (Lincoln Park, NJ) in Ham's F-12 media and harvested at confluence. Clone 5 MDCK cells (a generous gift of Dr. Mike Roth, Southwestern Medical School, Dallas, TX) were either maintained in Dulbecco's modified Eagle's medium in 10-cm Falcon dishes and harvested at confluence (nonpolarized cells) or maintained in polycarbonate transwell inserts (Corning, Cambridge, MA) and harvested 10 d after confluence (polarized cells). Caco-2 cells (a generous gift of Dr. Xavier Alvarez, Louisiana State University Medical Center [LSUMC], Shreveport, LA) were cultured on polycarbonate transwells as described for MDCK cells. Polarization and formation of tight junctions in MDCK and Caco-2 cells were assessed by measuring transepithelial resistance. 3T3-L1 preadipocytes were seeded into 6-cm Falcon dishes containing Dulbecco's modified Eagle's medium and grown to confluence. Differentiation was induced 2 d after confluence by exposure to isobutylmethylxanthine, dexamethasone, and insulin for 2 d followed by 4 d of incubation with insulin-containing media as described previously (Zuber *et al.*, 1985). Insulin was removed, and the cells were used after 2 additional days of incubation in insulin-free media. 3T3-L1 preadipocytes were cultured in parallel without exposure to insulin, isobutylmethylxanthine, or dexamethasone. Neurons and astrocytes were provided by Dr. Judson Chandler (LSUMC, Shreveport, LA). Pheochromocytoma (PC)-12 cells were provided by Dr. Donard Dwyer (LSUMC, Shreveport, LA).

Native Protein Binding Assays

His_6–GLUT1CBP and rabbit IgG were coupled to CNBr-activated Sepharose CL-4B (Pharmacia) according to the manufacturer's instructions at a density of 1 μg of protein per microliter of beads. Before use, the beads were blocked for 1 h at room temperature in PBS + 1% Triton X-100 (lysis buffer) + 1% BSA. For GLUT1 pull-down assays, one portion of beads was preincubated with 0.2 mg/ml peptide A (GLUT1 C-terminal 12 amino acids) for 1 h before the extract was added. Cell extracts were prepared by scraping confluent 10-cm dishes of CHO or MDCK cells into 0.5 ml/plate of

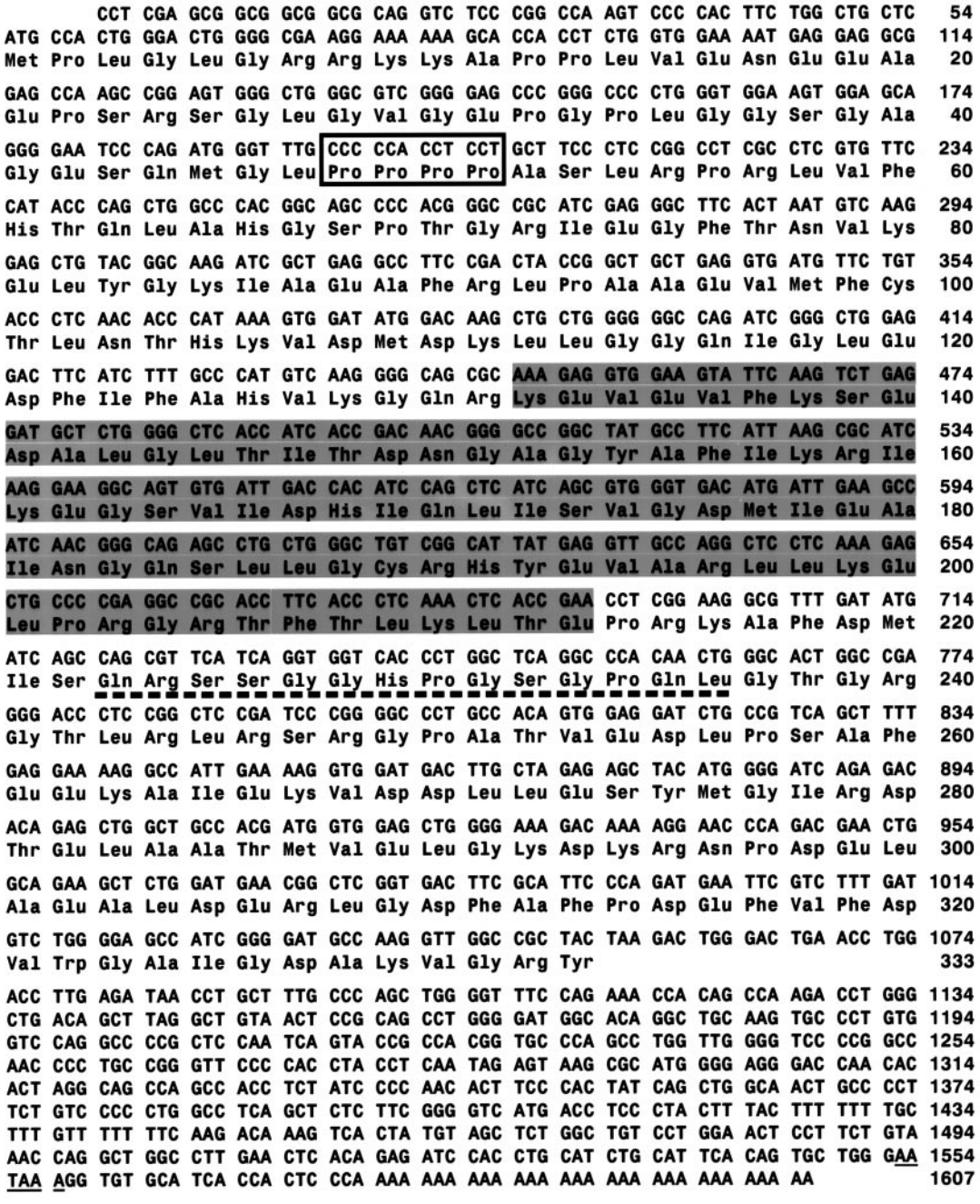

Figure 1. The nucleotide and deduced amino acid sequences of GLUT1CBP. The amino acid and nucleotide sequence numbering is indicated on the right. A box surrounds the four-proline repeat present in the proline-rich N terminus. The PDZ domain (amino acid residues 132–213) is shaded. The polyadenylation signal (solid lines) and the peptide sequence used to generate antibody LSU43 (dashed line) are indicated. The sequence has been entered into the GenBank database with accession number AF032120.

ice-cold PBS containing 1% Triton X-100 and protease inhibitors (0.2 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, 0.096 trypsin inhibitor unit/ml aprotinin). Lysates were treated eight times with 10 strokes each in a glass–teflon homogenizer with a total incubation time of 1 h on ice and then were centrifuged at 100,000 $\times$ *g* for 1 h at 4°C. One milliliter of clear supernatant was added to 100 μl of GLUT1CBP– or rabbit IgG–Sepharose beads and incubated for 2 h at 4°C. The beads were washed for 5 min at 37°C once with 1 ml of lysis buffer, twice with 1 ml of PBS containing 0.1% Triton X-100, and once with 1 ml of PBS. Bound proteins were eluted in 100 μl of gel-loading buffer containing 4% SDS by heating for 30 min at 37°C and 5 min at 80°C. Thirty microliters of the supernatant were resolved on an SDS-PAGE gel, and the separated proteins were transferred to a polyvinylidene fluoride (PVDF)

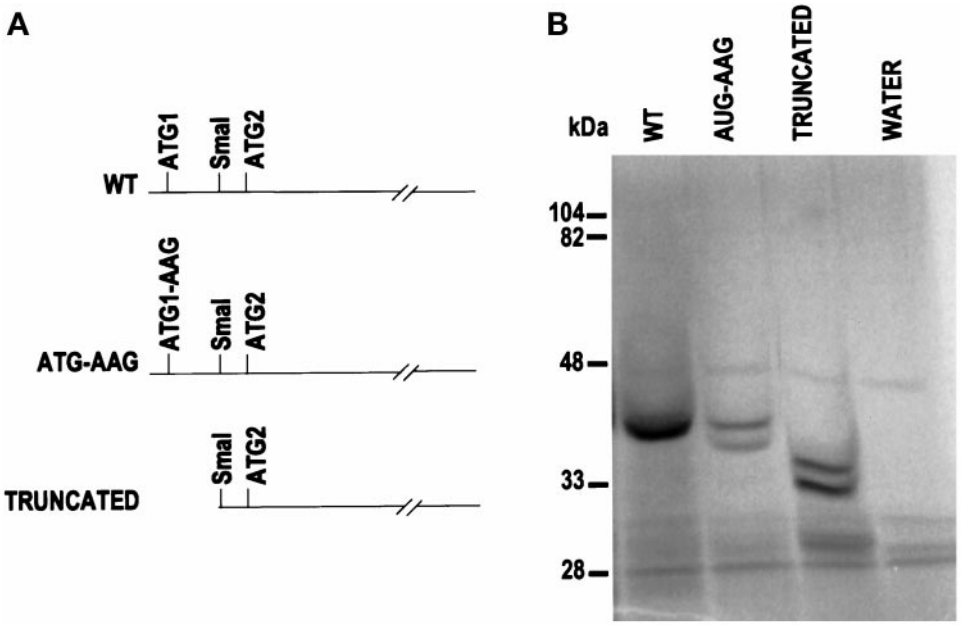

Figure 2. Identification of the authentic translation start site in GLUT1CBP mRNA. (A) Diagram of the cDNA constructs used for in vitro transcription and translation. Each indicated cDNA insert in pBSKII was transcribed in vitro with T3 RNA polymerase. (B) In vitro translation of native or mutant GLUT1CBP mRNA. Equivalent amounts of purified mRNA were translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The crude lysate was resolved by SDS-PAGE, and the gel was dried and exposed to a phosphorimager screen. Lane 1 (left), WT, full-length GLUT1CBP mRNA; lane 2, AUG-AAG, upstream AUG changed to AAG; lane 3, TRUNCATED, upstream AUG absent in GLUT1CBP mRNA; and lane 4, WATER, no mRNA.

membrane. Membranes were probed with RE11 (anti-GLUT1 C terminus, 1:2000 dilution), LSU43 (anti-GLUT1CBP, 1.5 μg/ml), anti-myosin VI (1 μg/ml, kindly provided by Dr. Tama Hasson), or anti-α-actinin (Sigma A 5044, 1:500 dilution). Detection was with ^{125}I-protein A for RE11, LSU43, and anti-myosin VI or with horse anti-mouse IgG (heavy + light) for anti-α-actinin followed by ECL.

RESULTS

GLUT1CBP Cloning

Approximately 500,000 yeast colonies were obtained by cotransformation with plasmids expressing both a Gal4 DNA binding domain–GLUT1 C terminus fusion protein (DBD–GT1) and the Gal4 activation domain fused to a library of proteins encoded by rat brain cDNAs (ACT–cDNA). Three colonies survived after selection for growth on synthetic media lacking histidine. ACT–cDNA plasmids from the three colonies were isolated, sequenced, and found to contain identical 1607-bp inserts. The sequence for the cDNA clones, designated GLUT1CBP, is presented in Figure 1. β-Galactosidase assays demonstrated that the activation of the *LACZ* reporter gene required the presence of both DBD–GT1 and ACT–GLUT1CBP plasmids (our unpublished results).

The cDNA contains two extended open reading frames that have coding potential for a protein containing either 333 or 289 amino acids. Two altered GLUT1CBP cDNAs were created to determine the authentic translation start codon (Figure 2A). In vitro translation of mRNA derived from the full-length cDNA produced a protein product migrating at 39 kDa as determined by SDS-PAGE (Figure 2B, WT). This is close to the predicted size of 36.1 kDa for a protein initiated from the first AUG and is identical in size to GLUT1CBP protein detected in Western blots of various tissue and cell extracts (see Figure 7). Translation of a truncated GLUT1CBP mRNA containing only the second AUG (missing sequence upstream of nucleotide 150, the *Sma*I site in the cDNA) produced shorter protein products (Figure 2B, TRUNCATED), indicating that the first AUG was used in the full-length construct. Mutation of the first AUG to an AAG codon, which does not initiate translation, significantly reduced the amount of full-length protein produced and induced the appearance of a shorter protein (Figure 2B, AUG-AAG). The doublets that appear with both mutants most likely arise from less efficient initiation from in-frame CUG codons that are used when the upstream AUG is absent.

An 82-amino-acid domain of the predicted 333-amino-acid GLUT1CBP protein product (Figure 1, shaded region, residues 132–213) is homologous to the PDZ domains in several proteins retrieved via a homology search of the GenBank database (Figure 3). Another notable feature is a proline-rich N terminus in which proline represents 11 of the first 56 amino acids (20%), with a four-proline repeat within this region (Figure 1, open box).

The PDZ Domain of GLUT1CBP Is Sufficient and the Four-Amino-Acid PDZ Recognition Motif of GLUT1 Is Required for the GLUT1CBP–GLUT1 Interaction

To delineate the region of GLUT1CBP that directs binding to the GLUT1 C terminus, we inserted various

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GLUT1CBP 132   KEVEVFKSED-ALGLTITDNGAGY-AFIKRIKEGSVIDHIQLISVGD
LIN2     545   RLVQFQKDTQEPMGITLKVNEDGR-CFVARIMHGGMIHRQATLHVGD
SAP102   385   RKIILHKGST-GLGFNIVGGEDGEGIFVSFILAGGPADLSGELRRGD
ZO-2     494   TKMVRFKKGD-SVGLRLAGGNDVG-IFVAGIQEGTSAEQEG-LQEGD
                     *   *           *   *  *          **

GLUT1CBP 177   MIEAINGQSLLGCRHYEVARLLKELPRGRTFTLKLTE
LIN2     591   EIREINGMSVANRSVESLQEMLRDARGQVTFKIIPSY
SAP102   431   RILSVNGVNLRNATHEQAAAALKRA--GQSVTIVAQY
ZO-2     538   QILKVNTQDFRGLVREDAVLYLLEIPKGEMVTILAQS
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Figure 3. Alignment of the GLUT1CBP PDZ domain with PDZ domains present in other proteins. An amino acid alignment of the PDZ domain of GLUT1CBP with PDZ domains from three representative proteins is shown. Shaded residues mark regions in which the chemical character of the residues is conserved in GLUT1CBP and one or more of the aligned proteins. Asterisks denote amino acid identity in all proteins. The amino acid sequence numbering is indicated to the left of the sequence. PDZ domains from Lin2 (*Caenorhabditis elegans*) (Hoskins *et al.*, 1996), SAP102 (NE-dlg) (*Homo sapiens*) (Makino *et al.*, 1997), and ZO-2 (*Canis familiaris*) (Jesaitis and Goodenough, 1994) are shown. Sequences outside the PDZ domain of GLUT1CBP fail to show significant homology to additional proteins with the exception of TIP-2 (GenBank accession number AF028824), a cDNA sequence with an open reading frame corresponding to a truncated human homologue of GLUT1CBP.

domains of the protein into pACT vectors. Using growth on minimal medium minus histidine as a qualitative assay for interactions, we tested the domains for their ability to bind to the Gal4 DBD fusions to the

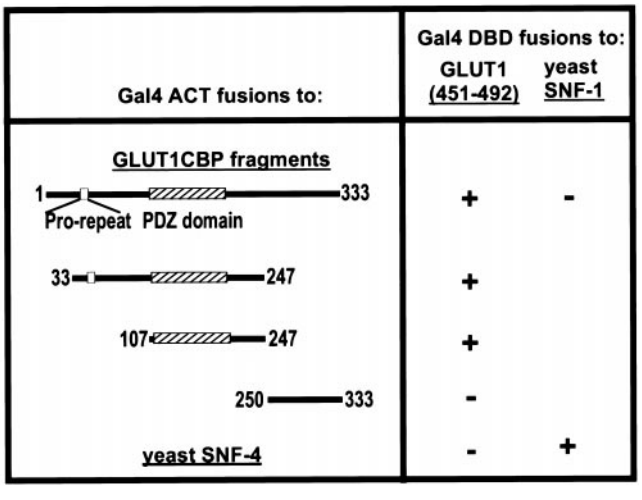

Figure 4. Interactions of GLUT1 with various domains of GLUT1CBP. Yeast HF7c was cotransformed with plasmids encoding various Gal4 DBD and Gal4 ACT fusion proteins. Transformations were plated onto synthetic dextrose plates lacking tryptophan and leucine. Three isolated colonies from each plate were then patched onto a synthetic dextrose plate lacking tryptophan, leucine, and histidine, and growth was monitored. +, three out of three of the patches grew; −, zero out of three of the patches grew. Yeast SNF-1 and SNF-4 interact in this system and serve as positive controls. Numbers in parentheses refer to the amino acids of each protein that are fused to the Gal4 DBD proteins.

GLUT1 C terminus (residues 451–492) in the two-hybrid system. The results presented in Figure 4 demonstrate that the solitary C-terminal domain (250–333) of GLUT1CBP failed to bind to the C terminus of GLUT1, while truncated forms of GLUT1CBP (33–347 and 107–247) retained binding activity equivalent to that of native GLUT1CBP (1–333). This illustrates that neither the proline-rich N-terminal domain nor the C terminus of GLUT1CBP is required for binding to GLUT1 and that amino acids 107–247 (the PDZ domain) are sufficient for GLUT1CBP to recognize and interact with the GLUT1 C terminus.

To confirm the two-hybrid results and to analyze the binding specificity of GLUT1CBP in more detail, protein overlay assays were performed using GST fusions of various transporter C-terminal constructs (Figure 5A). GLUT1CBP recognizes the GLUT1 C terminus (Figure 5B, left, GT1). The interactions are specific for the membrane distal one-half of the GLUT1 C terminus, because no binding to the homologous membrane proximal region of either the GLUT4 or GLUT1Δ24 C-termini was observed (Figure 5B, left, GT4 and GT1Δ24). Deletion of this motif to form the GLUT1Δ4 mutant abolishes GLUT1CBP binding (Figure 5B, left, GT1Δ4), further localizing the binding site to the GLUT1 PDZ recognition motif. The gel mobility and level of expression of the GST fusion proteins were similar (Figure 5B, right).

The PDZ motifs of some PDZ-containing proteins have a specific requirement for a C-terminal valine in the PDZ recognition motif. For instance, a valine-to-alanine mutation at the C terminus of the K_v 1.4 potassium channel abolishes its interaction with the PDZ domains of postsynaptic density (PSD)-95 (Kim *et al.*, 1995). However, a valine-to-alanine mutation in the GLUT1 C terminus increased the amount of GLUT1CBP bound to GLUT1 (Figure 5B, left, GT1V492A). The GLUT1CBP PDZ domain was unable to bind to human GLUT3, which possesses a potential PDZ recognition motif at the C terminus (Figure 5B, left, HGT3), thereby indicating further sequence discrimination. These characteristics of GLUT1CBP binding were confirmed in the two-hybrid system (our unpublished results). Thus, the GLUT1CBP PDZ domain exhibits distinct differences in sequence binding specificity compared with the specificity of other PDZ domains.

GLUT1CBP Binds to Endogenous GLUT1

Although indicative of direct interactions between GLUT1CBP and the C terminus of GLUT1, the two-hybrid system and protein overlay assay do not address the ability of GLUT1CBP to interact with the native, full-length GLUT1 molecule. GLUT1CBP must be capable of binding the C terminus of GLUT1 resident in the native transporter for the interaction to have potential physiological importance.

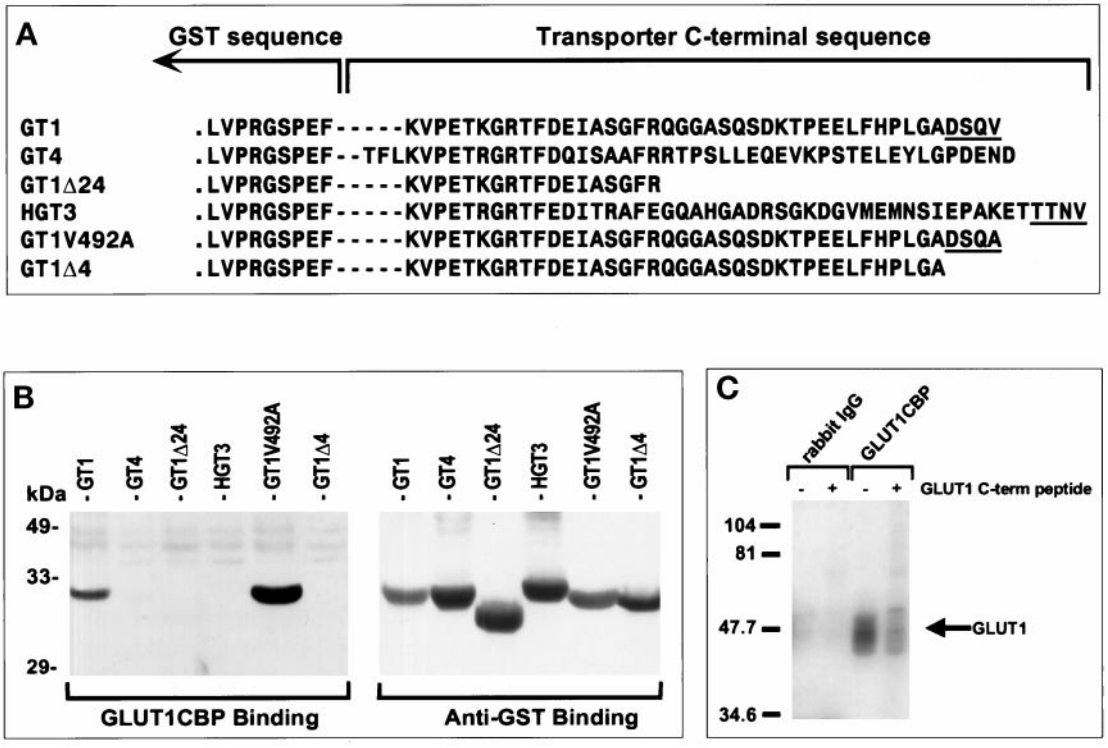

Figure 5. Analysis of His_6–GLUT1CBP binding specificity and ability to bind endogenous GLUT1. (A) Alignment of the C-terminal regions of GST fusion proteins containing the C-termini of GLUT1 (GT1), GLUT4 (GT4), GLUT1Δ24 (GT1Δ24), human GLUT3 (HGT3), GLUT1V492A (GT1V492A), and GLUT1Δ4 (GT1Δ4). The putative PDZ recognition motifs are underlined. (B) Binding of purified His_6–GLUT1CBP to GST fusions to GT1, GT4, GT1Δ24, HGT3, GT1V492A, and GT1Δ4 C-termini immobilized on a nitrocellulose membrane. Left, bound His_6–GLUT1CBP detected with HRP-conjugated S-protein and ECL. Right, comparison of the relative levels of each of the GST fusion proteins determined after quantitation of GLUT1CBP binding by stripping the blot and probing with an HRP-conjugated antibody to GST. Bound antibody was detected with ECL. (C) Binding of purified His_6–GLUT1CBP to endogenous GLUT1 from MDCK cells. Cells were solubilized with PBS + 1% Triton X-100. The extract was incubated with GLUT1CBP– or rabbit IgG–Sepharose beads. After washing, proteins that were bound to the beads were eluted with gel-loading buffer. Proteins were resolved on SDS-PAGE gels and transferred to a PVDF membrane. The membrane was probed with RE11 antibody against the C terminus of GLUT1. Bound antibody was detected with ^{125}I-protein A and phosphorimager analysis. +, the beads were preincubated with a peptide corresponding to the GLUT1 C terminus before the addition of extract.

This capability was tested using a technique in which purified His_6–GLUT1CBP was covalently attached to Sepharose beads, and the beads were subsequently incubated with MDCK cell lysates prepared with the nondenaturing detergent Triton X-100. After extensive washing of the beads, bound GLUT1 was detected by Western analysis using a GLUT1-specific antibody. The endogenous glucose transporter was bound to GLUT1CBP beads but not to rabbit IgG beads (Figure 5C). Preincubating the GLUT1CBP beads with a peptide consisting of the C-terminal 12 amino acids of GLUT1 eliminated >70% of GLUT1 binding, providing further confirmation of binding specificity. This indicates that GLUT1CBP can interact with the C terminus of GLUT1 in the context of the native, full-length transporter.

Comparison of the Tissue-specific Expression of GLUT1CBP and GLUT1 mRNA

Functional interactions between GLUT1CBP and GLUT1 require that both proteins be expressed in the same tissue and cell types in vivo. As a first step toward addressing this issue, Northern blots of poly(A)+ RNA were performed to measure the level of expression of mRNA for each gene (Figure 6). A GLUT1CBP antisense RNA probe hybridized to a 1.6-kb RNA from all tissues examined (Figure 6, top). A GLUT1 antisense RNA probe hybridized to a 2.6-kb RNA that was also present in most tissues examined (Figure 6, bottom). Among the tissues examined, the highest amount of both RNA species was observed in brain, and the lowest of both species was in liver, in accordance with previously published data regarding GLUT1 expression (Mueckler, 1994). GLUT1CBP mRNA was also present in MDCK, Caco-2 (our unpublished results), CHO, and 3T3-L1 (preadipocyte and adipocyte) cells. Only the MDCK and Caco-2 cell lines lack the 1.6-kb form of GLUT1CBP message, which is replaced by a slightly larger 2.0-kb form. GLUT1 mRNA was present at very high levels in MDCK cells and 3T3-L1 adipocytes and at lower levels in CHO and 3T3-L1 preadipocytes.

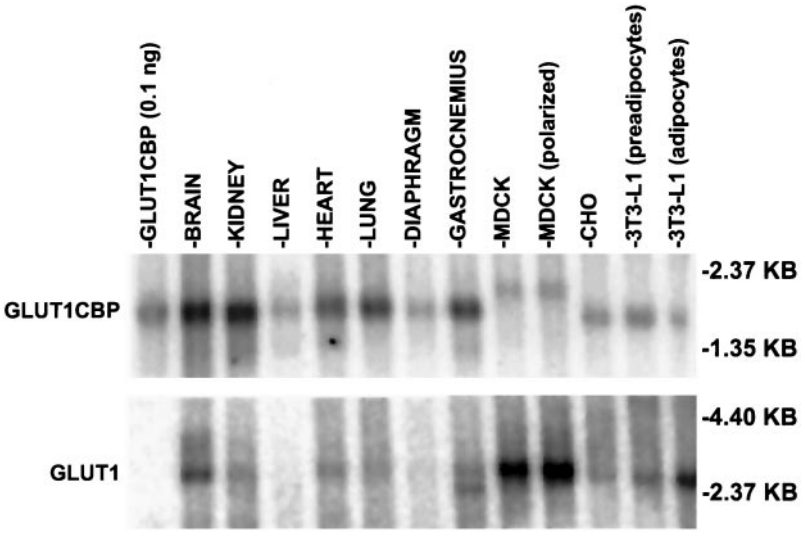

Figure 6. Comparison of GLUT1 and GLUT1CBP mRNA expression in various tissues and cells. Poly(A)+ RNA (2 μg) isolated from each of the indicated rat tissues or cell monolayers was resolved on a denaturing agarose gel and then transferred to a nylon membrane. Using ^{32}P-labeled antisense RNA probes, we first hybridized the message with the GLUT1CBP probe and then stripped and hybridized with the GLUT1 probe. Bound probe was detected in both cases by phosphorimager analysis. In vitro transcribed GLUT1CBP (0.1 ng) is included as a standard.

Analysis of GLUT1CBP Protein Expression

Tissue proteins reactive with GAb(249–333) antibody were detected by Western blot (Figure 7A, left). All tissues examined, with the exception of the small intestine, possessed a 39-kDa protein that was reactive with GAb(249–333). This protein was present at highest levels in brain, testis, and lung. LSU43 antibody also recognizes an identical pattern of expression for the 39-kDa GLUT1CBP (our unpublished results). However, LSU43 recognizes additional high molecular weight immunoreactive proteins. These apparently are unrelated to GLUT1CBP because they are not recognized by GAb(249–333).

To confirm that immunoreactive bands were specific for the GLUT1CBP epitope, we incubated the antibody with Sepharose beads or covalently coupled His_6–GLUT1CBP–Sepharose beads before use in Western blot analysis (Figure 7A, left and right, respectively). Preincubation of GAb(249–333) with beads did not affect the ability to detect any of the immunoreactive species on Western blots. However, preincubation of GAb(249–333) with GLUT1CBP beads eliminated most immunoreactive bands, indicating the presence of a conserved epitope between these proteins and GLUT1CBP. Some nonspecific interactions are evident in the diaphragm sample. The immunoreactive triplet evident below GLUT1CBP in the kidney sample, the high molecular weight singlet in 3T3-L1 preadipocytes, and the higher molecular weight doublet in diaphragm (Figure 7A) are most likely proteins unrelated to GLUT1CBP that contain epitopes recognized by GAb(249–333) because they are not recognized by antibody LSU43 (our unpublished results). Furthermore, the smaller triplet of proteins detected in kidney does not appear to represent proteolytic products of GLUT1CBP. Fragments of GLUT1CBP missing the epitope recognized by LSU43 and retaining that recognized by GAb(249–333) would be smaller than 12 kDa.

The 39-kDa GAb(249–333)-reactive protein was also present in primary cultures of rat cortical neurons, astrocytes, and human umbilical vein-endothelial (HUVEC) and artery-endothelial cells (HUAEC) (Figure 7, B and C, left). Several cell lines including 3T3-L1, CHO, PC-12, MDCK, and Caco-2 possess the 39-kDa GAb(249–333)-reactive protein. The relative level of expression of GLUT1CBP by neurons and 3T3-L1 preadipocytes is underrepresented in Figure 7B because only 40 μg of protein extract was applied to the gel, rather than the 100 μg used for all other cells and tissues presented. Interestingly, upon differentiation of preadipocytes to adipocytes, 3T3-L1 cells cease the expression of GLUT1CBP. Furthermore, as demonstrated with the tissue extracts, immunoreactive protein bands observed in the primary cultures and cell lines are not observed when GAb(249–333) is preincubated with GLUT1CBP beads (Figure 7, B and C, right).

Identification of Additional Proteins That Bind GLUT1CBP

PDZ-containing proteins often harbor additional sites for protein interactions outside the PDZ domain. To gain a better understanding of the protein interactions mediated by GLUT1CBP, we used the full-length protein in the two-hybrid system to screen a brain library for additional interacting proteins. Twenty-three independent ACT–cDNA clones were isolated, of which only a subset was identifiable by a search for homology to proteins in the sequence databases (Figure 8). Interestingly, GLUT1CBP was isolated in the screen,

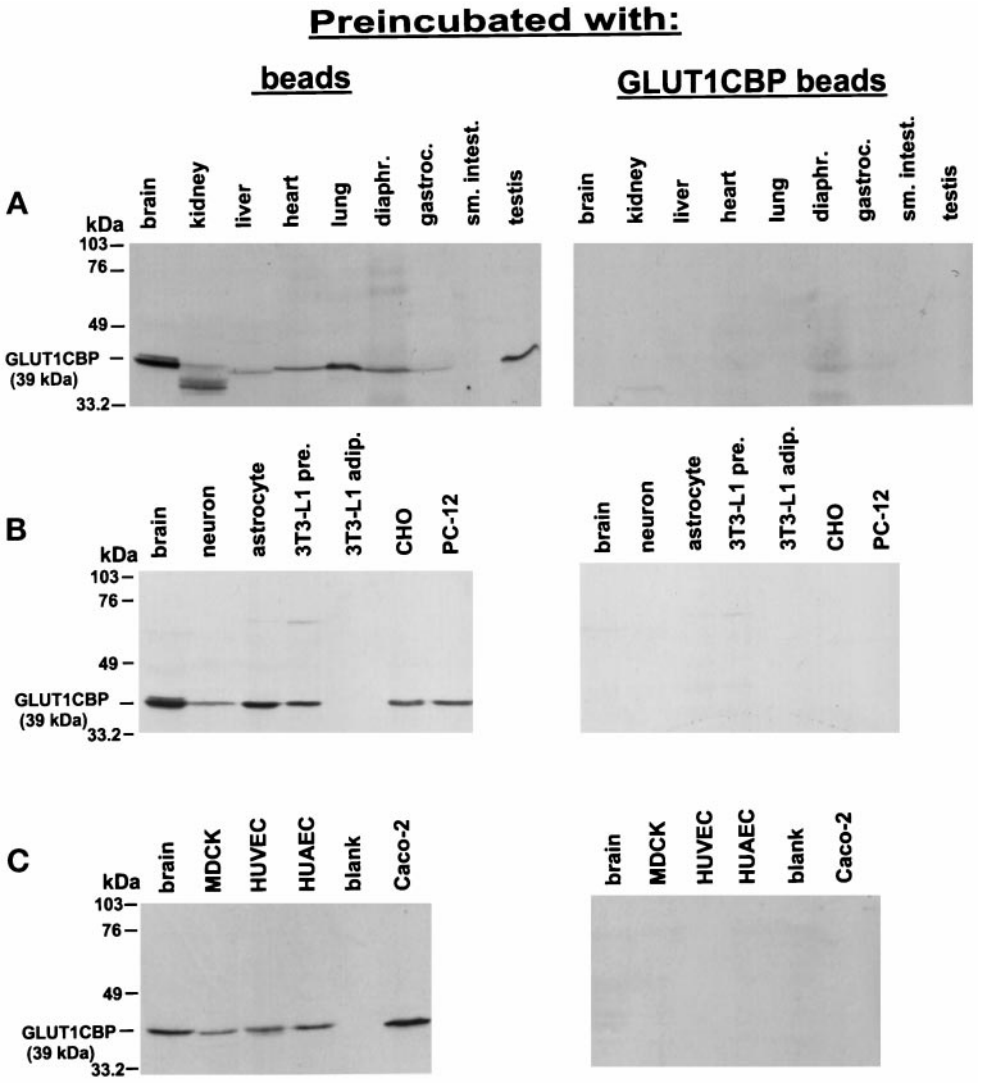

Figure 7. Analysis of GLUT1CBP protein expression in various tissues and cells. Tissue and cell extracts were prepared as described in MATERIALS AND METHODS. One hundred micrograms of each sample (40 μg for 3T3-L1 preadipocytes and neurons) were separated by SDS-PAGE, and the proteins were transferred to membranes. Analysis of GLUT1CBP expression in tissue samples (A) and in various cell lines (B and C) is indicated with brain controls to allow comparison between blots. Blots were probed with GAb(249–333) antibody raised against the C-terminal 85 amino acids of GLUT1CBP. The antibody was preincubated with Sepharose beads (left) or covalently coupled His_6–GLUT1CBP–Sepharose beads (right) before the antibody was added to the membrane. Bound antibody was detected by ECL. adip., adipocyte; diaphr., diaphragm; gastroc., gastrocnemius; pre., preadipocyte; sm. intest., small intestine.

indicating the potential of this protein to form homomultimers. Also, GLUT1CBP interacted with the proteins myosin VI, an unconventional myosin; the kinesin superfamily protein 1B (KIF-1B), a kinesin-like monomeric microtubule motor; and α-actinin-1, an actin cross-linking protein.

We next determined which of the interacting proteins bound to GLUT1CBP via its PDZ domain. Whereas Gal4 ACT fusions with myosin VI, KIF-1B, α-actinin-1, and GLUT1CBP support growth of yeast colonies expressing a Gal4 fusion to full-length GLUT1CBP, only KIF-1B and α-actinin-1 Gal4 ACT fusions interact with a Gal4 DBD fusion to amino acids 107–247 of GLUT1CBP (Figure 8). This indicates that, in addition to GLUT1, only KIF-1B and α-actinin-1 are capable of binding via the GLUT1CBP PDZ domain (Figure 8). The failure of myosin VI and GLUT1CBP to bind the PDZ domain indicates the presence of at least one other protein interaction domain in GLUT1CBP.

These interactions were tested outside the yeast two-hybrid environment. Purified His_6–GLUT1CBP was first assayed for its ability to bind endogenous GLUT1CBP from detergent-solubilized CHO cells (Figure 9A). In the absence of cell extract, only a portion of the larger molecular weight His_6–GLUT1CBP that escaped cross-linking to the beads is detected by antibody (LSU43) against GLUT1CBP (Figure 9A, left lane). Association of the lower molecular weight native endogenous GLUT1CBP with the His_6–GLUT1CBP cross-linked beads is detected when the beads are incubated with extract (Figure 9A, middle lane). This association is specific because no native GLUT1CBP binds to IgG cross-linked beads (Figure 9A, right lane). In addition, His_6–GLUT1CBP–Sepha-

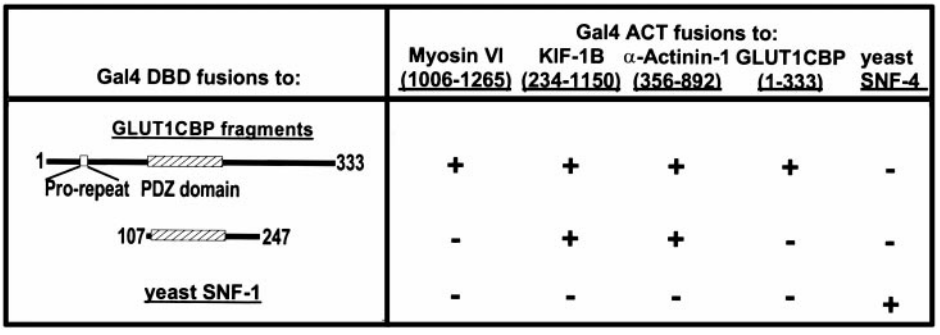

Figure 8. Interactions of GLUT1CBP with cellular proteins. Yeast HF7c was cotransformed with plasmids encoding the indicated Gal4 DBD and Gal4 ACT fusion proteins and assayed as described in Figure 4. Yeast SNF-1 and SNF-4 interact in this system and serve as positive controls. Numbers in parentheses refer to the amino acids of each protein that are fused to the Gal4 ACT domain.

rose beads precipitated myosin VI and α-actinin-1 from detergent-solubilized MDCK cells (Figure 9, B and C, respectively). Native protein binding assays have not been performed with KIF-1B because of our inability to obtain the antibody to this protein. The ability of GLUT1CBP to bind the intact, endogenous proteins suggests that these interactions are important physiologically. The fact that GLUT1CBP, KIF-1B (Nangaku *et al.*, 1994), myosin VI (Hasson and Mooseker, 1994), and α-actinin-1 (Puius *et al.*, 1998) are ubiquitously expressed further supports the possibility of functional interactions among these proteins.

DISCUSSION

The PDZ domain, which recognizes C-terminal amino acid motifs, places GLUT1CBP among a unique and interesting category of proteins involved in membrane protein organization. The PDZ designation, previously termed DHR or GLGF repeat, is derived from the names of three proteins initially noted to contain this domain: mammalian PSD protein PSD-95, *Drosophila* discs large tumor suppressor DLG, and mammalian tight junction protein ZO-1. Proteins that possess PDZ domains participate in a variety of cellular processes. Among these are receptor clustering (Kim *et al.*, 1995), organizing signal transduction cascades (Montell, 1998), anchoring proteins to the cytoskeleton (Short *et al.*, 1998), localizing proteins to specific regions of the plasma membrane (Simske *et al.*, 1996), modulating the activity of ion channels (Hall *et al.*, 1998), and determining the substrate specificity of some enzymes (Snow *et al.*, 1998).

A recent study of PDZ domain binding specificity identified at least two independent groups of PDZ domains that possess divergent recognition motif specificity (Songyang *et al.*, 1997). Each of the two groups can be subdivided on the basis of primary sequence determinants within the PDZ domain. Members of the first group, including PSD-95 and DLG, preferentially bind to peptides with a C-terminal sequence Xxx-Ser/Thr/Tyr-Xxx-Val/Ile, where Xxx represents any amino acid. The second group, which includes p55 and Lin2, bind preferentially to Xxx-Phe/Tyr-Xxx-Val/Phe/Ala. C-terminal sequences of pro-

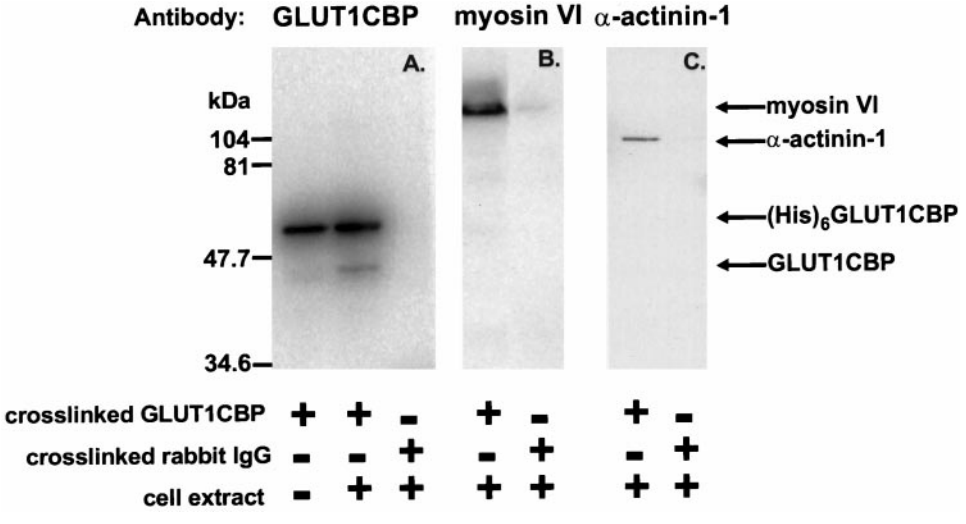

Figure 9. Interaction of purified GLUT1CBP with cellular GLUT1CBP, myosin VI, and α-actinin-1. Native protein binding assays were performed essentially as described in Figure 5C. Extracts were prepared from CHO cells for endogenous GLUT1CBP (A) and from MDCK cells for endogenous myosin VI (B) and α-actinin-1 (C). The presence (+) or absence (−) of extract, cross-linked His_6–GLUT1CBP beads, or cross-linked IgG beads in the incubation mixture is indicated. After separation of the bound proteins by SDS-PAGE and transfer to the PVDF membrane, blots were incubated with the appropriate antibodies. (A) LSU43 against GLUT1CBP. (B) Anti-myosin VI. (C) Anti-α-actinin-1.

Interacting protein	C-terminal sequence	Binds to GLUT1CBP PDZ domain
GLUT1	...FHPLGADSQV	YES
GLUT1V492A	...FHPLGADSQA	YES
α-actinin-1	...STALYGESDL	YES
KIF-1B	...NLKAGRETTV	YES
Myosin VI	...ATAMLQNLLK	NO
GLUT1CBP	...AIGDAKVGRY	NO
TAX	...SEKJFRETEV	YES

Figure 10. C-terminal sequences of proteins tested for their ability to bind to the PDZ domain of GLUT1CBP. The results reflect a summary of protein interactions tested in this article with the exception of TAX that was reported (Rousset *et al.*, 1998) to interact with the PDZ domain of TIP-2, a truncated form of the human homologue to GLUT1CBP.

teins known to require the PDZ domain of GLUT1CBP for interaction are included in Figure 10. An analysis of the recognized sequences indicates that the specificity of the PDZ domain of GLUT1CBP is most closely related to the first group of PDZ domains because it recognizes a C-terminal hydrophobic valine, alanine, or leucine, with a serine or threonine two amino acids upstream (position −2) in GLUT1, GLUT1V492A, KIF-1B, TAX, or α-actinin-1. It should be noted that the binding of GLUT1CBP to internal sequences in α-actinin-1 cannot be excluded, because PDZ domains can bind to the spectrin repeats of α-actinin-2 (Xia *et al.*, 1997).

These observations suggest that the GLUT1CBP PDZ domain can exhibit some plasticity in binding specificity as described previously for other PDZ domains (Songyang *et al.*, 1997). Such plasticity allows PDZ-containing proteins to participate in a variety of cellular functions by interacting with a limited, but not unique, class of partners. Nevertheless, the PDZ domain of GLUT1CBP can discriminate between other prospective targets within the same recognition group, as evidenced by its failure to bind to the C terminus of human GLUT3, which ends with the sequence Thr-Thr-Asn-Val. Furthermore, the GLUT1 C terminus is not spuriously recognized by PDZ domains because the two-hybrid screen with this domain failed to isolate any of the other known PDZ-containing proteins, such as PSD-95. This characteristic is consistent with the observation that a PDZ domain from one subgroup is not able to bind to a peptide specifically designed for another member of the same subgroup (Songyang *et al.*, 1997). These observations suggest that additional determinants within the PDZ domain and sequences adjacent to the terminal recognition motif may further refine the specificity of PDZ domain interactions (Shieh and Zhu, 1996).

This study demonstrates that GLUT1CBP can interact with multiple cellular proteins. GLUT1 is a strong candidate because of the overlapping expression of both mRNAs and protein. Brain, heart, and kidney tissue showed high relative levels of expression of both GLUT1 and GLUT1CBP mRNA species. However, all tissues examined possessed a detectable signal for both mRNAs with the exception of liver, which lacked detectable levels of GLUT1. The wide tissue distribution observed for GLUT1CBP in this study is supported by the numerous homologous GenBank expressed sequence tag sequences identified in diverse human and mouse tissues. The function of GLUT1CBP may therefore be important in a number of different cellular environments.

The significance of the larger form of GLUT1CBP mRNA observed in canine MDCK and human Caco-2 cells is not yet understood. This may reflect species- or tissue-specific variations in the length of the 5′- or 3′-untranslated regions or alternative splicing. In either case, the modifications do not significantly alter the size of the resultant GLUT1CBP detected by the goat anti-GLUT1CBP antibody.

High levels of GLUT1CBP mRNA expression correlate with high GLUT1CBP protein expression in heart, lung, and brain tissue, whereas low mRNA levels correlate with lower GLUT1CBP protein expression in liver tissue. However, the contrasting high message level and low protein levels exhibited in gastrocnemius and kidney tissue suggest that an altered translational and/or posttranslational regulatory state exists for these two tissues. Similar contrasts in translational and/or posttranslational control of GLUT1CBP expression apparently occur during the differentiation of 3T3-L1 preadipocytes. As illustrated in Figure 7B, upon differentiation of 3T3-L1 preadipocytes into adipocytes, the expression of GLUT1CBP protein is lost with only a slight decrease in the level of message (Figure 6).

What may be the function of GLUT1CBP? The membrane topology of GLUT1 and the wide tissue distribution of the cytosolic, soluble proteins GLUT1CBP, KIF-1B, α-actinin-1, and myosin VI suggest the formation of dimeric (or multimeric) complexes characteristic of PDZ-containing proteins. Such linkages have been reported for ion channels and other membrane proteins and may be important for localizing GLUT1 to specialized membrane sites or for regulating transport activity. GLUT1CBP binds to GLUT1, KIF-1B, and α-actinin-1 via its PDZ domain and to myosin VI or another molecule of GLUT1CBP via one or more adjacent domains. Although GLUT1CBP has only one PDZ domain, a dimeric form of the protein may exist because the protein interacts with itself. Furthermore, because the interaction is not via the PDZ domain, the dimer would expose two PDZ domains for binding to other cellular proteins. These characteristics make

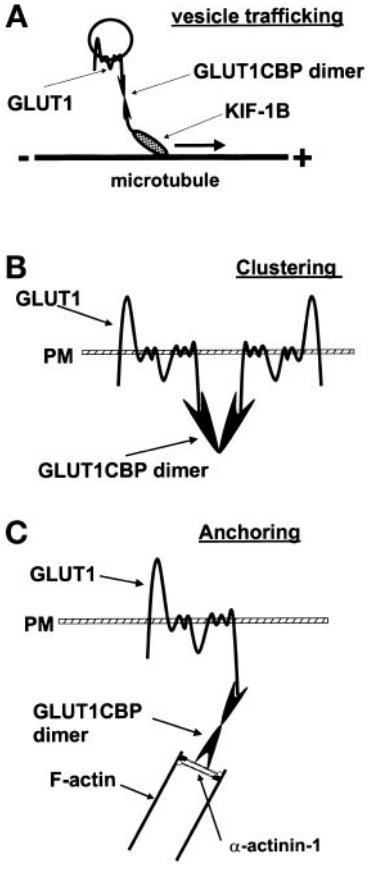

Figure 11. Models depicting the potential functions of GLUT1CBP. (A) GLUT1CBP tethers GLUT1-containing vesicles to cytoskeletal motor proteins. This particular model invokes dimerization of GLUT1CBP and linkage to KIF-1B and the microtubule. An alternative model could be proposed in which monomeric GLUT1CBP links GLUT1 vesicles to myosin VI and actin. (B) GLUT1 is linked to other GLUT1 molecules by GLUT1CBP, resulting in clustering of GLUT1 and perhaps stabilization of the transporter at a specific plasma membrane (PM) domain. (C) GLUT1 is anchored to α-actinin-1 via GLUT1CBP, thus providing linkage to the submembranous actin cytoskeleton.

GLUT1CBP an ideal candidate to serve as a bridging protein between GLUT1 monomers or between GLUT1 and cytoskeletal elements (Figure 11, A and B, respectively). By cross-linking GLUT1 monomers, GLUT1CBP could stabilize GLUT1 within specific domains of the plasma membrane, which is one proposed mechanism for localizing proteins to specialized membrane domains. Alternatively, linkage of GLUT1 monomers may affect the transport activity of GLUT1. There is a possibility for regulation of GLUT1 activity to occur via GLUT1CBP-mediated colocalization with an as yet unidentified regulatory protein present in its native cellular environment. Another mechanism for localizing GLUT1 to a specific membrane site may involve tethering the transporter to cytoskeletal motor proteins such as myosin VI and/or KIF-1B, which would then direct GLUT1-containing vesicles toward their proper location in the cell. Tethering GLUT1 to α-actinin-1 may be yet another way to restrict GLUT1 to specific membrane domains (Figure 11C), and disruption of this interaction could serve to regulate GLUT1 activity. This is consistent with the recent observation that disruption of the actin cytoskeleton in erythrocytes leads to activation of GLUT1 (Zhang and Ismail-Beigi, 1998). Importantly, the topology of the GLUT1 transporter in the plasma membrane and intracellular vesicles is appropriate in both cases to permit interaction of its C terminus with cytosolic GLUT1CBP in vivo.

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