Modulation of GLUT1 Intrinsic Activity in Clone 9 Cells by Inhibition of Oxidative Phosphorylation*

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Brief (1–2 h) exposure of Clone 9 cells to inhibitors of oxidative phosphorylation such as azide is known to markedly increase glucose uptake. Clone 9 cells express GLUT1 but not GLUT2, -3, and -4, and the azide effect was not accompanied by any increase in cellular or plasma membrane GLUT1 level. To identify the molecular event underlying this apparent increase in GLUT1 intrinsic activity, we studied the acute effects of azide on the substrate binding activity of GLUT1 in Clone 9 cells by measuring glucose-sensitive cytochalasin B binding. The glucose-displaceable, cytochalasin B binding activity was barely detectable in membranes isolated from Clone 9 cells under control conditions but was readily detectable after a 60-min incubation of cells in the presence of 5 mM azide showing a 3-fold increase in binding capacity with no change in binding affinity. Furthermore, the cytochalasin B binding activity of purified human erythrocyte GLUT1 reconstituted in liposomes was significantly reduced in the presence of cytosol derived from azide-treated Clone 9 cells but not in the presence of cytosol from control cells; this effect was heat-labile and abolished by the presence of the peptide corresponding to the GLUT1 COOH-terminal sequence. These results suggest that a cytosolic protein in Clone 9 cells binds to GLUT1 at its COOH-terminal domain and inhibits its substrate binding and that azide-induced metabolic alteration releases GLUT1 from this inhibitory interaction. Studying the binding of cytosolic proteins derived from 35S-labeled Clone 9 cells to glutathione S-transferase fusion protein containing glucose transporter COOH-terminal sequences, we identified 28- and 70-kDa proteins that bind specifically to the cytoplasmic domain of GLUT1 and GLUT4 in vitro. We also found a 32P-labeled, 85-kDa protein that binds to GLUT4 but not to GLUT1 and only in cytosol derived from azide-treated cells. The roles, if any, of these glucose transporter-binding proteins in the azide-sensitive modulation of GLUT1 substrate binding activity in Clone 9 cells are yet to be determined.

A family of six distinct intrinsic membrane proteins (facilitative glucose transporters) catalyze the glucose uptake and release in animal cells (1, 2). GLUT1, the member (isoform) of this family that is abundant in human erythrocytes and transformed cell lines, catalyzes glucose uptake in Clone 9 cells (2–4), a “nontransformed” rat liver cell line (4). As in nucleated avian erythrocytes (5), mammalian muscle, and adipose cells (6), the glucose utilization in Clone 9 cells is rate-limited and metabolically regulated at the transport step (3). A brief (1–2 h) exposure to cyanide or azide, inhibitors of oxidation phosphorylation, stimulates glucose uptake in Clone 9 cells 6–8-fold (3, 7). This acute effect of azide occurs without any increase in cellular GLUT1 content (3), indicating that it is due to a post-translational modulation. A longer (up to 24 h) incubation with azide causes an additional 2-fold stimulation of glucose uptake in these cells (7). In contrast to the acute effect, however, this later effect of azide is accompanied by 2- and 8-fold increases in GLUT1 and GLUT1 mRNA cellular content, respectively (8), indicating that it is largely due to a pretranslational modulation. The two well known post-translational mechanisms for glucose transport regulation in animal cells include changes in transporter intrinsic activity (2) and transporter redistribution from a microsomal storage pool to the cell surface (recruitment) (9, 10). A 2-h incubation with azide only slightly increases plasma membrane GLUT1 levels in Clone 9 cells (3), indicating that the acute effect of azide on glucose transport is due to an increased GLUT1 intrinsic activity rather than GLUT1 recruitment. Furthermore, kinetic studies have shown that the acute glucose transport stimulation by azide is due to an increased V_max with no change in K_m for glucose (4), suggesting that azide most likely increases GLUT1 catalytic turnover. Alternatively, however, it is also possible that azide converts GLUT1 from an inactive state to an active state in terms of substrate binding; GLUT1 in Clone 9 cells may be largely kept in an inactive state where it cannot bind substrate, and an azide-induced metabolic alteration may convert this inactive GLUT1 to an active (unmasked) state where it can now bind glucose with a given affinity and is hence functional.

In the present study, we tested the alternative possibility discussed above. We studied the substrate binding capacity of GLUT1 in Clone 9 cells by measuring o-glucose-sensitive CB binding and examined if it is increased after azide treatment in association with the transport stimulation. CB, a well known inhibitor of facilitative glucose transporters, binds to GLUT1, and this binding is readily and specifically displaced by o-glucose but not by l-glucose (11). This o-glucose-induced cytochalasin B displacement has been used extensively as an

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1 The abbreviations used are: CB, cytochalasin B; CE, cytochalasin E; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalnine chloromethyl ketone; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
assay for substrate binding activity of GLUT1 (12). We demonstrate here that the glucose-displaceable cytochalasin B binding activity is barely detectable in membranes isolated from control Clone 9 cells, but it is increased greatly after a 1-h incubation with azide. We further show that the glucose-displaceable cytochalasin B binding capacity of GLUT1 purified from human erythrocytes and reconstituted as proteoliposomes is significantly reduced in the presence of cytosol isolated from azide-treated Clone 9 cells but not from untreated Clone 9 cells. We also describe three distinct cytosolic proteins that bind to the glutathione S-transferase (GST) fusion proteins containing the COOH-terminal cytoplasmic domain of GLUT1 and GLUT4 but not to irrelevant fusion proteins. Possible roles of these putative glucose transporter-binding proteins in the regulation of glucose transporter function in response to metabolic alteration are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Clone 9 cells were obtained from the American Type Culture Collection (Rockville, MD) and used between the 24th and 34th passages. Dulbecco’s modified Eagle’s medium (DMEM), calf serum, and D/H2O Escherichia coli strain were purchased from Life Technologies, Inc. Untreated plastic culture dishes were from Corning Glass Works (Medfield, MA). [3H]Cytochalasin B Western blotting detection reagents were purchased from Amersham Life Science (Chicago, IL). Cytochalasin B, cytochalasin E (CE), dimethyl sulfoxide, phenylmethylsulfonyl fluoride, N′-p-tosyl-l-lysine chloromethyl ketone (TLCK), N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK), l-glutamine, HEPES, l-glutathione, ampicillin, glutathione-agarose bead, LB broth, and standard chemicals were all from Sigma. Nitrocellulose paper and Ecoscint diluent (80 Ci/mmol) were from Packard Instrument Co. (Downers Grove, IL). [3H]Cytochalasin B Western blotting detection reagents were purchased from Amersham Life Science (Chicago, IL). 

**Clone 9 cell culture—**Clone 9 cells were maintained on plastic culture dishes in DMEM containing 5.6 mM d-glucose supplemented with 10% calf serum at 37 °C in a 95% CO2-humidified atmosphere (pH 7.4). These cells formed confluent monolayers and exhibited density-dependent (contact) growth inhibition. For each experiment, cells were passaged at 1:8 dilution and seeded on 100-mm culture dishes in 8 ml of medium containing 10% calf serum. When the cells reached confluence (3–4 days), the medium was replaced with fresh medium containing 10% calf serum and used after 16–18 h of culture. Cells were treated with diluent (80 μl of DMEM) or 5 mM azide (dissolved in 80 μl of DMEM) for 1 h prior to use.

**Preparation of Total Membranes and Cytosol—**Clone 9 cells cultured to confluency on ten 100-mm plates were rinsed twice with 8 ml of PBS, harvested in PBS, and pooled in 3 ml of lysis buffer containing 1 mM Tris (pH 7.4) and 0.1 mM each of phenylmethylsulfonyl fluoride, TLCK, and TPCK. After 10 min of cell swelling on ice, cells were homogenized with 20 strokes by employing a tight-fitting Dounce homogenizer. The resulting lysates were centrifuged at 500 × g for 2 min per pellet the nuclei, and the resulting supernatants were centrifuged for 60 min at 185,000 × g at 4 °C. The pellets (total membranes) were resuspended in 1 ml of the above lysis buffer, and the supernatants (cytosols) were parted by addition of equal volumes of an isolation solution (100-fold or less). Both total membranes and cytosols were stored at −70 °C and used within 2 weeks of preparation. Preparation was performed by the method of Bradford (13) using γ-globulin as a standard. Azide at the concentrations present was shown to have no effect on the assay (7).

**Metabolic Labeling of Cellular Protein—**For [35S]methionine labeling, Clone 9 cells grown in twenty 100-mm culture plates were rinsed twice with methionine-free DMEM and reincubated in 5 ml of methionine-free DMEM. Then the cells on each plate were incubated in 2.5 ml of methionine-free DMEM containing 80 μl of TRAN-35S label. After 16 h of incubation, cells were treated with diluent or 5 mM azide (dissolved in methionine-free DMEM) for 1 h prior to harvest. Cells were then fractionated to total membrane and cytosol as described above. Radioactivity of protein labeling was measured by scintillation counting, and the efficiency of labeling was expressed as cpn/mg of protein.

For [35P]labeled, four 100-mm confluent plates of cells were rinsed four times with sodium phosphate-free DMEM and then suspended for 2 h with 2.5 ml of sodium phosphate-free DMEM containing 3.5 μl of 5 μCi of 32P (9,000 Ci/mmol). After 2 h, cells were treated with diluent or azide (dissolved in sodium phosphate-free DMEM) for 1 additional hour prior to harvest. All subsequent steps were as described except that 1 ml of lysis buffer was used per two plates of cells. Whole Cell Radioactivity Analysis—SDS-PAGE was performed according to the method of Laemmli (14) with the following modification. Cell fractions containing equal amounts of protein (50 μg) were solubilized in Laemmli buffer containing 5% 2-mercaptoethanol and incubated in 60 °C water bath for 30 min. Solubilized protein samples were separated by polyacrylamide gel electrophoresis and electroblotted on the nitrocellulose paper according to the standard methods (15). Blots were incubated for 2 h at room temperature with antibodies specific to GLUT1, GLUT2, GLUT3, or GLUT4 at 1:500 dilution. The antigen-antibody complexes were recognized using horseradish peroxidase-protein A diluted 1:4000 and then developed by Western blotting detection reagents. In experiments analyzing the GST fusion protein, 2 μg of each fusion protein eluted from 10 ml of glutathione was used.

**[3H]Cytochalasin B Binding Assay—**Equilibrium [3H]cytochalasin B binding was measured by a centrifugation method as described previously (11) with a slight modification. Total membranes (100 μg of protein) isolated from control and azide-treated cells were suspended in 1 ml of 10 mM Tris buffer containing a tracer amount (0.02 μCi) of [3H]Jyotochalasin B with a varying concentration (0–10 μM) of unlabeled cytochalasin B and 1.5% ethanol (pH 7.4) and incubated for 30 min at room temperature. The membranes were pelleted by centrifugation at 185,000 × g at 4 °C for 60 min, and aliquots (0.3 ml) of the supernatant were removed for radioactive measurement. The supernatant remaining in the tube was aspirated completely, and the inner wall of the tube was wiped with cotton swabs to remove any residual medium. Pellets thus obtained were dissolved in 1 ml of 0.1% SDS in 0.1 M HCL for 15 min at room temperature for radioactive measurement for bound ligand. Radioactivity was measured by a liquid scintillation spectrophotometer (Pharmacia Biotech Inc.) using 3 ml of Ecoscint as counting fluid.

**-Glucose Displaceable cytochalasin B binding was quantitated by calculating the difference in CB binding measured in the presence of 500 mM d-glucose and l-glucose. In experiments where the effects of Clone 9 cells cytosol on d-glucose-sensitive CB binding activity of purified GLUT1 were measured, purified GLUT1 (5 μg of protein) was preincubated with 0.5–1 ml of 10 mM Tris/150 mM NaCl buffer alone or the same buffer containing 150–200 μM of cytosol proteins for 1–2 h in room temperature. Samples were then subjected to d-glucose-sensitive CB binding assay as described above.

**Expression and Purification of GST Fusion Proteins—**The procedure of expression and purification of GST fusion proteins was essentially as described (16). The fragments of DNA encoding the carboxyl-terminal cytoplasmic domain of GLUT1 and GLUT4 were produced by PCR methods (17). PCR primers were designed according to the human cDNA sequence. For the needs of ligation, BamHI restriction site was added at the sense primer, and EcoRI restriction site was added at the antisense primer. A nonsense fusion protein whose open reading frame encoding from the 3′ end to the 5′ end of GLUT4 cytoplasmic COOH-terminal domain was also made and used as a negative control. In this case, BamHI was added at the antisense primer while the EcoRI was added at the sense primer. The DNA fragments produced by PCR were then cloned into pGEX.3X vector and introduced into E. coli (DH5α) by transformation. A small number of transformants were grown in liquid culture in the presence of IPTG to induce expression of the fusion protein and were screened for expression of the expected fusion protein by SDS-PAGE of total cell lysates and for the ability of the soluble fusion protein to bind to glutathione-agarose. The GST fusion proteins corresponding to the COOH-terminal domain of GLUT1 and GLUT4 were further confirmed by PCR methods and Western blotting. A suitable transformant was then grown on a large scale, and fusion proteins were purified by incubating soluble materials from lysed cells with glutathione-agarose beads. When specified, the fusion proteins were eluted from washed beads by 5–10 mM glutathione based on competition with free glutathione.

**Assay for Cytosolic Protein Binding to GST Fusion Proteins—**75 μg of each purified GST fusion protein was used for specific binding. GST fusion proteins coupled with glutathione beads were incubated with...
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Equilibrium Binding of Cytochalasin B to Clone 9 Cell Membranes and Effects of Acute Azide Treatment—Clone 9 cells were incubated without (A) or with (B) 5 mM sodium azide for 60 min, and total membranes were isolated from them. Membranes (100 μg protein) were incubated with a varying concentration (0–10 μM) of CB and a tracer amount (0.02 μCi) of [3H]CB in the absence or in the presence of 500 mM D-glucose (○), 100 μM cytochalasin E (●), or 500 mM D-glucose plus 100 μM cytochalasin E (□) for 20 min at room temperature and then separated from the medium by centrifugation. Both membrane-bound and free [3H]CB were measured, from which amounts of bound ligand were calculated as the percentages of the total (bound plus free) and plotted as a function of the CB concentration used for the binding experiment.

RESULTS

Equilibrium Binding of Cytochalasin B to Clone 9 Cell Membranes and Effects of Acute Azide Treatment—Clone 9 cells were incubated in the absence and presence of 5 mM sodium azide for 60 min, and the equilibrium binding of cytochalasin B to the total membranes prepared from these cells was measured as a function of cytochalasin B concentration in the absence and the presence of an excess of cytochalasin E and/or D-glucose (Fig. 1). With ligand concentrations up to 10⁻⁵ M, there was saturable and linear binding of cytochalasin B to the membranes of both control and azide-treated cells. An excess (0.1 mM) of cytochalasin E, the cytochalasin B analog that binds to cytoskeletal proteins with an high affinity but does not bind to GLUT1 in human erythrocytes (11, 19), greatly inhibited the saturable binding of the ligand to membranes of both control and azide-treated cells (Fig. 1). An excess (500 mM) of D-glucose, on the other hand, affected the saturable CB binding very little if at all in membranes isolated from control cells, although this glucose effect was slightly but significantly greater in the presence of an excess of CE (Fig. 1A). More importantly, this glucose effect was clearly demonstrable in membranes of azide-treated cells even in the absence of CE (Fig. 1B), and an excess of CE did not affect this glucose effect. For both control and azide-treated cells, a significant portion of the saturable, cytochalasin B binding was seen even in the presence of both D-glucose and cytochalasin E in excess (Fig. 1). These findings indicate that there are at least three distinct, membrane-associated, saturable CB binding components in Clone 9 cells, namely, a glucose-sensitive component, a cytochalasin E-sensitive component, and a component that is insensitive to both glucose and CE. Of importance is the finding that the acute azide treatment increases the glucose-sensitive CB binding component. The apparent increase in the glucose-sensitive CB binding to control cell membranes by an excess of CE observed (Fig. 1A) is intriguing; it is reminiscent of our previous observation (19) that in human erythrocyte membranes the displacement of CB by glucose plus CE exceeds the sum of their independent displacements.

The CE-sensitive and CE-insensitive CB binding components mentioned above were analyzed (not illustrated) according to Scatchard (20). The CE-sensitive CB binding was estimated by measuring the difference in ligand binding observed in the presence and in the absence of 10⁻⁴ M CE. The CE-insensitive binding component was estimated by measuring CB binding in the presence of 10⁻⁴ M CE and subtracting linear binding component. The CE-sensitive binding in Scatchard plot revealed a single binding component with apparent K_D and B_T (total binding capacity) of approximately 2 μM and 80 pmol/mg protein, respectively, for both control and azide-treated cells. The CE-insensitive CB binding also revealed an apparent linearity in Scatchard plot, yielding an apparent K_D of 0.2 μM for both control and azide-treated cells. The plot, however, gave B_T values of approximately 15 and 20 pmol/mg protein for control and azide-treated cells, respectively, indicating that azide treatment selectively increases the high affinity, CE-sensitive CB binding in Clone 9 cell membranes. It should be noted that the K_D value for this apparently azide-sensitive, high affinity binding component is similar to the K_D value known for cytochalasin B binding to GLUT1 (11). We next quantitated the glucose-sensitive cytochalasin B binding to Clone 9 cell membranes that is stereospecifically displaced by D-glucose, the physiological substrate of GLUT1, by quantitating difference in cytochalasin B binding observed between the presence of 500 mM D- and L-glucose (Fig. 2). For control cells, CB bound less in the presence of D-glucose than in the presence of L-glucose, but the difference was so slight that exact quantitation was rather difficult (Fig. 2). However, when we repeated these measurements with membranes derived from azide-treated cells, cytochalasin B binding was clearly less in the presence of D-glucose compared with that in the presence of L-glucose (Fig. 2), demonstrating that a significant portion of the ligand bound to membranes derived from azide-treated cells is readily displaceable by D-glucose but not by L-glucose. This D-glucose-sensitive CB binding was a saturable function of CB concentrations for both control and azide-treated cells (Fig. 2). Scatchard analysis of the D-glucose-sensitive, CB binding data (not illustrated) revealed a single CB binding component with an apparent K_D and B_T value of 0.26 ± 0.03 μM and 1.8 ± 1.6 pmol/mg protein for control cells (n = 3) and 0.25 ± 0.03 μM and 6.7 ± 1.2 pmol/mg protein for azide-treated cells (n = 4), respectively. These K_D values are similar to the K_D values of the CE-insensitive, CB binding component, but an order of magnitude smaller than that of the CE-sensi-
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![Graph: Effects of acute azide treatment (5 mM) on d-glucose-sensitive CB binding in Clone 9 cells.](image)

**Fig. 2.** Effects of acute azide treatment (5 mM) on d-glucose-sensitive CB binding in Clone 9 cells. Clone 9 cell total membranes (100 μg of protein) were incubated with 500 mM D-glucose or L-glucose for 20 min, and the equilibrium binding of cytochalasin B was measured in the presence of 500 mM D-glucose and L-glucose, respectively, using 0.02 μCi of [3H]CB as tracer in the presence of a varying CB concentration (0–10 μM). The d-glucose-sensitive CB binding was calculated as the difference in CB binding observed in the presence of L- and D-glucose for Clone 9 cells before (●) and after (○) sodium azide (5 mM) treatment for 60 min and plotted as a function of CB concentration. Each data point represents an average of data obtained in three independent measurements with S.D. shown as vertical bars.

![Graph: Glucose-Sensitive CB Binding (pmol Protein/mg).](image)

**Fig. 3.** Western blot analysis of glucose transporters in Clone 9 cells. Total membranes (40 μg of proteins) of Clone 9 cells before (Con) or after (Azide) azide treatment (5 mM for 60 min) were applied for each lane and immunoblotted using anti-peptide antibodies specific to GLUT1, 2, and 4. Also included are purified human erythrocyte GLUT1 (HEGT, 1 μg of protein), rat liver cell total membranes (Hep, 40 μg of protein) and rat epididymal adipocyte microsomes (Adip, 40 μg of protein) as controls to confirm isoform specificities of the antibodies. These were reproduced in two other experiments, whereas the positive blot with GLUT4 IgG of an apparent molecular mass of 50 kDa seen in hepatocyte membranes was not reproducible.

![Graph: Effect of Clone 9 Cell Cytosol on Cytochalasin B Binding.](image)

**Fig. 4.** Effect of Clone 9 cell cytosol on cytochalasin B binding to human erythrocyte GLUT1 (HEGT) and rat liver cell total membranes (Hep). Cytosol and membranes were incubated with purified GST fusion proteins (Fig. 5). The GST fusion proteins were then purified from bacterial lysates based on their molecular mass of 34 kDa as revealed by protein staining in a SDS-PAGE gel. The GST fusion proteins were then separated by centrifugation at 185,000 g protein). Total membranes (40 μg of protein), rat liver cell total membranes (Hep, 40 μg of protein) and rat epididymal adipocyte microsomes (Adip, 40 μg of protein) were incubated with 500 mM D-glucose or L-glucose, respectively, indicating that the glucose effect was not affected in the presence of an excess of CE.

We next examined expression of glucose transporter isoforms in Clone 9 cells. GLUT1 is the only isoform detectable in Northern blot of Clone 9 cells (7). Our Western blot analysis shows that Clone 9 cells express GLUT1 protein in abundance without any detectable amounts of GLUT2, GLUT4 (Fig. 3), or GLUT3 (not illustrated). Again, the acute treatment (2 h) with azide did not affect Clone 9 cell GLUT1 content to any significant degree (Fig. 3).

These findings strongly suggest that the d-glucose-sensitive portion of the saturable CB binding is due to GLUT1 and that the acute azide treatment increases the GLUT1 CB binding capacity without GLUT1 content in Clone 9 cells. The exact nature of this azide-induced apparent increase in GLUT1 CB binding, particularly its relevance to GLUT1 substrate (glucose) binding function, can only be speculated at this time.

Effect of Clone 9 Cell Cytosol on Cytochalasin B Binding Activity of Purified GLUT1—An interesting possibility for the observed increase in glucose-sensitive CB binding in azide-treated cells is that GLUT1 in Clone 9 cells may largely be kept in such a (masked) state that it can not bind glucose and CB and that azide treatment converts this inactive GLUT1 to functional GLUT1 in terms of both the substrate and CB binding activities. A certain cytosolic factor or factors may be responsible for this azide-induced modulation of GLUT1 glucose and CB binding activities.

To test this possibility, we examined whether the cytochalasin B binding activity of purified GLUT1 and/or its sensitivity to d-glucose are affected by cytosol derived from basal and azide-treated Clone 9 cells (Fig. 4). Human erythrocyte GLUT1 was purified and reconstituted in liposomes (18). Equilibrium binding of cytochalasin B to this preparation was measured as a function of CB concentrations and analyzed in a Scatchard plot. When suspended in Tris buffer, purified GLUT1 typically bound CB with Kd and Bmax of 0.2–0.4 μM and 14–16 nmol/mg protein, respectively (Fig. 4A). The incubation of purified GLUT1 in Tris buffer containing 5 mM azide for up to 60 min prior to measurement did not affect its CB binding activity (not illustrated). When suspended in cytosol of control Clone 9 cells, GLUT1 bound CB equally well, showing apparent Kd and Bmax values that were practically identical to those measured in buffer alone (Fig. 4A, inset). In the presence of the cytosol derived from azide-treated cells, however, CB binding was significantly reduced compared with those in buffer or in the presence of the cytosol derived from control cells (Fig. 4A), and this reduction was due to a decrease in Bmax without any significant change in Kd (Fig. 4, A (inset) and C). Furthermore, this reduction in Bmax by azide-treated cytosol was largely (50% or more) abolished in the presence of the peptide corresponding to the cytoplasmic COOH-terminal domain of GLUT1 (Fig. 4C). These findings suggest the presence of a factor in azide-treated cell cytosol that interacts with purified GLUT1 at its COOH-terminal domain and inhibits its CB binding activity and that this factor is virtually missing in cytosol obtained from control cells. There was no reduction in CB binding when this experiment was repeated using azide-treated cytosol preheated for 15 min at 70°C (Fig. 4B), indicating that the factor is heat-labile, thus most likely a protein.

Identification of Glucose Transporter-binding Proteins in Clone 9 Cell Cytosol—To identify this putative, GLUT1-binding protein proposed above, we studied possible binding of Clone 9 cell cytosol proteins to GLUT1 GST fusion proteins (Fig. 5). The peptides corresponding to the cytoplasmic COOH-terminal domain of GLUT1 and GLUT4 (amino acid sequences of 427–492 and 428–493, respectively) were fused with GST (GST-G1C and GST-G4C, respectively) by molecular cloning. For negative control, a “nonsense” fusion protein whose open reading frame encodes the COOH-terminal portion of GLUT4 in the 3’ to 5’ direction (GST-G4CR) was also prepared. The GST fusion proteins were then purified from bacterial lysates based on their ability to bind to glutathione-agarose beads. The GST-G1C and GST-G4C were 85 and 95% pure, respectively, with apparent molecular mass of 34 kDa as revealed by protein staining in SDS-PAGE (Fig. 5), and their identities were further confirmed by Western blotting (not illustrated) using appropriate antibodies (specific to a COOH-terminal 13 amino-acid sequence of GLUT1 and GLUT4, respectively).

The Clone 9 cells were metabolically labeled with 35S, and Cytochalasin B (CB) binding activity was measured as the difference in CB binding observed in the presence of L- and D-glucose for Clone 9 cells before (●) and after (○) sodium azide (5 mM) treatment for 60 min and plotted as a function of CB concentration. Each data point represents an average of data obtained in three independent measurements with S.D. shown as vertical bars.
To prevent nonspecific binding, the GST fusion protein beads were washed three times with PBS before use. Bound proteins were eluted from the beads by adding 5 mM glutathione. The eluted proteins were then separated on 10% SDS-PAGE and subjected to autoradiography.

A typical result of such an experiment revealed two cytosolic proteins with apparent molecular masses of 70 and 28 kDa that bind to GLUT1 and GLUT4 GST fusion proteins but not to GST (Fig. 6) or to the GST fusion protein of “incorrectly orientated GLUT4 COOH terminus” (not illustrated). The 70-kDa protein binding was much more intense for GLUT4 fusion protein than for GLUT1 fusion protein (Fig. 6). Azide treatment (5 mM for 1 h) did not reproducibly affect either the 28- or the 70-kDa protein binding appreciably in these experiments. It is interesting to note that there is a 70-kDa protein in rat adipocyte cytosol that binds to the GST fusion proteins of GLUT1 and 4, although the relationship if any of this adipocyte protein to the 70-kDa Clone 9 cell protein is yet to be determined. The 28-kDa protein binding, on the other hand, appeared unique to Clone 9 cells; no 28-kDa protein binding to GLUT1 or GLUT4 fusion proteins was detectable when rat adipocyte or hepatocyte cytosols were employed in these experiments (not illustrated).

To test if either the 70- or the 28-kDa GLUT1-binding protein is a phosphoprotein, we repeated these experiments using 32P-labeled Clone 9 cell cytosol (Fig. 7). Neither 70- nor 28-kDa protein binding was detected with 32P-labeled cytosols for GLUT1 and GLUT4 fusion proteins, indicating that the 70- and 28-kDa GLUT1-binding protein are not phosphoproteins. Instead, there was a 32P-labeled, 85-kDa protein that bound to GLUT4 fusion protein but not to GLUT1 fusion protein. Furthermore, this 85-kDa phosphoprotein binding was seen only in cytosol of azide-treated cells and not of control cells (Fig. 7). The 85-kDa binding was not always (two in four independent experiments) reproducible. The failure in detection of this protein by 35S-labeled cytosol would suggest that its metabolic turnover is rather slow.

Percentage bound as a function of CB concentrations and also analyzed (insets in A and B) according to Scatchard (20) in Tris buffer (pH 7.4), in control cytosol (○), and in azide-treated cytosol (●). B, equilibrium binding of CB to GLUT1-containing liposomes suspended in control cytosol (○) and in azide-treated cytosol preheated for 15 min at 70 °C (●). The GLUT1 preparation used here shows a slightly higher Kd value for CB binding than that used in A. Experiments were otherwise similar to that in A. These results were reproduced in three independent sets of experiments. C, CB binding to purified GLUT1 (5 μg) suspended in 500 μl of control and azide-treated Clone 9 cytosols were measured in the presence or absence of the synthetic peptide (10 μg) corresponding to GLUT1 COOH-terminal sequence (residues 451–492) using a CB concentration of 10−7 M. Values for bound/free are means of three measurements with S.D. shown as vertical bars.
namely, a cytochalasin E-sensitive site, a D-glucose-sensitive
membrane at three distinct classes of saturable binding sites,
B, a potent inhibitor of GLUT1 function, binds to Clone 9 cell
muscle cells (25), avian erythrocytes (3, 26), and yeast (27) as
Clone 9 cells studied here, but also in rat myocardial cells (24),
such an adaptive response has been described not only in the
include a substantial enhancement of glucose transport (4).
adaptiveresponsetoareductioninoxidativemetabolismmust
under basal condition and glycogen storage is minimal (23), the
increased need of glucose consumption by glycolysis. Thus, for
little protection against a reduced oxidative pathway unless
ever, an intrinsic facilitation of glycolysis itself would provide
findings are reminiscent of the characteristics of the CB bind-
ing sites in human erythrocyte membranes (19), where a D-
glucose-sensitive site (site I), a CE-sensitive site (site II), and a
site (site III) insensitive to both D-glucose and CE exist and
where site I is GLUT1 and site II is cytoskeletal.

We have shown in the present study that the amount of the
CE-insensitive, D-glucose-sensitive CB binding site, presumably
GLUT1 (GLUT2, 3, and 4 were not detectable in Western
blots), is very small in Clone 9 cells, amounting to a low per-
centage of the total CB binding sites in membranes and thus
barely detectable. This corresponds to 1–2 pmol/mg membrane
protein. The GLUT1 content in human erythrocyte membranes
has been estimated to be 300 pmol/mg protein (19). The binding
capacity of this site, however, increased in Clone 9 cells after
acute treatment with azide to 5–7 pmol/mg membrane protein.
This increase is large enough to account for most of the acute
increase in glucose transport by azide (5). Incidentally, the
azide treatment also increases CE-insensitive CB binding ca-
pacity, and this increase is almost stoichiometric to the in-
crease in glucose-sensitive CB binding capacity. It is interest-
ing in this regard to recall that in human erythrocytes CB
binding to site III becomes glucose-sensitive when site II is
saturated or selectively removed by extraction with EDTA (18),
suggesting that site III is an altered form of GLUT1 inactivated
by molecular association with a cytoskeletal component.

Most revealing and intriguing in the present study is the
finding that the CB binding activity of purified GLUT1 is
inhibited by cytosol of azide-treated Clone 9 cells. This effect is
specific to the cytosol isolated from azide-treated cells and was
absent in cytosol from control cells. This effect was largely
abolished by the peptide corresponding to the COOH-terminal
cytoplasmic domain of GLUT1. This effect was lost after heat-
treatment, suggesting that it is due to protein. One simple
interpretation of these observations is that there is a cytosolic
protein, X, in Clone 9 cells that interacts with the COOH-
terminal cytoplasmic domain of GLUT1 to form a reversible
X-GLUT1 complex where GLUT1 is incompetent to bind CB
and glucose. It is further possible that the total amount of X in
Clone 9 cells is rather limited and is available in copy number.
similar to that of GLUT1, such that practically all of X are in complex with GLUT1 in control cells and that azide treatment effectively dissociates X-GLUT1 complex releasing X almost quantitatively to the cytosol.

The biochemical or physicochemical basis of the “masking” and “unmasking” of GLUT1 by X proposed above is unknown. X could be an adaptor protein whose physical interaction with GLUT1 makes GLUT1 sterically unable to bind substrate. Alternatively, X could be an enzyme that modulates GLUT1 activity via a covalent change such as phosphorylation and dephosphorylation (28, 29). The chemical identity of X is unknown at present. It could be an intermediate of glucose metabolism that responds to the metabolic alteration secondary to known at present. It could be an intermediate of glucose dephosphorylation (28, 29). The chemical identity of X is unknown.

We have identified three proteins with apparent molecular masses of 28, 70, and 85 kDa in Clone 9 cell cytosol that bind to the COOH-terminal cytoplasmic domains of glucose transporters expressed as GST fusion proteins. They did not bind to irrelevant fusion proteins, indicating that the binding is specific to glucose transporters. The 28- and 70-kDa proteins were labeled by \( ^{35}S \) but not by \( ^{32}P \), suggesting they are not phosphoproteins. The 28-kDa protein bound more to GLUT1 than GLUT4, whereas the 70-kDa protein binding was more intense to GLUT4. Azide treatment did not affect these protein bindings significantly. The 85-kDa protein was labeled by \( ^{32}P \), indicating that it is a phosphoprotein. The 85-kDa protein bound only to GLUT4 fusion protein but not to GLUT1 fusion protein. One cannot rule out the possibility, however, that this protein may bind to the GLUT1 fusion protein under more appropriate experimental conditions or that it may bind to intact GLUT1. The \( ^{32}P \)-labeling of this 85-kDa protein is seen only in azide-treated cytosol but not in control cytosol, strongly suggesting that the binding is sensitive to metabolic alteration induced by azide treatment. This is particularly interesting because the putative inhibitory factor that inactivates purified GLUT1 glucose binding is seen only in azide-treated cytosol. More experiments are needed to establish the functional significance, if any, of these glucose transporter-binding proteins. These would include purification, partial sequencing, and cloning of this protein. Once purified protein is available in a large quantity, its role in GLUT1 modulation would be directly studied by injecting the protein into Clone 9 cells.

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