Cytochalasin B Interferes with Conformational Changes of the Human Erythrocyte Glucose Transporter Induced by Internal and External Sugar Binding†

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ABSTRACT: To gain insight into the mechanism of facilitated sugar transport and possible mechanisms by which glucose transporter intrinsic activity might be altered, we have investigated conformational changes of the human erythrocyte glucose transporter induced by internal and external sugar binding and by the transport inhibitor, cytochalasin B. Changes in the ability of thermolysin to digest glucose transporters present in erythrocyte ghosts were used to monitor conformational changes of the glucose transporter. The degree of protease digestion was determined by the amount of undigested glucose transporter remaining after the protease treatment, as assessed in Western blots using the glucose transporter specific monoclonal antibody 7F7.5. D-Glucose, the physiological substrate of the transporter, increased the transporter's susceptibility to cleavage by thermolysin. Nontransportable glucose analogues which bind specifically to either an internal or external glucose transporter sugar binding site also altered susceptibility of the transporter to thermolysin. Both methyl and propyl glucoside, which preferentially bind the internal sugar site, increased thermolysin susceptibility of the glucose transporter in a manner similar to that of D-glucose. In contrast, 4,6-*O*-ethylideneglucose, which preferentially binds the external sugar site, protected the transporter from thermolysin digestion. These results suggest that sugar binding to internal and external sugar sites induces distinct conformational changes and that the observed D-glucose effect on the susceptibility of the glucose transporter to thermolysin is due to D-glucose at equilibrium predominantly forming a complex with the internal sugar site. The protection from cleavage by thermolysin caused by external sugar binding is attenuated by the addition of an internally binding sugar. Cytochalasin B at 10 μM, a concentration that inhibits transport maximally, had no effect on transporter proteolysis, in marked contrast to the effects of externally or internally binding sugars. However, cytochalasin B blocked both the protective effect of ethylideneglucose and the potentiating effect of D-glucose and methyl glucoside on transporter proteolysis. Thus, cytochalasin B does not induce the same conformational change as sugar binding to either site. Rather than locking the glucose transporter into either the inward- or outward-facing conformation, cytochalasin B appears to inhibit transport by locking the transporter into a conformation that prevents induction of either sugar-induced conformation. These data argue against a ternary complex of the transporter with an externally bound sugar and cytochalasin B.

The facilitated diffusion of sugars across the membranes of mammalian cells is mediated by specific transmembrane transport proteins, the glucose transporters. Several of these proteins have now been cloned (Mueckler et al., 1985; Kayano et al., 1988; Birnbaum et al., 1989; Fukumoto et al., 1989; James et al., 1989a) and show a high degree of residue similarity, particularly in the transmembrane regions. Therefore, they are expected to have a similar mechanism of transport. Understanding this mechanism is of particular importance in light of recent evidence which suggests that the intrinsic activity of glucose transporters in the cell membrane may be modulated by insulin and other hormones, such as the β-adrenergic agonist isoproterenol (Joost et al., 1986; Kuroda et al., 1987; Mühlbacher et al., 1988; James et al., 1989b).

However, the molecular mechanism(s) by which transporter intrinsic activity may be modulated, like the actual mechanism by which the transporter translocates sugars across the cell membrane, is (are) a matter of controversy. In the present work we have studied the transport mechanism of the human erythrocyte glucose transporter. Because of their relative abundance of glucose transporters, human erythrocytes have been used for many years as a model system to study sugar transport. On the basis of extensive kinetic studies several models of sugar transport have been proposed. Central to the mechanisms implied in all the various kinetic models are substrate-induced conformational changes [for review see Carruthers (1990)]. The alternating conformation or "one-site" model, in which the transporter alternates between conformations that expose a glucose binding site on either side of the membrane, proposes that the change of the bound transporter from one conformation to the other is the mechanism by which a sugar molecule is translocated (Widdas, 1952; Barnett et al., 1975; Gorga & Lienhard, 1982). Various "two-site" models for transport, in which the transporter may be bound by sugars on both sides of the membrane simultaneously, invoke conformational changes as gating phenomena that open or close a pore in the transport protein secondary structure through which selected hexoses can travel (Holman, 1980; Carruthers, 1986). Kinetic evidence has shown the inward and outward sugar binding sites to have asymmetric binding requirements (Holman et al., 1981), and studies of the interaction of various side-specific transport inhibitors and nontransportable side-specific sugar analogues have provided kinetic evidence of inward- and outward-facing conformations of the transporter (Barnett et al., 1975; Baker et al., 1978; Krupka, 1985; May, 1988).

Noncovalent binding of glucose, glucose analogues that preferentially bind the transporter's external sugar site, and

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inhibitors of glucose transport such as cytochalasin B and phloretin has been shown to affect transporter intrinsic fluorescence spectra and fluorescence quenching (Gorga & Lienhard, 1982; Carruthers, 1986, Pawagi & Deber, 1990), while D-glucose and cytochalasin B has been shown to affect circular dichroism spectra (Chin et al., 1987; Pawagi & Deber, 1987, 1990). However, interpretations of the results of these studies have been contradictory. Studies of proteolyzed transporter labeled with cytochalasin B and an externally binding mannose derivative have also demonstrated conformational changes marked by increased and decreased thermolysin proteolysis, respectively (Holman & Rees, 1987). However, conformational changes induced by covalent binding of photoaffinity labels cannot be assumed to be the same as those induced by equilibrium binding of sugar or inhibitor to the transporter.

The present study characterizes sugar and cytochalasin B induced conformational changes in the human erythrocyte transporter. Susceptibility of the transporter to cleavage by the protease thermolysin in the presence of one or more ligands of the transporter and immunodetection of intact transporter remaining after the protease treatment were used to detect conformational changes of the transporter. This technique has the advantage that changes in transporter secondary structure induced by the noncovalent binding of sugars and inhibitors can be studied. Conformational changes induced by the actual transport of D-glucose were compared to those induced by nontransportable glucose analogues specific for the internal or external sugar site, and by the transport inhibitor cytochalasin B.

The technique of immunodetection of partially digested transporters in the presence of sugars and inhibitors may prove useful in the study of the transport mechanisms of other glucose transporters, such as the transporter isoform most prevalent in muscle and adipose (GLUT-4) and that most prevalent in liver (GLUT-2). Furthermore, this technique can also be used to investigate transporter interactions with other molecules that may alter the intrinsic activity of glucose transporters.

Experimental Procedures

Materials. *n*-Propyl β-D-glucopyranoside (PG) was a kind gift of Dr. Gustav Lienhard, Dartmouth. Cytochalasin B, methyl α-D-glucopyranoside (MG), and 4,6-*O*-ethylideneglucose (EG) were purchased from Aldrich. Thermolysin, D-glucose, maltose, and molecular weight standards were purchased from Sigma. Blood was obtained from the Detroit Chapter of the American Red Cross. Monoclonal antibody 7F7.5 was made against partially purified glucose transporters from human erythrocytes (Tai & Carter-Su, 1988).

Preparation of Alkali-Stripped Erythrocyte Membranes. Human erythrocyte ghosts were prepared according to the method of Dodge et al. (1963). Leaky, alkaline-stripped ghosts were made by exposing the ghosts to pH 11.5 to remove cytoskeletal proteins (Gorga & Lienhard, 1981).

Enzyme Proteolysis. Thermolysin digestions were performed in 50 mM Tris, pH 7.4. Alkali-stripped ghosts (225 μg/75 μL) were incubated for 30 min at 25 °C with various concentrations of sugars or inhibitors. They were then incubated for 15 min with thermolysin (2.25 units/mL). Proteolysis was stopped by diluting the 150-μL sample with 75 μL of buffer consisting of 167 mM Tris-HCl, 10 mg/mL EDTA, 33% glycerol, and 3.3% SDS (w/v), pH 6.8. The entire sample containing the digested proteins was added directly to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels.

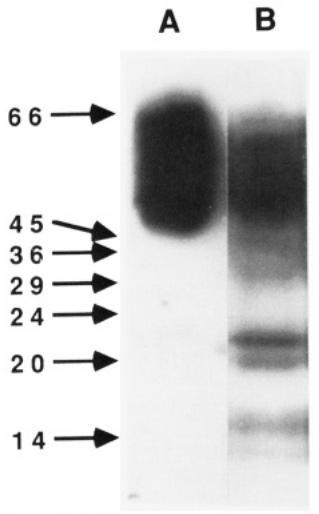

FIGURE 1: Digestion of the human erythrocyte glucose transporter by thermolysin. Human erythrocyte ghosts were incubated in the absence (lane A) or presence (lane B) of 0.4 unit/mL thermolysin. Proteins were separated by SDS–PAGE and Western blotted with monoclonal antibody (MAb) 7F7.5.

SDS–PAGE and Immunoblotting. SDS–PAGE was performed according to the two-phase system of Laemmli (1970) with a 11.25% or 15% polyacrylamide separating gel and a 3.5% polyacrylamide stacking gel [30:0.08 acrylamide:bisacrylamide]. Proteins were transferred electrophoretically for 3 h at 1.8 A (4 °C) onto nitrocellulose (Towbin et al., 1979). The blots were incubated first with blocking buffer, consisting of 2% milk proteins (Carnation Instant Milk) and 0.05% Tween-20 in PBS, followed by 10% (v/v) hybridoma cell supernatant containing monoclonal antibody 7F7.5 in blocking buffer overnight at room temperature. After being washed, the blots were incubated with ^{125}I-protein A (125 000 cpm/lane) for 2 h at room temperature. Antibody binding was visualized by autoradiography. The amount of intact glucose transporter was determined by antibody binding to the M_r = 45K–65K region of the blot, as assessed by laser densitometry using a BioMed Instruments scanning laser densitometer employing an Apple IIE microcomputer and Videophoresis II program. Estimates of cleavage rates in the presence of sorbitol, glucose, and inhibitors were obtained from the fraction of the total transporters that were digested, assuming pseudo-first-order kinetics.

Results

Effect of D-Glucose on Thermolysin Proteolysis of the Glucose Transporter. We first characterized the ability of thermolysin to proteolyze the glucose transporter. Glucose transporters present in alkali-stripped ghosts were partially digested using thermolysin and then blotted with monoclonal antibody 7F7.5 (Figure 1). When ghosts had not been treated with thermolysin, 7F7.5 antibody identified only intact glucose transporter, which migrates as a broad band of M_r = 45 000–65 000, as reported previously (Tai & Carter-Su, 1988) (Figure 1, lane A). Ghosts exposed to a "mild" thermolysin treatment (0.4 units/mL for 15 min) show reduced signal from the band 4.5 region of the Western blots, indicating less intact transporter remaining after proteolysis. Several lower molecular weight proteolytic fragments that contain the 7F7.5 binding site also appear, with M_r = 30 000, 20 000, 19 000, 15 000, and 13 500 (Figure 1, lane B). The cleavage fragments produced by partial digestion appear to be considerably more susceptible to digestion than the intact transporter, since even after extensive treatment with thermolysin (2.25 units/mL,

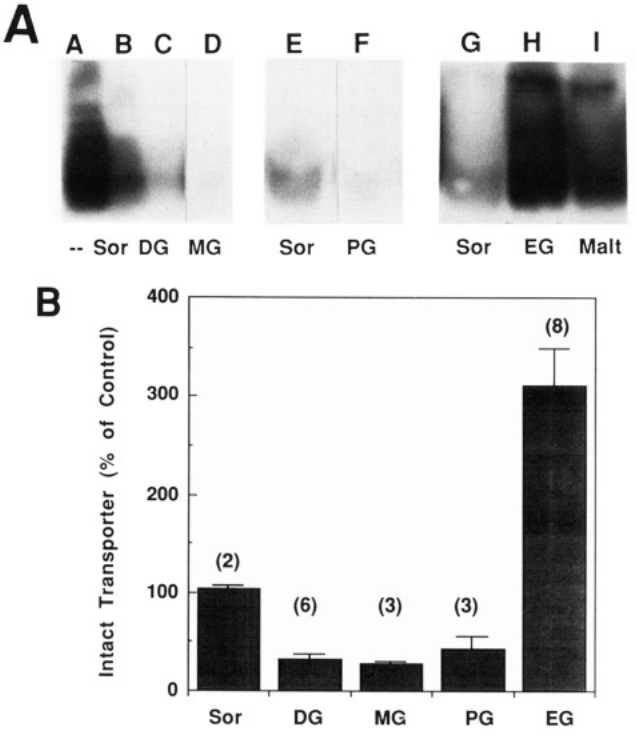

FIGURE 2: Effect of D-glucose and side-specific glucose analogues on thermolysin digestion of the glucose transporter. Panel A: Erythrocyte ghosts were equilibrated with 100 mM sorbitol (Sor, lanes B, E, and G), 100 mM D-glucose (DG, lane C), 100 mM methyl glucoside (MG, lane D), 50 mM propyl glucoside (DG, lane F), 100 mM ethylideneglucose (EG, lane H), or 100 mM maltose (Malt, lane I) and treated with 2.25 units/mL thermolysin for 15 min. Proteins were separated by SDS–PAGE and blotted with MAb 7F7.5. Lane A is glucose transporter from unproteolyzed erythrocyte ghosts. Panel B: Erythrocyte ghosts were treated with 100 mM sorbitol (Sor), 100 mM D-glucose (DG), 100 mM methyl glucoside (MG), 50 mM propyl glucoside (PG), and 100 mM ethylidene glucose (EG) and subjected to thermolysin digestion and blotting with 7F7.5 antibody. Amount of intact transporter was analyzed by densitometry of the transporter band in the blots. The sorbitol data are expressed as a percentage of the amount of intact transporter present after digestion in the absence of any analogue. All other data are expressed as a percentage of the amount of intact transporter present after digestion in the presence of sorbitol, which served as an osmotic control. Data represent means ± SE for separate experiments. When $n = 2$, data represent mean ± range (n is indicated by numbers in parentheses above each bar).

15 min), some intact transporter can still be detected, while the fragments cannot (data not shown). This is expected if cleavage at an initial site or sites results in exposure of additional cleavage sites that were previously embedded in the membrane or otherwise sterically protected from the protease. Given the relative instability of the cleavage fragments, we have determined the degree of transporter proteolysis by quantifying the amount of intact transporter remaining after the digestion.

When ghosts were preincubated with D-glucose (Figure 2A, lane C) before the addition of thermolysin, much smaller amounts of intact transporter remained after the digestion than when ghosts were incubated with sorbitol (Figure 2A, lane B), indicating higher levels of transporter proteolysis. D-Sorbitol is not transported by the glucose transporter and does not inhibit the binding of [^{3}H]cytochalasin B (Carter-Su et al., 1982), and hence it was used as an osmotic control in these experiments. At concentrations up to 100 mM, sorbitol had no significant effect on transporter proteolysis by thermolysin (Figure 2B). In contrast, a maximally effective concentration of D-glucose increased the degree of cleavage by thermolysin at 15 min by approximately 3-fold (Figure 2B). The effect

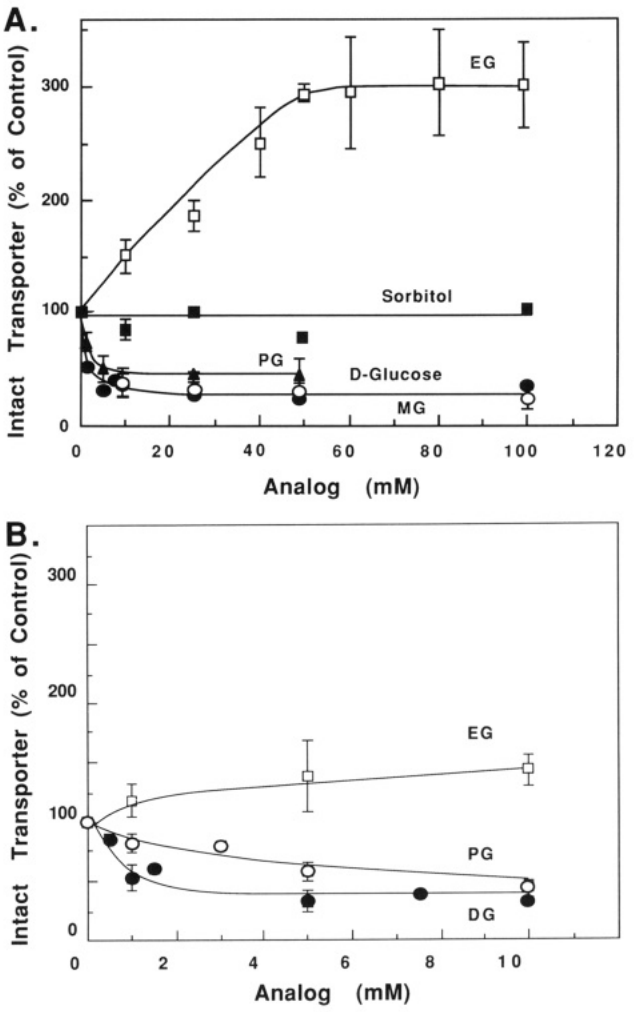

FIGURE 3: Dose dependence of modulation of thermolysin proteolysis of glucose transporter by D-glucose and side-specific analogues. Ghosts were equilibrated with various concentrations of sorbitol (filled squares), ethylideneglucose (EG, empty squares), D-glucose (filled circles), methyl glucoside (MG, empty circles), or propyl glucoside (PG, filled triangles) and treated with 2.25 units/mL thermolysin. Proteins were separated by SDS–PAGE and blotted with 7F7.5. When sorbitol was used, data show amount of intact transporter remaining after digestion in the presence of sorbitol expressed as a percentage of the amount remaining after digestion in the absence of all sugars or alcohols. All other curves show amount of intact transporter remaining after digestion in the presence of the noted sugar, expressed as a percentage of amount remaining after digestion in the presence of 100 mM sorbitol. All data points represent at least 2 separate experiments (most represent 3 or more) and show means ± SE when $n \geq 3$, ± range when $n = 2$. Panel A shows data points when sugar concentrations are from 0 to 100 mM, in panel B, the graph is expanded to show data points when sugar concentrations are from 0 to 10 mM.

of D-glucose on thermolysin proteolysis was found to be saturable and dependent upon the concentration of D-glucose (Figure 3). The concentration at which D-glucose was half-maximally effective was estimated graphically to be ~1 mM. Glucose (100 mM) increased the estimated rate of cleavage by 1.6-fold (Table I). This is consistent with reported D-glucose-induced increases in tryptic cleavage of the transporter (Gibbs et al., 1988).

Effect of Side-Specific Glucose Analogues on Thermolysin Proteolysis of the Glucose Transporter. To determine if the increase in the proteolysis of the transporter to thermolysin caused by D-glucose required the actual translocation of glucose by the transporter, or whether sugar binding to one or both of the sugar binding sites was sufficient to cause this effect, the effects of side-specific, nontransportable glucose

Table I: Effects of Substrates and Inhibitors on the Rate of Thermolysin Digestion of the Human Erythrocyte Glucose Transporter[a]

substrate/inhibitor	10^3 × estimated rate constant (min^{-1})	control	10^3 × estimated rate constant (min^{-1})	% of control[b]
D-glucose (100 mM)	163 ± 31	sorbitol (100 mM)	104 ± 26 (n = 4)	156[c]
ethylideneglucose (100 mM)	28 ± 7	sorbitol (100 mM)	113 ± 22 (n = 5)	25[c]
propyl glucoside (50 mM)	180 ± 8	sorbitol (50 mM)	98 ± 6 (n = 2)	183[d]
methyl glucoside (50 mM)	111 ± 29	sorbitol (50 mM)	58 ± 7 (n = 3)	189[c]
cytochalasin B (10 μM, 1% EtOH)	77 ± 19	EtOH (1% v/v)	77 ± 16 (n = 4)	100

[a] Rate constants were estimated assuming pseudo-first-order kinetics from the relative amount of undigested transporter remaining after digestion with thermolysin in the presence of substrate or inhibitors, or sorbitol or EtOH (control) at a single time point (15 min), as compared to total undigested transporter. [b] Percent (%) of control for the test substrate/inhibitors was considered statistically different from 100 if $p < 0.05$ by the one-tailed one-sample t test. [c] Statistically different from 100. [d] n not large enough for statistical analysis.

analogues on the susceptibility of the transporter to thermolysin digestion was studied. PG and MG are nontransportable glucose analogues that preferentially bind to the internal glucose site (Barnett et al., 1973, 1975; Deves & Krupka, 1978). Both MG and PG increased the cleavage of the transporter by thermolysin (Figure 2A, lanes D and F; Figure 2B) in a dose-dependent and saturable manner (Figure 3). The half-maximally effective concentrations were estimated graphically to be approximately 3 mM for PG and <10 mM for MG. PG (100 mM) and MG (50 mM) increased the estimated rate of cleavage by 1.8- and 1.9-fold, respectively (Table I). Thus, binding to the internal sugar site without actual translocation is sufficient to cause an increase in thermolysin susceptibility.

In contrast to the actions of glucose analogues that preferentially bind the internal sugar site, a nontransportable glucose analogue that preferentially binds the external site, EG (Baker & Widdas, 1973; Baker et al., 1978; Deves & Krupka, 1978), protects the transporter from proteolysis by thermolysin (Figure 2A, lane H; Figure 2B). The protective effect of EG is also dose-responsive and saturable (Figure 3), with half-maximal protection occurring at 25 mM EG. EG decreases the estimated rate of cleavage approximately 4-fold (Table I). The nontransportable disaccharide maltose, which inhibits glucose transport when present on the outside of cells (Lacko & Burger, 1962; Krupka, 1985a), also protects the transporter from thermolysin (Figure 2A, lane I). In the one experiment tested, the effect of maltose was also dose-dependent. A 350 mM maltose concentration gave a result similar to that of 100 mM EG, but the protection from proteolysis effected by 100 mM maltose was only 77% of this maximum (data not shown).

We tested whether analogues that bind different sugar sites compete with each other for induction of their respective conformations. When ghosts were equilibrated in the presence of increasing concentrations of EG and a saturating concentration of MG or D-glucose, MG and D-glucose attenuated the protection from proteolysis caused by EG (Figure 4). Thus it appears that occupation of the internal sugar site can interfere with the conformational change induced or stabilized by external sugar binding that results in protection from thermolysin.

The ability of these analogues to alter the degree of proteolysis appears to be specific to the glucose transporter and does not appear to involve altering the activity of the protease in general. D-Glucose and all other analogues used in this study do not alter the pattern of thermolysin proteolysis of other membrane proteins, as determined by Coomassie blue staining of SDS–polyacrylamide gels (data not shown).

Effect of Cytochalasin B on Thermolysin Proteolysis of the Glucose Transporter. To determine if cytochalasin B mimics the effects of glucose analogues that bind either the internal or external sugar binding site, we studied the effect of cytochalasin B binding on proteolysis of the transporter by thermolysin. At concentrations (1–25 μM) consistent with its inhibitory effect on glucose uptake, cytochalasin B had no significant effect on the proteolysis of the transporter by thermolysin, nor did cytochalasin E, a structurally related molecule that does not inhibit glucose transport (data not shown). When ghosts were equilibrated in the presence of 10 μM cytochalasin B with various concentrations of D-glucose or D-glucose analogues and then subjected to thermolysin digestion, cytochalasin B blocked both the protection from proteolysis caused by EG (Figure 5A) and the potentiation of proteolysis caused by either MG (Figure 5B) or D-glucose (Figure 5C). In contrast, cytochalasin E had no effect on the D-glucose-induced increase in cleavage (data not shown), indicating that the effects of cytochalasin B are not due to a nonspecific interaction with the transporter or the protease. Thus, cytochalasin B interferes with the effects of sugars that bind to both the internal and external glucose sites.

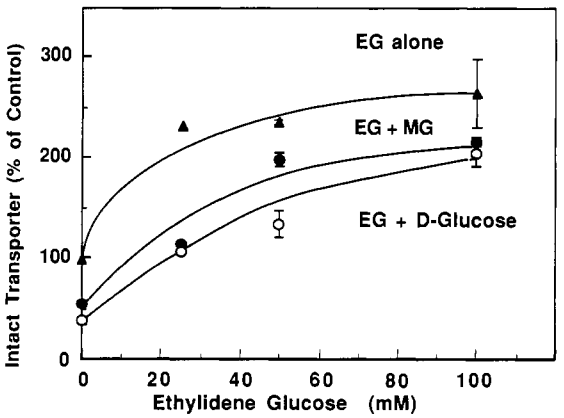

FIGURE 4: Effects on thermolysin digestion of the glucose transporter of ethylideneglucose alone, in the presence of D-glucose, or in the presence of methyl glucoside. Ghosts were equilibrated with various concentrations of ethylideneglucose, and with 50 mM sorbitol (EG alone, triangles), D-glucose (EG + D-glucose, empty circles), or methyl glucoside (EG + MG, filled circles). They were treated with 2.25 units/mL thermolysin for 15 min. Proteins were separated by SDS–PAGE and Western blotted with MAb 7F7.5. The graph shows amount of intact transporter remaining after digestion in the presence of the noted sugars, expressed as a percentage of transporter remaining after digestion in the presence of 100 mM sorbitol (control). Results are shown for 3 separate experiments. Data are means ± SE.

Discussion

D-Glucose Induces a Conformational Change in the Glucose Transporter. D-Glucose was found to increase the proteolysis of the glucose transporter by thermolysin in a dose-dependent and saturable manner, while D-sorbitol had no effect on thermolysin proteolysis of the transporter. Therefore, the observed D-glucose potentiation of proteolysis is not likely to be due to a change in osmolarity or a nonspecific interaction of sugars with the lipid membrane, the transporter, or the

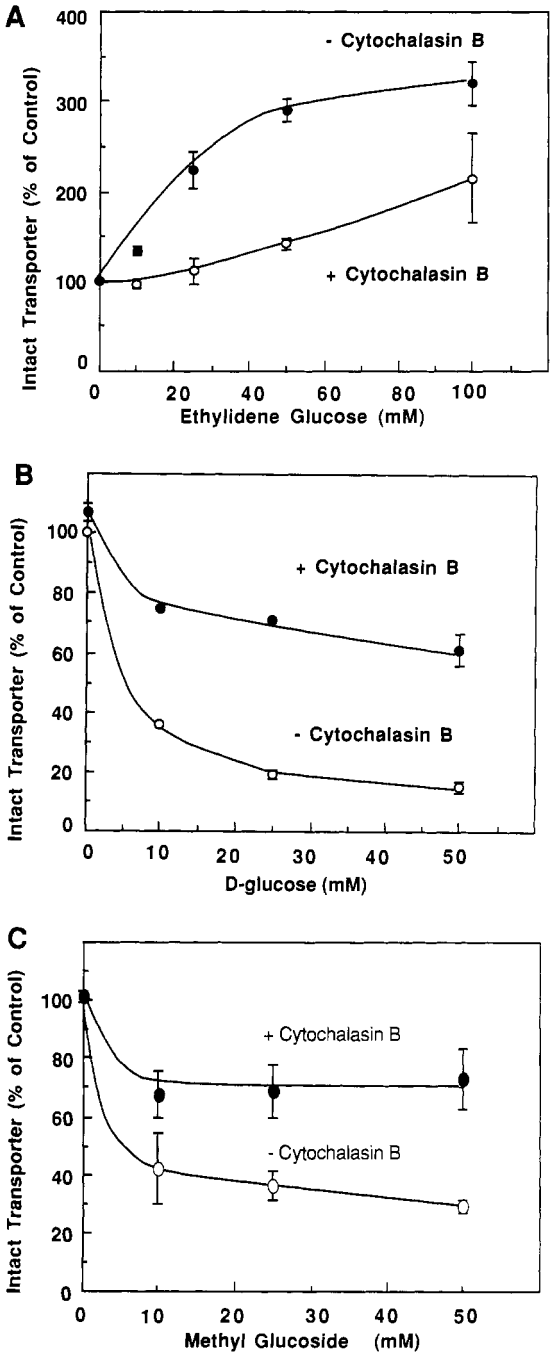

FIGURE 5: Cytochalasin B attenuation of the effects of ethylidene-glucose (panel A), D-glucose (panel B), and methyl glucoside (panel C) on thermolysin proteolysis of the glucose transporter. Ghosts were equilibrated with various concentrations of the indicated sugars, with or without 10 μM cytochalasin B, and digested with 2.25 units/mL thermolysin. Proteins were separated by SDS–PAGE and Western blotted with MAb 7F7.5. The graphs show amount of intact transporter remaining after digestion in the presence of the various analogues expressed as a percentage of that remaining after digestion in the presence of 100 mM sorbitol, as determined by densitometry. Data are means ± SE, n = 3 separate experiments.

protease. Further evidence that glucose and other analogues used in this study do not directly alter the activity of the protease is provided by the absence of a similar effect on the digestion of other membrane proteins. Rather, the observed potentiation of proteolysis by D-glucose appears to result from a D-glucose-induced conformational change of the transporter that makes thermolysin cleavage sites more accessible to the protease. No intact transporter was detectable in autoradiographs after longer (30-min) incubations of ghosts with thermolysin when tested in the presence of sorbitol, consistent with the effect of the sugars being on the rate and not the extent of proteolysis. Thermolysin cleaves proteins on the amino side of hydrophobic residues (Umezawa & Aoyagi, 1977). However, it appears to cleave the intact glucose transporter only on the cytoplasmic face, since virtually no thermolysin cleavage of transporters was observed when the protease was added to sealed erythrocyte ghosts (Shanahan & D'Artel-Lewis, 1984; Holman & Rees, 1987). Therefore, the D-glucose-induced conformational change must involve cytoplasmic domains of the transporter.

Internal Sugar Binding and External Sugar Binding Induce Distinct Conformational Changes in the Transporter. D-Glucose is known to bind to both an internal and an external binding site of the transporter (Barnett et al., 1975; Baker et al., 1978; Holman et al., 1981). Therefore, the question of whether the observed D-glucose effect is due primarily to binding at one of these sites, or to the actual translocation process, was investigated. The nontransportable glucose analogue EG binds the transporter preferentially at the external sugar site and, in the present study, was found to protect the transporter from proteolysis by thermolysin. The protective effect of EG on proteolysis of the transporter exhibited a half-maximally effective concentration of approximately 25 mM, in good agreement with measured K_i values for EG inhibition of glucose transport when EG is present on the outside of cells (Baker & Widdas, 1973; Baker et al., 1978). Binding to the external site appears therefore to induce a conformation of the transporter distinct from that induced by D-glucose, in which the thermolysin cleavage sites are made less accessible to thermolysin. This finding is consistent with the finding of Holman and Rees (1987) that the mannose derivative *N*-(4-azidosalicyloyl)-1,3-bis(D-mannos-4′-yloxy)-propylamine protects the transporter from proteolysis by thermolysin when it is covalently bound to the external substrate site of the transporter, and also with recent evidence from Gibbs et al. (1989) that EG decreases the rate of proteolysis of the transporter by trypsin. The disaccharide maltose has been used as an inhibitor of glucose transport and a probe for the external glucose binding site by virtue of the fact that it does not appear to penetrate red cells (Lacko & Burger, 1962). However, it could ostensibly have significant affinity for the internal glucose site since one glucose moiety of the disaccharide contains the C-4 and C-6 hydroxyls that appear to be required for binding the internal, but not external, glucose site (Barnett et al., 1973; Holman et al., 1981). Interestingly, maltose acts like an analogue which preferentially binds the external site, since it protects the transporter from thermolysin.

In contrast to the protection afforded by EG and maltose, the inward-binding nontransportable glucose analogues MG and PG were both found to increase the degree of proteolysis of the transporter by thermolysin in a manner similar to that of D-glucose. Binding to the internal sugar site, without actual transport of sugar, thus appears to be sufficient to induce a conformational change in the transporter that increases its proteolysis by thermolysin. D-Glucose acts similarly to nontransportable glucose analogues that bind only the internal sugar site, which is consistent with D-glucose, like the internally binding PG and MG, increasing the rate of inactivation of glucose transport by 1-fluoro-2,4-dinitrobenzene (FDNB), while EG and maltose protect the transporter from inactivation (Krupka, 1971; Barnett et al., 1975). The conformational state of the transporter favored at equilibrium with glucose thus shares physical characteristics (susceptibility to thermolysin cleavage and inactivation by FDNB) with the conformation

induced by the binding of nontransportable glucose analogues (MG and PG) and is different from that of the unbound transporter. The simplest explanation of these findings is that D-glucose at equilibrium predominantly forms a complex with the transporter's internal sugar site and thus induces or stabilizes the "inward-facing" conformation of the transporter. This has been proposed previously to explain the effects of sugars on the action of FDNB (Barnett et al., 1975; Gorga & Lienhard, 1982). However, glucose causing an increase in the proportion of transporters in the inward-facing conformation is not predicted by several kinetic studies of glucose transport (Regen & Tarpley, 1973; Baker et al., 1978; Lowe & Walmsley, 1985). These kinetic studies predict that the transporter resides predominately in the inward-facing conformation in the absence of glucose and that glucose increases the proportion of transporters in the external-facing conformation. For our data to be consistent with the interpretation of the kinetic data, one must conclude that the conformational state induced by glucose must be distinct from both that of the transporter bound at the external site by a nontransportable analogue (EG) and the proposed inward-facing conformational state of the unbound transporter. However, since glucose acts like MG both in increasing cleavage and in having this effect blocked by cytochalasin B, the favored conformational state of the transporter in equilibrium with glucose (and presumably undergoing transport) must have structural similarities to the conformation of the transporter while bound at the internal site by a nontransportable glucose analogue (MG or PG). It seems most likely that glucose bound to the transporter at equilibrium stabilizes an inward-facing conformation that is not necessarily the same as that of the unbound transporter when its reactive glucose site is facing inward.

Cytochalasin B Interferes with Conformational Changes Induced by Internal and External Sugar Binding. Cytochalasin B is a competitive inhibitor of sugar efflux, but a noncompetitive inhibitor of influx, and has therefore been assumed to bind an internal site of the transporter (Deves & Krupka, 1978; Baldwin et al., 1980). The location of the cytochalasin B binding site on the glucose transporter is unknown. It has been hypothesized to affinity label tryptophan 412 (Cairns et al., 1987), which is located on proposed membrane-spanning region 12, using the nomenclature of Mueckler et al. (1985). More recent evidence suggests that this is unlikely, since substituting Trp 412 with a leucine does not alter the ability of cytochalasin B to affinity-label the mutant glucose transporters expressed in CHO cells (Katagiri & Oka, 1990). Because cytochalasin B appears to compete with glucose for the internal sugar site, several groups have proposed that the cytochalasin B binding site is identical to or overlaps the internal sugar site (Jung & Rampal, 1977; Basketter & Widas, 1978). However, there is also evidence to suggest that cytochalasin B binds a site distinct from the internal sugar site (Krupka & Deves, 1980). Cytochalasin B has also widely been assumed to induce the same conformational change as that of sugar binding to the internal sugar site (Gorga & Lienhard, 1981, 1982; Carruthers, 1986).

In striking contrast to the potentiation of proteolysis caused by glucose analogues that bind to the internal substrate site (PG and MG), cytochalasin B, used at a concentration (10 μM) that shows maximal inhibition of glucose transport, had no effect on thermolysin cleavage of the transporter. Thus, cytochalasin B appears not to induce the same conformational change as either internal or external sugar binding. Furthermore, cytochalasin B attenuated both the increase in proteolysis caused by internal sugar (MG) or D-glucose binding and the protection from proteolysis caused by external sugar binding (EG). These findings are consistent with cytochalasin B alone causing no change in circular dichroism spectra (Chin et al., 1987; Pawagi & Deber, 1988), but reversing the change in the spectrum induced by D-glucose (Pawagi & Deber, 1988), and with the finding that D-glucose, but not cytochalasin B, increases the rate of tryptic digestion of the transporter (Gibbs et al., 1989). Since cytochalasin B itself has little effect on transporter cleavage, it may stabilize a conformation similar to the "unbound" inward-facing conformation, which is predicted to predominate at the temperature tested. Cytochalasin B could then inhibit transport by preventing the transporter from undergoing conformational changes induced by binding to either the internal or external binding site. This is consistent with kinetic evidence (Krupka & Deves, 1985) that cytochalasin B binds a site distinct from the internal sugar site and allosterically competes with internal sugar binding. The present findings therefore indicate that studies which have assumed that cytochalasin B induces the same conformational change as sugar binding to the internal site need to be reevaluated. Holman and Rees (1987) showed that covalently bound cytochalasin B potentiates formation of a cytochalasin B labeled thermolysin proteolysis fragment, which would appear to disagree with the present study. However, covalent binding of cytochalasin B may induce a novel conformation of the transporter that results in increased proteolysis which is not induced by equilibrium binding of cytochalasin B, which the present study examined.

This new insight into the mechanism of cytochalasin B inhibition of glucose transport is intriguing in light of evidence that the glucose transport inhibitors androstenedione and androstanedione bind an internal site that appears to be distinct from the internal sugar site but identical to the cytochalasin B site (Krupka, 1985b; May, 1988a). It is possible that these and other endogenous inhibitors of glucose transport may share mechanistic features with cytochalasin B and that the cytochalasin B binding site represents an endogenous inhibitory regulation site on the transporter.

Does the Transporter Form Ternary Complexes with Sugars and/or Inhibitors? Although an alternating conformation model of glucose transport was proposed by Widdas in 1952, the complex kinetics of glucose transport have left the mechanism a matter of controversy. The present study provides evidence for ligand-induced conformational changes that are predicted by both the "one-site" and "two-site" kinetic models of glucose transport. The central prediction of the alternating conformation model is that the internal and external binding sites are mutually exclusive, as opposed to "two-site" models (Holman, 1980; Carruthers, 1986) in which the transporter forms a ternary complex with two sugars bound simultaneously on both sides of the membrane.

If the sites are mutually exclusive, as in the one-site model, then nontransportable side-specific analogues would be expected to compete for binding the glucose transporter from opposite sides of the membrane and should compete for induction of the associated conformation in a manner determined by their affinities to the respective binding site.

In a recent modification of the two-site model (Carruthers, 1986) it was proposed that EG binds the external sugar site with higher affinity (K_d = 1.1 mM) than had been determined from transport inhibition studies (Baker & Widdas, 1973; Baker et al., 1978) and that EG binds the internal, rather than the external, sugar site with K_d of approximately 25 mM. This assumption allowed reinterpretation of an earlier study (Gorga & Lienhard, 1982) and led to the conclusion that a ternary

complex of EG·transporter·cytochalasin B exists. However, the results of the present study are inconsistent with EG binding the external site with K_d of 1.1 mM and the internal site with K_d of approximately 25 mM. A 1 mM concentration of EG does not protect the transporter from thermolysin cleavage. The protection from proteolysis by EG which shows a half-maximum at 25 mM is almost certainly due to EG binding at the external, and not the internal, sugar site, since sugars that bind the internal sugar site potentiate digestion. The protective effect of EG cannot be explained by a novel conformation induced by EG binding to both the internal and external sites simultaneously; were this the case, analogues that bind the internal site would be expected to increase the protective effect of EG when present at the same time. In contrast, internally bound analogues were found to reduce EG's protective effect. This conclusion that EG binds the external site with K_d of 25 mM is consistent with studies of EG inhibition of glucose transport which show K_i values of 12–25 mM when EG is outside of the cells, and K_i = 110–>200 mM when EG is on the inside of cells (Baker & Widdas, 1973; Baker et al., 1978).

Studies which have attempted to resolve whether or not the transporter forms a ternary complex have examined the ability of side-specific glucose analogues to displace cytochalasin B (Gorga & Lienhard, 1982; Helgerson & Carruthers, 1987), which has been assumed to induce the same inward-facing conformation as glucose. As noted before, the present data argue that cytochalasin B does not induce the same conformation as sugar binding to the internal site. Furthermore, the transporter does not appear to form a ternary complex with EG and cytochalasin B, since cytochalasin B interferes with the ability of EG to induce or stabilize the outward-facing conformation in which the transporter is protected from thermolysin. The conclusion that the transporter does not form a ternary complex with EG and cytochalasin B is consistent with reports that EG and other agents that bind to the external faces of the transporter displace cytochalasin B from an internal site (Gorga & Lienhard, 1981; Krupka, 1985a,b; May, 1988b).

The question of whether the transporter forms a ternary complex with an outward- and inward-bound sugar is more difficult to answer from examination of the present data. The finding that EG binding to a site on the external face causes changes in the secondary structure of cytoplasmic domains of the transporter that contain the thermolysin cleavage sites and are thought to be near the internal substrate site (Holman & Rees, 1988) is consistent with the external and internal sugar sites being mutually exclusive. However, other data are more consistent with a ternary complex. Although cytochalasin B (10 μM) completely blocked protection of the transporter by EG at a maximally effective concentration (100 mM), glucose and MG at 50 mM were less effective. If the binding sites were mutually exclusive, the effect of 50 mM glucose (about 50 times its half-maximally effective concentration) would be expected to predominate over that of 100 mM EG (i.e., about 4 times its half-maximal concentration), and thus overall increased rather than decreased cleavage would be expected. Thus although the present study argues against a ternary complex of the transporter with EG and cytochalasin B, a complex with an outward-bound (EG) and inward-bound (MG) sugar cannot be excluded.

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Development of Affinity Labeling Agents Based on Nonsteroidal Anti-inflammatory Drugs: Labeling of the Nonsteroidal Anti-inflammatory Drug Binding Site of 3α-Hydroxysteroid Dehydrogenase[†,‡]

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their effect by inhibiting the target enzyme cyclooxygenase (prostaglandin H_2 synthase); however, little is known about the peptides comprising its NSAID binding site. Hydroxyprostaglandin dehydrogenases also bind NSAIDs, but their NSAID binding sites have not been well characterized. Using existing synthetic strategies, we have incorporated the bromoacetoxy affinity labeling moiety around the perimeter of two potent NSAIDs, indomethacin and mefenamate, a *N*-phenylanthranilate. The compounds synthesized were 1-(4-(bromoacetamido)benzyl)-5-methoxy-2-methylindole-3-acetic acid (**1**), 3-(2-(2-bromoacetoxy)ethyl)-1-(4-chlorobenzyl)-5-methoxy-2-methylindole (**2**), 4-(bromoacetamido)-*N*-(2,3-dimethylphenyl)anthranilic acid (**3**), *N*-(3-(bromoacetamido)phenyl)-anthranilic acid (**4**), and *N*-(4-(bromoacetamido)phenyl)anthranilic acid (**5**). To access whether these compounds have general utility in labeling NSAID binding sites, the compounds were evaluated as affinity labeling agents for 3α-hydroxysteroid dehydrogenase (3α-HSD) from rat liver cytosol. This enzyme displays 9-, 11-, and 15-hydroxyprostaglandin dehydrogenase activity, is inhibited potently by NSAIDs, and is homologous to bovine lung prostaglandin F synthase. Compounds **1–5** were shown to affinity label the NSAID binding site of 3α-HSD. They inactivated 3α-HSD through an E·I complex in a time- and concentration-dependent manner with $t_{1/2}$ values ranging from seconds to hours. Ligands that compete for the active site of 3α-HSD (NAD^+ and indomethacin) afforded protection against inactivation, and the inactivators could demonstrate competitive kinetics against 3α-hydroxysteroid substrates by forming an E·NAD^+·I complex. Further, when compounds **1–3** were radiolabeled with [^{14}C]bromoacetate, inactivation of 3α-HSD was accompanied by a stoichiometric incorporation of inactivator, indicating the labeling of discrete amino acids at the enzyme active site. This analysis suggests that these NSAID analogues may have general utility in affinity labeling the drug binding site of NSAID target enzymes.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of arthritis, rheumatism, and other inflammatory disorders. The presumed target enzyme for these drugs is cyclooxygenase (prostaglandin H_2 synthase, EC 1.14.99.1) (Vane, 1971, 1974; Flower, 1974). This enzyme is responsible for the initial step in the biosynthesis of the primary prostaglandins, one class of inflammatory mediators. Cyclooxygenase has been purified to homogeneity from ram and bovine seminal vesicles (Miyamoto et al., 1976; van Der Ouderra et al., 1977), and its cDNA has been cloned and the protein sequence has been deduced (DeWitt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988). Despite the wealth of information about the protein, little is known about the peptides that comprise the NSAID binding site. Aspirin is known to acetylate Ser_{530}, 70 amino acids from the C-terminus (Roth et al., 1983); however, site-directed mutagenesis

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