Ligand-induced conformational changes modify proteolytic cleavage of the adipocyte insulin-sensitive glucose transporter

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The transport conformation of the human erythrocyte glucose transporter (GLUT1) modifies rates of proteolytic cleavage of this protein by a variety of enzymes. We investigated the effects of ligand-induced conformational change on the susceptibility to enzymic cleavage of the insulin-sensitive rat adipocyte glucose transporter (GLUT4). A GLUT4-enriched slow sedimenting microsomal fraction was prepared from basal adipocytes and subjected to PAGE and immunoblotting. The GLUT4 protein was detected in these immunoblots with a C-terminal-specific antiserum as an M_r -46000–50000 doublet. GLUT1 protein was not detected by a GLUT1-specific antiserum in these membranes. Tryptic digestion caused loss of the GLUT4 signal in immunoblots in a time- and concentration-dependent fashion. Low- M_r membrane-bound fragments were not observed in electrophoretic gels, whether detection was attempted by immunoblotting or by counting radioactivity in gel slices following photolabelling with

[3 H]cytochalasin B. Transport-specific ligands known to induce an outward-facing conformation in the human erythrocyte GLUT1 protein retarded cleavage of the GLUT4 protein by submaximal concentrations of trypsin, whereas ligands known to induce an inward-facing conformation increased the extent of cleavage. The transported substrate D-glucose retarded tryptic cleavage of GLUT4. This result contrasts with the known behaviour of GLUT1, in which D-glucose accelerates cleavage. Cleavage of GLUT4 by thermolysin was also retarded by the outward-binding analogue 4,6-*O*-ethylidene glucose. These results show that the conformational sensitivity to proteolysis of GLUT4 mirrors that of GLUT1, except that the glucose-loaded GLUT4 has a different steady-state configuration, which may reflect underlying kinetic differences between the two proteins.

INTRODUCTION

The family of glucose transporters mediating facilitated diffusion of glucose have many similarities in their primary amino acid sequences [1]. For example, the human erythrocyte GLUT1 and rat adipocyte GLUT4 proteins have 65% (amino acid) identity and 76% (nucleotide) sequence similarity [2,3]. The similarity in sequences for all the isoforms is much less in the extramembrane hydrophilic regions, which have been suggested to contribute to functional differences between the proteins [1]. Enzymic proteolytic cleavage of such hydrophilic domains has helped to establish not only the transmembrane and loop structure of the human erythrocyte GLUT1 protein, but also the regions involved in transport- or ligand-induced conformational change. In the best studied example, trypsin has been shown to cleave the GLUT1 protein at several locations in a large cytoplasmic loop, as well as on a C-terminal cytoplasmic tail [4,5]. This results in two major membrane-bound fragments upon electrophoresis: a broadly migrating glycosylated M_r 24000–40000 N-terminal fragment and an M_r 18000–19000 C-terminal fragment. The susceptibility to cleavage of GLUT1 with trypsin or other enzymes at the cytoplasmic loop and C-terminal tail is determined by the conformation of the intact transporter in the membrane [6–9]. Sugars known from kinetic transport studies to bind to the transporter in its outward-facing conformation decrease the extent of cleavage [6–8], whereas inward-binding sugars and ligands either do not modify [6] or increase the extent of cleavage [7]. The transported sugar, D-glucose, accelerates the rate of proteolytic cleavage [6–8]. These data suggest that ligand binding to, or transport through, the membrane-spanning domains

modifies the structure of the large cytoplasmic loop or C-terminal tail of the GLUT1 protein. If so, and if sequence mismatches between transporters in these regions do contribute to kinetic transport differences [1], then the sensitivity to proteolytic cleavage should also differ between transporters.

On the basis of the above rationale, in the present work we have studied the effects of ligand-induced conformational change on the extent of proteolytic cleavage of the insulin-sensitive GLUT4 transporter present in slow-sedimenting membranes of rat adipocytes. We compare these results with published results from similar treatments of the erythrocyte GLUT1 protein.

EXPERIMENTAL

Materials

Trypsin, thermolysin (Type X), and *Staphylococcus aureus* V8 proteinase (Type XVIIB) were purchased from Sigma. Boehringer-Mannheim supplied the N-glycosidase F (PGNase F). Phenyl β -D-glucopyranoside (PGlc) and 4,6-*O*-ethylidene glucose (EGlc) were purchased from Aldrich Chemical Co. Radionuclides were obtained from New England Nuclear (125 I-Protein A) and from Amersham International ([4- 3 H]cytochalasin B). Polyclonal antibodies were raised in rabbits against the C-terminal 15 amino acids of the human GLUT1 and rat GLUT4 proteins as described by Goto et al. [10].

Membrane preparation

Isolated rat adipocytes were prepared by collagenase digestion [11] from epididymal and perirenal adipose tissue of 180–240 g

Abbreviations used: EGlc, 4,6-*O*-ethylidene glucose; PGlc, phenyl β -D-glucopyranoside; GLUT1, human erythrocyte glucose transporter; GLUT4, insulin-sensitive rat adipocyte glucose transporter; PGNase F, N-glycosidase F.

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male Sprague–Dawley rats. Isolated adipocytes were suspended in Krebs–Henseleit/Hepes buffer [12], containing 1.3 mM CaCl_2 and 20 mg/ml BSA (Fraction V) at 37 °C. Plasma-membrane and slow-sedimenting ('low density') microsomal fractions were prepared by method of Kono et al. [13]. Protein was determined by the BCA method (Pierce Chemical Co.).

Enzymic digestions

Slow-sedimenting adipocyte membranes (6 mg/ml) were digested for 20 min at room temperature with the indicated amount of trypsin in 100 mM sodium phosphate buffer, pH 7.5, in the presence or absence of sugars or inhibitors. The digestion was terminated with the addition of soybean trypsin inhibitor to a concentration of 6 $\mu\text{g}/\text{ml}$. Thermolysin digestion was performed at an enzyme concentration of 8 units/ml in 50 mM Tris/HCl buffer, pH 7.4, for 30 min at room temperature. Proteolysis was stopped by the addition of the same volume of electrophoresis sample buffer containing 2% SDS (w/v), 20% glycerol (v/v), 2 mM EDTA, 40 mM dithiothreitol, and 50 mM Tris/HCl at pH 6.8. Treatment with *S. aureus* V8 proteinase was performed at an enzyme concentration of 2 units/ml in 5 mM sodium phosphate buffer, pH 7.8, for 30 min at 37 °C. The digestion was terminated with the addition of electrophoresis sample buffer. Digestion with PGNase F was performed by adding 1.5 units of the enzyme to 15 μl of 10 mM Tris/HCl buffer, pH 7.5, containing 30 μg of membranes, and incubating overnight at room temperature. Digestion was again terminated with the addition of electrophoresis sample buffer.

Cytochalasin B photolabelling

Slow-sedimenting adipocyte membranes (300 μg) were incubated with 3 μM [^3H]cytochalasin B (15 Ci/mol) and 20 μM cytochalasin E for 15 min at 4 °C. The membrane sample (1 mg/ml) was placed in a quartz cuvette and irradiated for 5 min at 254 nm with a short-wave u.v. lamp (Foto-Dyne, model 3-3000). Following irradiation, the membranes were diluted with 10 ml of 100 mM sodium phosphate buffer, pH 7.5, and centrifuged at 150000 g for 60 min at 3 °C. The pellets were resuspended to a protein concentration of 6 mg/ml in the phosphate buffer, and tryptic digestion was carried out as described above using 3 $\mu\text{g}/\text{ml}$ trypsin for 20 min at room temperature. Following enzyme digestion, the samples were dissolved in an equal volume of electrophoresis sample buffer, and electrophoresis on 14% acrylamide gels was carried out as described below. Three membrane-containing gel lanes were cut into 1 mm slices, the locations of prestained M_r markers run in adjacent lanes being noted. The slices were incubated overnight at room temperature with 400 μl of Soluene 300 (Packard Instrument Co.) in scintillation vials to elute labelled proteins. A 5 ml portion of scintillation fluid (Hionic-Fluor, Packard Instrument Co.) was then added to each vial. The contents of the vials were vigorously mixed and left several hours before the radioactivity was counted in a Packard CA2000 liquid-scintillation spectrometer.

Gel electrophoresis and immunoblotting

Transporter proteins were detected using the immunoblotting method described by Saltis et al. [14]. Briefly, membrane proteins were separated by SDS/PAGE by the method of Laemmli [15], using 4% stacking and 10% or 14% resolving gels as indicated. Protein bands were electrotransferred for 60 min at 4 °C to poly(vinylidene difluoride) membranes (Millipore) using a Bio-Rad Mini-Protein II Trans Blot apparatus. Following transfer,

non-specific protein-binding sites were blocked by incubating the membrane for 60 min at room temperature in 5% Carnation non-fat dry milk in a Tris/saline buffer containing 10 mM Tris/HCl, 154 mM NaCl, 0.02% NaN_3 (w/v) and 0.1% (v/v) Tween-20, pH 7.5. The membrane was washed once in 20–30 ml of this Tris/saline/Tween buffer for about 1 min. It was then incubated with the primary antibody at a 1:1000 dilution in a buffer made up of 10 mM Tris/HCl/154 mM NaCl/0.02% NaN_3 /50 mg/ml BSA (Fraction V) for 2 h at room temperature, followed by six washes over several minutes, each with 20–30 ml of 10 mM Tris/HCl/154 mM NaCl, pH 7.5. The membrane was then incubated with ^{125}I -Protein A (0.5 $\mu\text{Ci}/\text{ml}$) for 30 min at room temperature in the Tris/saline/albumin buffer, and washed six more times in 20–30 ml of Tris/saline buffer without albumin or azide. The membrane was air-dried and placed for overnight autoradiography using Kodak XAR film. Prestained M_r markers ('Rainbow' standards; Amersham International) were run in adjacent gel lanes and transferred with the adipocyte proteins. The relative amount of immunoreactive glucose transporter was determined by laser densitometry (LKB Ultrascan XL, using GELSCAN software) of the autoradiogram.

RESULTS

The GLUT4 transporter typically appeared as a broad single band or doublet of M_r 46000–50000 in immunoblots, although the relative amounts of each band were variable in different

Figure 1 Effects of trypsin concentration on the extent of cleavage of GLUT4 in slow-sedimenting microsomal membranes

Membranes were incubated with the indicated concentration of trypsin (in $\mu\text{g}/\text{ml}$) for 20 min as detailed in the Experimental section. Proteins (30 $\mu\text{g}/\text{gel}$ lane) were separated by SDS/PAGE (10% gel) and detected by immunoblotting using the GLUT4 antibody. (a) Immunoblot of membranes (CON, control, no trypsin). (b) Plot of the relative intensity of bands in (a) obtained from densitometry as a function of the concentration of trypsin.

Figure 2 Effects of glucose-transporter-specific ligands on high-dose trypsin cleavage of GLUT4

Slow-sedimenting microsomal membranes were incubated with or without the indicated transport-specific agent for 5 min at room temperature, followed by addition of trypsin to the concentration noted below and digestion as described in the Experimental section. Each gel lane contained 30 μ g of protein. Lane 1, control, no trypsin; lane 2, 3 μ g/ml trypsin alone; lane 3, 200 mM EGlc + 3 μ g/ml trypsin; lane 4, 200 mM D-glucose + 3 μ g/ml trypsin; lane 5, 100 mM maltose + 3 μ g/ml trypsin; lane 6, 10 μ M cytochalasin B + 3 μ g/ml trypsin; lane 7, 10 μ M cytochalasin E + 3 μ g/ml trypsin; and lane 8, 100 μ M phloretin + 3 μ g/ml trypsin. SDS/PAGE on 10% acrylamide gels and immunoblotting with the GLUT4 antiserum were carried out as described in the Experimental section. The data from several experiments, including that shown, were quantified by laser densitometry and the results expressed relative to the intensity of the undigested control band. The following are means $\pm$ S.D. for *n* experiments performed at 3 μ g/ml trypsin: trypsin alone, 0.32 ± 0.2^a (*n* = 6); 200 mM EGlc, 1.03 ± 0.12^b (*n* = 7); 200 mM D-glucose, 0.64 ± 0.16^b (*n* = 4); 100 mM maltose, 0.6 ± 0.05^b (*n* = 4); 100 μ M phloretin, 0.5 ± 0.1^b (*n* = 3); 10 μ M cytochalasin B, 0.3 ± 0.08 (*n* = 4); and 200 mM EGlc + 10 μ M cytochalasin B, 0.37 ± 0.24^c (*n* = 4). Since there was variability in the extent of cleavage between experiments, comparisons were made within an experiment. Statistical comparisons were performed using the Mann-Whitney Rank-Sum test, accepting $P \leq 0.02$ as a significant difference: ^a, versus control; ^b, versus trypsin alone; ^c, versus EGlc + trypsin.

Figure 3 Effects of transporter-specific ligands on low-dose trypsin cleavage of GLUT4

Conditions were those described in the legend to Figure 2: lane 1, control, no trypsin; lane 2, 1.5 μ g/ml trypsin alone; lane 3, 5 mM PGlc + 1.5 μ g/ml trypsin; lane 4, 10 mM PGlc + 1.5 μ g/ml trypsin; lane 5, 20 mM PGlc + 1.5 μ g/ml trypsin; and lane 6, 10 μ M cytochalasin B + 1.5 μ g/ml trypsin.

membrane preparations. Digestion with increasing amounts of trypsin resulted in a progressive decrease in band intensity in immunoblots (Figure 1a), with a half-maximal effect at approx. 1.5 μ g/ml (Figure 1b). Low- M_r fragments containing the C-terminal tail were not detected, even at the lowest enzyme concentrations used. Loss of the GLUT4 band in immunoblots at a given trypsin concentration was also time-dependent, although usually not complete, leaving 5–10% of the original band at the end of 3 h of digestion with 3 μ g/ml trypsin (results not shown). This suggests that most of the cytoplasmic surface of the slow-sedimenting microsomal fraction was accessible to the enzyme.

The effects of incubation of the purified membranes with various transporter-specific ligands before and during digestion with trypsin was investigated, with results of two experiments at different trypsin concentrations shown in Figures 2 and 3. At 3 μ g/ml trypsin, EGlc, D-glucose, maltose and phloretin consistently decreased the extent of tryptic cleavage of the immunoreactive GLUT4 bands (Figure 2). Agents without effect at this

Figure 4 Effects of various concentrations of EGlc on the extent of tryptic cleavage of the GLUT4 protein

(a) Slow-sedimenting microsomes were incubated without trypsin (lane 1), with 3 μ g/ml trypsin alone (lane 2) or with 3 μ g/ml trypsin and the following concentrations of EGlc: 25 mM (lane 3); 50 mM (lane 4); 100 mM (lane 5); 200 mM (lane 6); 400 mM (lane 7); and 800 mM (lane 8). Proteins (30 μ g/lane) were separated by SDS/PAGE (10% gel) and immunoblotted using GLUT4 antisera as described in the Experimental section. (b) The immunoblot of (a) was scanned by laser densitometry and the amount of intact GLUT4 remaining after cleavage is expressed relative to control. The data were fitted to a hyperbolic model using the non-linear curve-fitter in the graphics programme Sigmaplot 5.0 (Jandel Scientific). The line through the data points represents the fitted results.

trypsin concentration were cytochalasins B and E (Figure 2), and the sugars L-glucose and PGlc (results not shown). The results of several experiments at the higher trypsin dose with the different ligands were quantified by densitometry and were expressed relative to the intensity of the control band, with results provided in the Figure 2 legend. The protective effect of EGlc, even at 3 μ g/ml trypsin, was virtually complete in this series of experiments. Maltose, phloretin, and D-glucose each resulted in significant protection over the combined experiments. The effect of D-glucose was about 50%. Whereas 10 μ M cytochalasin B did not affect the extent of cleavage at this relatively high concentration of trypsin, cytochalasin B did block the protective effect of EGlc, as indicated in the legend to Figure 2. Moreover, when the concentration of trypsin was decreased to 1.5 μ g/ml, cytochalasin B was found to increase the extent of tryptic cleavage, which is shown in lane 6 of Figure 3. Additionally, PGlc also increased the extent of cleavage under these conditions in a dose-dependent fashion (Figure 3).

Since the strongest protection against tryptic cleavage was afforded by EGlc, dose-response experiments were performed and a representative immunoblot and densitometric scan are shown in Figure 4. As Figure 4(a) shows, EGlc protected against tryptic cleavage in a concentration-dependent manner. In this experiment, tryptic digestion under the standard conditions was extensive, leaving only about 10% of the transporter intact. This may account for the fact that protection by EGlc was incomplete. The immunoblot was scanned by laser densitometry, and a plot of band intensity as a function of the EGlc concentration is

Figure 5 Proteolysis of GLUT4 by enzymes other than trypsin

Slow-sedimenting microsomes were treated by the methods described in the Experimental section for each enzyme, and 30 μ g of membrane protein was loaded in each gel lane. (a) lane 1, control; lane 2, thermolysin; lane 3, 200 mM EGlc + thermolysin; (b) lane 1, control; lane 2, *S. aureus* V8 proteinase; (c) lane 1, control; lane 2, PGNase F alone; lane 3, 200 mM EGlc plus PGNase F. Proteins were subjected to SDS/PAGE on 10% gels and immunoblotted using GLUT4 antisera as described in the Experimental section. Unrelated lanes were cut from (a) and (c).

Figure 6 Tryptic digestion of [3 H]cytochalasin B-photolabelled GLUT4

Slow-sedimenting microsomes were photoaffinity-labelled with [3 H]cytochalasin B and 50 μ g of protein per lane subjected to SDS/PAGE as described in the Experimental section. Individual gel slices were cut across three gel lanes. The closed circles indicate the controls, and the open triangles indicate trypsin-treated membranes. Positions of M_r standards are shown at the top of the Figure.

shown in Figure 4(b). When these data were fitted to a hyperbolic model using non-linear regression, the protective effect of the sugar was half-maximal at a concentration of 15 ± 4 mM.

The effects of other proteolytic enzymes with different cleavage specificities were investigated. Cleavage with thermolysin resulted in a decrease in the intensity of the GLUT4 band (Figure 5a), without appearance of smaller immunoreactive fragments (results not shown). As was observed with trypsin, digestion in the presence of 200 mM EGlc prevented the decrease in intensity of the immunoreactive band (Figure 5a). Staphylococcal V8 proteinase also cleaved the transport protein, leaving an M_r -21 000 immunoreactive band (Figure 5b). However, increasing amounts of EGlc had no effect on the rate of cleavage by V8 proteinase under these conditions (results not shown). Finally, the effects of PGNase cleavage on the banding pattern were evaluated and are shown in Figure 5(c). In this experiment the control band was converted into a triplet with two faster-migrating components, as

might be expected if the glycosylation had been at least partially removed. Addition of 200 mM EGlc during the digestion with PGNase had no effect on the banding pattern.

In the tryptic-cleavage experiments the actual site of cleavage is unknown, since lower- M_r immunoreactive fragments were not evident on electrophoretic gels. This indicates that cleavage of the C-terminal antibody-recognition sequence had occurred. Whether cleavage had also occurred at one or more sites in the large cytoplasmic loop cannot be ascertained from the data presented thus far. In order to determine whether the cytoplasmic loop was also cleaved by trypsin, we first photolabelled the GLUT4 transporter with [3 H]cytochalasin B before performing tryptic digestion and electrophoresis, then counted radioactivity in electrophoretic gel slices. It is evident from Figure 6 that control membranes had a small amount of an M_r -21 000 proteolytic fragment containing the cytochalasin B-labelling site, but not the C-terminal portion of the GLUT4 protein (otherwise this fragment would have been detected in the immunoblotting experiments above). Tryptic cleavage decreased the radioactivity of the cytochalasin B-labelled M_r -46 000–50 000 band, but failed to generate any smaller labelled fragments detectable on 14%-acrylamide gels. These results suggest that, under the digestion conditions used in our studies, not only is the C-terminal cytoplasmic tail removed, but cleavage likely occurs at the large cytoplasmic loop and elsewhere in the C-terminal half of the protein, generating labelled fragments too small to be resolved on 14% gels (i.e. $M_r < 10 000$).

We attempted to determine whether tryptic cleavage affected the GLUT1 transporter isoform in adipocyte membranes. However, in neither plasma nor slow-sedimenting membranes were we able to reliably detect a GLUT1 signal with two different C-terminal antibodies in immunoblots (not shown).

DISCUSSION

Despite the fact that the human erythrocyte transporter (GLUT1) and the rat insulin-sensitive transporter (GLUT4) are similar in primary and higher-order structure, they do show functional differences with regard to transport. The kinetic features of the GLUT1 protein in human erythrocytes have been extensively studied, but there is much less information regarding GLUT4 kinetics. GLUT4 transport kinetics are often complicated by the presence of substantial amounts of the GLUT1 protein in the plasma membrane in cultured cells. Use of the *Xenopus* oocyte system for expression of GLUT4 has potential, but has been hampered by poor expression of the GLUT4 isoform [16,17], which is probably related to intracellular sequestration [18]. The most reliable data come from studies of insulin-stimulated rat adipocytes, in which GLUT4 constitutes 90% or more of the functioning transporters [19]. The adipocyte GLUT4 has been reported to have equal K_m values for entry and exit [20,21], indicating a symmetrical transport mechanism by the usual definition. On the other hand, the erythrocyte GLUT1 is known to be asymmetric, with differing K_m values for entry and exit [22–24]. Given the obvious structural asymmetry of both transporters relative to the plane of the membrane, asymmetry of some aspect of the GLUT4 transport mechanism might well be expected. In this regard, as pointed out by Devés and Krupka [25], the apparent transport K_m or inhibitor K_i is a function of both the equilibrium dissociation constant of substrate or inhibitor (K_D) and the steady-state orientation of the transporter orientation in the membrane (which determines binding-site availability). The latter is dependent on the relative magnitude of the rate constants of the relevant steps in the kinetic model [23].

When considered in this context, there is asymmetry in the GLUT4 transporter mechanism.

Holman and Rees [26] studied the effects on adipocyte GLUT4 transport of carrier ligands known to bind the GLUT1 protein asymmetrically. They found that binding of these inhibitors to GLUT4 was also asymmetric and was dependent on the conformation of the transporter. Instead of monitoring transport, we assessed changes in adipocyte GLUT4 conformation by measuring the ability of trypsin to cleave the C-terminal cytoplasmic tail of the protein. In erythrocytes this type of approach was successful in defining distinct conformations of the GLUT1 transporter. For example, ligands known from kinetic transport studies to bind to the outward-facing GLUT1 transporter have been shown to retard rates of cleavage [6–9]. In the GLUT4 protein, the non-transported outward-binding ligands, EGlc, maltose and phloretin, also decreased the extent of tryptic cleavage (Figure 2). On the basis of the transport studies of Holman and Rees [26], it is likely that these ligands bind to the same conformation of the GLUT1 and GLUT4 proteins [26]. Therefore, an outward-facing conformation protects against tryptic cleavage in both proteins. The effect was not limited to trypsin, since a high concentration of EGlc also decreased the extent of thermolysin cleavage (Figure 5a), again as in erythrocytes [7]. Moreover, when the concentration of trypsin was adjusted to produce relatively little cleavage, it was possible to show that both PGlc and cytochalasin B increased the extent and presumably the rate of trypsin cleavage (Figure 3). Both of these ligands are thought to bind selectively to the inward-facing conformation of the erythrocyte [27] and adipocyte [26] transporters. In the erythrocyte GLUT1 transporter, Gibbs et al. [6] showed that cytochalasin B accelerated tryptic cleavage, but only at relatively high temperatures. They found no effect of PGlc. King et al. [7] reported that thermolysin cleavage of the erythrocyte GLUT1 protein was accelerated by the inward-binding ligand *N*-propyl β -D-glucoside, but not affected by cytochalasin B alone. Our results that both cytochalasin B and PGlc increased the extent of tryptic cleavage at room temperature may reflect a greater tendency of the unloaded GLUT4 transporter to assume an outward-facing conformation than observed for the erythrocyte GLUT1 protein.

Another difference between GLUT1 and GLUT4 was the effect of D-glucose on the extent of tryptic cleavage. In erythrocytes D-glucose accelerates rates of GLUT1 cleavage with trypsin [6], thermolysin [7] and papain [8]. In the adipocyte GLUT4, on the other hand, D-glucose slowed the rate of tryptic cleavage. This observation suggests that the D-glucose-bound GLUT4 transporter has a different conformational stability than the unliganded protein, and that this differs from the erythrocyte GLUT1 protein. Lowe and Walmsley [23] have shown that, at 20 °C, the glucose-bound erythrocyte transporter is oriented about 70% inwardly, a trypsin-sensitive conformation. One possible explanation for our results is that the glucose-transporting GLUT4 is oriented in an outward-facing trypsin-resistant conformation. Such conformational asymmetry is in accord with the results of a previous study by Vinten [28]. He found in insulin-stimulated rat adipocytes that rates of zero-*trans* efflux were greater than those observed under equilibrium exchange conditions [28]. He interpreted this to indicate that inward movement of the substrate-loaded transporter is the rate-limiting step of the transport cycle for GLUT4. If so, then the steady-state distribution of the transporter under maximal flux conditions (i.e. in the presence of 200 mM D-glucose) is mostly outward-facing. However, it should be noted that Vinten's [28] efflux studies were performed using initial intracellular 3-*O*-methylglucose concentrations at, or well above, the derived K_m

for efflux [28]. When lower initial intracellular 3-*O*-methylglucose concentrations have been used, several-fold lower zero-*trans* [20,26] and exchange [21,26] efflux K_m and V_{max} values have been reported, which equalled the respective influx parameters. Such a symmetrical mechanism for GLUT4 requires that substrate loading alone induces a trypsin-resistant conformation. Direct measurements of the rate constants and binding affinities of the GLUT4 protein will be required to establish which of the two explanations is correct.

The GLUT1 and GLUT4 proteins also appear to differ in the bind pattern on electrophoretic gels after tryptic digestion. Under mild digestion conditions (3 μ g/ml trypsin for 20 min at room temperature), we found that trypsin cleaves the GLUT4 protein, but does not produce electrophoretic fragments over about M_r 10000. This was evident both in immunoblots (Figure 1a), and in cytochalasin B-photolabelled protein (Figure 6). Oka et al. [29] also failed to observe low- M_r C-terminal tryptic fragments derived from cytochalasin B-photolabelled adipocyte GLUT4, although they did observe an M_r -18000 fragment when erythrocyte ghosts were digested under identical conditions (10 μ g/ml trypsin for 90 min at 37 °C). In erythrocyte membranes, even 10-fold higher trypsin concentrations have been shown to leave cytochalasin B-labelled C-terminal fragments of M_r 15000–24000 in electrophoretic gels [30,31]. On the other hand, in a study using GLUT4 photolabelled with an iodinated derivative of forskolin, Hellwig et al. [32] found that the expected low- M_r C-terminal fragments were generated by tryptic digestion of immunoprecipitated and denatured GLUT4. Whereas our results and those of Oka et al. [29] suggest that the C-terminal half of GLUT4 in the native membrane may be more susceptible to tryptic cleavage than that of GLUT1, the choice of digestion conditions also appears to be important in determining whether detectable C-terminal fragments are observed.

The effects of staphylococcal V8 proteinase on the cleavage pattern of GLUT4 (Figure 4b) contrast with those of trypsin. Although the extent of cleavage of the transporter by the V8 proteinase was not sensitive to transport inhibitors under the conditions examined, such cleavage did produce an M_r -21000 electrophoretic fragment containing the cytoplasmic C-terminal tail. The presence of a fragment cleaved at the cytoplasmic loop, but containing the C-terminal tail, suggests that the C-terminal tail is relatively resistant to cleavage with the V8 proteinase. This pattern resembles that observed for the erythrocyte GLUT1 protein, in which an M_r -24000 fragment was found which required digestion with relatively high V8-proteinase concentrations to produce a residual M_r -15000 fragment [33].

In conclusion, our results show that rat adipocyte GLUT4 is quite sensitive to proteolytic digestion with trypsin and that its conformation determines its susceptibility to cleavage by trypsin and thermolysin. In several respects this sensitivity mirrors that observed for the erythrocyte GLUT1 protein, except that the conformation of GLUT4 appears to be more outwardly oriented than that of GLUT1. These results likely indicate differences between the two proteins in the transport mechanism. They also point out the usefulness of measuring conformation-induced proteolytic changes as an adjunct to kinetic transport experiments in defining the transport mechanism.

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