Analysis of the structural features of the C-terminus of GLUT1 that are required for transport catalytic activity

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C-terminally truncated and mutated forms of GLUT1 have been constructed to determine the minimum structure at the C-terminus required for glucose transport activity and ligand binding at the outer and inner binding sites. Four truncated mutants have been constructed (CTD24 to CTD27) in which 24 to 27 amino acids are deleted. In addition, point substitutions of R468 → L, F467 → L and G466 → E have been produced. Chinese hamster ovary clones which were transfected with these mutant GLUT1s were shown, by Western blotting and cell-surface carbohydrate labelling, to have expression levels which were comparable with the wild-type clone. Wild-type levels of 2-deoxy-D-glucose transport activity were retained only in the clone transfected with the construct in which 24 amino acids were deleted (CTD24). The CTD25, CTD26 and CTD27 clones showed markedly reduced transport activity. From a kinetic comparison of the CTD24 and CTD26 clones it was found that the reduced transport was mainly associated with a reduced $V_{max.}$ value for 2-deoxy-D-glucose uptake but with a slight lowering of the K_m. These data establish that the 24 amino acids at the C-terminus of GLUT1 are not required for the transport catalysis. However, the point mutations of F467L and G466E (26 and 27 residues from the C-terminus) did not significantly perturb the kinetics of 2-deoxy-D-glucose transport. The substitution of R468L produced a slight, but significant, lowering of the K_m. The ability of the truncated GLUT1s to bind the exofacial ligand, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yl-oxy)-2-propylamine (ATB-BMPA), and the endofacial ligand, cytochalasin B, were assessed by photolabelling procedures. The ability to bind ATB-BMPA was retained only in the CTD24 truncated mutant and was reduced to levels comparable with those of the non-transfected clone in the other mutant clones. Cytochalasin B labelling was unimpaired in all four mutated GLUT1s. These data establish that a minimum structure at the C-terminus of GLUT1, which is required for the conformational change to expose the exofacial site, includes amino acids at positions Phe-467 and Arg-468; however, these amino acids are not individually essential.

INTRODUCTION

Sequence alignment of members of the sugar transporter superfamily suggest that a gene encoding an ancestral six-membrane-spanning helical protein may have produced, in the evolutionary process, a two-domain 12-membrane-spanning helical structure [1–4]. Domain assembly experiments [5,6], and mutagenesis experiments [7] suggest that both the N- and C-terminal halves of the transport proteins are necessary for function. However, photolabelling studies involving active-site ligands suggest that the bis-mannose compound, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) (an exofacial ligand), cytochalasin B (an endofacial ligand) and 3-iodo-4-azidophenethylamino-7-*O*-succinyl-deacetyl-forskolin (IAPS-forskolin) interact only with the C-terminal half of GLUT1 [8–11]. The N-terminal half of GLUT1 may therefore be responsible for stabilizing the C-terminal half in a ligand-binding conformation [6].

Mutagenesis and proteolysis can stabilize either inward-directed or outward-directed conformational states of the GLUT1 protein. We have recently shown [12] that substitution of Tyr-293 by isoleucine stabilizes an outward-directed conformation which retains high affinity for the exofacial ligand ATB-BMPA, but which totally loses the ability to bind cytochalasin B at the endofacial site. C-terminal truncation of 37 amino acids from GLUT1 has been shown [13] to produce a protein that is stabilized in an inward-directed conformation. This mutant GLUT1 could not bind the exofacial ligand ATB-BMPA, but retained cytochalasin B binding. Similarly, the 18 kDa proteolytic fragment of GLUT1, which has lost an approx. 4 kDa portion of the C-terminus, has reduced affinity for ATB-BMPA but binds cytochalasin B well [14]. Stabilizing the GLUT1 in either the outward-directed or inward-directed conformational state results in reduced glucose transport catalysis.

We have suggested [15] that conformational flexibility in the glycine- and proline-rich transmembrane segment (TM) 10 is essential for the transport catalysis process. Substitution of isoleucine for Pro-385 sterically reduces flexibility and glucose transport while retention of flexibility, by substitution of glycine for Pro-385, allows conformational alternation between the

Abbreviations used: ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine; CHO, Chinese hamster ovary; $C_{12}E_9$, nonaethylene glycol dodecyl ether; IAPS-forskolin, 3-iodo-4-azidophenethylamino-7-*O*-succinyldeacetyl-forskolin; KRPB, Krebs–Ringer phosphate buffer; TM, transmembrane segment.

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outward-directed and inward-directed conformations. The proline- and glycine-rich region in TM 10 may provide a pivotal point which allows TM 11 and TM 12 to either pack against the outside (ATB-BMPA-binding) site in TM 7, 8 and 9 or against the inner (cytochalasin B-binding) site at the base of TM 10. These data and molecular modelling (P. A. Hodgson, D. J. Osguthorpe and G. D. Holman, unpublished work) suggest that in the outward-directed conformation, the base of TM 12 (the C-terminal segment) packs against the base of TM 10 and thereby blocks cytochalasin B binding. Thus deletion of critical residues in the C-terminus may prevent the closing of the inner site and the consequent opening of the outer site.

The deletion of 37 amino acids but not 12 amino acids locks the GLUT1 in the inward-facing conformation [13,16]. In order to establish the minimum size of truncation that would produce a perturbation of glucose transport activity and the associated inability to expose the outward-facing site, we have constructed a series of C-terminal mutants of GLUT1 which have from 24 to 37 amino acid deletions and we have studied the transport and ligand-binding characteristics in CHO-K1 clones expressing these constructs. Preliminary studies indicated that truncated GLUT1s in which 31, 34 and 37, but not 24, amino acids were deleted from the C-terminus were expressed in the cell, as detected by Western blotting, at levels comparable with the wild type, but that they had impaired 2-deoxy-D-glucose transport activity. Subsequent studies, described here, focused on the region around the highly conserved residues Phe-467 and Arg-468 (the CTD26 and CTD25 deleted GLUT1s, respectively) for further study. During the course of this work, a preliminary study by Due and May suggested that 21 amino acids could be deleted from the C-terminus of GLUT1 without loss of function [17].

The residues Phe-467 and Arg-468 have been mutated to leucine to test the possibility that these amino acids are individually critical for glucose transport. In addition we have also tested the possibility that the introduction of the glutamate in GLUT2 (a high K_m transporter) at the equivalent of Gly-466 in GLUT1 may alter the K_m.

EXPERIMENTAL

Materials

Phloretin, cytochalasin E, galactose oxidase, neuraminidase, Protein A–Sepharose and molecular-mass markers were from Sigma. 2-Deoxy-[2,6-^{3}H]D-glucose, [4-^{3}H]cytochalasin B and ^{125}I-Protein A were from Amersham International. The detergent, nonaethylene glycol dodecyl ether ($C_{12}E_9$) was from Boehringer. ATB-[2-^{3}H]BMPA (specific radioactivity 10 Ci/mmol) was synthesized as described elsewhere [14].

Site-directed mutagenesis and construction of human GLUT1 cDNA and expression in Chinese hamster ovary (CHO) cells

A full-length human glucose transporter (GLUT1) cDNA was kindly provided by Dr. G. I. Bell (University of Chicago, Chicago, IL, U.S.A.). Point mutations which led to termination of translation were introduced according to the method of Kunkel [18]. The template for mutagenesis was prepared in *Escherichia coli* RZ1032. Construction of truncation mutants was carried out by using the mutagenic primer, 5′-GCTCCCC-CCTACCGGAAGCCG-3′ for Gln-469, 5′-CTCCCCCCTGCT-AGAAGCCG-3′ for Arg-468, 5′-CCCTGCCGCTAGCCGGA-AGC-3′ for Phe-467 and 5′-CTGCCGGAATCAGGAAGCG-ATC-3′ for Gly-466 thereby converting the base triplets coding for these amino acids into stop codons. For point mutations G466E, F467L and R468L the oligonucleotides 5′-CCCCTG-CCGGAATTCGGAAGCGATC-3′, 5′-CCCTGCCGGAGGC-CGGAAGCGATC-3′ and 5′-CCCCCCTGCAGGAAGCCGG-AAG-3′ were used. Suitable mutant clones were selected and their sequences were confirmed by dideoxy-nucleotide sequencing in M13. Resultant cDNAs were designated CTD24, CTD25, CTD26 and CTD27, respectively. The mutated fragments were reinserted either into the *Nhe*I/*Xba*I site (for CTD27, CTD25, CTD24, G466E, F467L and R468L) or the *Bst*EII/*Xba*I site (for CTD26) of the previously constructed pRC/CMV-GT1(WT) [19]. pRC/CMV-GT1(WT) contains the vector pRC/CMV containing the human cytomegalovirus IE1 promoter and the bacterial neomycin resistance gene fused to the SV40 promoter. Mutant GLUT1 cDNAs were transfected by the calcium phosphate method into CHO-K1 cell lines, which were maintained in Ham's F-12 medium containing 10% (v/v) fetal-calf serum. The clones which obtained neomycin resistance were selected with 600 μg/ml of the neomycin derivative G418 (GIBCO) and these clones were subsequently subjected to Western blotting analysis using anti-peptide antibody against the intracellular loop portion of human GLUT1 to identify clones which express similar amounts of protein to the wild-type clone.

Western blot analysis

This was carried out essentially as described previously [19] except that both an anti-(C-terminal) antibody [19] and an antibody raised against the peptide CLINRNEENRAKS (corresponding to residues 215–226 in the central loop region [13]) were used.

Assays of glucose transport activity in CHO-K1 cells

Cells in 24-well (16-mm-diam.) plates were grown to confluence for 2–3 days and then washed three times in Krebs–Ringer phosphate buffer (KRPB) containing 130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM Na_2HPO_4, pH 7.4. 2-Deoxy-[2,6-^{3}H]D-glucose was then added to give a final assay concentration of 0.1–10 mM in 0.5 ml of KRPB and incubated at 37 °C. Uptake was terminated at 10 min, or 1 min (for experiments in which K_m and $V_{max.}$ were determined), by an addition of 1 ml/well of ice-cold PBS containing 0.3 mM phloretin, followed by three rapid washes in ice-cold PBS/phloretin. Following the arrest of transport, cells were solubilized with 0.4 ml of 0.1% SDS and the extract was added to scintillant for estimation of radioactivity. Zero-time uptake was determined by adding phloretin before the transported substrate.

Labelling of transporter cell-surface carbohydrate with tritiated sodium borohydride

This procedure was carried out as previously described in detail [19]. In brief, confluent cells in 35-mm-diam. dishes were simultaneously treated with 10 units of galactose oxidase and 1 unit of neuraminidase followed by 1 mCi of tritiated sodium borohydride (specific radioactivity 13.4 Ci/mmol). The cells were solubilized in detergent buffer consisting of 2% $C_{12}E_9$ in PBS, pH 7.2, with the proteinase inhibitors antipain, aprotinin, pepstatin A and leupeptin, each at 1 μg/ml. The solubilized material was then subjected to immunoprecipitation by Protein A–Sepharose coupled with anti-(GLUT1 central loop) serum. The immunoprecipitates were solubilized in electrophoresis sample buffer containing 10% SDS, 6 M urea and 10% mercaptoethanol and were subjected to electrophoresis on 10% acrylamide gels using a discontinuous buffer system [20]. Radioactivity in the gel slices was extracted and quantified as described previously [19].

ATB-BMPA and cytochalasin B photolabelling of GLUT1-transfected CHO-K1 cells

Confluent cells in 35-mm-diam. dishes were washed four times in PBS buffer and were then incubated at 18 °C with 100 μCi of ATB-[2-^{3}H]BMPA or, 1.4 μCi of cytochalasin B and 100 μM cytochalasin E, in 250 μl of PBS for 2 min and then irradiated for 1 min in a Rayonet RPR-100 photochemical reactor with 300 nm lamps as described [14,21]. Following irradiation the dishes were washed five times in PBS and directly solubilized in SDS/electrophoresis sample buffer, subjected to electrophoresis on 10% acrylamide gels and then the radioactivity associated with the gel peaks was determined.

RESULTS

The four mutant GLUT1 cDNAs, encoding the CTD24, CTD25, CTD26 and CTD27 constructs were transfected into CHO-K1 cell lines by the calcium phosphate method and the stable transformants were selected by their resistance to neomycin. Western blot analysis (Figure 1a) of crude membranes isolated from neomycin clones, using an anti-peptide antibody directed against residues 215–226 of the intracellular loop portion of GLUT1, enabled clones to be selected which had levels of expression comparable with the wild-type clone previously isolated [19]. Quantification of the Western blots (Table 1) showed that the expression levels in these clones were approx. 10-fold higher than for the non-transfected CHO-K1 clone. The bands corresponding to the truncated glucose transporter proteins showed only slight differences in their migration and apparent molecular mass on SDS/PAGE in comparison with the wild-type proteins. When Western blotting was carried out with an anti-peptide antibody directed against residues 478–492 of the C-terminus of GLUT1 [19] then only the wild-type GLUT1 protein was observed and no bands were detected for the four mutant clones lacking the C-terminal epitope (Figure 1b).

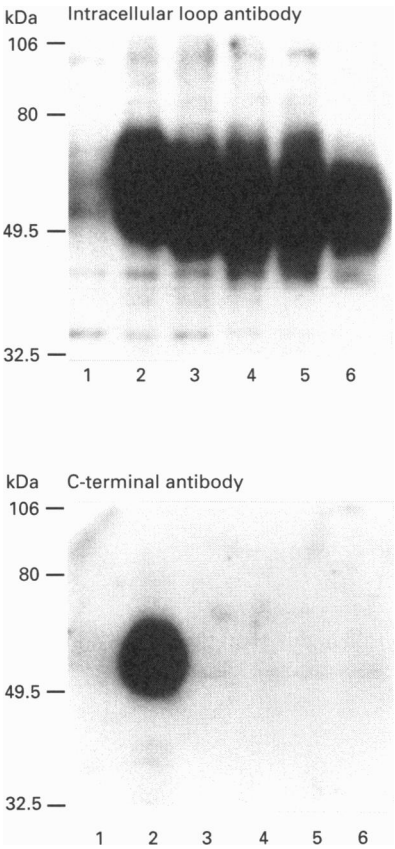

Figure 1 Western blot analysis of truncated GLUT1 transporters

CHO-K1 cells were transfected with cDNA coding for the wild type (lane 2) and clones in which 24 amino acids (CTD24; lane 3), 25 amino acids (CTD25; lane 4), 26 amino acids (CTD26; lane 5), and 27 amino acids (CTD27; lane 8) were deleted, and compared with the non-transfected clone (lane 1). A crude membrane fraction was obtained from stably transfected cells and subjected to Western blotting with (**a**) anti-(GLUT1 central-loop) antibody and (**b**) anti-(GLUT1 C-terminus) antibody.

A representative experiment showing the results following cell-surface labelling of the CTD24 and CTD26 clones is shown in Figure 2. The collected results from labelling all four clones in a series of experiments are shown in Table 1. Using this technique, all four truncated GLUT1 clones were confirmed to be expressed at the cell surface at levels that were similar to that occurring in the wild-type clone. By contrast, the CHO-K1 clone was labelled to a level which was only 13% of the wild-type clone.

2-Deoxy-D-glucose uptake measurements in the truncated clones revealed that only the CTD24 clone retained transport activity which was similar to that of the wild-type clone (Table 1). The remaining three mutant clones showed reductions in hexose transport. These were $55.3 \pm 3.4\%$ ($n = 4$) for CTD25, $36.3 \pm 1.7\%$ ($n = 4$) for CTD26, and $22.6 \pm 0.8\%$ ($n = 4$) for CTD27 of that occurring in the wild type. The transport activity in the CTD27 clone was similar to that observed in the non-transfected clone. To analyse the change in transport in more detail we investigated the kinetics of 2-deoxy-D-glucose net uptake at a range of substrate concentrations in the CTD24 and CTD26 clones (Figure 3). The most marked kinetic change due to the deletion was a reduction in the $V_{max.}$ value. In this series of experiments, the $V_{max.}$ of the wild type was 80.6 ± 10.6 nmol/min per mg of protein. The $V_{max.}$ for 2-deoxy-D-glucose transport in the CTD24 clone (68.2 ± 5.9 nmol/min per mg of protein) was similar to that of the wild type. By contrast the $V_{max.}$ for the CTD26 clone was 17.1 ± 2.3 nmol/min per mg of protein. The K_m values, which were similar in the wild-type and CTD24 clones, were 1.7 ± 0.2 mM and 1.30 ± 0.4 mM respectively, and were slightly lower in the CTD26 clone, in which the K_m was 0.75 ± 0.04 mM. These results are the mean ± S.E.M. from three separate experiments.

In a separate series of experiments (Table 1) amino acid substitutions were introduced, on the basis of these results and their strong conservation in the glucose transporter superfamily, into presumably important amino acids, i.e. Gly-466, Phe-467, and Arg-468. Also the effects on the kinetics of 2-deoxy-D-glucose uptake of point mutations at G466 → E, F467 → L and R468 → L were examined to further analyse the possibility that these amino acids are individually critical for glucose transport. Surprisingly, no large reductions in 2-deoxy-D-glucose transport were observed. However, a small but consistent reduction in the K_m of 2-deoxy-D-glucose transport was observed for the R468 → L mutant.

To analyse further the characteristics of the truncated transporters with perturbed function, the ligand-binding ability of these transporters was assessed using photolabelling procedures with the exofacial-site-specific reagent, ATB-BMPA, and with the internal-site-specific reagent, cytochalasin B. The labelling of the CTD24 clone by ATB-BMPA was similar to that of the wild type (Figure 4a). The ligand labelling at the exofacial site was only markedly reduced as a result of the deletion of the Arg-468 residue, and the CTD25 clone was labelled by ATB-BMPA to a level which was $12.5 \pm 1.6\%$ ($n = 3$) of that observed with the

Table 1 Analysis of expression, transport activity and ligand labelling of C-terminally truncated and mutated GLUT1

Abbreviation: 2-DG, 2-deoxy-D-glucose.

	Western blotting (%)	Borohydride labelling (%)	Transport at 100 μM 2-DG (%)	K_m (mM)	$V_{max.}$ (nmol/min per mg)	Cytochalasin B labelling (%)	ATB-BMPA labelling (%)
Wild type	100	100	100	1.7±0.2 (n = 3) *1.42±0.34 (n = 5)	80.6±10.6 (n = 3) *69.0±12.8 (n = 5)	100	100
CTD24	115.7±2.9 (n = 3)	93.8±6.3 (n = 4)	117.6±2.2 (n = 4)	1.3±0.4 (n = 3)	68.2±5.9 (n = 3)	102.2±12.0 (n = 3)	67.5±1.6 (n = 3)
CTD25	123.3±7.6 (n = 3)	151.5±12.6 (n = 4)	55.3±3.4 (n = 4)	0.92±0.14 (n = 3)	20.2±1.15 (n = 3)	84.7±4.8 (n = 3)	12.5±1.6 (n = 3)
CTD26	139.7±27.4 (n = 3)	130.5±11.7 (n = 4)	36.3±1.7 (n = 4)	0.75±0.04 (n = 3)	17.1±2.3 (n = 3)	96.1±8.6 (n = 3)	6.4±1.5 (n = 3)
CTD27	101.0±7.1 (n = 3)	118.3±17.7 (n = 4)	22.6±0.8 (n = 4)	0.72±0.10 (n = 3)	15.4±0.52 (n = 3)	83.2±11.5 (n = 3)	3.8±1.0 (n = 3)
R468L	126.0±17.8 (n = 3)	–	108.2±17.0 (n = 3)	0.92±0.1 (n = 4)	40.5±3.0 (n = 4)	–	–
F467L	107.3±21.1 (n = 3)	–	93.7±7.6 (n = 3)	2.51±0.26 (n = 4)	51.8±2.5 (n = 4)	–	–
G466E	106.6±3.9 (n = 3)	–	102.5±2.1 (n = 3)	1.55±0.22 (n = 4)	65.3±14.6 (n = 4)	–	–
Non-transfected	11.3±1.4 (n = 3)	13.0±0.8 (n = 4)	20.6±0.7 (n = 3)	–	–	13.7±2.1 (n = 3)	6.8±0.4 (n = 3)

*Experiments carried out for comparison with point mutations, G466E, F467L and R468L.

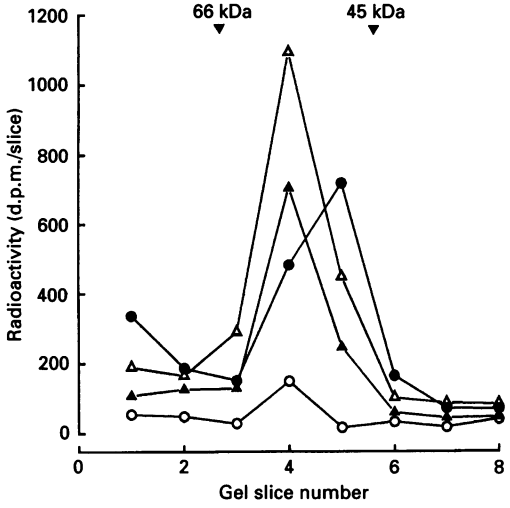

Figure 2 Cell-surface carbohydrate labelling of truncated GLUT1 transporters

Cells of the wild type (▲), CTD24 (●) and CTD26 (△) were compared with those of the non-transfected CHO-K1 clone (○) in a representative experiment. Cells in 35-mm-diam. dishes were simultaneously treated with galactose oxidase and neuraminidase and then 1 mCi of tritiated sodium borohydride. Immunoprecipitation of the labelled transporters was then carried out using anti-(central-loop) antibody. The labelled proteins were then analysed by electrophoresis. The positions of the radioactive peaks in the gel lanes are compared with molecular-mass markers (arrowheads).

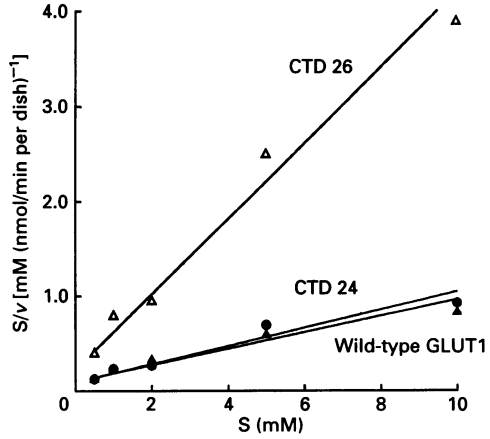

Figure 3 2-Deoxy-D-glucose transport in truncated GLUT1 transporters

The kinetic properties of the 2-deoxy-D-glucose transport activity in the wild-type (▲), CTD24 (●) and CTD26 (△) clones are compared. The data points are the means from three independent experiments. Least-squares regression using the Michaelis–Menten equation gave the kinetic parameters described in the Results section.

wild-type clone. A further reduction occurred on removal of Phe-467, i.e. the CTD26 clone. In this clone the labelling was 6.4±1.5% (n = 3) of that of the wild type and was comparable with that occurring in the non-transfected CHO-K1 clone (6.8±0.4%, n = 3).

As shown in the representative experiment in Figure 4(b), the cytochalasin B labelling of the CTD24 and CTD26 clones was unperturbed as a result of the C-terminal truncation. The collected results from a series of experiments on all four truncated clones (Table 1) showed cytochalasin B labelling in all these clones was similar to that of the wild-type clone and this labelling was approx. 7-fold higher than that of the non-transfected CHO-K1 clone.

DISCUSSION

The data presented here suggest that the C-terminal 24 amino acids of GLUT1 are not required for the transport catalysis process or the exofacial ligand-binding function of this protein. This does not necessarily exclude the possibility that these amino acids may be required as part of a targeting sequence that may be recognized by a possible subcellular sorting system in other cell types, as has been suggested for the N-terminal [22] and C-terminal sections of GLUT4 [23,24]. If such a system is present in CHO cells then the ability of the C-terminally truncated mutants of GLUT1 to be expressed at the cell surface at similar levels to the wild-type protein suggests that these C-terminal

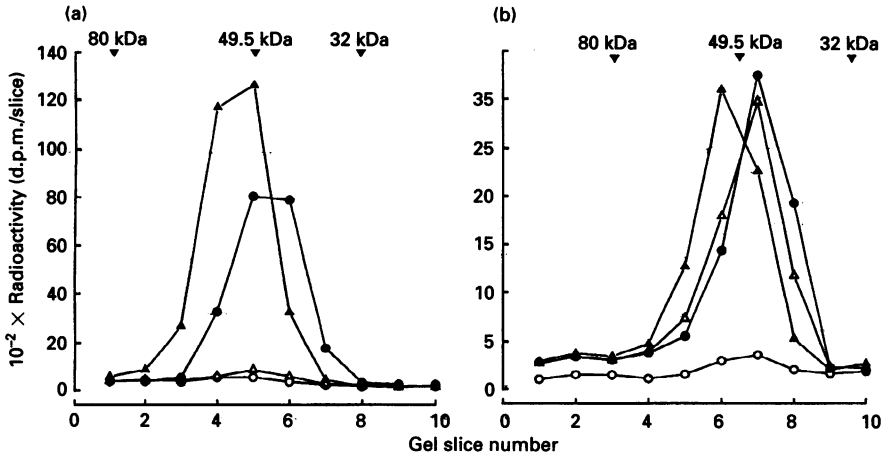

Figure 4 Photolabelling of truncated GLUT1 transporters by the exofacial reagent, ATB-BMPA, and the endofacial reagent, cytochalasin B

Cells of the wild-type (▲), CTD24 (●), CTD26 (△) and non-transfected (○) clones in 35-mm-diam. dishes were labelled with (**a**) 100 μCi of ATB-[2-^{3}H]BMPA or (**b**) 1.4 μCi of [4-^{3}H]cytochalasin B with 100 μM cytochalasin E in 250 μl of PBS at 18 °C by irradiation for 1 min in a Rayonet photochemical reactor. Following irradiation, the cells were washed five times in PBS and directly solubilized in SDS/PAGE sample buffer and subjected to electrophoresis. The positions of the radioactive peaks are compared with molecular-mass markers (arrowheads). Results in (**a**) and (**b**) are from experiments which are representative of three similar experiments.

amino acids of GLUT1 do not constitute part of the targeting sequence. It is possible that more proximal regions are involved in such targeting or in the maintenance of membrane protein stability, as deletion of 37 amino acids results in a mutant GLUT1 that is only labelled by the cell-surface-carbohydrate-labelling technique to a level which is approximately one-third of the wild-type level, despite an equal expression within the cell as detected by Western blotting [13]. A reduced stability in the membrane is the more likely consequence of deletion of 37 amino acids from GLUT1 as the two basic amino acids, Lys-456 and Arg-458 at the end of the highly conserved sequence P^{453}ETKGR are absent.

The transition between full and impaired functional activity is acute and not gradual as amino acids Arg-468 and Phe-467 are deleted. However, the individual amino acids Arg-468 and Phe-467 are not essential, as point mutations in which these residues are converted into leucine residues produce GLUT1 clones with fast rates of transport. This suggests that the truncation at these positions may perturb transport because the C-terminus is too short to produce the normal conformational change associated with alternating between the inner- and outer-facing sites in the transport catalysis cycle. We have suggested [15] that, in order to produce the outward-directed conformation, the base of TM 12, the C-terminal segment packs against the base of TM 10. Studies in which stable inward- and outward-directed conformations result from binding side-specific hexose ligands, from proteolytic digestion and from mutagenesis, all support the alternating conformational model [25,26] as a mechanistic basis of transport catalysis. It has been suggested that some kinetic alterations of this basic mechanism could occur in an oligomeric form of the transporter [27]. Whatever the details of the conformational change associated with transport catalysis, results presented here lead to the suggestion that a minimum structure required for this process is an extension to residues 467 and 468, although these residues are not individually essential.

Katagiri et al. [28] have reported that substitution of the C-terminal 39 amino acids of GLUT1 by the corresponding region of GLUT2 results in glucose transport kinetics that resemble those of the GLUT2 isoform. The data presented here allow

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                    454              468
human GLUT1;   FKVPE/TKGRTFDEIASGFR//QGG
rabbit GLUT1;  ...../..............//...
human GLUT2;   ...../...KS.E...AE.Q//KKS
rat GLUT2;     ...../...KS.....AE../KKS
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Figure 5 C-terminal sequences of GLUT1 and GLUT2 in the critical region around residues Phe-467 and Arg-48

further interpretation of these results. The C-terminal sequences of GLUT1 and GLUT2 in the critical region around residues Phe-467 and Arg-468 are as shown in Figure 5.

The chimeric protein is rabbit GLUT1 up to Glu-454 and then has the sequence of human GLUT2. The truncations of GLUT1 that have no effect on function occur after Arg-468. Apart from conservative substitutions, the major difference between the wild-type GLUT1 and the GLUT1/GLUT2 chimeric protein occurs at the SG residues in GLUT1 which are AE in the chimeric protein. In order to test whether the possibility that the substitution of a neutral residue by an acidic residue at position 466 could be responsible for the high K_m and high $V_{max.}$, a GLUT1 G466 → E point mutation was constructed. However, this mutation did not produce a high K_m phenotype. This leaves the possibility that residues of GLUT2 that correspond to the deleted 24 amino acids of GLUT1 may be involved. If this effect occurs then it may be due to the additional basic residues, Lys-Lys, occurring after Phe-467 and Arg-468. Consistent with this possibility we note that replacing the basic Arg-468 by leucine and truncation of Arg-468 (the CTD 25–CTD27 mutants) produces a small but consistent reduction in the K_m for transport. The basic arginine residue, Arg-468, may become less critical when Lys-Lys occurs in this C-terminal region as Gln can substitute for Arg in human GLUT2. An alternative explanation for the high K_m phenotype may be that the increased length of the GLUT2 C-terminus beyond positions 467 and 468 could be

required for producing an altered packing of the inner site with a consequent lowering in affinity for substrates.

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