Possible domains responsible for intracellular targeting and insulin-dependent translocation of glucose transporter type 4

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Translocation of the type 4 glucose transporter (GLUT4) to the cell surface from an intracellular pool is the major mechanism of insulin-stimulated glucose uptake in insulin-target cells. We developed a highly sensitive and quantitative method to detect GLUT4 immunologically on the surface of intact cells, using c-myc epitope-tagged GLUT4 (GLUT4myc). We constructed c-myc epitope-tagged glucose transporter type 1 (GLUT1myc) and found that the GLUT1myc was also translocated to the cell surface of Chinese hamster ovary cells, 3T3-L1 fibroblasts and NIH 3T3 cells, in response to insulin, but the degree of translocation was less than that of GLUT4myc. Since GLUT1 and GLUT4 have different intracellular distributions and different degrees of insulin-stimulated translocation, we examined the domains of GLUT4, using c-myc epitope-tagged chimeric glucose transporters between these two isoforms. The results indicated that, (1) all the cytoplasmic N-terminal region, middle intracellular loop and cytoplasmic C-terminal region of GLUT4 have independent intracellular targeting signals, (2) these sequences for intracellular targeting of GLUT4 were not sufficient to determine GLUT4 translocation in response to insulin, and (3) the N-terminal half of GLUT4 devoid both of cytoplasmic N-terminus and of middle intracellular loop seems to be necessary for insulin-stimulated GLUT4 translocation.

INTRODUCTION

Insulin is a hormone that is required for the regulation of blood glucose levels [1]. The molecular mechanisms involved in insulin effects need to be closely examined to determine the cause of non-insulin-dependent diabetes mellitus [2]. Glucose uptake by cells is influenced mainly by the number of glucose transporters present on the cell surface and possibly by their intrinsic activities [3]. The type of glucose transporters (there are at least seven) and cellular factors also have effects on glucose uptake [3–6]. The type 4 glucose transporter (GLUT4) is exclusively expressed in muscle and fat cells and has been reported to be rapidly translocated to the cell surface from an intracellular pool, in response to insulin [3–9]. The translocation of GLUT4 is thought to be a major mechanism of insulin-stimulated glucose uptake in these cells [3–9]. Insulin also stimulates glucose uptake in cells which do not express GLUT4 but do have the type 1 glucose transporter (GLUT1) [10–17]. However, there are conflicting reports as to whether GLUT1 is translocated to the cell surface in response to insulin [14–18]. Since conventional approaches used to measure the translocation leave much to be desired, we developed a highly sensitive and quantitative method to detect immunologically the translocation of GLUT4 in intact cells using c-myc epitope-tagged GLUT4 (GLUT4myc) [19]. We now report construction of c-myc epitope-tagged GLUT1 (GLUT1myc). We inserted the c-myc 14 amino acid sequence into the first external loop, without disrupting the subcellular localization or function of GLUT1. We found that the GLUT1myc translocated in response to insulin in various species of cells but that the degree of GLUT1myc translocation was less than that of GLUT4myc.

When GLUT4 and GLUT1 were expressed exogenously in some cells, the majority of GLUT4 was sequestered into an intracellular region but GLUT1 was located predominantly on the cell surface [15,17,20–28]. However, analyses to identify the different sorting signals between GLUT1 and GLUT4 produced contradicting results [22–28]. In addition, it was not clear whether the intracellular targeting domains determine the insulin-stimulated translocation of GLUT4, because precise and highly-sensitive methods to estimate the translocation were not available.

Here, we report that the domain responsible for the insulin-stimulated translocation of GLUT4 differs from the domains that determine the intracellular targeting of GLUT4. We examined the translocation of chimeric c-myc-tagged glucose transporters in which each reciprocal region between GLUT1 and GLUT4 was exchanged and identified the domains responsible for intracellular targeting and the possible domain required for insulin-dependent translocation of GLUT4.

EXPERIMENTAL

Cells and materials

Chinese hamster ovary (CHO) cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum (FCS; Gibco/BRL), while NIH3T3 cells, 3T3-L1 fibroblasts and COS-7 cells were grown in Dulbecco's modified essential medium supplemented with 10% FCS. Mouse monoclonal antibodies to the human c-myc epitope of 14 amino acids (9E10) [29] and to the rat insulin-regulatable glucose transporter, GLUT4 (1F8) [30], were purchased from Oncogene Science Inc. and Genzyme respectively. A mouse antiserum against the C-terminal 12 amino acids of GLUT4 and a monoclonal antibody to the C-terminal

Abbreviations used: GLUT4, glucose transporter type 4; GLUT1, glucose transporter type 1; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; HIR, human insulin receptor; FCS, fetal calf serum.

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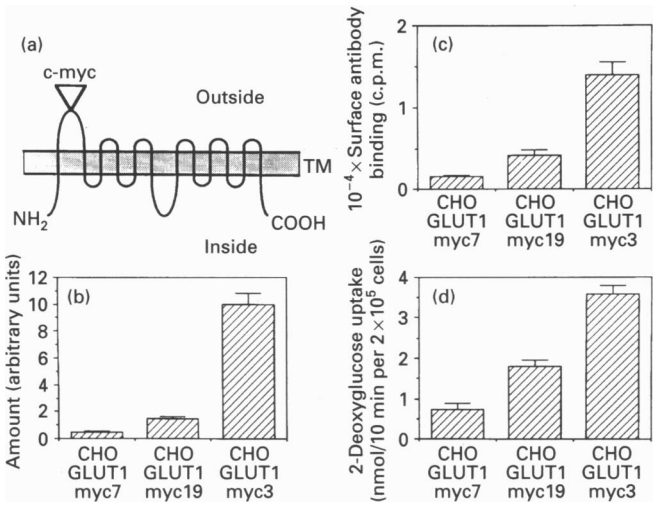

Figure 1 Relationship among levels of total GLUT1myc (b), surface GLUT1myc (c) and 2-deoxyglucose uptake (d) in CHO cells

(**a**) Construction of GLUT1myc. The c-myc epitope is inserted in the first extracellular region. TM indicates transmembrane regions. The cytoplasmic side of the membrane faces downwards. (**b**) CHO cells expressing various levels of GLUT1myc (CHO.GLUT1myc7, CHO.GLUT1myc19 and CHO.GLUT1myc3) were labelled with [^{35}S]Met and immunoprecipitated with a monoclonal antibody specific to c-myc epitope (9E10), as described in the Experimental section. The quantities of total immunoprecipitated GLUT1myc were measured by a Bio-image-analyzer BAS 2000. (**c**) The quantities of surface GLUT1myc in these cells were measured by the anti-c-myc antibody-binding assay, as described in the Experimental section. (**d**) Glucose uptake of these cells was measured and represented after subtracting that of parent CHO cells (1.68 nmol/10 min per 10^5 cells), as described in the Experimental section.

15 amino acids of GLUT1 (MAb 37-4) were prepared as described by other workers [9–11,31]. ^{125}I-Labelled goat anti-(mouse IgG) was purchased from ICN. All other reagents were of analytical grade.

Construction of c-myc epitope-tagged glucose transporters

The cDNA encoding rat brain GLUT1 was kindly provided by Dr. M. J. Birnbaum (Harvard Medical School, MA, U.S.A.) [11]. The c-myc epitope-tagged GLUT1 (GLUT1myc) was prepared and used for the construction of GLUT4myc, as described previously [19]. The GLUTmyc has the 14 amino acid sequence of human c-myc epitope (Ala-Glu-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Leu-Lys) [29] inserted between Ile-56 and Pro-57, in the predicted first exofacial loop (Figure 1a). Various chimeric glucose transporters between rat GLUT1myc and rat GLUT4myc were constructed by exchanging the reciprocal domains of the cDNAs [9,11] at appropriate restriction enzyme sites. The restriction enzyme sites were either endogenous or newly created by PCR-based mutagenesis [32,33], such that the amino acid sequences were not altered, except for creating a *Bgl*II site at Ile-461–Ala-462 in GLUT1 (Ala-462 was replaced with Ser). The first series of chimera was constructed by exchanging the cytoplasmic N-termini, middle intracellular loops and cytoplasmic C-termini between GLUT1myc and GLUT4myc. Endogenous restriction enzyme sites *Bam*HI at Gly-22–Ser-23 in GLUT1 and *Bgl*II at Ile-479–Ser-480 in GLUT4, and newly created restriction enzyme sites *Bam*HI at Gly-34–Ser-35 in GLUT4 and *Bgl*II at Ile-461–Ala-462 (Ala-462 was replaced with Ser) in GLUT1 were used to switch their cytoplasmic N-termini or C-termini. To construct chimeric transporters, exchanged with the other middle intracellular loops between 6th and 7th transmembrane regions, *Nru*I sites were introduced at Ser-210–Arg-269 in GLUT1myc by deleting Pro-211–Tyr-268 of GLUT1 (GLUT1mycΔL) and at Ser-226–Arg-285 in GLUT4myc by deleting Pro-227–Tyr-284 of GLUT4 (GLUT4mycΔL) with no changes in amino acid sequences. Finally, the fragments coding Pro-227–Tyr-284 of GLUT4 and Pro-211–Tyr-268 of GLUT1 were created by PCR and inserted into the *Nru*I sites of GLUT1mycΔL and GLUT4mycΔL respectively. The constructions of the chimeras (see Figure 4a) are as follows: GLUT1mycN, GLUT4(1–34)/GLUT1myc(23–492); GLUT1mycL, GLUT1myc(1–210)/GLUT4(227–284)/GLUT1-(269–492); GLUT1mycC, GLUT1myc(1–461)/GLUT4(480–509); GLUT1mycNC, GLUT4(1–34)/GLUT1myc(23–461)/GLUT4(480–509); GLUT1mycNLC, GLUT4(1–34)/GLUT1-myc(23–210)/GLUT4(227–284)/GLUT1(269–461)/GLUT4-(480-509); GLUT4mycN, GLUT1(1–22)/GLUT4myc(35–509); GLUT4mycL, GLUT4myc(1–226)/GLUT1(211–268)/GLUT4-(285–509); GLUT4mycC, GLUT4myc(1–479)/GLUT1(462–492).

The second series of chimera was constructed by exchanging the other halves between GLUT1myc and GLUT4myc. GLUT1mycΔL and GLUT4ΔL were exchanged with the other halves at each *Nru*I site. The resulting chimeric transporters were designated GLUTmyc14ΔL, GLUT1myc(1–210)/GLUT4(285–509) and GLUTmyc41ΔL, GLUT4myc (1–226)/GLUT1(269–492). Finally, the fragment coding the middle intracellular loop of GLUT4(Pro-227–Tyr-284) was inserted into the *Nru*I sites of GLUTmyc14ΔL or GLUTmyc41ΔL. The constructions of the chimeras (see Figure 6a) are as follows: GLUTmyc144, GLUT1myc(1–210)/GLUT4(227–509); GLUT-myc441, GLUT4myc(1–284)/GLUT1(269-492). All of the constructions were confirmed by sequencing through the changed areas. The cDNAs were subcloned into the mammalian expression vector pcDL-SRα [34]. These plasmids were transiently expressed in COS-7 cells by the LIPOFECTIN Reagent (GIBCO/BRL) according to the manufacturer's protocol, and identified by immunoprecipitation, using anti-c-myc, anti-GLUT1 and anti-GLUT4 antibodies. The plasmids were also transfected with pSV2neo by calcium phosphate precipitation into the various cell lines. Many independent clones stably expressing the glucose transporters were selected by cell-surface anti-c-myc antibody-binding assay or immunoprecipitation using antibodies to GLUT1 and GLUT4, as described below.

Cell-surface anti-c-myc antibody-binding assay

Cells in 24-well plates were incubated in KRH buffer (136 mM NaCl/4.7 mM KCl/1.25 mM $MgSO_4$/1.25 mM $CaCl_2$/20 mM Hepes/2 mg/ml BSA) for 30 min at 37 °C and then treated with the indicated concentrations of insulin, phorbol 12-myristate 13-acetate (PMA) or NaF in KRH buffer for 30 min at 37 °C. They were washed with ice-cold KRH buffer and the quantities of cell surface glucose transporters were detected with the anti-c-myc antibody and ^{125}I-labelled goat anti-(mouse IgG), as described previously [19].

Cell labelling and immunoprecipitation

Cells were metabolically labelled for 4 h in 0.15 mCi/ml Tran ^{35}S-label (ICN) and lysed with lysis buffer (50 mM Hepes, pH 7.5/150 mM NaCl/1% Triton X-100/20 μM *p*-amidinophenylmethanesulphonyl fluoride hydrochloride/1 mg/ml bacitracin/5 mM EDTA/5 mM EGTA/20 mM sodium pyrophosphate/1 mM orthovanadate/20 mM NaF). Cell lysates were immunoprecipitated with the anti-c-myc, anti-GLUT1 or anti-GLUT4 antibodies. The samples were extensively washed, re-

solved by 10% SDS/PAGE and then fluorographed, as described previously [35]. The quantities of glucose transporters were measured by a Bio-image-analyzer BAS 2000 (FUJI Film Institution).

Immunofluorescence microscopy

CHO cells stably expressing specific glucose transporters were grown in 35-mm tissue culture dishes. The cells were washed once with PBS (137 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4, pH 7.4), fixed in 2% paraformaldehyde/PBS at room temperature for 20 min and washed three times with PBS. The fixed cells were then treated with 100 mM glycine/PBS at room temperature for 15 min and permeabilized with 0.2% Triton X-100/PBS at room temperature for 15 min. After an extensive wash in PBS, the cells were incubated with 5% non-fat dry milk/PBS at room temperature for 1 h, and then incubated with the anti-c-myc, anti-GLUT1 or anti-GLUT4 antibodies (each 1:20 dilution with 5% non-fat dry milk/PBS) at 37 °C for 1 h. The cells were further incubated with fluorescein isothiocyanate-conjugated goat anti-(mouse IgG) at 37 °C for 1 h, washed five times with 0.05% Tween 20/PBS at room temperature for 5 min each and visualized under a microscope.

Sucrose density gradient analysis

Sucrose density gradient analyses of CHO cells stably expressing GLUT1myc, GLUT4myc, GLUT1 and GLUT4 were carried out according to the method of Haney et al. [20]. The cells of ten confluent 90 mm dishes were scraped, homogenized in 0.9 ml of HES buffer (20 mM Hepes/250 mM sucrose/1 mM sodium EDTA) and loaded onto 8.7 ml of 15–37% (w/v) sucrose gradients. The gradients were centrifuged overnight at 100000 *g* at 4 °C in a swing rotor (type RPS 40T; Hitachi Co., Ltd.). The samples were fractionated into 19 aliquots (0.5 ml each) from top to bottom of the tubes. The fractions were subjected to 10% SDS/PAGE, transferred to nitrocellulose paper and probed with the anti-c-myc, anti-GLUT1 or anti-GLUT4 antibodies. The protein bands were detected with ^{125}I-labelled goat anti-(mouse IgG) and the radioactivities of the bands were quantified by a Bio-image-analyzer BAS 2000.

Assay of 2-deoxyglucose uptake

Cells in 24-well plates, either treated with 100 nM insulin for 10 min or not treated with insulin, were incubated with 0.1 mM 2-deoxy-D-[1,2-^{3}H]glucose (Du Pont NEN Products) for 10 min at 37 °C, washed in ice-cold KRH buffer containing 0.1 mM phloretin and solubilized with 0.05% SDS. The radioactivity was measured as described in our previous reports [36,37].

RESULTS

Expression of GLUT1myc in CHO cells without disrupting the GLUT1 functions

We inserted the synthetic oligonucleotides of c-myc epitope (14 amino acids) into the first external loop of GLUT1 cDNA to detect GLUT1 on the cell surface, directly and immunologically (Figure 1a). The CHO cells were transfected with an expression vector of the c-myc epitope-tagged GLUT1 (GLUT1myc) and pSV2neo. Cell clones resistant to G418 were assayed for the surface binding of anti-c-myc antibody. We obtained many clones of CHO cells stably expressing various levels of GLUT1myc (CHO.GLUT1myc). Three independent clones (CHO.GLUT1myc7, -myc19 and -myc3), which showed different degrees of surface binding of anti-c-myc antibody (Figure 1c), were used for further experiments. To examine the total amount of GLUT1myc, the GLUT1myc expressed in CHO cells was labelled with [^{35}S]Met, immunoprecipitated with a monoclonal anti-c-myc antibody (9E10) and subjected to 10% SDS/PAGE. Total GLUT1myc proteins, which were electrophoresed around M_r 55000, were quantified in a Bio-image-analyzer (Figure 1b). The immunoprecipitation with anti-GLUT1 antibody (MAb37-4) gave almost the same results (not shown). The amount of surface GLUT1myc was determined by anti-c-myc antibody binding (Figure 1c). According to the total GLUT1myc protein levels, the amount of surface GLUT1myc increased, though the surface GLUT1myc levels did not strictly reflect the total GLUT1myc levels (Figures 1b and 1c). The rates of basal glucose uptake of these clones after subtracting that of parent CHO cells, were almost in proportion to the counts of surface binding of anti-c-myc antibody (Figure 1d). These results indicate that the surface GLUT1myc takes up glucose in accordance with GLUT1myc levels on the cell surface and that the c-myc epitope insertion did not disrupt the function of glucose uptake of GLUT1.

Next, we examined the effects of the c-myc epitope insertion on subcellular distributions, using sucrose density gradient and immunofluorescence microscopy (Figure 2). Total cell homogenates of CHO cells expressing high levels of GLUT1myc (CHO.GLUT1myc3), GLUT4myc (CHO.GLUT4myc14), GLUT1 or GLUT4 were layered onto 15–37% linear sucrose gradients and centrifuged to equilibrium. Gradient fractions were collected and immunoblotted with anti-c-myc epitope (9E10), anti-GLUT1 (MAb 37-4) or anti-GLUT4 (1F8) antibodies respectively. The blotting pattern was visualized for radioactivity, measured using a Bio-image-analyzer BAS 2000. As shown in Figure 2(a), both GLUT1myc and GLUT1 were concentrated in high density fractions 14–16 (mean density 32%) in each gradient examined ($n = 3$). This presumably reflects the distribution of plasma membrane in these gradients [20,21]. The shoulder seen around fraction number 11 of GLUT1myc and GLUT1 may relate to the presence of a small amount of GLUT1myc and GLUT1 in an intracellular region [Figure 2a (I) and (II)]. In contrast, both GLUT4myc and GLUT4 were in the low density fractions 10–12 (mean density 27%) in each gradient examined ($n = 3$). These fractions were thought to correspond to intracellular microsome fractions [20,21]. A shoulder was also seen around fraction number 14 of GLUT4myc and GLUT4 [Figure 2a (III) and (IV)]. Some of the exogenously expressed GLUT4myc or GLUT4 seemed to distribute to the plasma membrane. The subcellular distributions of GLUT1myc and GLUT4myc were also examined by immunofluorescence microscopy, using the anti-c-myc antibody (Figure 2b). The plasma membrane of CHO cells expressing GLUT1myc (CHO.GLUT1myc3) was labelled most prominently, but weak staining was observed in the cytoplasm. This staining pattern was similar to GLUT1 labelled with an antibody against the GLUT1. On the other hand, GLUT4myc (CHO.GLUT4myc14) was localized predominantly within cytoplasmic structures. The plasma membrane was also stained, albeit weakly. The staining was concentrated in a discrete perinuclear region and was also observed in a punctuate pattern throughout the cytoplasm. The staining pattern was similar to GLUT4 labelled with the antibody against GLUT4. The subcellular distributions of these glucose transporters are consistent with findings in previous reports [20,21]. Taken together with the above results, the c-myc epitope insertion in the first external loop of GLUT1 did not affect the immunoreactivity to the anti-GLUT1 antibody, the glucose transport activity or the subcellular distribution. Therefore, we

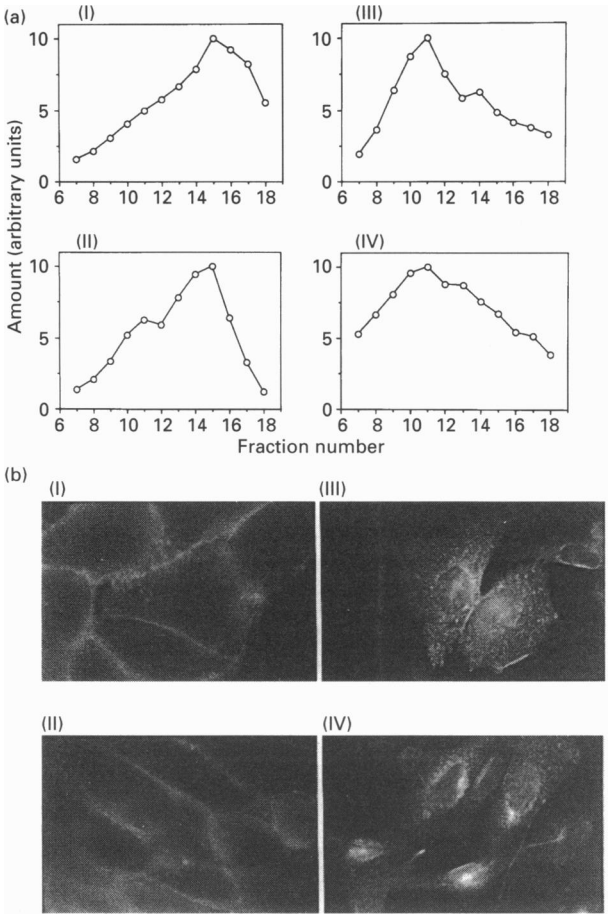

Figure 2 Subcellular localization of GLUT1myc and GLUT4myc in CHO cells

(**a**) Sucrose density gradient analysis of CHO cells expressing (I) GLUT1myc (CHO.GLUT1myc3), (II) GLUT1, (III) GLUT4myc (CHO.GLUT4myc14) or (IV) GLUT4. The cell homogenates were layered onto a 15–37% sucrose gradient. After centrifugation to equilibrium, gradient fractions were collected and subjected to 10% SDS/PAGE and immunoblotting using anti-c-myc for (I) and (III), anti-GLUT1 for (II) or anti-GLUT4 for (IV) antibodies. The radioactivities of the protein bands were quantified using a Bio-image-analyzer BAS 2000. The numbers below the panels represent the fractions collected beginning from the top of the gradient tube. (**b**) Immunohistochemical localization of GLUT1myc (CHO.GLUT1myc3), GLUT1, GLUT4myc (CHO.GLUT4myc14) and GLUT4 in CHO cells. The cells were grown in a 35 mm dish and processed for immunofluorescence microscopy, as described in the Experimental section. (I) CHO cells overexpressing GLUT1myc (CHO.GLUT1myc3) were visualized with use of the anti-c-myc antibody, (II) CHO cells overexpressing GLUT1 were visualized with use of the anti-GLUT1 antibody, (III) CHO cells overexpressing GLUT4myc (CHO.GLUT4myc14) were visualized with the anti-c-myc antibody and (IV) CHO cells overexpressing GLUT4 were visualized with the anti-GLUT4 antibody. Under these conditions, normal mouse serum exhibited almost no immunofluorescence in the cells. Control CHO cells had almost no immunofluorescence with the anti-c-myc or anti-GLUT4 antibodies but had a weak staining on the plasma membrane with the anti-GLUT1 antibody.

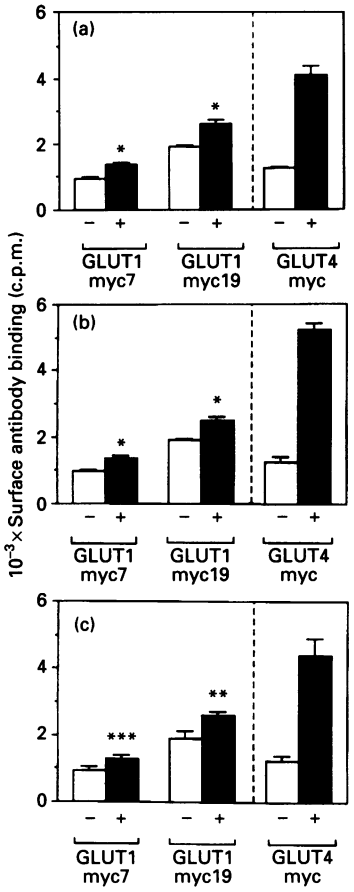

Figure 3 Translocation of GLUT1myc and GLUT4myc by insulin (a), PMA (b) and NaF (c) in CHO cells

CHO.GLUT1myc7, CHO.GLUT1myc19 and CHO.GLUT4myc were incubated, with (+) or without (−) 100 nM insulin (**a**), 1 mM PMA (**b**) and 10 mM NaF (**c**) for 30 min at 37 °C, and then subjected to the anti-c-myc antibody-binding assay, as described in the Experimental section. Values represent means ± S.E. of six determinations. Asterisks show significant difference (* $P < 0.001$; ** $P < 0.01$; *** $P < 0.05$) from values obtained with no stimulation.

used GLUT1myc to evaluate the translocation of GLUT1 in various cells.

Insulin-, PMA- and NaF-dependent translocation in CHO cells

Insulin [3–9] stimulates glucose transport in various cell lines. In muscle and fat cells, GLUT1 as well as GLUT4 was translocated to the cell surface in response to insulin [38–41]. However, it is ambiguous that GLUT1 is translocated to the cell surface in cells other than muscle and fat cell, in response to insulin. There have been contradictory reports on insulin-stimulated GLUT1 translocation, using sucrose density gradient analysis [20], immunofluorescence microscopy [15,20], chemical labelling such as cytochalasin B and 2-*N*-[4-(1-azi-2,2,2,-trifluoroethyl)-benzoyl]-1,3-bis-(D-mannose-4-yloxy)-2-propylamine [15,16,42] or an exofacial antibody to GLUT1 [17], probably because these methods are not adequate to evaluate the translocation, sensitively and quantitatively. We first examined the effect of insulin on the translocation of GLUT1myc in CHO cells. This method can assess exogenous GLUT1myc, independently of endogenous glucose transporters, and can be used for almost all cells by transfection. As shown in Figure 3(a), insulin (100 nM) increased the number of GLUT1myc on the cell surface by 1.4–1.5-fold (translocation). On the other hand, the number of GLUT4myc on the cell surface was increased 3.3-fold in response to 100 nM insulin. The degree of translocation between GLUT1myc and GLUT4myc was different in CHO cells. As shown in Figure 2, most of the exogenously expressed GLUT1myc was localized at the plasma membrane, but some seemed to be sequestered in an intracellular region. The intracellular GLUT1myc was translocated by insulin stimulation. Insulin-stimulated translocation of GLUT1myc and GLUT4myc was also observed in 3T3-L1

Table 1 Translocation of GLUT1myc and GLUT4myc by insulin in CHO cells, NIH3T3 cells and 3T3-L1 fibroblasts

NIH3T3 cells and 3T3-L1 fibroblasts were transfected with mammalian expression plasmids, SRα-GLUT1myc or SRα-GLUT4myc with pSV2neo. Several independent clones stably expressing GLUT1myc or GLUT4myc were established and examined for anti-c-myc antibody binding after treatment with (+) or without (−) 100 nM insulin for 30 min at 37 °C, as described in the Experimental section. The radioactivities (c.p.m.) obtained from one clone, with basal (without insulin) antibody bindings almost the same, are shown here, because other clones gave almost the same results. For CHO cells, the data obtained from the CHO.GLUT1myc7 and CHO.GLUT4myc33 are shown in Figure 3(a). Values represent means ± S.E. ($n = 3$–6). Fold-increases over basal values (without insulin) are also shown.

		CHO	NIH3T3	3T3-L1 fibroblast
GLUT1myc	Ins(−)	940 ± 23	1914 ± 127	1672 ± 86
	Ins(+)	1369 ± 58	3079 ± 341	2157 ± 46
	Translocation (fold-increase)	1.5	1.6	1.3
GLUT4myc	Ins(−)	1246 ± 30	2221 ± 252	1053 ± 18
	Ins(+)	4093 ± 264	6790 ± 234	3550 ± 29
	Translocation (fold-increase)	3.3	3.1	3.4

fibroblasts and NIH3T3 cells (Table 1); more GLUT4myc was translocated to the cell surface than GLUT1myc in these cells. Therefore, the ubiquitously-expressed GLUT1 translocates in response to insulin in several cell lines. The insulin-stimulated translocation of GLUT1 could play an important role in insulin-stimulated glucose transport in various species of cell other than muscle and fat cells.

We next examined the effect of PMA and NaF on GLUT1myc translocation in CHO cells, because PMA [41,43] and NaF [44] as well as insulin cause GLUT4myc translocation [19]. PMA and NaF stimulated the GLUT1myc translocation by 1.3–1.4-fold, as shown in Figures 3(b) and 3(c). These data suggest the involvement of protein kinase C and GTP-binding protein(s) [45] in GLUT1 translocation as well as GLUT4 translocation.

When we overexpressed the human insulin receptor (HIR) in the CHO.GLUT1myc cells, the sensitivity of GLUT1myc translocation in response to insulin increased, but the maximum increase of GLUT1myc translocation was unchanged (results not shown). This means that the endogenous insulin receptors in CHO cells are sufficient to fully translocate the GLUT1myc in the presence of 100 nM insulin and we used CHO cells instead of CHO–HIR cells to examine differences between GLUT1 and GLUT4.

Construction of c-myc epitope-tagged chimeric glucose transporters exchanging the cytoplasmic N-termini, middle intracellular loops and cytoplasmic C-termini between GLUT1 and GLUT4

As shown in Figure 2, exogenous expression of GLUT1 and GLUT4 in some cell lines indicated that GLUT1 was prominently targeted to the plasma membrane and that GLUT4 was mainly sequestered into an intracellular compartment [15,16,20,21]. To identify the different sorting signals between GLUT1 and GLUT4, other researchers constructed chimeric transporters in which reciprocal domains were exchanged between the two isoforms [22–28]. However, analyses of the subcellular distributions of these chimeras revealed various results. The c-myc epitope-tagged chimeric glucose transporters seem to be useful to identify the primary sequences responsible for subcellular distributions and insulin-stimulated translocation of GLUT1 and GLUT4. These different observations may relate to the different expression systems or assay systems used. In addition, we found no documentation concerning the GLUT4 domain which determines the insulin-stimulated translocation of GLUT4.

We constructed chimeric glucose transporters (similar to those of other researchers) in which reciprocal domains conferring intracellular sequestration were exchanged between the two isoforms [22–28]). In addition, we also constructed transporters in which the middle intracellular loops were exchanged, because the middle intracellular loops as well as the cytoplasmic N-terminus and the cytoplasmic C-terminus are divergent between GLUT1 and GLUT4. Combined chimeras carrying the cytoplasmic N-terminus, middle loop and C-terminus were also prepared because these complicated conformations could confer signals of intracellular targeting or insulin-stimulated translocation. The precise recombination sites are shown in Figure 4(a) and are described in the Experimental section. To confirm the recombinant construction, these c-myc-tagged chimeric transporters were expressed transiently in COS-7 cells and the ^{35}S-labelled transporters were immunoprecipitated with anti-c-myc (9E10), anti-GLUT1 C-terminus (MAb 37-4) or anti-GLUT4 C-terminus (1F8) antibodies. As shown in Figure 4(b), all the c-myc-tagged chimeric transporters were detected around M_r 55000 by the anti-c-myc antibody, and GLUT4 was electrophoresed somewhat more slowly than GLUT1 because of the longer N-terminus. The transporters containing the N-terminus of GLUT4 (GLUT4myc, GLUT1mycN, GLUT1mycNC, GLUT1mycNLC, GLUT4mycL and GLUT4mycC) were electrophoresed somewhat slower than transporters containing the N-terminus of GLUT1 (GLUT1myc, GLUT1mycL, GLUT1mycC and GLUT4mycN). Transporters with the C-terminus of GLUT1 (GLUT1myc, GLUT1mycN, GLUT1mycL and GLUT4mycC) were detected using the anti-GLUT1 C-terminus antibody but not the anti-GLUT4 C-terminus antibody. On the other hand, the transporters possessing the C-terminus of GLUT4 (GLUT4myc, GLUT1mycC, GLUT1mycNC, GLUT1mycNLC, GLUT4mycN and GLUT4mycL) were detected using the anti-GLUT4 C-terminus antibody but not the anti-GLUT1 C-terminus antibody. These observations showed that the chimeric transporters were properly constructed, based on their transient expression in COS-7 cells, although the levels of expression of these chimeric transporters differed because of different transfection efficiencies in each experiment. We then expressed stably the chimeric glucose-transporters in CHO cells, because GLUT1myc and GLUT4myc showed different subcellular localizations and different insulin-stimulated translocations in CHO cells, as in 3T3-L1 adipocytes [21,38,39] (Figures 2 and 3).

Subcellular distribution and insulin-stimulated translocation of the chimeric glucose transporters exchanging the cytoplasmic regions between GLUT1 and GLUT4 in CHO cells

These chimeric transporters were expressed stably in CHO cells, and several independent clones (at least four clones) for each chimera, whose expression levels were different, were established and analysed to avoid clonal deviations. As shown in Figure 5, we examined their cellular distributions by immunofluorescence in cells expressing at high levels each chimeric glucose transporter (see Table 2, upper half) because it is difficult to identify by immunofluorescence the precise locations of chimeras in cells expressing low levels of the chimeras. The total levels of expression of chimeras were determined by the amount of ^{35}S-labelled chimeric glucose transporters immunoprecipitated with

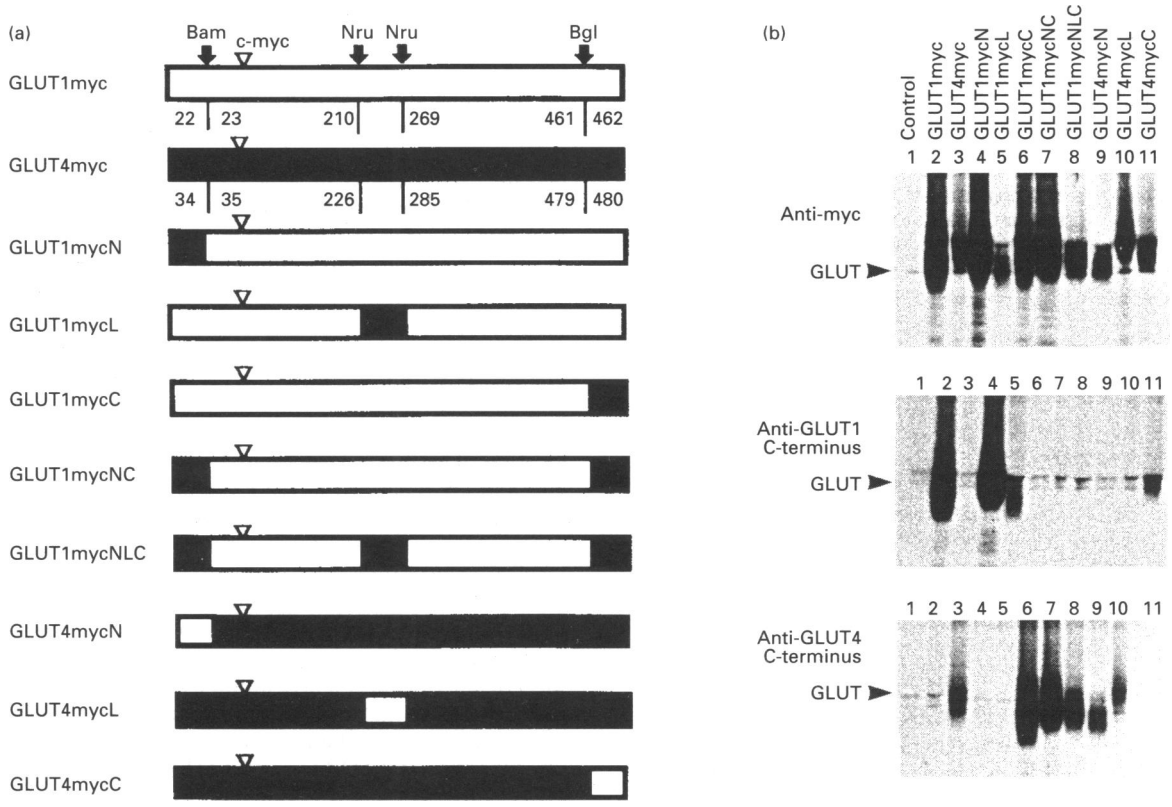

Figure 4 Construction (a) and expression (b) of chimeric transporters exchanging the cytoplasmic N-termini, middle intracellular loops and cytoplasmic C-termini between GLUT1 and GLUT4 in COS-7 cells

(**a**) Structures of the chimeric glucose transporters. The open and solid boxes represent rat GLUT1 [11] and rat GLUT4 [9] sequences respectively. The triangle indicates the inserted c-myc 14 amino acids epitope. The divergent cytoplasmic N-terminal regions, middle intracellular loops and cytoplasmic C-terminal regions of the two glucose transporters were exchanged with other reciprocal regions. The restriction enzyme sites used for the chimera construction are shown [Bam, *Bam*HI sites at Gly-22–Ser-23 in GLUT1, or Gly-34–Ser-35 in GLUT4; Bgl, *Bgl*II sites at Ile-461–Ala-462 (the Ala-462 was replaced with Ser) in GLUT1, or Ile-479–Ser-480 in GLUT4; Nru, *Nru*I sites introduced by deleting the middle intracellular loops, Pro-211–Tyr-268 of GLUT1 (GLUT1mycΔL) and Pro-227–Tyr-284 of GLUT4 (GLUT4mycΔL)]. The deleted fragments of GLUT1 and GLUT4 were created with PCR and inserted into the *Nru*I sites of GLUT4mycΔL and GLUT1mycΔL respectively, as described in the Experimental section. The names of the chimeric glucose transporters are given on the left. (**b**) Chimeric glucose transporters in COS-7 cells. COS-7 cells transiently expressing each chimera (lanes 2–11) were labelled with [^{35}S]Met and immunoprecipitated with the anti-c-myc monoclonal antibody (anti-myc, upper panel), the anti-C-terminus of GLUT1 monoclonal antibody (anti-GLUT1 C-terminus, middle panel) or the anti-C-terminus of GLUT4 monoclonal antibody (anti-GLUT4 C-terminus, lower panel), as described in the Experimental section. COS-7 cells transfected with pcDL-SRα vector plasmid only were used as a control (lane 1). The locations of glucose transporters (GLUT, around M_r 55000) are indicated.

the anti-c-myc antibody. Figure 5 shows the typical clones expressing nearly equal levels of chimeric glucose transporters in the whole cell. The substitutions of N-terminus (GLUT1mycN), middle intracellular loop (GLUT1mycL) and C-terminus (GLUT1mycC) of GLUT1myc with those of GLUT4, changed the subcellular distributions in immunofluorescence microscopy. GLUT1mycN, GLUT1mycL, GLUT1mycC, GLUT1mycNC and GLUT1mycNLC showed almost the same intracellular distributions as GLUT4myc, but not as GLUT1myc. In addition, substitutions of the middle intracellular loop (GLUT4mycL) and C-terminus (GLUT4mycC) of GLUT4myc for those of GLUT1, showed an intracellular distribution similar to that of GLUT4myc. Although the GLUT4myc chimera with the cytoplasmic N-terminus substituted with that of GLUT1 (GLUT4mycN) was expressed transiently in COS-7 cells (Figure 4), we could not obtain CHO cells stably expressing this chimera; this chimeric protein may be unstable in CHO cells. These results indicate that the N-terminus, middle loop and C-terminus of GLUT4 have independent intracellular targeting signals.

Next, we examined the amount of chimeric transporters on the cell surface and glucose uptake in the same clones (Table 2, upper half). Although the chimera examined here were mainly targeted to an intracellular pool (Figure 5), some smaller portion of each chimera was expressed on the cell surface, as deduced from surface anti-c-myc antibody binding. All the cells expressing the chimeric glucose transporters took up more glucose than did the parent CHO cells. In addition, the rate of glucose uptake increased in proportion to the amount of surface transporter for each chimera (results not shown). These observations suggest that each chimera newly synthesized in the cell was folded properly, targeted to an intracellular region (main portion) and then moved to the cell surface (some portion) to transport glucose, as discussed by others [46]. However, a comparison of the amount of surface chimeric transporters with the rate of glucose uptake among different chimeras raises problems, as different chimeras could have different catalytic activities related to glucose transport.

We then examined the relationship between subcellular distribution and insulin-stimulated translocation. To evaluate insulin-stimulated translocation of the chimeric glucose transporters, we examined all clones (at least four clones for each chimera) for the amount of surface glucose transporters before

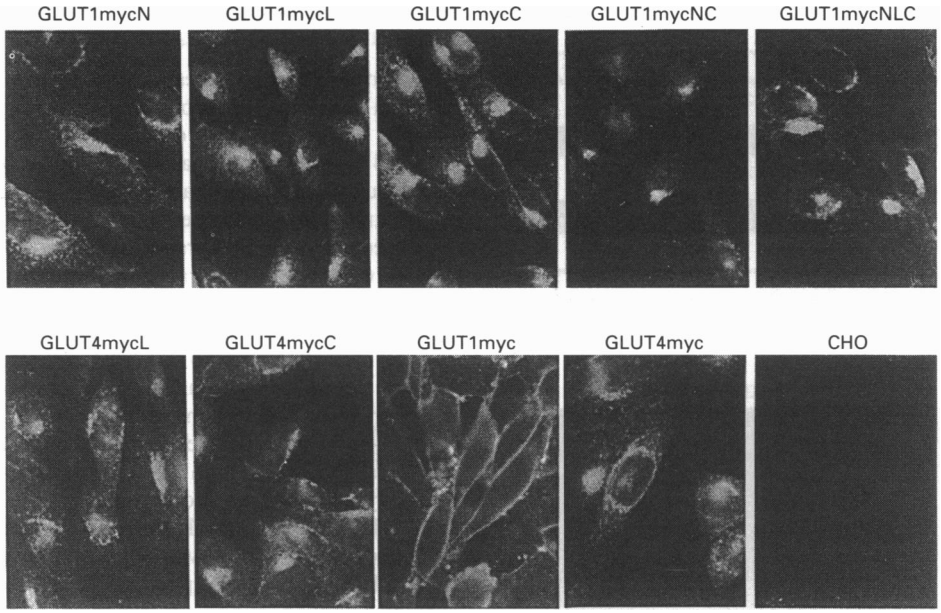

Figure 5 Subcellular distribution of the chimeric glucose transporters exchanging the cytoplasmic regions between GLUT1 and GLUT4 in CHO cells

Immunohistochemical localization of the chimeric glucose transporters (see Figure 4a) expressed in CHO cells. CHO cells expressing nearly equal levels of chimeric glucose transporters in whole cells, determined by the label of [^{35}S]Met (Table 2), were grown in 35 mm dishes and processed for immunofluorescence microscopy with the anti-c-myc antibody as described in the Experimental section.

Table 2 Functional expression and translocation of the chimeric transporters

	Glucose transporter										
	GLUT1 myc	GLUT4 myc	GLUT1 mycN	GLUT1 mycL	GLUT1 mycC	GLUT1 mycNC	GLUT1 mycNLC	GLUT4 mycL	GLUT4 mycC	GLUT myc144	GLUT myc441
Cells	Clone 3	Clone 14	Clone 40	Clone 70	Clone 79	Clone 152	Clone 56	Clone 121	Clone 4	Clone 23	Clone 48
Total expression level* (arbitrary unit)	9.06	10.07	6.80	7.19	12.08	8.81	6.81	9.13	6.65	7.22	3.00
Surface GLUTmyc† (c.p.m.)	6994	7089	6327	6342	7024	6671	5781	9010	8335	5272	6456
Glucose uptake‡ (nmol/10 min per 2×10^5cells)	3.58	2.53	2.70	2.95	4.10	2.34	1.17	3.74	3.69	2.91	0.51
Subcellular distribution§	Mem.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.	Cyt.
Cells	Clone 19	Clone 29	Clone 23	Clone 44	Clone 90	Clone 169	Clone 47	Clone 88	Clone 172	Clone 69	Clone 40
Total expression level* (arbitrary units)	1.96	2.03	1.50	2.63	2.36	2.29	2.48	2.81	0.82	2.74	0.64
SurfaceGLUTmyc‖											
Ins (−) (c.p.m.)	1931	1744	1449	2305	2201	1717	2173	2323	1159	1952	2090
Ins (+) (c.p.m.)	2781	4185	2130	2973	2927	2747	3194	5087	2723	2538	4661
Translocation¶ (fold-increase)	1.4	2.4	1.5	1.3	1.3	1.6	1.5	2.2	2.4	1.3	2.2

*Total expression level of the chimera was represented by the amount of ^{35}S-labelled chimeras immunoprecipitated with the anti-c-myc antibody.
† Surface GLUTmyc was represented as the surface binding of the anti-c-myc antibody in the absence of insulin.
‡ Glucose uptake of each chimera was measured using 2-deoxyglucose in the absence of insulin and was shown after subtracting that of parent CHO cells (1.68 nmol/10 min per 2×10^5 cells).
§ Subcellular distribution was examined by immunofluorescence as Figures 5 and 6(b). Cyt., mainly distributed in a cyoplasmic region; Mem., mainly distributed in the plasma membrane.
‖ Surface GLUTmyc was measured in the absence and presence of insulin.
¶ Translocation was shown as fold-increase over basal value (without insulin).

and after insulin treatment. As shown in Figure 3(a), it is not difficult to differentiate the degree of translocation between GLUT1myc and GLUT4myc in cells expressing transporters at low to middle levels. Table 2, lower half, shows the insulin-stimulated translocation of the typical clone expressing each chimera at low to middle levels. The substitutions of reciprocal domains of GLUT1myc by GLUT4 had little effect on their insulin-stimulated translocation in CHO cells. The GLUT1-mycN, GLUT1mycL, GLUT1mycC, GLUT1mycNC and GLUT1mycNLC showed almost the same extent of insulin-

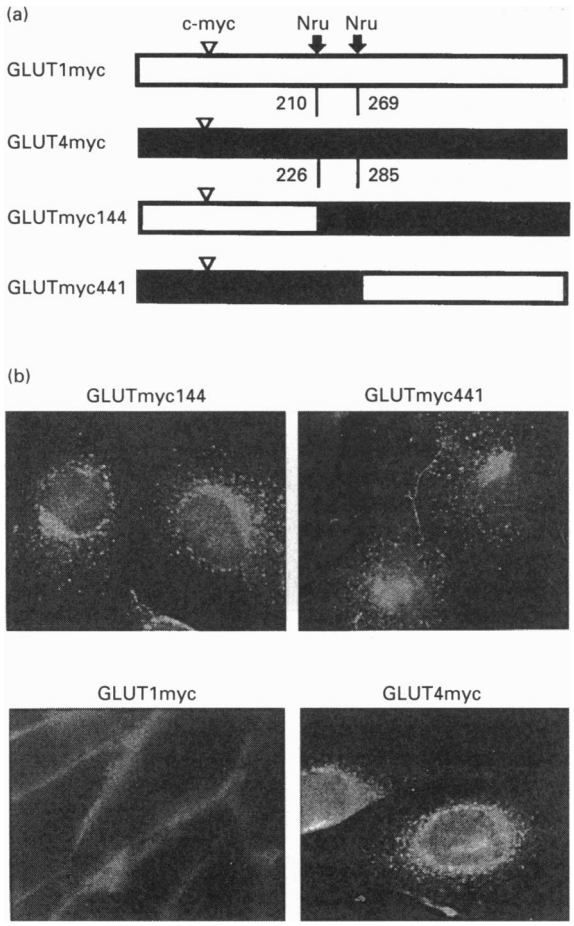

Figure 6 Construction (a) and subcellular distribution (b) of chimeric glucose transporters exchanging the other halves between GLUT1 and GLUT4 in CHO cells

(**a**) Structures of the chimeric transporters. The open and solid boxes represent rat GLUT1 [11] and rat GLUT4 [9] sequences respectively. The triangle indicates the inserted c-myc 14 amino acids epitope. Nru shows the restriction enzyme sites used for the chimera construction. The glucose transporters with the middle intracellular loops deleted, GLUT1mycΔL (GLUT1myc with Pro-211–Tyr-268 deleted, introduced an *Nru*I site at Ser-210–Arg-269) and GLUT4mycΔL (GLUT4myc with Pro-227–Tyr-284 deleted, introduced an *Nru*I site at Ser-226–Arg-285) were exchanged with the other half at their *Nru*I sites. The deleted fragment of GLUT4 coding Pro-227–Tyr-284 was inserted into their *Nru*I sites respectively, as described in the Experimental section. The names of the chimeric glucose transporters are given on the left. (**b**) Immunohistochemical localization of the chimeric transporters expressed in CHO cells. CHO cells expressing almost the same levels of chimeric transporters in whole cells, determined by the [^{35}S]Met label, were grown in 35 mm dishes and processed for immunofluorescence microscopy with the anti-c-myc antibody, as described in the Experimental section.

stimulated translocation as GLUT1myc (1.3–1.6-fold increase), although these chimeric transporters were targeted to an intracellular region as was GLUT4myc. Therefore, domains conferring intracellular GLUT4 sequestration, such as the cytoplasmic N-terminus, middle intracellular loop and cytoplasmic C-terminus, were not sufficient to ensure the full characteristics of GLUT4. On the other hand, GLUT4mycL and GLUT4mycC which were targeted to an intracellular region as was GLUT4myc, showed the same extent of insulin-stimulated translocation as GLUT4myc (2.2–2.4-fold increase). Thus, the middle intracellular loop or cytoplasmic C-terminus of GLUT4 is apparently not essential for insulin-stimulated GLUT4 translocation.

The c-myc epitope-tagged chimeric glucose transporters exchanging the other halves between GLUT1 and GLUT4

To determine the region responsible for insulin-stimulated translocation of GLUT4, we constructed two further chimeric transporters exchanging the other halves between GLUT1 and GLUT4 (Figure 6a) and analysed their subcellular distribution and insulin-stimulated translocation (Figure 6b, and Table 2, right-hand side), using the same procedure as described above. As expected, both chimeras GLUTmyc144 and GLUTmyc441 were targeted to an intracellular region as was GLUT4myc (Figure 6b) because both had the middle intracellular loop and cytoplasmic C-terminus, and the cytoplasmic N-terminus and middle intracellular loop of GLUT4, respectively. Unfortunately, we were not able to obtain CHO cells expressing GLUT4myc441 at a high level, as determined by ^{35}S-labelling experiments (Table 2, right-hand side). However, the CHO.GLUT4myc441 (clone 48) cells showed a high level of binding to the anti-c-myc antibody and took up more glucose than did the parent CHO cells. Therefore, we think the GLUT4myc441 chimera newly sythesized in the cell was folded properly, targeted to an intracellular pool (main portion), and then moved to the cell surface (some portion) to transport glucose; even a minor conformational change could increase the affinity to the anti-c-myc antibody. As shown in Table 2, right-hand side, the chimeric transporter possessing the N-terminal half of GLUT1 and C-terminal half of GLUT4 (GLUTmyc144) showed almost the same insulin-stimulated translocation as GLUT1myc (about a 1.3-fold increase). On the other hand, the chimeric transporter possessing the N-terminal half of GLUT4 and C-terminal half of GLUT1 (GLUTmyc441) showed almost the same insulin-stimulated translocation as GLUT4myc (about a 2.2-fold increase). Thus the N-terminal half of GLUT4 seemed to be necessary and sufficient for insulin-stimulated GLUT4 translocation. However, we could not rule out the possibility that a minor conformational change in GLUTmyc441 directed it to the same insulin-stimulated translocation as GLUT4myc.

DISCUSSION

Using the c-myc epitope-tagged GLUT1 and GLUT4, we obtained evidence that insulin triggers the translocation of GLUT1 as well as GLUT4 in several cell lines (Table 1). Since these glucose transporters showed different intracellular distributions (Figure 2) and different degrees of insulin-stimulated translocation (Figure 3a and Table 1), they seemed to have independent trafficking pathways.

The relationship among levels of total exogenously expressed GLUT1myc, cell surface GLUT1myc and 2-deoxyglucose uptake was measured in CHO cells expressing different levels of GLUT1myc, as shown in Figure 1. The amount of GLUT1myc on the cell surface, judged by the binding of anti-c-myc antibody, was almost in proportion to the rates of 2-deoxyglucose uptake (Figures 1c and 1d). This good correlation indicates that the GLUT1myc on the cell surface is functional for 2-deoxyglucose uptake. However, there is controversy as to whether insulin stimulates the translocation of GLUT1 in CHO cells and whether the translocated GLUT1 takes up more glucose [16,17]. As shown in Table 1 and Figure 3a, insulin stimulated the translocation of exogenously expressed GLUT1myc in CHO cells, by the cell surface binding of anti-c-myc antibody. Using a GLUT1 antibody (δ-antibody) which recognizes the external region of GLUT1 on the cell surface, Harrison et al. did not detect insulin-stimulated translocation of either endogenous GLUT1 or exogenously expressed GLUT1 in CHO cells [17]. Since the degree of insulin-stimulated translocation of GLUT1myc is not large

(1.3–1.5-fold) compared with that of GLUT4 (2.4–3.3-fold) as shown in Figure 3(a) and Tables 1 and 2, the different results concerning insulin-stimulated GLUT1 translocation may be caused by the differences in specificity, affinity and sensitivity between the anti-c-myc antibody and the δ-antibody against the exofacial region of GLUT1, assuming that the epitope-tagged GLUT1myc is functionally indistinguishable from the endogenous GLUT1 (Figures 1 and 2).

The exogenously expressed GLUT1myc takes up glucose in accordance with GLUT1myc levels on the cell surface (Figures 1c and 1d). Insulin treatment stimulated cell surface antibody binding (Figure 3a) and 2-deoxyglucose uptake in these CHO.GLUT1myc cells, but the absolute increases of cell surface GLUT1myc and 2-deoxyglucose uptake by insulin were much the same in all cells, despite different levels of expression of GLUT1myc (results not shown). We also observed that the absolute increases in glucose uptake by insulin in these CHO.GLUT1myc cells were almost the same as that in parent CHO cells, and that the maximum response of insulin-stimulated glucose uptake was not increased, even after overexpression of exogenous insulin receptors in these cells (results not shown). The number of GLUT1myc (or GLUT1) which can be translocated by insulin, seemed to be limited and to be already saturated by endogenous GLUT1 in CHO cells. A small part of the exogenously expressed GLUT1myc (or GLUT1) located in an intracellular region was probably translocated by insulin. The translocation machinery or unknown cellular factors which participate in insulin-stimulated GLUT1 translocation may be limited in CHO cells. Thus, the translocatable GLUT1myc level (or GLUT1) is constant in CHO cells, despite different levels of expression of GLUT1myc, and the absolute increases in cell surface GLUT1myc and 2-deoxyglucose uptake by insulin are also constant in these CHO cells. Our findings of insulin-stimulated glucose uptake by exogenously expressed GLUT1myc (or GLUT1) are consistent with the results of Harrison et al. [17]. On the other hand, Asano et al. reported that exogenously expressed GLUT1 increased to some extent the absolute insulin-stimulated increment of 2-deoxyglucose uptake. GLUT1 translocation was not given attention in their report [16], therefore, our proposal that GLUT1 translocates in response to insulin still stands.

Chimeric transporters between GLUT1 and GLUT4 have been constructed, but various results were obtained [22–28]. Piper et al. [22,23] reported that the cytoplasmic N-terminus of GLUT4 is necessary and sufficient for intracellular sequestration. In contrast, Czech et al. [25], Verhey et al. [26,27] and Marshall et al. [28] reported that the cytoplasmic C-terminus of GLUT4 confers intracellular sequestration. Furthermore, Asano et al. [24] identified two non-contiguous domains located on either side of the middle loop of GLUT4. Our immunofluorescence data on the chimeric glucose transporters, indicate that the cytoplasmic N-terminus, loop and C-terminus of GLUT4 have independent intracellular targeting signals (Figures 5 and 6b). Although chimeras containing the targeting signals were mainly sequestered into an intracellular region, part of each chimera was also expressed in the plasma membrane. The amount of surface anti-c-myc binding increased according to the levels of expression of chimeric transporters. Therefore, it is difficult to discriminate the targeting of each chimera only by the amount of surface anti-c-myc binding in the absence of insulin. Some of our data are not consistent with data in other reports [22–28]; different expression systems or assay systems may have subtle effects on the subcellular distribution of chimeric glucose transporters.

This present report is the first on translocation of chimeric glucose transporters. Although we were not able to show evidence that intracellularly targeted chimeric glucose transporters resided on the same intracellular vesicle of GLUT4myc, other than in the immunofluorescence microscopy study (Figures 5 and 6b), we were able to measure the amount of translocation by comparing the amounts of surface binding in the presence and absence of insulin (Table 2). As the amount of insulin-stimulated translocation of GLUT4myc was greater than that of GLUT1myc (Figure 3a, Table 2), each chimera was classified into two types of translocation. We reached the following conclusions concerning the relationship between the intracellular targeting domains of GLUT4myc and the insulin-stimulated translocation. (1) All the cytoplasmic N-terminal region, middle intracellular loop and cytoplasmic C-terminal region of GLUT4 have independent intracellular targeting signals (from the results shown in Figure 5). (2) The intracellular targeting domains (cytoplasmic N-terminus, intracellular middle loop and cytoplasmic C-terminus) of GLUT4 were not sufficient to confer the same translocation as GLUT4 (from the results for GLUT1mycN, GLUT1mycL and GLUT1mycC shown in Table 2). (3) The cooperation of intracellular targeting domains (cytoplasmic N-terminus, middle intracellular loop and cytoplasmic C-terminus) was not sufficient for insulin-stimulated GLUT4 type translocation (from the results for GLUT1mycNC and GLUT1mycNLC given in Table 2). (4) The middle intracellular loop or cytoplasmic C-terminus of GLUT4 was not essential for insulin-stimulated GLUT4 translocation (from the results for GLUT4mycL and GLUT4mycC given in Table 2). (5) The N-terminal half of GLUT4, devoid of both cytoplasmic N-terminus and of middle intracellular loop seemed to be necessary for insulin-stimulated GLUT4 translocation (from the results for GLUTmyc144 and GLUTmyc441, GLUT1mycN, GLUT1mycL and GLUT1mycNLC in Table 2).

The use of CHO cells to study the insulin-stimulated translocation of glucose transporters has been questioned [47], yet this same line of cells has been widely used for the study of insulin-stimulated glucose transport. 3T3-L1 adipocytes provide a cell culture model for analysing insulin-stimulated glucose uptake, since they express the major insulin-regulatable glucose transporter (GLUT4) and a large number of insulin receptors. In contrast with adipocytes, CHO cells have a few endogenous insulin receptors and endogenous GLUT1, but not GLUT4. Incubation of 3T3-L1 adipocytes and CHO cells with insulin results in approximately 15- and 1.5-fold increases in glucose uptake respectively. Using conventional methods, exogenously expressed GLUT1 and GLUT4 in CHO cells showed no apparent translocation in response to insulin [16,17,22]. Therefore, Quon et al. considered that CHO cells are not equipped with machinery for the insulin-stimulated translocation of GLUT1 and GLUT4 [47]. However, based on our previous [19] and present results, the majority of exogenously expressed GLUT4myc was targeted to an intracellular region in both 3T3-L1 adipocytes and CHO cells. In 3T3-L1 adipocytes, translocation of the intracellular GLUT4myc in response to insulin could be detected not only by surface binding of anti-c-myc antibody but also by immunoblot after sucrose gradient analysis [19]. The translocated GLUT4myc results in an increase in glucose uptake in adipocytes [19], although the fold-increase of surface binding of anti-c-myc antibody in response to insulin did not precisely coincide with the fold-increase of glucose uptake, because of the presence of large amounts of endogenous GLUT4 and GLUT1 in 3T3-L1 adipocytes [48].

In CHO cells, however, a relatively small amount of intracellular GLUT4myc could be translocated to the cell surface and most of the intracellular GLUT4myc was retained intracellularly even after insulin treatment, because the GLUT4myc

translocated by insulin on the plasma membrane could be detected only by a sensitive surface-binding assay using anti-c-myc antibody, but not by sucrose gradient analysis or by immunofluorescence microscopy. Therefore, the fold-increase in glucose uptake in response to insulin did not precisely coincide with the fold-increase in surface binding of anti-c-myc antibody in CHO.GLUT4myc cells, because the increase in surface binding of anti-c-myc antibody is determined by the amount of surface GLUT4myc, but the increase in glucose uptake is determined by the total amount of GLUT4myc and endogenous GLUT1 on the cell surface; (1) most of endogenous GLUT1 and a small part of exogenously expressed GLUT4myc were on the cell surface of CHO.GLUT4myc cells, in the absence of insulin, and (2) in response to insulin, intracellular endogenous GLUT1 and a small part of exogenously expressed intracellular GLUT4myc would be translocated. However, the amount of translocatable GLUT4myc is greater than GLUT1myc, because the absolute increase of 2-deoxyglucose uptake by insulin in CHO cells expressing GLUT4myc (CHO.GLUT4myc) is greater than in parent CHO cells and CHO cells expressing GLUT1myc (CHO.GLUT1myc) (K. Ishii, H. Hayashi and Y. Ebina, unpublished work). In addition, the absolute count of surface binding increased by insulin-stimulation in CHO.GLUT4myc was greater than that of CHO.GLUT1myc (Figure 3a). On the other hand, insulin, PMA and NaF stimulated GLUT1myc translocation as well as GLUT4myc in adipocytes and CHO cells [19,38,39,41–43] (Figure 3). However, insulin, PMA or NaF did not stimulate GLUT1myc translocation in some cell lines, although these reagents did stimulate GLUT4myc translocation in the same cell lines (K. Ishii, H. Hayashi and Y. Ebina, unpublished work). Taking all of these results together, we think that the mechanism of translocation of GLUT1 differs from that of GLUT4 in CHO cells. Although CHO cells might contain smaller amounts of the machinery or factor(s) required for the translocation of GLUT4 and GLUT1 than adipocytes and although the efficiencies of translocation of these glucose transporters are low, we think that CHO cells do have the same essential translocation machinery for GLUT1 and GLUT4 as 3T3-L1 adipocytes.

CHO cells seem to be useful for the study of the insulin-stimulated glucose uptake and translocation of glucose transporters, for the following reasons. (1) Exogenously expressed GLUT4 and GLUT1 have different intracellular distributions (Figure 2) and GLUT4 was translocated to the cell surface in response to insulin more efficiently than was GLUT1 in CHO cells (Figure 3), as also noted with adipocytes [21,38,39]. (2) PMA, NaF and GTP[γS] (apart from insulin), which are able to trigger GLUT4 translocation in adipocytes [41–44], also trigger GLUT4 translocation in CHO cells [19]. Although the degree of GLUT4 translocation observed in CHO cells was less than that observed in adipocytes, CHO cells possess basic machinery for GLUT4 translocation, which mimics that of adipocytes. (3) CHO cells are clonally stable even after long-term culture or transfection by expression plasmids. However, 3T3-L1 adipocytes are relatively clonally unstable compared with CHO cells and do not provide an ideal cell culture model for the introduction of exogenous cDNAs. (4) CHO cells have been widely used for the study of insulin signal transduction, including glucose transport [1,36,37].

In the present study, we have detected different translocations of GLUT1myc and GLUT4myc in response to insulin, sensitively and quantitatively. We suggest for the first time that the possible domain responsible for the insulin-stimulated translocation of GLUT4 differs from domains which determine the intracellular targeting of GLUT4.

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