# Roles of insulin, guanosine 5′-[γ-thio]triphosphate and phorbol 12-myristate 13-acetate in signalling pathways of GLUT4 translocation

Mikio TODAKA*†, Hideki HAYASHI*, Takanobu IMANAKA*, Yasumasa MITANI*, Seika KAMOHARA*, Kazuhiro KISHI*, Keisuke TAMAOKA*, Fumihiko KANAI*, Motoaki SHICHIRI†, Narito MORII‡, Shuh NARUMIYA‡ and Yousuke EBINA*§

*Department of Enzyme Genetics, Institute for Enzyme Research, University of Tokushima, 3-18-15 Kuramoto-cho 770, †Department of Metabolic Medicine, Kumamoto University, School of Medicine, Honjo 1-1-1 Kumamoto 860, and ‡Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

Insulin, guanosine 5′-[γ-thio]triphospate (GTP[S]) and phorbol 12-myristate 13-acetate (PMA) trigger the translocation of GLUT4 (type 4 glucose transporter; insulin-sensitive glucose transporter) from an intracellular pool to the cell surface. We have developed a highly sensitive and quantitative method to detect GLUT4 immunologically on the surface of intact 3T3-L1 adipocytes and Chinese hamster ovary (CHO) cells, using c-myc epitope-tagged GLUT4 (GLUT4myc). We examined the roles of insulin, GTP[S] and PMA in the signalling pathways of GLUT4 translocation in the CHO cell system. Among small molecular GTP-binding proteins, ras, rab3D, rad and rho seem to be candidates as signal transmitters of insulin-stimulated GLUT4 translocation. Overexpression of wild-type H-ras and the dominant negative mutant H-ras$^{S17N}$ in our cell system respectively enhanced and blocked insulin-stimulated activation of mitogen-activated protein kinase, but did not affect insulin-stimulated GLUT4 translocation. Overexpression of rab3D or rad in the cells did not affect GLUT4 translocation triggered by insulin, GTP[S] or PMA. Treatment with *Botulinum* C3 exoenzyme, a specific inhibitor of rho, had no effect on GLUT4 translocation induced by insulin, GTP[S] or PMA. Therefore these small molecular GTP-binding proteins are not likely to be involved in GLUT4 translocation. In addition, insulin, GTP[S] and PMA apparently stimulate GLUT4 translocation through independent pathways.

## INTRODUCTION

Insulin binds to the α-subunit of its receptor and activates the tyrosine kinase of the β-subunit. The receptor tyrosine kinase phosphorylates insulin receptor substrate-1 (IRS-1) (for reviews see [1,2]). Tyrosine-phosphorylated IRS-1 forms a complex with several proteins containing src homology 2 (SH2) domain(s), such as phosphatidylinositol (PI) 3-kinase [3,4], GRB2 [5,6], Nck [7] and SHPTP2 [8]. IRS-1-bound GRB2 ultimately activates ras/mitogen-activated protein kinase (MAP kinase) [9], and SHPTP2 activated by IRS-1 also enhances DNA synthesis induced by insulin [10]. One of the major physiological effects of insulin is the stimulation of glucose uptake in target cells [1,2,11]. Insulin-stimulated glucose uptake in the cells is caused mainly by translocation of GLUT4 (type 4 glucose transporter; insulin-sensitive glucose transporter) from an intracellular pool to the plasma membrane [11–13]. To understand the causes of non-insulin-dependent diabetes mellitus, it is important to elucidate the mechanism of GLUT4 translocation [1,2]. In order to examine the mechanisms of GLUT4 translocation, we developed a novel and sensitive method to measure directly the quantities of c-myc epitope-tagged GLUT4 (GLUT4myc) on the cell surface of 3T3-L1 adipocytes and Chinese hamster ovary (CHO) cells [14]. Using this novel cell system we demonstrated that PI 3-kinase plays an important role in the signal transduction of GLUT4 translocation induced not only by insulin but also by platelet-derived growth factor and epidermal growth factor [15–17]. Other groups, using other methods, also reported that insulin-activated PI 3-kinase is involved in insulin-stimulated GLUT4 translocation [18–23].

The use of CHO cells to study the insulin-stimulated translocation of GLUT4 has been questioned, because CHO cells do not express GLUT4 endogenously, and exogenously expressed GLUT4 in the cells showed no apparent translocation in response to insulin when assessed by conventional methods [24]. However, using our novel approach we have shown that exogenously expressed GLUT4myc is translocated from an intracellular pool to the cell surface in response to insulin in CHO cells, as well as in 3T3-L1 adipocytes [14,25]. The high sensitivity of our method made feasible the detection of GLUT4 on the cell surface following insulin-induced translocation in CHO cells. In 3T3-L1 adipocytes, translocation of the intracellular GLUT4myc in response to insulin could be detected not only by surface binding of an anti-c-myc antibody but also by conventional immunoblotting after sucrose gradient analysis [14,26–28]. In CHO cells, however, a relatively small amount of intracellular GLUT4myc was translocated to the cell surface and most was retained intracellularly, even after insulin treatment, as the GLUT4myc translocated to the plasma membrane in response to insulin could be detected by a sensitive surface binding assay using anti-c-myc antibody, but not by sucrose gradient analysis or immunofluorescence microscopy [25].

Although the machinery for insulin-stimulated GLUT4 translocation in CHO cells is not identical with that in 3T3-L1 adipocytes, CHO cells seem to possess a basic machinery for the insulin-stimulated translocation of exogenously expressed

Abbreviations used: IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; GLUT4, type 4 glucose transporter; GLUT4myc, c-myc epitope-tagged GLUT4; CHO cells, Chinese hamster ovary cells; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; PMA, phorbol 12-myristate 13-acetate; MAP kinase, mitogen-activated protein kinase; PDBu, phorbol 12,13-dibutyrate.

§ To whom correspondence should be addressed.

GLUT4 which mimics that of adipocytes, based on the following evidence. Firstly, exogenously expressed GLUT4myc and GLUT1myc have different intracellular distributions, and GLUT4myc was translocated to the cell surface in response to insulin more efficiently than was GLUT1myc in CHO cells, using our novel method, as has also been noted in adipocytes [14,25]. Secondly, phorbol 12-myristate 13-acetate (PMA), NaF and guanosine 5′-[$\gamma$-thio]triphosphate (GTP[S]), agents other than insulin that are able to trigger GLUT4 translocation in adipocytes [29–32], also trigger GLUT4 translocation in CHO cells [14]. Furthermore, CHO cells are clonally stable even after long-term culture or transfection by expression plasmids. On the other hand, 3T3-L1 adipocytes are relatively clonally unstable compared with CHO cells, and do not provide an excellent cell culture model for the introduction of exogenous cDNAs. Therefore CHO cells seem to be useful for the study of the insulin-stimulated translocation of GLUT4.

The addition of GTP[S] to permeabilized adipocytes or CHO cells induces GLUT4 translocation [14,33]. It seems to be important to identify the target GTP-binding protein(s) in order to elucidate the molecular mechanisms of insulin-stimulated GLUT4 translocation. Since some small GTP-binding proteins play important roles in intracellular trafficking or secretion [34–36], which is similar to GLUT4 translocation from an intracellular pool to the plasma membrane, we examined the effects of the small GTP-binding proteins ras, rab3D, rho and rad on insulin-, GTP[S]- and PMA-triggered GLUT4 translocation. Finally, we examined cross-talk in GLUT4 translocation stimulated by these agents.

## EXPERIMENTAL

### Cell culture and antibodies

CHO-GLUT4myc·IR cells are CHO cells expressing GLUT4myc and insulin receptors [14]. CHO-GLUT4myc·IR-WT, CHO-GLUT4myc·IR-Asn, CHO-GLUT4myc·IR-rab3D and CHO-GLUT4myc·IR-rad are CHO-GLUT4myc·IR cells stably overexpressing wild-type H-ras$^{WT}$, the dominant negative mutant H-ras$^{S17N}$, rab3D and rad respectively. These cells were cultured in F-12 medium supplemented with 10% fetal calf serum (Gibco). The wild-type and mutant H-ras cDNAs were kindly provided by Dr. G. M. Cooper, Harvard Medical School, Boston, MA, U.S.A., and were expressed under the mouse metallothionein promoter [37]. An anti-(pan ras) antibody (Ag-2), an anti-phosphotyrosine antibody (PY20) and an anti-(MAP kinase) antibody were purchased from Oncogene Science Inc., ICN ImmunoBiologicals and Upstate Biotechnology Inc. respectively. Anti-rab3D and anti-rad antibodies were prepared by immunizing rabbits with glutathione S-transferase-fused rab3D and rad proteins respectively, as described previously [38,39].

### Expression of ras, rab3D and rad in CHO-GLUT4myc·IR cells

CHO-GLUT4myc·IR cells were transfected with the mammalian expression vectors pMT-H-ras$^{WT}$ (WT), pMT-H-ras$^{S17N}$ (Asn), SR$\alpha$-rab3D or SR$\alpha$-rad and pSV2·bsr (blasticidin S deaminase) by calcium phosphate co-precipitation, and selected with 10 $\mu$g/ml blasticidin S hydrochloride (Funakoshi Co. Ltd.) for at least 2 weeks. Several independent clones expressing the H-ras proteins were established after screening by Western blotting with the anti-(pan ras) antibody (Ag-2). The stable cell lines were induced with 75 $\mu$M $ZnSO_4$ in the medium for 20 h at 37 °C before assays were carried out, because the pMT plasmid carries the mouse metallothionein promoter [37]. Several independent clones expressing rab3D or rad were established after screening by immunoprecipitating the $^{35}$S-labelled proteins with an anti-rab3D antibody or an anti-rad antibody respectively.

### Immunoblotting

Cells were lysed in Laemmli sample buffer and separated by SDS/PAGE. Proteins were transferred to nitrocellulose filters and incubated with the anti-phosphotyrosine (PY20) or anti-(pan ras) (Ag-2) antibody, followed by $^{125}$I-goat anti-mouse IgG, as described previously [4].

### Cell labelling and immunoprecipitation

$^{35}$S-labelling of cellular proteins and immunoprecipitation were carried out as described previously [4]. Briefly, cells were incubated with Tran$^{35}$S label (ICN) in methionine-free medium, and lysed with lysis buffer containing 1% Triton X-100. The lysates were immunoprecipitated with anti-rab3D or anti-rad antibody and Protein G–Sepharose (Zymed), and analysed by SDS/12.5%-PAGE.

### PI 3-kinase assay

Cells were stimulated with 10 nM insulin or 1 $\mu$M PMA in KRH (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 20 mM Hepes, pH 7.4, and 2 mg/ml BSA) for 10 min, and then lysed in lysis buffer containing 1% Nonidet P40, as described previously [4]. The cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody (PY20) or an anti-p85 antibody and Protein G-Sepharose. The immunoprecipitates were subjected to a PI 3-kinase assay as described previously [4]. The radioactive spots were quantified using a Bio-image analyser BAS2000 (Fuji Film Institution).

### MAP kinase assay

Cells were treated for 10 min with or without 10 nM insulin, and scraped into suspension buffer [25 mM Tris/HCl, pH 7.5, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 20 $\mu$M (*p*-amidinophenyl)methanesulphonyl fluoride hydrochloride, 0.5 mM EGTA]. The suspensions were sonicated by 4 × 15 s bursts on ice, and centrifuged at 18 500 ***g*** for 20 min at 4 °C. The supernatant (100 $\mu$g of total protein) was immunoprecipitated with an anti-(MAP kinase) antibody, and the kinase reaction was carried out by incubating with 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EGTA, 1 mg/ml myelin basic protein (Sigma) and 40 $\mu$M [$\gamma$-$^{32}$P]ATP (Amersham) for 20 min at 24 °C [40]. The phosphorylated myelin basic protein was visualized by autoradiography after separation on SDS/15%-PAGE, and quantified using a Bio-image analyser BAS2000.

### Assay of cell surface anti-c-myc antibody binding to measure GLUT4myc translocation

The cell surface binding assay was carried out as described previously [14]. (i) For intact cells, confluent cells in 24-well dishes were pretreated with KRH buffer for 30 min at 37 °C, and stimulated with indicated concentrations of insulin or PMA in KRH for 10 min at 37 °C. (ii) For permeabilized cells, confluent cells in 24-well dishes were preincubated with GK buffer (20 mM Hepes, 125 mM potassium glutamate, 5 mM EGTA, 5 mM $MgCl_2$, 15 mM KCl, 5 mM NaCl and 1 mg/ml BSA) for 30 min

at 37 °C, and then incubated with 0.05 unit/ml streptolysin O (Nissui Pharmaceutical Co. Ltd.) in GK buffer for 10 min at 37 °C [14]. The permeabilized cells were stimulated with the indicated concentrations of insulin, GTP[S] or PMA in GK buffer for 10 min at 37 °C. The stimulated cells (either intact or permeabilized) were incubated with an anti-c-myc antibody (mAb 9E10) in KRH for 2 h on ice, and then with $^{125}$I-labelled goat anti-mouse IgG in KRH for 2 h on ice. After washing with ice-cold KRH buffer, bound $^{125}$I-labelled goat anti-mouse IgG was solubilized and the radioactivity was determined in a $\gamma$-radiation counter. Translocation of GLUT4myc was shown as the fold increase over control (without ligands) after subtracting the background values measured in CHO cells.

### ADP-ribosylation with *Botulinum* C3 exoenzyme

Recombinant *Botulinum* C3 exoenzyme was prepared essentially as described elsewhere [41]. Cells permeabilized with streptolysin O as described above were treated with 400 ng/ml C3 exoenzyme in GK buffer for 10 min at 37 °C, and then stimulated with the indicated concentrations of insulin, GTP[S] or PMA in GK buffer for 10 min. The ligand-stimulated translocation of GLUT4myc was determined as described above. To confirm that the rho protein in the cells was ADP-ribosylated by this C3 exoenzyme treatment *in vivo*, we measured the residual rho protein which had not been ribosylated during this initial treatment by again ribosylating the cell lysates (prepared by sonication) with 1 $\mu$g of C3 exoenzyme and 10 $\mu$M [$^{32}$P]NAD$^+$ (NEN) *in vitro*, as described previously [41,42]. The [$^{32}$P]ADP-ribosylated rho was precipitated with trichloroacetic acid, subjected to SDS/12.5 %-PAGE and autoradiographed.

## RESULTS

### Effects of the small GTP-binding proteins ras, rab3D, rho and rad on GLUT4 translocation

Some small GTP-binding proteins are involved in the intracellular sorting of secretory vesicles in yeast and mammalian cells [36]. Thus a small GTP-binding protein might be a target for GTP[S] in GTP[S]-stimulated GLUT4 translocation. It has already been reported that the ras/MAP kinase pathway is unlikely to play a key role in GLUT4 translocation in adipocytes [43–47]. Using our sensitive and direct method to detect the translocation of GLUT4myc to the cell surface, we sought to confirm that ras/MAP kinase activation is not involved in insulin-stimulated GLUT4 translocation, as Merrall et al. [48], Kozma et al. [49] and Manchester et al. [50] have presented contradictory results obtained with *Xenopus* oocytes, 3T3-L1 adipocytes and cardiac myocytes respectively. We overexpressed wild-type H-ras (p21ras$^{WT}$) or a dominant negative mutant of H-ras (p21ras$^{S17N}$) under the mouse metallothionein promoter [37] in CHO-GLUT4myc·IR cells. Figure 1 shows the data obtained with two typical, independent, clones for each construct. Zinc treatment enhanced the expression of p21H-ras proteins by about 5–20-fold, as determined by immunoblotting with an anti-(pan ras) antibody (Figure 1A). As shown in Figure 1(B), the over-expression of ras$^{WT}$ enhanced insulin-stimulated MAP kinase activation (WT40 and WT44) about 4–5-fold compared with findings in the parent CHO-GLUT4myc·IR cells. In contrast, insulin-stimulated MAP kinase activation in cells overexpressing ras$^{S17N}$ (Asn52 and Asn59 in Figure 1) was completely inhibited. However, the enhancement or repression of insulin-stimulated MAP kinase activity did not affect insulin-stimulated GLUT4 translocation (Figure 1C). These results indicate that insulin-

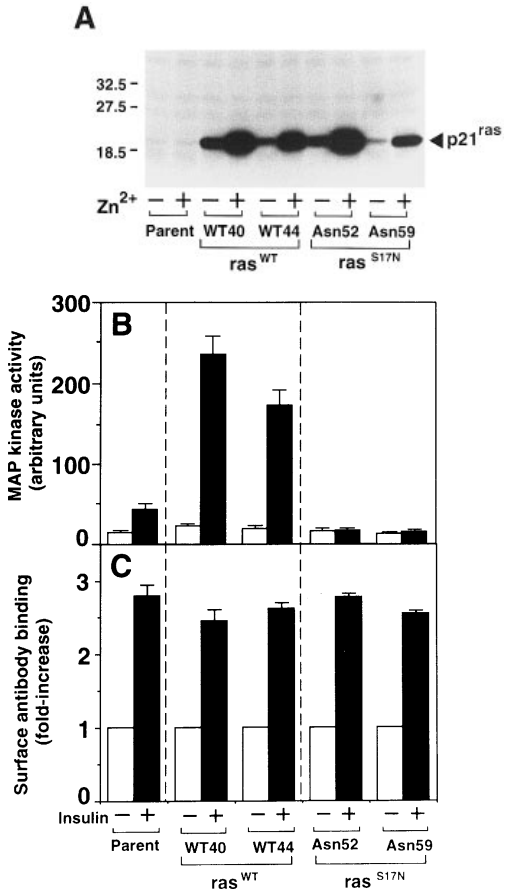


**Figure 1 Expression of wild-type (WT) or a dominant negative mutant (Asn) H-ras in CHO-GLUT4myc·IR cells (A), and insulin-dependent activation of MAP kinase activity (B) and GLUT4myc translocation (C)**

(**A**) Parent CHO-GLUT4myc·IR cells were transfected with plasmids containing the mouse metallothionein promoter [pMT-H-ras$^{WT}$ (WT) or pMT-H-ras$^{S17N}$ (Asn)] and pSV2-bsr (blasticidin S deaminase). After selection with 10 $\mu$g/ml blasticidin S for 2 weeks, two independent clones stably expressing wild-type H-ras$^{WT}$ (WT40 and WT44) or the dominant negative mutant H-ras$^{S17N}$ (Asn52 and Asn59) were established. The cells were exposed to 75 $\mu$M $ZnSO_4$ in medium for 20 h and the lysates were prepared and immunoblotted with an anti-(pan ras) antibody and $^{125}$I-labelled-goat anti-mouse IgG, as described in the Experimental section. In the autoradiogram, p21H-ras proteins are indicated by an arrowhead. Molecular mass markers (kDa) are shown on the left of the gel. (**B**) and (**C**) Cells exposed to 75 $\mu$M $ZnSO_4$ for 20 h were treated with 10 nM insulin (+) or buffer alone (−) for 10 min. The cells were subjected to a MAP kinase assay (**B**) or a cell surface anti-c-myc antibody binding assay (**C**), as described in the Experimental section. Values represent means ± S.E.M. of three separate experiments.

stimulated GLUT4 translocation is independent of ras/MAP kinase activation, and are consistent with findings reported by Robinson et al. [43], van den Berghe et al. [44], Hausdorff et al. [45], Gould et al. [46] and Wiese et al. [47], who used 3T3-L1 adipocytes.

Next, we examined the role of rab3D in GLUT4 translocation, as Baldini et al. [35] reported that rab3D could be a candidate to transmit the GLUT4 translocation signal in 3T3-L1 adipocytes. We obtained two independent clones (Rab3D2 and Rab3D27) stably overexpressing rab3D by transfecting a rab3D-expressing plasmid into CHO-GLUT4myc·IR cells (Figure 2A). As shown in Figure 2(B), the concentrations of insulin producing maximal and half-maximal responses were respectively 1–10 nM and 0.020 nM (parent cell line); 1–10 nM and 0.028 nM (Rab3D2 ); and 1 nM and 0.026 nM (Rab3D27). The maximal increases in

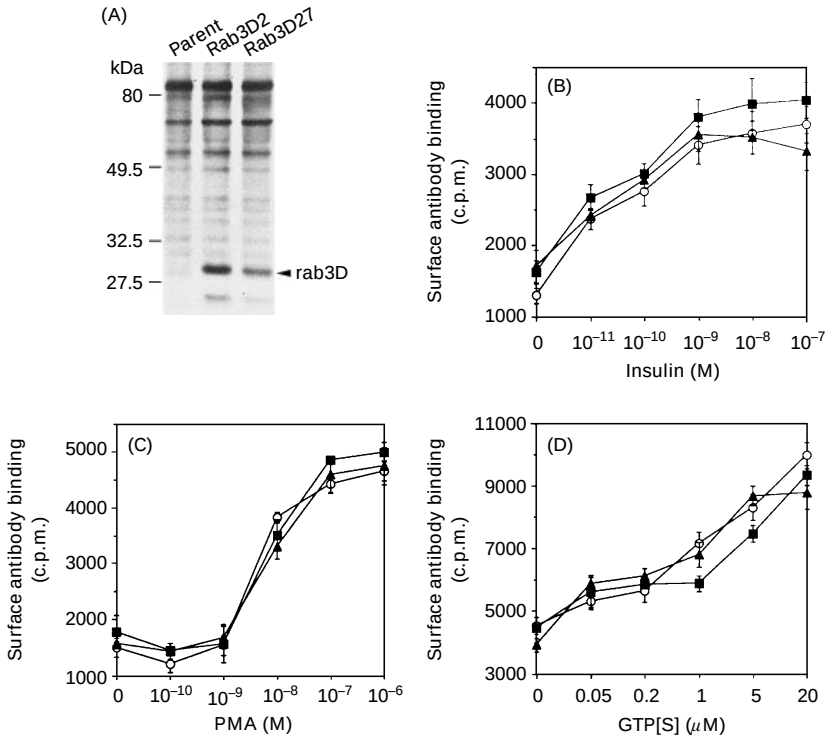


**Figure 2 Expression of rab3D in CHO-GLUT4myc·IR cells, and effect on ligand-stimulated GLUT4 translocation**

Parent CHO-GLUT4myc·IR cells were transfected with mammalian expression vector SRα-rab3D and pSV2-bsr, and selected with blasticidin S. Two independent clones (Rab3D2 and Rab3D27) stably expressing rab3D protein were established. (**A**) Cells were labelled with Tran$^{35}$S label and immunoprecipitated with an anti-rab3D antibody, as described in the Experimental section. The immunoprecipitates were separated by SDS/12.5%-PAGE. The autoradiogam indicates the location of rab3D. (**B**)–(**D**) The surface anti-c-myc antibody binding assay was carried out after treatment with various concentrations of insulin (**B**), PMA (**C**) or GTP[S] (**D**) for 10 min, as described in the Experimental section. ○, Parent cell line; ■, Rab3D2; ▲, Rab3D27. Values represent means ± S.E.M. of six determinations.

GLUT4myc translocation (buffer alone as control) were 2.8-fold (parent), 2.5-fold (Rab3D2) and 2.2-fold (Rab3D27). As shown in Figure 2(C), the concentrations of PMA producing maximal and half-maximal responses were respectively 0.1–1.0 μM and 4.5 nM (parent cell line); 0.1–1.0 μM and 6.5 nM (Rab3D2); and 0.1–1.0 μM and 6.5 nM (Rab3D27). The maximal increases in GLUT4myc translocation (buffer alone as control) were 2.6-fold (parent), 2.8-fold (Rab3D2) and 3.0-fold (Rab3D27). The concentrations of GTP[S] producing maximal and half-maximal responses were respectively 20 μM and 1.1 μM (parent cell line); 20 μM and 2.3 μM (Rab3D2); and 5–20 μM and 0.3 μM (Rab3D27), as shown in Figure 2(D). The maximal increases in GLUT4myc translocation (buffer alone as control) were 2.2-fold (parent), 2.1-fold (Rab3D2) and 2.2-fold (Rab3D27). Therefore the overexpression of rab3D in CHO-GLUT4myc·IR cells did not change significantly the sensitivity or magnitude of GLUT4myc translocation in response to insulin, GTP[S] or PMA. In addition, overexpression of rabGDI (GDP dissociation inhibitor) to inhibit the dissociation of GDP from the GDP-bound rab family, including rab3D [51,52], had no effect on GLUT4myc translocation (results not shown). These results suggest that rab3D is not involved in insulin-, PMA- or GTP[S]-stimulated GLUT4 translocation.

Rho, another family of small GTP-binding proteins, is thought to regulate the assembly of actin stress fibres and focal adhesions in various cells [34,42]. Since GLUT4 translocation seems to require cytoskeletal reorganization, we examined the role of rho proteins in GLUT4 translocation using *Botulinum* C3 exoenzyme, which inhibits rho function by ADP-ribosylation [41,42]. We first examined whether C3 exoenzyme treatment *in vivo* sufficiently ADP-ribosylates the 25 kDa rho protein in CHO-GLUT4myc·IR cells (Figure 3A). The apparently decreased *in vitro* labelling of the 25 kDa rho protein in lane 2 (with *in vivo* C3 treatment) compared with lane 1 (without *in vivo* C3 treatment) indicates that C3 treatment *in vivo* is sufficient to ADP-ribosylate the rho protein.

We then examined the effects of C3 exoenzyme treatment *in vivo* on insulin-, PMA- and GTP[S]-stimulated GLUT4myc translocation (Figures 3B–3D). Such treatment lowered to some extent the amount of surface GLUT4myc, but this was not statistically significant. The concentrations of insulin that stimulated maximal and half-maximal GLUT4myc translocation were 1–10 nM and 0.14 nM (without C3 treatment) and 1–10 nM and 0.19 nM (with C3 treatment) respectively (Figure 3B). The concentrations of PMA that stimulated maximal and half-maximal GLUT4myc translocation were 0.1–1.0 μM and 4.0 nM (without C3 treatment) and 0.1–1.0 μM and 4.1 nM (with C3 treatment) respectively (Figure 3C). The concentrations of GTP[S] that stimulated maximal and half-maximal GLUT4myc translocation were 20 μM and 1.7 μM (without C3 treatment) and 20 μM and 2.1 μM (with C3 treatment) respectively (Figure 3D). C3 treatment did not change significantly the magnitudes of these ligand-stimulated GLUT4myc translocations. Therefore it seems that rho is not involved in the signal transduction pathway of GLUT4 translocation.

Rad, a small novel GTP-binding protein, has been suggested

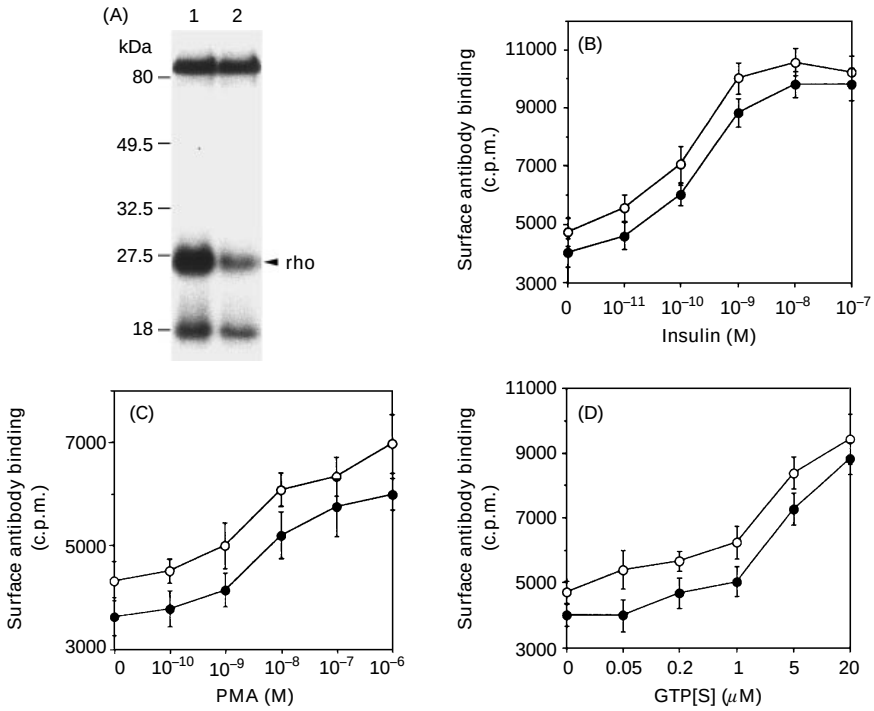


**Figure 3 Effects of ADP-ribosylation of rho by *Botulinum* C3 exoenzyme on ligand-stimulated GLUT4 translocation in CHO-GLUT4myc·IR cells**

(**A**) To determine whether rho protein is ADP-ribosylated by *Botulinum* C3 exoenzyme in streptolysin O-permeabilized CHO-GLUT4myc·IR cells *in vivo*, cells treated with (lane 2) or without (lane l) 400 ng/ml *Botulinum* C3 exoenzyme for 10 min *in vivo* were scraped, sonicated and ribosylated again with 1 $\mu$g of C3 exoenzyme and [$^{32}$P]NAD$^{+}$ *in vitro*. The [$^{32}$P]ADP-ribosylated rho was electrophoresed and autoradiographed, as described in the Experimental section. (**B**)–(**D**) Streptolysin O-permeabilized CHO-GLUT4myc·IR cells were treated with (●) or without (○) 400 ng/ml C3 exoenzyme for 10 min *in vivo* and then stimulated with various concentrations of insulin (**B**), PMA (**C**) or GTP[S] (**D**) for 10 min. The surface anti-c-myc antibody binding assay was carried out as described in the Experimental section. Values represent means ± S.E.M. of six determinations.

as a candidate to block insulin-stimulated glucose uptake, as this protein is expressed at several-fold higher levels in muscle of non-insulin-dependent diabetes mellitus patients compared with that of healthy subjects [53]. Therefore we examined whether overexpression of rad inhibits insulin-, GTP[S]- or PMA-stimulated GLUT4myc translocation in CHO-GLUT4myc·IR cells. We obtained two independent clones (Rad23 and Rad39) stably overexpressing rad by transfecting a rad-expressing plasmid into CHO-GLUT4myc·IR cells (Figure 4A). As shown in Figure 4(B), the concentrations of insulin producing maximal and half-maximal responses were respectively 1–10 nM and 0.02 nM (parent cell line); 1–10 nM and 0.01 nM (Rad23); and 1–10 nM and 0.035 nM (Rad39). The maximal increases in GLUT4myc translocation (buffer alone as control) were 2.8-fold (parent), 3.4-fold (Rad23) and 3.0-fold (Rad39). As shown in Figure 4(C), the concentrations of PMA producing maximal and half-maximal responses were respectively 0.1–1.0 $\mu$M and 4.5 nM (parent cell line); 0.1–1.0 $\mu$M and 4.5 nM (Rad23); and 0.1–1.0 $\mu$M and 4.4 nM (Rad39). The maximal increases in GLUT4myc translocation (buffer alone as control) were 2.6-fold (parent), 2.8-fold (Rad23) and 2.9-fold (Rad39). The concentrations of GTP[S] producing maximal and half-maximal responses were respectively 20 $\mu$M and 1.1 $\mu$M (parent cell line); 20 $\mu$M and 1.5 $\mu$M (Rad23); and 20 $\mu$M and 1.0 $\mu$M (Rad39) (Figure 4D). The maximal increases in GLUT4myc translocation (buffer alone as control) were 2.2-fold (parent), 2.1-fold (Rad23) and 1.8-fold (Rad39). Therefore the overexpression of rad protein in the cells did not affect significantly the sensitivity or magnitude of GLUT4myc translocation in response to insulin, GTP[S] or PMA. The results suggest that rad is not involved in the GLUT4 translocation pathway. Therefore ras, rab3D, rho and rad do not seem to play an important role in GTP[S]-stimulated GLUT4 translocation.

### Insulin, GTP[S] and PMA stimulate GLUT4 translocation through independent pathways

To investigate further the relationships between insulin, PMA and GTP[S] in the signalling pathways of ligand-stimulated GLUT4 translocation, we investigated whether PMA or GTP[S] can stimulate PI 3-kinase activity. In contrast to insulin treatment, treatment with PMA or GTP[S] had no effect on PI 3-kinase activity immunoprecipitated with an anti-phosphotyrosine antibody or with an anti-p85 antibody, although all treatments evoked GLUT4myc translocation in permeabilized CHO-GLUT4myc·IR cells (Table 1). This indicates that PI 3-kinase is not downstream of protein kinase C (activated by PMA) or unknown GTP-binding protein(s) (activated by GTP[S]) that stimulate(s) GLUT4 translocation in CHO cells.

Next we examined PMA-, insulin- and GTP[S]-stimulated GLUT4myc translocation after down-regulation of protein kinase C by phorbol 12,13-dibutyrate (PDBu) pretreatment [54]. PDBu pretreatment inhibited PMA-stimulated GLUT4myc translocation, but had little effect on insulin- or GTP[S]-stimulated GLUT4myc translocation (results not shown). This suggests that protein kinase C is not downstream of PI 3-kinase

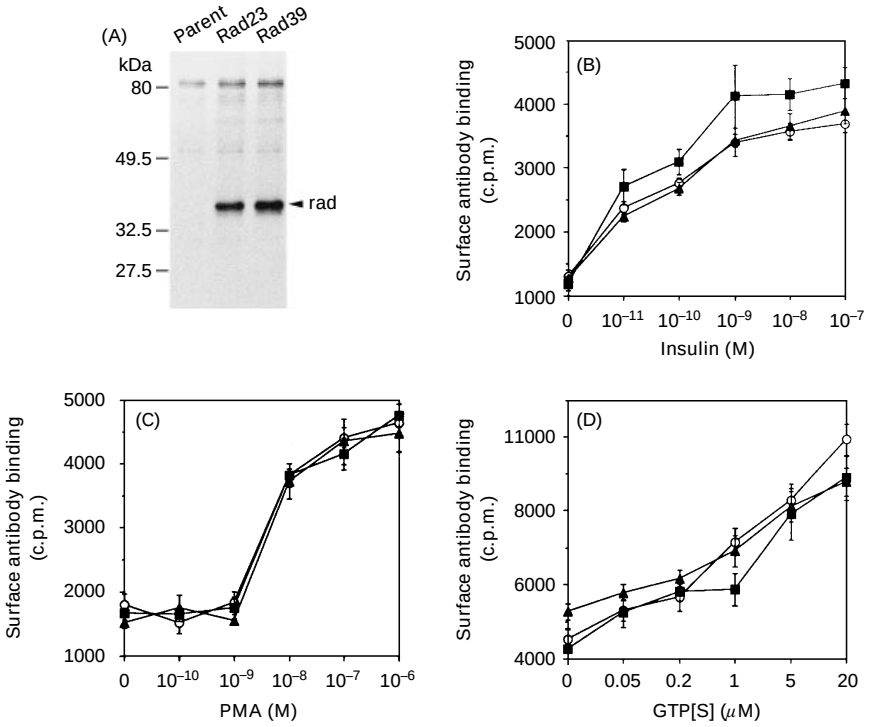


**Figure 4 Expression of rad in CHO-GLUT4myc·IR cells, and effect on ligand-stimulated GLUT4 translocation**

Parent CHO-GLUT4myc·IR cells were transfected with mammalian expression vector SRα-rad and pSV2-bsr and selected with blasticidin S. Two independent clones (Rad23 and Rad39) stably expressing rad protein were established. (**A**) Cells were labelled with Tran$^{35}$S label and immunoprecipitated with an anti-rad antibody, as described in the Experimental section. The immunoprecipitates were separated by SDS/12.5%-PAGE, and location of rad on the autoradiogam is shown. (**B**)–(**D**) The surface anti-c-myc antibody binding assay was carried out after treatment with various concentrations of insulin (**B**), PMA (**C**) or GTP[S] (**D**) for 10 min, as described in the Experimental section. ○, Parent cell line; ■, Rad23; ▲, Rad39. Values represent means ± S.E.M. of six determinations.

**Table 1 Effects of insulin, PMA and GTP[S] treatments on GLUT4myc translocation and PI 3-kinase activity**

CHO-GLUT4myc·IR cells were permeabilized with 0.05unit/ml streptolysin O for 10 min, and then stimulated with buffer alone, 10 nM insulin, 1 mM PMA or 10 μM GTP[S] for 10 min, as described in the Experimental section. GLUT4myc translocation was measured using the surface anti-c-myc antibody binding assay, and is expressed as the fold increase over the control (in the absence of ligands). Values represent means ± S.E.M. of three determinations. For measurement of PI 3-kinase activity, cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody (αpTyr) and with an anti-p85 antibody (αp85). The immunoprecipitates were subjected to PI 3-kinase assays and the kinase activity was quantified. Values represent means ± S.E.M. of three determinations.

| Parameter | Control (buffer) | + Insulin | + PMA | + GTP[S] |
|---|---|---|---|---|
| Surface antibody binding (c.p.m.) | 3525 ± 133 | 7740 ± 134 | 5877 ± 271 | 7585 ± 367 |
| Translocation (fold increase) | | 2.2 | 1.7 | 2.2 |
| PI 3-kinase activity (αpTyr) (arbitrary units) | 8.1 ± 1.3 | 281.6 ± 19.9 | 10.2 ± 2.6 | 8.6 ± 1.4 |
| PI 3-kinase activity (αp85) (arbitrary units) | 473 ± 46 | 1433 ± 210 | 504 ± 65 | 526 ± 15 |

or of the GTP-binding protein(s) involved in GLUT4 translocation.

Finally, we examined the effects of guanosine 5′-[β-thio]diphosphate (GDP[S]) on insulin-, PMA- and GTP[S]-stimulated GLUT4 translocation. Pretreatment with 100 μM GDP[S] inhibited 10 μM GTP[S]-stimulated GLUT4myc translocation by 27.5 ± 7.0% ($P < 0.05$), and the inhibition occurred dose-dependently, as expected. However, the same GDP[S] treatment had no significant effects on insulin- (3.7 ± 12.7% inhibition) or PMA- (3.6 ± 6.7% inhibition) stimulated GLUT4 translocation. This suggests that the GTP-binding protein(s) involved in GLUT4 translocation is not downstream of the insulin receptor (specifically PI 3-kinase) or of protein kinase C.

Figure 5 summarizes the above data, and also shows the working sites of wortmannin, okadaic acid and a cAMP analogue (8-bromo-cAMP). Wortmannin and okadaic acid inhibit insulin-stimulated but not PMA-stimulated GLUT4myc translocation; and 8-bromo-cAMP inhibits MAP kinase activity but not PMA- or insulin-stimulated GLUT4myc translocation. We concluded that insulin (via PI 3-kinase), GTP[S] [possibly via heterotrimeric GTP-binding protein(s)], and PMA (via protein kinase C) trigger GLUT4 translocation through independent pathways.

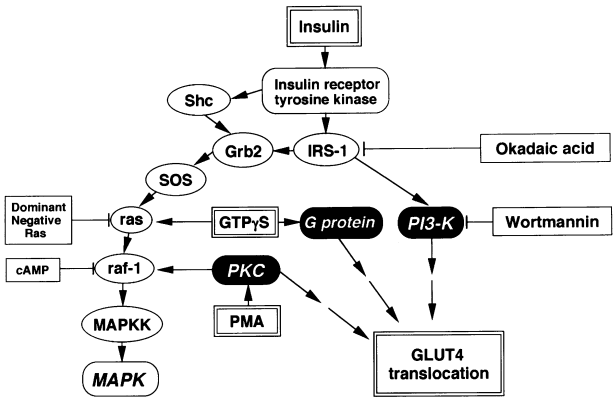


**Figure 5 Putative model for GLUT4 translocation triggered by insulin, GTP[S] and PMA**

Insulin-stimulated GLUT4 translocation is caused by tyrosine phosphorylation of IRS-1 and PI 3-kinase (PI3-K) activation, but apparently not by ras/MAP kinase (MAPK) activation. GTP[S] (GTP$\gamma$S in Figure) and PMA activate protein kinase C (PKC) or unknown GTP-binding protein(s) (G protein) respectively and trigger GLUT4 translocation through pathways other than PI 3-kinase. Stimulatory effects are indicated by arrows and inhibitory effects are indicated by lines. Other abbreviations: MAPKK, MAPK kinase; SOS, son of sevenless.

## DISCUSSION

Insulin binding to its receptor on the cell surface activates PI 3-kinase, GRB2 and SHPTP2 through phosphorylation of IRS-1 and Shc, and eventually stimulates glucose uptake by the cell [1,2]. However, stimulation of glucose uptake by recruiting GLUT4 from an intracellular pool to the cell surface in adipocytes is not a specific action of insulin [11–14]. PMA and GTP[S], which activate protein kinase C and GTP-binding proteins respectively, also stimulate GLUT4 translocation [14–17,29–33,54]. Using CHO-GLUT4myc cells and 3T3-L1 adipocytes expressing GLUT4myc, we found that PI 3-kinase plays a key role in insulin-stimulated GLUT4 translocation [15–17]. The manner by which GTP[S] stimulates GLUT4 translocation is poorly understood. Here we examined the role of ras, rab3D, rho and rad in GLUT4 translocation, since these proteins seem to play important roles in intracellular traffic of secretory proteins and vesicles [34–36,42,53]. We were obliged to use CHO-GLUT4myc·IR cells instead of 3T3-L1 adipocytes expressing GLUT4myc in order to obtain many independent stable clones expressing wild-type or dominant negative mutants of ras, rab3D, rho and rad. We have shown that CHO cells carry the basic machinery for GLUT4 translocation which mimics that of adipocytes, as discussed in the Introduction section. Although our methods do not completely rule out the possibility of an involvement of these small molecular GTP-binding proteins in GLUT4 translocation, we conclude that ras, rab3D, rho and rad do not seem to play important roles in GTP[S]-stimulated GLUT4 translocation. In addition, GTP[S] stimulates heterotrimeric GTP-binding proteins as well as small molecular GTP-binding proteins. We observed that NaF stimulates GLUT4 translocation [14], thereby suggesting an involvement of heterotrimeric GTP-binding protein(s) [55]. Ligand stimulation of a heterotrimeric G-protein-coupled receptor triggers GLUT4 translocation in 3T3-L1 adipocytes and CHO-GLUT4myc cells (K. Kishi, H. Hayashi and Y. Ebina, unpublished work). Therefore a heterotrimeric G-protein may be a target of GTP[S] in the GTP[S]-stimulated translocation of GLUT4.

The next question concerns cross-talk of the pathways of insulin-, PMA- and GTP[S]-stimulated GLUT4 translocation. As shown in Table 1, PI 3-kinase (composed of p110 and p85) was not activated by PMA or GTP[S] in CHO-GLUT4myc·IR cells. In PC12 cells, however, PI 3-kinase was reported to be a direct target of ras [56]. This discrepancy may derive from differences in cell systems or assay conditions. Recently two other PI 3-kinases (G-protein-coupled PI 3-kinase and phosphatidylinositol-specific 3-kinase) have been cloned [57,58], in addition to conventional PI 3-kinase. The G-protein-coupled PI 3-kinase does not seem to be important in GLUT4 translocation in CHO cells, since pertussis toxin pretreatment, which blocks the coupling between PI 3-kinase and G-proteins, had no effect on insulin- or GTP[S]-stimulated GLUT4myc translocation (K. Kishi, H. Hayashi and Y. Ebina, unpublished work). The phosphatidylinositol-specific PI 3-kinase needs to be examined concerning possible involvement in GLUT4 translocation, as the yeast homologue plays a crucial role in vacuolar transport of some lysosomal proteins [59].

On the other hand, a product of PI 3-kinase, phosphatidylinositol 3,4,5-trisphosphate, has been reported to activate the $\zeta$-isoform of protein kinase C [60]. However, our data did not indicate that protein kinase C was downstream of PI 3-kinase. CHO cells may not contain $\zeta$-type protein kinase C, or activation of this isoform by phosphatidylinositol 3,4,5-trisphosphate or down-regulation by PDBu may not be sufficient *in vivo*. Other researchers have also noted that insulin stimulates GLUT4 translocation independent of protein kinase C activation in adipocytes [29,31].

In conclusion, we have shown that insulin (via PI 3-kinase), GTP[S] [possibly via heterotrimeric GTP-binding protein(s)] and PMA (via protein kinase C) trigger GLUT4 translocation through independent pathways, as depicted in Figure 5.

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