G_i-mediated translocation of GLUT4 is independent of p85/p110 α and p110 γ phosphoinositide 3-kinases but might involve the activation of Akt kinase

Lihong WANG*, Hideki HAYASHI*, Kazuhiro KISHI*, Luping HUANG*, Akifumi HAGI*, Keisuke TAMAOKA*, Phillip T. HAWKINS† and Yousuke EBINA*¹

*Division of Molecular Genetics, Institute for Enzyme Research, University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan, and †Inositol Laboratory, Department of Signalling, Babraham Institute, Cambridge CB2 4AT, U.K.

Activation of phosphoinositide 3-kinase (PI-3K) is essential for insulin-stimulated translocation of GLUT4 and glucose transport in insulin target tissues. A novel p110 γ PI-3K was reported to be activated by G_i-coupled receptors via G $\beta\gamma$ subunits. We asked whether the stimulation of G_i-coupled receptors would trigger GLUT4 translocation and glucose uptake by the activation of G $\beta\gamma$ -dependent p110 γ PI-3K. We find that this translocation and glucose uptake can be induced by the ligand stimulation of G_i-coupled α_{2A} adrenergic receptor and fMet-Leu-Phe receptor in cells stably expressing these receptors. The noradrenaline ('noradrenaline')- and fMet-Leu-Phe-stimulated GLUT4 translocations were abolished by pretreatment with pertussis toxin. Pretreatment with wortmannin or genistein also inhibited the G_i-mediated GLUT4 translocation. On ligand stimulation of these two kinds of G_i-coupled receptor, although there was a slight increase in PtdIns(3,4,5)P₃ production, activation of either the p85/p110 α PI-3K or G $\beta\gamma$ -dependent p110 γ PI-3K was not

observed even in Chinese hamster ovary cells stably overexpressing exogenous p101/p110 γ . The G_i-mediated GLUT4 translocation was accompanied by activation of the serine-threonine kinase Akt; the inhibitory effects of pertussis toxin, wortmannin and genistein on G_i-mediated GLUT4 translocation paralleled their inhibitory effects on Akt activation. In contrast, the activation of some other G_i-coupled receptors, such as prostaglandin EP3 α receptor and platelet-activating factor receptor, did not cause either pertussis-toxin-sensitive translocation of GLUT4myc or activation of Akt kinase. These results indicate that the ligand stimulation of some G_i-coupled receptors triggers GLUT4 translocation that occurs independently of p85/p110 α -type and p110 γ -type PI-3Ks but might involve the activation of Akt kinase.

Key words: glucose transporter, glucose uptake, trimeric G-proteins.

INTRODUCTION

Insulin increases glucose uptake, mainly by recruiting the insulin-responsive glucose transporter GLUT4 from an intracellular pool to the plasma membrane in adipocytes and muscle cells [1,2]. Activation of the insulin receptor (IR) results in the phosphorylation of insulin receptor substrate 1 (IRS-1) [3] and subsequently activates p85/p110 α phosphoinositide 3-kinase (PI-3K). We have reported that the activation of p85/p110 α -type PI-3K by IRS-1 is important in insulin-stimulated GLUT4 translocation, as determined by using the *c-myc* epitope-tagged GLUT4 (GLUT4myc) [4,5]. However, we also found that the translocation of GLUT4 is divergently regulated not only by insulin but also by other hormones [6]. We reported that stimulation of the G_i-coupled α_{1B} adrenergic receptor caused GLUT4 translocation through an insulin-independent and wortmannin-insensitive pathway [6]. These findings suggested a possible mechanism for the exercise-stimulated GLUT4 translocation in heart muscle [7] and cold-stimulated GLUT4 translocation in brown adipocytes [8]. Noradrenaline ('noradrenaline') is released from sympathetic nerve termini during physical exercise; exposure to cold activates adrenergic receptors present on the cell surface. We also reported that bradykinin triggers GLUT4 translocation in L6 myotubes through trimeric G-protein G_q [9]. Bradykinin is released from muscle cells during physical exercise [10] and might be one factor responsible for exercise-stimulated glucose uptake in skeletal muscles. Mech-

anisms of insulin-independent GLUT4 translocation therefore require more attention. In contrast, the stimulation of G_i-coupled prostaglandin EP3 α receptor does not cause GLUT4 translocation and the G_q/G_i-coupled platelet-activating factor (PAF) receptor-stimulated GLUT4 translocation is not inhibited by pretreatment with pertussis toxin (PTX) [6]. However, these findings do not rule out the possibility that G_i-coupled receptors might transmit signals for GLUT4 translocation because each receptor couples to different subtypes of G α and G $\beta\gamma$ subunits, and that different signals are transmitted (reviewed in [11]). A better understanding of the mechanism by which G-protein mediates glucose transport should pave the way for identifying signalling pathways for the mediation of insulin-stimulated glucose transport.

An active form of Akt kinase, possibly located downstream of PI-3K, has been reported to cause GLUT4 translocation [12–16]. In addition, a novel p110 γ [17] or p101/p110 γ [18] PI-3K has been reported to be regulated by G_i α -linked receptors in a manner that permits the activation of Akt [18]. We therefore investigated whether the activation of G_i-coupled receptors would trigger GLUT4 translocation and, if so, whether the activation of p101/p110 γ PI-3K and Akt is involved. Here we report that stimulation of the G_i-coupled α_{2A} adrenergic receptor and fMet-Leu-Phe receptor can trigger GLUT4 translocation and that glucose uptake is enhanced in cells stably expressing α_{2A} adrenergic and fMet-Leu-Phe receptors. Noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocation accompanied

Abbreviations used: CHO, Chinese hamster ovary; IR, insulin receptor; IRS-1, insulin receptor substrate 1; MAP, mitogen-activated protein; PAF, platelet-activating factor; PTX, pertussis toxin; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate.

¹ To whom correspondence should be addressed (e-mail ebina@ier.tokushima-u.ac.jp).

Figure 1 GLUT4myc translocations induced by activation of the G_i -coupled receptors in CHO cells

CHO-GLUT4myc cells expressing EP3 α prostaglandin E_2 receptor, PAF receptor, α_{2A} adrenergic receptor and fMet-Leu-Phe (fMLP) receptor were treated with each ligand for 10 min at 37 °C after pretreatment without (—) or with 100 ng/ml PTX. The GLUT4myc translocations were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three separate experiments done in triplicate. Abbreviation: NE, noradrenaline.

Akt activation with no evident increase in PI-3K activities (p85/p110 α -type and p110 γ -type). The noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocation and Akt activation were inhibited by PTX, wortmannin and genistein. In addition, ligand stimulation of the G_i -coupled EP3 α receptor and PAF receptor did not lead to Akt activation or to PTX-sensitive GLUT4 translocation. These findings suggest that the noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocations are mediated by some type of G_i -protein and might involve the activation of Akt kinase.

MATERIALS AND METHODS

Cells and materials

The parent cell lines used in this study were CHO-GLUT4myc, a Chinese hamster ovary (CHO) cell line stably expressing GLUT4myc [constructed by inserting a human *c-myc* epitope (14 amino acid residues) into the first ectodomain of GLUT4] 3T3-L1-GLUT4myc (a 3T3-L1 fibroblast line stably expressing GLUT4myc) [4]. The 3T3-L1-GLUT4myc fibroblasts were induced to differentiate into adipocytes as described [4]. CHO-GLUT4myc-EP3 α and CHO-GLUT4myc-PAFR cells were CHO-GLUT4myc cells stably expressing prostaglandin EP3 α receptor [19] and PAF receptor [20] respectively. All other reagents were of analytical grade.

Establishment of stable cell lines specifically expressing G_i -coupled receptors

The human adrenergic receptor α_{2A} [21] and fMet-Leu-Phe receptor [22] were subcloned into a mammalian expression vector, pCXN2 [23]. These plasmids were co-transfected into CHO cells, CHO-GLUT4myc cells and 3T3-L1-GLUT4myc cells with pSV2-*bsr*, a blasticidin S deaminase expression plasmid, and selected

with blasticidin S hydrochloride. Several independent clones stably expressing each kind of receptor were established and designated as follows: CHO-GLUT4myc- α_{2A} AR and CHO-GLUT4myc-fMet-Leu-PheR cells were CHO-GLUT4myc cells stably expressing the α_{2A} adrenergic receptor and fMet-Leu-Phe receptor respectively; 3T3-L1-GLUT4myc- α_{2A} AR and 3T3-L1-GLUT4myc-fMet-Leu-PheR cells were 3T3-L1-GLUT4myc cells stably expressing the α_{2A} adrenergic receptor and fMet-Leu-Phe receptor respectively. 3T3-L1-GLUT4myc-PAFR cells were obtained as described [6]. To express p110 γ stably, CHO-GLUT4myc- α_{2A} AR and CHO-GLUT4myc-fMet-Leu-PheR cells were transfected with pCXN2-p110 γ and pSV2-hph (a hygromycin B phosphotransferase expression plasmid) and selected with hygromycin B. The resultant CHO-GLUT4myc- α_{2A} AR-p110 γ and CHO-GLUT4myc-fMet-Leu-PheR-p110 γ cells stably expressed p110 γ with the α_{2A} adrenergic and fMet-Leu-Phe receptors respectively. To express a constitutively active mutant Ha-ras^{G12V} and a dominant inhibitory mutant Ha-ras^{S17N} under control of the metallothionein promoter, pMT64AA-Ha-ras^{G12V} and pMT64AA-Ha-ras^{S17N} [24] were used for transfection. CHO-GLUT4myc-IR- α_{2A} AR-Ha-ras^{G12V} and CHO-GLUT4myc-IR- α_{2A} AR-Ha-ras^{S17N} stably expressed Ha-ras^{G12V} and Ha-ras^{S17N} under control of the metallothionein promoter respectively. The cells were incubated with 50 μ M ZnSO₄ in medium for 24 h to induce the expression of Ha-ras^{G12V} and Ha-ras^{S17N} before experiments with these cells.

Cell-surface anti-*c-myc* antibody binding assay (GLUT4myc translocation assay)

The CHO-GLUT4myc cells expressing various receptors in 24-well plates were incubated in 500 μ l of Krebs-Ringer/Hepes buffer [4] for 30 min at 37 °C, and then with indicated concentrations of ligands for indicated periods at 37 °C. GLUT4myc translocation was measured as described [4]. The 3T3-L1-GLUT4myc- α_{2A} AR and 3T3-L1-GLUT4myc-fMet-Leu-PheR adipocytes in 24-well plates were incubated in 500 μ l of Krebs-Ringer/Hepes buffer for 20 min at 37 °C and then treated with the indicated concentrations of ligands for the indicated periods at 37 °C. GLUT4myc translocation was measured after fixation with 2% (w/v) paraformaldehyde as described [4].

Measurement of 2-deoxyglucose uptake

Cells in 24-well plates were treated with the indicated concentrations of ligands for the indicated periods at 37 °C. 2-Deoxyglucose uptake was measured as described [4].

Down-regulation of protein kinase C with phorbol 12,13-dibutyrate (PDBu) and pretreatment with PTX, genistein or wortmannin

Cells were pretreated with or without PDBu (100 ng/ml) for 24 h at 37 °C in medium for down-regulating protein kinase C, and with or without PTX (100 ng/ml) for 24 h at 37 °C in medium that abolished G_i -coupled pathway(s). The cells were pretreated with the indicated concentrations of wortmannin or genistein for 20 min at 37 °C before stimulation with ligand.

PI-3K assay

Cell lysates were prepared in a buffer containing 1% (v/v) Nonidet P40, after treatments with the indicated reagents, for 5 min. The cell lysates (200 μ g) were incubated with anti-p110 α antibody (a C-terminal 20-residue peptide), anti-p110 γ antibodies (a peptide, residues 738–756, ENLQNSQLPESFRVPYDPG), and a C-terminal peptide, QFNWFLHLVLGIKQGEKHS),

Figure 2 Dose- and time-dependent GLUT4myc translocation and glucose uptake in response to ligand stimulation in CHO-GLUT4myc and 3T3-L1GLUT4myc cells expressing α_{2A} adrenergic receptor and fMet-Leu-Phe (fMLP) receptor

(A–D) The parent cells (CHO-GLUT4myc cells) and CHO-GLUT4myc- α_{2A} AR/fMet-Leu-Phe cells were incubated with various concentrations of noradrenaline (NE) (A) or fMet-Leu-Phe (C) for 10 min at 37 °C, or with 1 μ M noradrenaline (B) or 300 nM fMet-Leu-Phe (D) for the indicated periods. The GLUT4myc translocations were measured as described in the Materials and methods section. (E–H) 3T3-L1 adipocytes and 3T3-L1GLUT4myc- α_{2A} AR/fMet-Leu-PheR adipocytes were incubated with various concentrations of noradrenaline (E) or fMet-Leu-Phe (G) for 10 min at 37 °C, or with 1 μ M noradrenaline (F) or 300 nM fMet-Leu-Phe (H) for the indicated periods. The uptake of 2-deoxyglucose was measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three separate experiments performed in triplicate.

and with anti-phosphotyrosine antibody (PY20). PI-3K assay was performed by adding the reaction buffer containing 0.2 mg/ml PtdIns, 0.2 mg/ml phosphatidylserine, 120 μ M adenosine, 20 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$ and 10 μ M [γ - ^{32}P]ATP to the extensively washed immune complexes. PI-3K activities are represented as the amount of ^{32}P -labelled PtdIns3P separated on TLC plates [25].

PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 *in vivo*

Cells were labelled with 25 μ Ci [^{32}P]P_i for 90 min and stimulated for 50 s with the indicated ligands. The ^{32}P -labelled phospholipids were extracted with chloroform/methanol/1% (w/v) $HClO_4$ (10:5:3.6, by vol.) and deacylated with methylamine [25,26]. The glycerol-PtdIns(3,4) P_2 and glycerol-PtdIns(3,4,5) P_3 were separated by using a potent anion-exchange SAX HPLC column in a gradient of 0.04–0.85 M $NH_4H_2PO_4$, pH 3.8, for 85 min (pump A, water; pump B, 0.85 M $NH_4H_2PO_4$; flow rate 0.5 ml/min) (a continuous gradient of 5% B at 0 min, 13% B at 20 min, 33% B at 21 min, 44% B at 60 min, 70% B at 61 min, 100% B at 72 min and 100% B at 85 min), then counted in a scintillation counter. The deacylated forms of PtdIns3P, PtdIns4P, PtdIns(3,4) P_2 , PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 were eluted at approx. 23, 27, 48, 52 and 81 min respectively.

Akt kinase assay

Cells were lysed in Laemmli's sample buffer after treatments with the indicated reagents for 10 min. Cell lysates (10 μ g) were analysed with an anti-Akt antibody and an enhanced chemiluminescence system after separation by SDS/PAGE [7% (w/v) gel]. The anti-Akt antibody was prepared by immunizing a rabbit with a C-terminal peptide (SLELDQRTHFPQFSYSASIRE) of rat Akt2. Akt kinase activation was determined as the ratio of phosphorylated Akt (p-Akt) to non-phosphorylated Akt (Akt) plus phosphorylated Akt (p-Akt) on the gel, with the use of a densitometer.

Mitogen-activated protein (MAP) kinase assay

Cells were lysed in Laemmli's sample buffer after treatments with the indicated reagents for 5 min. Cell lysates (10 μ g) were analysed with an anti-(activated MAP kinase) antibody (which detects only phosphorylated MAP kinase), and an enhanced chemiluminescence system, after separation by SDS/PAGE [10% (w/v) gel]. The intensity of the phosphorylated band was measured with a densitometer.

RESULTS

Stimulation of G_i -coupled α_{2A} adrenergic and fMet-Leu-Phe receptors triggered GLUT4myc translocations and glucose uptake by cells expressing these receptors

We found previously that stimulation of the G_i -coupled EP3 α receptor by prostaglandin E_2 did not lead to GLUT4 translocation in CHO-GLUT4myc cells stably expressing prostaglandin EP3 α receptor, and that PAF-stimulated GLUT4 translocation was not inhibited by pretreatment with PTX [6]. We suggested that stimulation of G_i -coupled receptors did not cause GLUT4 translocation. In the present study we further examined ligand-stimulated GLUT4 translocation in cells expressing two other kinds of G_i -coupled receptor, α_{2A} adrenergic and fMet-Leu-Phe receptors. GLUT4myc translocation was triggered by noradrenaline in CHO-GLUT4myc- α_{2A} AR cells and by fMet-Leu-Phe in CHO-GLUT4myc-fMet-Leu-PheR cells (Figure 1).

Figure 3 Effects of PTX, PDBu and wortmannin on insulin-, noradrenaline- and fMet-Leu-Phe (fMLP)-stimulated GLUT4myc translocations in CHO cells

CHO-GLUT4myc- α_{2A} AR or CHO-GLUT4myc-fMet-Leu-PheR cells were pretreated with PTX, PDBu or wortmannin, as described in the Materials and methods section; GLUT4myc translocations were then measured after treatment with 1 μ M noradrenaline (A) or 300 nM fMet-Leu-Phe (B) at 37 °C for 10 min. The GLUT4myc translocations were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for at least three determinations. Abbreviations used: Ins, insulin; NE, noradrenaline.

PTX, an inhibitor of G_i , abolished the noradrenaline- or fMet-Leu-Phe-stimulated GLUT4myc translocation, thus indicating that these translocations were mediated by G_i protein. In addition, the noradrenaline- or fMet-Leu-Phe-stimulated GLUT4myc translocation and glucose uptake occurred in a dose-dependent manner in CHO-GLUT4myc- α_{2A} AR/fMet-Leu-PheR cells and 3T3-L1GLUT4myc- α_{2A} AR/fMet-Leu-PheR adipocytes respectively (Figures 2A, 2C, 2E and 2G). The parent cells, which lack these receptors, showed no response to either noradrenaline or fMet-Leu-Phe. Over the time course, noradrenaline- and fMet-Leu-Phe-stimulated GLUT4myc translocation and glucose uptake occurred within 1 min and reached a maximum in 10 min but the effect of fMet-Leu-Phe was comparatively transient (Figures 2B, 2D, 2F and 2H).

These observations suggest that noradrenaline and fMet-Leu-Phe can stimulate GLUT4 translocation and glucose uptake through a G_i -dependent pathway. To explore signal-transduction mechanisms by which the GLUT4 translocation was mediated by G_i , we used several inhibitors.

Figure 4 Tyrosine phosphorylation of IRS-1, PI-3K activity and PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ production *in vivo* induced by activation of the G_i-coupled receptors and insulin in CHO-GLUT4myc- α_2AAR and CHO-GLUT4myc-fMet-Leu-Phe cells

Tyrosine phosphorylation of IRS-1 was measured by immunoblotting with an anti-phosphotyrosine antibody using the lysate of two cell lines after treating the cells with 1 μ M noradrenaline (NE), 300 nM fMet-Leu-Phe (fMLP) or 300 nM insulin (Ins) at 37 °C for 10 min (A). PI-3K activity was determined in immunoprecipitates by using an anti-phosphotyrosine antibody (B), an anti-p110 α antibody (C) or an anti-p110 γ antibody (D) after treatment of the cells as in (A). For PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ production *in vivo*, the cells were labelled with [³²P]P_i for 90 min at 37 °C and exposed to 1 μ M noradrenaline, 300 nM fMet-Leu-Phe or 300 nM insulin for 50 s at 37 °C. Determination of the amount of PtdIns(3,4,5)P₃ (E) and PtdIns(3,4)P₂ (F) were performed as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three determinations. Significance, as assessed by the Student's *t* test, is indicated as follows: * *P* < 0.05 in comparison with the appropriate control.

Figure 5 Effects of exogenously expressed p110 γ and p101/p110 γ on G_i-mediated GLUT4 translocations and PI-3K activities

Noradrenaline (NE)- and fMet-Leu-Phe (fMLP)-stimulated PI-3K activities precipitated with anti-p110 γ (A, D) or anti-myc-p101 (B, E) antibodies and GLUT4myc translocations were determined in CHO-GLUT4myc- α_{2A} AR and CHO-GLUT4myc-fMet-Leu-PheR cells with or without p110 γ expression. Values are means $\pm$ S.E.M. for at least three determinations.

Effects of PTX, PDBu and wortmannin on G_i-mediated GLUT4myc translocation

As confirmed previously, PI-3K is necessary for insulin-stimulated GLUT4 translocation, and protein kinase C is involved in phorbol 12-myristate 13-acetate (PMA)-stimulated GLUT4 translocation [27]. We next wished to determine which signal pathway is involved in G_i-mediated GLUT4 translocation. In

CHO-GLUT4myc- α_{2A} AR/fMet-Leu-PheR cells, as shown in Figures 3(A) and 3(B), PTX abolished noradrenaline- and fMet-Leu-Phe-stimulated GLUT4myc translocation; pretreatment with the selective PI-3K inhibitor wortmannin (100 nM) inhibited the G_i-mediated GLUT4myc translocation to a large extent. No significant inhibitory effect occurred after pretreatment with PDBu. In contrast, the insulin- and PMA-stimulated GLUT4myc translocations were inhibited by wortmannin and PDBu pre-

Figure 6 Effects of a constitutively active mutant, Ha-ras^{G12V}, and dominant inhibitory Ha-ras^{S17N} expression on noradrenaline-stimulated PI-3K activity, PtdIns(3,4,5)P₃ production and GLUT4myc translocation

CHO-GLUT4myc-IR- α_{2A} AR-Ha-ras^{G12V} and CHO-GLUT4myc-IR- α_{2A} AR-ras^{S17N} cells were incubated with 50 μ M ZnSO₄ in medium for 24 h to induce the expression of Ha-ras^{G12V} and Ha-ras^{S17N}, then stimulated with insulin (Ins) or noradrenaline (NE) for 10 min. PI-3K activity

treatment respectively. These observations suggested that PI-3K might be involved in G_i-mediated GLUT4 translocation; we therefore next examined the noradrenaline- and fMet-Leu-Phe-stimulated PI-3K activity.

Effect of G_i-mediated GLUT4myc translocation on p85/p110 α and p110 γ PI-3K activity and on the production of PtdIns(3,4,5)P₃

The PI-3K family includes several isoforms and p85/p110 α -type PI-3K is important in insulin-stimulated GLUT4 translocation. An isoform of PI-3K, p110 γ [17] or p101/p110 γ [18], was found to be activated by G $\beta\gamma$, including $\beta_1\gamma_2$, G $\beta\alpha$ and G α . Our investigation focused on these two types of PI-3K and their product, PtdIns(3,4,5)P₃. The PI-3K activity induced by G_i-coupled receptors was determined and compared with insulin-stimulated activity. Insulin treatment led to the phosphorylation of IRS-1 (Figure 4A), activated p85/p110 α -type PI-3K (Figures 4B and 4C) and increased the amount of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ (Figures 4E and 4F). In contrast, noradrenaline and fMet-Leu-Phe had no detectable effect on the phosphorylation of IRS-1, p85/p110 α -type PI-3K activation or PtdIns(3,4)P₂ production but did cause a slight increase in the amount of PtdIns(3,4,5)P₃ ($P < 0.05$). In contrast, there was no detectable activation of p101/p110 γ PI-3K after treatment with insulin, noradrenaline or fMet-Leu-Phe (Figure 4D). It might be that the endogenous p110 γ level was too low to detect or that CHO cells have no endogenous p110 γ .

We next overexpressed p110 γ in CHO-GLUT4myc- α_{2A} AR/fMet-Leu-PheR cells to study the role of p110 γ PI-3K in G_i-mediated GLUT4 translocation. As shown in Figure 5, noradrenaline and fMet-Leu-Phe did not stimulate p110 γ activation, and overexpression of p110 γ had no apparent effect on G_i-mediated GLUT4myc translocations (Figures 5C and 5F). Stephens et al. [18] indicated that p101 might be necessary to activate p110 γ fully with G $\beta\gamma$. The lack of activation of p110 γ by noradrenaline or fMet-Leu-Phe can be explained if CHO cells have no p101. To address this possibility, p101 and p110 γ were stably co-expressed in CHO-GLUT4myc- α_{2A} AR/fMet-Leu-PheR cells. Even though p101/p110 γ was overexpressed, as shown in Figure 5, there was no detectable increase in p101/p110 γ activity by stimulation with noradrenaline or fMet-Leu-Phe; overexpression of p101/p110 γ had no detectable effect on the GLUT4myc translocation induced by noradrenaline and fMet-Leu-Phe, which means that p110 γ was not activated by noradrenaline or fMet-Leu-Phe in CHO cells.

It has been suggested that G_i protein might activate PI-3K by activating Ras, on the basis of findings that G_i-coupled α_{2A} AR can stimulate Ras-dependent MAP kinase activation and Ras can probably regulate PI-3K [28,29]. To confirm that the G_i-mediated GLUT4 translocations occurred without the involvement of activation of p85/p110 α -type PI-3K via Ras, we examined G_i-mediated GLUT4 translocation after expressing a constitutively active mutant Ha-ras^{G12V} or a dominant inhibitory mutant Ha-ras^{S17N} in CHO-GLUT4myc-IR- α_{2A} AR cells [24]. The expression of Ha-ras^{G12V} elevated basal MAP kinase activity and the expression of Ha-ras^{S17N} inhibited insulin- and noradrenaline-stimulated MAP activation (results not shown) but the expression of Ha-ras^{G12V} or Ha-ras^{S17N} had no significant effects on the noradrenaline-stimulated p85/p110 α PI-3K activity, PtdIns(3,4,5)P₃ accumulation or GLUT4myc trans-

(A), PtdIns(3,4,5)P₃ production (B) and GLUT4 translocation (C) were determined as described in the Materials and methods section. Values are means $\pm$ S.E.M. for at least three determinations.

Figure 7 Effects of PTX, wortmannin and genistein on Akt activation induced by ligand stimulation of G_i -coupled receptors

CHO-GLUT4myc cells (A–D) or 3T3-L1-GLUT4myc adipocytes (E, F) expressing prostaglandin EP3 α R, PAF, α_{2A} adrenergic and fMet-Leu-Phe (fMLP) receptors were treated with each ligand for 10 min after pretreatment without (–) or with PTX, wortmannin or genistein as described in the Materials and methods section. Immunoblotting was performed with rabbit anti-(rat Akt 2) antibody (A, C) or anti-(phospho-Akt) antibody (B, D, E, F). Abbreviation: noradrenaline, NE.

location (Figure 6). These observations indicated that the G_i -mediated GLUT4 translocations occurred without involving the activation of p85/p110 α -type PI-3K through Ras.

Taken together, these observations suggest that the stimulation of G_i -coupled α_{2A} AR and fMet-Leu-PheR triggers GLUT4 translocation without the involvement of the activation of p85/p110 α -type and p101/p110 γ -type PI-3Ks. However, a slight increase in PtdIns(3,4,5) P_3 production was observed, an increase that might not be vital for G_i -mediated GLUT4 translocation (see the Discussion section).

G_i -mediated GLUT4myc translocation is closely related to Akt activation

It has been shown [15] that a serine–threonine kinase activation of Akt is involved in insulin-stimulated GLUT4 translocation and glucose uptake. We next investigated the effect of noradrenaline and fMet-Leu-Phe on Akt phosphorylation and the relationship between Akt activation and G_i -mediated GLUT4 translocation. As shown in Figures 7(A) and 7(B), ligand stimulation of α_{2A} adrenergic and fMet-Leu-Phe receptors led to a mobility shift of Akt kinase on the SDS/PAGE gel. Slowly migrating forms indicate phosphorylated or activated Akt [30,31]; the upper and lower bands represent the phosphorylated

Akt (p-Akt) and unphosphorylated Akt (Akt) respectively. Wortmannin is an inhibitor of PI-3K; genistein, a relatively specific inhibitor of tyrosine kinase, inhibits the G_i -mediated activation of some tyrosine kinase(s) [32]. Pretreatment with PTX (Figures 7A and 7B), wortmannin or genistein completely inhibited Akt phosphorylation (Figures 7C and 7D), paralleling its inhibitory effect on ligand-stimulated GLUT4 translocation (Figure 8), whereas ligand stimulation of EP3 α receptor and PAF receptor had no effect on either the activation of Akt kinase or the PTX-sensitive translocation of GLUT4 (Figure 1 and Figures 7A and 7B). In the 3T3-L1-adipocyte cell lines, Akt phosphorylation by noradrenaline or fMet-Leu-Phe was observed; these activations were inhibited by pretreatment with PTX, wortmannin or genistein (Figures 7E and 7F). Furthermore, the noradrenaline- or fMet-Leu-Phe-stimulated Akt activation inhibited by genistein pretreatment with no effect on PtdIns(3,4,5) P_3 production and by wortmannin with a decrease in PtdIns(3,4,5) P_3 production (Figure 9). Therefore the activation of Akt kinase might be involved in G_i -mediated GLUT4 translocation and that noradrenaline or fMet-Leu-Phe stimulate Akt activation by a signal pathway sensitive to wortmannin and genistein.

In contrast, noradrenaline or fMet-Leu-Phe stimulated GLUT4myc translocation to the same extent as the insulin-stimulated

Figure 8 Effects of genistein and wortmannin on insulin-, noradrenaline- and fMet-Leu-Phe (fMLP)-stimulated GLUT4myc translocations in CHO cells

GLUT4myc translocations were measured after treatment with buffer (○), 300 nM insulin (Δ) or 1 μM noradrenaline (●) for 10 min in CHO-GLUT4myc- α_{2A} AR cells after preincubation with various concentrations of genistein (A) or wortmannin (B) for 20 min. In CHO-GLUT4myc-fMet-Leu-PheR cells, treatment with buffer (○), 300 nM insulin (Δ) or 300 nM fMet-Leu-Phe (●) for 10 min was applied after preincubation with genistein (C) or wortmannin (D) for 20 min. In (B) and (D) the insulin-, noradrenaline- or fMet-Leu-Phe-stimulated GLUT4myc translocations in the absence of inhibitors are set at 100%. Values are means $\pm$ S.E.M. for at least three determinations.

translocation but stimulated Akt phosphorylation to a smaller extent (Figures 10B and 10C). As shown in Figures 8(A) and 8(C), pretreatment with genistein inhibited the noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocations in a dose-dependent manner but the inhibitory effect of genistein was incomplete (90%) even at 150 μM. Pretreatment with genistein up to 150 μM had only a limited inhibitory effect (20%) on insulin-stimulated GLUT4myc translocation. Wortmannin or genistein did not completely inhibit the G_i-mediated GLUT4myc translocation (Figure 8), which means that an additional signalling pathway might be involved in mediating the noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocation. As shown in Figures 7(E) and 7(F), Akt phosphorylation stimulated by noradrenaline or fMet-Leu-Phe were also observed in the 3T3-L1 adipocyte cell lines; the time course was similar to that shown in Figure 10(B) (results not shown). The noradrenaline- and insulin-stimulated GLUT4 translocations were approx. 4-fold and 17-fold respectively 10 min after stimulation of the adipocyte cell line.

The G_i-mediated GLUT4 translocation might involve a tyrosine-phosphorylation process, because genistein inhibited the noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocations. To determine which proteins showed an increase in tyrosine phosphorylation on G_i stimulation in CHO cells, we performed immunoblotting with anti-phosphotyrosine antibodies. As shown in Figure 11, stimulation with noradrenaline

and fMet-Leu-Phe induced an increase in phosphorylation of two proteins of molecular masses 85 and 180 kDa between 60 and 200 kDa. The time course showed that the two phosphorylated bands appeared 40 s after stimulation with agonist and were attenuated from 300 s. Pretreatment with PTX and wortmannin completely inhibited the tyrosine phosphorylation of the 180 kDa band and attenuated p85 phosphorylation, thus correlating their inhibitory effects on G_i-mediated GLUT4 translocation. These observations suggest that wortmannin also inhibits one or more other tyrosine kinases in addition to PI-3K. Interestingly, genistein had practically no inhibitory effects on p180 and p85; genistein might therefore inhibit the molecular signalling events downstream of these two proteins.

DISCUSSION

Our evidence suggests that the activation of G_i-coupled α_{2A} adrenergic and fMet-Leu-Phe receptors can trigger GLUT4 translocation and glucose uptake through an insulin-independent pathway. It has been proposed that insulin-stimulated GLUT4 translocation is mediated by the signal transduction pathway in which IRS proteins and PI-3K (p85/p110) as well as Akt (protein kinase B) are involved. Our study suggests that: (1) GLUT4 translocation can be triggered by the activation of G_i-coupled α_{2A} adrenergic and fMet-Leu-Phe receptors; (2) this G_i-mediated GLUT4 translocation occurs independently of p85/p110 α -type

Figure 9 Effects of genistein and wortmannin on noradrenaline- and fMet-Leu-Phe-stimulated PtdIns(3,4,5)P₃ production and Akt phosphorylation in CHO cells

CHO-GLUT4myc-α_{2A}AR cells (**A, C**) or CHO-GLUT4myc-fMet-Leu-PheR cells (**B, D**) were pretreated with buffer (—), wortmannin or genistein as described in the Materials and methods section, then treated with 1 μM noradrenaline (NE) or 300 nM fMet-Leu-Phe (fMLP) at 37 °C for 10 min. PtdIns(3,4,5)P₃ (PI-3,4,5-P₃) production (**A, B**) and Akt phosphorylation (**C, D**) were determined as described in the Materials and methods section. Values are means ± S.E.M. for at least three determinations.

and p110γ-type PI-3Ks; (3) Akt kinase might be involved in G_i-mediated GLUT4 translocation. We used mainly the CHO cell system; although these cells have no endogenous GLUT4, they do seem to possess basic machinery for the insulin-stimulated translocation of exogenously expressed GLUT4 that mimics that of adipocytes [33–35].

PI-3K, a term that generally refers to the p85/p110 type, is required for insulin-stimulated glucose transport. We developed a novel method of detecting the translocation of GLUT4 directly and simply by using c-myc epitope-tagged GLUT4 (GLUT4myc) and showed that PI-3K is important in insulin-stimulated GLUT4 translocation [4,5]. Several lines of evidence support the requirement for PI-3K in insulin-stimulated GLUT4 translocation in adipocytes [36,37]; most evidence has been derived from findings that wortmannin at 100 nM [26] and LY294002 at 100 μM [38] were relatively selective inhibitors of PI-3K. In the present study, G_i-mediated GLUT4 translocation was sensitive to wortmannin; the ligand stimulation of G_i-coupled α_{2A} adrenergic and fMet-Leu-Phe receptors slightly increased the amount of PtdIns(3,4,5)P₃ in the cells (Figure 4E). These results suggest an involvement of PI-3K; however, this kinase apparently does not have an important role in G_i-mediated GLUT4 translocation. On ligand stimulation there was no significant increase in either p85/p110α PI-3K activity with CHO-GLUT4myc-α_{2A}AR/fMet-Leu-PheR cells or p110γ-type PI-3K activity with CHO-

GLUT4myc-α_{2A}AR/fMet-Leu-PheR cells expressing exogenous p101/p110γ. The expression of p101/p110γ had no effect on G_i-mediated GLUT4 translocation, indicating that p85/p110α-type PI-3K and p110γ-type PI-3K are not regulated by G_i-coupled α_{2A}AR and fMet-Leu-PheR; neither are they involved in G_i-mediated GLUT4 translocation in these cells. Wortmannin at 100 nM also inhibited the noradrenaline- and fMet-Leu-Phe-induced tyrosine phosphorylation of some proteins (Figure 11), implying that wortmannin can inhibit tyrosine kinase as well as PI-3K. It was reported that the amount of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ paralleled PI-3K's capacity to activate the downstream target [16,39]. In the present study the activation of G_i had no effect on the accumulation of PtdIns(3,4)P₂ (Figure 4F) and stimulated a very limited increase in amount of PtdIns(3,4,5)P₃ compared with that stimulated by insulin, even though both insulin and G_i triggered GLUT4 translocation to similar extents (Figure 10). Genistein inhibited the noradrenaline- or fMet-Leu-Phe-induced activation of Akt and translocation of GLUT4 with no inhibitory effect on the accumulation of PtdIns(3,4,5)P₃ (Figure 9). Taken together, these results indicate that the effect of PI-3K is very limited.

Other studies [15,40–42] have reported data supporting the role for Akt in insulin-stimulated GLUT4 translocation. Our results indicate that noradrenaline and fMet-Leu-Phe can induce Akt activation through a wortmannin-sensitive or genistein-

Figure 10 Relationships between PtdIns(3,4,5)P₃ production, Akt activation and GLUT4myc translocation induced by insulin (▲), noradrenaline (○) and fMet-Leu-Phe (△) in CHO cells

In CHO-GLUT4myc cells expressing α_{2A} adrenergic receptor and fMet-Leu-Phe receptor, the time courses for PtdIns(3,4,5)P₃ (PI-3,4,5-P₃) production (A), Akt kinase activation (B) and GLUT4myc translocation (C) induced by insulin, noradrenaline or fMet-Leu-Phe were determined as described in the Materials and methods section. Values are means $\pm$ S.E.M. for at least three determinations.

sensitive pathway. The activated Akt kinase was also involved in G_i-mediated GLUT4 translocation, which was accompanied by Akt activation; the inhibitory effects of PTX, wortmannin and genistein on this translocation occurred with their inhibitory effects on Akt activation. In contrast, one or more other signal pathways besides the Akt pathway might mediate noradrenaline- and fMet-Leu-Phe-stimulated GLUT4 translocation, because noradrenaline or fMet-Leu-Phe stimulated less Akt activity than

insulin did, even though both insulin and G_i triggered GLUT4 translocation to similar extents (Figures 10B and 10C) and partial GLUT4 translocation remained even though Akt activity was completely inhibited (Figures 7C, 7D and 8).

Genistein, a tyrosine kinase inhibitor, inhibited the insulin-stimulated GLUT4 translocation as well as guanosine 5'-[γ -thio]triphosphate-stimulated GLUT4 translocation [43,44], indicating that tyrosine kinase is involved in both insulin-stimulated and G-protein-mediated GLUT4 translocation. In our study, pretreatment with 150 μ M genistein inhibited the noradrenaline/fMet-Leu-Phe-stimulated GLUT4 translocation by 90% and insulin-stimulated GLUT4 translocation by 20% in CHO cells (Figures 8A and 8C). The different sensitivities to this inhibitor suggested that different signalling pathways are involved in mediating insulin- and noradrenaline/fMet-Leu-Phe-stimulated GLUT4 translocations.

In immunoblotting with anti-phosphotyrosine antibody, stimulation with noradrenaline and fMet-Leu-Phe resulted in an increase in the phosphorylation of two proteins of molecular mass 85 and 180 kDa in the range 60–200 kDa (Figure 11). Pretreatment with PTX and wortmannin, but not genistein, inhibited the tyrosine phosphorylation of 180 and 85 kDa. These two proteins might be candidates for the signal transduction of G_i-mediated GLUT4 translocation. By using an assay with a glutathione S-transferase fusion protein, we observed that the p85 protein showed a significant increase in tyrosine phosphorylation after treatment with noradrenaline or fMet-Leu-Phe. The increased tyrosine phosphorylation of p85 created binding sites that were recognized *in vitro* by the SH2 domain of CrkII (results not shown), a key adaptor protein involved in cell differentiation and transformation [45]. Further investigation is required for the identification of these two kinds of protein and their roles in G_i-mediated GLUT4 translocation.

We examined four kinds of G_i-coupled receptor, prostaglandin EP3 α , PAF, adrenergic α_{2A} and fMet-Leu-Phe receptors. As with PTX-sensitive GLUT4 translocation and Akt activation, EP3 α and PAF receptors differed from α_{2A} and fMet-Leu-Phe receptors (Figure 1). Ligand stimulation of α_{2A} and fMet-Leu-Phe receptors caused membrane ruffling (H. Hayashi, L. Wang, A. Hagi, L. Huang and Y. Ebina, unpublished work) and enhanced glycogen synthesis (H. Hayashi, L. Wang, A. Hagi, L. Huang, T. Ohnishi and Y. Ebina, unpublished work), but the stimulation of EP3 α and PAF receptors had almost no effect on membrane ruffling or glycogen synthesis. There are numerous other G_i-coupled receptors in addition to these four [11]. The G α_i class (PTX-sensitive) is composed of at least eight different molecules [11]. Five G β and eleven G γ types are reported to show binding specificity, e.g. β_1 interacts with γ_1 , γ_2 and γ_3 ; β_2 interacts with γ_2 and γ_3 but not with γ_1 ; and β_3 does not interact with γ_1 , γ_2 or γ_3 [11,46]. However, there is limited information on which receptor couples to which G α_i and which G $\beta\gamma$ combination [11]. Our results indicate that the G_i-coupled receptors could be classified into at least two groups on the basis of their capacities to activate Akt kinase, translocate GLUT4, cause ruffling and enhance glycogen synthesis. In addition, these differences aid in identifying G α_i types and G $\beta\gamma$ combinations coupling to specific receptors.

Some types of G_i-coupled receptor, including α_{2A} adrenergic receptor and GLUT4, are expressed in endothelial cells lining capillaries of skeletal muscles [47]. The GLUT4 in capillaries translocates from an intracellular compartment to the luminal plasma membrane in response to insulin [47]. Therefore the translocation of GLUT4 to the luminal plasma membrane by noradrenaline via α_{2A} adrenergic receptor and G_i during physical exercise could contribute to some extent to the uptake of glucose

Figure 11 Tyrosine phosphorylation of discrete proteins induced by ligand-stimulation of G_i -coupled α_{2A} adrenergic and fMet-Leu-Phe receptors in CHO cells

CHO-GLUT4myc- α_{2A} AR cells (**A**) and CHO-GLUT4myc-fMet-Leu-PheR cells (**B**) were pretreated with buffer (—), PTX, wortmannin or genistein as described in the Materials and methods section, then treated with $1 \mu\text{M}$ noradrenaline (NE) or 300 nM fMet-Leu-Phe (fMLP) at 37°C for the indicated periods. Cell lysates were prepared and subjected to Western blots with the use of the PY20 monoclonal anti-phosphotyrosine antibody.

required for energy supply during exercise. We have obtained evidence that GLUT1 also translocates from an intracellular pool (albeit a small portion) to the cell surface in response to fMet-Leu-Phe and noradrenaline in cells expressing α_{2A} adrenergic and fMet-Leu-Phe receptors respectively (A. Hagi, H. Hayashi and Y. Ebina, unpublished work). This also suggests that G_i -proteins are involved in glucose metabolism. G_i -mediated GLUT1 translocations might explain the fMet-Leu-Phe-stimulated glucose uptake in leucocytes for energy supply on chemotaxis [48].

We found that the activation of G_i -coupled α_{2A} adrenergic and fMet-Leu-Phe receptors can trigger GLUT4 translocation and glucose uptake through an insulin-independent pathway. In addition to the physiological role of G_i -mediated GLUT4 translocation, it is important to elucidate mechanisms of insulin-independent GLUT4 translocation because clarifying the mechanism by which G_i mediates the glucose transport will lead to a better understanding of signalling pathways that mediate insulin-stimulated glucose transport. The effective activation of insulin-independent pathways can be considered when designing therapy for insulin-resistant states of non-insulin-dependent diabetes mellitus [49].

We thank Dr. J. Miyazaki, Dr. G. Tsujimoto, Dr. R. J. Lefkowitz, Dr. H. Kurose, Dr. F. Boulay, Dr. T. Shimizu and Dr. G. M. Cooper for providing pCXN2 plasmid, cDNA species encoding α_{2A} adrenergic receptor, fMet-Leu-Phe receptor, PAF receptor and Ha-ras^{S17N}, and Dr. M. D. Waterfield, Dr. B. A. Hemmings, Dr. O. Hazeki and Dr. M. Ohara for comments. This work was supported by research grants from the Ministry

of Education, Science, Sports and Culture of Japan; for diabetes research from the Ministry of Health and Welfare; for diabetes research from Otsuka Pharmaceutical, Tokushima, Japan; from 'The Mitsubishi Foundation' (to Y.E.); and from 'The Kato Memorial Bioscience Foundation' (to H.H.).

REFERENCES

- 1 Suzuki, K. and Kono, T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2542–2545
- 2 Cushman, S. W. and Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762
- 3 Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) *Nature (London)* **352**, 73–77
- 4 Kanai, F., Nishioka, Y., Hayashi, H., Kamohara, S., Todaka, M. and Ebina, Y. (1993) *J. Biol. Chem.* **268**, 14523–14526
- 5 Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993) *Biochem. Biophys. Res. Commun.* **195**, 762–768
- 6 Kishi, K., Hayashi, H., Wang, L., Kamohara, S., Tamaoka, K., Shimizu, T., Ushikubi, F., Narumiya, S. and Ebina, Y. (1996) *J. Biol. Chem.* **271**, 26561–26568
- 7 Fischer, Y., Thomas, J., Holman, G. D., Rose, H. and Kammermeier, H. (1996) *Am. J. Physiol.* **270**, C1204–C1210
- 8 Omatsu-Kanbe, M. and Kitasato, H. (1992) *FEBS Lett.* **314**, 246–250
- 9 Kishi, K., Murotomo, N., Nakaya, Y., Miyata, I., Hagi, A., Hayashi, H. and Ebina, Y. (1998) *Diabetes* **47**, 550–558
- 10 Rett, K., Wicklmayr, M., Fink, E., Maerker, E., Dietze, G. and Mehnert, H. (1989) *Biol. Chem. Hoppe Seyler* **370**, 445–449
- 11 Neer, E. J. (1995) *Cell* **80**, 249–257
- 12 Bellacosa, A., Franke, T. F., Gonzalez-Portal, M. E., Datta, K., Taguchi, T., Gardner, J., Cheng, J. Q., Testa, J. R. and Tsichlis, N. (1993) *Oncogene* **8**, 745–754
- 13 Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) *Cell* **81**, 727–736
- 14 Burgering, B. M. T. and Coffey, P. J. (1995) *Nature (London)* **376**, 599–602

- 15 Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996) *J. Biol. Chem.* **271**, 31372–31378
- 16 Franke, T. F., Kaplan, D. R., Cantley, L. C. and Toker, A. (1997) *Science* **275**, 665–668
- 17 Stoyanov, B., Volina, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhan, R., Nürnberg, B. et al. (1995) *Science* **269**, 690–693
- 18 Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Cadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P. and Hawkins, P. T. (1997) *Cell* **89**, 105–114
- 19 Sugimoto, Y., Negishi, M., Hayashi, Y., Namba, T., Honda, A., Watabe, A., Hirata, M., Narumiya, S. and Ichikawa, A. (1993) *J. Biol. Chem.* **268**, 2712–2718
- 20 Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T. and Shimizu, T. (1991) *Nature (London)* **349**, 342–346
- 21 Kobilka, B. K., Matsui, H., Kobilka, T. S., Yang-Feng, T. L., Francke, U., Caron, M. G., Lefkowitz, R. J. and Regan, J. W. (1987) *Science* **238**, 650–656
- 22 Boulay, F., Tardif, M., Bouchon, L. and Vignais, P. (1990) *Biochemistry* **29**, 11123–11133
- 23 Niwa, H., Yamamura, K. and Miyazaki, J. (1991) *Gene* **108**, 193–199
- 24 Szeberényi, J., Cai, H. and Cooper, J. M. (1990) *Mol. Cell. Biol.* **10**, 5324–5332
- 25 Hayashi, H., Miyake, N., Kanai, F., Shibasaki, F., Takenawa, T. and Ebina, Y. (1991) *Biochem. J.* **280**, 769–775
- 26 Okada, T., Sakuma, L., Fukui, Y., Hazeki, O. and Ui, M. (1994) *J. Biol. Chem.* **269**, 3563–3567
- 27 Todaka, M., Hayashi, H., Imanaka, T., Mitani, Y., Kamohara, S., Kishi, K., Tamaoka, K., Kanai, F., Shichiri, M., Morii, N. et al. (1996) *Biochem. J.* **315**, 875–882
- 28 Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M. and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 19125–19132
- 29 Rodríguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D. and Downward, J. (1994) *Nature (London)* **370**, 527–532
- 30 Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) *Nature (London)* **378**, 785–789
- 31 Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X. F., Han, J. W. and Hemmings, B. A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5699–5704
- 32 Touhara, K., Hawes, B. E., van Bisen, T. and Lefkowitz, R. J. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9284–9287
- 33 Clark, A. E. and Holman, G. D. (1990) *Biochem. J.* **269**, 615–622
- 34 Baldini, G., Hohman, R., Charron, M. J. and Lodish, H. F. (1991) *J. Biol. Chem.* **266**, 4037–4040
- 35 Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992) *J. Cell Biol.* **111**, 1001–1007
- 36 Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Shibasaki, Y., Kikuchi, M., Yazaki, Y. and Oka, Y. (1996) *J. Biol. Chem.* **271**, 16987–16990
- 37 Frevert, E. U. and Kahn, B. B. (1997) *Mol. Cell. Biol.* **17**, 190–198
- 38 Vlahos, C. V., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) *J. Biol. Chem.* **269**, 5241–5248
- 39 Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997) *Science* **277**, 567–570
- 40 Summers, S. A. and Birnbaum, M. J. (1997) *Biochem. Soc. Trans.* **25**, 981–988
- 41 Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I. and Quon, M. J. (1997) *Mol. Endocrinol.* **11**, 1881–1890
- 42 Summers, S. A., Garza, L. A., Zhou, H. and Birnbaum, M. J. (1998) *Mol. Cell. Biol.* **18**, 5457–5464
- 43 Chen, D., Elmendorf, J. S., Olson, A. L., Li, X., Earp, H. S. and Pessin, J. E. (1997) *J. Biol. Chem.* **272**, 27401–27410
- 44 Elmendorf, J. S., Chen, D. and Pessin, J. E. (1998) *J. Biol. Chem.* **273**, 13289–13296
- 45 Petruzzelli, L., Takami, M. and Herrera, R. (1996) *J. Biol. Chem.* **271**, 7796–7801
- 46 Ray, K., Kunsch, C., Bonner, L. M. and Robinshaw, J. D. (1995) *J. Biol. Chem.* **270**, 21765–21771
- 47 Vilaro, S., Palacin, M., Pilch, P. F., Testar, X. and Zorzano, A. (1989) *Nature (London)* **342**, 798–800
- 48 Okuno, Y. and Gliemann, J. (1988) *Biochim. Biophys. Acta* **941**, 157–164
- 49 Kahn, C. R. (1994) *Diabetes* **43**, 1066–1084

Received 17 May 1999/11 October 1999; accepted 12 November 1999