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Platelet-derived growth factor triggers translocation of the insulin-regulatable glucose transporter (type 4) predominantly through phosphatidylinositol 3-kinase binding sites on the receptor

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ABSTRACT **Insulin is the only known hormone which rapidly stimulates glucose uptake in target tissues, mainly by translocation to the cell surface of the intracellular insulin-regulatable glucose transporter (glucose transporter type 4, GLUT4). We have developed a cell line for direct, sensitive detection of GLUT4 on the cell surface. We have suggested that insulin-activated phosphatidylinositol (PI) 3-kinase may be involved in the signaling pathway of insulin-stimulated GLUT4 translocation. We report that platelet-derived growth factor (PDGF), which stimulates PI 3-kinase activity, triggers GLUT4 translocation in Chinese hamster ovary (CHO) cells stably overexpressing the PDGF receptor and in 3T3-L1 mouse adipocytes. Using mutant PDGF receptors that cannot bind to Ras-GTPase-activating protein, phospholipase C-γ, and PI 3-kinase, respectively, we obtained evidence that PI 3-kinase binding sites play a key role in the signaling pathway of PDGF-stimulated GLUT4 translocation in the CHO cell system.**

The insulin signaling pathway mediating the translocation of the insulin-regulatable glucose transporter (glucose transporter type 4, GLUT4) is poorly understood (1–4). A defect in this pathway is thought to be one major cause of non-insulin-dependent diabetes mellitus. We developed a simple, direct, sensitive immunological method to detect GLUT4 on the cell surface (5). cDNA encoding GLUT4 with an insert of a c-Myc epitope (14 amino acids) in the first ectodomain (GLUT4myc) was constructed and was stably expressed in 3T3-L1 adipocytes (3T3-L1-GLUT4myc) and in Chinese hamster ovary (CHO) cells (CHO-GLUT4myc). Translocation of GLUT4myc to the cell surface was detected in both cell lines, in binding assays with the anti-c-Myc antibody (5). The chimeric GLUT4myc was translocated to the cell surface in the same manner as GLUT4 in response to insulin, and translocation of GLUT4myc to the cell surface was detectable by a binding assay in CHO cells and 3T3-L1 adipocytes after treatment with insulin, phorbol 12-myristate 13-acetate (PMA), NaF, or guanosine 5′-[γ-thio]triphosphate (5). Therefore, the basic mechanisms involved in GLUT4 translocation in CHO cells seem to be similar to those in 3T3-L1 adipocytes (5).

Wortmannin is an inhibitor of phosphatidylinositol (PI) 3-kinase (6) and inhibits both PI 3-kinase activation and GLUT4myc translocation in response to insulin in the CHO cell system. We suggested that activation of PI 3-kinase may be involved in the signaling pathway of insulin-stimulated GLUT4 translocation (7).

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Platelet-derived growth factor (PDGF) activates PI 3-kinase in mammalian cells (8), and we asked whether PDGF would trigger GLUT4 translocation by PI 3-kinase activation. We report here that PDGF, as well as insulin, triggers GLUT4 translocation in CHO cells stably overexpressing the PDGF receptor (PDGFR) and in 3T3-L1 adipocytes. Use of mutant PDGFRs in the CHO cell system revealed that PI 3-kinase plays a key role in the signaling pathway of PDGF-stimulated GLUT4 translocation.

MATERIALS AND METHODS

Cells and Materials. The parent cell lines used in this study were CHO-GLUT4myc, a CHO cell line that overexpresses GLUT4myc, constructed by inserting a human c-Myc epitope (14 amino acids) into the first ectodomain of GLUT4 (5), and 3T3-L1-GLUT4myc, a 3T3-L1 fibroblast line that overexpresses GLUT4myc and that, like 3T3-L1, can be induced to differentiate into adipocytes (5). Reagents were from commercial sources and were of analytical grade.

Construction of Cell Lines Expressing Wild-Type and Mutant PDGFRs. The parent cells were cotransfected with mouse wild-type or mutant PDGFRβ expression plasmid pSVRI-PDGFR (9, 10) and the hygromycin B phosphotransferase expression plasmid pSV2-hph (11). The candidate clones were selected with hygromycin B. The numbers of wild-type and mutant PDGFRs were estimated from binding of ^{125}I-labeled PDGF and from immunoblot analysis using an anti-PDGFR antibody.

PI 3-Kinase Assay. Confluent CHO-GLUT4myc-PDGFR cells were treated with or without PDGF-BB (50 ng/ml) for 10 min at 37°C, lysed in buffer containing 1% (vol/vol) Nonidet P-40 (12), and immunoprecipitated with an anti-phosphotyrosine antibody (7) and protein A-Sepharose. The immunoprecipitates were washed and subjected to the PI 3-kinase assay as described (12).

GLUT4myc Translocation Assay Using Cell Surface Anti-c-Myc Antibody Binding. CHO-GLUT4myc-PDGFR cells were grown overnight in 24-well plates and the subconfluent cells were incubated in 1 ml of KRH buffer (5) for 30 min at 37°C and then with the indicated concentrations of PDGF-BB for 30 min at 37°C. GLUT4myc translocation was measured as described (5). The GLUT4myc translocation in 3T3-L1-GLUT4myc adipocytes was measured after fixation with 2% paraformaldehyde (5).

Abbreviations: GLUT4, glucose transporter type 4; PI, phosphatidylinositol; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PMA, phorbol 12-myristate 13-acetate; PBt_2, phorbol 12,13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; GAP, GTPase-activating protein; IRS-1, insulin receptor substrate 1.

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Downregulation of Protein Kinase C (PKC) by Phorbol 12,13-Dibutyrate (PBt_2) and Wortmannin. CHO-GLUT4myc-PDGFR cells were grown overnight in 24-well plates and the subconfluent cells were incubated in the absence or presence of PBt_2 (100 ng/ml) for 20 hr at 37°C in medium for PKC downregulation (13, 14). For wortmannin treatment, the cells were incubated first with 0.1 μM wortmannin for 30 min at 37°C and then with PMA (1 μM) or PDGF (50 ng/ml) for 30 min at 37°C. GLUT4myc translocation was determined by anti-c-Myc antibody binding assay, as described above.

2-Deoxyglucose Uptake Measurements. Cells in 24-well plates were treated with various concentrations of PDGF or insulin for 30 min at 37°C and 2-deoxyglucose uptake was measured as described (5).

RESULTS AND DISCUSSION

PI 3-Kinase Activation and GLUT4myc Translocation by PDGF. To examine the effect of PDGF on GLUT4 translocation, we used CHO cells and 3T3-L1 adipocytes expressing GLUT4myc, in which a c-Myc epitope (14 amino acids) had been inserted in the first ectodomain of GLUT4, without disrupting GLUT4 function (5). When PDGFRβ (hereafter referred to as PDGFR) was stably overexpressed in the parent CHO-GLUT4myc cells, PDGF autophosphorylated the overexpressed receptors (Fig. 1*A*) and activated PI 3-kinase in these cells (Fig. 1*B*). PDGF-triggered GLUT4myc translocation was observed in cells stably overexpressing PDGFR (Fig. 1*C*). We analyzed several independent CHO-GLUT4myc clones stably overexpressing PDGFR, and the results with these clones were much the same as those shown in Fig. 1.

To examine whether the GLUT4myc translocated by PDGF contributes to glucose uptake by the cells, we prepared CHO cells expressing PDGFR (CHO-PDGFR) or both GLUT4myc and PDGFR (CHO-GLUT4myc-PDGFR). These clones had almost the same number of PDGFRs. The GLUT4myc translocated to the cell surface resulted in stimulation of glucose uptake in response to PDGF (Fig. 1*D*). In addition, the PDGF-stimulated glucose uptake in CHO-GLUT4myc-PDGFR cells increased according to the level of expression of GLUT4myc (S.K., unpublished data). The phenomenon of PDGF-stimulated glucose uptake by human fibroblasts has been reported by Allard *et al.* (15). This glucose uptake is presumably mediated by GLUT1, because human fibroblasts have GLUT1 but no GLUT4 (3, 4, 16). We obtained evidence that PDGF triggers GLUT4myc translocation and stimulates glucose uptake by the translocated GLUT4myc (Fig. 1 *C* and *D*).

We next examined the effects of PDGF on GLUT4myc translocation and glucose uptake in 3T3-L1 adipocytes with endogenous GLUT4 and PDGFR. We previously showed that GLUT4myc in 3T3-L1 adipocytes was translocated in response to insulin and that the translocated GLUT4myc stimulated the uptake of glucose (5). As observed in CHO cells, PDGF triggered GLUT4myc translocation and stimulated glucose uptake in 3T3-L1 adipocytes stably expressing GLUT4myc (3T3-L1-GLUT4myc adipocytes) (Fig. 2). PDGF dose-dependently stimulated GLUT4myc translocation, and the translocation reached a maximum (≈3.5-fold) with PDGF at 50 ng/ml. Insulin also dose-dependently stimulated GLUT4myc translocation, and the translocation reached a maximum (≈7.5-fold) at 100 nM insulin. Insulin at 3 nM led to almost the same degree of GLUT4myc translocation as seen with PDGF at 50 ng/ml. In parallel to the GLUT4myc translocation, PDGF at 50 ng/ml stimulated glucose uptake (≈3-fold), but with 3 nM insulin, a greater amount of glucose was taken up (≈7.5-fold). These results may relate to

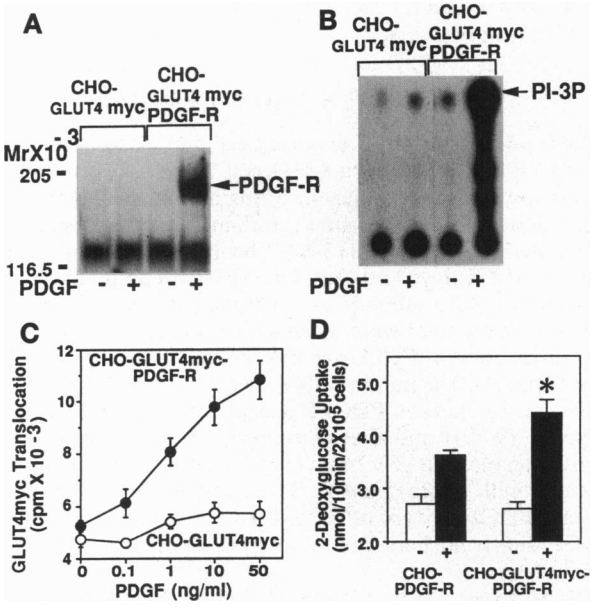

FIG. 1. PDGF-dependent receptor autophosphorylation (*A*), PI 3-kinase activation (*B*), GLUT4myc translocation (*C*), and glucose uptake (*D*). (*A*) PDGF-dependent autophosphorylation of PDGFR. Quiescent CHO-GLUT4myc parent cells and cells overexpressing PDGFR (CHO-GLUT4myc-PDGFR cells) were treated with PDGF-BB (50 ng/ml) for 10 min. The cell lysates (30 μg each) were analyzed by immunoblotting with an anti-phosphotyrosine antibody. (*B*) PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates. The same lysates (100 μg each) as in *A* were immunoprecipitated with the anti-phosphotyrosine antibody and subjected to PI 3-kinase assay. PI-3P, PI 3-phosphate. (*C*) Translocation of GLUT4myc. The CHO-GLUT4myc parent cells (○) and those overexpressing PDGFR (●) were treated with various concentrations of PDGF for 30 min. The amount of cell surface GLUT4myc was determined by anti-c-Myc antibody binding assay. Values are means ± SE for three separate experiments done in triplicate. (*D*) 2-Deoxyglucose uptake in CHO-PDGFR cells (CHO cells overexpressing PDGFR) and CHO-GLUT4myc-PDGFR cells. The two cell lines have almost the same number of PDGFRs. Cells were treated with (+) or without (−) PDGF-BB (50 ng/ml) for 30 min before 2-deoxyglucose uptake assay. Values are means ± SE of three determinants. Asterisk shows significant difference ($P < 0.01$) from PDGF-stimulated 2-deoxyglucose uptake by CHO-PDGFR.

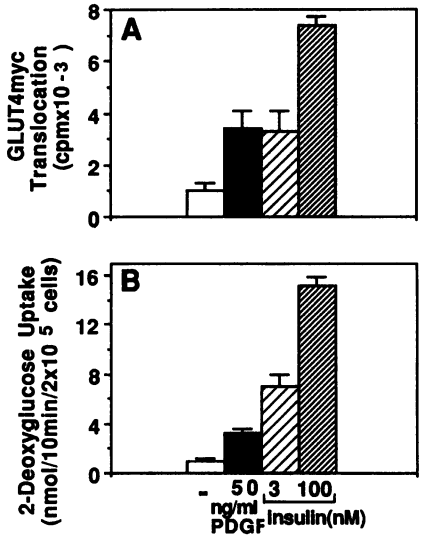

FIG. 2. Functional translocation of GLUT4myc in 3T3-L1 adipocytes. The amount of GLUT4myc on the cell surface (*A*) and 2-deoxyglucose uptake (*B*) were measured after treatment of 3T3-L1-GLUT4myc adipocytes with buffer alone (−), PDGF-BB (50 ng/ml), or 3 or 100 nM insulin for 30 min. Values are means ± SE for three separate experiments done in triplicate. Nonspecific binding was subtracted in *A*.

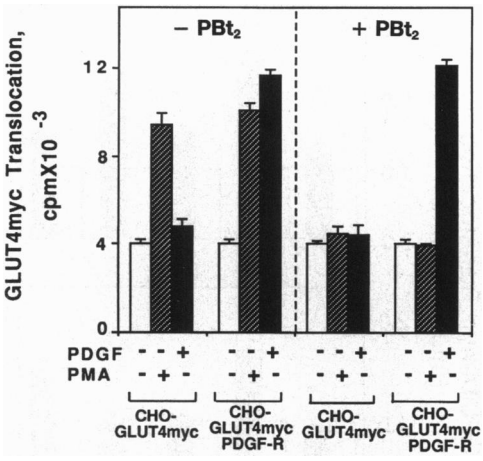

FIG. 3. PDGF-stimulated GLUT4myc translocation after downregulation of PKC by PBt_2. The parental cell line and cells stably overexpressing wild-type PDGFR were treated with PMA (1 μM) or PDGF-BB (50 ng/ml) for 30 min at 37°C after pretreatment with (*Right*) or without (*Left*) PBt_2 (100 ng/ml) for 20 hr at 37°C. The amount of cell surface GLUT4myc was determined. Values are means ± SE for two separate experiments done in triplicate.

activation of the intrinsic activity of GLUT4myc by insulin (16, 17). In addition, both PDGF and insulin activated PI 3-kinase in 3T3-L1 adipocytes (S.K., unpublished data).

We examined molecular mechanisms of signaling pathways that mediate PDGF-stimulated GLUT4 translocation. Ligand-activated PDGFR forms a complex that includes proteins containing the Src homology 2 domain, such as PI 3-kinase (18, 19), phospholipase C-γ (PLCγ) (19, 20), and Ras-GTPase-activating protein (GAP) (18, 19). On the other hand, the insulin-activated insulin receptor phosphorylates insulin receptor substrate 1 (IRS-1) (21–23) and the p85 subunit of PI 3-kinase (24) at tyrosine residues. The phosphorylated IRS-1 forms a complex with PI 3-kinase (12, 21–23), but not with PLCγ (22, 23) or GAP (22, 23). PI 3-kinase is a common molecule which forms complexes with PDGFR or with IRS-1. As yeast PI 3-kinase regulates the intracellular transport of vacuolar proteins (25), this kinase may also play a role in the trafficking of GLUT4 in mammalian cells.

PDGF-Stimulated GLUT4myc Translocation After Downregulation of PKC by PBt_2. We have shown that insulin-activated PI 3-kinase may be involved in insulin-stimulated GLUT4 translocation (7). However, treatment with PMA, an activator of PKC (13, 14), triggers GLUT4 translocation in adipocytes (26, 27) and in our CHO cell system (5). Moreover, PDGF induces phosphorylation of PLCγ at tyrosine residues, increases its activity, and stimulates PI turnover, resulting in PKC activation (8, 13, 14, 19). Therefore, one signaling pathway of PDGF-stimulated GLUT4 translocation is possibly mediated by PLCγ and PKC. When we examined GLUT4myc translocation after treatment with PBt_2, which downregulates PKC (13, 14), effects of PMA on GLUT4myc translocation in CHO cells overexpressing PDGFR were abolished (Fig. 3). Despite the absence of PKC activation in these cells, PDGF continued to trigger GLUT4myc translocation (Fig. 3). Therefore, a signaling pathway(s) other than that mediated by PKC in PDGF-stimulated GLUT4myc translocation may be involved.

PI 3-Kinase Activation and GLUT4myc Translocation Mediated by Wild-Type and Mutant PDGFRs. To identify the molecule which transmits the PDGF-stimulated GLUT4myc translocation, we used CHO-GLUT4myc cells, as they have much smaller amounts of endogenous PDGFR than do 3T3-L1 adipocytes. We stably overexpressed four kinds of mutant mouse PDGFRs in the parent (CHO-GLUT4myc) cells (Fig. 4*A*): (*i*) GAP^-, which cannot bind GAP, due to substitution of Phe for Tyr^{739} (18, 19); (*ii*) $PLC\gamma^-$, which cannot bind PLCγ,

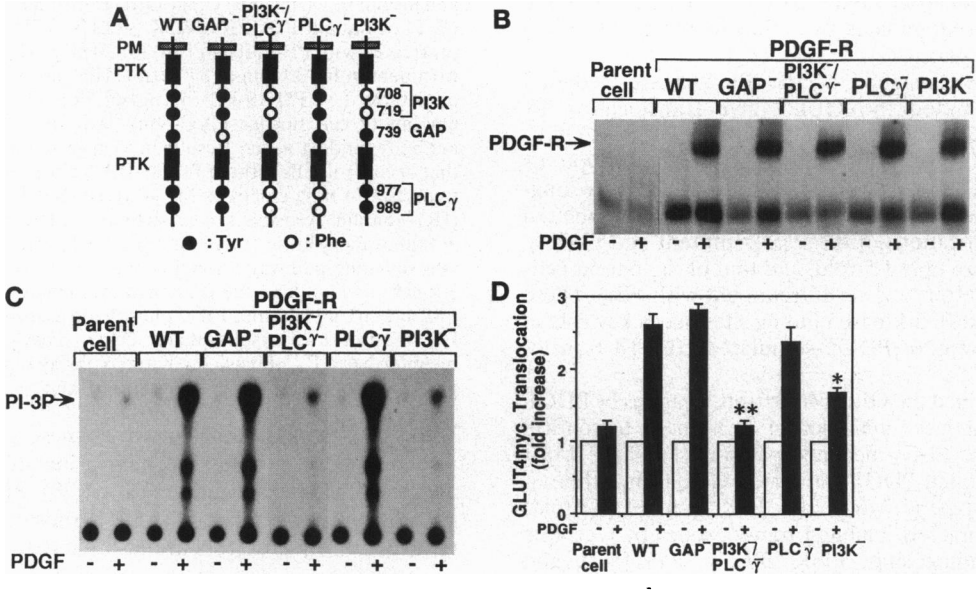

FIG. 4. Wild-type and mutant PDGFRs (*A*), PDGF-dependent receptor autophosphorylation (*B*), PI 3-kinase activation (*C*), and GLUT4myc translocation (*D*) in CHO-GLUT4myc cells expressing those receptors. (*A*) Filled circles represent the tyrosine residues in the mouse PDGFRβ cytoplasmic domain. The proteins that bind to these sites are indicated to the right of the residue number. Open circles represent Tyr → Phe substitutions. The mutant PDGFRs were designated by their binding specificities, being unable to bind GAP (GAP^-), neither PI 3-kinase nor PLCγ ($PI3K^-/PLC\gamma^-$), PLCγ ($PLC\gamma^-$), or PI 3-kinase ($PI3K^-$). WT, wild type; PM, plasma membrane; PTK, protein-tyrosine kinase domain. (*B*) PDGF-dependent receptor autophosphorylation. CHO-GLUT4myc cells overexpressing wild-type or mutant PDGFRs were incubated with (+) or without (−) PDGF-BB (50 ng/ml) for 10 min. The cell lysates (30 μg each) were analyzed by immunoblotting with an anti-phosphotyrosine antibody. (*C*) PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates of the same lysates (100 μg each) as for *B*. PI-3P, PI 3-phosphate. (*D*) Translocation of GLUT4myc. CHO-GLUT4myc cells overexpressing wild-type or mutant PDGFRs were incubated with (+) or without (−) PDGF-BB (50 ng/ml) for 30 min, and the amount of cell surface GLUT4myc was determined by anti-c-Myc antibody binding assay. Results are expressed as fold increases over that of control cells without PDGF-BB. Values are means ± SE for five to nine independent clones. *, $P < 0.01$; **, $P < 0.001$ compared with wild-type PDGFR.

due to substitutions of Phe for Tyr^{977} and Tyr^{989} (19, 20); (*iii*) $PI3K^-$, which cannot bind PI 3-kinase, due to substitutions of Phe for Tyr^{708} and Tyr^{719} (18, 19); and (*iv*) $PI3K^-/PLC\gamma^-$, which cannot bind either PI 3-kinase or PLCγ, due to substitutions of the above four tyrosine residues. We obtained several clones expressing each mutant PDGFR which had about the same number of PDGFRs. Receptor number was determined by ^{125}I-PDGF binding assay and by immunoblot analysis with an anti-PDGFR antibody. All the mutant PDGFRs showed much the same PDGF-stimulated autophosphorylation seen with the wild-type PDGFR in cultured cells (Fig. 4*B*), as was noted by other workers (19, 28). The cells stably overexpressing wild-type, GAP^-, or $PLC\gamma^-$ PDGFRs showed much the same PDGF-stimulated PI 3-kinase activity, but the PDGF-stimulated PI 3-kinase activities in the cells stably overexpressing $PI3K^-/PLC\gamma^-$ or $PI3K^-$ PDGFRs were reduced (Fig. 4*C*). As for GLUT4 translocation, Fig. 4*D* shows the average fold increase of several independent clones for each PDGFR mutant. The GAP^- mutant fully transmitted the GLUT4myc translocation signal, as did the wild-type receptor, but the $PI3K^-/PLC\gamma^-$ mutant hardly transmitted the signal as seen in the parent cells. Therefore, the GAP binding site has little effect on GLUT4myc translocation, and PI 3-kinase and/or PLCγ binding sites play an important role in GLUT4myc translocation. To determine the importance of the binding sites, the PI 3-kinase binding sites and PLCγ binding sites were added back to the $PI3K^-/PLC\gamma^-$ mutant. When the PI 3-kinase binding sites were added back ($PLC\gamma^-$ mutant), the GLUT4myc translocation was almost completely recovered with PI 3-kinase activity (Fig. 4*D*). However, when the PLCγ binding sites were added back ($PI3K^-$ mutant), only partial recovery of the GLUT4myc translocation was seen. There seem to be at least two signaling pathways involved in PDGF-stimulated GLUT4myc translocation: one mediated by the PI 3-kinase binding sites, and one mediated by the PLCγ binding sites (Fig. 5*C*). The pathway of the PDGF-stimulated GLUT4myc translocation mediated by PI 3-kinase binding sites apparently predominates over that mediated by PLCγ binding sites (Fig. 4*D*).

To confirm the existence of the PI 3-kinase-mediated pathway, we examined the GLUT4myc translocation by PDGF, after PKC downregulation (Fig. 5*A*). The PDGF-stimulated GLUT4myc translocation by the wild-type or $PLC\gamma^-$ mutant PDGFR (2.5-fold) was not affected by long-term treatment with PBt_2. However, the PDGF-stimulated GLUT4myc translocations of the $PI3K^-$ mutant and $PI3K^-/PLC\gamma^-$ mutant were only 1.5-fold, and that of the parent cells was only 1.2-fold, after long-term treatment with PBt_2. These results indicate that PI 3-kinase binding sites play a key role in the signaling pathway of PDGF-stimulated GLUT4 translocation.

Finally, we examined the GLUT4myc translocations by PDGF after wortmannin-induced inhibition of PI 3-kinase, to confirm the existence of the PLCγ-mediated pathway (Fig. 5*B*). Wortmannin partly inhibited PDGF-stimulated GLUT4myc translocation by the wild-type (1.7-fold) and $PI3K^-$ mutant (1.5-fold) receptors, and completely inhibited translocations by receptors lacking PLCγ binding sites, $PI3K^-/PLC\gamma^-$ (1.0-fold), and $PLC\gamma^-$ (1.0-fold) mutants. In addition, wortmannin had little effect on PMA-stimulated GLUT4myc translocations, in all mutants studied. Therefore, translocation by the wild type and the $PI3K^-$ mutant after wortmannin treatment was presumably mediated by PLCγ and PKC.

Epidermal growth factor triggers the activation of PI 3-kinase (29, 30) and the GLUT4myc translocation in CHO-GLUT4myc cells stably overexpressing epidermal growth factor receptors (31). Translocation of the insulin-regulatable glucose transporter (GLUT4) may not be specifically triggered only by insulin. While triggering of GLUT4 translocation may be a common property of growth factors which activate PI 3-kinase, PI 3-kinase activated by these growth factors has diverse effects on cellular signaling, including DNA synthesis and receptor internalization (18, 19, 28). PI 3-kinase is essential for insulin-stimulated GLUT4 translocation and glucose uptake, but other factors are also likely to be involved.

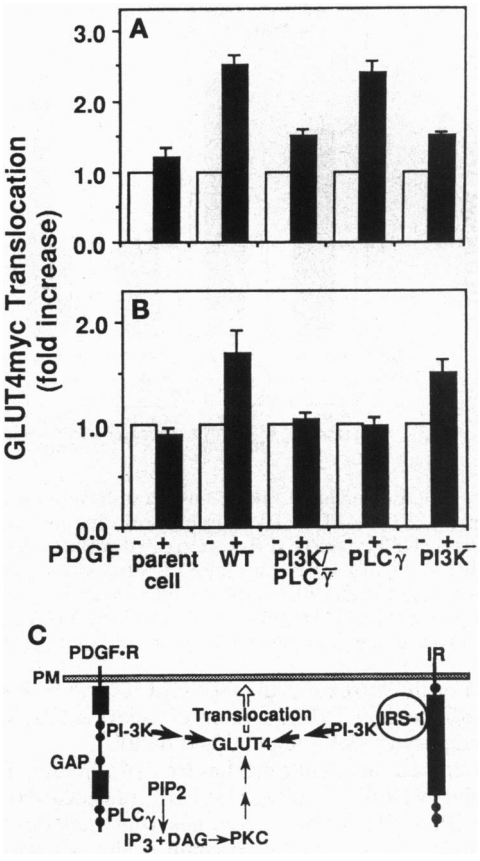

FIG. 5. Effects of PBt_2 (*A*) and wortmannin (*B*) on PDGF-stimulated GLUT4myc translocation by PDGFR mutants, and a schematic model of GLUT4 translocation stimulated by PDGF-BB and insulin (*C*). (*A* and *B*) CHO-GLUT4myc cells expressing wild-type (WT) or mutant PDGFRs ($PI3K^-/PLC\gamma^-$, $PLC\gamma^-$, or $PI3K^-$) were pretreated with PBt_2 (100 ng/ml) for 20 hr at 37°C (*A*) or with 0.1 μM wortmannin for 30 min at 37°C (*B*). The cells were treated with (+) or without (−) PDGF-BB (50 ng/ml) for 30 min at 37°C, and the amount of cell surface GLUT4myc was determined by anti-c-Myc antibody binding assay. Results are expressed as fold increases over that of control cells without PDGF-BB. Values are means ± SE for the same five to nine clones as Fig. 4. (*C*) PDGFR and insulin receptor (IR) contain tyrosine kinase activity (filled box) that undergoes autophosphorylation (filled circle) after ligand binding. PDGFR has two signaling pathways: a major one (thick arrows) mediated by PI 3-kinase and a minor one (thin arrows) mediated by PLCγ and PKC. PM, plasma membrane; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol. The insulin receptor has a PI 3-kinase-mediated pathway through IRS-1 tyrosine phosphorylation but has no effects on PLCγ.

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