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Biochemistry

1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells

(glucose transporters/insulin receptor substrate 1/translocation)

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ABSTRACT **Insulin stimulation drives the formation of a complex between tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) and 1-phosphatidylinositol 3-kinase (PI 3-kinase; ATP:1-phosphatidyl-1D-*myo*-inositol 3-phosphotransferase, EC 2.7.1.137), a heterodimer consisting of regulatory 85-kDa (p85) and catalytic 110-kDa (p110) subunits. This interaction takes place via the phosphorylated YMXM motifs of IRS-1 and the Src homology region 2 (SH2) domains of p85. In this study, the stable overexpression in a Chinese hamster ovary (CHO) cell line of a mutant p85α (Δp85) protein, which lacks a binding site for p110, disrupted the complex formation between IRS-1 and the catalytic subunit of PI 3-kinase in intact cells during insulin stimulation. Activation of insulin receptor kinase and the tyrosine phosphorylation of IRS-1 remained unaffected. In this cell line, both insulin-stimulated accumulation of phosphatidylinositol 3,4,5-trisphosphate and the insulin-stimulated glucose uptake due to the translocation of GLUT1 glucose transporters were markedly impaired, whereas neither phorbol 12-myristate 13-acetate-stimulated glucose uptake nor the insulin-stimulated activation of RAS was impaired. These results suggest that PI 3-kinase is required for glucose transport in insulin signaling in CHO cells.**

1-Phosphatidylinositol 3-kinase (PI 3-kinase; ATP:1-phosphatidyl-1D-*myo*-inositol 3-phosphotransferase, EC 2.7.1.137) phosphorylates, the D-3 position of the inositol ring of phosphatidylinositol (PtdIns), PtdIns 4-phosphate [PI(4)*P*] and PtdIns 4,5-bisphosphate [PI(4,5)P_2] to produce PtdIns 3-phosphate [PI(3)*P*], PtdIns 3,4-bisphosphate [PI(3,4)P_2], and PtdIns 3,4,5-trisphosphate [PI(3,4,5)P_3], respectively (1). These D-3 phosphorylated PtdIns probably act as second messenger molecules distinct from those in the classical PtdIns pathway (1). PI 3-kinase is a heterodimer consisting of 85-kDa (p85) and 110-kDa (p110) subunits. Bovine, murine, and human p85 (2–4) have been cloned. cDNA cloning revealed two highly homologous isoforms of p85—α and β. Amino acid sequence analysis of p85 showed that the protein contains two Src homology region 2 (SH2) domains and one SH3 domain. The cDNA of bovine p110 was also cloned and analysis of recombinant protein revealed that p110 is the catalytic subunit of PI 3-kinase (5). It has been established that SH2 domains are involved in interactions with tyrosine-phosphorylated proteins (6). Therefore, p85 appears to be the subunit that links PI 3-kinase activity in p110 to the tyrosine-phosphorylated proteins.

The insulin receptor belongs to the family of structurally related transmembrane growth factor receptors with ligand-activated protein-tyrosine kinase activity (7, 8). Insulin treatment of cells has been found to increase PI 3-kinase activity in immunoprecipitates made by using antibody to phosphotyrosine (9, 10). Insulin treatment of various intact cells causes rapid tyrosine phosphorylation of a high molecular weight protein (M_r 160,000–185,000) (11, 12) termed insulin receptor substrate 1 (IRS-1) and its sequence was deduced by cDNA cloning (13). Insulin drives the formation of a complex between tyrosine-phosphorylated IRS-1 and SH2 domains of several proteins including p85 (14–16). However, the role of the binding of PI 3-kinase to IRS-1 in insulin signal transduction is not clear. To address this issue, we disrupted complex formation between the catalytic p110 subunit of PI 3-kinase and IRS-1 by overexpressing mutant p85α (Δp85), which lacks a binding site for p110.

MATERIALS AND METHODS

Cell Cultures and Antibodies. CHO-IR cells were maintained and cultured as described (14). The antibodies used were as follows: monoclonal antibodies (mAbs) against the bovine p85α (F12 and G12) (14); polyclonal antipeptide antibodies against a synthetic C-terminal peptide of bovine p85α (residues 713–724) or bovine p85β (residues 707–724); a polyclonal anti-p110 antibody against a glutathione *S*-transferase (GST) fusion protein containing residues 441–605 of bovine p110 (Transduction Laboratory, Lexington, KY); polyclonal anti-IRS-1 antibodies against a synthetic rat IRS-1 peptide (pep80) corresponding to residues 489–507 (13) or a GST fusion protein containing N-terminal residues 1–240 of rat IRS-1 (generously provided by Alan Saltiel, Warner–Lambert, Ann Arbor, MI); a mAb against rat IRS-1 (ID6) (17); a mAb (py20; ICN) and a polyclonal antibody (Upstate Biotechnology) against phosphotyrosine residues.

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Abbreviations: IRS-1, insulin receptor substrate 1; PtdIns, phosphatidylinositol(s); SH2, Src homology region 2; mAb, monoclonal antibody; PI(3,4,5)P_3, PtdIns 3,4,5-trisphosphate; PI(4)*P*, PtdIns 4-phosphate; PI(4,5)P_2, PtdIns 4,5-bisphosphate; PI(3)*P*, PtdIns 3-phosphate; PI(3,4)P_2, PtdIns 3,4-bisphosphate; PI 3-kinase, 1-PtdIns 3-kinase; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine; PMA, phorbol 12-myristate 13-acetate.

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Construction of Plasmids and Expression of Wild-Type and Mutant p85s in CHO Cells. For a wild-type bovine p85α (Wp85), an *Eco*RI/*Stu* I fragment containing an entire coding sequence of bovine p85α was subcloned into the SRα plasmid (18) (SRα-Wp85). For a mutant bovine p85α (Δp85), two PCR products, A_1 (nucleotides 988–1434) and A_2 (nucleotides 1540–2175), were amplified from p85α cDNA. Three fragments—that is, an *Eco*RI/*Xho* I fragment of p85α (from 81 bp upstream of ATG to 1014 bp), an *Xho* I/*Xba* I fragment of A_1, and an *Xba* I/*Kpn* I fragment of A_2—were ligated into the SRα plasmid (SRα-Δp85). Expression of this mutant results in the deletion of 35 amino acids from residues 479–513 of p85α and the insertion of two other amino acids (Ser-Arg) in this deleted position. To obtain CHO-IR cells stably overexpressing Wp85 or Δp85, CHO-IR cells were cotransfected with pSV40-hgh, a plasmid conferring hygromycin resistance and either the SRα-Wp85 or SRα-Δp85 plasmid. Selection was started by addition of hygromycin B (final concentration, 250 μg/ml) to the culture medium. Resistant CHO-IR cells expressing each protein were screened by Western blotting with anti-p85α antibodies and subcloned by limiting dilution. At least two independent cell lines of CHO-IR/Wp85 and CHO-IR/Δp85 cells were tested in the following experiments. These cells proliferated almost normally in response to serum.

Cell Labeling and Immunoprecipitation. Confluent 60-mm plates of cells were incubated with 0.15 mCi (1 Ci = 37 GBq) of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN) as described (14). These cells were then lysed and immunoprecipitated with an anti-p85α mAb (F12 or G12) as described (14). The immunoprecipitates were analyzed by SDS/8.5% PAGE and autoradiography.

Biological Assays. The PI 3-kinase assay was carried out as described (9). Analysis of insulin-stimulated accumulation of 3-phosphorylated inositol phospholipids was performed as described (19). Analysis of RAS-bound guanine nucleotides was performed as described (17). Insulin-stimulated 2-deoxy-D-glucose uptake and photolabeling of cell-surface GLUT1 glucose transporters with 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)-benzoyl]-1,3-bis-[2-^{3}H](D-mannos-4-yloxy)-2-propylamine (ATB-[2-^{3}H]BMPA) was performed as described (20).

RESULTS

Overexpression of Mutant p85α Lacking a Region Necessary for Its Association with PI 3-Kinase Activity in CHO-IR Cells. Thirty-five amino acids (residues Met^{479} to Lys^{513} of bovine p85α) in the inter-SH2 region have been defined as necessary for the binding of p110 subunit of PI 3-kinase (21). CHO-IR cells were transfected with cDNA encoding Δp85 or Wp85. Several stable cell lines overexpressing each protein, designated as CHO-IR/Wp85 or CHO-IR/Δp85 cells, were cloned. While our anti-p85α mAb F12 recognizes both hamster and bovine p85α, another anti-p85α mAb (G12) recognizes only bovine p85α (14). ^{35}S labeling experiments revealed that mAb F12 immunoprecipitated two endogenous p85s from CHO-IR cells (Fig. 1, lane 1). The upper band is hamster p85α, since this is reacted with polyclonal antibodies against a synthetic C-terminal peptide of bovine p85α. The lower band is possibly hamster p85β, since this is reacted with polyclonal antibodies against a synthetic C-terminal peptide of bovine p85β but its molecular mass is different from that of bovine p85β. mAb G12 immunoprecipitated the overexpressed bovine Wp85 and Δp85 proteins but not endogenous hamster p85α from CHO-IR cells (lanes 2–4). Wp85 and Δp85 proteins were overexpressed in 2- to 3- and 4- to 5-fold excess, respectively, compared with the endogenous hamster p85s (p85α + p85β) [however, the immunoblotting with anti-C-terminal peptide antiserum revealed that Wp85 and Δp85 proteins were overexpressed in 10- and 20-fold excess, respectively, compared with the endogenous hamster p85α (data not shown)]. In addition, F12 specifically immunoprecipitated another doublet of 110- and 120-kDa proteins in CHO-IR cells (lane 1). The 110-kDa protein was the bovine p110, since anti-bovine p110 antiserum recognized this protein. The 120-kDa protein may be the hamster p110β as described recently (22). Bovine Wp85 protein was coimmunoprecipitated with the 110- and 120-kDa proteins from CHO-IR/Wp85 cells. In contrast, in CHO-IR/Δp85 cells, bovine Δp85 protein was not coimmunoprecipitated with 110- and 120-kDa proteins, indicating that the overexpressed bovine Δp85 protein did not associate with the catalytic subunit of PI 3-kinase.

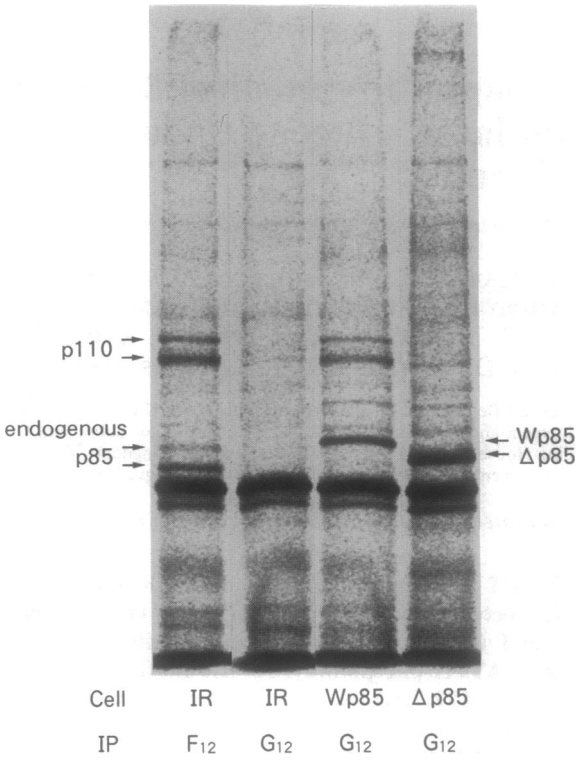

FIG. 1. Overexpression of Wp85 or Δp85 and its association with PI 3-kinase in CHO-IR cells (IR). Endogenous or overexpressed p85s were immunoprecipitated (IP) with anti-p85 antibody F12 or G12 bound to protein G-agarose from metabolically ^{35}S-labeled CHO-IR, CHO-IR/Wp85, or CHO-IR/Δp85 cells. Immunoprecipitates were electrophoresed and autoradiographed.

Inhibition of Insulin-Stimulated Association of PI 3-Kinase Activity with IRS-1 in CHO-IR/Δp85 Cells. With insulin stimulation, CHO-IR, CHO-IR/Wp85, and CHO-IR/Δp85 cells showed similar levels of tyrosine phosphorylation on the insulin receptor β subunit and IRS-1 (data not shown). The level of both tyrosine phosphorylation of Shc and the binding of Syp to IRS-1 was not different between the three cell lines (data not shown). The Δp85 was also tyrosine phosphorylated upon insulin stimulation in CHO-IR/Δp85 cells (data not shown).

To define the interaction between overexpressed p85s with IRS-1 after insulin treatment, Wp85 or Δp85 protein was immunoprecipitated with mAb G12 from nontreated and insulin-treated cells and immunoblotted with a polyclonal anti-IRS-1 antibody (pep80). The mAb G12 precipitated nearly the same amount of IRS-1 from insulin-treated CHO-IR/Wp85 or CHO-IR/Δp85 cells as well as the mAb F12 immunoprecipitated from insulin-treated CHO-IR cells (Fig. 2). This suggests that bovine Wp85 or Δp85 protein binds to IRS-1 in response to insulin. To confirm this, cells were labeled with ^{35}S, treated with or without insulin, and then immunoprecipitated with the anti-N-terminal IRS-1 antibody. The antibody coimmunoprecipitated endogenous p85

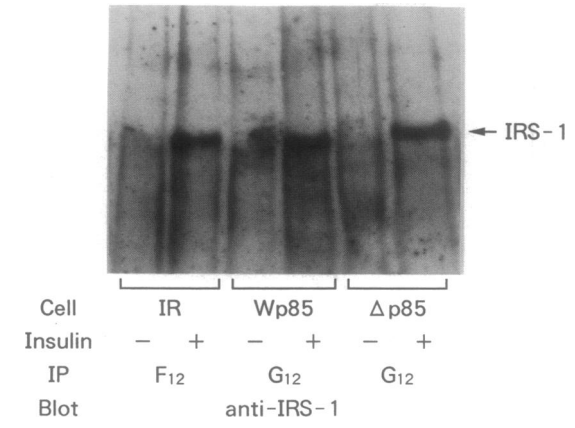

FIG. 2. Insulin-dependent formation of a complex between overexpressed Wp85 or Δp85 and IRS-1. Cells were treated with or without 0.1 μM insulin for 1 min at 37°C and then lysed. Lysates were immunoprecipitated (IP) with an anti-p85 mAb (F12 or G12). Immunoprecipitates were Western blotted with a polyclonal anti-IRS-1 antibody (pep80).

proteins and the 110- and 120-kDa proteins from CHO-IR cells after insulin treatment. It coimmunoprecipitated the bovine Wp85 protein and the 110- and 120-kDa proteins from CHO-IR/Wp85 cells. However, from CHO-IR/Δp85 cells, it coimmunoprecipitated only Δp85 protein and the 110- and 120-kDa proteins were not coimmunoprecipitated (data not shown). Consistent with this observation, the insulin-stimulated increase in PI 3-kinase activity in anti-IRS-1 (1D6) immunoprecipitates was markedly reduced in CHO-IR/Δp85 cells, whereas the insulin dose–response of IRS-1-associated PI 3-kinase activity in CHO-IR/Wp85 cells was similar to that of CHO-IR cells (Fig. 3). The same results were obtained in immunoprecipitates by using a polyclonal anti-IRS-1 antibody (pep80) or a monoclonal anti-phosphotyrosine antibody (py20) (data not shown). These results suggest that the binding site for p85 of PI 3-kinase on IRS-1 is occupied almost entirely by the bovine Δp85 proteins in CHO-IR/Δp85 cells.

Insulin-Stimulated Accumulation of PI(3,4,5)P_3 in CHO-IR Cells Overexpressing p85s. CHO-IR cells were labeled with $^{32}P_i$ and exposed to 1 μM insulin for the times indicated at 37°C,

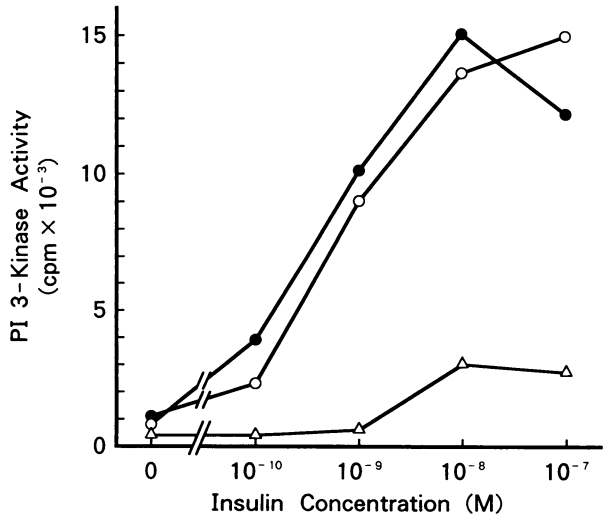

FIG. 3. Inhibition of insulin-stimulated association of PI 3-kinase activity with IRS-1 in CHO-IR/Δp85 cells. PI 3-kinase activities were determined in anti-IRS-1 (1D6) immunoprecipitates made from lysates of CHO-IR (○), CHO-IR/Wp85 (●), or CHO-IR/Δp85 (△) cells treated with the indicated concentrations of insulin for 1 min at 37°C. Data are representative of three separate experiments.

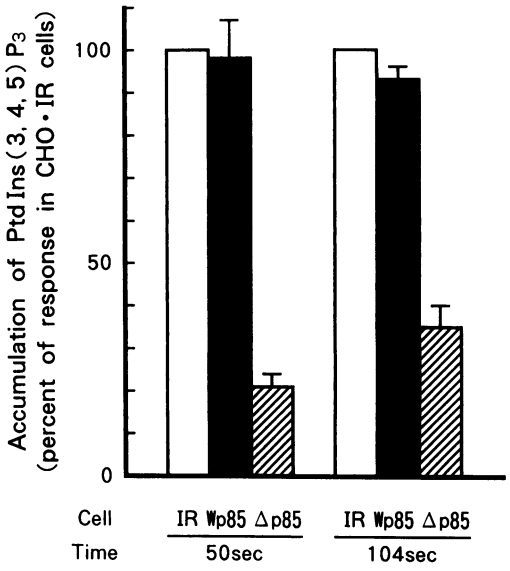

FIG. 4. Insulin-stimulated accumulation of PI(3,4,5)P_3 in CHO-IR cells overexpressing p85s. CHO-IR (□), CHO-IR/Wp85 (■), or CHO-IR/Δp85 (▨) cells were prelabeled with $^{32}P_i$, washed, and stimulated with 1 μM insulin for the indicated times at 37°C. Incubations were terminated, and lipids were extracted and analyzed (19). Response of [^{32}P]PI(3,4,5)P_3 above control in CHO-IR/Wp85 or CHO-IR/Δp85 cells is presented as percentage of response in CHO-IR cells. Data are means ± range (n = 2).

and ^{32}P-labeled phospholipids were analyzed. The insulin-stimulated accumulation of PI(3,4,5)P_3 was markedly reduced in CHO-IR/Δp85 cells, whereas the insulin-stimulated PI(3,4,5)P_3 accumulation in CHO-IR/Wp85 cells was similar to that of CHO-IR cells (Fig. 4). Insulin-stimulated accumulation of PI(3,4)P_2 in these cell lines was similar to that of PI(3,4,5)P_3, but the levels of PI(3)P, PI(4)P, and PI(4,5)P_2 did not increase with insulin stimulation (data not shown).

Insulin-Stimulated RAS Activation in CHO-IR Cells Overexpressing p85s. In serum-starved CHO-IR cells, 7–8% of the RAS protein was complexed with GTP and upon stimulation with 0.1 μM insulin, the percentage of RAS–GTP reached ≈30% after 2 min, as described (17, 23) (Fig. 5). Insulin

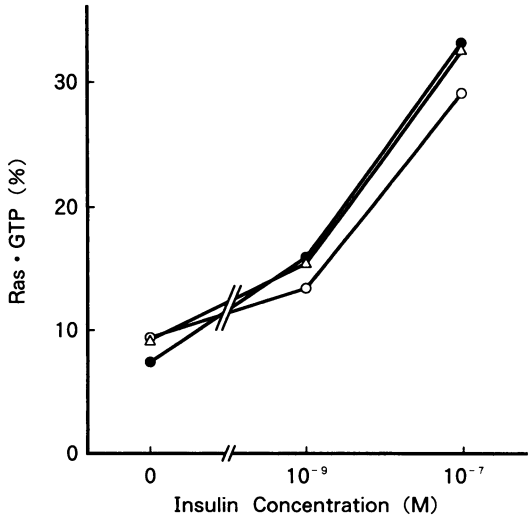

FIG. 5. Insulin-stimulated RAS activation in CHO-IR cells overexpressing p85s. Quantitative analysis of the accumulation of RAS–GTP complex in CHO-IR (○), CHO-IR/Wp85 (●), or CHO-IR/Δp85 (△) cells was performed as described (17). A representative of three separate experiments is shown.

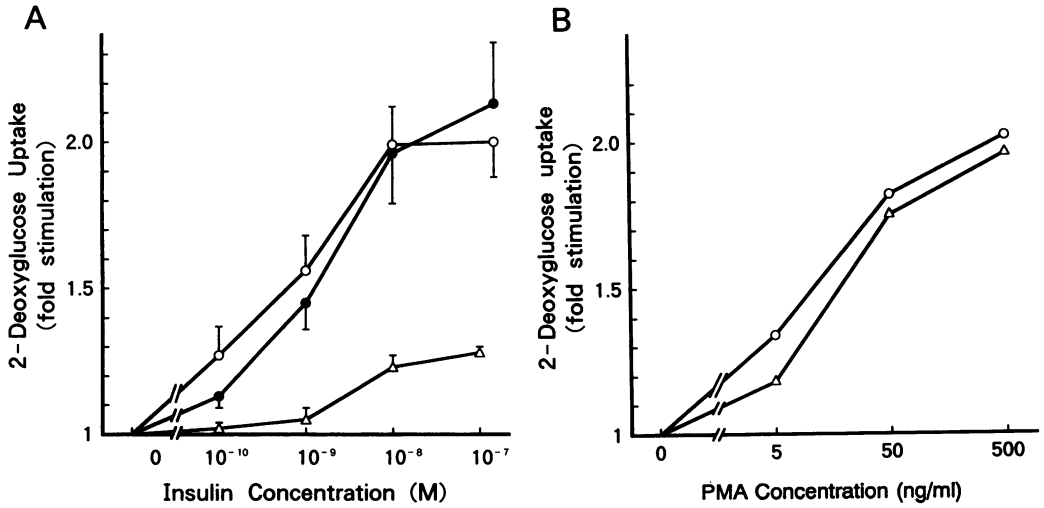

FIG. 6. 2-Deoxyglucose uptake in CHO-IR cells overexpressing p85s. (*A*) CHO-IR (○), CHO-IR/Wp85 (●), and CHO-IR/Δp85 (△) cells were stimulated with the indicated concentrations of insulin for 20 min at 37°C and incubated for another 10 min in the presence of 2-deoxy-D-[1,2-^{3}H]glucose. Then the cells were washed and solubilized, and the incorporated radiolabeled 2-deoxy-D-glucose was determined. Data are presented as means ± SE from three separate experiments ($n = 3$). (*B*) CHO-IR (○) and CHO-IR/Δp85 (△) cells were stimulated with the indicated concentration of PMA for 30 min at 37°C and then 2-deoxyglucose uptake was determined as described in *A*. A representative of two separate experiments is shown. In both *A* and *B*, results are expressed as -fold stimulation over basal (in the absence of added insulin) 2-deoxyglucose uptake in each cell line.

stimulated the formation of an active RAS–GTP complex in a similar manner in the three cell lines (Fig. 5).

Insulin-Stimulated Glucose Uptake and GLUT1 Transporter Translocation in CHO-IR Cells Overexpressing p85s. In CHO-IR cells, exposure to insulin stimulation for 20 min resulted in a dose-dependent enhancement of the uptake of labeled 2-deoxyglucose. The insulin dose dependence of 2-deoxyglucose uptake in CHO-IR/Wp85 cells was similar to that of CHO-IR cells (Fig. 6*A*). In contrast, in CHO-IR/Δp85 cells, the maximal response to insulin was markedly reduced (Fig. 6*A*). It has been proposed that the mechanism of insulin-stimulated glucose uptake is mainly due to translocation of glucose transporters from an intracellular microsomal compartment to the plasma membrane (24, 25). Therefore, we performed the photolabeling of cell-surface GLUT1 glucose transporters with a cell-impermeant photoaffinity label, ATB-BMPA (20), since GLUT1 is the main glucose transporter contributing to the observed glucose transport in CHO cells (data not shown). The total amounts of GLUT1 transporters photolabeled after permeabilization with digitonin were similar in the basal and insulin-stimulated states in all three cell lines (data not shown). As shown in Fig. 7, the insulin-stimulated appearance of cell-surface GLUT1 glucose transporters was also impaired in CHO-IR/Δp85 cells compared to that of CHO-IR or CHO-IR/Wp85 cells. The cell-surface levels of GLUT1 found in the basal state of CHO-IR/Wp85 and CHO-IR/Δp85 cells were reduced to 60% of that found in the basal state of CHO-IR cells. This is consistent with the basal 2-deoxyglucose uptake in these cells. At present, the reason for this is not clear. In contrast to insulin-stimulated glucose transport, the ability of CHO-IR/Δp85 cells to stimulate 2-deoxyglucose uptake (Fig. 6*B*) and the translocation of GLUT1 glucose transporter (data not shown) in response to phorbol 12-myristate 13-acetate (PMA) was normal compared with that of CHO-IR cells, suggesting that PMA may stimulate the translocation of glucose transporters through a mechanism different from insulin.

DISCUSSION

The binding of heteromeric PI 3-kinase (p85 and p110) was prevented by the saturation of phosphorylated IRS-1 binding sites for p85 by Δp85, which does not associate with p110, resulting in reducing the insulin-stimulated accumulation of PI(3,4,5)P_3 in CHO-IR/Δp85 cells. This suggests that the

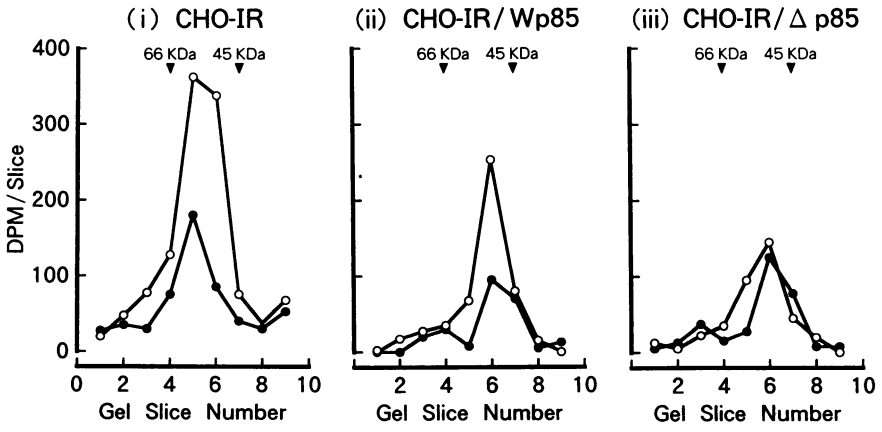

FIG. 7. Photolabeling of cell-surface GLUT1 glucose transporters with ATB-BMPA in CHO-IR cells overexpressing p85s. Cells were labeled with ATB-BMPA, in either the basal (●) or insulin-stimulated (0.1 μM for 20 min at 37°C) (○) state. GLUT1 was then immunoprecipitated and analyzed by electrophoresis with acrylamide gels. Radioactivities of gel slices were then analyzed.

association of IRS-1 with PI 3-kinase is required for activation of PI 3-kinase not only *in vitro* (16) but also *in vivo*. The failure of the overexpression of Wp85 to inhibit the PI 3-kinase activity associated with IRS-1 may be due to the association of bovine Wp85 with endogenous p110, since the free form of p110 exists in CHO cells (unpublished observation). The activation of the insulin receptor kinase, the tyrosine phosphorylation of IRS-1 and Shc, the binding of Syp to IRS-1, and insulin-stimulated RAS activation were not affected in CHO-IR/Δp85 cells. However, insulin-stimulated translocation of GLUT1 glucose transporter was markedly decreased in CHO-IR/Δp85 cells.

The most straightforward interpretation of our data is that the association of PI 3-kinase activity with IRS-1 and the accumulation of PI(3,4,5)P_3 is required for insulin-stimulated translocation of GLUT1 glucose transporters from the intracellular vesicle pool to the plasma membrane in CHO cells. Recently wortmannin was reported to be an inhibitor of PI 3-kinase activity (26, 27). We found that wortmannin inhibited insulin-stimulated PI 3-kinase activity associated with IRS-1 and the translocation of GLUT1 in a similar dose-dependent manner in CHO cells (data not shown). Although we have characterized CHO-IR/Δp85 cells extensively, it is difficult to exclude completely the possibility that overexpressed Δp85 may interact with proteins other than IRS-1. Alternatively, CHO-IR/Δp85 cells may have certain somatic mutations, which affect some functions of cells. Wortmannin is a relatively uncharacterized substance and may interact with proteins other than PI 3-kinase. However, the fact that two totally different approaches reached the same conclusion strongly suggests that PI 3-kinase activity is involved in insulin-stimulated translocation of GLUT1 glucose transporters. The validity of our approach using Δp85 has also been documented in that insulin-induced membrane ruffling was inhibited by three different approaches: microinjection of phosphorylated peptides containing YMXM motifs, which inhibit the association of IRS-1 with p85; treatment with wortmannin; and overexpression of Δp85 (CHO-IR/Δp85 cells) (28).

GLUT1 and GLUT4 were reported to be translocated in response to insulin (29). The latter isoform is expressed only in tissues in which hexose transport is very sensitive to insulin and appears to be the major insulin-responsive glucose transporter isoform (30). The mechanisms of GLUT1 and GLUT4 translocation in response to insulin can be different. However, wortmannin was recently reported to inhibit insulin-stimulated glucose uptake in rat adipocytes (27) and the translocation of GLUT4 glucose transporters (31). These results suggest that PI 3-kinase may be required not only for the translocation of GLUT1 but also for that of GLUT4.

The recent cloning of the 110-kDa catalytic subunit of PI 3-kinase has provided some interesting insights into the possible function of this enzyme (5). The p110 subunit has a region of significant homology with the yeast VPS34 gene product, a protein that has been shown to be involved in targeting of proteins to the yeast vacuole as well as vacuole morphogenesis during budding (32). In addition, the VPS34 protein has recently been shown to possess intrinsic PI 3-kinase activity (33), suggesting that the mammalian PI 3-kinase could be involved in vesicle trafficking. Our findings are consistent with this notion and may provide the opportunity to investigate the mechanisms of the complex cellular functions regulated by PI 3-kinase activity in mammalian cells.

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