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The Pleckstrin Homology (PH) Domain-Interacting Protein Couples the Insulin Receptor Substrate 1 PH Domain to Insulin Signaling Pathways Leading to Mitogenesis and GLUT4 Translocation

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Receptor-mediated tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) is required for the propagation of many of insulin's biological effects. The amino-terminal pleckstrin homology (PH) domain of IRS-1 plays a pivotal role in promoting insulin receptor (IR)–IRS-1 protein interactions. We have recently reported the isolation of a PH domain-interacting protein, PHIP, which selectively binds to the IRS-1 PH domain and is stably associated with IRS-1 in mammalian cells. Here we demonstrate that overexpression of PHIP in fibroblasts enhances insulin-induced transcriptional responses in a mitogen-activated protein kinase-dependent manner. In contrast, a dominant-negative mutant of PHIP (DN-PHIP) was shown to specifically block transcriptional and mitogenic signals elicited by insulin and not serum. In order to examine whether PHIP/IRS-1 complexes participate in the signal transduction pathway linking the IR to GLUT4 traffic in muscle cells, L6 myoblasts stably expressing a myc-tagged GLUT4 construct (L6GLUT4myc) were transfected with either wild-type or dominant-interfering forms of PHIP. Whereas insulin-dependent GLUT4myc membrane translocation was not affected by overexpression of PHIP, DN-PHIP caused a nearly complete inhibition of GLUT4 translocation, in a manner identical to that observed with a dominant-negative mutant of the p85 subunit of phosphatidylinositol 3-kinase (Δp85). Furthermore, DN-PHIP markedly inhibited insulin-stimulated actin cytoskeletal reorganization, a process required for the productive incorporation of GLUT4 vesicles at the cell surface in L6 cells. Our results are consistent with the hypothesis that PHIP represents a physiological protein ligand of the IRS-1 PH domain, which plays an important role in insulin receptor-mediated mitogenic and metabolic signal transduction.

As a major substrate of the insulin receptor, insulin receptor substrate 1 (IRS-1) plays a central role in transducing insulin-dependent signals that regulate biological processes such as cell growth and cellular uptake of glucose. IRS-1 is a modular protein comprised of an N-terminal region harboring a pleckstrin homology (PH) domain, followed by a phosphotyrosine-binding (PTB) domain that cooperatively ensures selective recognition and efficient substrate phosphorylation by the activated insulin receptor (IR) (35, 55, 65). The C-terminal portion contains multiple tyrosine phosphorylation motifs which serve as docking sites for the recruitment of various SH2 (Src-homology 2) domain containing signaling molecules, such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb-2 adaptor protein, and SHP2 (SH2 containing phosphatase 2) tyrosine phosphatase, which in turn elicit the activation of biochemical cascades that promote the metabolic and growth responses to insulin (61).

Studies have shown that IRS-1 is enriched in intracellular low-density microsomes (LDM) and that this state of subcellular localization is critical for proper signaling (2, 19, 20, 28). An important question that remains to be addressed is the mechanism by which IRS-1 intersects the plasma membrane-associated IR. In vitro studies and yeast two-hybrid systems revealed that, after insulin stimulation, the PTB domain of IRS-1 mediates direct binding to the phosphorylated tyrosine residue within the NPEY motif in the juxtamembrane region of the activated IR (11, 63). However, a direct interaction between the IR and the IRS-1 PH domain has not been detected (8, 18, 37). It is noteworthy that deletion of the IRS-1 PH domain selectively impairs the ability of IRS-1 to undergo in vivo insulin-dependent tyrosine phosphorylation, whereas the capacity of this deletion mutant to serve as an in vitro substrate for the IR remains unaffected (55). This has led to the idea that interactions of the IRS-1 PH domain with membrane phospholipids in intact cells may help localize IRS-1 to the plasma membrane compartment. Indeed, recent studies have demonstrated the importance of the IRS-1 PH domain in signal-dependent targeting of IRS-1 to the cell surface and that this function is impaired by PH domain mutations that abrogate lipid binding in vitro (44, 53). Despite these findings, there are several hints that something other than phosphoinositide binding is involved in IRS-1 PH domain-mediated receptor coupling. First, although mutations of the lipid-binding sites in

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the IRS-1 PH domain cause significant impairment of IRS-1 plasma membrane translocation in response to insulin, there is no detectable impairment of IRS-1 tyrosine phosphorylation or associated PI 3-kinase activation (23, 53). This suggests that the lipid-binding function of the IRS-1 PH domain and IR/IRS-1 substrate recognition may be uncoupled. Second, chimeric IRS-1 molecules in which the IRS-1 PH domain has been substituted with heterologous PH domains that exhibit phospholipid-binding profiles similar to that of IRS-1 fail to functionally restore IRS-1-dependent signaling (5). Third, modification of IRS-1 with membrane anchoring sequences, such as the p21Ras CAAX motif, results in decreased levels of IRS-1 tyrosine phosphorylation and impairment of p85-PI3K binding after insulin stimulation, suggesting that constitutive association of IRS-1 with the lipid microenvironment of the plasma membrane, compromises IRS-1–receptor interactions (28). This raises the possibility that association of the activated IR with IRS-1 may occur at intracellular sites outside the plasma membrane. Consistent with this view, biochemical studies have demonstrated that IRS-1 is associated and preferentially tyrosine phosphorylated by the IR in low-density microsomal fractions where it is anchored not to membrane components but rather to the cytoskeleton (2, 6, 7, 19, 20, 28, 51, 62). Conceivably, the IRS-1 PH domain may interact with a specific protein ligand(s) in the LDM that regulates the accessibility of IRS-1 to the IR in response to insulin. We have recently reported the isolation of such a putative ligand, namely, the protein referred to as PHIP, that selectively binds to the PH domain of IRS-1 in vitro and is stably associated with IRS-1 in vivo (12). We showed that PHIP could modulate insulin-dependent tyrosine phosphorylation of IRS-1 without affecting the status of IR kinase activity or SHC tyrosine phosphorylation (12).

We attempt to explore herein the functional significance of PHIP–IRS-1 PH domain interactions in IR signal transduction. We provide evidence that supports the role of PHIP as a physiological IRS-1 PH domain ligand that functions as a critical component of insulin-mediated gene transcription, mitogenesis, and glucose transport and in cellular processes that induce actin remodeling.

MATERIALS AND METHODS

Constructs. All constructs of PHIP and IRS-1 have been previously described (12). The pSG5p85SH2-N construct was a generous gift from Julian Downward (Imperial Cancer Research Fund [United Kingdom]).

Antibodies. Anti-PHIP antibodies were produced as previously described (12). Anti-IRS-1^{PCT} (generated against a 16-amino-acid pre-C-terminal polypeptide sequence) was purchased from Upstate Biotechnology, Inc. The monoclonal anti-hemagglutinin (HA) antigen 12CA5 was from Babco, and anti-myc (9E10) and monoclonal IRS-1 (E-12) antibodies were from Santa Cruz Biotechnology. Anti-chloramphenicol acetyltransferase (CAT) antibodies and mouse antibody to bromodeoxyuridine (BrdU) were purchased from 5 Prime-3 Prime, Inc., and Sigma, respectively. Rhodamine-conjugated phalloidin was obtained from Molecular Probes.

Reporter gene assays. COS-1 cells were transiently transfected in triplicate samples with a 5X serum responsive element (SRE)-fos luciferase reporter gene (5X SRE-LUC) and the indicated plasmids. At 24 h after transfection, the cells were serum starved for 16 h. Serum-starved cells were either left untreated or treated with Mek-1 inhibitor (50 μM; NEB) for 2 h. Cells were incubated for 10 h with or without insulin (0.2 μM; Sigma). Luciferase activity was then analyzed in cell lysates (Roche) and normalized to protein concentrations.

Microinjection assays. NIH 3T3 cells overexpressing IR (NIH/IR) or Rat-1 fibroblasts were plated onto gridded glass coverslips, serum starved for 30 h, and microinjected with the indicated plasmids with or without 5X SRE-CAT reporter gene. For the reporter assay, 2 h after injection, cells were treated with 0.5 μM insulin or serum (20%) as indicated and incubated for 5 h before fixation. For the mitogenesis assay, cells were treated 3 h after injection with 10 μM BrdU (Roche), followed by the addition of either 0.5 μM insulin or 20% serum. Cells were incubated for 36 h before fixation. Anti-CAT and anti-BrdU antibodies were then used to analyze reporter gene expression or DNA synthesis levels, respectively.

Metabolic labeling of cells. L6GLUT4myc cells were grown in α-minimal essential medium lacking methionine and cysteine for 2 h; 100 μCi of Pro-Mix [^{35}S]methionine (Amersham Pharmacia Biotech) was the added per ml of medium. Cells were incubated for 4 h before they were lysed in PLC lysis buffer and processed for immunoprecipitation.

Immunoprecipitation and immunoblotting. L6GLUT4myc cells were serum starved for 4 h and either left untreated or treated with 100 nM insulin for 10 min. Cells were then lysed in PLC lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1.5 mM $MgCl_2$, I mM EGTA, 10 mM sodium pyruvate, 10 μM sodium orthophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, and 10 μg of leupeptin/ml. Cleared lysates were immunoprecipitated with the corresponding antibodies for 2 h at 4°C, separated on sodium dodecyl sulfate-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes and immunoblotted with the corresponding antibodies.

GLUT4myc translocation assay. L6GLUT4myc stable cell lines were generated as previously described (25, 27, 33). Cells growing on coverslips were transfected with the indicated constructs according to the Effectene protocol manual (Qiagen). At 43 h after transfection, cells were deprived of serum in culture medium for 3 h and either left untreated or treated with 100 nM insulin for 20 min. Indirect immunofluorescence for expression of cDNA constructs and GLUT4myc translocation was carried out on intact cells as previously described (42). Several representative images of at least three separate experiments were quantified with the use of National Institutes of Health (NIH) image software.

Actin labeling. Growing L6GLUT4myc cells on coverslips were left untreated or treated with 100 nM insulin for 10 min after serum deprivation. Cells were rinsed with ice-cold phosphate-buffered saline (PBS; 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM NaH_2PO_4-Na_2HPO_4 [pH 7.4]) before being fixed with 3% paraformaldehyde in PBS for 30 min (initiated at 4°C for 5 min and shifted immediately to room temperature). The rest of the procedure was performed at room temperature. The cells were the rinsed once with PBS, and unreacted fixative was quenched with 100 nM glycine in PBS for 10 min. Permeabilized cells (0.1% Triton X-100 in PBS for 3 min) were washed quickly with PBS and blocked with 5% goat serum in PBS for 10 min. To detect filamentous actin, cells were incubated in the dark with rhodamine-conjugated phalloidin for 1 h. Rinsed coverslips were then mounted and analyzed with the Leica TCS 4D fluorescence microscope (Leica Mikroscoipe Systeme GmbH, Wetzlar, Germany).

RESULTS

PHIP potentiates insulin-stimulated 5X SRE-LUC transactivation. One of the mitogenic signaling events initiated downstream of the IR is activation of mitogen-activated protein (MAP) kinase, leading to the stimulation of immediate-early gene expression and, in particular, c-fos transcription (10). To evaluate the involvement of PHIP in insulin-mediated transcriptional responses, we examined its ability to induce transcription from a synthetic reporter, 5X SRE-LUC, which contains five copies of the SRE from the human c-fos promoter (16). Insulin stimulation of COS-1 cells transiently transfected with the reporter plasmid 5X SRE-LUC led to a marginal increase (1.3-fold) in luciferase activity (Fig. 1). In contrast, cotransfection of cells with pCGN-hPHIP, an expression plasmid encoding full-length PHIP, led to a marked dose-dependent increase in basal levels of transcription in untreated cells, which was further enhanced 2.7- to 4-fold in response to insulin. These results are consistent with a synergistic effect between insulin and PHIP, as reflected by the ability of insulin to

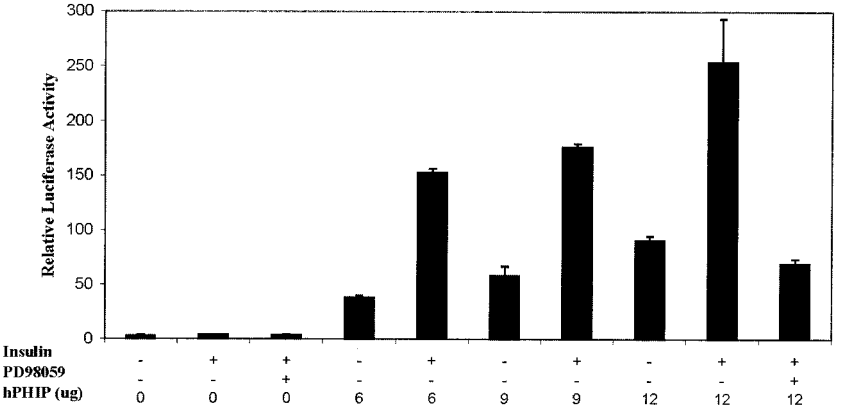

FIG. 1. Effect of PHIP on insulin-induced transcriptional activation of the SRE-Fos promoter. COS-1 cells were transiently transfected with increasing amounts of pCGN/hPHIP (6, 9, or 12 μg) or empty vector as control (12 μg), together with 3 μg of 5X SRE-fos luciferase reporter construct (5X SRE-LUC). Serum-starved cells were either left untreated or treated with Mek-1 inhibitor. Cells were incubated with or without insulin, and relative luciferase activity was measured in cell lysates. Results are expressed as the mean ± the standard deviation of triplicates from a representative experiment.

stimulate gene expression in the PHIP overexpressing cell lines but not in the parental cell lines.

In order to investigate the relative importance of the MAP kinase pathway as a downstream effector of PHIP-mediated gene expression, we used the MEK1 inhibitor, PD98059 (1), to block MAP kinase activation during insulin stimulation of COS-1 cells that had been transiently transfected with the highest levels of pCGN-hPHIP. The complete sensitivity of ligand-dependent PHIP SRE-LUC activity to PD98059 suggests that the MAP kinase cascade is an important component of insulin-stimulated PHIP transcriptional responses.

Expression of the IRS-1 PH domain blocks PHIP-mediated transcriptional responses. To determine whether IRS-1 PH binding is required for the ability of PHIP to potentiate insulin responses, we evaluated the effect of overexpressing the N-terminal IRS-1 PH domain on PHIP-stimulated SRE-LUC activity in COS-1 cells. We reasoned that overexpression of the isolated PH domain of IRS-1 would transdominantly interfere with PHIP binding to endogenous IRS-1. As shown in Fig. 2A, increasing expression of the IRS-1 PH domain progressively blocked the PHIP signal, supporting the notion that PH domain-directed interaction between PHIP and IRS-1 is required for PHIP-mediated transcriptional responses. To demonstrate that the inhibitory effect of the IRS-1 PH domain was a specific consequence of decreased IRS-1 activity and not secondary to nonspecific titration of cellular components, we cotransfected the cells with increasing concentrations of an expression plasmid for full-length IRS-1. Coexpression of excess IRS-1 caused reversal of the IRS-1 PH domain blockade on PHIP-stimulated SRE-LUC activity in a dose-dependent manner, confirming the functional specificity of the IRS-1 PH domain, and is in accordance with the view that wild-type IRS-1 outcompetes the IRS-1 PH domain for PHIP complex formation (Fig. 2B).

Expression of DN-PHIP blocks insulin but not serum-stimulated transcriptional responses. The simplest model deduced from the experiments described above would assume that PHIP is an intermediate in the signaling pathway used by IR to regulate SRE activity. To further explore this possibility, we used a HA-tagged PHIP construct that encodes the IRS-1 PH-binding region alone (DN-PHIP), which has previously been shown to function in a dominant inhibitory fashion in Rat-1 fibroblasts, by competing with the endogenous PHIP for the IRS-1 PH domain (12). Insulin treatment of parental Rat-1 cells microinjected with the reporter plasmid 5X SRE-CAT resulted in expression of CAT protein detectable by immunofluorescence staining with anti-CAT antibodies (Fig. 3). However, cells coinjected with the construct expressing HA-tagged DN-PHIP completely blocked insulin-stimulated CAT expression (Fig. 3A and B). To assess the specificity of the inhibitory phenotype associated with expression of DN-PHIP, we examined its effect on serum-induced transactivation of the c-fos serum response element. The experiments were conducted in a manner analogous to those described above, except that after microinjection of DN-PHIP cells were treated with 10% serum. The Rat-1 cells responded to serum stimulation by increasing expression of SRE-CAT to the same levels as that of insulin; however, this signal-dependent response was completely refractile to the effects of DN-PHIP (Fig. 3A and B). Thus, the inhibition of signal transduction to the c-fos SRE by DN-PHIP is due to specific pertubation of the signaling pathway downstream of the IR.

In order to address whether the effects of DN-PHIP on SRE-CAT in response to insulin occur at the level of IRS-1 or at some distal point in the IR signaling pathway, we coinjected Rat-1 cells expressing DN-PHIP with excess IRS-1. Consistent with the idea that PHIP functions upstream of IRS-1, we found that overexpression of IRS-1 fully restored SRE-CAT expression in DN-PHIP-expressing cells (Fig. 3C).

Expression of DN-PHIP blocks insulin but not serum-stimulated mitogenesis. Although insulin is a weaker mitogen than many other growth factors, it is nevertheless essential for growth and differentiation of many tissues and cell types. A variety of experimental approaches including the use of antisense RNA, neutralizing antibodies, or expression of dominant inhibitory constructs of IRS-1 have been used to implicate IRS-1 and its association with SH2 proteins as key elements in insulin-stimulated mitogenic responses (13, 15, 45, 46, 56, 59). To examine the role of PHIP in the growth-stimulatory effects

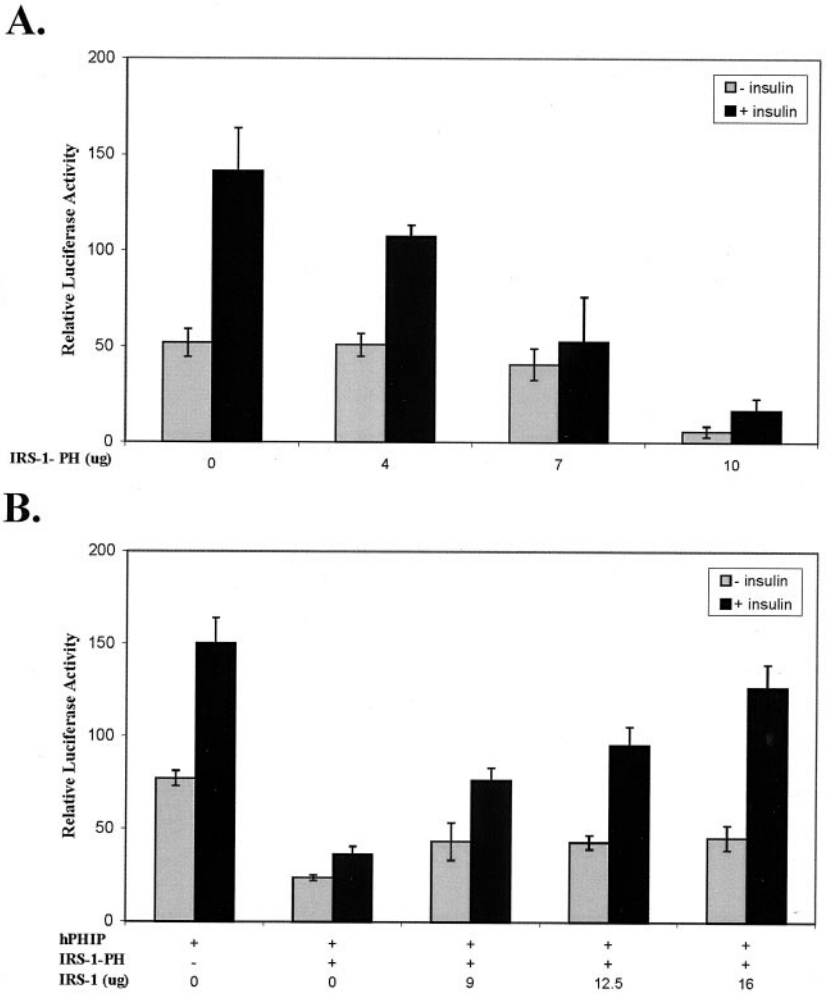

FIG. 2. Effect of IRS-1 PH domain on PHIP-mediated transcriptional activation of the SRE-Fos promoter. (A) IRS-1 PH domain inhibits PHIP-induced SRE-LUC activity. COS-1 cells were cotransfected with pCGN/hPHIP (4 μg) and the indicated amount of pCGN/IRS-1 PH domain, together with 2 μg of 5X SRE-LUC. Cells were treated with or without insulin, and the relative luciferase activity was measured in cell lysates. (B) IRS-1 PH domain-mediated inhibition of PHIP-stimulated luciferase activity is restored by wild-type IRS-1 in a dose-dependent manner. COS cells were cotransfected with 1 μg of pCGN/hPHIP, 2 μg of 5X SRE-LUC, either 1 μg of pCGN/IRS-1 PH or of vector DNA, and increasing amounts of pCGN/IRS-1 cDNA as indicated. Cells were then treated and processed as for panel A.

of insulin, DN-PHIP was microinjected into NIH fibroblasts overexpressing IR (NIH/IR) cells to evaluate its effect on insulin-stimulated DNA synthesis. Compared to quiescent cells, stimulation with insulin or serum led to a marked increase in the proportion of cells staining positive for BrdU incorporation (Fig. 4A). In contrast, microinjection of NIH/IR cells with DN-PHIP markedly attenuated insulin-induced stimulation of DNA synthesis, whereas there was no significant effect on serum-stimulated BrdU incorporation (Fig. 4A). These results are consistent with the notion that PHIP–IRS-1 PH domain interactions specifically promote the proliferative actions of insulin.

Effect of PHIP on GLUT4 translocation in L6 myoblasts. One of the main metabolic effects of insulin action on fat and muscle cells is the stimulation of glucose uptake. This process involves a redistribution of the glucose transporter GLUT4 from intracellular compartments to the plasma membrane (9). Activation of the p85/p110 isoform of PI 3-kinase through its recruitment to phosphotyrosine sites on IRS-1 is a necessary component of insulin-stimulated GLUT4 translocation (40, 47). The role of IRS-1 in this process is somewhat controversial, with some studies, indicating that IRS-1 tyrosine phosphorylation can be blocked without any effect on GLUT4 transport (34, 46). In order to examine whether PHIP/IRS-1 complexes participate in the signal transduction pathway linking the IR to GLUT4 traffic in muscle cells, we first sought to examine the interaction of PHIP and IRS-1 in L6 myoblasts. We have previously reported that endogenous PHIP is found associated with IRS-1 under basal and insulin-stimulated conditions in fibroblast cells, indicating that PHIP and IRS-1 form a stable complex (12). To address the stoichiometry of the PHIP–IRS-1 interaction in cells expressing endogenous levels of these proteins, we analyzed metabolically labeled extracts from L6 myoblasts under basal conditions by immunoprecipitation with either anti-PHIP or anti-IRS-1^{CT} antibodies (Fig. 5A). A 104-kDa [^{35}S]methionine-labeled protein observed in

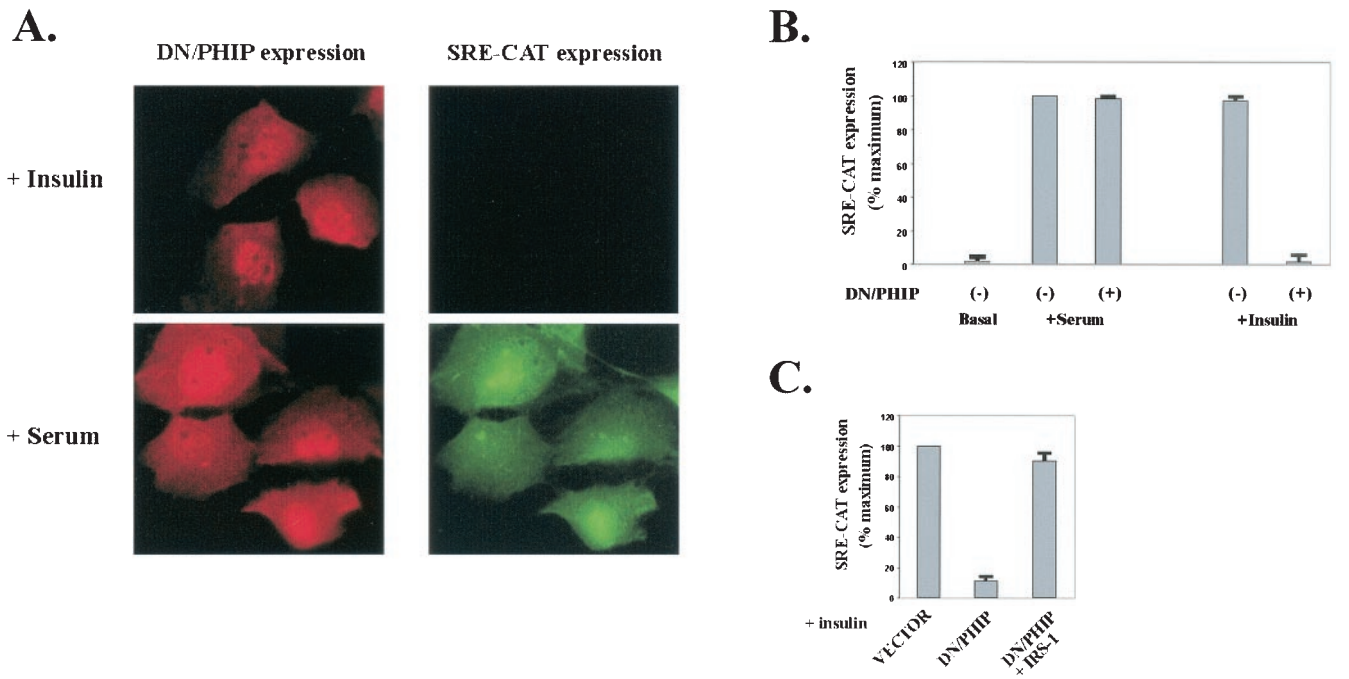

FIG. 3. Effect of DN-PHIP on insulin-mediated transcriptional activation of the SRE-Fos promoter. (A) DN-PHIP inhibits insulin-induced 5X SRE-CAT. Serum-deprived Rat-1 cells were coinjected with constructs expressing HA-tagged DN-PHIP and the 5X SRE-CAT reporter construct. Cells were treated with insulin or serum. The expression of CAT or DN-PHIP was monitored by immunostaining with anti-CAT or anti-HA antibodies, respectively. (B) The results shown in A are expressed as the percentage of maximum, which represents the average number of cells expressing SRE-CAT upon serum stimulation. The values represent averages of five independent experiments in which at least 50 cells were injected for each condition. (C) IRS-1 rescues SRE-CAT expression. Serum-deprived Rat-1 cells were coinjected with plasmids encoding 5X SRE-CAT and either pCGN vector or HA-tagged DN-PHIP in the presence or absence of HA-tagged IRS-1 as indicated. The results are expressed as the percent maximum that represents the average number of cells expressing SRE-CAT upon insulin stimulation. The values represent the average of five independent experiments in which at least 50 cells were injected for each condition.

anti-PHIP but not in preimmune serum immunoprecipitates was identified as PHIP by immunoblotting and by immunodepletion experiments with anti-PHIP antibodies (data not shown). Immunoprecipitation with the anti-IRS-1 antibody resulted in the coprecipitation of 50% of the total cellular PHIP that was directly immunoprecipitated by use of the anti-PHIP antibody (Fig. 5A). Conversely, immunoprecipitation with anti-PHIP antibodies that are directed against the PHIP PBR domain showed no discernible precipitation of IRS-1, as assessed by [^{35}S]methionine incorporation or Western blot analysis with anti-IRS antibodies (Fig. 5A, lane 1, and B, lane 3). This is in accordance with our previous observation with anti-IRS-1 antibodies directed to the PH domain, in which immunoreactive PHIP signals were not detected in anti-IRS-1^{PH} immune complexes (12). One possible explanation for these findings is that antibodies raised to structural determinants defining the PHIP PBR–IRS-1 PH domain interface may disrupt preformed PHIP/IRS-1 complexes.

Next, to evaluate the relative proportions of cellular PHIP associated with IRS-1, lysates prepared from unstimulated L6 myoblasts were immunodepleted of IRS-1 by subjecting them to immunoprecipitation with anti-IRS-1^{CT} antibodies. Immune complexes were analyzed by Western blotting with anti-IRS-1 and anti-PHIP antibodies, and the IRS-1 immunodepleted supernatant was subsequently subjected to a second round of immunoprecipitation with anti-PHIP antibodies. As shown in Fig. 5B, anti-IRS-1^{CT} antibodies quantitatively depleted L6 cell lysates of PHIP proteins in support of the idea that PHIP constitutes a major IRS-1-associated protein.

To evaluate the effect of PHIP on insulin-mediated GLUT4 mobilization, L6 myoblasts stably expressing a myc-tagged GLUT4 construct (L6GLUT4myc) (25, 27, 33) were transiently transfected with either wild-type or dominant-interfering forms of PHIP. Coexpression of green fluorescent protein (GFP) cDNA was used to facilitate recognition of transfected cells. As previously shown, insulin treatment of L6GLUT4myc myoblasts generates a twofold gain in cell surface GLUT4myc detected by immunofluorescence labeling of the exofacial myc epitope (42, 52) (Fig. 6A). Ectopic expression of DN-PHIP caused a nearly complete inhibition of insulin-dependent GLUT4myc membrane translocation (>90%), in a manner identical to that observed with a dominant-negative mutant of the p85 subunit of PI 3-kinase (Δp85) (40, 58) (Fig. 6B). The effect of DN-PHIP was specific for the insulin-stimulated state, since the content of cell surface GLUT4myc in unstimulated cells was not altered by the PHIP mutant (Fig. 6C).

We next examined whether expression from a plasmid encoding the IRS-1 PH domain, which would uncouple IRS-1 from PHIP, could also mitigate insulin's effects on GLUT4 translocation. Our data demonstrate that the IRS-1 PH domain caused a significant reduction in insulin-dependent GLUT4myc incorporation into the plasma membrane, albeit this was somewhat less robust (60%) than that induced by DN-PHIP (Fig. 7A). The incomplete inhibition may be ac-

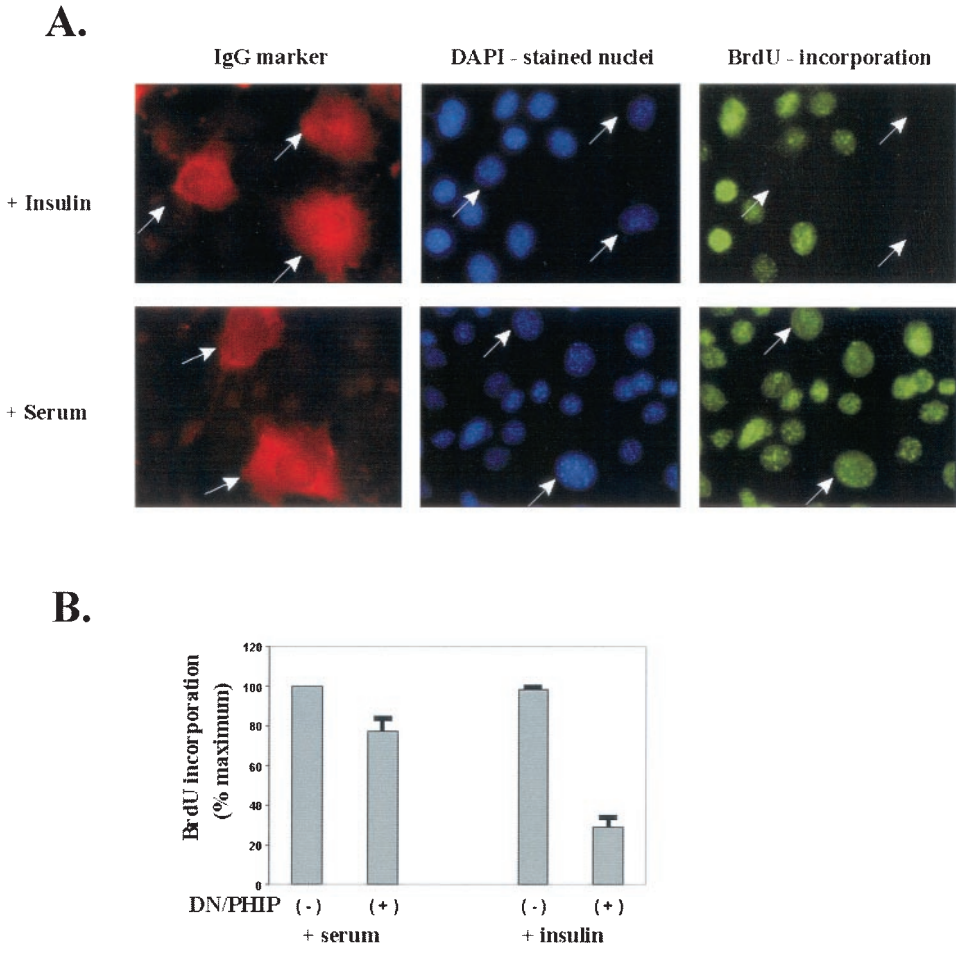

FIG. 4. Effect of DN-PHIP on insulin-mediated mitogenesis. (A) DN-PHIP inhibits insulin-induced DNA synthesis. Serum-deprived NIH/IR cells were coinjected with HA-tagged DN-PHIP and rabbit anti-rat immunoglobulin G. The injected cells are indicated by arrows. Cells were treated with BrdU, followed by the addition of insulin or serum. DNA synthesis was observed by immunostaining with antibody to BrdU. (B) The results shown in panel A are expressed as the percent maximum that represents the average number of cells incorporating BrdU upon serum stimulation. The values represent averages of five independent experiments in which at least 50 cells were injected for each condition.

counted for in part by the presence of other IRS proteins that may partially substitute for IRS-1 function. These findings are in accordance with previous reports that overexpression of the IRS-1 PH domain in 3T3-L1 adipocytes can inhibit insulin's ability to stimulate IRS-1 tyrosine phosphorylation and activate the PI 3-kinase/AKT pathway (15). In contrast, neither full-length PHIP nor full-length IRS-1 caused any measurable change in GLUT4myc redistribution under basal or insulin-stimulated conditions (Fig. 6B and C and 7). Taken together, these results support the idea that PHIP/IRS-1 complex formation is necessary but not sufficient in promoting the metabolic effects of insulin in L6 muscle cells.

Effect of PHIP on the actin cytoskeleton in L6 myoblasts. Recent evidence points to the potential participation of the actin microfilament network not only in promoting insulin-dependent redistribution of PI 3-kinase to GLUT4-containing vesicles but also in mobilizing GLUT4 to the cell surface (26, 49, 57). In light of previous evidence supporting the requirement of functional IRS-1 for insulin-stimulated actin cytoskeletal rearrangement (34), we sought to examine the role of PHIP in this process. To this end, we used rhodamine-conjugated phalloidin to detect changes in the pattern of filamentous actin in L6GLUT4myc cells ectopically expressing either wild-type PHIP or DN-PHIP. Whereas actin staining in the basal state exhibits a filamentous pattern that runs along the longitudinal axis of the cell, a marked reorganization of actin into dense structures throughout the myoplasm was observed upon insulin stimulation (Fig. 8). This effect was dramatically decreased by the expression of DN-PHIP but not by the empty vector or wild-type PHIP. Intriguingly, overexpression of wild-type PHIP alone appeared to induce a pattern of F-actin staining under basal conditions that was similar to that induced by treatment with insulin (Fig. 8A, top panel). Taken together, our observations implicate PHIP in the regulation of cellular processes that promote cytoskeletal remodeling and accompany incorporation of GLUT4 vesicles at the plasma membrane surface of muscle cells.

DISCUSSION

The conventional model for PH domain function is the targeting of host signaling proteins to membrane structures by binding acidic phospholipids (29). Indeed, most PH domain-

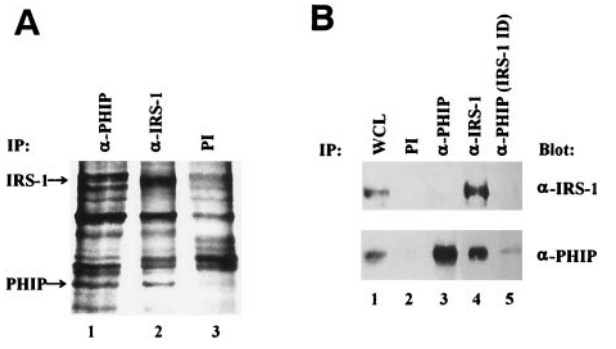

FIG. 5. PHIP and IRS-1 are stably associated in L6 myoblasts. (A) Stoichiometric analysis of the PHIP-IRS-1 interactions. Lysates from metabolically [^{35}S]methionine-labeled L6GLUT4myc cells were immunoprecipitated with preimmune serum (PI), anti-PHIP, or anti-IRS-1^{PCT} antibodies and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Bands corresponding to PHIP and IRS-1-labeled proteins are denoted by an arrow. (B) PHIP stably associates with IRS-1 in L6 cells. One milligram of lysate from basal unstimulated L6GLUT4myc cells was used in immunoprecipitation analysis with preimmune serum, anti-PHIP, and anti-IRS-1^{CT} antibodies (lanes 2 to 4), and complexes were immunoblotted with the indicated antibodies. The anti-IRS-1 immunodepleted (ID) supernatant was subsequently subjected to a second round of immunoprecipitation with anti-PHIP antibodies and immunoblot analysis with the indicated antibodies (lanes 5). As control for total protein levels of PHIP and IRS-1, 50 μg of whole-cell lysate (WCL) was subjected to Western blotting with anti-IRS-1 or anti-PHIP antibodies (lane 1).

containing proteins have a functional requirement for membrane association but do not contain recognizable membrane-anchoring motifs. However, with the exception of PLCδ, AKT, BTK, and a few other proteins whose PH domains have been shown to bind with high affinity to phospholipid products, most PH domains bind phosphoinositides weakly and nonselectively, which raises questions regarding the physiological relevance of PH domain-lipid interactions (21). Several studies have now established that certain PH domains bind to specific protein ligands to drive membrane association. For example, the β-adrenergic receptor kinase (βARK) PH domain must bind to Gβγ subunits of heterotrimeric G-proteins and phosphatidylinositol 4,5-P_2 in concert to ensure effective membrane association and subsequent activation of βARK (38). PH domains from pleckstrin and BTK have been shown to bind to filamentous actin, a newly defined PH domain-protein ligand implicated in directing the localization of its host proteins to sites of cytoskeletal rearrangement at the plasma membrane (30–32, 64). It is noteworthy that the Shc PTB and WASP enabled/VASP homology 1 domains which adopt the PH fold, although known to participate in low-affinity interactions with phosphoinositides, rely on in high-affinity interactions with protein ligands to ensure proper localization and function of the host protein (41, 43, 48, 50). More recently, a modeling study of *Caenorhabditis elegans* PH domains found in Dbl homology domain-containing guanine nucleotide exchange factors, identified a number of PH domains predicted not to bind to membrane phospholipids due to a strong overall negative electrostatic potential (13). Thus, the PH domain fold may be considered as a functionally plastic platform for binding a wide range of ligands.

In the present study we provide evidence that PHIP represents a novel physiological protein ligand of the IRS-1 PH domain. PHIP is a ubiquitously expressed protein which is found stably associated with IRS-1 in several cell types, including fibroblasts and insulin responsive tissues such as myoblasts and adipocytes (12; also data not shown). Our findings with [^{35}S]methionine-labeled myoblasts indicate that a significant proportion of PHIP proteins are found complexed with IRS-1. This association, which is mediated through the N-terminal PH binding region of PHIP appears to be specific for IRS proteins. Notably, in vitro pull-down assays with isolated PH domains from a variety of other signaling molecules including βARK, p120 RasGAP, son-of-sevenless 1 (Sos-1), and ECT fail to bind to the PH binding region domain of PHIP (12). Conceivably, the selective engagement of PHIP by the IRS-1 PH domain may account for the specificity and sensitivity displayed by the PH domain in coupling IRS-1 to the activated IR.

We have described a dominant-negative N-terminal truncation mutant of PHIP, DN-PHIP, which potently inhibits transcriptional and proliferative responses downstream of the IR. This blockade is remarkably specific for insulin, since serum induced transactivation and DNA synthesis is unaffected by DN-PHIP. Indeed, the inhibitory phenotype of DN-PHIP parallels the effects seen with neutralizing anti-IRS-1 antibodies in selectively inhibiting insulin and not serum-mediated mitogenesis (45). Moreover, the transdominant effects of DN-PHIP on DNA synthesis were fully rescuable by wild-type IRS-1. This argues well for the role of PHIP functioning at the first steps of insulin action, i.e., at the level of substrate recognition.

This notion is further strengthened by our observations that overexpression of DN-PHIP inhibits insulin-dependent tyrosine phosphorylation of IRS-1 without affecting the status of IR kinase activity (12). Taken together, our data suggest that regions of PHIP implicated in interactions with the IRS-1 PH domain can disengage IR from IRS-1 and subsequently decrease sensitivity to growth-promoting responses of insulin.

To extend these observations we evaluated the effects of overexpression of full-length PHIP in potentiating insulin response pathways involved in the regulation of c-fos expression. A clear MAP kinase-dependent induction by insulin of the c-fos promoter was observed in cells expressing PHIP but not in the parental cell lines. Such behavior would be consistent with models whereby PHIP is activating either some integral component of the insulin/MAP kinase pathway itself or an alternative route eventually converging onto it. The observation that coexpression of the IRS-1 PH domain dominantly interfered with c-fos SRE activity induced by PHIP suggests that the first possibility is more likely and that PHIP is probably acting upstream of IRS-1 in the signaling cascade involved in insulin-induced gene expression.

The role of IRS-1 in insulin action on glucose transport is less clear. Several lines of evidence support the involvement of IRS-1 for GLUT4 externalization. For example, expression of antisense ribozyme directed against rat IRS-1 significantly reduces GLUT4 translocation to the plasma membrane of rat adipose cells in response to insulin (39). Moreover, mutations of IR Tyr960 that do not alter receptor kinase activity but are critical for IRS-1 binding and phosphorylation abolish glucose transport (3, 24, 60). However, in contrast to these findings, other reports indicate that microinjection of anti-IRS-1 anti-

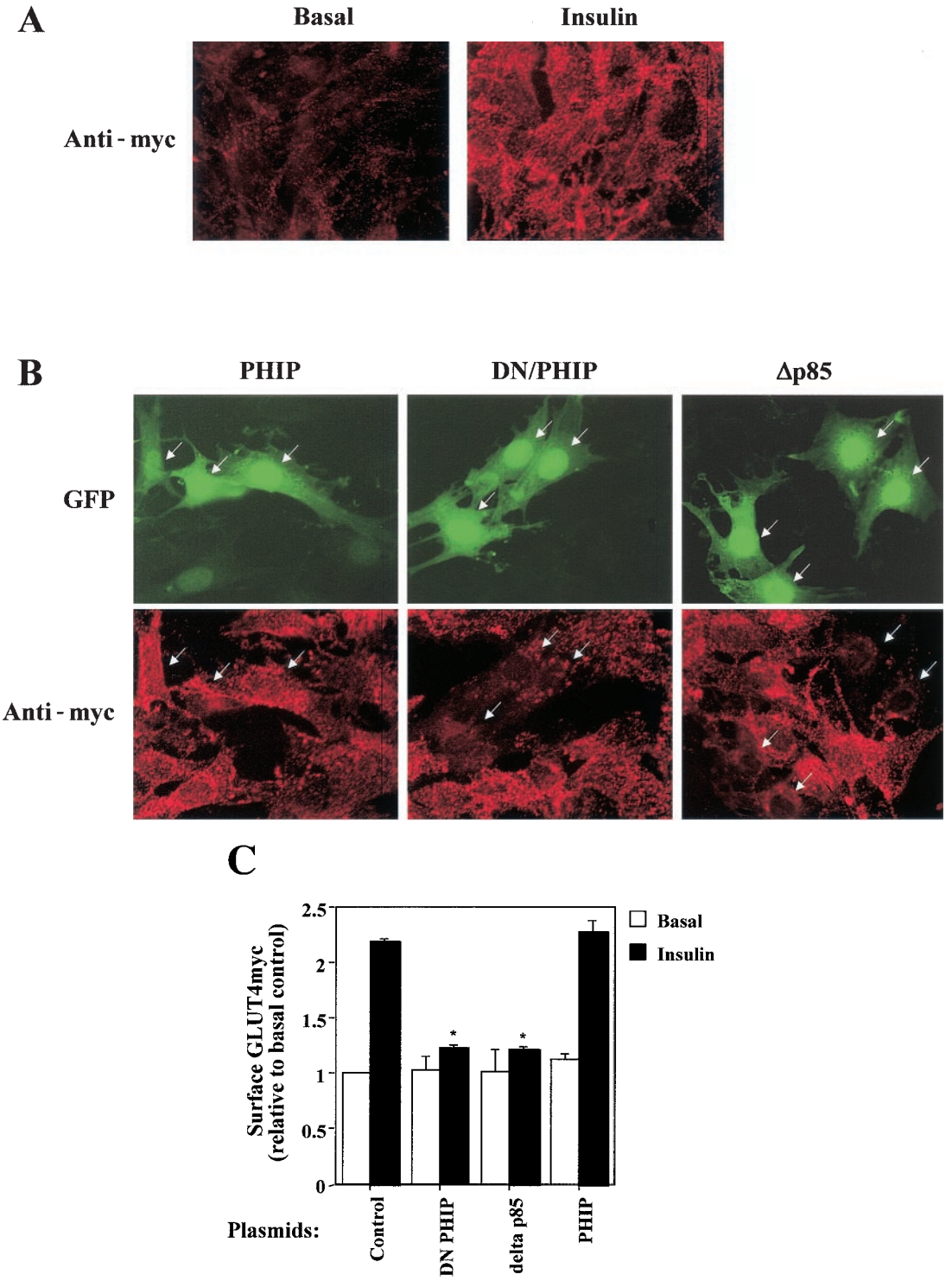

FIG. 6. Effect of DN-PHIP on insulin-induced GLUT4 translocation. (A) Insulin promotes GLUT4 translocation to the plasma membrane. Serum-starved L6 myoblast cells ectopically expressing myc-GLUT4 (L6GLUT4myc) were either left untreated or treated with insulin and then immunostained with anti-myc antibodies to monitor cell surface incorporated myc-GLUT4. (B) L6GLUT4myc cells were transiently cotransfected with 0.3 μg of pEGFP and 0.9 μg of either pCGN/hPHIP, pCGN/DN-PHIP, or Δp85 plasmid containing the SH2 domain of the p85 subunit of PI 3-kinase. Insulin-stimulated cell surface incorporated GLUT4 was then monitored as for panel A. Arrows point toward the transfected cells as monitored by GFP expression. Shown are representative images from at least three independent experiments. (C) Membrane GLUT4myc translocation from unstimulated and insulin-treated transfectants are expressed as the fold stimulation relative to basal levels of surface GLUT4myc from nontransfected cells (control). The intensity of the fluorescent GLUT4myc label was quantitated with the use of NIH Image Software. The pixel intensity of GLUT4myc staining was measured in similar numbers of untransfected and transfected cells. A value of 1.0 was assigned to untransfected, unstimulated cells within each field. The values presented are means ± the standard error of raw data from at least three independent experiments under each set of conditions.

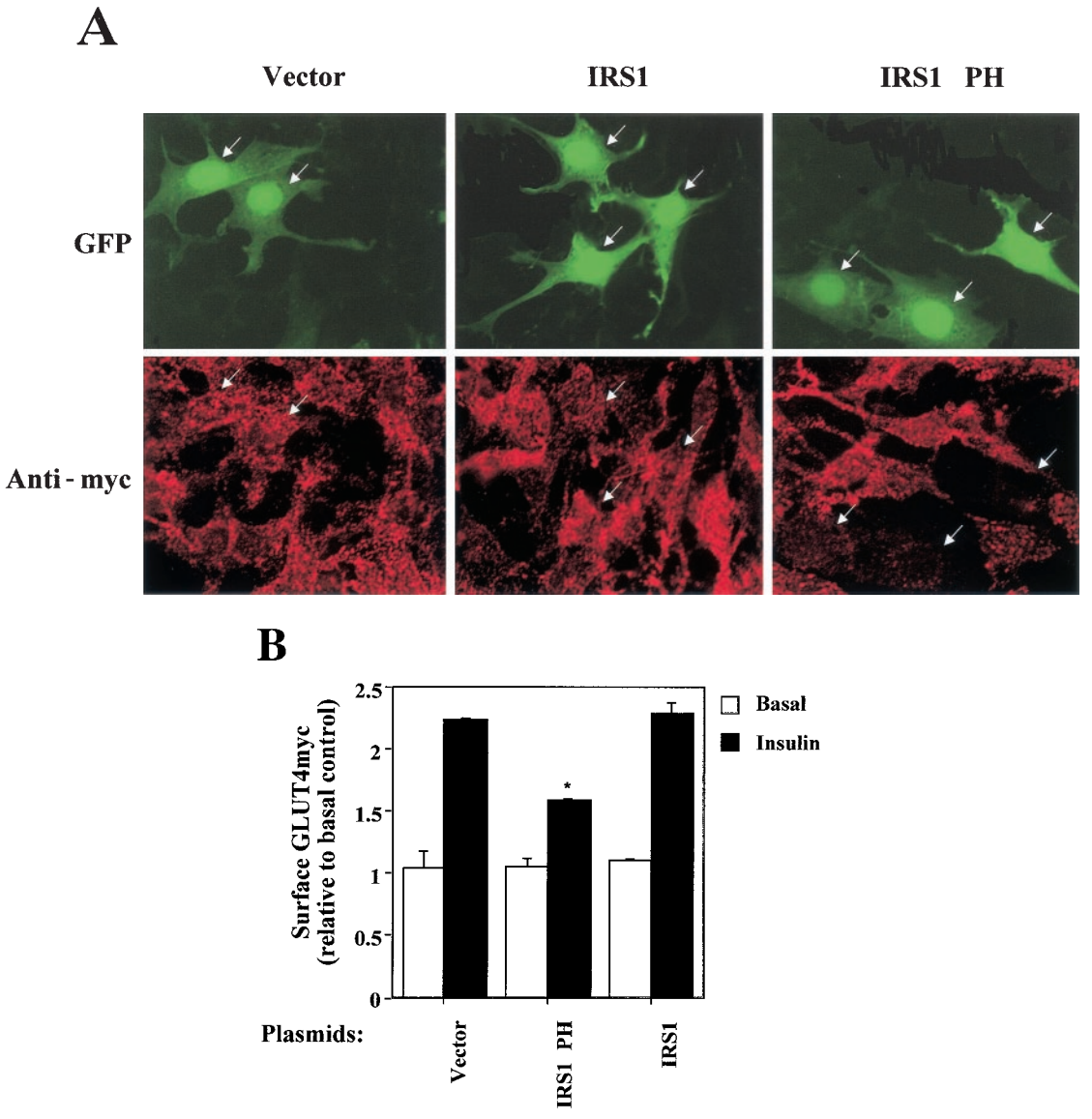

FIG. 7. Effect of IRS-1 PH domain on insulin-induced GLUT4 translocation. (A) IRS-1 PH domain inhibits insulin-stimulated GLUT4 membrane translocation. L6GLUT4myc cells were transiently cotransfected with 0.3 μg of pEGFP (enhanced GFP) and 0.9 μg of either pCGN vector control, pCGN/IRS-1, or pCGN/IRS-1 PH. At 48 h after transfection, cells were starved for 3 h and either left untreated or treated with insulin. Shown are representative images from at least three independent experiments from insulin-stimulated transfectants. Arrows point toward the transfected cells as monitored by GFP expression. (B) Membrane GLUT4myc translocation from unstimulated and insulin-treated transfectants are expressed as the fold stimulation relative to basal levels of surface GLUT4myc from untransfected cells. Quantification of fluorescent surface label was measured as described in Fig. 6. The values presented are means ± the standard errors of raw data from at least three independent experiments under each set of conditions.

bodies or expression of dominant inhibitory PTB domains of IRS-1 are able to block the mitogenic effects of insulin in fibroblasts but not GLUT4 trafficking in cultured adipocytes (34, 46). Interpretation of the results in adipocytes is confounded by the observation that insulin-stimulated glucose uptake proceeds unabated in IRS-1 PTB-expressing cells, despite a nearly complete inhibition of not only IRS-1 tyrosine phosphorylation but also IR kinase activity (46).

In the present study we demonstrate that overexpression of either PHIP or IRS-1 alone in muscle cells was not sufficient in promoting transport of GLUT4 to plasma membrane surfaces. This is consistent with other observations, indicating that activation of IRS-1-associated signaling effectors such as PI 3-kinase, although necessary, is not sufficient for GLUT4 activation. Notably, growth factors such as platelet-derived growth factor and interleukin-4 can activate PI 3-kinase as efficiently as insulin and yet fail to stimulate glucose transport in insulin-sensitive cells (17, 22). One possible explanation is that additional PHIP/IRS-1/PI 3-kinase-independent pathways are required to coordinate GLUT4 intracellular routing. Indeed, recent evidence points to a novel insulin-responsive pathway that recruits flotillin/CAP/CBL complexes to IR-associated

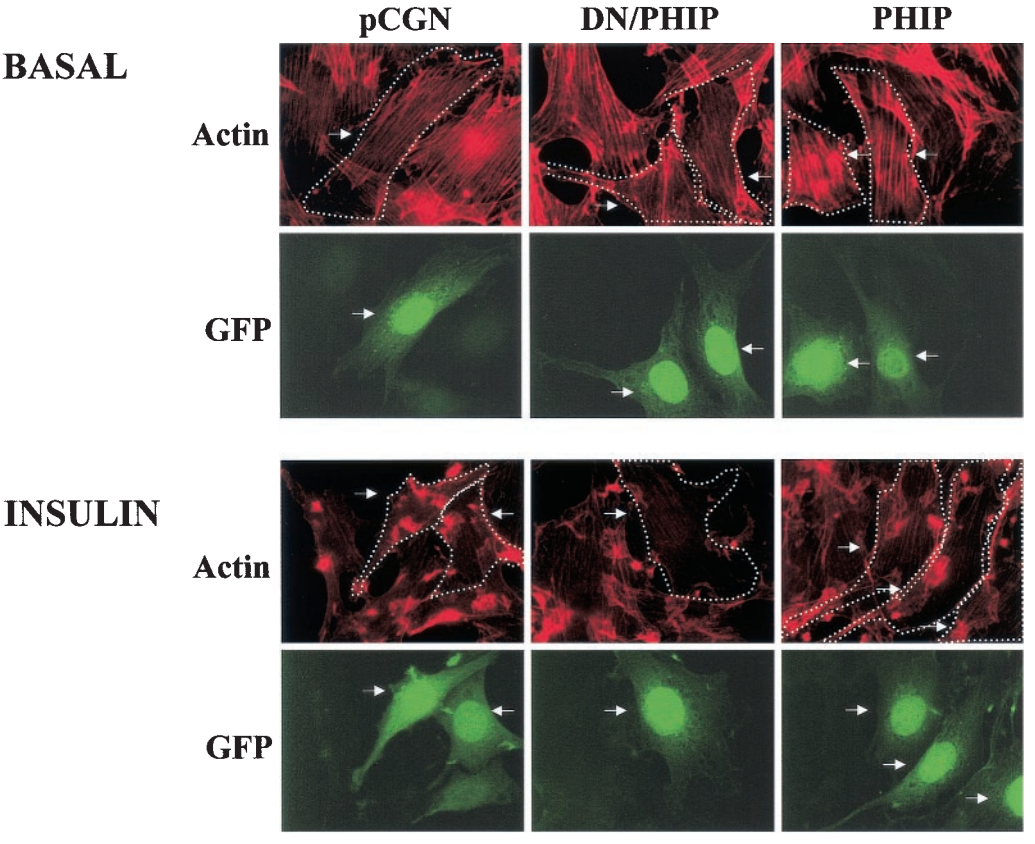

FIG. 8. Effect of DN-PHIP on insulin-induced actin remodeling. L6GLUT4myc cells were transiently cotransfected with 0.3 μg of pEGFP and 0.9 μg of either pCGN vector control, pCGN/hPHIP, or pCGN/DN-PHIP. Serum-starved cells were either left unstimulated or stimulated with insulin. GFP expression was used to monitor transfected cells. Filamentous actin was detected by using rhodamine-conjugated phalloidin.

lipid rafts in the plasma membrane, an event which is thought to potentiate GLUT4 docking to the cell surface after IR activation (4).

Our data, however, provide support for the involvement of PHIP/IRS-1 complexes in glucose transporter GLUT4 translocation in muscle cells. Specifically, the use of DN-PHIP or IRS-1 PH domain constructs known to interfere with efficient IR–IRS-1 protein interaction, and hence productive signal transduction from IRS-1 to PI 3-kinase, blocked the ability of insulin to stimulate GLUT4 mobilization in L6 myoblasts and inhibited insulin-stimulated actin cytoskeletal reorganization, a process required for the productive incorporation of GLUT4 vesicles at the cell surface. Moreover, this inhibition did not coincide with changes in the autophosphorylation status of the IR (12, 15). The mechanism by which overexpression of the IRS-1 PH domain may functionally interfere with insulin-mediated responses remains unclear. One simple explanation is that the isolated PH domain, by virtue of its lipid-binding properties, blocks signaling in a nonspecific manner by sequestering phosphoinositides at cellular membranes. While this is one possibility, there is evidence that suggests that the IRS-1 PH domain retains biological specificity when expressed in cells. Notably, the ability of the *Xenopus* IRS-1 PH domain to block insulin and not progesterone-mediated MAP kinase activation and subsequent germinal vesicle breakdown (GVBD) in *Xenopus* oocytes suggests that the IRS-1 PH domain is interacting with a component(s) of the tyrosine kinase signaling pathway specific to insulin-induced GVBD (36). In accordance with these findings, biochemical assays in 3T3-L1 adipocytes revealed that dominant inhibitory effects of the IRS-1 PH are very specific to the IR/IRS-1 cascade such that expression of the IRS-1 PH domain selectively blocks IRS-1 phosphorylation and PI 3-kinase association without any effects on Shc phosphorylation or Shc-mediated activation of prenyltransferases downstream of the IR (15).

A commonly held view to account for the specificity of insulin signaling on glucose transport is that biological specificity is conferred at the level of cellular compartmentalization of signaling intermediates. Indeed, subcellular fractionation studies in 3T3-L1 adipocytes and IR-overexpressing CHO cells have revealed that activated IRS-1/PI 3-kinase complexes are found predominantly in the LDM after insulin treatment, whereas activation of PI 3-kinase in response to platelet-derived growth factor in the same cells occurs at the plasma membrane (6, 7). Analogously, differences in the pattern of intracellular distribution have been documented among the four members of the IRS protein family (IRS1 to IRS4) and may account for differences in their ability to engage downstream signaling elements that may ultimately contribute to their functional specifity in vivo (2, 14, 20). The molecular basis for sequestration of IRS-1 to internal low-density microsomal fractions remains unclear. Clark et al. have demonstrated that

majority of IRS-1 is not anchored to membrane components but rather to an insoluble protein matrix enriched in actin filaments (6). This indicates that IRS-1 must be maintained at this location by specific association with other protein(s). In intact cells, PHIP and IRS-1 coimmunolocalize to punctate structures in the cytoplasm as detected by immunofluorescence microscopy (data not shown). Thus, it is possible that PHIP, through its association with the IRS-1 PH domain, may direct the targeting of IRS-1 to specific subcellular compartments and serve as a key determinant for the signaling specificity of IRS-1 with respect to the IR pathway. However, we have observed that an IRS-1 mutant devoid of PH domain sequences is still targeted to the LDM fraction (data not shown), suggesting that proteins other than PHIP may serve as receptors regulating the subcellular localization of IRS-1. One such putative adaptor is the σ3A subunit of AP-3 which has been reported to regulate the localization of IRS-1 to the LDM (54).

What might then be the mechanism by which PHIP may influence the coupling efficiency of IRS-1 to the IR? We have observed that ectopic expression of PHIP can induce filamentous actin reorganization at discrete sites in the myoplasm, implicating PHIP in the spatial control of actin assembly. It has yet to be determined whether PHIP itself can bind directly or indirectly to actin. Conceivably, PHIP, through direct association with the IRS-1 PH domain, may promote the assembly of IRS-1 onto a cytoskeletal scaffold that is in close apposition to plasma membrane-localized IR, thereby providing a kinetic advantage in IRS-1 substrate recognition after receptor ligation (6).

In conclusion, these studies point to an important role of IRS-1 PH domain–PHIP interactions in insulin-mediated mitogenic and metabolic signal transduction and may provide new insights in understanding the specificity of IRS-1 function in insulin sensitive cells.

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