THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 10, Issue of March 8, pp. 8091–8098, 2002

Printed in U.S.A.

Specificity of Interleukin-2 Receptor γ Chain Superfamily Cytokines Is Mediated by Insulin Receptor Substrate-dependent Pathway*

Received for publication, July 16, 2001, and in revised form, January 10, 2002
Published, JBC Papers in Press, January 11, 2002, DOI 10.1074/jbc.M106650200

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Interleukins 9 (IL-9) and 4 are cytokines within the IL-2 receptor γ chain (IL-2Rγ) superfamily that possess similar and unique biological functions. The signaling mechanisms, which may determine cytokine specificity and redundancy, are not well understood. IRS proteins are tyrosine-phosphorylated following IL-9 and IL-4 stimulation, a process in part mediated by JAK tyrosine kinases (Yin, T. G., Keller, S. R., Quelle, F. W., Witthuhn, B. A., Tsang, M. L., Lienhard, G. E., Ihle, J. N., and Yang, Y. C. (1995) *J. Biol. Chem.* 270, 20497–20502). In the present study, we used 32D cells stably transfected with insulin receptor (32D^{IR}), which do not express any IRS proteins, as a model system to study the requirement of different structural domains of IRS proteins in IL-9- and IL-4-mediated functions. Overexpression of IRS-1 and IRS-2, but not IRS-4, induced proliferation of 32D^{IR} cells in response to IL-9. The pleckstrin homology (PH) domain of IRS proteins is required for IRS-mediated proliferation stimulated by IL-9. The phosphotyrosine binding and Shc and IRS-1 NPXY binding domains are interchangeable for IRS to transduce the proliferative effect of IL-4. Therefore, the PH domain plays different roles in coupling IRS proteins to activated IL-9 and IL-4 receptors. The role of IRS proteins in determining cytokine specificity was corroborated by their ability to interact with different downstream signaling molecules. Although phosphatidylinositol 3′-kinase (PI3K) and Grb-2 interact with tyrosine-phosphorylated IRS proteins, Shp-2 only binds to IRS proteins following IL-4, but not IL-9, stimulation. Although PI3K activity is necessary for the IRS-1/2-mediated proliferative effect of IL-9 and IL-4, Akt activation is only required for cell proliferation induced by IL-4, but not IL-9. These data suggest that IRS-dependent signaling pathways work by recruiting different signaling molecules to determine specificity of IL-2Rγ superfamily cytokines.

IL-9 and IL-4[1] are two multifunctional cytokines within the IL-2 receptor γ chain (IL-2Rγ) superfamily along with IL-2, IL-7, and IL-15, which share IL-2Rγ as the common receptor component (1–4). IL-9 and IL-4 are secreted by Th2 cells in response to infection and induce a growth-promoting response on T cell clones *in vitro* and an anti-apoptotic effect on lymphoma cells treated with glucocorticoid (5, 6). However, IL-9 and IL-4 also possess distinct physiological functions. IL-4 acts as a major determinant for Th2 cell differentiation (7) and immunoglobulin class switch (8). IL-9 is essential for mast cell proliferation (9) and is involved in asthma development and airway inflammation (10–12). We have previously shown that IL-9 and IL-4 have common and distinct functions in supporting the proliferation and differentiation of a hematopoietic stem cell line, EMLC1, in synergy with stem cell factor and erythropoietin (13).

Upon ligand binding, IL-9- and IL-4-specific receptor α chains heterodimerize with the IL-2Rγ chain, leading to the activation of Jak1 and Jak3. Despite activating the same Janus kinases, IL-9 and IL-4 elicit common and distinct signaling pathways in hematopoietic cells. Both cytokines stimulate phosphorylation of signal transducers and activators of transcription (STATs) and insulin receptor substrate (IRS) proteins (14, 15). IL-9 and IL-4 activate STAT3 and STAT6, respectively, which may in part be responsible for the expression of different primary response genes. IRS proteins (IRS-1 through -4) with a highly conserved N terminus and divergent C terminus are involved in many physiological functions, such as cell growth, insulin response and reproduction *in vivo* (16, 17). The N terminus, consisting of pleckstrin homology (PH), phosphotyrosine binding (PTB), and Shc and IRS-1 NPXY binding (SAIN) domains, has been proposed to couple IRS proteins to activated receptors and mediate subsequent tyrosine phosphorylation of IRS proteins. After tyrosine phosphorylation at the C terminus, which contains about twenty potential tyrosine phosphorylation sites, IRS proteins interact with SH2-containing signaling proteins, such as the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI3K), Grb-2, Shp-2, Nck, and PLCγ.

The PH domain is present in over 120 proteins, including serine/threonine kinases, tyrosine kinases, phospholipases and cytoskeletal proteins (18). The PTB domain was originally identified in Shc as a phosphotyrosine-binding motif (NPXY binding motif) different from the SH2 domain and has been characterized in many molecules, including IRS proteins (19, 20). Although primary sequences do not reveal any homology, PH and PTB domains have similar three-dimensional structures, consisting of seven anti-parallel β-sheets, a C-terminal

* This work was supported by National Institutes of Health Grants DK50570, CA78433, and HL48819 (to Y.-C. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: IL-9, interleukin-9; IL-2Rγ, IL-2 receptor γ chain; STAT, signal transducers and activators of transcription; IR, insulin receptor; IRS, insulin receptor substrate; PH, pleckstrin homology; PTB, phosphotyrosine binding domain; SAIN, Shc and IRS-1 NPXY binding domain; PI3K, phosphatidylinositol 3′-kinase; CMV, cytomegalovirus; MAPK, mitogen-activated protein kinase; PVDF, polyvinylidene difluoride.

α-helix, plus an α-helix in one of the surface loops (21). They are electrostatically polarized and contain a pocket capable of binding to the ligand. The SAIN domain (amino acids 313–462) was identified as the minimum region essential for IRS-1 interaction with the insulin receptor (IR) in yeast two-hybrid studies, and this region shows a high degree of similarity with the Shc PTB domain (amino acids 42–200) (22, 23). Overexpression of the SAIN domain of IRS-1 abrogates tyrosine phosphorylation of IRS-1 and Shc following stimulation by insulin (24), probably through competitive binding to the juxtamembrane region of IR, indicating a role for the SAIN domain in mediating IRS-1 interaction with IR.

The PH domain is often involved in the attachment of proteins to membranes, either by direct binding to phospholipids and/or by protein-protein interactions. Interaction of PH domain with the kinase domain of Bruton's tyrosine kinase regulates its kinase activity (25). The PH domain of IRS proteins binds to phospholipids and proteins, whereas the PTB domain binds to NP*X*Y motifs in the IR and insulin-like growth factor-1 receptor (26). Besides growth factors like insulin and insulin-like growth factor-1, IRS proteins are tyrosine-phosphorylated following stimulation of cells by cytokines, such as IL-2, IL-4, IL-9, interferons, oncostatin M, and leukemia inhibitory factor (27, 28). Among these cytokine receptors, only the IL-4 receptor α chain contains NP*X*Y motifs.

To elucidate the mechanisms by which IRS proteins integrate into different cytokine signaling, we used IL-4 and IL-9 as models to explore the functional roles of IRS N-terminal domains in coupling IRS proteins to cytokine receptors with and without NP*X*Y. Through examining the tyrosine phosphorylation and growth-promoting ability of N-terminal deletion mutants of IRS-1/2 in IL-9- and IL-4-stimulated cells, we found that the PH domain is essential for the activation of IRS signaling induced by IL-9, but not IL-4. These results suggest the important role of the PH domain in mediating IRS coupling to non-NP*X*Y motif-containing cytokine receptors. In addition, tyrosine-phosphorylated IRS proteins in IL-4- and IL-9-stimulated cells recruited different SH2-containing molecules, further suggesting IRS-mediated signaling specificity between these two cytokines.

MATERIALS AND METHODS

Reagents and Antibodies—The anti-phosphotyrosine (Sc-7020), anti-IRS-1 (Sc-559), anti-PI3K p85α (Sc-4016), anti-Grb-2 (Sc-255), anti-p-p70 S6 kinase (Sc-11759) and anti-p70 S6 kinase (Sc-8418) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-IRS-2 (06-506), anti-Shp-2 (06-118), and anti-phospho-Akt (06-678) were from Upstate Biotechnology, Inc. (Lake Placid, NY); wortmannin and LY294002 were from Sigma Chemical Co. Murine IL-9 and IL-4 were from R&D Systems (Minneapolis, MN).

Plasmid Construction—pcDNA3 (Invitrogen) was digested with *Nde*I and *Eco*RV to remove part of the CMV promoter and multiple cloning sites. To restore the functions of the CMV promoter in pcDNA3, we ligated the *Nde*I-*Sma*I fragment that contains part of the CMV promoter, FLAG epitope sequence and multiple cloning sites derived from pCMV-FLAG-2 vector. We renamed the construct pcDNA-FLAG. This contains the CMV promoter, FLAG epitope-tag, and G418-resistant gene. Mouse IRS-1 cDNA (29) cloned at *Hin*dIII site of pRc/CMV (Invitrogen) served as wild type IRS-1. For the IRS-$1^{\Delta PH}$ construct, mouse IRS-1 cDNA cloned at the *Hin*dIII site of pRc/CMV was digested with *Xho*I/*Apa*I to remove amino acids 1–65 (30) that contain the PH domain and subcloned into pcDNA-FLAG vector at the *Sal*I/*Apa*I site. IRS-$1^{\Delta(PH+PTB)}$ was constructed by *Nsi*I digestion of bluescript-IRS-1 to remove amino acids 27–244 (31) and subcloning remaining pBluescript IRS-1 molecule into pcDNA3 at *Hin*dIII site. IRS-$1^{\Delta SAIN}$ was constructed by *Xba*I digestion of pcDNA3 containing the full-length of IRS-1 cDNA to remove amino acids 248–582 that is required for the functions of the SAIN domain (31) in IRS-1. The remaining IRS-1 was religated in-frame to pcDNA3. IRS-$1^{\Delta(SAIN+PH+PTB)}$ was generated by *Bam*HI and *Eco*RV digestion to delete amino acids 1–859 that contain the PH, PTB, and SAIN domains. The *Bam*HI-*Eco*RV fragment (amino acids 860–1231) was subcloned into pcDNA-FLAG at the *Bgl*II and *Eco*RV sites. All plasmid constructs were confirmed by DNA sequencing and protein expression in COS-1 cells and TS1 T lymphocytes. IRS-1 constructs used to generate $32D^{IR}$ transfectants have been described previously (32).

Cell Culture and Transfections—$32D^{IR}$ and $32D^{IR}$ transfectants expressing wild type and mutated forms of IRS-1/2 were grown in RPMI supplemented with 5% WEHI-conditioned medium as a source of murine IL-3. For IRS-1/2 transfectants, 5 mM histidinol was included in the medium. TS1 cells were maintained in Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with murine IL-9 (0.1 ng/ml). Electroporation was performed to transfect IRS-1 mutants into TS1 cells as described previously (33). Briefly, 10 μg of IRS-1 mutant constructs was transfected into TS1 cells by a single 250-V/960-microfarad (μF) pulse in a Bio-Rad Gene Pulser. After selection in G418 (0.6 mg/ml) for 10–14 days, resistant cells were further cloned by limiting dilution. The dominant negative Akt mutant (34) was cotransfected with pBabe-puro (35) (DNA ratio 10:1) into $32D^{IR}$/IRS-1 cells by a single 250-V/500-μF pulse. Following transfection, cells were plated into 96-well plates and selected with 1.5 μg/ml of puromycin for 10 days. For each construct, at least 24 clones were picked and analyzed for expression of IRS-1 or dominant negative Akt by immunoblotting. Three independent clones from each of the constructs were used for further experiments.

Cytokine Stimulation, Immunoprecipitation, and Immunoblotting—$32D^{IR}$ cells were starved in Dulbecco's modified Eagle's medium medium without serum and WEHI-conditioned medium for 4 h, while TS1 cells were starved in Dulbecco's modified Eagle's medium without serum and IL-9 for 7 h. Following starvation, cells (2×10^7/ml) were stimulated with IL-9 and IL-4 (100 ng/ml) at 37 °C for 5 min. Cells were collected by centrifugation and lysed in 1 ml of TNE lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5% glycerol, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium vanadate) containing 1% Nonidet P-40 for immunoprecipitation or 0.5% Nonidet P-40 for coimmunoprecipitation. Solubilized proteins were collected for immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as described previously (36).

[^{3}H]Thymidine Uptake Assay—IL-9- and IL-4-stimulated [^{3}H]thymidine incorporation in $32D^{IR}$ cell lines was assayed as described previously (33). Briefly, $32D^{IR}$ cells were washed and seeded into 96-well plates at 2×10^5/ml in RPMI with 10% fetal bovine serum alone or containing various concentrations of IL-9 and IL-4, or 5% IL-3 containing WEHI-conditioned medium, followed by incubation at 37 °C for 48 h. [^{3}H]Thymidine (Amersham Biosciences, Inc., specific activity 5 Ci/mmol) was added at a concentration of 2 μCi/ml for the final 4 h. Cells were collected onto glass microfiber filters and counted in scintillation fluid using a liquid scintillation counter (Beckman Instruments, LS6000IC).

RESULTS

Tyrosine Phosphorylation of Wild Type and Mutant IRS-1—We have previously shown that IRS-1 and IRS-2 are tyrosine-phosphorylated by Janus kinases following IL-9 and IL-4 stimulation (33, 36). The mechanisms involved in tyrosine phosphorylation of IRS proteins by Janus kinases and other receptor tyrosine kinases are unclear. The NP*X*Y (Tyr-497) motif within the human IL-4 receptor is critical for the activation of the IRS pathway (20). We found that Box1 and a downstream region (amino acids 338–422) within the human IL-9 receptor, which do not contain NP*X*Y motifs, are necessary for IRS-2 tyrosine phosphorylation (33). It is possible that IL-9 and IL-4 receptors may use different mechanisms to recruit IRS proteins following ligand binding. To explore such mechanisms, we examined the functional roles of the structural domains of IRS-1 in TS1 cells following stimulation with IL-4 or IL-9.

A series of N-terminal deletion mutants of IRS-1 (Fig. 1*A*) were stably transfected into TS1 cells. The expression levels of these IRS-1 mutants were relatively equal as determined by Western blot analysis (Fig. 1*B*). We analyzed different domains in coupling IRS-1 to activated receptors by examining the tyrosine phosphorylation of these deletion mutants after cytokine stimulation. As shown in Fig. 1*C*, following IL-9 stimulation, wild type IRS-1 and the IRS-$1^{\Delta SAIN}$ deletion mutant were tyrosine-phosphorylated, suggesting that the SAIN domain is not

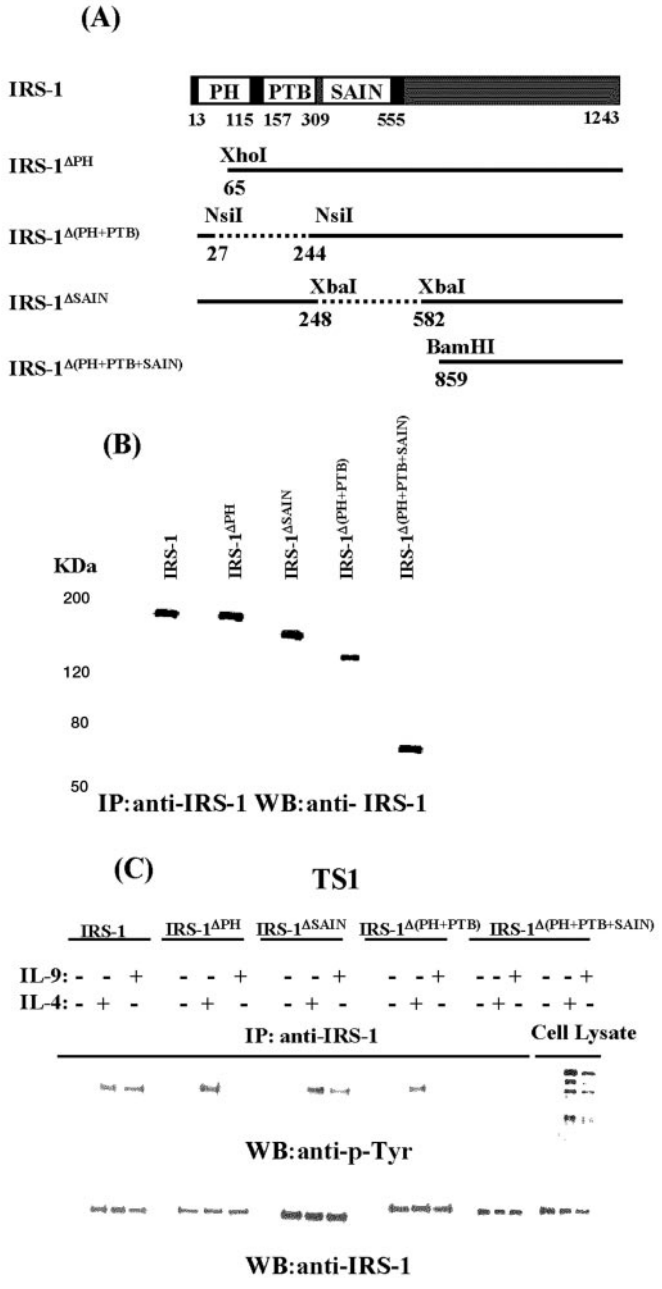

FIG. 1. **Tyrosine phosphorylation of wild type and mutant forms of IRS-1 in TS1.** *A*, schematic representation of wild type and deletion mutants of IRS-1. *B*, expression levels of various IRS-1 mutants in TS1 cells. *C*, 2×10^7 TS1 cells were starved and stimulated, and cell lysates were immunoprecipitated with 20 μl of anti-IRS-1. After extensive washing, immunoprecipitated proteins were separated by 10% SDS-PAGE. Proteins were transferred onto a PVDF membrane. After immunoblotting with anti-phosphotyrosine, the membrane was stripped and re-blotted with anti-IRS-1. These experiments were repeated at least twice with similar results.

necessary for IL-9-induced tyrosine phosphorylation of IRS-1. However, the IRS-$1^{\Delta PH}$ mutant lacking half of the PH domain was not tyrosine-phosphorylated, indicating that the PH domain is required for IL-9-induced tyrosine phosphorylation. IRS-$1^{\Delta(PH+PTB)}$ and IRS-$1^{\Delta(PH+PTB+SAIN)}$ mutants, which lack PH domain, were not tyrosine-phosphorylated, further demonstrating the essential role of the PH domain in IL-9-induced IRS-1 tyrosine phosphorylation. In contrast, IL-4 was able to stimulate tyrosine phosphorylation of all of the IRS-1 deletion mutants tested except IRS-$1^{\Delta(PH+PTB+SAIN)}$, suggesting that the PH, PTB, or SAIN domains are sufficient to mediate IRS-1 tyrosine phosphorylation induced by IL-4. These data demonstrate that the PH domain plays different roles in coupling IRS-1 to IL-9 and IL-4 receptors.

Proliferation of 32D^{IR} Cells Stably Transfected with IRS-1/2 Mutants—We have previously shown that overexpression of IRS-1 in TS1 cells, which express IRS-2 constitutively, enhanced the sensitivity of TS1 cells to IL-9 (36), suggesting a possible role for IRS proteins in IL-9-mediated proliferation. In this study, we used myeloid progenitor 32D cells, which do not express any IRS proteins, as a model system to explore the functions of IRS proteins and the roles of N-terminal domains in mediating the proliferative effects of IL-9 and IL-4.

We transfected 32D^{IR} cells with wild type and deletion mutants of IRS-1/2 (Fig. 2*A* (32)). 32D^{IR} cells transfected with these mutants expressed comparable levels of the IRS proteins (32). As in TS1 cells, deletion of the PH domain, but not the PTB domain, abrogated tyrosine phosphorylation of IRS-1 induced by IL-9 but not IL-4 (Fig. 2*B*). These consistent data, based on results using two independent PH deletion mutants characterized in two different cell lines, suggest that the PH domain is necessary for coupling IRS-1 to activated IL-9 but not IL-4 receptor. Despite high homology between IRS-1 and IRS-2, different mechanisms are involved in the binding of IRS-1/2 to the insulin receptor. Although IRS-1 binds to the phosphorylated NP*X*Y (Tyr-960) motif of the insulin receptor juxtamembrane region, mainly through the PTB domain (23), IRS-2 can also bind to insulin receptor through the 591–786 region, which is absent in IRS-1 (38). To test whether the functions of the PH, PTB, and SAIN domains of IRS-2 are different from those of IRS-1 in IL-9 and IL-4 signaling, we examined tyrosine phosphorylation of wild type and serial deletion mutants of IRS-2 in 32D^{IR} cells following immunoprecipitation with anti-IRS-2. As shown in Fig. 2*C*, 32D^{IR} cell lines overexpressing IRS-2 deletion mutants yielded results similar to those obtained with IRS-1. This suggests that similar mechanisms are utilized to couple IRS-1 and IRS-2 to activated IL-9 and IL-4 receptors.

To determine the physiological effects of IRS-1/2 deletion mutants, we measured the proliferation of 32D^{IR} cell lines by [^{3}H]thymidine incorporation. First, we investigated the ability of wild type IRS proteins to mediate the proliferative effects of IL-9 and IL-4 in 32D^{IR} cells. As shown in Fig. 3*A*, IRS-1/2 enhanced IL-9- and IL-4-induced 32D^{IR} cell proliferation. IRS-4 only promoted cell proliferation induced by IL-4, but not IL-9, suggesting that IRS-4 plays different roles in IL-9 and IL-4 signaling. Further studies on IRS-1/2 deletion mutants demonstrated that growth promotion mediated by IRS-1/2 mutants correlates with their tyrosine phosphorylation status in 32D^{IR} cells (Fig. 3*B*).

IRS-1/2-interacting Proteins in IL-9- and IL-4-stimulated Cells—The mechanisms involved in IRS-1/2 tyrosine phosphorylation may vary depending on cytokines studied. We hypothesized that these differences affect the ability of phosphorylated IRS proteins to interact with downstream signaling molecules. To test this possibility, we examined interaction of PI3K, Grb-2, and Shp-2 with IRS proteins activated by IL-4 and IL-9. As shown in Fig. 4, in TS1/IRS-1 and 32D^{IR}/IRS-1/2 cells, tyrosine-phosphorylated IRS-1 stimulated by IL-9 and IL-4 recruited the p85 regulatory subunit of PI3K and Grb-2. We detected Shp-2 binding to IRS-1 in TS1/IRS-1 cells following stimulation by IL-4 but not IL-9. Although Shp-2 association with IRS-1/2 was not detectable in 32D^{IR}/IRS-1/2 cells following stimulation with IL-9 or IL-4 (Fig. 4), Shp-2 tyrosine phosphorylation was IRS-1/2-dependent and only induced by IL-4 but not IL-9 (Fig. 5).

To explore the role of PI3K in IRS protein-mediated cell proliferation, we examined whether PI3K inhibitors, wortman-

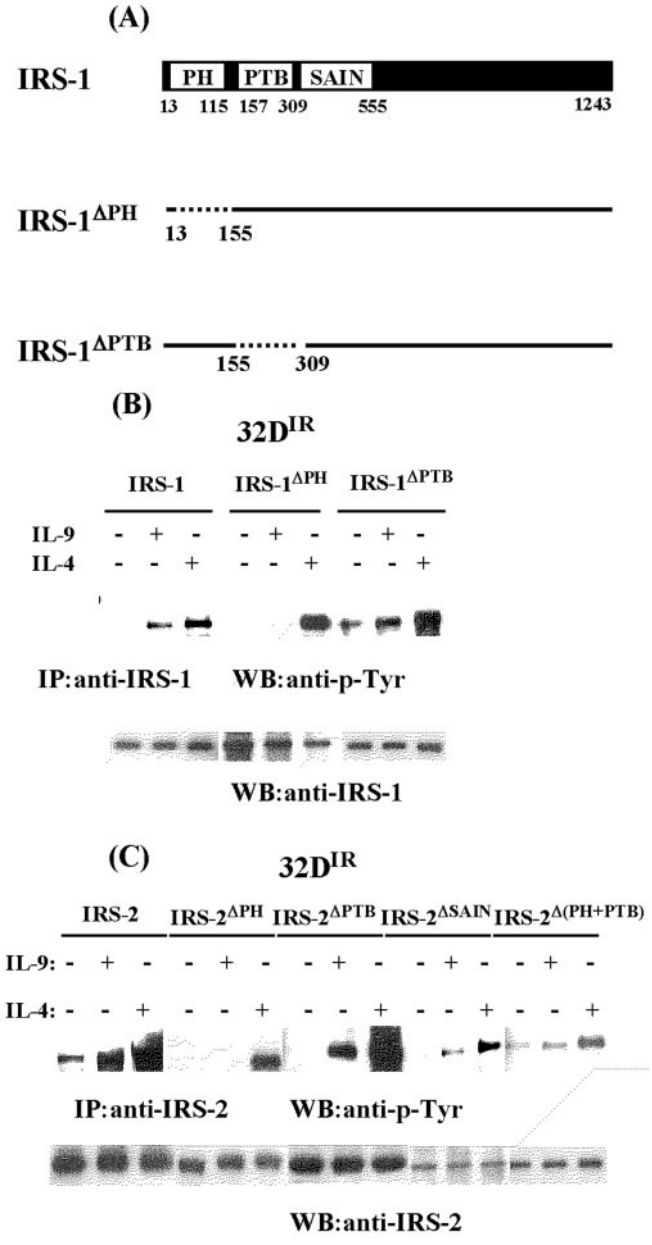

FIG. 2. **Tyrosine phosphorylation of wild type and mutant forms of IRS-1/2 in 32D^{IR}.** *A*, schematic representation of wild type and deletion mutants of IRS-1. The locations of PH, PTB, and SAIN are indicated. 1×10^8 32D^{IR} cells were starved, stimulated, and immunoprecipitated with 20 μl of anti-IRS-1 (*B*) or 5 μl of anti-IRS-2 (*C*). After extensive washing, immunoprecipitated proteins were separated by 10% SDS-PAGE. After immunoblotting with anti-phosphotyrosine, the membrane was stripped and re-blotted with anti-IRS-1 (*B*) or anti-IRS-2 (*C*). These experiments were repeated at least twice with similar results.

nin and LY294002, could inhibit proliferation stimulated by IL-9 and IL-4 in TS1/IRS-1 and 32D^{IR}/IRS-1 cells. As shown in Fig. 6*A*, wortmannin and LY294002 inhibited cell proliferation induced by IL-9 and IL-4, suggesting that PI3K plays an essential role in IRS-1-mediated cell proliferation stimulated by both cytokines. Although PI3K could be recruited to IRS, the downstream signaling events mediated by PI3K might be different following IL-9 and IL-4 stimulation. Previous studies have shown that the Akt threonine/serine kinase is a downstream target for activated PI3K. Thus we tested whether Akt can be activated by IL-9. As shown in Fig. 6*B*, although Akt was phosphorylated at Thr-308 site in response to IL-4 stimulation in 32D^{IR}/IRS-1 cells, we could not detect Akt phosphorylation after IL-9 stimulation.

To further address whether Akt plays a specific role in IL-4- and IL-9-induced cell proliferation, we established 32D^{IR}/IRS-1 cell lines that express dominant negative Akt. As shown in Fig. 7*A*, IL-4-induced cell proliferation was attenuated in three independent dominant negative Akt-expressing clones. Furthermore, the activation of p70 S6 kinase, which was inhibited in the presence of PI3K inhibitor, was reduced by the expression of dominant negative Akt in IL-4-stimulated cells (Fig. 7*B*), demonstrating that PI3K-dependent activation of Akt and p70 S6 kinase is important for IL-4 signaling. In contrast, dominant negative Akt did not attenuate IL-9-stimulated cell proliferation in 32D^{IR}/IRS-1 cells (Fig. 7*A*) and p70 S6 kinase activation was not detected in IL-9-stimulated 32D^{IR}/IRS-1 cells (Fig. 7*B*).

DISCUSSION

IL-9 and IL-4 promote the growth of T lymphoma cells *in vitro*, but the mechanism by which the proliferative signals are transduced is not well understood. Activation of Jak/STAT and IRS-1 pathways has been shown to be important for IL-4- and IL-9-stimulated proliferation and anti-apoptosis in hematopoietic cells (39, 40). In this study, we showed that IRS-1/2, but not IRS-4, enhances proliferation in response to IL-9. Thus IRS family members play different physiological functions in cytokine signaling.

Many multimodular molecules engage signaling pathways through various structural domains or different interface of the same domain to interact with diverse proteins. Shc associates with the IR through the PTB domain binding to the NP*X*Y motifs, but primarily uses an SH2 domain to interact with activated epidermal growth factor receptor (41). In insulin signaling, both the PH and PTB domains of IRS-1, as well as the KRLP domain of IRS-2 are important in mediating insulin-induced IRS activation (38, 42, 43). Previous studies demonstrated that the NPAY motif in the IL-4 receptor is required for IRS tyrosine phosphorylation stimulated by IL-4, and the PTB domain can directly bind to this motif *in vitro*, suggesting the important role for the PTB domain in IL-4 signaling (20). In this study, we showed that none of the N-terminal domains is absolutely essential for the activation of the IRS pathway by IL-4, and the SAIN domain appears to be sufficient to couple IRS proteins to the IL-4 receptor. Considering the structural and functional similarities between the PTB and SAIN domains, we propose that the PTB and/or SAIN domains could be the major adaptors for coupling IRS proteins to cytokine receptors containing the NP*X*Y or NP*X*Y-like motifs.

Our data also suggest that the PH domain, but not PTB or SAIN domains, is essential for coupling IRS proteins to IL-9 receptor. This PH domain-coupling model in IL-9 signaling may be widely employed by other cytokine receptors that do not contain the NP*X*Y motifs, such as interferon, IL-2, and IL-7 receptors. The PH domain may interact with phospholipids or membrane proteins to localize IRS proteins to the plasma membrane to facilitate interactions with the IL-9 receptor or bind with the IL-9 receptor-associated proteins. Because the PH domain plays different roles in IL-9 and IL-4 signaling, the mechanisms involved in IRS activation could be due to specific interactions with IL-9 receptor-associated proteins. So far, many proteins have been shown to interact with the PH domain of IRS proteins, such as PHIP (PH domain-interacting protein), acidic proteins as well as 14-3-3 (44–46). 14-3-3 plays important roles in signal transduction pathways involved in cell cycle regulation and induction of apoptosis, functioning as a chaperone by binding conserved phosphoserine/threonine motifs in Raf and cdc25. Interestingly, 14-3-3 also interacts with

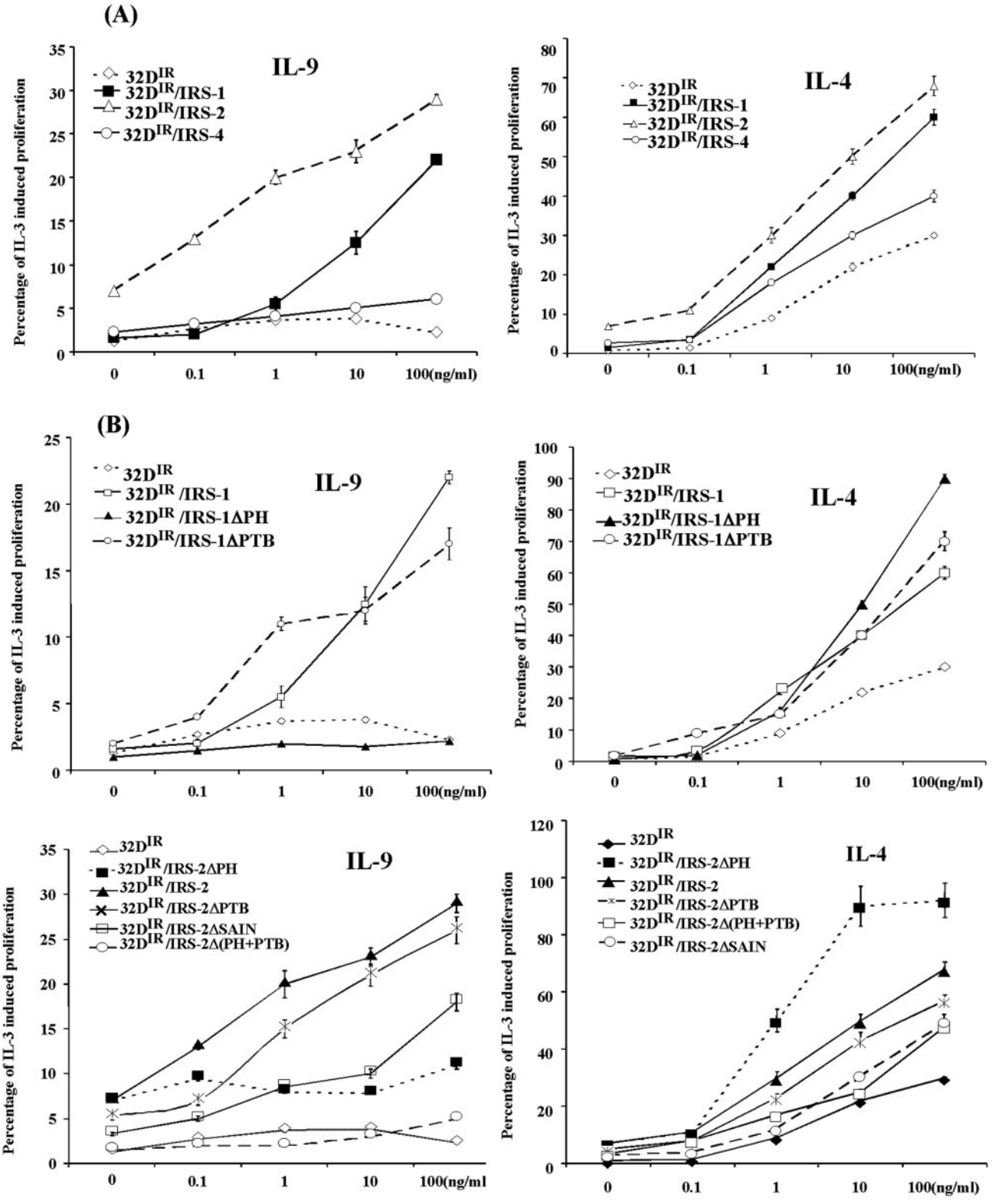

FIG. 3. **Cytokine-induced proliferation of 32D^{IR} cell lines expressing different members of IRS proteins (*A*) or mutant forms of IRS-1/2 proteins (*B*).** 32D^{IR} cells and 32D^{IR} transfectants were seeded into 96-well plates (2×10^4 cells/well) after deprivation of IL-3 and incubated with various concentrations of murine IL-9 or IL-4 for 48 h. Cells were pulsed with [^{3}H]thymidine (0.5 μCi/per well) for the last 4 h, harvested, and counted. Data points are the mean of triplicate samples in one experiment. Proliferation induced by IL-9 and IL-4 in different 32D^{IR} cell lines is presented as a percentage of proliferation of corresponding cells in 5% WEHI-conditioned medium. Each experiment was repeated at least three times and similar results were obtained.

integrin and cytokine receptors, raising the possibility that 14-3-3 may function as a bridge between the receptors and other signaling molecules (47–50). Furthermore, x-ray structure study provides evidence that 14-3-3 dimer forms two large acidic grooves that can associate with two different molecules. We have shown that 14-3-3 interacts with the IL-9 but not IL-4 receptor α chain (51), further suggesting that 14-3-3 may link IRS proteins to the IL-9 receptor.

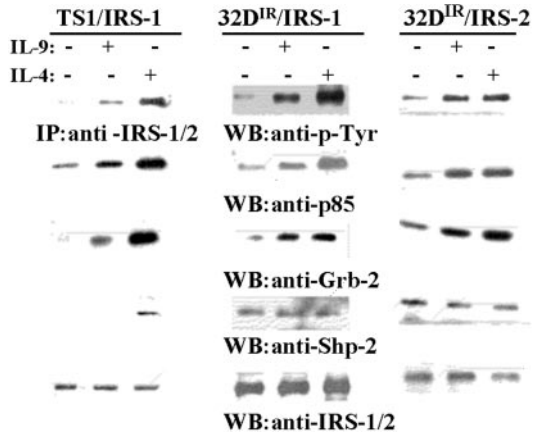

FIG. 4. **Coimmunoprecipitation of SH2-containing molecules with IRS-1/2.** 5×10^7 cells were starved for 6 h and then stimulated with IL-9 or IL-4 (100 ng/ml) for 10 min. Cell lysates were prepared in TNE buffer containing 0.5% Nonidet P-40 and immunoprecipitated with 30 μl of anti-IRS-1 or 5 μl of anti-IRS-2. After immunoprecipitation, proteins were separated by 12% SDS-PAGE, and transferred onto a PVDF membrane. The PVDF membrane was blotted with anti-phosphotyrosine, anti-p85, anti-Shp-2, anti-Grb-2, or anti-IRS-1/2 antibodies, respectively. The experiment was repeated at least twice with similar results.

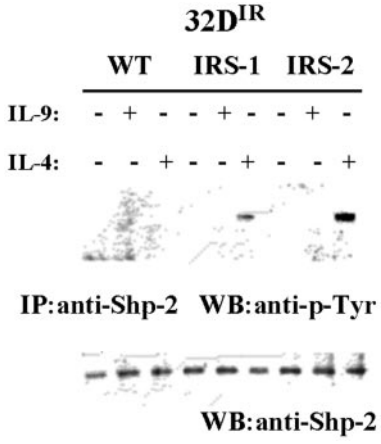

FIG. 5. **Tyrosine phosphorylation of Shp-2 stimulated by IL-9 and IL-4 in different 32D^IR cell lines.** 4×10^7 cells were starved for 4 h prior to stimulation for 10 min by IL-9 or IL-4. Cell lysates were immunoprecipitated with 5 μl of anti-Shp-2. Protein samples were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with anti-phosphotyrosine, anti-Shp-2, and anti-IRS-1, respectively. The same results were obtained from three independent experiments.

We also investigated the role of signaling molecules, such as PI3K, Grb-2, and Shp-2, in cell proliferation mediated by IL-9 and IL-4. By using PI3K inhibitors and dominant negative Akt, we have confirmed that the PI3K-Akt pathway is necessary for IRS protein-mediated cell proliferation in IL-4 signaling. Although PI3K activity is required for cell proliferation stimulated by IL-9 in 32D cells (Fig. 6*A*), we and others have failed to detect Akt activation in this cell line (52). In addition, p70 S6 kinase, one of the downstream substrates of PI3K, is not activated by IL-9 stimulation, indicating there might be other pathways activated by PI3K that mediate the proliferative effect of IL-9. Although Grb-2 can activate MAPK upon binding to IRS-1 following insulin stimulation, Grb-2/Sos binding to IRS-1 does not result in MAPK activation by IL-4 (37) or IL-9 (data not shown). Therefore, Grb-2 may bind other unknown proline-rich proteins through the SH3 domains to transduce IRS-dependent signals following IL-9 and IL-4 stimulation. Thus, PI3K, but not MAPK, is likely to be an important downstream signaling molecule in IRS-mediated cell proliferation in IL-9- and IL-4-stimulated cells. Divergent signaling pathways downstream of PI3K most likely further contribute to the signaling and functional specificity of these two cytokines. We also found that Shp-2, an SH2-containing tyrosine phosphatase,

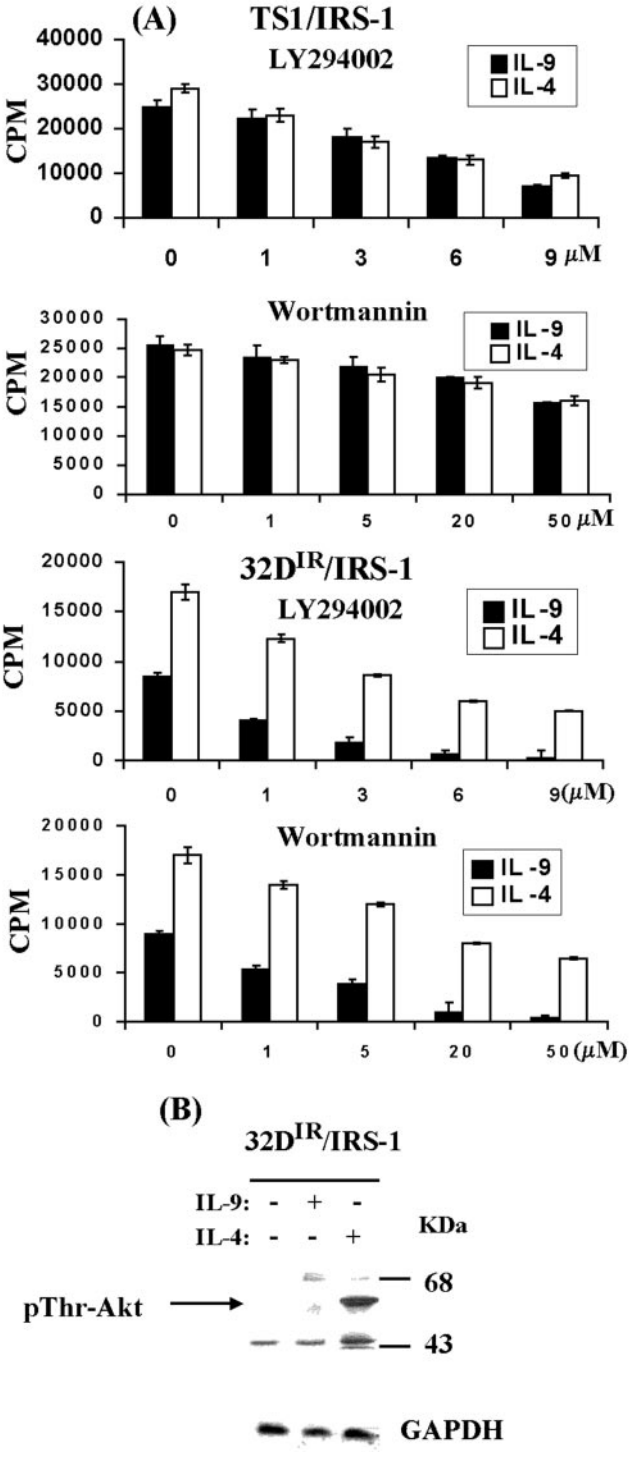

FIG. 6. **PI3K and Akt activation by IL-4 and IL-9.** *A*, cell proliferation assay measured by [^{3}H]thymidine incorporation. The same amount of Me_2SO was added instead of inhibitors in control cells. *B*, cells were starved and stimulated as described under "Materials and Methods." After lyses in TNE buffer, cell lysates from 2×10^6 cells/sample were separated by 10% SDS-PAGE. After proteins were transferred onto a PVDF membrane, anti-phospho-threonine (Thr-308) was used to examine Akt activation. The data are representative of three independent experiments.

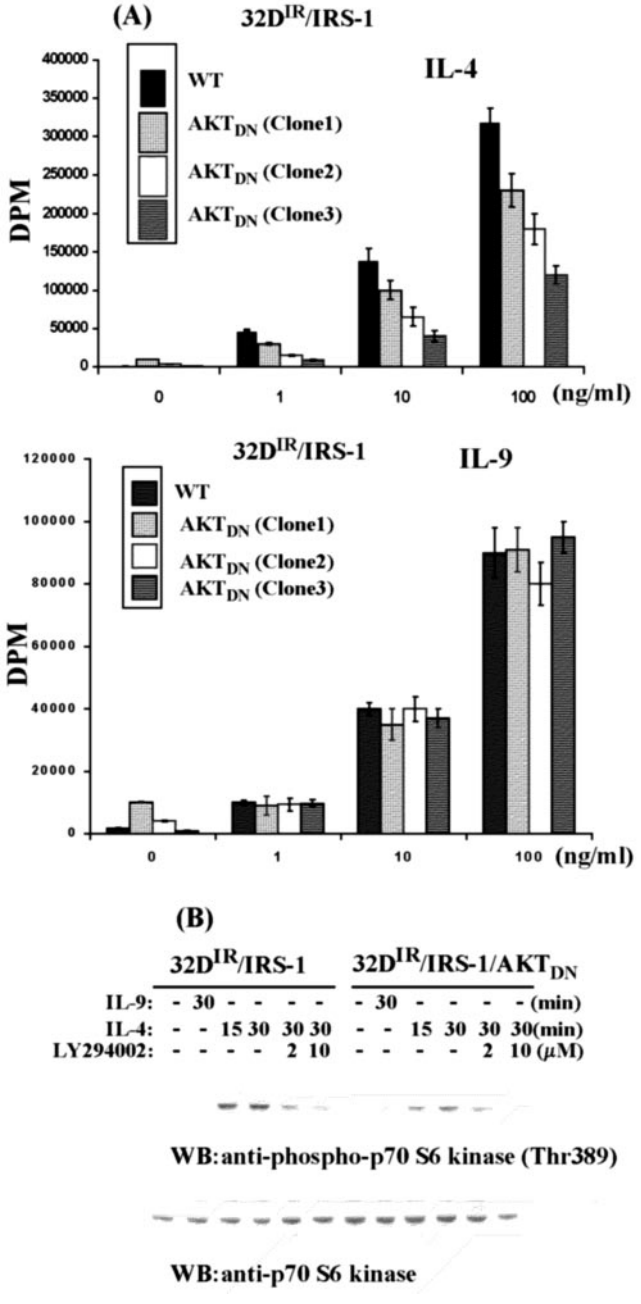

FIG. 7. **IL-4-induced cell proliferation is attenuated by dominant negative Akt.** *A*, three independent clones that express dominant negative Akt were used for [^{3}H]thymidine incorporation assay. *B*, the same amount of Me_2SO or LY294002 was added to starved cells 30 min prior to stimulation by either IL-4 or IL-9 (50 ng/ml) for indicated time period. 2×10^6 cells were lysed, and proteins were separated by 10% SDS-PAGE. After proteins were transferred onto a PVDF membrane, anti-phospho-p70 S6 kinase (Thr-389) and anti-p70 S6 kinase were used for immunoblotting, respectively. These experiments were repeated twice with similar results.

physically associates with tyrosine-phosphorylated IRS-1 induced by IL-4 but not by IL-9. Shp-2 recruited by IRS proteins may mediate downstream signaling in the IL-4-induced IRS signaling pathway. However, in the present study, we could not detect IRS protein-dependent MAPK activation in IL-4 signaling (data not shown), suggesting that other uncharacterized pathways may be activated by IRS/Shp-2 in IL-4 signaling. These data support the hypothesis that different tyrosine motifs within the C terminus of IRS proteins are phosphorylated to transduce specific functions of different stimuli.

In summary, our attempts to explore the roles of different N-terminal domains in coupling IRS proteins to IL-9 and IL-4 receptors reveal that different structural domains are utilized to couple different cytokine receptors. The PTB and/or SAIN domains may anchor IRS proteins to receptors with the NP*X*Y or NP*X*Y-like motifs, whereas the PH domain is essential for IRS association with receptors without the NP*X*Y motifs. Our data also demonstrate that the specific physiological functions of IL-9 and IL-4 might be mediated by IRS through the recruitment of different SH2-containing molecules. Furthermore, PI3K-dependent, but Akt/p70 S6 kinase-independent, pathways may play an important role in transducing the proliferative effect of IL-9.

Acknowledgments—We thank Dr. Richard A. Roth for generously providing dominant negative Akt mutant and Dr. David Donner for reading the manuscript and helpful discussions.

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