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Association between GRB2/Sos and Insulin Receptor Substrate 1 Is Not Sufficient for Activation of Extracellular Signal-Regulated Kinases by Interleukin-4: Implications for Ras Activation by Insulin

W. PRUETT,[1] Y. YUAN,[1] E. ROSE,[1] A. G. BATZER,[1] N. HARADA,[2] AND E. Y. SKOLNIK[1]*

Skirball Institute for Biomolecular Medicine, Department of Pharmacology, New York University Medical Center, New York, New York,[1] and Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, California[2]

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Insulin receptor substrate 1 (IRS-1) mediates the activation of a variety of signaling pathways by the insulin and insulin-like growth factor 1 receptors by serving as a docking protein for signaling molecules with SH2 domains. We and others have shown that in response to insulin stimulation IRS-1 binds GRB2/Sos and have proposed that this interaction is important in mediating Ras activation by the insulin receptor. Recently, it has been shown that the interleukin (IL)-4 receptor also phosphorylates IRS-1 and an IRS-1-related molecule, 4PS. Unlike insulin, however, IL-4 fails to activate Ras, extracellular signal-regulated kinases (ERKs), or mitogen-activated protein kinases. We have reconstituted the IL-4 receptor into an insulin-responsive L6 myoblast cell line and have shown that IRS-1 is tyrosine phosphorylated to similar degrees in response to insulin and IL-4 stimulation in this cell line. In agreement with previous findings, IL-4 failed to activate the ERKs in this cell line or to stimulate DNA synthesis, whereas the same responses were activated by insulin. Surprisingly, IL-4's failure to activate ERKs was not due to a failure to stimulate the association of tyrosine-phosphorylated IRS-1 with GRB2/Sos; the amounts of GRB2/Sos associated with IRS-1 were similar in insulin- and IL-4-stimulated cells. Moreover, the amounts of phosphatidylinositol 3-kinase activity associated with IRS-1 were similar in insulin- and IL-4-stimulated cells. In contrast to insulin, however, IL-4 failed to induce tyrosine phosphorylation of Shc or association of Shc with GRB2. Thus, ERK activation correlates with Shc tyrosine phosphorylation and formation of an Shc/GRB2 complex. Previous studies have indicated that activation of ERKs in this cell line is dependent upon Ras since a dominant-negative Ras (Asn-17) blocks ERK activation by insulin. Our findings, taken in the context of previous work, suggest that binding of GRB2/Sos to Shc may be the predominant mechanism whereby insulin as well as cytokine receptors activate Ras.

Insulin stimulates a diverse array of cellular responses, including cell growth and metabolic responses such as glucose uptake and glycogen synthesis. Ligand activation of the tyrosine kinase domain of the insulin receptor is probably the central event in mediating a cellular insulin response (16, 34). One function of the tyrosine kinase is to autophosphorylate the insulin receptor itself, which leads to activation of the tyrosine kinase catalytic domain (37, 58). A second important function of the tyrosine kinase is to stimulate tyrosine phosphorylation of several intermediate proteins, including the insulin receptor substrate 1 (IRS-1) (48, 55, 57).

Recent studies have suggested that IRS-1 may be critical in linking the insulin receptor to cellular responses. IRS-1 is a 185-kDa cytoplasmic protein that is a major target of the insulin and insulin-like growth factor 1 (IGF-1) receptors (48). IRS-1 contains 20 potential tyrosine phosphorylation sites, many of which are contained within consensus SH2 binding motifs. SH2 domains are found in a variety of distinct signaling molecules and mediate protein-protein interaction by binding phosphotyrosine moieties in the context of short amino acid sequences (31, 43). It has been hypothesized that binding of signaling molecules, via their SH2 domains, to IRS-1 couples the insulin receptor to downstream signaling pathways.

Several observations support the idea that IRS-1 is important in insulin and IGF-1 receptor signaling. In response to insulin stimulation, IRS-1 simultaneously binds and activates a variety of signaling molecules with SH2 domains including p85-associated phosphatidylinositol 3-kinase (PI3 kinase), GRB2, SYP, and Nck (3, 4, 24, 25, 45). For example, the binding of p85 to IRS-1 results in enzymatic activation of the 110-kDa catalytic domain of PI3 kinase (3). In addition, IRS-1 has been shown to directly link the insulin receptor to DNA synthesis; overexpression of IRS-1 results in an increase in the proliferative response to insulin, and inhibition of IRS-1 inhibits the mitogenic response (36, 47, 53). Further evidence supporting the idea that IRS-1 is important for insulin-stimulated mitogenesis was provided by the finding that the introduction of IRS-1 into a myeloid progenitor cell line restored the ability of insulin to stimulate DNA synthesis in this cell line (52). More recently, IRS-1 knockout mice have been generated and reveal that IRS-1 is important not only for mitogenesis, but also for metabolic response; although IRS-1 knockout mice are viable, they exhibit growth retardation and insulin resistance (1, 49).

A problem with the previous studies is their inability to distinguish whether tyrosine phosphorylation of IRS-1 is sufficient to mediate responses from the insulin receptor, such as mitogenesis, or whether activation of IRS-1-independent sig-

* Corresponding author. Mailing address: New York University Medical Center, Skirball Institute for Biomolecular Medicine, Department of Pharmacology, 550 First Ave., New York, NY 10016. Phone: (212) 263-7458. Fax: (212) 263-5711.

naling pathways must also occur for the insulin receptor to signal. Moreover, the role of IRS-1 as distinct from that of other intermediate molecules, such as Shc, in mediating certain insulin responses has not been assessed. For example, it has previously been shown that GRB2 couples the Ras guanine nucleotide exchange factor, Son of Sevenless (Sos), to IRS-1 and Shc in insulin-stimulated cells (4, 33, 44). Shc is an SH2 domain-containing protein that is thought to be important in Ras activation; in addition to binding GRB2/Sos, overexpression of Shc induces differentiation of PC12 cells and transformation of 3T3 fibroblasts (32, 38). On the basis of these findings, it has been proposed that GRB2 activates Ras by functioning as an adaptor molecule to couple Sos, bound to the SH3 domains of GRB2, to IRS-1 and/or Shc in insulin-stimulated cells (4, 44, 45). Although there is much evidence to support this model, we still do not know the relative contributions of IRS-1 and Shc to Ras activation, or even whether the functional consequences of GRB2 interacting with IRS-1 and GRB2 interacting with Shc are similar.

Recently, it has been shown that interleukin-4 (IL-4) also phosphorylates IRS-1 and an IRS-1-related molecule, 4PS, in a hematopoietic cell line, 32D (52). The IL-4 receptor (IL-4R) is a member of the cytokine receptor superfamily. Under physiological conditions, the IL-4R requires the expression of two subunits to reconstitute high-affinity binding for IL-4, the IL-4Rα subunit and a common IL-2Rγ subunit that is shared among several cytokine receptors such as the IL-2, IL-4, and IL-7 receptors (12, 39). For the most part, only hematopoietic cells contain both subunits of the IL-4R; therefore, the biological effects of IL-4 are predominantly restricted to cells of hematopoietic lineages (12). As for other cytokine receptors, the IL-4R signals cells by activation of a cytoplasmic tyrosine kinase of the Janus kinase family (Jak) (21, 60). However, in contrast to other cytokine receptors, only the IL-4R together with the insulin and IGF-1 receptors have thus far been shown to tyrosine phosphorylate IRS-1 (55). These three receptors share a common IRS-1 binding motif that functions to juxtapose IRS-1 with a tyrosine kinase, thereby enabling IRS-1 to become phosphorylated (22, 55).

We were interested in determining whether we could reconstitute a functional IL-4R in an insulin-responsive tissue. We reasoned that if activation of specific signaling molecules by IRS-1 in response to insulin stimulation is sufficient to elicit certain biological responses, IL-4 and insulin should function similarly in an insulin-responsive cell line, such as an L6 myoblast. In this report, we show that IRS-1 is tyrosine phosphorylated to similar degrees in response to insulin and IL-4 stimulation in L6 myoblasts overexpressing both the IL-4Rα and IL-2Rγ subunits (L6/IL-4R). In agreement with previous findings, IL-4 failed to activate the extracellular signal-regulated kinases (ERKs) in this cell line or to stimulate DNA synthesis, whereas the same responses were activated by insulin. Surprisingly, IL-4's failure to activate ERKs was not due to a failure to stimulate the association of tyrosine-phosphorylated IRS-1 with GRB2/Sos; the amounts of GRB2/Sos associated with IRS-1 were similar in insulin- and IL-4-stimulated cells. In contrast to insulin, however, IL-4 failed to induce tyrosine phosphorylation of Shc or association of Shc with GRB2. These data suggest that the association of GRB2/Sos with IRS-1 plays little if any role in ERK activation in L6 myoblasts. Furthermore, the finding that insulin but not IL-4 stimulates the association of Shc with GRB2/Sos suggests that binding of GRB2/Sos to Shc may be the predominant mechanism whereby insulin activates Ras in muscle.

MATERIALS AND METHODS

Growth factors, cell lines, and stimulation. L6 rat myoblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (44). CHO/IR cells are Chinese hamster ovary cells which overexpress the wild-type human insulin receptor. To stimulate cells, cells were serum starved for 48 h in DMEM containing 0.2% FBS and then stimulated for 5 min with either 100 nM bovine insulin (Sigma) or 100 ng of human recombinant IL-4 (Intergen) per ml.

Antibodies. Polyclonal rabbit antibodies to a peptide corresponding to amino acid residues 36 to 50 of GRB2 (Ab 86) and to a full-length GRB2–glutathione *S*-transferase (GST) fusion protein (Ab 50) were used for GRB2 immunoblotting and immunoprecipitation (26). IRS-1 antibodies were produced by immunizing rabbits with baculovirus-produced rat IRS-1 (3). Antibodies to p85 were raised against a GST fusion protein containing amino acids 265 to 523, and antiphosphotyrosine immunoblots were performed with a rabbit polyclonal antibody (19). Shc antibody is a rabbit polyclonal antibody raised against an Shc-SH2-GST fusion protein (45). Antibodies against ERK2 were kindly provided by Melanie Cobb, University of Texas Southwestern, Dallas (6).

Expression vectors and transfections. The cDNA for the full-length human IL-4Rα subunit (18) was subcloned into the eukaryotic expression vector pME18S containing the neomycin resistance gene, while the human IL-2Rγ subunit cDNA was subcloned into pME18S lacking the neomycin resistance gene (39). (The human IL-2Rγ cDNA was kindly provided by Warren Leonard, National Institutes of Health, Bethesda, Md.) To generate L6 myoblast cell lines overexpressing both chains of the functional IL-4R, L6 cells were cotransfected with the IL-2Rγ subunit together with the IL-4Rα subunit at a ratio of 20:1 by calcium phosphate precipitation (11). Neomycin-resistant clones were isolated, and IL-4R overexpression was assessed by determining whether stimulation of neomycin-resistant clones with human IL-4 resulted in tyrosine phosphorylation of IRS-1. In addition, overexpression of the IL-4Rα subunit was determined by cross-linking experiments using ^{125}I-IL-4 (see below) and overexpression of the IL-2Rγ subunit was confirmed by Western blotting (immunoblotting) (data not shown) (39).

Affinity labeling of IL-4R with ^{125}I-IL-4. Recombinant human IL-4 was iodinated by using Iodogen (Pierce) as previously described (39). Parental L6 cells or L6 cells transfected with the human IL-4Rα and IL-2Rγ chains were incubated with 150 pM ^{125}I-IL-4 and disuccinimidyl suberate in the absence or presence of a 100-fold molar excess of unlabeled IL-4. After cells were washed, cross-linking of ^{125}I-IL-4 was carried out as described elsewhere (39). Cell lysates were then analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 12% polyacrylamide gel), and after the gels were dried, bound IL-4 was determined by autoradiography.

Cell lysis, immunoprecipitation, and immunoblotting. Parental L6 cells or L6 cells overexpressing functional human IL-4Rs were stimulated as described above. Cell lysis, immunoprecipitation, and immunoblotting were performed as previously described (44).

Protein kinase assays. PI3 kinase assays were performed as previously described by Whitman et al. (59). To assay for ERK activity, after serum starvation L6 cells overexpressing the IL-4R were either left untreated or treated with insulin or IL-4 as appropriate. Cells were then lysed in lysis buffer {1% Triton X-100, 10 mM Tris [pH 7.4], 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 50 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40}, and 100 μg of total protein was immunoprecipitated with antibodies to ERK2. The immune complexes were isolated with protein A-Sepharose beads, washed twice with lysis buffer and twice with kinase buffer (10 mM Tris [pH 7.4], 10 mM $MgCl_2$) containing 4 μCi of [γ-^{32}P]ATP, 20 μM ATP, and 0.5 mg of myelin basic protein (MBP). After a 30-min incubation at 30°C, reactions were terminated by boiling the mixtures in sample buffer for 5 min and the reaction products were separated by SDS-PAGE (15% polyacrylamide gel) as previously described (44). The gel was then dried and subjected to autoradiography for 1 h.

Cellular proliferation assays. Cells (10^5) were plated in triplicate in 96-well plates in DMEM containing 10% FBS. Cells were growth arrested for 48 h as described above and then incubated with various concentrations of insulin or IL-4 for 36 h. The cells were pulsed with 0.5 μCi of [^{3}H]thymidine per ml for the final 16 h and harvested. [^{3}H]thymidine uptake was determined by liquid scintillation counting (46).

RESULTS

Generation of stable L6 cell lines overexpressing the IL-4R (L6/IL-4R). The human IL-4Rα subunit and the human IL-2Rγ subunit were cotransfected into an L6 myoblast cell line by calcium phosphate precipitation. Neomycin-resistant L6 cell lines expressing both the IL-4Rα and the IL-2Rγ subunits (L6/IL-4R) were generated. Overexpression of the IL-4Rα subunit was demonstrated by cross-linking experiments using ^{125}I-IL-4 (Fig. 1). In addition, overexpression of the IL-2Rγ subunit was confirmed by Western analysis (data not shown).

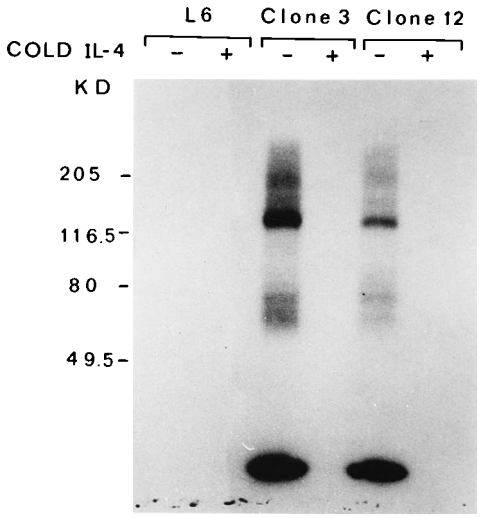

FIG. 1. Cross-linking of ^{125}I-IL-4 to L6 myoblasts transfected with the IL-4Rα and IL-2Rγ subunits. Two clones of L6 cells (clones 3 and 12) transfected with cDNAs encoding the human IL-4Rα and IL-2Rγ subunits, or mock-transfected L6 cells, were incubated with 150 pM ^{125}I-IL-4 either in the presence (+) or in the absence (−) of a 100-fold molar excess of cold IL-4. After cross-linking of ^{125}I-IL-4, lysates were analyzed directly on an SDS-PAGE gel (4 to 12% polyacrylamide) and, after drying of the gel, IL-4 bound to the IL-4Rα was determined by autoradiography.

IL-4 stimulation of L6/IL-4R cells results in tyrosine phosphorylation of IRS-1. It has been shown previously that, in response to ligand binding, IL-4 phosphorylates IRS-1 or an IRS-1 homolog, 4PS, in several hematopoietic cell lines (52). To assess whether a functional IL-4R was reconstituted into L6 cells, tyrosine phosphorylation of IRS-1 in L6/IL-4R cells was measured after stimulation with either 100 nM insulin or 100 ng of IL-4 per ml. Stimulation of L6 cells overexpressing the IL-4R with either insulin or IL-4 led to tyrosine phosphorylation of a 185-kDa protein (Fig. 2A). In contrast, stimulation of parental L6 cells with IL-4 did not result in an increase in tyrosine phosphorylation (data not shown). To confirm that the 185-kDa tyrosine-phosphorylated protein is IRS-1, IRS-1 was immunoprecipitated from L6 cell lysates by using a polyclonal antibody raised against baculovirus IRS-1 (3). The IRS-1 immunoprecipitates were then immunoblotted by using antibodies to phosphotyrosine. These results confirmed that IL-4 stimulates tyrosine phosphorylation of IRS-1; the same 185-kDa protein was immunoprecipitated from both insulin- and IL-4-stimulated L6 cell lysates (Fig. 2B, lanes 2 and 6). Moreover, this protein comigrated on SDS gels with tyrosine-phosphorylated IRS-1 that was immunoprecipitated from CHO cells overexpressing IRS-1 (Fig. 2B, lane 4).

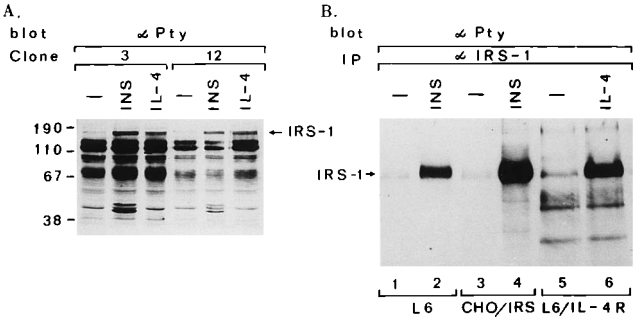

FIG. 2. IL-4 stimulates tyrosine phosphorylation of IRS-1 in L6/IL-4R cells. (A) Two clones of L6/IL4R cells (clones 3 and 12) were either left unstimulated (−) or stimulated for 5 min with 100 nM insulin or 100 ng of recombinant human IL-4 per ml and lysed. Cell lysates were then separated by SDS-PAGE (10% polyacrylamide gel) and, after transfer to nitrocellulose filters, immunoblotted with antibodies to phosphotyrosine. (B) Parental L6 cells (lanes 1 and 2) or geneticin-resistant pools of L6 cells that were transfected with IL-4Rα and IL-2Rγ subunits (lanes 5 and 6) were either left unstimulated (−) or stimulated as described in the legend for panel A, lysed, and immunoprecipitated with antibodies to IRS-1. The washed immunoprecipitates were then immunoblotted with antiphosphotyrosine antibodies. As a control, IRS-1 was immunoprecipitated from CHO cells overexpressing IRS-1 that were either left unstimulated or stimulated with 100 nM insulin (lanes 3 and 4). INS, insulin; αPty, antibody to phosphotyrosine; αIRS-1, antibody to IRS-1; IP, immunoprecipitate.

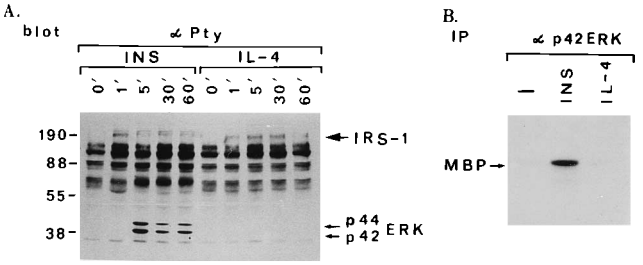

FIG. 3. IL-4 does not activate ERK2 in L6/IL-4R cells. (A) L6/IL-4R cells were treated for various times (given in minutes above the lanes) with either 100 nM insulin or 100 ng of IL-4 per ml and lysed. A 25-μg amount of cell lysates was separated by SDS-PAGE and immunoblotted with antibodies to phosphotyrosine. p44 ERK1 and p42 ERK2 are indicated by arrows. (B) A portion of cell lysates from the experiment shown in panel A was immunoprecipitated with antibodies to ERK2. An in vitro kinase reaction was then performed with the immunoprecipitates by using [γ-^{32}P]ATP and MBP as a substrate. The reaction products were then separated by SDS-PAGE (15% polyacrylamide gel) and, after drying of the gel, phosphorylated MBP was visualized by autoradiography. INS, insulin; αPty, antibody to phosphotyrosine; IP, immunoprecipitate; αp42 ERK, antibody to p42 ERK2.

Stimulation of L6/IL-4R with insulin, but not IL-4, results in the tyrosine phosphorylation and activation of p42 ERK2 and p44 ERK1. Previous studies have shown that IL-4 stimulation does not activate Ras and ERKs or mitogen-activated protein kinases in hematopoietic cells (42, 54). We have previously shown that GRB2 couples the Ras guanine nucleotide exchange factor, Sos, to tyrosine-phosphorylated IRS-1 in insulin-stimulated cells and have proposed that the interaction between GRB2, IRS-1, and Sos is important for the activation of Ras by insulin (44, 45). Ras activation initiates the activation of a kinase cascade that eventually results in the activation of ERKs (5). We have previously shown that activation of p42 ERK2 and p44 ERK1 by insulin in L6 cells is dependent upon Ras activation (44). Therefore, the finding that IL-4 also stimulated IRS-1 phosphorylation in L6 cells led us to determine whether IL-4 activated ERK1 and ERK2 in this cell line. While insulin- and IL-4-treated cells showed equal tyrosine phosphorylation of IRS-1, only insulin stimulation led to tyrosine phosphorylation of two proteins of 42 and 44 kDa that comigrated on SDS-PAGE gels with p42 ERK2 and p44 ERK1 (Fig. 3A and data not shown). To confirm that stimulation with insulin but not IL-4 resulted in an increase in ERK enzymatic activity, ERK1 and ERK2 were immunoprecipitated from either unstimulated or stimulated cell lysates and ERK enzymatic activity was determined in an in vitro kinase reaction using MBP as a substrate. These results showed that stimulation of L6/IL-4R cells with insulin but not stimulation with IL-4 led to enzymatic activation of ERK2 (Fig. 3B) and ERK1 (data not shown). The inability of IL-4 to activate ERKs was due neither to the amount of tyrosine phosphorylation of IRS-1 nor to differences in the time course of activation of IRS-1; the

time course of IRS-1 phosphorylation and the amount of tyrosine phosphorylation, as determined by antiphosphotyrosine immunoblotting, was the same whether cells were stimulated with insulin or IL-4 (Fig. 3A). Moreover, IL-4 did not activate signaling pathways that antagonized ERK1 and ERK2 activation; insulin activation of ERK1 and ERK2 was not affected by costimulation of L6/IL-4R cells with both IL-4 and insulin (data not shown).

IL-4 stimulation leads to a complex between GRB2/Sos and IRS-1 but not between GRB2 and Shc in L6/IL-4R cells. In order to determine why stimulation of L6 cells with insulin but not stimulation with IL-4 led to ERK activation, we assessed whether IL-4 stimulation resulted in a complex between IRS-1 and GRB2/Sos. We have shown previously that GRB2 binds phosphotyrosine 895 in IRS-1 (45). One possible reason for IL-4's failure to activate ERKs may be the inability of IL-4 to stimulate phosphorylation of IRS-1 at Y-895. Therefore, if IRS-1 phosphorylation by IL-4 is qualitatively different from IRS-1 phosphorylation by insulin, GRB2 may associate with IRS-1 in cells stimulated with insulin but not IL-4.

To assess whether IL-4 stimulation results in the stable association of IRS-1 with GRB2, L6 cells overexpressing the IL-4R were stimulated with IL-4 and the association of IRS-1 and GRB2 was assessed by determining whether the two proteins coimmunoprecipitate. Stimulation of parental L6 cells with IL-4 resulted in neither detectable tyrosine phosphorylation of IRS-1 nor association between GRB2 and IRS-1 (data not shown). However, IL-4 stimulation of L6/IL-4R cells resulted in tyrosine phosphorylation of IRS-1 and the formation of an IRS-1/GRB2 complex, as demonstrated by the coimmunoprecipitation of these proteins by anti-IRS-1 or anti-GRB2 antibodies (Fig. 4A). In addition, GRB2 coupled Sos to IRS-1 in IL-4-stimulated cells; anti-Sos immunoprecipitates resulted in the coimmunoprecipitation of tyrosine-phosphorylated IRS-1 from IL-4- and insulin-stimulated cells (Fig. 4C). These results demonstrate that the failure of IL-4 to activate ERKs is not due to the failure of IL-4 to induce the formation of a complex between GRB2/Sos and IRS-1.

We have previously shown that insulin stimulation results in tyrosine phosphorylation of Shc and the formation of a complex between Shc and GRB2 and have proposed that GRB2 binding of Shc may be an alternative route whereby insulin activates Ras. Therefore, if binding of GRB2 to Shc is more important than binding of GRB2 to IRS-1 for Ras activation by insulin, IL-4's failure to activate ERKs may be due to the inability of IL-4 to stimulate tyrosine phosphorylation of Shc and the formation of a complex between Shc and GRB2.

Antibodies to Shc recognize three proteins of 46, 52, and 66 kDa (32). To determine whether IL-4 stimulates tyrosine phosphorylation of Shc in L6/IL-4R cells, anti-Shc immunoprecipitates from IL-4- or insulin-stimulated cell lysates were immunoblotted with either antibodies to Shc or antibodies to phosphotyrosine. While equivalent amounts of Shc protein were immunoprecipitated from IL-4- and insulin-stimulated cell lysates (Fig. 4B), tyrosine phosphorylation of the 66- and 52-kDa Shc proteins was detected only in lysates from insulin-stimulated cells (Fig. 4B). Moreover, insulin but not IL-4 stimulation resulted in formation of a complex between GRB2 and Shc as demonstrated by coimmunoprecipitation of these proteins (Fig. 4B). While a minor increase in tyrosine phosphorylation of the 66-kDa Shc protein was seen with the IL-4-stimulated cells in some experiments, association between Shc and GRB2 was not seen after IL-4 stimulation. These observations suggest that IL-4's inability to activate ERKs in L6 cells may be due to the failure of IL-4 to stimulate tyrosine phosphorylation of Shc.

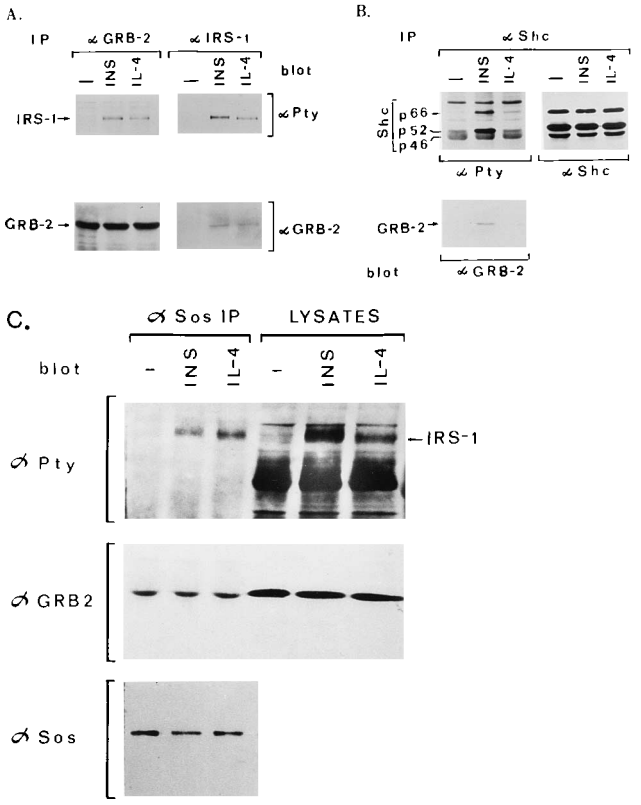

FIG. 4. IL-4 stimulates the association of GRB2/Sos with IRS-1 but not with Shc. L6/IL-4R cells were treated as described in the legend to Fig. 2, lysed, and immunoprecipitated with antibodies to IRS-1 or GRB2 (A), Shc (B), or Sos (C) as indicated. The washed immunoprecipitates were then separated by SDS-PAGE (10% polyacrylamide gel) and immunoblotted with antibodies as indicated. As a control, a 50-ng sample of total cell lysates was run alongside the anti-Sos immunoprecipitates in the experiment shown in panel C. To ensure that equal amounts of GRB2 and Sos were immunoprecipitated, the nitrocellulose filter was stripped and reprobed in the experiments shown in panels A and C with antibodies to GRB2 and Sos, respectively. αGRB-2, antibody to GRB2; αIRS-1, antibody to IRS-1; αShc, antibody to Shc; αPty, antibody to phosphotyrosine; αSos, antibody to Sos; IP, immunoprecipitate; INS, insulin.

Stimulation of L6/IL-4R cells with insulin but not IL-4 results in DNA synthesis. Stimulation of some hematopoietic cell lines with IL-4 leads to mitogenesis, and this increase in proliferation occurs independently of ERK activation (54). To determine whether IL-4 is mitogenic for L6 cells overexpressing the IL-4R, cells were stimulated with either IL-4 or insulin and induction of DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into DNA. While stimulation of L6 cells with insulin resulted in a severalfold increase in [^{3}H]thymidine uptake, stimulation of the same cells with IL-4 did not (Fig. 5). Thus, these results demonstrate that IL-4 does not activate a Ras-independent mitogenic pathway(s) in L6 cells.

PI3 kinase activations by IL-4 and insulin are similar in L6/IL-4R cells. As discussed above, it is possible that tyrosine phosphorylation of IRS-1 by IL-4 is qualitatively different from tyrosine phosphorylation of IRS-1 by insulin. Thus, IRS-1 may associate with distinct sets of signaling molecules in insulin- and IL-4-stimulated L6/IL-4R cells. One signaling molecule that is activated by binding IRS-1 and has been proposed to be important in linking the insulin receptor as well as other receptor tyrosine kinases to Ras activation and mitogenesis is PI3 kinase (10, 17, 41, 50). We were, therefore, interested in de-

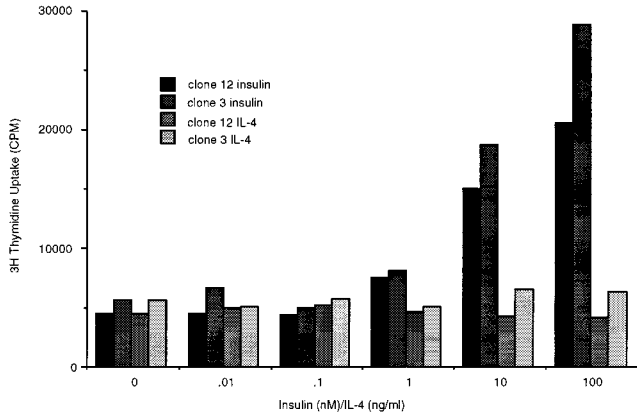

FIG. 5. Insulin, but not IL-4, stimulates proliferation of L6/IL-4R cells. Two clones of L6/IL-4R cells were stimulated with various concentrations of insulin and IL-4. [^{3}H]thymidine uptake was determined as described in Materials and Methods.

termining whether the inability of IL-4 to activate ERKs or to stimulate DNA synthesis was secondary to an inability of IL-4 to activate PI3 kinase in this cell line. To determine whether IL-4 activates PI3 kinase in L6/IL-4R cells, we assessed whether p85 and PI3 kinase activity coimmunoprecipitate with IRS-1 (45). Both insulin stimulation and IL-4 stimulation result in the association of p85 with IRS-1, as demonstrated by the coimmunoprecipitation of these proteins by either anti-p85 or anti-IRS-1 antibodies (Fig. 6A). In addition, insulin stimulation and IL-4 stimulation resulted in similar amounts of PI3 kinase activity being detected in anti-IRS-1 immunoprecipitates (Fig. 6B). Thus, these findings suggest that IL-4's inability to stimulate DNA synthesis and to activate ERKs is not secondary to IL-4's failure to activate PI3 kinase. In addition, while these results do not rule out a possible role for PI3 kinase in mediating Ras activation and mitogenesis by insulin, they suggest that activation of PI3 kinase via IRS-1 is not sufficient to mediate these responses.

DISCUSSION

To dissect IRS-1's role in mediating insulin-stimulated biological responses, we have reconstituted the IL-4R in an L6 myoblast cell line. Because the IL-4R also phosphorylates IRS-1, we reasoned that comparing the responses elicited by the IL-4R with those stimulated by the insulin and IGF-1 receptors might allow the delineation of specific signaling pathways that are regulated by IRS-1. One difficulty in evaluating the role of IRS-1 in insulin receptor signaling has been that of separating the manifestations of IRS-1 stimulation from those of Shc stimulation. Shc is ubiquitously expressed in cells; therefore, wherever IRS-1 is phosphorylated, Shc is also phosphorylated. Until now, this has prevented a clear assessment of the relative contributions of Shc and IRS-1 to Ras activation. The ability of the IL-4R to activate IRS-1 without activating Shc has given us a unique opportunity to evaluate IRS-1 signaling independent of the effects of Shc. The results presented in this paper suggest that binding of GRB2/Sos to IRS-1 plays little, if any, role in Ras activation. In contrast to insulin, IL-4 fails to activate p42 ERK2 and p44 ERK1, yet it is able to induce the formation of a complex between GRB2/Sos and IRS-1 that is of a magnitude equal to that observed after insulin stimulation. The observation that insulin, but not IL-4, induces tyrosine phosphorylation of Shc and the formation of a Shc/GRB2 complex suggests that phosphorylation of Shc is the primary mechanism whereby insulin activates ERKs in muscle.

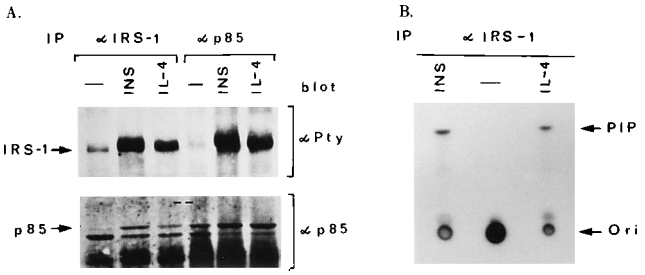

FIG. 6. p85 and PI3 kinase activity bind IRS-1 in IL-4-stimulated cells. (A) L6/IL-4R cells were stimulated as described in the legend to Fig. 2, lysed, and immunoprecipitated with anti-p85 or anti-IRS-1 antibodies as indicated. The immunoprecipitated proteins were separated by SDS-PAGE (10% polyacrylamide gel) and immunoblotted with antiphosphotyrosine or anti-p85 antibodies as indicated. (B) Anti-IRS-1 immunoprecipitates were subjected to a PI3 kinase assay. PIP, phosphatidylinositol phosphate; INS, insulin; IP, immunoprecipitate; αPty, antibody to phosphotyrosine; αp85, antibody to p85; αIRS-1, antibody to IRS-1.

The inability of IL-4 to activate Ras and ERKs has been reported previously (42, 54). While only activation of ERKs in L6 cells is shown in this paper, we are confident that activation of ERKs correlates with activation of Ras, on the basis of our previous observation that activation of ERKs by insulin in the L6 cell line used in this study is dependent upon Ras (44). We have excluded the possibility that IL-4 activates an inhibitory molecule or signaling pathway that antagonizes Ras's ability to activate ERKs; IL-4 failed to inhibit the activation of ERKs by insulin. These results, coupled with the demonstration that activation of Ras is sufficient to mediate activation of ERKs, indicate that Ras is not activated by IL-4 in this cell line (5). Direct measurements of Ras activation (Ras loading experiments) are not shown here because of our inability to detect increased Ras-GTP even in insulin-stimulated cells (unpublished results), despite the fact that activation of ERKs by insulin is clearly dependent on Ras in the L6 cell line used; expression of a dominant-negative Ras mutant blocks activation of Ras in this cell line (44). While we do not know why we do not detect an increase in Ras-GTP, others have reported difficulty detecting increases in Ras loading in other cell lines after growth factor stimulation (8), despite the fact that mediation of several biological responses by growth factor receptors in these cells clearly depends on Ras activation. Thus, our findings indicate that IL-4's inability to activate ERKs cannot be explained by its failure to stimulate the formation of a GRB2/Sos–IRS-1 complex. The finding that Shc is tyrosine phosphorylated by other cytokine receptors that activate Ras (13, 14, 27), but not by IL-4, suggests that Shc plays a critical role in linking cytokine receptors to Ras activation.

The results presented here are in agreement with other studies that have suggested that Shc may be more important than IRS-1 in coupling the insulin receptor to Ras activation (33, 40, 61). In addition, insulin has been shown to activate Ras in the 32D cell line lacking IRS-1 or the IRS-1-related molecule 4PS. The observation that insulin stimulation leads to tyrosine phosphorylation of Shc and to association of Shc with GRB2 in this cell line reinforces the idea that Shc is an intermediate between the insulin receptor and Ras activation. However, while there is a strong correlation between the association of tyrosine-phosphorylated Shc and GRB2/Sos with activation of Ras, other proteins are also likely to be important in coupling the insulin receptor to Ras activation. For example, PTP1D (SYP) has recently been shown to be important in

mediating Ras activation by insulin (28, 30), although the mechanism whereby PTP1D activates Ras is not yet known.

It is not clear why the association of GRB2/Sos with IRS-1 is not sufficient to activate ERKs in L6 myoblasts. It has been proposed that binding of GRB2/Sos to IRS-1 results in the translocation of a constitutively active Sos from the cytosol to the plasma membrane in insulin-stimulated cells, where it can then interact with Ras. Support for this hypothesis of Ras activation by Sos has been substantiated; targeting of Sos to the plasma membrane by the addition of either farnesylation or myristoylation signals is sufficient to activate Ras (2). However, unlike what is observed with the epidermal growth factor receptor, which binds GRB2 and Shc directly and thus can function to relocalize Sos to the plasma membrane (7), only a small amount of IRS-1 translocates to the plasma membrane in insulin-stimulated cells, presumably by interacting with the activated insulin receptor (23, 47). This may explain why IRS-1 does not couple to ERK activation in this cell line. Shc, on the other hand, may activate Ras by more efficiently localizing GRB2/Sos to the plasma membrane. However, the localization of Shc in insulin-stimulated cells, as well as the mechanism whereby Shc may couple Sos to the plasma membrane, is not known; Shc does not directly bind the insulin receptor or IRS-1 in insulin-stimulated cells (33, 45). Our findings, in contrast to those of others (33, 40), do not support the idea that the inability of GRB2 to link Sos to IRS-1 accounts for IRS-1's inability to couple to ERK activation in L6 cells; we found more Sos associated with IRS-1 than with Shc in L6 myoblasts stimulated with insulin (unpublished observation).

While the results we have obtained with L6 myoblasts using IL-4 suggest that formation of a complex between IRS-1 and GRB2/Sos is not important for Ras activation, Myers et al. suggested that IRS-1 may play a role in activation of Ras by insulin (29). Overexpressing IRS-1, but not an IRS-1 mutant that is unable to associate with GRB2, resulted in a twofold increase in p42 ERK2 activation over that in untransfected controls. Several explanations may account for the differences between that report and our results. First, muscle and hematopoietic (32D) cells may have different requirements for Ras activation in response to insulin stimulation; IRS-1 may play a role in Ras activation in hematopoietic but not in muscle cells. Alternatively, IRS-1 may not be biologically significant to Ras activation by insulin; the increase in ERK activity observed in the 32D cells transfected with IRS-1 was not shown to be secondary to an increase in Ras activity. ERK is activated via Ras-independent pathways under some circumstances (9), suggesting that the increase in ERK activity in the 32D cells observed may not be due to the activation of Ras by IRS-1. It is also possible that given physiological levels of IRS-1 expression, IRS-1 may add little to the effect of other signaling molecules, such as Shc, that activate Ras.

Although it might be argued that IRS-1 functions differently in insulin-stimulated and IL-4-stimulated cells, and therefore that GRB2/Sos binding to IRS-1 may actually be important in Ras activation by insulin, we think that this is unlikely. First, all of the known SH2 domain-containing signaling molecules that associate with IRS-1 in insulin-stimulated cells also associate with IRS-1 in IL-4-stimulated cells (PI3 kinase [Fig. 6], PTP1D [unpublished observation], and Nck [unpublished observation]). Second, it might be thought that IRS-1 could localize to the plasma membrane in insulin- but not IL-4-stimulated cells, if IRS-1 functions to activate Ras by coupling GRB2/Sos to the plasma membrane. However, while the cellular localization of IRS-1 in IL-4-stimulated cells has not been determined, it has recently been shown that IRS-1 coimmunoprecipitates with the IL-4Rα in IL-4-stimulated cells and binds a similar amino acid sequence motif present in the IL-4, insulin, and IGF-1 receptors (22). This leads us to predict that the relative affinities of IRS-1 for the IL-4, insulin, and IGF-1 receptors are similar. Third, we considered the possibility that association of IRS-1 with GRB2/Sos is necessary, but not sufficient, to activate Ras. However, there is currently no evidence to support the idea that activation of two signaling pathways is necessary for Ras activation by receptor tyrosine kinases (5, 50).

In agreement with previous studies showing that IL-4 activates PI3 kinase (20, 51), stimulation of L6 myoblasts overexpressing the IL-4R led to the activation of PI3 kinase. Several studies using mutant receptor tyrosine kinases have suggested that activation of PI3 kinase by these receptors is necessary for both mitogenesis and Ras activation in some cell lines (10, 17, 41, 50). In addition, a recent study has suggested that PI3 kinase activation may also be necessary for insulin-stimulated mitogenesis; inhibition of PI3 kinase with the inhibitor LY294002 blocks insulin-induced mitogenesis in L13T3 adipocytes (10). The findings reported here suggest that while activation of PI3 kinase may be necessary for Ras activation and mitogenesis by receptor tyrosine kinase under some conditions, PI3 kinase activation by itself is not sufficient to mediate these responses in muscle. Rather, in agreement with studies addressing the role of PI3 kinase in transformation by polyomavirus middle T antigen, it is likely that for PI3 kinase to mediate mitogenesis and Ras activation by insulin, it must cooperate with other signaling molecules; transformation by polyomavirus middle T antigen requires that middle T antigen bind both Shc and PI3 kinase (15). It is important to point out that PI3 kinase activation was determined indirectly by measuring the amount of p85 and PI3 kinase activity in anti-IRS-1 immunoprecipitates from IL-4- and insulin-stimulated cells. While many researchers have relied on similar assays to determine PI3 kinase activity (3, 17, 45, 50) the association of PI3 kinase with phosphotyrosine-containing proteins does not always correlate with the production of PI3 products in vivo (35). Moreover, a recent report has shown that PI3 kinase may also be activated by Ras (35). Thus, the finding that IL-4, in contrast to insulin, fails to activate Ras suggests that in spite of similar recruitments of PI3 kinase to IRS-1 in insulin- and IL-4-stimulated cells, IL-4 and insulin do not activate PI3 kinase equally; IL-4 stimulation may result in lower levels of PI3 kinase products in cells.

A general paradigm that has emerged over the past several years is that the interaction of SH2 domain-containing proteins with an autophosphorylated receptor or with intermediate phosphotyrosine-containing proteins, such as IRS-1 or Shc, is critical for regulation of the SH2 domain-containing molecules. This model, coupled with the demonstration that the functions served by a variety of signaling molecules can be determined by identifying which proteins they contact within a cell, has led to the assignment of signaling functions solely on the basis of these interactions (31, 43). For example, the finding that two proteins important in Ras activation, GRB2 and Sos, bind IRS-1 has led to the suggestion that this interaction is critical for coupling the insulin receptor to Ras activation. The results presented in this paper suggest that the biological functions predicted to occur on the basis of such interactions may not hold up to closer scrutiny. These interactions may prove to be important to insulin-mediated Ras activation only in certain defined situations. Therefore, clarification of the full implications of the interactions mediated by SH2 domain-containing proteins awaits a more detailed understanding of how insulin activates Ras.

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