

The Insulin Receptor Substrate-1-Related 4PS Substrate But Not the Interleukin-2R γ Chain Is Involved in Interleukin-13-Mediated Signal Transduction

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Interleukin-13 (IL-13) induced a potent mitogenic response in IL-3-dependent TF-1 cells and DNA synthesis to a lesser extent in MO7E and FDC-P1 cells. IL-13 stimulation of these lines, like IL-4 and insulin-like growth factor-1 (IGF-1), resulted in tyrosine phosphorylation of a 170-kD substrate. The tyrosine-phosphorylated 170-kD substrate strongly associated with the 85-kD subunit of phosphoinositol-3 (PI-3) kinase and with Grb-2. Anti-4PS serum readily detected the 170-kD substrate in lysates from both TF-1 and FDC-P1 cells stimulated with IL-13 or IL-4. These data provide evidence that IL-13 induces tyrosine phosphorylation of the 4PS substrate, providing an essential interface between the IL-13 receptor and signaling molecules containing SH2 domains.

HUMAN INTERLEUKIN-13 (IL-13) is a recently described cytokine that, like IL-4, is produced by activated T cells.¹⁻⁴ It has been demonstrated to have effects on both B cells and monocytes that are strikingly similar to those induced by IL-4.⁵ IL-13 induces proliferation and regulates expression of various cell surface antigens on activated B cells.^{1,4} It also facilitates immunoglobulin E (IgE) and IgG₂ production and decreases IgA secretion by B cells,^{1,3} a function previously attributed only to IL-4.⁶ IL-13 induces monocytes to differentiate into dendritic-like cells.^{2,5} It also upregulates cell surface expression of the low-affinity receptor for IgE (CD23 or Fc ϵ RII), several members of the integrin superfamily, and class II major histocompatibility complex (MHC) antigens and downregulates Fc γ receptors on human monocytes.^{5,7} The similarity in functional responses induced by these two cytokines may be partially explained by the finding that they share approximately 30% amino acid sequence homology and many structural features are conserved between the two proteins.^{3,8} The human IL-13 gene has been mapped to a region upstream within 50 kilobases of IL-4, suggesting the possibility of gene duplication.⁹

The cDNAs encoding single chain high-affinity human and murine IL-4 receptors (IL-4R α) have been molecularly cloned.¹⁰⁻¹³ This 120 to 140 kilodalton (kD) receptor has

IL-13 and IL-4 stimulation of murine L cell fibroblasts, which endogenously express the IL-4 receptor (IL-4R α) and lack expression of the IL-2 receptor γ subunit (IL-2R γ), resulted in tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1)/4PS. Enhanced tyrosine phosphorylation of IRS-1/4PS was observed in response to IL-4, but not IL-13 treatment of L cells transfected with the IL-2R γ chain. These results indicate that IL-13 does not use the IL-2R γ subunit in its receptor complex and that expression of IL-2R γ enhances, but is not absolutely required for mediating IL-4-induced tyrosine phosphorylation of IRS-1/4PS.

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been categorized as a member of the hematopoietin receptor superfamily based on consensus sequences in its extracellular domain.^{13,14} Unlike many members of this family, the intracellular domain of IL-4R α is quite large, consisting of over 500 amino acids. It was previously assumed that expression of this single chain molecule was sufficient for eliciting all IL-4-mediated signals. However, a recent report provided evidence that the IL-4 can use at least two subunits as part of its receptor complex, and the second subunit was identified as the γ chain of the IL-2 receptor (IL-2R γ).¹⁵ A primary argument for the existence of distinct receptors for IL-4 and IL-13 is that certain cell lines that responded to IL-4 failed to do so in response to IL-13.⁵ Although IL-13 may share some structural homology with IL-4, there is no evidence that IL-13 binds directly to IL-4R α or IL-2R γ . A study involving a human IL-4 mutant, designated hIL-4.Y124D, suggested that some component of the IL-4R complex interacts with IL-13.⁸ The hIL-4.Y124D mutant bound to but did not activate the IL-4 receptor complex on TF-1 cells and, thereby, acted as a potent IL-4 antagonist. The hIL-4.Y124D mutant also blocked IL-13 action on TF-1 cells.⁸ A possible explanation is that the shared homology between these two hematopoietins may allow both to bind to either IL-2R γ or IL-4R α and cause competitive antagonism between the two cytokines. Alternatively, hIL-4.Y124D might bind directly to an unknown IL-13 receptor subunit. Thus, it would also act as an antagonist of IL-13 action.

Although the known components of IL-4 receptor complex do not possess intrinsic tyrosine kinase activity, IL-4 stimulation was demonstrated to result in rapid and pronounced tyrosine phosphorylation of a 170-kD substrate, designated 4PS, in the factor-dependent cell lines, FDC-P1 and FDC-P2.¹⁶ This substrate was also phosphorylated on tyrosine residues in response to insulin and insulin-like growth factor-1 (IGF-1) stimulation of these same cell lines. Moreover, IL-4, insulin, and IGF-1 treatment induced potent DNA synthesis of the FDC lines. Interestingly, 4PS was found to share functional and structural characteristics in common with insulin receptor substrate-1 (IRS-1), including similarity in molecular size and striking association with the

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85-kD (p85) subunit of phosphoinositol-3 (PI-3) kinase after factor stimulation.¹⁶ IRS-1 was originally described as the principal substrate phosphorylated on tyrosine residues in response to insulin and IGF-1 stimulation in cells of connective tissue origin.¹⁷ IRS-1 has been demonstrated to associate with many SH2-containing molecules including p85, Grb-2, Syp, Nck, and Crk.¹⁸ The ability of IRS-1 to bind a wide variety of SH2-containing molecules suggests that it plays a crucial role in signal transduction. Several studies have indicated that IRS-1 expression is crucial for mediating insulin-induced mitogenesis and oocyte maturation.¹⁹⁻²³ Although IRS-1 and 4PS were shown to share weak immunologic crossreactivity, V8 digestion showed that the sizes of certain phosphopeptides were shared between the two molecules, while others were distinct.¹⁶ Moreover, Northern blot analysis and reverse transcriptase polymerase chain reaction analysis of lines expressing 4PS with IRS-1-specific probes indicated that they were not identical molecules (L.-M. Wang, J.H. Pierce, X.-J. Sun and M.F. White, unpublished observations). In the present study, we analyze whether IL-13 activates a signal transduction cascade involving 4PS that is similar to that mediated by IL-4 in factor-dependent hematopoietic cell lines.

MATERIALS AND METHODS

Growth factors and antibodies. Recombinant human and murine IL-3 and IL-4 and human IGF-1 were purchased from Peptotech (Nutley Hill, NJ), murine IL-13 was purchased from R&D Systems (Minneapolis, MN), and human IL-13 was provided by Sanofi Recherche (Labege, France). Transferrin and selenium from Collaborative Research (Boston, MA) were used for formulation of serum-free medium. Rabbit anti-IRS-1 and anti-4PS serum were generated by immunization of rabbits with baculovirus-expressed rat IRS-1 protein or a glutathione-S-transferase (GST) fusion protein containing 100 amino acid of 4PS (X.-J. Sun and M.F. White, unpublished observations), respectively. The anti-IRS-1 serum weakly recognizes 4PS,¹⁶ while anti-4PS serum does not recognize IRS-1 (X.-J. Sun and L.-M. Wang, unpublished observations). The murine antiphosphotyrosine monoclonal antibody (anti-pTyr), rabbit anti-PI-3 kinase serum (anti-p85), and rabbit anti-Grb2 serum were obtained from UBI (Lake Placid, NY). Rabbit antihuman IL-2R γ serum was a kind gift from Dr Warren Leonard, and the GST and GST-Grb-2 fusion proteins were from Drs Toshio Ishibashi and William Wong, respectively. Affinity-purified rabbit anti-GST serum was a kind gift from Drs Toru Miki and William Wong.

Cell lines and proliferation assays. The murine IL-3-dependent hematopoietic cell lines, FDC-P1 and FDC-P2, have been previously described.¹⁶ The human IL-3-dependent hematopoietic cell lines, TF-1 and MO7E, were obtained from Dr Alan Musson (Holland Laboratory, American Red Cross, Rockville, MD) and Dr Steven Clark (Genetic Institute, Cambridge, MA), respectively. The murine cell lines were cultured in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 15% fetal bovine serum and 5% WEHI-3B (ATCC, Rockville, MD) conditioned medium as a source of murine IL-3. Human cell lines were cultured in RPMI 1640 medium supplement with 15% fetal bovine serum and recombinant human IL-3 (10 ng/mL). The murine L cell line and IL-2R γ -transfected L cells were a kind gift from Dr Achsa Keegan (Holland Laboratory, American Red Cross, Rockville, MD). The L cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% calf serum.

Incorporation of [³H] thymidine was used to quantitate factor-

induced DNA synthesis of the factor-dependent hematopoietic cells. Briefly, exponentially growing cells were washed two times and aliquoted (2×10^5 cells/mL) in RPMI 1640 containing 10% fetal bovine serum in 24-well costar plates (1 mL/well) in the absence or presence of various concentrations of recombinant factors. Samples were incubated for 48 hours at 37°C, and 0.5 μ Ci [³H] thymidine was added per well for the last 4 hours of the incubation period. Cells were harvested on an automated cell harvester (Skatron; Vienna, VA) and triplicate samples were counted in a Beckman Beta counter (Beckman Instruments, Irvine, CA). Analysis of factor-dependent long-term growth properties of the various cell lines were performed in serum-free RPMI 1640 medium containing transferrin (5 μ g/mL), selenium (10 nmol/L) and 1% bovine serum albumin (BSA). Recombinant growth factors were added at 10 ng/mL. Cells were initially plated at 5×10^4 cells/mL in serum-free medium with or without added factors. They were transferred 1:10 and 1:50 biweekly. Cells for successive transfers were always obtained from cultures that had been similarly treated in the previous passages.

Immunoprecipitation and immunoblot analysis. Hematopoietic cells were washed and starved in DMEM with 50 μ mol/L Na₃VO₄ for 2 hours and resuspended in a small volume of medium (2 to 4×10^7 cells/mL) for factor treatment. L-cell lines were washed twice and starved overnight in DMEM containing 5 μ g/mL transferrin and 10 nmol/L selenium. After stimulation with the various factors (500 ng/mL) for 10 minutes at 37°C, the cells were diluted in phosphate-buffered saline containing 100 μ mol/L Na₃VO₄. The cells were then pelleted and lysed in a Lysis buffer containing 20 mmol/L Tris.Cl, pH 7.5, 1% NP-40, 10 mmol/L NaCl, 50 mmol/L NaF, 10 mmol/L NaPPi, 2.5 mmol/L EDTA, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, and 4 mmol/L diisopropyl fluorophosphate (DFP).

The total protein content of the lysates were determined by the Bio-Rad protein assay. Routinely, equal amounts of clarified hematopoietic cell lysates (2 to 5 mg) or nonhematopoietic cell lysates (0.5 to 1 mg) were immunoprecipitated with 25 μ L of agarose-conjugated anti-pTyr (4 mg/mL settled beads), or with other antisera (1:500) plus 30 μ L of protein G-coupled sepharose (10 mg/mL settled beads; Pharmacia, Piscataway, NJ). The immunoprecipitates were washed three times with Lysis Buffer minus DFP, solubilized with Laemmli buffer, boiled, and resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In some experiment, proteins (200 μ g) were directly resolved by SDS-PAGE without prior immunoprecipitation.

Immunoblot analysis was performed by transferring proteins from a polyacrylamide gel onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA) in Tris-glycine buffer containing 20% methanol. The membrane was then treated for 1 to 2 hours with 3% nonfat dry milk in TTBS (20 mmol/L Tris.Cl, pH 7.5, 154 mmol/L NaCl, 0.05% Tween, 0.05% NaN₃), incubated with antibodies in TTBS containing 0.5% BSA (TTBS-BSA) for 1 to 2 hours (concentration of antibodies: 2 μ g/mL for anti-pTyr, 1 μ g/mL for anti-GST, and 1:500 for the remaining antisera) and reacted with [¹²⁵I] protein A (3×10^5 cpm/mL) in TTBS-BSA for 1 hour. All the procedures were done at room temperature, and membranes were washed extensively with TTBS after each treatment. After the final wash, the membrane was rinsed with distilled water, air dried, and autoradiographed with intensifying screens at -70°C. For the Grb-2 in vitro binding assay, the membrane was first incubated with a GST-Grb2 fusion protein (2 μ g/mL) or GST (2 μ g/mL) in TTBS-BSA for 1 hour at room temperature, followed by extensive washes with TTBS. The binding of GST-Grb2 or GST was then detected by incubating the membrane with anti-GST serum and [¹²⁵I] protein A as described in the previous paragraph.

Surface expression of IL-4 and IL-13 receptors. The level of expression of IL-4 and IL-13 receptors on the parental and

Table 1. Mitogenic and Long-Term Growth Potential of Various IL-3-Dependent Cell Lines in Response to IL-13, IL-4, or IGF-1

Cell Line	Mitogenic Response*			Long-Term Growth†		
	IL-13	IL-4	IGF-1	IL-13	IL-4	IGF-1
FDC-P1	+	+++	+++	-	-	-
FDC-P2	-	+++	+++	-	-	-
TF-1	+++	+++	+	+	+	-
MO7E	+	++	-	-	-	-

* The mitogenic response of the different lines was analyzed using recombinant murine or human growth factors depending on the species of origin of the line tested as described in Materials and Methods. The data are the average of triplicate samples and represented as follows: fold stimulation above background greater than 50 (+++), greater than 10 (++), greater than 2 (+), and no increase (-).

† The ability of the different lines to grow indefinitely in various factors was performed as described in Materials and Methods. Cell lines that continued to divide for over 2 months were scored as positive (+). Cell lines that did not survive were retested twice before being scored as negative (-). No line could be maintained in medium without added growth factor.

transfected L-cell lines was determined by saturation binding analysis using [¹²⁵I]-labeled murine IL-13 or murine IL-4. Cells were incubated at $5 \times 10^5/100 \mu\text{L}$ in Hanks' Balanced Salt Solution (HBSS), 1 mg/mL BSA, and 25 mmol/L HEPES, pH 7.4 with 50 ng/mL of [¹²⁵I]-labeled factor for 2 hours at 16°C. The radioactivity associated with the cell pellet was determined by the phthalate oil procedure and was corrected for nonspecific binding. Nonspecific binding was determined by including a 100-fold molar excess of unlabeled factor. The number of molecules bound per cell was calculated using 15 kD as the molecular weight of IL-4 and 10 kD for IL-13. The reported receptor values represent the average of the calculated receptor numbers from two separate experiments.

RESULTS

IL-13 induces mitogenesis and tyrosine phosphorylation of a 170-kD substrate in certain factor-dependent hematopoietic cell lines. IL-4 and IGF-1 have previously been shown to induce potent DNA synthesis in the IL-3-dependent FDC-P1 and FDC-P2 cell lines.¹⁶ Murine IL-13 induced a weak mitogenic response in FDC-P1 cells, and FDC-P2 cells did not detectably respond to this cytokine (Table 1). Analysis of a human IL-3-dependent cell line, TF-1, showed that it readily responded to IL-13 (Table 1 and Fig 1). IGF-1 stimulation also evoked DNA synthesis in the TF-1 line, although to a lesser extent (Table 1 and Fig 1). TF-1 cells could be adapted to propagate indefinitely in serum-free medium containing either recombinant human IL-4 or IL-13, but not IGF-1 (Table 1). Another IL-3-dependent human megakaryocytic line, MO7E, was also found to modestly respond mitogenically to human IL-13 and IL-4, but not to IGF-1 (Table 1). However, neither the MO7E or the FDC-P1 lines could be adapted to grow long-term in IL-13, IL-4, or IGF-1 (Table 1).

The ability of IL-13 to induce tyrosine phosphorylation events in responsive cell lines had not been previously examined. Therefore, quiescent TF-1 cells were either untreated or stimulated with IL-4, IL-13, IGF-1, or IL-3. Cell lysates were prepared and immunoprecipitated with a monoclonal

antibody directed against phosphotyrosine (anti-pTyr). Immunoprecipitated proteins were electrophoretically separated, and transferred proteins were subsequently immunoblotted with the identical anti-pTyr monoclonal antibody. As shown in Fig 2, stimulation of TF-1 cells with human IL-4, IL-13, or IGF-1 resulted in readily detectable tyrosine phosphorylation of a 170-kD protein. While a tyrosine-phosphorylated protein of 97 kD was observed after IL-3 treatment of TF-1 cells, IL-3 stimulation did not induce the phosphorylation of a protein in the 170-kD size range (Fig 2). Stimulation of the FDC-P1 and MO7E cell lines with murine IL-13 or human IL-13, respectively, lead to weak but reproducible tyrosine phosphorylation of the 170-kD substrate under the same conditions employed for TF-1 cells (data not shown).

IL-13 stimulation of responsive cells results in association of PI-3 kinase and Grb-2 with the tyrosine-phosphorylated 170-kD substrate. We previously demonstrated that 4PS associates with the SH2-containing p85 regulatory subunit of PI-3 kinase on IL-4 or IGF-1 stimulation of FDC-P1 and FDC-P2 cells.¹⁶ Because IL-13 stimulation also resulted in tyrosine phosphorylation of a 170-kD protein in TF-1 cells, we investigated whether p85 would coprecipitate with this substrate after IL-13 treatment. To directly address this question, cell lysates from untreated or factor-treated TF-1 cells were immunoprecipitated with anti-p85 serum and subsequently immunoblotted with anti-pTyr. The 170-kD substrate was readily detected in lysates of cells stimulated by IL-13, IL-4, and IGF-1, but not in the untreated sample (Fig 3A). An immunoblot analysis of identical samples with anti-p85 showed that levels of p85 were the same in each lysate (Fig 3B). These results further suggested that the 170-kD

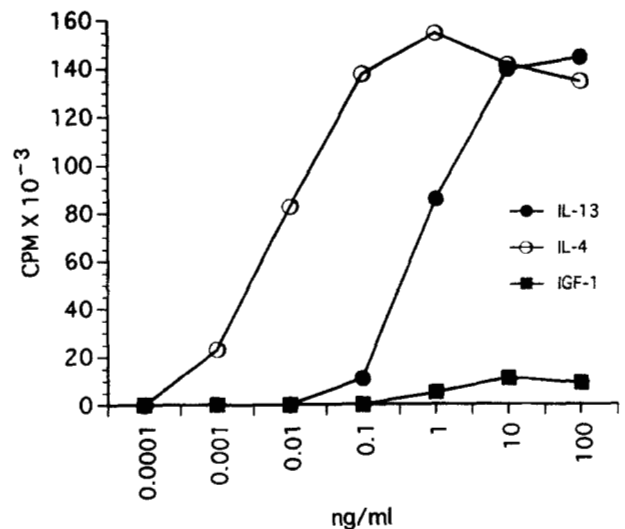


Fig 1. Mitogenic dose response of TF-1 cells to IL-13, IL-4, or IGF-1. TF-1 cells were incubated for 48 hours at 37°C with various concentrations of IL-13 (●), IL-4 (○), or IGF-1 (■). [³H] Thymidine (0.5 μCi per well) was added for the last 4 hours of the incubation period and triplicate samples were collected by an automatic harvester and counted. Results are expressed as counts per minute (CPM) in stimulated samples minus background CPM and are representative of three separate experiments.

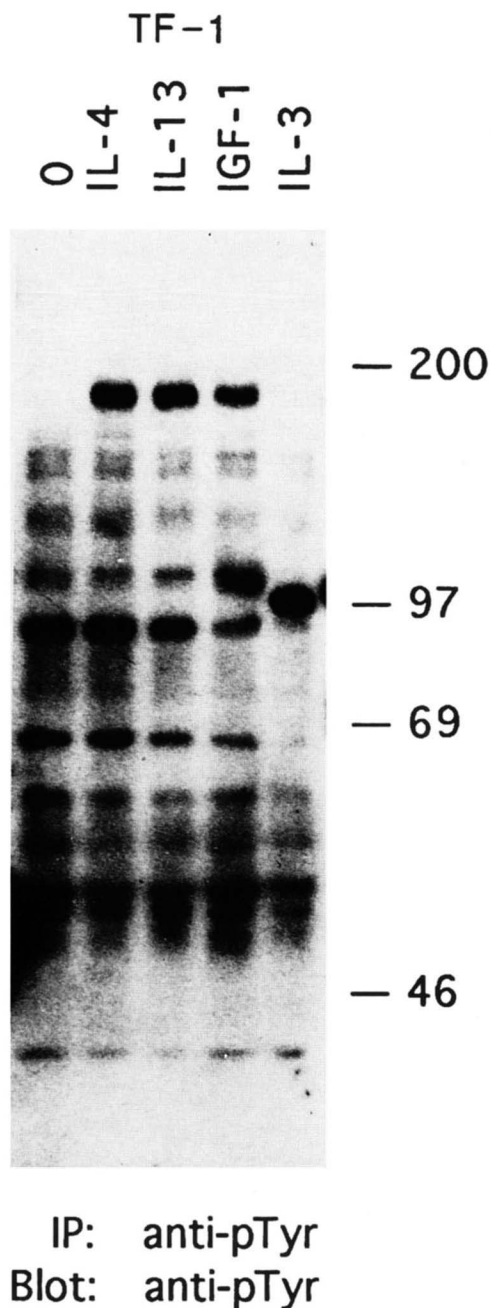


Fig 2. Detection of a phosphotyrosine-containing 170-kD protein in TF-1 cells after stimulation with IL-4, IL-13, or IGF-1 but not IL-3. Quiescent cells were untreated (0) or treated with the various factors as illustrated. Cell lysates were immunoprecipitated (IP) with anti-pTyr, resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted (Blot) with anti-pTyr as indicated. Molecular sizes of marker proteins are indicated in kD.

protein phosphorylated in response to IL-13, IL-4, and IGF-1 stimulation of TF-1 cells was the human counterpart of the 4PS substrate previously identified in the murine FDC lines.

While it has been shown that tyrosine phosphorylation of IRS-1 mediates its association with Grb-2,²⁴ the ability of

4PS to bind Grb-2 had not been analyzed. To further determine the functional similarities between IRS-1 and 4PS, we first analyzed whether Grb-2 would associate with 4PS after treatment of the murine FDC lines with either IL-4 or IGF-1. Lysates from FDC-P1 and FDC-P2 cells stimulated with IL-4, IGF-1, or IL-3 were immunoprecipitated with anti-Grb-2 serum, immune complexes were separated by SDS-PAGE, and transferred proteins were subsequently immunoblotted with anti-pTyr. As shown in Fig 4A, 4PS could be detected after IL-4 and IGF-1, but not IL-3 treatment of each line, providing evidence that Grb-2 associates with tyrosine-phosphorylated 4PS in vivo. Immunoblot analysis with anti-Grb-2 serum provided evidence that similar levels of Grb-2 protein were detected in each sample (Fig 4B).

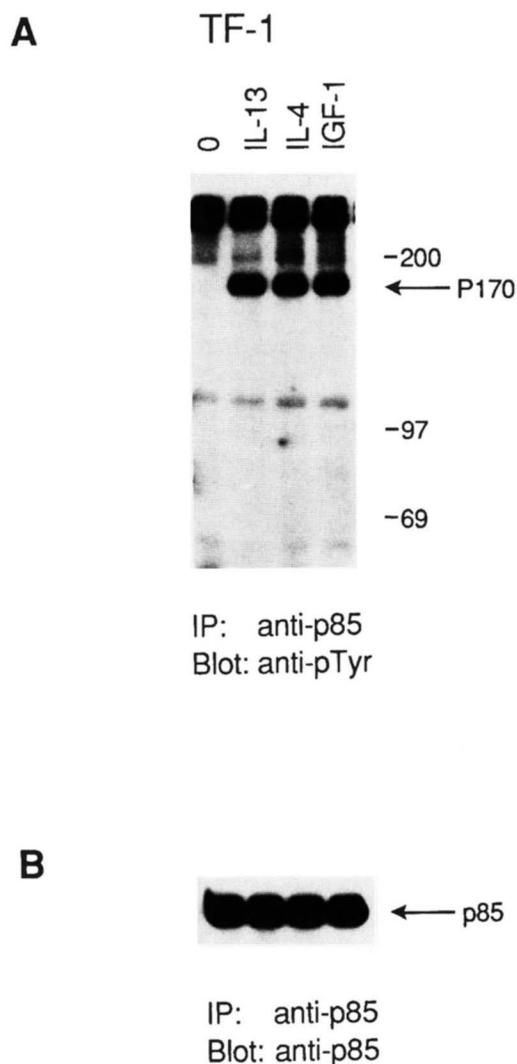


Fig 3. Association of the p85 subunit of PI-3 kinase with the 170-kD substrate in TF-1 cells after stimulation with IL-4, IL-13, or IGF-1, but not IL-3. Quiescent cells were either unstimulated (0) or stimulated with IL-4, IL-13, or IGF-1 as illustrated. Cell lysates were immunoprecipitated (IP) with anti-p85 serum, subjected to SDS-PAGE and transferred proteins were subsequently immunoblotted (Blot) with (A) anti-pTyr or (B) anti-p85 serum. Molecular sizes are given in kD.

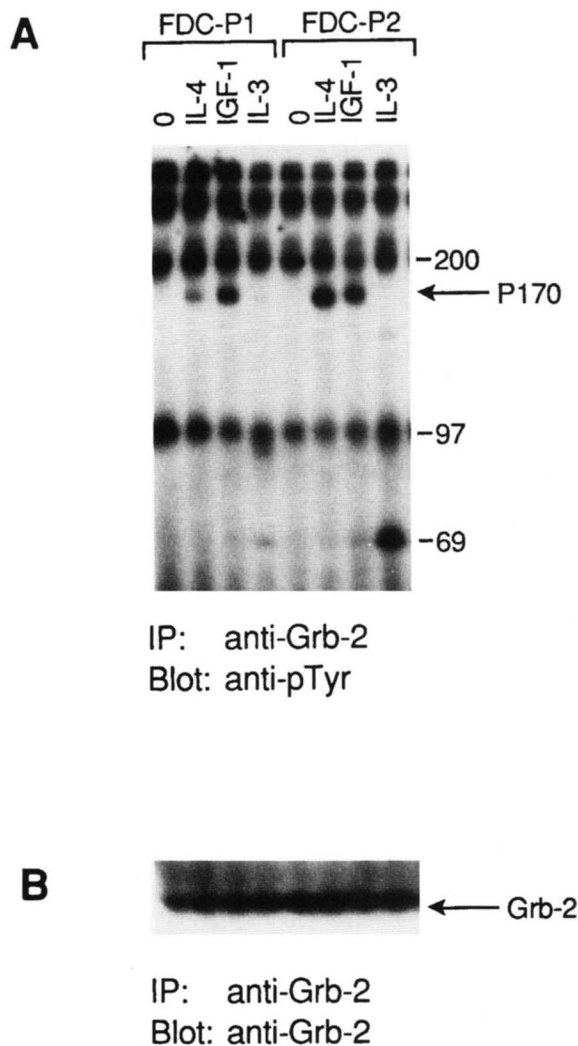


Fig 4. In vivo association of Grb-2 with 4PS in FDC cell lines after IL-4 and IGF-1, but not IL-3 stimulation. Serum-starved cells were either untreated (0) or treated with the various factors as illustrated. Cell lysates were immunoprecipitated (IP) with anti-Grb-2 serum, subjected to SDS-PAGE, and transferred proteins were subsequently immunoblotted (Blot) with (A) anti-pTyr or (B) anti-Grb-2. Molecular weight markers are shown in kD.

Because the phosphotyrosine content of the 170-kD protein induced by IL-13 and IL-4 treatment of TF-1 cells was much less than that observed in response to IL-4 and IGF-1 in the FDC lines, the above method proved to be unsuccessful for demonstrating Grb-2 association with the 170-kD substrate in response to factor treatment of TF-1 cells. Therefore, we investigated whether in vitro binding of a GST-Grb-2 fusion protein to the tyrosine-phosphorylated 170-kD protein could be detected after IL-13, IL-4, or IGF-1 stimulation of TF-1 cells. Cell lysates from the untreated or factor-stimulated TF-1 or FDC-P2 lines were immunoprecipitated with anti-pTyr, and proteins were separated by SDS-PAGE. Transferred proteins were probed with purified GST-Grb-2 or GST alone and subsequently immunoblotted

with anti-GST serum. As shown in Fig 5A, GST-Grb-2 readily bound to tyrosine-phosphorylated 4PS after IL-4 and IGF-1 treatment of FDC-P2 cells. GST alone did not bind to 4PS under the same conditions (data not shown). GST-Grb-2, but not GST alone, also specifically associated with the 170-kD substrate after IL-13, IL-4, and IGF-1 treatment of the TF-1 line (Fig 5B and C). These results indicate that the 170-kD substrate can bind Grb-2 after it becomes phosphorylated on tyrosine by IL-13, IL-4, or IGF-1 stimulation of responsive cells.

Anti-4PS serum recognizes the 170-kD substrate phosphorylated by IL-13 in TF-1 and FDC-P1 cells. The above data strongly suggested that the substrate phosphorylated in response to IL-13 stimulation of TF-1 cells was human 4PS. We recently generated a rabbit polyclonal antiserum that specifically detects 4PS and does not recognize IRS-1.²⁵ To directly demonstrate that the tyrosine-phosphorylated substrate induced by IL-13 treatment of TF-1 and FDC-P1 cells was 4PS, we analyzed whether the 170-kD protein could be immunoprecipitated by anti-4PS serum. TF-1 cells stimulated with human IL-4 or IL-13 or FDC-P1 cells treated with murine IL-4 or IL-13 were lysed, and lysates were immunoprecipitated with anti-4PS serum. Proteins eluted from immune complexes were separated by SDS-PAGE and transferred for subsequent immunoblot analysis with anti-pTyr. As shown in Fig 6A, anti-4PS serum readily detected the tyrosine-phosphorylated 170-kD substrate in samples from both TF-1 and FDC-P1 cells stimulated with either factor. In contrast, an antipeptide serum directed against the carboxy-terminal domain of IRS-1 that does not recognize 4PS did not detect the 170-kD substrate under the same conditions (data not shown). The specificity of the anti-4PS serum was confirmed by comparing its ability to recognize 4PS in FDC-P1 and FDC-P2 cells and 32D cells transfected with IRS-1 (32D-IRS-1). As shown in Fig 6B, 4PS was readily detected in IL-4- or IGF-1-stimulated FDC-P1 and FDC-P2 cell lysates, but not in 32D-IRS-1 cell lysates after immunoprecipitation with anti-4PS followed by immunoblot analysis with anti-pTyr. In contrast, anti-IRS-1 serum recognized IRS-1 in lysates from the 32D-IRS-1 line, but not in the lysates from FDC-P1 and FDC-P2 lines under the same conditions (Fig 6C). Thus, anti-4PS serum specifically recognized the 170-kD substrate in both the human and murine systems, suggesting that the 4PS molecule is conserved between species. Moreover, the above results provide conclusive evidence that IL-13 mediates tyrosine phosphorylation of 4PS in both murine and human cells.

IL-13 does not appear to use the IL-2R γ chain to mediate tyrosine phosphorylation of IRS-1/4PS. The nonhematopoietic murine L cell line normally expresses the murine IL-4R α chain, but is devoid of the IL-2R γ subunit. A recent study demonstrated that the ability of IL-4, but not insulin, to induce tyrosine phosphorylation of a 170-kD molecule, presumably IRS-1/4PS, was dependent on the transfection of a human IL-2R γ expression vector into the parental L cell line.¹⁵ We reasoned that if L cells expressed at least one component of the murine IL-13 complex and required the IL-2R γ chain as a second component, similar results would be obtained when the parental and IL-2R γ -transfected L

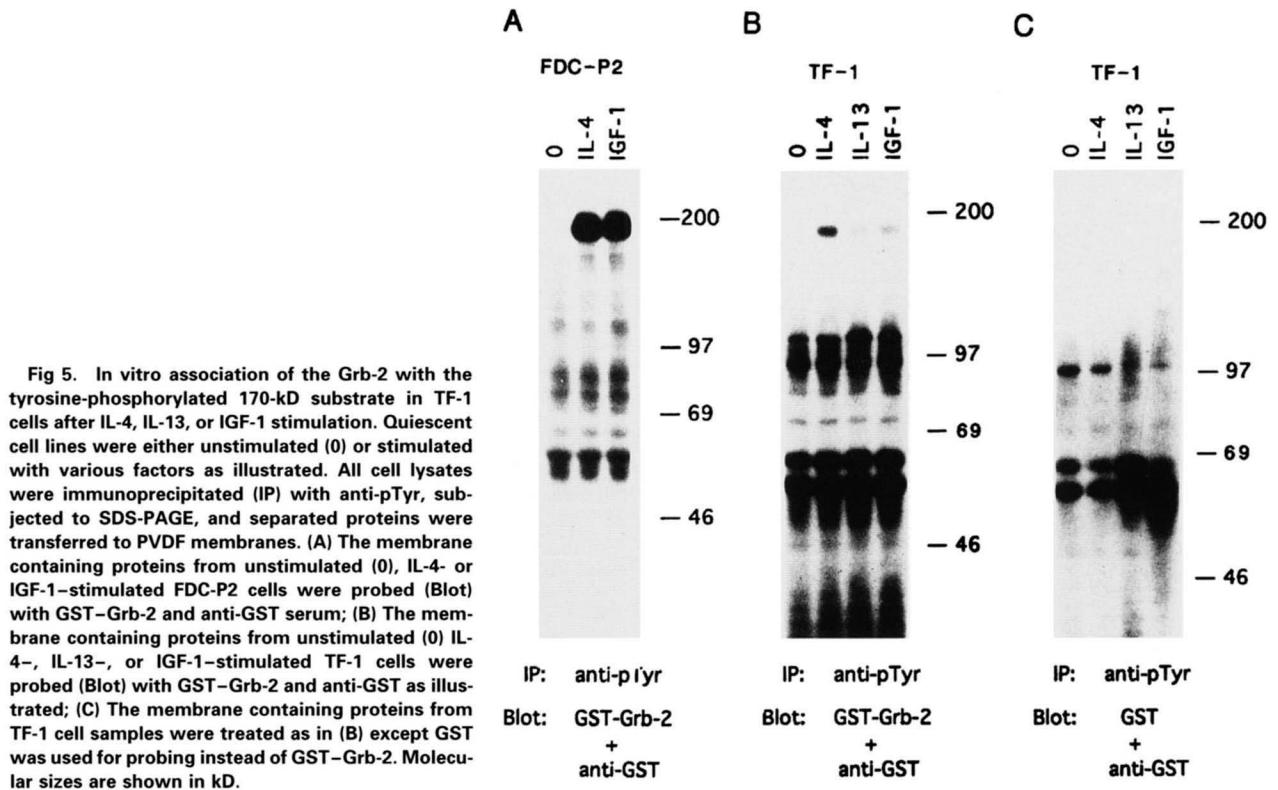


Fig 5. In vitro association of the Grb-2 with the tyrosine-phosphorylated 170-kD substrate in TF-1 cells after IL-4, IL-13, or IGF-1 stimulation. Quiescent cell lines were either unstimulated (0) or stimulated with various factors as illustrated. All cell lysates were immunoprecipitated (IP) with anti-pTyr, subjected to SDS-PAGE, and separated proteins were transferred to PVDF membranes. (A) The membrane containing proteins from unstimulated (0), IL-4- or IGF-1-stimulated FDC-P2 cells were probed (Blot) with GST-Grb-2 and anti-GST serum; (B) The membrane containing proteins from unstimulated (0) IL-4-, IL-13-, or IGF-1-stimulated TF-1 cells were probed (Blot) with GST-Grb-2 and anti-GST as illustrated; (C) The membrane containing proteins from TF-1 cell samples were treated as in (B) except GST was used for probing instead of GST-Grb-2. Molecular sizes are shown in kD.

cells (L-IL-2R γ) were analyzed after IL-13 stimulation. Serum-starved untransfected L or L-IL-2R γ cells were stimulated with murine IL-4, IL-13, or IGF-1, and cell lysates were immunoprecipitated with a potent polyclonal anti-IRS-1 serum that recognizes both IRS-1 and 4PS. Immunoprecipitated proteins were electrophoretically separated, and transferred proteins were immunoblotted with anti-pTyr. As shown in Fig 7A, IL-4 and IL-13 treatment led to readily detectable tyrosine phosphorylation of the IRS-1/4PS proteins in the untransfected L-cell line. However, no increase in phosphotyrosine content was observed after IL-13 stimulation of the L-IL-2R γ transfectant. In contrast, the phosphotyrosine content of IRS-1/4PS was substantially increased after IL-4 treatment of L-IL-2R γ cells. IGF-1 stimulation resulted in equal and pronounced phosphorylation of IRS-1/4PS in both lines (Fig 7A). Other proteins possessing molecular sizes of approximately 110 and 130 kD were also observed after stimulation of both L-cell lines with all three factors (Fig 7A). This suggests that these molecules associate with IRS-1/4PS and are tyrosine phosphorylated when stimulated with IL-4, IL-13, and IGF-1. These results indicate that neither IL-4 nor IL-13 absolutely require expression of IL-2R γ to mediate a certain degree of tyrosine phosphorylation of IRS-1/4PS and other unknown proteins. However, IL-2R γ expression greatly enhances the ability of IL-4, but not IL-13, to induce such an effect. The expression of IL-2R γ protein in the L-IL-2R γ transfectant, but not in the parental line, was confirmed by direct immunoblot analysis of cell lysates with anti-IL-2R γ serum (Fig 7B).

Saturation binding assays were performed to confirm that

L and L-IL-2R γ cells express murine IL-13 and IL-4 receptors. Results demonstrated that receptors for murine IL-13 (147 binding sites/cells) and murine IL-4 (1,144 binding sites/cells) were present on the cell surface of the parental L-cell line. The levels of IL-13 receptors (190 binding sites/cell) and IL-4 receptors (1,299 binding sites/cell) expressed on the L-IL-2R γ transfectant were similar to those expressed on the parental line. Unfortunately, the L-cell line is highly transformed, and attempts to determine the ability of IL-4 and IL-13 to induce DNA synthesis in these cells were unsuccessful. However, the results obtained do indicate that IL-13 does not use IL-2R γ to evoke certain tyrosine phosphorylation events in the L-cell system.

DISCUSSION

In the present study, we demonstrate that IL-13 is capable of evoking a potent mitogenic response in TF-1 cells and inducing DNA synthesis to a lesser extent in the MO7E and FDC-P1 cell lines. The TF-1 line could be adapted to grow continuously in serum-free medium containing either recombinant human IL-4 or IL-13. In contrast, neither FDC-P1 nor MO7E cells could be continuously propagated under similar conditions. IL-13 stimulation of the IL-3-dependent human cell line, TF-1, resulted in tyrosine phosphorylation of a 170-kD substrate. A protein of this molecular size, designated 4PS, has previously been demonstrated to be the principal substrate phosphorylated on tyrosine residues in response to IL-4, insulin, and IGF-1 treatment of the murine myeloid cell lines, FDC-P1 and FDC-P2.¹⁶ In accordance with its undetectable mitogenic response to murine IL-13,

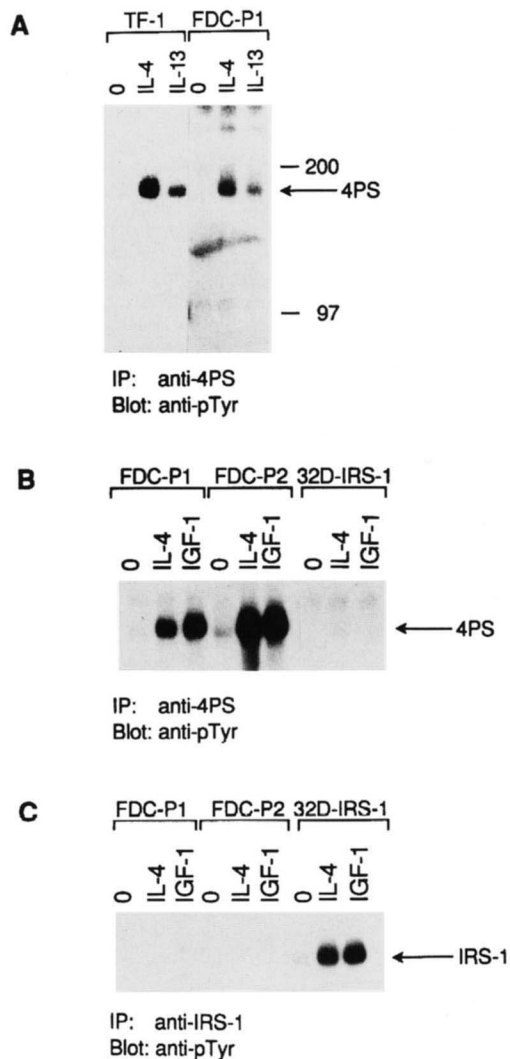


Fig 6. The tyrosine-phosphorylated 170-kD substrate induced by IL-4 and IL-13 is recognized by an anti-4PS serum. (A) Quiescent TF-1 or FDC-P1 cells were untreated (0) or stimulated with IL-4 or IL-13 as shown. Cell lysates were immunoprecipitated (IP) with anti-4PS serum and subsequently immunoblotted (Blot) with anti-pTyr. (B) The FDC-P1, FDC-P2 and 32D-IRS-1 lines were either untreated or stimulated with IL-4 or IGF-1 as shown. Cell lysates were immunoprecipitated (IP) with anti-4PS serum and immunoblotted (Blot) with anti-pTyr. (C) The same cell lines were treated as described in (B) except anti-IRS-1 serum was used for immunoprecipitation (IP). Molecular markers are shown in kD.

this cytokine did not induce detectable phosphorylation of the 170-kD protein in FDC-P2 cells. In contrast, FDC-P1 and MO7E cells did respond mitogenically to IL-13, and IL-13 did induce weak but detectable phosphorylation of the 170-kD molecule in these lines (L-M. Wang and J.H. Pierce, unpublished observation).

Our previous study suggested that 4PS is functionally related to IRS-1.¹⁶ The 4PS substrate, like IRS-1, was rapidly phosphorylated on tyrosine residues by IL-4, insulin, or IGF-1 treatment of the FDC cell lines. Moreover, 4PS strongly associated with PI-3 kinase after factor stimulation.¹⁶ In this

report, we demonstrate that the 170-kD substrate phosphorylated in response to IL-13 stimulation of TF-1 cells also readily associated with the p85 subunit of PI-3 kinase. Stimulation of TF-1 cells with IL-4, IL-13, and IGF-1 or FDC cell lines with IL-4 and IGF-1 also induced association of the 170-kD substrate with Grb-2, another property of IRS-1.²⁴ Finally, an antiserum that specifically recognizes 4PS, but not IRS-1, readily detected the 170-kD substrate in lysates from both TF-1 and FDC-P1 cells stimulated with IL-4 and IL-13 after immunoprecipitation with anti-4PS serum followed by immunoblot analysis with anti-pTyr. In contrast, an anti-IRS-1-specific serum did not recognize this substrate under the same conditions. Taken together, these data provide strong evidence that the 170-kD substrate that becomes phosphorylated on tyrosine residues by IL-13 stimulation of TF-1 and FDC-P1 cells is 4PS. A very recent study by M.J. Welham et al²⁷ has indirectly confirmed our finding that IL-13 induces 4PS phosphorylation.

It is known that association of Grb-2 with tyrosine-phosphorylated proteins induces its association with the GTP/GDP exchange protein, SOS, and this ultimately leads to Ras activation.^{28,29} Therefore, it is difficult to explain why several studies have failed to demonstrate that IL-4 is able

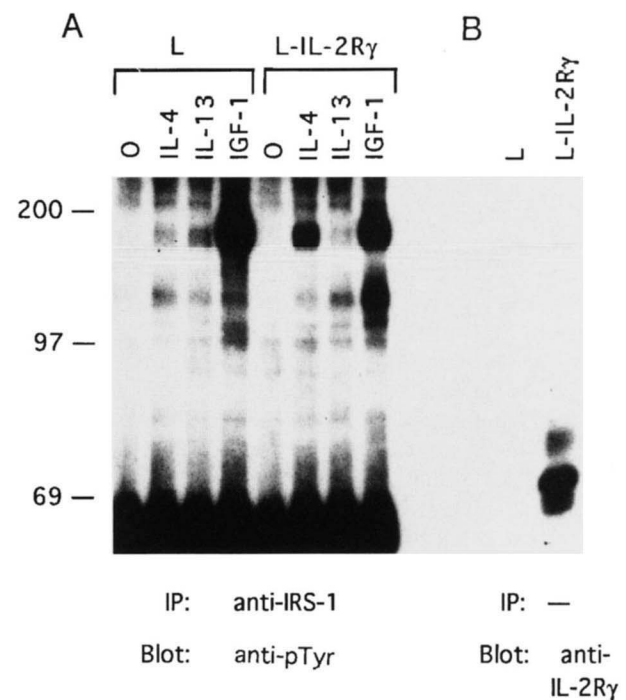


Fig 7. Stimulation of the L-cell fibroblast line with IL-4, IL-13, or IGF-1 results in tyrosine phosphorylation of IRS-1/4PS before expression of the IL-2R γ chain. (A) Serum-starved L or L-IL-2R γ cells were untreated (0) or treated with IL-4, IL-13, or IGF-1. Cell lysates were immunoprecipitated (IP) with anti-IRS-1 serum, subjected to SDS-PAGE, and transferred proteins were subsequently immunoblotted (Blot) with anti-pTyr; (B) Cell lysates (200 μ g) from L cells or L-IL-2R γ transfectants were subjected to SDS-PAGE, separated proteins were transferred to PVDF membranes, and subsequently immunoblotted (Blot) with anti-IL-2R γ serum. Molecular markers are shown in kD.

to activate Ras or downstream signaling molecules such as Raf or MAP kinase.³⁰⁻³² Although many factor-dependent cell lines respond mitogenically to IL-4, very few have been successfully propagated on a long-term basis in IL-4 or IL-13. Thus, we are currently investigating whether Ras, Raf and MAP kinase are activated by these two cytokines in cell lines adapted to grow continuously in their presence.

Recent studies have demonstrated that IL-2 and IL-4 induce tyrosine phosphorylation of two members of the Janus kinase family, Jak-1 and Jak-3.³³⁻³⁷ Jak-3 has also been shown to specifically associate with the IL-2R γ subunit, while Jak-1 appears to associate with the IL-2 β R and IL-4R α chains.³⁵⁻³⁷ A separate study showed that the Fes tyrosine kinase associates with IL-4R α and becomes phosphorylated on tyrosine residues after IL-4 stimulation.³⁸ It has been suggested that activation of these kinases might mediate tyrosine phosphorylation of the intracellular substrates that are detected after IL-4 treatment. While we did not observe tyrosine phosphorylation of proteins in the size range of Jak-1, Jak-3, or Fes after stimulation of TF-1 cells with either IL-4 or IL-13, it is possible that the levels of expression of these kinases in this line are insufficient to detect their autophosphorylation under the conditions employed in our study. A recent study demonstrated that Jak-1 not only associates with IL-4R α but is also found in a complex with 4PS/IRS-1.³⁷ Interestingly, we did observe IL-4- and IL-13-dependent tyrosine-phosphorylated proteins associated with anti-pTyr immunoprecipitates in the size range of JAK-1 from L-cell lysates. While it is still not clear whether Jak-1 or another tyrosine kinase is responsible for directly phosphorylating IRS-1 or 4PS in response to IL-4, the ability of IL-13 to mediate tyrosine phosphorylation of 4PS suggests that it may activate the same tyrosine kinase as that activated by IL-4.

Evidence obtained using the nonhematopoietic L-cell line suggests that IL-13 does not use the IL-2R γ chain in mediating phosphorylation of IRS-1/4PS. Surprisingly, our data also indicated that phosphorylation of this substrate(s) by IL-4 is not entirely dependent on the expression of IL-2R γ . While the study by Russel et al¹⁵ indicated that IL-2R γ expression was absolutely required for IL-4-mediated phosphorylation of IRS-1/4PS in L cells, they used anti-pTyr for immunoprecipitation in their study rather than the anti-IRS-1/4PS serum, which strongly recognized IRS-1 and also detects 4PS. It is likely that the use of the anti-IRS-1 serum increased our ability to detect specific phosphorylation of this substrate(s) in the untransfected cells. Nevertheless, it is clear that the ability of IL-4 to induce IRS-1/4PS phosphorylation was greatly enhanced by IL-2R γ expression, while this was not the case for IL-13. However, it is possible that unlike the murine IL-4R α /human IL-2R γ complex, the murine IL-13 receptor is unable to form a functional complex with the human IL-2R γ . Unfortunately, we have been unable to obtain a murine IL-2R γ expression vector to address this question. In contrast to the results obtained concerning IL-4-mediated phosphorylation of IRS-1/4PS in L cells, we found that phosphorylation of the IL-4R α chain was dependent on the expression of IL-2R γ (L.-M. Wang and J.H. Pierce, unpublished observation). However, a previous study

suggested that tyrosine phosphorylation of IL-4R α may not be required for any signaling events mediated by IL-4.³⁹

The ability of the IL-2R γ chain to mediate biological responses to IL-2, IL-4, IL-13, and IL-15 has been analyzed in X-linked severe combined immunodeficiency (X-SCID) B cells in which the IL-2R γ chain is disrupted.⁴⁰ Interestingly, B cells from X-SCID mice did not respond to IL-2 or IL-15, but responded as well or better than normal to IL-4 and IL-13 in B-cell activation, proliferation, and IgE secretion assays. A second study demonstrated that IL-2R γ did not appear to be associated with IL-4R α in human renal carcinoma cell lines.⁴¹ Moreover, even in the absence of the IL-2R γ chain, IL-4 and IL-13 were able to upregulate intracellular adhesion molecule-1 antigen on these carcinoma lines.⁴¹ Another study provided evidence that the B9 plasmacytoma cell line proliferated in response to both IL-4 and IL-13 and also did not express IL-2R γ .⁴² IL-4 and IL-13 were also shown to cross-compete on B9 cells.⁴³ Therefore, it is likely that the proposed receptor subunit shared between IL-4 and IL-13 is not IL-2R γ . However, it is still possible that IL-4R α is shared between these two cytokines. Because it has been demonstrated that Jak-3 resides in association with the IL-2R γ chain and is activated by ligand binding,³²⁻³⁵ our data suggest that IL-13 would not activate Jak-3 by this mechanism. This result is supported by two recent studies.^{27,44} The ability of other cytokines, either those that use the common IL-2R γ chain as part of their receptor complex or those that do not, to induce IRS-1/4PS phosphorylation should shed light on which tyrosine kinases are involved in mediating phosphorylation of these crucial substrates.

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