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Ling-Mei Wang

National Cancer Institute

Achsah D. A D Keegan

National Institute of Allergy and Infectious Diseases

Weiqun Li

National Cancer Institute

Gustav E. Lienhard

Dartmouth College

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Common elements in interleukin 4 and insulin signaling pathways in factor-dependent hematopoietic cells

(insulin receptor substrate 1/tyrosine phosphorylation/signal transduction)

LING-MEI WANG*, ACHSAH D. KEEGAN†, WEIQUN LI*, GUSTAV E. LIENHARD‡, STEFANIA PACINI§, J. SILVIO GUTKIND¶, MARTIN G. MYERS, JR.¶, XIAO-JIAN SUN¶, MORRIS F. WHITE¶, STUART A. AARONSON*, WILLIAM E. PAUL†, AND JACALYN H. PIERCE*,**

*Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892; †Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; ‡Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844; §Institute of General Pathology, University of Florence, Florence, Italy; ¶Laboratory of Cellular Development and Oncology, National Institute of Dental Research, Bethesda, MD 20892; and ||Joslin Diabetes Center, Department of Medicine, Harvard Medical School, Boston, MA 02215

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ABSTRACT Interleukin 4 (IL-4), insulin, and insulin-like growth factor I (IGF-I) efficiently induced DNA synthesis in the IL-3-dependent murine myeloid cell lines FDC-P1 and FDC-P2. Although these factors could not individually sustain long-term growth of these lines, a combination of IL-4 with either insulin or IGF-I did support continuous growth. The principal tyrosine-phosphorylated substrate observed in FDC cells stimulated with IL-4, previously designated 4PS, was of the same size (170 kDa) as the major substrate phosphorylated in response to insulin or IGF-I. These substrates had phosphopeptides of the same size when analyzed by digestion with *Staphylococcus aureus* V8 protease, and each tightly associated with the 85-kDa component of phosphatidylinositol 3-kinase after factor stimulation. IRS-1, the principal substrate phosphorylated in response to insulin or IGF-I stimulation in nonhematopoietic cells, is similar in size to 4PS. However, anti-IRS-1 antibodies failed to efficiently precipitate 4PS, and some phosphopeptides generated by V8 protease digestion of IRS-1 were distinct in size from the phosphopeptides of 4PS. Nevertheless, IL-4, insulin, and IGF-I were capable of stimulating tyrosine phosphorylation of IRS-1 in FDC cells that expressed this substrate as a result of transfection. These findings indicate that (i) IL-4, insulin, and IGF-I use signal transduction pathways in FDC lines that have at least one major feature in common, the rapid tyrosine phosphorylation of 4PS, and (ii) insulin and IGF-I stimulation of hematopoietic cell lines leads to the phosphorylation of a substrate that may be related to but is not identical to IRS-1.

Interleukin 4 (IL-4) is a pluripotent cytokine that is intimately involved in determining the nature of an immune response to a given pathogen (reviewed in ref. 1). The cDNAs encoding both the murine and human IL-4 receptors have been cloned (2–4). Although there are no consensus sequences in the receptor that suggest any intrinsic tyrosine kinase activity, IL-4 does induce striking tyrosine phosphorylation of a 170-kDa protein, designated IL-4-induced phosphotyrosine substrate (4PS) (5). In a previous study, 4PS was shown to strongly associate with the 85-kDa subunit (p85) of phosphatidylinositol (PI) 3-kinase immediately after IL-4 stimulation (5). This association is reminiscent of PI 3-kinase interaction with insulin receptor substrate 1 (IRS-1), which leads to PI 3-kinase activation (6–8). Although functions of the metabolites produced by this enzyme are not understood, evidence suggests that PI 3-kinase association with receptors or oncogene products correlates with cell proliferation (reviewed in ref. 9).

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IRS-1, the most prominent substrate phosphorylated in response to stimulation with insulin or insulin-like growth factor I (IGF-I), is a hydrophilic protein of 160–185 kDa (10–13). The cDNAs for rat and mouse IRS-1 have been cloned (14, 15). The inferred amino acid sequence contains 20 potential tyrosine and 30 potential serine and threonine phosphorylation sites. Nine putative tyrosine phosphorylation sites are in YMXM or YXXM motifs (16). Tyrosine phosphorylation of these motifs is thought to result in association with proteins containing Src homology 2 domains, such as p85 (reviewed in refs. 9, 17, and 18). The unique properties of IRS-1 suggest that it could potentially serve as a docking molecule for many key proteins involved in signal transduction (14, 16). Since insulin receptor mutants that are defective for mitogenic signaling do not mediate phosphorylation of IRS-1 (19–22), it has been suggested that IRS-1 is a crucial component of insulin and IGF-I signaling.

In the present study we demonstrate that insulin and IGF-I act as mitogens for certain IL-4-responsive hematopoietic cell lines and that the characteristics of the principal substrate tyrosine-phosphorylated in cells treated with each of these factors are identical. The relationship between insulin- and IL-4-mediated signal transduction pathways in these factor-dependent cell lines is investigated.

MATERIALS AND METHODS

Cell Lines and Growth Assays. The CHO/IR/IRS-1 cells have been described (7, 22). The IL-3-dependent murine hematopoietic cell lines FDC-P1 (23) and FDC-P2 (24) were cultured in RPMI 1640 medium with 10% fetal bovine serum and 5% WEHI-3B conditioned medium. The FDC-P1/IRS-1 line was generated by electroporation of the rat IRS-1 expression vector after selection in the presence histidinol (2 mM) as described (7, 25). Incorporation of [³H]thymidine was used to quantitate factor-induced DNA synthesis in FDC lines as described (5). Analysis of long-term growth properties was performed in RPMI 1640 medium with 10% fetal bovine serum and recombinant IL-4 (10 nM), insulin (1 μM), or IGF-I (10 nM). Cells (5 × 10⁴ per ml) were plated in growth medium with one or more factors. They were transferred at a split ratio of 1:10 biweekly. Cell viability was determined by trypan blue exclusion.

Immunoprecipitation and Immunoblot Analysis. FDC or CHO cells were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) with 50 μM Na₃VO₄ for

Abbreviations: IGF-I, insulin-like growth factor I; IL, interleukin; IRS-1, insulin receptor substrate 1; PI, phosphatidylinositol; 4PS, IL-4-induced phosphotyrosine substrate.

**To whom reprint requests should be addressed.

2 hr. After stimulation with either IL-4 (100 nM), insulin (10 μ M), or IGF-I (100 nM) for 10 min at 37°C, cells were lysed in lysis buffer as described (5). Equal amounts of clarified FDC lysates (2 mg) or CHO/IR/IRS-1 lysates (0.5–1 mg) were immunoprecipitated with 20 μ l of agarose-conjugated anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), anti-p85 (1:500; Upstate Biotechnology), or anti-IRS-1 (1:500; ref. 7) plus 30 μ l of protein G-coupled Sepharose. Immunoprecipitates were washed with lysis buffer, solubilized with Laemmli buffer, boiled, and resolved by SDS/8% PAGE for immunoblot analysis (5).

Peptide Mapping Using *Staphylococcus aureus* V8 Protease. Cells were cultured in serum-free DMEM containing 50 μ M Na_3VO_4 for 2 hr and labeled with [^{32}P]orthophosphoric acid (500 $\mu\text{Ci/ml}$; 1 μCi = 37 kBq) in phosphate-free medium for 4 hr. Untreated or factor-treated cells were lysed in lysis buffer and immunoprecipitated with anti-phosphotyrosine or anti-IRS-1. The immunoprecipitates were resolved by SDS/8% PAGE and the 170-kDa bands (present only in samples from factor-treated cells) were located by autoradiography and excised. Peptides from the 170-kDa bands were generated by *in situ* proteolytic cleavage within the stacking gel (26).

RESULTS

Growth Potential of IL-4, Insulin, and IGF-I in IL-3-Dependent FDC Lines. IL-4, insulin, and IGF-I each induced dose-dependent [^3H]thymidine incorporation in the myeloid progenitor lines FDC-P1 and FDC-P2 under serum-free conditions. IL-4, insulin, and IGF-I stimulated half-maximal responses in FDC-P1 cells at approximately 100 pM, 10 nM, and 20 pM, respectively (Fig. 1). The maximum level of [^3H]thymidine incorporation achieved by each factor was similar to that of IL-3, implying that they were capable of inducing as potent a mitogenic response as IL-3 in FDC-P1 cells. FDC-P2 cells also responded mitogenically to IL-4, insulin, and IGF-I, with half-maximal responses at 25 pM, 100 nM, and 1 nM, respectively (data not shown). These results provide evidence that the FDC lines possess cell surface receptors and intracellular components required to allow short-term mitogenic signaling through the IL-4, insulin, and IGF-I pathways.

While IL-4, insulin, and IGF-I did stimulate DNA synthesis, they failed to support long-term growth of the FDC cells. Although cells from both lines survived for over 72 hr in growth medium containing recombinant IL-4, insulin, or

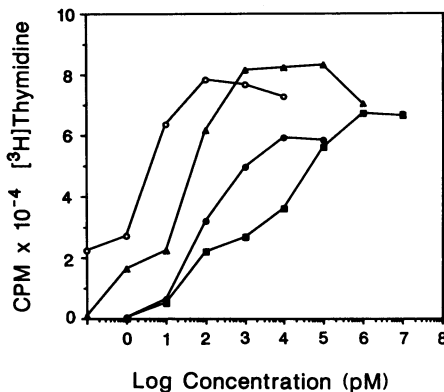


FIG. 1. Mitogenic dose-response of FDC-P1 cells to various growth factors. FDC-P1 cells were incubated for 36 hr in serum-free medium with various concentrations of IL-4 (●), IL-3 (○), insulin (■), or IGF-I (▲). [^3H]Thymidine (0.5 μCi per well) was added for 4 hr and samples were then collected by an automatic harvester. Results are expressed as counts per minute (cpm) in stimulated samples minus background cpm.

IGF-I, they did not remain viable when transferred to new culture dishes containing the same factor. By contrast, a combination of IL-4 with either insulin or IGF-I yielded sustained growth of both lines. Insulin and IGF-I together did not support continuous growth of either line. Even after propagation for over 4 months in medium containing both IL-4 and insulin, neither line continued to proliferate when switched to medium containing only one factor.

Relationship of the Tyrosine-Phosphorylated Substrates Induced by IL-4, Insulin, and IGF-I in FDC Lines. The capacity of insulin or IGF-I in combination with IL-4 to allow FDC cells to grow long-term could reflect either the use of complementing signaling pathways or the surpassing of a threshold response through a common signaling pathway in which receptor numbers were limiting. To help distinguish between these two alternatives, we compared the tyrosine phosphorylation patterns induced by the three factors in FDC-P1 and FDC-P2 cells. In both cell lines, each factor induced rapid tyrosine phosphorylation of a major band migrating at 170 kDa (Fig. 2). Stimulation with IL-4 also resulted in the phosphorylation of other less prominent bands in each line, whereas no other substrates were detected after insulin or IGF-I treatment. To determine whether the 170-kDa substrates phosphorylated by the three factors were identical, we performed V8 protease digestion analysis. The phosphopeptide patterns of the 170-kDa bands tyrosine-phosphorylated by IL-4, insulin, or IGF-I were indistinguishable (Fig. 3). Taken together, these results provide strong evidence that insulin and IGF-I cause phosphorylation of the IL-4-induced phosphotyrosine substrate 4PS.

Association of the p85 Subunit of PI 3-Kinase with 4PS in Response to IL-4, Insulin, and IGF-I Stimulation of FDC Lines. In a previous study, we observed that PI 3-kinase activity was greatly enhanced in the phosphotyrosine fraction of lysates of IL-4-stimulated cells (5). A substantial increase in the amount of PI 3-kinase activity was also observed in the

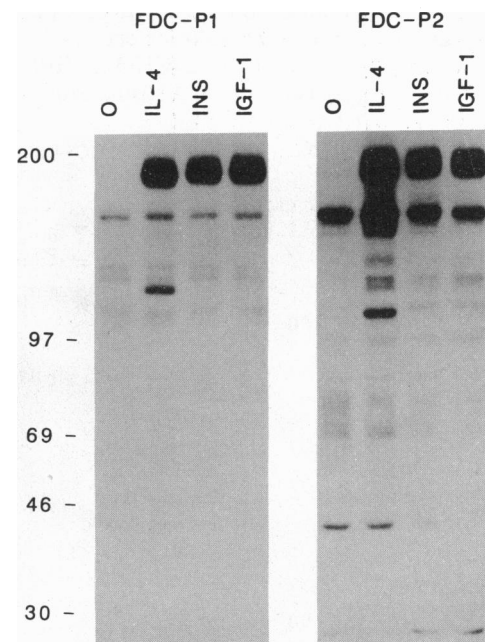


FIG. 2. Detection of phosphotyrosine-containing proteins in FDC lines after stimulation with IL-4, insulin, or IGF-I. Quiescent FDC-P1 and FDC-P2 cells were treated with IL-4, insulin (INS), or IGF-I as illustrated. Cell lysates were immunoprecipitated with anti-phosphotyrosine, resolved by SDS/PAGE, transferred to a poly(vinylidene difluoride) (PVDF) membrane, and probed with anti-phosphotyrosine. Molecular masses of marker proteins are indicated in kilodaltons.

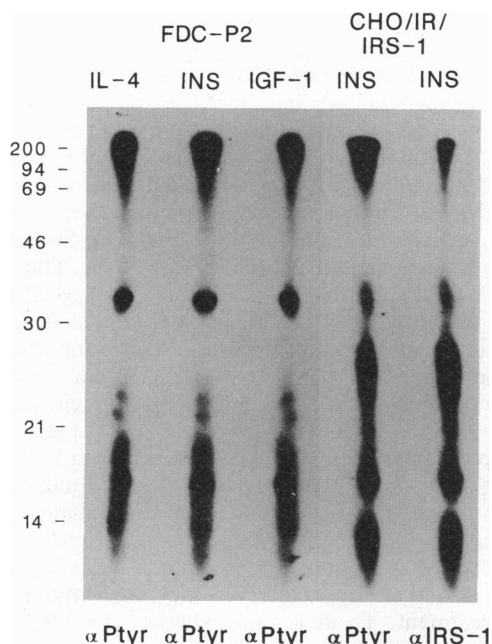


FIG. 3. V8 digestion pattern of tyrosine-phosphorylated 170-kDa proteins after factor stimulation of FDC-P2 and CHO/IR/IRS-1 cells. Partial V8 digestion was performed on IL-4-, insulin (INS)-, or IGF-I-treated [32 P]orthophosphate-labeled 170-kDa proteins immunoprecipitated with anti-phosphotyrosine (α Ptyr) or anti-IRS-1 (α IRS-1) from FDC-P2 and CHO/IR/IRS-1 cells.

phosphotyrosine fraction of lysates of insulin- or IGF-I-treated FDC-P1 or FDC-P2 cells as determined by the conversion of PI to PI 3-phosphate in an *in vitro* reaction (data not shown). To more specifically define the interaction of PI 3-kinase with the tyrosine-phosphorylated substrates induced in FDC lines, lysates were first immunoprecipitated with anti-p85 and then immunoblotted with anti-phosphotyrosine. Chinese hamster ovary cells transfected with both the human insulin receptor and rat IRS-1 (CHO/IR/IRS-1) were also analyzed for comparison (7, 22). A strong band migrating

at 170 kDa was observed when either FDC line was treated with IL-4, insulin, or IGF-I or when CHO/IR/IRS-1 was treated with insulin (Fig. 4A). When lysates were immunoprecipitated with anti-phosphotyrosine and immunoblotted with anti-p85, the amount of p85 was substantially increased in the IL-4-, insulin-, or IGF-I-treated FDC and insulin-stimulated CHO/IR/IRS-1 samples when compared with the lysates of untreated cells (Fig. 4B). Thus, 4PS strongly associates with the p85 subunit of PI 3-kinase in response to insulin, IGF-I, or IL-4 stimulation of hematopoietic cells just as IRS-1 does in nonhematopoietic cells after insulin stimulation.

Relationship of 4PS to IRS-1. To determine the relationship of 4PS to IRS-1, lysates from factor-stimulated FDC-P2 and CHO/IR/IRS-1 cells were immunoprecipitated with a polyclonal antibody directed against baculovirus-generated rat IRS-1 or with anti-phosphotyrosine and subsequently immunoblotted with anti-phosphotyrosine (Fig. 5). Although anti-IRS-1 immunoprecipitated a modest amount of 4PS from lysates of IL-4- or insulin-treated FDC-P2 cells, it was much less than that immunoprecipitated by anti-phosphotyrosine. By contrast, anti-IRS-1 immunoprecipitated a much greater fraction of that recognized by anti-phosphotyrosine from the insulin-stimulated CHO/IR/IRS-1 lysate. Moreover, when rabbit antibodies directed against either amino- or carboxyl-terminal peptides of rat/mouse IRS-1 (13, 27) were utilized in similar experiments or for direct immunoblot analysis, 4PS was not detected whereas IRS-1 was readily observed (data not shown).

V8 protease digestion analysis was carried out on 4PS immunoprecipitated with anti-phosphotyrosine from lysates of IL-4- or insulin-stimulated FDC-P2 cells and on IRS-1 immunoprecipitated with anti-IRS-1 or anti-phosphotyrosine from insulin-treated CHO/IR/IRS-1 cells. Although the patterns of phosphopeptides derived from 4PS (from FDC-P2 cells) and IRS-1 (from CHO/IR/IRS-1 cells) showed similarities, they were not identical (Fig. 3). While a 34-kDa peptide as well as other less distinct peptides in the 12- to 24-kDa range were found in digests from both substrates, the IRS-1 digest contained a prominent 25- to 29-kDa peptide not found in the 4PS digest (Fig. 3). Although these results further

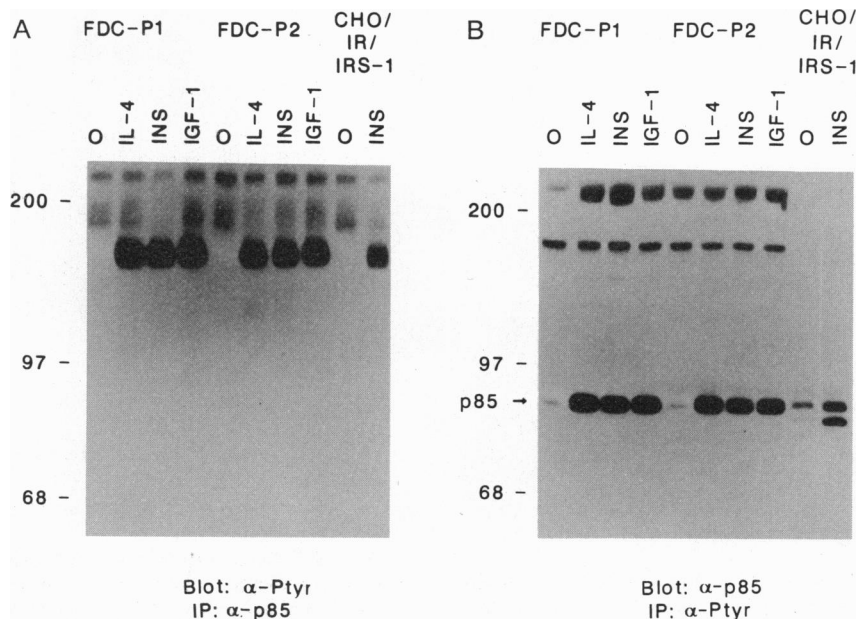


FIG. 4. Association of the p85 subunit of PI 3-kinase with 4PS in FDC lines after stimulation with IL-4, insulin (INS), or IGF-I. (A) Lysates of IL-4-, insulin-, or IGF-I-treated FDC-P2 and insulin-stimulated CHO/IR/IRS-1 cells were immunoprecipitated (IP) with anti-p85, subjected to SDS/PAGE, and immunoblotted with anti-phosphotyrosine (α Ptyr). (B) The same lysates were immunoprecipitated with anti-phosphotyrosine, and transferred proteins were immunoblotted with anti-p85.

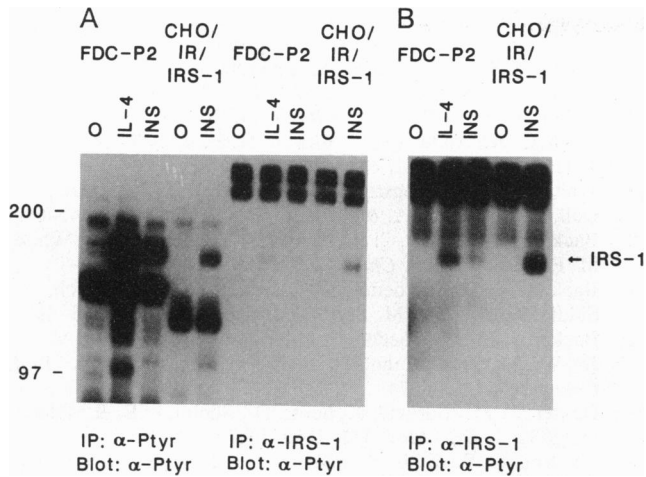


FIG. 5. Weak recognition of 4PS by an IRS-1-specific antibody. (A) Lysates of IL-4- or insulin (INS)-treated FDC-P2 or insulin-treated CHO/IR/IRS-1 cells were immunoprecipitated (IP) with anti-phosphotyrosine (α -Ptyr) or anti-rat IRS-1, resolved by SDS/PAGE, and immunoblotted with anti-phosphotyrosine. (B) Same anti-phosphotyrosine immunoblot of anti-IRS-1 immunoprecipitates as in A, except that the autoradiograph was exposed for 72 hr instead of 24 hr.

indicate that 4PS and IRS-1 are not identical, the finding of phosphopeptides of common size and the weak recognition of 4PS by polyclonal anti-rat IRS-1 suggest that they may be related to one another.

IL-4 Induces Tyrosine Phosphorylation of Rat IRS-1 in FDC-P1 Cells Transfected with an IRS-1 Expression Vector. To more fully investigate the relationship between insulin- and IL-4-mediated signals, we sought to determine whether IL-4 could induce tyrosine phosphorylation of the protein encoded by the cloned IRS-1 gene in the FDC background. An expression vector containing rat IRS-1 was introduced into the FDC-P1 line by electroporation. Bands of 170 kDa were readily detected in lysates of IL-4- and insulin-treated FDC-P1/IRS-1 cells after immunoprecipitation with anti-IRS-1 followed by immunoblot analysis with anti-phosphotyrosine (Fig. 6B). The relative amount of tyrosine-phosphorylated IRS-1 compared to total tyrosine-phosphorylated protein rec-

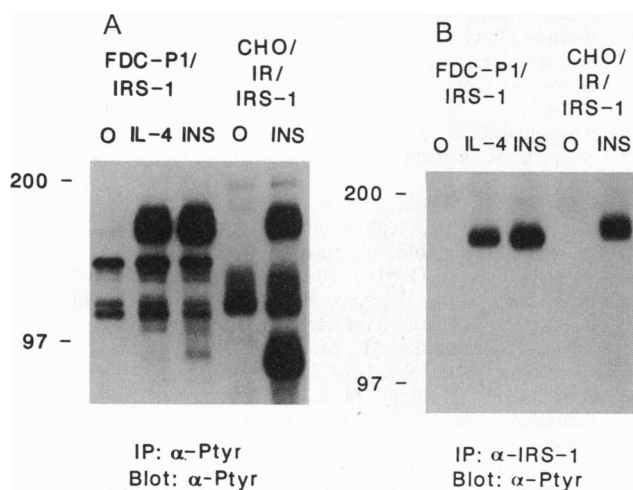


FIG. 6. Detection of tyrosine-phosphorylated IRS-1 in response to IL-4 or insulin (INS) stimulation of the FDC-P1/IRS-1 transfectant. (A) Lysates from factor-stimulated FDC-P1/IRS-1 or CHO/IR/IRS-1 cells were immunoprecipitated (IP) and subsequently immunoblotted with anti-phosphotyrosine (α -Ptyr). (B) Identical lysates were immunoprecipitated with anti-rat IRS-1 and immunoblotted with anti-phosphotyrosine.

ognized in FDC-P1/IRS-1 lysates was equivalent to that observed in the CHO/IR/IRS-1 lysate (Fig. 6). These results imply that IRS-1 can be efficiently phosphorylated on tyrosine in response to IL-4 or insulin stimulation in the FDC hematopoietic cell background.

DISCUSSION

The results demonstrate that IL-4, insulin, and IGF-I each induce pronounced tyrosine phosphorylation of a 170-kDa substrate in murine IL-3-dependent myeloid progenitor cell lines. We have also observed tyrosine phosphorylation of a 170-kDa substrate in megakaryocytic, mast cell, and B lymphoid lines in response to IL-4, insulin, or IGF-I treatment and in lipopolysaccharide-stimulated B cells and Con A-stimulated T cells after treatment with IL-4 (unpublished work). Thus, IL-4 and insulin induce similar tyrosine phosphorylation events in very different hematopoietic cell lineages. Interestingly, the concentrations of both IL-4 and insulin required to detect phosphorylation of and PI 3-kinase association with 4PS correlated precisely with those needed to evoke DNA synthesis (data not shown), suggesting that this substrate may play a crucial role in hematopoietic cell signal transduction.

Our results indicate that the 170-kDa protein phosphorylated in response to insulin and IGF-I in the FDC lines is identical to that phosphorylated in response to IL-4. The substrate strongly associated with the 85-kDa subunit of PI 3-kinase after stimulation with each factor. Moreover, the phosphopeptide patterns generated by V8 digestion of the 170-kDa proteins were identical to each other. Thus, it appears that IL-4, insulin, and IGF-I activate tyrosine kinases in FDC cells (and presumably other hematopoietic-lineage cells) which phosphorylate an identical substrate, 4PS. The results obtained suggest that 4PS is probably related to but not identical to IRS-1. While antibodies directed against amino- and carboxyl-terminal peptides of rat/mouse IRS-1 did not recognize 4PS (data not shown), a polyclonal antibody directed against the entire rat IRS-1 protein was able to weakly detect 4PS. V8 digestion of 4PS and IRS-1 generated some peptides with similar mobilities and others which were distinct. Although 4PS and IRS-1 do not appear to be identical proteins, IL-4 was able to induce readily detectable tyrosine phosphorylation of rat IRS-1 expressed in the FDC-P1/IRS-1 transfectant. Thus, the tyrosine kinase activated by IL-4 stimulation is capable of phosphorylating IRS-1, just as insulin or IGF-I receptor activation can mediate tyrosine phosphorylation of 4PS. These results suggest that 4PS and IRS-1 may be encoded by evolutionarily related genes which are preferentially expressed in different cell types and may play similar functional roles in signal transduction.

To date, no other factor has been shown to induce phosphorylation of 4PS or IRS-1. While IL-3 stimulated pronounced tyrosine phosphorylation of several substrates in the FDC lines, it did not detectably phosphorylate 4PS (5). We have recently observed weak IL-3-induced phosphorylation of an \approx 170-kDa protein in certain cell lines. Whether this substrate is related to 4PS remains to be determined. Other cytokines whose receptors are members of the hematopoietin superfamily, including granulocyte/macrophage-colony-stimulating factor, IL-2, IL-7, and erythropoietin, have also been reported to induce tyrosine phosphorylation in various cell lines (28–33). However, no substrate in the 170-kDa size range was described in any of these studies. These results suggest that the tyrosine kinase activated by IL-4 stimulation differs from those most commonly induced by other cytokines.

An important question which arises from this study is how IL-4-mediated signaling leads to the tyrosine phosphoryla-

tion of 4PS. In contrast to insulin and IGF-I receptors, the IL-4 receptor possesses no known tyrosine kinase domain (2-4, 34, 35). A previous study demonstrated that tyrosine residues found in the YXXM motifs of IRS-1 were excellent substrates for an activated insulin receptor kinase (16). Thus, it is likely that activated insulin or IGF-I receptors directly phosphorylate 4PS in the FDC lines. It is tempting to speculate that IL-4 might channel its signal by transactivating either insulin or IGF-I receptor tyrosine kinases. While we were able to observe insulin-dependent insulin receptor autophosphorylation in the FDC lines, IL-4 treatment did not detectably affect this receptor (data not shown). Thus, it is more likely that IL-4 induces phosphorylation of 4PS by activating an independent tyrosine kinase.

Although our results provide evidence that IL-4, insulin, and IGF-I share similarities in some of the biological and biochemical cascades they induce in the FDC lines, it remains to be determined whether these factors activate other overlapping signaling events. Insulin has been demonstrated to stimulate tyrosine phosphorylation of mitogen-activated protein kinase, phosphorylation of Raf kinase, and activation of the cellular Ras protein (36-41). By contrast, others have reported that IL-4 does not affect any of these critical signaling molecules (42-45). Thus, it seems likely that IL-4 and insulin signaling cascades share some coincident and other divergent elements that account for their distinct physiological roles.

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