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An IL-4 Receptor Region Containing an Insulin Receptor Motif Is Important for IL-4-Mediated IRS-1 Phosphorylation and Cell Growth

Achsah D. Keegan,* Keats Nelms,* Morris White,† Ling-Mei Wang,‡ Jacalyn H. Pierce,‡ and William E. Paul*
*Laboratory of Immunology
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892
†Joslin Diabetes Center
Department of Medicine
Harvard Medical School
Boston, Massachusetts 02215
‡Laboratory of Cellular and Molecular Biology
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Summary

Interleukin-4 (IL-4) treatment of 32D cells overexpressing insulin receptor substrate 1 (IRS-1) causes prompt tyrosine phosphorylation of IRS-1. Transfection of truncation mutants of the human IL-4 (huIL-4) receptor into 32D–IRS-1 cells demonstrated that the region from amino acid 437–557 is important for IL-4 signaling. This region of the IL-4 receptor (IL-4R) contains the motif ^{488}PL-X4-NPXYXSXSD^{502} (insulin/IL-4R [I4R]) found in the insulin and insulin-like growth factor 1 receptors. Mutation of Y497 to F yielded receptors that caused little or no IRS-1 phosphorylation in response to huIL-4 when expressed in 32D–IRS-1 cells. Most cell lines expressing Y497F also failed to proliferate in response to huIL-4. Furthermore, a glutathione-S-transferase fusion protein containing the I4R motif-bound IRS-1, tyrosine kinase(s), and other unidentified phosphoproteins with molecular sizes of 140, 80, and 55 kd. Thus, the central tyrosine of the I4R motif has a major role in IL-4-mediated signal transduction in 32D cells.

Introduction

Interleukin-4 (IL-4) is a cytokine produced by T cells (Howard et al., 1982), mast cells (Plaut et al., 1989), and basophils (Seder et al., 1991). Among its diverse array of biologic functions (reviewed by Paul, 1991) are the ability to induce or enhance the expression of major histocompatibility complex class II molecules (Noelle et al., 1984) and CD23 on B cells (Conrad et al., 1987), its own receptor on lymphocytes (Ohara and Paul, 1988), and vascular cell adhesion molecule 1 on endothelial cells (Schleimer et al., 1992); the ability to induce the secretion of immunoglobulin G1 (Vitetta et al., 1985) and immunoglobulin E (Coffman et al., 1986) while diminishing the immunoglobulin secretion of other isotypes; and the ability to act as a growth factor for B cells, T cells, and mast cells (Lee et al., 1986).

This diverse array of IL-4-mediated effects is reflected in the broad distribution of IL-4 receptors (IL-4Rs). A single class of high-affinity binding sites (K_d, 20–300 pM) have been detected on a wide variety of hematopoietic and nonhematopoietic cell types at levels ranging from 100–5000 sites/cell (Ohara and Paul, 1987; Park et al., 1987; Lowenthal et al., 1988). The molecular cloning of cDNA encoding both the murine IL-4R (mulL-4R) (Mosley et al., 1989; Harada et al., 1990) and human IL-4R (hulL-4R) (Idzerda et al., 1990; Galizzi et al., 1990) and their expression in COS7 cells indicated that a single chain of ~139,000 daltons constituted a high-affinity IL-4-binding site. Recently, it has been shown that the IL-2R γ chain (common γ [γ_c]) is associated with IL-4R in the presence of IL-4 (Russell et al., 1993) and that its presence contributes a 2- to 3-fold increase in binding affinity. The IL-4R contains the characteristic pairs of cysteine residues and the WSXWS motif found in other members of the hematopoietin receptor superfamily, and it bears one of the largest cytoplasmic domains, comprised of over 500 amino acids (Cosman, 1993).

Like other members of this receptor family, the IL-4R does not contain any consensus sequences characteristic of tyrosine or serine/threonine kinases. However, activation of the IL-4R with IL-4 induces the rapid and striking tyrosine phosphorylation of a 170 kd protein (Morla et al., 1988; Isfort and Ihle, 1990; Wang et al., 1992), called 4PS, as well as phosphorylation of the receptor itself (Wang et al., 1992; Izuhara and Harada, 1993), suggesting a linkage among the IL-4R, 4PS, and a tyrosine kinase. Phosphorylation of 4PS results in its association with the p85 subunit of phosphotidylinsitol-3-kinase (PI-3K) and the subsequent induction of PI-3K activity (Wang et al., 1992). Interestingly, it was found that 4PS is antigenically related to a prominent substrate of the insulin receptor kinase, called insulin receptor substrate 1 (IRS-1) (Sun et al., 1991; Wang et al., 1993a). Addition of insulin or insulin-like growth factor 1 (IGF-1) resulted in the tyrosine phosphorylation of 4PS in myeloid cell lines. Furthermore, IL-4 stimulation of a myeloid cell line transfected with the cDNA encoding IRS-1 results in the tyrosine phosphorylation of IRS-1 and its subsequent association with the p85 subunit of PI-3K, suggesting that the IL-4R and the insulin receptor have partially overlapping signal transduction pathways and that IRS-1 and 4PS are functionally similar.

It has recently been shown that IL-4 treatment can lead to phosphorylation of IRS-1 in nonhematopoietic cells (Russell et al., 1993). L cells express both IL-4R and insulin receptor; however, they lack the γ_c. L cells demonstrated IRS-1 phosphorylation in response to insulin but not in response to IL-4. However, stable L cell transfectants that express the γ_c showed clear IRS-1 phosphorylation in response to IL-4. These results indicate that IRS-1 can be a natural substrate for IL-4-mediated signaling, and they show that the γ_c is not only associated with the IL-4R but is essential for certain forms of IL-4-mediated signal transduction.

The importance of IRS-1 in mediating the IL-4 signal

transduction that results in a biological response has recently been examined in the myeloid progenitor cell line, 32D (Wang et al., 1993b). Although 32D cells express approximately 1000 IL-4Rs per cell, they do not mount a strong mitogenic response to IL-4. In addition, IL-4 does not induce detectable tyrosine phosphorylation of 4PS in these cells. However, expression of the cDNA encoding rat IRS-1 in 32D cells significantly increases their mitogenic response to IL-4. The induction of tyrosine phosphorylation of IRS-1 by IL-4 is easily observed in the transfected cells, which are designated 32D–IRS-1. These results indicate that IRS-1 is critical for mediating the IL-4-induced proliferative response in these cells.

We wished to identify the regions of the IL-4R that interact with its signal transducing molecules. As one approach, we analyzed the ability of deletion mutants of the huIL-4R to signal growth and phosphorylation of IRS-1 in the 32D–IRS-1 cell line. In this study, we have demonstrated that the region between amino acids 437 and 557 plays an important role in the IL-4-mediated proliferative response and the tyrosine phosphorylation of IRS-1 in 32D–IRS-1 cells. Furthermore, we show that the IL-4R and a domain of the receptor consisting of residues 424–561 interact with IRS-1 and a tyrosine kinase. This region of the IL-4R contains a sequence motif, the insulin/IL-4R (I4R) motif, also expressed in the juxtamembrane region of insulin and IGF-1 receptors, suggesting that this motif is evolutionarily conserved among receptors that signal via the IRS-1/4PS pathway. The importance of this motif in IL-4 signaling was demonstrated by mutation of Y497, which is in the I4R motif, to F. This mutant receptor causes little or no tyrosine phosphorylation of IRS-1 in response to IL-4 and generally fails to signal a proliferative response in 32D–IRS-1 cells.

Results

Expression of IRS-1 Increases the Mitogenic Response of 32D Cells to IL-4

The myeloid cell line, 32D, shows very modest proliferation to IL-4, and no detectable tyrosine phosphorylation occurs in these cells. As shown in Figure 1 and in a recent study (Wang et al., 1993b), 32D–IRS-1 cells, which stably express IRS-1 as a result of transfection, respond to IL-4 with striking proliferation. IL-4-induced tyrosine phosphorylation of IRS-1 was also easily observed in 32D–IRS-1 cells (Figure 1, top). Since the expression of IRS-1 is required for the mitogenic response of 32D cells to IL-4 (Wang et al., 1993b), we studied the ability of huIL-4Rs to transduce signals in the 32D–IRS-1 cell line.

Identification of a Region of the IL-4R Important for IL-4-Dependent Proliferation and IRS-1 Phosphorylation

To understand the contribution of the large cytoplasmic domain of the IL-4R to its ability to transduce signals, a series of mutants of the huIL-4R were constructed (Figure 2). Since huIL-4 does not bind the mouse IL-4R, we could study the signaling properties of huIL-4R mutants in the presence of the endogenous mouse receptor. The constructs were cotransfected into the 32D–IRS-1 line with a guanine phosphoribosyl transferase–expressing (gpt-expressing) plasmid, and stable transfectants were selected. IL-4R-positive cells were screened by flourescence-activated cell sorting analysis. The number of IL-4 binding molecules per cell of these same lines was calculated by saturation binding analysis with ^{125}I–huIL-4 (data not shown). Receptor levels ranged from ~500 molecules/cell for the wild-type (wt) receptor to ~1500 for the frameshift and d657 mutants. Both the d557 and d437 mutants were expressed at ~900 molecules/cell. Scatchard analysis of binding data indicated that all mutants bound huIL-4 with high affinity, and analysis of the mutant receptors by cell surface cross-linkage of ^{125}I–huIL-4 demonstrated that truncated huIL-4Rs of the appropriate size were indeed expressed at the cell surface (data not shown).

The transfected 32D–IRS-1 cells were analyzed for the induction of IRS-1 phosphorylation in response to huIL-4

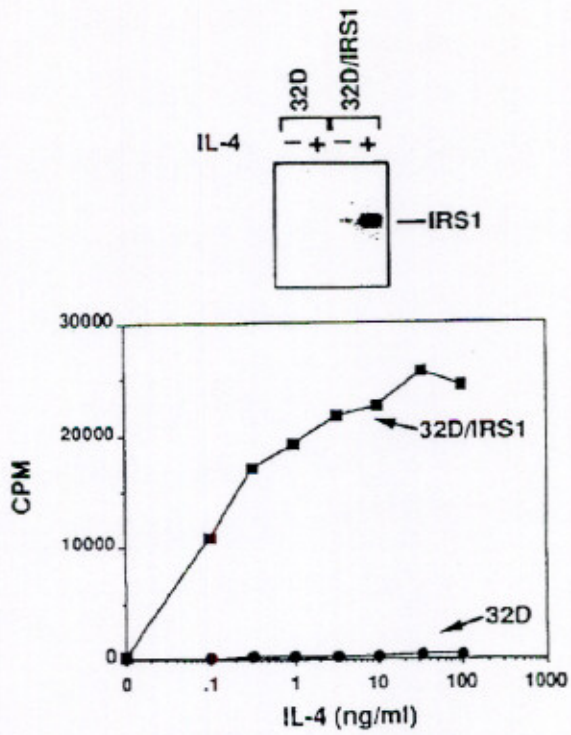

Figure 1. Response of 32D Cells Transfected with Rat IRS-1 to MuIL-4

(Top) 32D cells or 32D cells transfected with the cDNA encoding rat IRS-1 were deprived of IL-3 and serum before treatment with muIL-4 (plus sign) or media alone (minus sign) for 10 min. Subsequently, cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine. The precipitates were separated by SDS–PAGE and transferred to nitrocellulose. The blots were probed with anti-phosphotyrosine, followed by ^{125}I–protein A as described in the Experimental Procedures. The migration of phosphorylated IRS-1 is indicated.

(Bottom) 32D cells (circles) or 32D cells transfected with the cDNA encoding rat IRS-1 (squares) were incubated with various doses of muIL-4 or media alone in triplicate wells for 48 hr, as described in Experimental Procedures, before [^{3}H]thymidine uptake was analyzed. A representative experiment is shown.

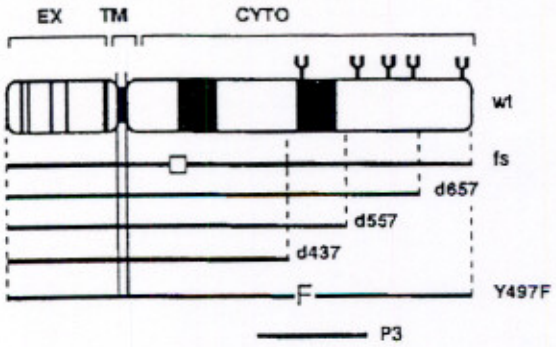

Figure 2. Diagram of IL-4R Constructs

Potentially important domains of the IL-4R are illustrated on the diagram of the constructs used in this study. The amino acid numbering begins (+1) at the methionine start of the signal peptide. The conserved pairs of cysteine residues are indicated by the thin lines. The WSXWS motif is indicated by the thick line. The two acidic regions in the cytoplasmic domain of the IL-4R are indicated by the stippled boxes. The acid region proximal to the transmembrane domain is considered the first acidic domain, and the more distal region is considered the second acidic domain. The positions of the five tyrosine residues that are conserved among the human, mouse, and rat IL-4Rs are indicated by Y. The open box represents the disrupted wt sequence found in the frameshift mutant of the huIL-4R. The deletion mutants are truncated at amino acids 657 (d657), 557 (d557), and 437 (d437). The GST–fusion protein of the muIL-4R containing amino acids 424–561 is referred to as P3. The mutant with Y497 replaced by F is also shown (Y497F).

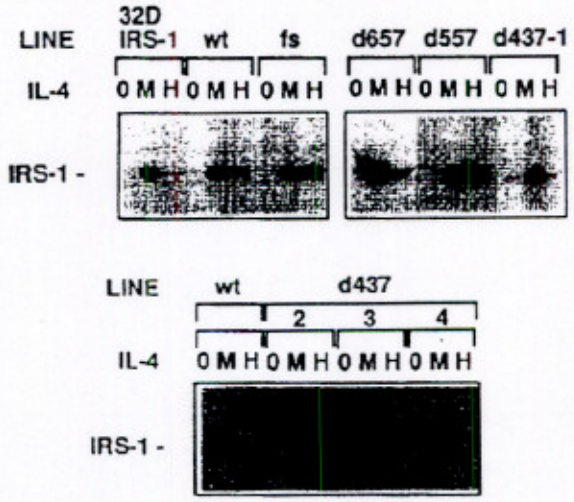

Figure 3. Phosphorylation of IRS-1 by IL-4 in the HuIL-4R Transfectants

(Top) The indicated huIL-4R-expressing lines were deprived of IL-3 and serum before treatment with huIL-4 (10 ng/ml), muIL-4 (2 ng/ml), or media for 10 min. Subsequently, cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine. The precipitates were separated by SDS–PAGE (7.5%) and transferred to nitrocellulose. The blots were probed with anti-phosphotyrosine, followed by ^{125}I–protein A as described in Experimental Procedures. The migration of phosphorylated IRS-1 is indicated.

(Bottom) Additional lines expressing the d437 construct were analyzed for IRS-1 phosphorylation as described above.

and muIL-4 (Figure 3). Tyrosine phosphorylation of IRS-1 in response to muIL-4 was observed in the parental 32D–IRS-1 line, as well as in all the huIL-4R-expressing lines. Phosphorylation of IRS-1 in response to huIL-4 was observed in the wt-, frameshift-, d657-, and d557-expressing lines, but not in the parental or the d437-expressing line. Additional lines expressing d437 also failed to demonstrate the tyrosine phosphorylation of IRS-1 in response to huIL-4 (Figure 3, bottom). Thus, the region between amino acids 437 and 557 is important in signaling the tyrosine phosphorylation of IRS-1 in 32D–IRS-1 cells.

The ability of the receptor mutants to signal proliferation in the 32D–IRS-1 cell line was also examined (Figure 4). Several cell lines transfected with each of the indicated mutants were incubated with various concentrations of huIL-4 or an optimal concentration (10 ng/ml) of muIL-4. HuIL-4 was able to signal a proliferative response in cell lines transfected with the wt, frameshift, d657, and d557 constructs of the huIL-4R. The response to huIL-4 varied somewhat from line to line; however, the maximal response to huIL-4 was similar to the response generated by muIL-4 for a given line. By contrast, the d437 mutant of the huIL-4R failed to signal a significant proliferative response to huIL-4 in five stable transfectants, although these same lines responded vigorously to muIL-4. We also transfected d557 and d437 into parental 32D cells (data not shown). Although the overall mitogenic response to huIL-4 (and mouse IL-4) was low, d557 transfectants demonstrated a weak response to huIL-4, while d437-expressing lines were unresponsive. These data strongly suggest that the region of the IL-4R cytoplasmic domain between amino acids 437 and 557 plays a central role in signaling proliferation and the tyrosine phosphorylation of IRS-1 in 32D cells.

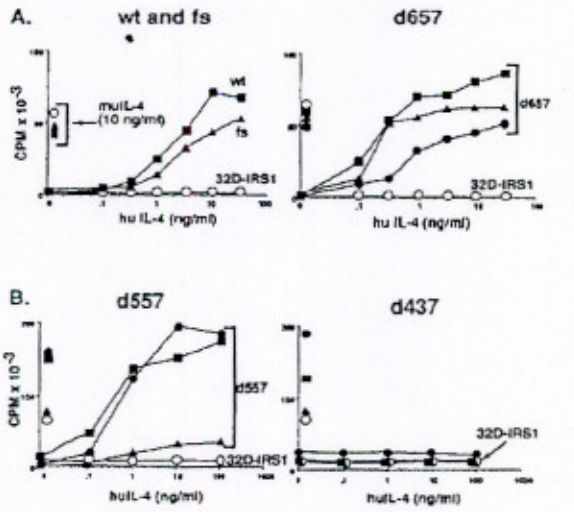

Figure 4. Proliferation of HuIL-4R Transfectants

The ability of the transfectants to proliferate in response to various doses of huIL-4 and an optimal dose of muIL-4 (10 ng/ml) was assessed as described in Experimental Procedures. Several lines expressing the different constructs were tested as indicated. The results presented in (A) were obtained from the same experiment, and those presented in (B) were obtained from a separate experiment. In both cases, the parental 32D–IRS-1 line was included (open circles).

Tyrosine Kinase Activity and IRS-1 Coprecipitate with the IL-4R and Bind to a Region of the IL-4R Containing the Sequence between Amino Acids 437 and 557

The observation that the region between amino acids 437 and 557 of the IL-4R is important for 32D proliferation and IRS-1 phosphorylation suggests the possibility that this region may interact with IRS-1, a tyrosine kinase, or both. Since the level of expression of the hulL-4R mutants in 32D cells was low, making immunoprecipitation studies difficult, we analyzed the ability of IRS-1 and tyrosine kinase activity to associate with mulL-4Rs in several cell lines and their ability to associate with a domain of the mulL-4R that contains amino acids 424–561 (P3 domain) expressed as a fusion protein with glutathione-S-transferase (GST) (see Figure 2). IRS-1-transfected FDC-P1 cells (FDC-P1–IRS-1) (Wang et al., 1993a) were treated with media alone or with mulL-4. After lysis, precipitations with control rabbit serum, anti-phosphotyrosine, or anti-IL-4R antisera were performed (Figure 5A). Immunoblots with anti-phosphotyrosine showed that IL-4 induces the tyrosine phosphorylation of a 170 kd protein in these cells and that this phosphorylated protein specifically coprecipitated with the IL-4R. We have confirmed that this protein is IRS-1 by immunoprecipitation and immunoblotting with anti-IRS-1 sera (Wang et al., 1993a; data not shown). In addition, phosphorylated IRS-1 was found bound to the P3 domain of the IL-4R as a result of the incubation of GST–P3-bearing beads with extracts of IL-4-treated FDC-P1–IRS-1 cells (Figure 5A). These data indicate that IRS-1 interacts with the IL-4R and that this interaction may be an integral part of the IL-4 signaling pathway.

We wished to determine whether tyrosine kinase activity would also coprecipitate with the IL-4R or with the P3 domain of the receptor. Preliminary immune complex kinase reactions of anti-IL-4R precipitates from an IL-4R-overexpressing line demonstrated the association of kinase activity with the IL-4R (data not shown). Overall, similar patterns of phosphorylated substrates were observed in precipitates from cells treated or untreated with IL-4. Therefore, cell lysates were prepared from untreated FDC-P2 cells and were precipitated with control rabbit serum, anti-IL-4R antiserum, GST–P3, or GST coupled to Affigel-10. After washing the precipitates, immune complex kinase reactions were performed with unlabeled ATP. The samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose filters, and then probed with anti-phosphotyrosine. Both the anti-IL-4R anti-serum and the P3 fusion protein precipitated tyrosine kinase activity, as demonstrated by the in vitro transfer of phosphate to tyrosine(s) on proteins present in the precipitates (Figure 5B). Proteins with molecular weights of ~140, 80, and 55 kd were phosphorylated in vitro. A small amount of the 140 kd molecule was also observed in the control rabbit serum precipitate. Thus, a domain of the IL-4R required for proliferation and IRS-1 tyrosine phosphorylation interacts with IRS-1 and with a tyrosine kinase. It is possible that one of these proteins represents the IL-4R-associated tyrosine kinase.

The Region between Amino Acids 437 and 557 Contains a Sequence Motif Found in the Insulin and IGF-1 Receptors (I4R Motif) That Is Important for IL-4-Mediated IRS-1 Phosphorylation and Growth

Examination of the hulL-4R revealed that the region between amino acids 437 and 557 contains a sequence that is homologous to a sequence found in the juxtamembrane region of the human insulin receptor (Figure 6). This ho-

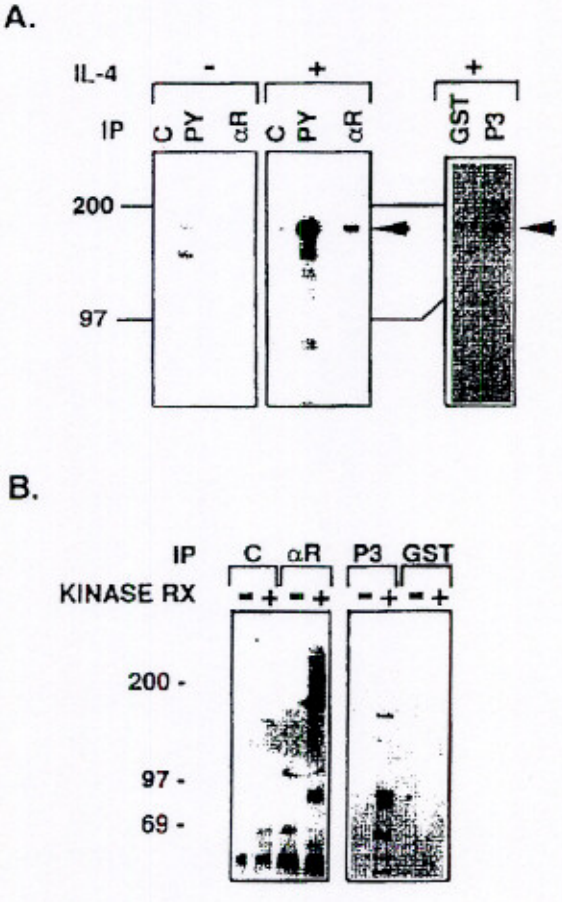

Figure 5. Interaction of IRS-1 with the MulL-4R

(A) FDC-P1 cells transfected with *IRS-1* cDNA were deprived of IL-3 and serum before treatment with mulL-4 (10 ng/ml) or media as indicated. Lysates from the treated cells were immunoprecipitated with a control rabbit serum (C), anti-phosphotyrosine (PY), rabbit anti-mulL-4R serum, (αR) or with GST–Affigel (GST) or GST–P3–Affigel (P3). After SDS–PAGE (7.5%) and Western blotting, the blots were probed with anti-phosphotyrosine and developed with ^{125}I–protein A (left panel) or the enhanced chemiluminescene system (ECL) (right panel). A representative of four separate experiments are shown.

(B) Kinase activity associates with the mulL-4R. Lysates were prepared from untreated FDC-P2 cells and precipitated with a control rabbit serum (C), rabbit anti-mulL-4R serum (αR), or with GST–Affigel (GST) or GST–P3–Affigel (P3). In vitro kinase reactions were performed using unlabeled ATP as described in Experimental Procedures. The precipitates were subjected to SDS–PAGE analysis (7.5%), followed by Western blot transfer. Tyrosine phosphorylated proteins were detected by probing with anti-phosphotyrosine and developing with the ECL system.

The I4R Motif
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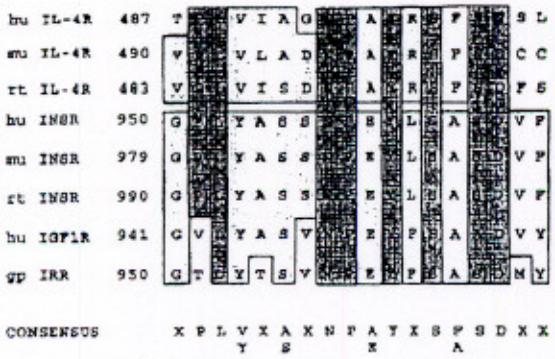

Figure 6. A Common Sequence Motif Found in the IL-4R and Insulin Receptor

The amino acid sequences of receptors that induce IRS-1 phosphorylation were aligned around the Y960 of the human insulin receptor. Consensus regions (I4R motif) are shown in the stippled box. Regions conserved among subfamily members are shown in the lightly hatched boxes.

mology, consisting of a consensus sequence of PL–X4–NPXYXSXSD is also exhibited by the rat and mouse IL-4Rs, the rat and mouse insulin receptors, the human IGF-1 receptor, and the guinea pig insulin receptor–related receptor, as well as by other members of the insulin receptor family. These receptors can be grouped into two subfamilies based on the intervening residues, designated X. The rat IL-4R, mouse IL-4R, and huIL-4R are highly homologous to one another in this region, and the insulin receptors, IGF-1 receptors, and the insulin receptor–related receptors are homologous to each other. Using the consensus sequence to search GenBank, significant alignments were found only for the IL-4Rs and a variety of insulin and IGF-1 receptors. This suggests that the I4R motif is preferentially expressed in receptors that use IRS-1/4PS as a substrate.

It has previously been demonstrated that mutation of Y960 of the human insulin receptor to F abolished IRS-1 phosphorylation in response to insulin in CHO cells and either abolished or substantially diminished insulin-induced proliferation, glucose metabolism, and amino acid uptake (White et al., 1988). Since Y960 is in the I4R motif, we prepared a Y to F mutation of the huIL-4R (within the I4R motif) at position 497 (Y497F). Stable lines of 32D–IRS-1 cells expressing this receptor mutant were examined. The level of receptor expression varied from 1030–6400 molecules/cell; for comparison, cells transfected with wt huIL-4R expressed 6400 receptors/cell. Cells expressing wt huIL-4R showed similar IRS-1 tyrosine phosphorylation in response to huIL-4 and muIL-4. By contrast, none of the Y497F-expressing lines displayed detectable tyrosine phosphorylation of IRS-1 in response to huIL-4, although each demonstrated comparable phosphorylation in response to muIL-4. Figure 7A shows a representative experiment for three of these lines. The seven Y497F-expressing cell lines were also tested for [^{3}H]thymidine

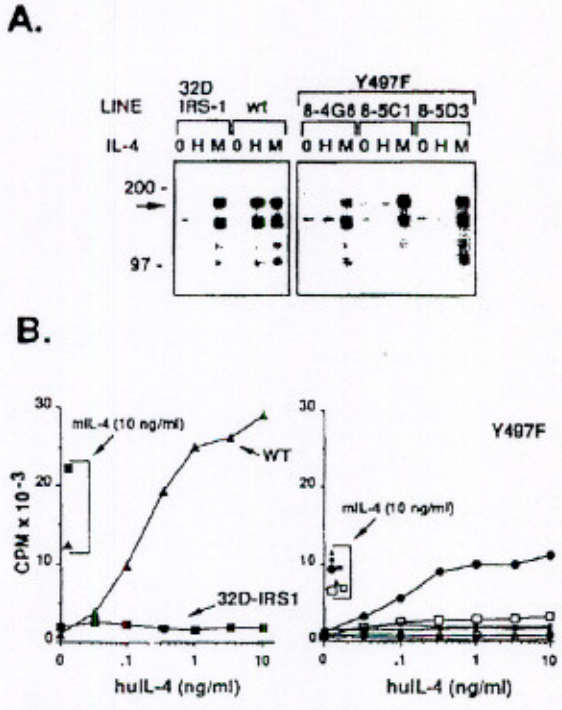

Figure 7. The HuIL-4R Mutant, Y497F, Fails To Signal IRS-1 Phosphorylation and Generally Fails To Signal Proliferation in Response to HuIL-4

(Top) The parental 32D–IRS-1 line, a wt-expressing line, and three Y497F-expressing lines were deprived of IL-3 and serum before treatment with muIL-4 (M) or huIL-4 (H). IL-4 treatment and sample processing were performed as described for Figure 3. The migration of IRS-1 is indicated by the arrow. Lower molecular weight bands are variably seen and represent degradation products of IRS-1.

(Bottom) The ability of the parental and wt- and Y497F-expressing lines to proliferate in response to various concentrations of huIL-4 and at optimal concentrations of muIL-4 was assessed as described in Experimental Procedures. Seven different Y497F-expressing lines were examined: the two lines that demonstrated some response to huIL-4 are shown in large symbols, 8–5D3 (circle) and 8–5B6 (open square); the five lines that failed to mount a significant response are shown in the small symbols, 8–4F4 (open square), 8–4G6 (open triangle), 8–5C1 (open square with plus sign), 8–6C1 (open square with diagonal line), and 8–5F2 (closed triangle). Levels of receptor expression are as follows: wt, 6400 molecules/cell; 8–5D3, 3,550 molecules/cell; 8–5B6, 3540 molecules/cell; 8–4F4, 1030 molecules/cell; 8–4G6, 4380 molecules/cell; 8–5C1, 2300 molecules/cell; 8–5F1, 1080 molecules/cell; and 8–5F2, 1500 molecules/cell.

uptake in response to muIL-4 and huIL-4 (Figure 7B). All showed responses to muIL-4: 5 of the 7 lines failed to proliferate in response to huIL-4; however, two lines were able to proliferate. In one line (8–5B6), the response was modest but in the other line (8–5D3), the response was rather striking. The ability to proliferate apparently did not correlate with the level of receptor expression. It should be noted that among the unresponsive lines, at least one (8–4G6) had a level of receptor expression (4380/cell) equivalent to that of 8–5D3 (3550/cell). These results indicate that Y497 in the I4R motif plays an important role in the transduction of IL-4–mediated signals that lead to IRS-1

tyrosine phosphorylation. In the majority of lines, this is coupled with a failure to proliferate.

Discussion

The 170 kd protein 4PS is a major substrate whose tyrosine phosphorylation is induced by the binding of IL-4 to its receptor in many hematopoietic cells, including B cells, T cells, myeloid cell lines, and mast cell lines (Wang et al., 1992; A. D. K., unpublished data). 4PS is also phosphorylated on tyrosine residues in response to insulin or IGF-1 in hematopoietic cells that bear insulin/IGF-1 receptors (Wang et al., 1993a), indicating that 4PS is a substrate of the insulin receptor and IGF-1 receptor kinases. Interestingly, the 4PS molecule is functionally and antigenically related to IRS-1, the major substrate of the insulin receptor tyrosine kinase in nonhematopoietic cells (Sun et al., 1991). The tyrosine phosphorylation of 4PS (Wang et al., 1992) and IRS-1 (Backer et al., 1992) results in their association with the regulatory subunit of PI-3K and thereby induces its enzyme activity.

The tyrosine phosphorylation of 4PS and IRS-1 as a result of engagement of the IL-4R and insulin receptor with their ligands has been shown to be important for mediating biologic responses characteristic for each growth factor. It has recently been shown that proliferation of the 4PS-deficient myeloid progenitor cell line 32D in response to insulin or IL-4 is dependent on the presence of transfected IRS-1 (Wang et al., 1993b). Indeed, it is in this system that we have analyzed the ability of mutants of the huIL-4R to signal the phosphorylation of IRS-1 and proliferation.

The failure of the cell lines expressing the d437 mutant of the huIL-4R described in this study either to proliferate or to phosphorylate IRS-1 detectably indicates that the domain of the receptor between amino acids 437 and 557 is important for both these functions. Our results are consistent with the report of Koettnitz and Kalthoff (1993) that the deletion of amino acids 487–605 (ΔR3), a deletion that removes the I4R motif, results in an IL-4R that does not signal proliferation in Ba/F3 cells. However, Harada et al., (1992) and Seldin and Leder, (1994) reported that mutants truncated at amino acids 431 and 455, respectively, were capable of signaling Ba/F3 proliferation in response to huIL-4. The explanation for the differences in these results is unclear. It does not appear that cell type can be responsible since Koettnitz and Kalthoff, (1993), Harada et al., (1992), and Seldin and Leder, (1994) used Ba/F3 for expression, although it is possible that different sublines of Ba/F3 may have different requirements for signal transduction. A resolution of this matter awaits further investigation.

Recently, we have observed that transfection of a mutant huIL-4R truncated at amino acid 455 (Seldin and Leder, 1994) into a different parental 32D–IRS-1 line than the one used in these studies resulted in lines that can proliferate in response to huIL-4, although less well so than cells transfected with the wt huIL-4R. One of these lines showed weak phosphorylation of IRS-1 in response to huIL-4, ~10–20% of that observed in transfectants expressing the wt huIL-4R. Thus, it seems most likely that several regions of the IL-4R as identified by deletion mutant analysis are important in signaling a strong proliferative response to IL-4. The requirement for several discontinuous regions for signal transduction has been demonstrated for other hematopoietin receptors. The β chain of the IL-2R has a serine-rich domain required for *c-myc* induction and proliferation of Ba/F3 cells, and it has an acidic domain required for tyrosine kinase activation as well as *c-fos* and *c-jun* induction (reviewed by Taniguchi and Minami, 1993). The gp130 chain of the IL-6R requires two short sequences, separated from one another by 30 or more residues, termed box 1 and box 2 to mediate proliferation and phosphorylation of gp130 in response to IL-6 (Murakami et al., 1991). The IL-4R contains a box 1–like sequence, although its importance in IL-4 signal transduction has not been analyzed. The common β subunit of the granulocyte/macrophage–colony-stimulating factor receptor, IL-3R, and IL-5R also demonstrates two functional domains. One domain located between amino acids 456–487 is required for granulocyte/macrophage–colony-stimulating factor–induced proliferation. The other domain (amino acids 518–626) is required for signaling the major tyrosine phosphorylation of 95 and 60 kd proteins, and it enhances the proliferative response (Sakamaki et al., 1992).

Our results demonstrate that phosphorylated IRS-1 is associated with the IL-4R after IL-4 treatment. In addition, phosphorylated IRS-1 binds to the fusion protein containing the P3 domain of the receptor. However, a much greater proportion of phosphorylated IRS-1 is immunoprecipitated by anti-phosphotyrosine antibody than by anti-IL-4R antibody. This is consistent with only a small proportion of tyrosine-phosphorylated IRS-1 being associated with the receptor; however, it cannot be excluded that the association is a weak or indirect one that is disrupted by the conditions of extraction. Similar results have been obtained from studies of the immunoprecipitation of phosphorylated IRS-1 with antibodies to the insulin receptor (Sun et al., 1992). A reasonable explanation might be that phosphorylated IRS-1/4PS dissociates from the IL-4R or insulin receptor soon after the substrate has been phosphorylated on some critical residue. We have previously demonstrated that after IL-4 treatment, PI-3K enzyme activity coprecipitates with the IL-4R (Wang et al., 1992). Again, when compared with the total activity found in the phosphotyrosine fraction, the amount of enzyme associated with the receptor is small. It is possible that, as has been shown for the insulin receptor (Backer et al., 1993), PI-3K interacts with the IL-4R indirectly by virtue of its ability to bind phosphorylated IRS-1/4PS.

One important difference between the IL-4R and the insulin/IGF-1 receptors is that the β chains of the latter are protein tyrosine kinases, while the cytosolic domain of the IL-4R lacks the consensus sequences established for tyrosine kinases. This suggests that the IL-4R must be associated with one or more kinases. Indeed, immunoprecipitates of the IL-4R are able to phosphorylate a series of associated substrates on tyrosine residues in the presence of unlabeled ATP. Similarly, the fusion protein containing the P3 domain of the receptor can precipitate a

kinase(s) capable of phosphorylating substrates associated with the domain, as well as purified IRS-1 (A. D. K. and K. N., unpublished data). These results suggest that the P3 domain of the receptor contains sites to which both IRS-1 and a tyrosine kinase can bind, directly or indirectly. The identity of the IL-4R-associated kinase is not known, although it is interesting to speculate that it may be related to the insulin receptor kinase since they share IRS-1 and 4PS as substrates.

The P3 domain contains two tyrosines and the second acidic domain of the receptor. However, the more COOH-terminal tyrosine does not appear to be essential since it is deleted in the d557 mutant without impairment of signaling. In examining the sequence surrounding tyrosine 497, as shown in Figure 6, we observed a striking homology between the human, mouse, and rat IL-4Rs, the human, mouse, and rat insulin receptors, the human IGF-1 receptor, and the guinea pig insulin receptor–related receptor. Sequence homology in this region between the human insulin and IGF-1 receptors has been previously described (White et al., 1988). We propose that this motif be termed the I4R motif.

None of the other hematopoietin receptors for which sequence information is available contain the I4R motif, in keeping with their failure to cause IRS-1/4PS tyrosine phosphorylation. The I4R motif contains within it a sequence (NPXY) required for coated pit-mediated internalization of the low density lipoprotein receptor (Chen et al., 1990). However, the sequences flanking NPXY in the low density lipoprotein receptor and in other transmembrane molecules, such as the epidermal growth factor receptor, are unrelated to the remainder of the I4R motif. Nor is there any evidence that these other NPXY-containing molecules induce the tyrosine phosphorylation of IRS-1/4PS. Indeed, the IL-4R is thus far unique in the hematopoietin receptor family in its relationship to the insulin receptor family. It is possible that new receptors containing the I4R motif will be described and that these receptors will utilize the IRS-1/4PS pathway.

Mutation of Y960 in the I4R motif of the insulin receptor results in its inability to signal the phosphorylation of IRS-1, although it does not impair the capacity of the receptor to undergo autophosphorylation (White et al., 1988). Here we have shown that mutation of Y497 of the huIL-4R, which is within the I4R motif, prevents IRS-1 tyrosine phosphorylation in response to huIL-4. In most, but not all, cell lines expressing Y497F, [^{3}H]thymidine uptake in response to huIL-4 is abrogated. Thus, Y497 of the huIL-4R, like Y960 of the human insulin receptor, is important in receptor-mediated signal transduction. This strongly suggests that the I4R motif plays a major role in ligand-stimulated signaling. Whether F497 can mediate some of the functions of Y497, whether other sites of the receptor replace Y497 in a comparable function, or whether an entirely different growth pathway is used by these cell lines remains to be established. Further, the weak tyrosine phosphorylation of IRS-1 observed in a cell line expressing the amino acid 455 deletion mutant indicates that the I4R motif, although important, may not be essential for such phosphorylation. The ability to detect IRS-1 phosphorylation may be explained by overexpression of signaling substrates or by other sequences in the IL-4R or in the γ_c that are capable of partially replacing the functions of the I4R motif. Indeed, overexpression of IRS-1 in cells expressing the Y960F mutation of the human insulin receptor restores the ability of the mutant receptor to transmit biological responses to insulin (White and Kahn, 1994).

Based on the results presented in this study and elsewhere (Wang et al., 1992, 1993a; Sun et al., 1992; Backer et al., 1992, 1993; Skolnik et al., 1993; Russell et al., 1993), we propose that IL-4-mediated signaling normally proceeds through phosphorylation of the IL-4R at position 497 by kinases that are associated with the receptor (Figure 8). Indeed, it has already been shown that the receptor becomes tyrosine phosphorylated as a result of ligand engagement, although the phosphorylation of Y497 has not been directly demonstrated. We envision that phosphorylation of Y497 would create a site to which 4PS or IRS-1

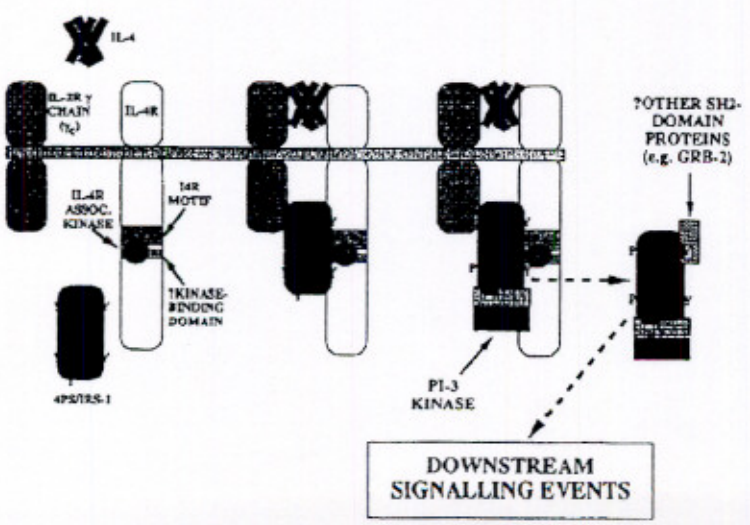

Figure 8. Scheme for Signal Transduction by the IL-4R

The binding of IL-4 to its receptor may induce heterodimerization between the IL-4R and the γ_c (Russell et al., 1993), which would cause an activation of a receptor-associated tyrosine kinase, resulting in the tyrosine phosphorylation of the I4R motif of the receptor. This would allow the association of IRS-1/4PS with the receptor complex and its subsequent phosphorylation. The phosphorylation of IRS-1/4PS creates a high-affinity binding site for the SH2 domain of p85 subunit of PI-3K, leading to the induction of its enzymatic activity. It is possible that, as has been shown in the case of the insulin receptor system for IRS-1 (Skolnik et al., 1993), IL-4-activated IRS-1/4PS would also interact with the SH2 domain of GRB-2 or other as yet unidentified signaling molecules.

can dock directly, or it could possibly dock through an adaptor molecule. The substrate would now be accessible to the action of receptor-associated kinases, leading to its tyrosine phosphorylation and dissociation from the receptor complex. Phosphorylated IRS-1/4PS would then interact with SH2 domain–containing proteins, such as the regulatory subunit of PI-3K (p85), potentially GRB-2, as well as other presently unidentified molecules, to initiate the IL-4-induced signaling pathway. Whether these early signaling events are directly linked to classic IL-4-induced differentiative effects, such as immunoglobulin class switching, remains to be determined.

Experimental Procedures

Cells and Reagents

The IL-3-dependent cell lines 32D, FDC-P1, and FDC-P2 were maintained in RPMI supplemented with 2 mM glutamine, 100 μg/ml penicillin–streptomycin, 5×10^{-5} M 2-mercaptoethanol, 10% FCS, and 5% WEHI-3B conditioned medium. FDC-P1 and 32D cells expressing rat IRS-1 were prepared as described (Wang et al., 1993a, 1993b) by electroporation of *IRS-1* cDNA and selection in histididinol. Recombinant muIL-4 expressed in the baculovirus system was affinity purified. An engineered form of recombinant huIL-4 that lacks N-linked glycosylation sites was expressed in the yeast system as described for muIL-4 (Mosley et al., 1989). Rabbit antisera specific for the carboxy-terminal region of the muIL-4R was prepared by immunizing rabbits with the peptide QTTEVPVGALGIAVS coupled to keyhole limpet hemocyanin. Monoclonal antibodies specific for the huIL-4R were generous gifts of the Immunex Corporation.

Expression Vectors and Transfections

The cDNA encoding the huIL-4R was cloned into the mammalian expression vector pDC302 and was obtained from the Immunex Corporation. This vector is a derivative of pDC201 described in Sims et al. (1988). Stop codons were introduced in the *IL-4R* cDNA by site-directed mutagenesis. These mutations resulted in the production of receptors with truncations in the cytoplasmic domain, starting from amino acids 657, 557, and 437 (amino acid numbering begins at the methionine start). One of the mutagenesis protocols resulted in a base deletion and a subsequent frameshift. This gave rise to a mutant receptor with a sequence different from the wt sequence between amino acids 351–365 as shown in parentheses, KTV(PGQRAS)EAPV.

The Y497F mutation was accomplished using recombinant PCR techniques as previously described (Higuchi, 1990). The huIL-4R cDNA from the DraIII site at nucleotide 834 to the stop codon was resynthesized in two sections. Initially, a sense oligonucleotide encompassing the nucleotides 823–838 (4RDRA5') and an antisense oligonucleotide (YMUT3') encompassing nucleotides 1655–1675 and containing a T→A mutation at nucleotide 1665 were used to generate an 852 bp fragment with a mutation of codon 497 from TAC (tyrosine) to TTC (phenylalanine). Similarly, sense oligonucleotide YMUT5', complementary to YMUT3', and antisense oligonucleotide 4RBGL3', encompassing nucleotides 2638–2657 (which included BglII and HindIII sites following the stop codon to facilitate cloning), were used to generate a 1002 bp PCR fragment that also contained the Y497F mutation. PCR reactions were performed with thermostable Pfu polymerase (Strategene), which has a proofreading exonuclease activity to limit PCR-generated mutations. The 852 and 1002 bp PCR fragments were gel purified, combined, and amplified in a third PCR reaction in which the 4RDRA5' and 4RBGL3' oligonucleotides were used as primers. The resulting 1.85 kb fragment of the huIL-4R (nucleotides 823–2657), which contained a mutated codon 497, was digested with DraIII and HindIII and gel purified. To facilitate cloning, the full huIL-4R cDNA was subcloned into the Asp718 (5') and BamHI (3') of pUC19, which lacks DraIII sites, and the resulting plasmid, pUC4R, was digested with DraIII and HindIII. A 3.5 kb fragment containing pUC19 sequences and huIL-4R sequences from nucleotide 1 (Asp718) to nucleotide 834 (DraIII) was isolated and ligated to the DraIII–HindIII-digested 1.82 kb PCR fragment. Resulting clones were sequenced to verify that the mutation of codon 497 was incorporated and that no PCR-induced mutations were present. The full-length mutated receptor cDNA was cut out with Asp718 and BglII and cloned into the pDC302 expression vector as described above for use in transfections.

DNA transfection was performed by electroporation using 10 μg of IL-4R-bearing plasmid and 1 μg of the LTR-2 plasmid (DiFiore et al., 1987), bearing the marker gene *ECOgpt*. Mass populations of stably transfected cells were selected by their ability to survive in growth medium containing HAT and mycophenolic acid (80 μM). Cells that survived the selection process were tested for expression of the huIL-4R by FACS analysis using a cocktail of two anti-huIL-4R monoclonal antibodies, M8 and M10, followed by a phycoerythrin-conjugated goat anti-rat immunoglobulin G. Rabbit immunoglobulin G was used to block Fc binding. Positive lines were expanded and grown in the presence of IL-3.

Production of the GST–IL-4R Fusion Protein

A fusion protein containing the IL-4R cytoplasmic region spanning amino acids 424–561 (P3) was generated using a GST gene fusion system (Pharmacia LKB Biotechnology). Oligonucleotides containing appropriate 5' restriction sites and complementary receptor sequences were synthesized and used in PCRs to amplify the 424–561 amino acid region of the IL-4R cytoplasmic tail. Amplified products were cut with BamHI and EcoRI, purified, and cloned into the pGEX2T vector. After analysis, vectors were transformed into Escherichia coli DH5α, and fusion proteins were purified from large-scale cultures essentially as described by the manufacturer using glutathione–Sepharose (Pharmacia). Preparations of the P3 fusion protein or GST protein were eluted from glutathione–Sepharose using 5 mM reduced glutathione (Calbiochem) in 50 mM Tris (pH 8.0), dialyzed against 200 vol of PBS containing 0.5 mM PMSF for 12 hr at 4°C, and coupled to Affigel-10 beads (Bio-Rad) according to the protocol of the manufacturer.

Surface Expression of Receptor

The level of expression of transfected huIL-4Rs was determined by saturation binding analysis using ^{125}I-labeled huIL-4. Recombinant huIL-4 was labeled with Na^{125}I (Amersham) using Enzymobeads (Bio-Rad) exactly as described (Idzerda et al, 1990). The transfectants were incubated at 5×10^6/ml in HBSS, 2% FCS, and 20 mM HEPES with 50 ng/ml of ^{125}I-huIL-4 for 1 hr at 4°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 either including a 50-fold molar excess of unlabeled huIL-4 or binding of radiolabeled huIL-4 to untransfected 32D–IRS-1 cells; the levels of nonspecific binding detected by these two methods were similar. The number of huIL-4 molecules bound per cell was calculated using 15000 daltons as the molecular weight of huIL-4. The reported receptor values represent the average of the calculated receptor numbers from four separate experiments.

Cellular Proliferation Assays

For the study of cellular proliferation, 32D–IRS-1 transfectants were incubated at 25000 cells/well in a final volume of 0.2 ml of complete RPMI in the presence or absence of various doses of huIL-4 or muIL-4 for 36 hr at 37°C. The wells were pulsed with 1 μCi/well [^{3}H]thymidine for the final 16 hr of culture before harvesting using a Skatron cell harvester followed by liquid scintillation counting.

Immunoprecipitation and Immunoblotting

Analysis of phosphotyrosine-containing proteins was performed essentially as previously described (Wang et al., 1992). Cells were preincubated in RPMI without serum or IL-3 for 2 hr at 37°C. After washing, the cells were resuspended in RPMI with 50 μM Na_3VO_4 and incubated in the presence or absence of muIL-4 (2 ng/ml) or huIL-4 (20 ng/ml) for 10 min at room temperature. The reaction was terminated by 10-fold dilution in ice-cold PBS with 100 μM Na_3VO_4. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5% NP-40, 1 mM Na_3VO_4, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, protease inhibitor cocktail) and clarified. The soluble fraction (~2 mg) was immunoprecipitated with a monoclonal anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology), control rabbit serum, or anti-IL-4R antisera (1:500), and the complexes were recovered with protein G (Pharmacia), or the lysate was precipitated with GST–Affi or P3–

Affi. The precipitates were washed three times in lysis buffer and solubilized in SDS sample buffer. SDS–PAGE analysis (7.5% acrylamide), Western blot transfer, and immunoblotting with 4G10 were performed as described (Wang et al., 1992). Where indicated, 4G10 binding was detected with ^{125}I–protein A (Amersham) or the enhanced chemiluminescence system (ECL, Amersham).

Immune Complex Kinase Reaction

Cell lysates were prepared and precipitations performed as described above. Before the kinase reactions, the precipitates were washed two times in kinase buffer (50 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 0.01% Brij 35, 0.1 mg/ml BSA, 10 mM DTT [pH 7.2]) and resuspended in 20 μl of kinase buffer. The kinase reaction was initiated by adding 20 μl of kinase buffer containing 100 μM ATP and 48 mM $MgCl_2$. The reactions were performed at 25°C for 20 min. Reactions were terminated by adding 15 μl of kinase buffer containing 30 mM EDTA and 300 mM ATP. The precipitates were washed as indicated above before SDS–PAGE analysis. The samples were subsequently transferred to nitrocellulose, and the blots were probed with anti-phosphotyrosine as described above.

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