A Role for the Insulin-Interleukin (IL)-4 Receptor Motif of the IL-4 Receptor α-Chain in Regulating Activation of the Insulin Receptor Substrate 2 and Signal Transducer and Activator of Transcription 6 Pathways

ANALYSIS BY MUTAGENESIS*

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The interleukin (IL)-4 receptor α-chain (IL-4Rα) contains a sequence motif (488PLVIAGNYPAYRSFSD) termed the insulin IL-4 receptor motif (I4R motif). Mutation of the central Tyr497 to Phe blocks the tyrosine phosphorylation of the insulin receptor substrate 1 (IRS1) and diminishes proliferation in response to IL-4. Recent data suggest that the I4R motif encodes binding sites for several protein tyrosine binding (PTB) domain-containing proteins such as IRS1 and Shc and potentially for the Src homology 2 domain of signal transducer and activator of transcription 6 (STAT6). To analyze the function of the I4R motif in regulating IL-4 signaling, we changed conserved residues upstream and downstream of the central Tyr to Ala in the human IL-4Rα. We analyzed the ability of these constructs to signal the tyrosine phosphorylation of IRS2 and STAT6, the induction of DNA binding activity, and CD23 induction in response to human IL-4 (huIL-4) in transfected M12.4.1 cells. Mutagenesis of residues downstream of Tyr497, such as Arg489 or Phe500, to Ala had no effect on any of these responses, suggesting that the I4R motif may not be important for functional Src homology 2 domain interactions. However, mutagenesis of Pro488 to Ala (P488A) greatly diminished the tyrosine phosphorylation of IRS2 and abolished tyrosine phosphorylation of STAT6, induction of DNA binding activity, and CD23 induction in response to huIL-4. By contrast, a P488G mutant signaled these responses to huIL-4. Mutagenesis of hydrophobic amino acids previously shown to contact the PTB domain of IRS1, Leu489 or Ile491, to Ala had only minimal effects on responses to huIL-4. However, changing both Leu489 and Ile491 to Ala greatly diminished the tyrosine phosphorylation of IRS2 and abolished STAT6 activation. Taken together, these results indicate the important role of the I4R motif in regulating IRS docking and suggest that I4R docking to a PTB domain-containing protein regulates activation of the STAT6 pathway.

Interleukin (IL)-41 evokes a wide variety of biological responses by binding to a high affinity receptor complex (1). In murine lymphoid cells, the receptor complex predominantly consists of a 140-kDa, high affinity binding chain (IL-4Rα) and the common γ-chain (γc) (2) that is also a component of the receptors for IL-2, IL-7, IL-9, and IL-15 (3). Both chains of the IL-4 receptor complex are members of the hematopoietin receptor superfamily (4). These receptor subunits do not contain any consensus sequences encoding tyrosine or serine/threonine kinases. However, it has been shown that the IL-4Rα associates with the Janus family kinase JAK-1 (5) and the γc associates with JAK-3 (6, 7). Binding of IL-4 to its receptor results in the tyrosine phosphorylation of several molecules, including JAK1, JAK3, and the IL-4Rα (2).

IL-4 treatment also results in the tyrosine phosphorylation of the insulin receptor substrate-1 (IRS1) and IRS2 (8–10). IRS1 and IRS2 are large cytoplasmic proteins (170–180 kDa) that contain numerous potential tyrosine and serine/threonine phosphorylation sites. Tyrosine-phosphorylated sites within IRS1 and IRS2 associate with SH2 domains found in cytoplasmic signaling molecules, including the p85 subunit of phosphatidylinositol 3-kinase and the growth factor receptor-bound protein 2. IRS1 and IRS2 have been shown to regulate both the proliferation and the protection from apoptosis of a factor-dependent myeloid cell line 32D in response to IL-4 (10–12).

Deletional studies of the IL-4Rα have led to the identification of a sequence motif (488PLXNYRFSFSD) termed the insulin IL-4 receptor motif (I4R motif) (13). Mutation of the central Tyr497 to Phe blocked the tyrosine phosphorylation of IRS1 and diminished proliferation in response to IL-4 in 32D cells. It has been shown that IRS1 contains a protein tyrosine binding (PTB) domain, also called a phosphotyrosine interaction domain (14–16), which is important for the interaction of the I4R motif with IRS1. Several independent studies have demonstrated that the I4R motif encodes a binding site for the PTB domain of IRS1/2 and SHc (17, 18). More recently, solution structure analyses of the binding of a phosphopeptide derived from the I4R motif of the huIL-4Rα with the PTB domain derived from IRS1 (19) have indicated that amino acid residues at the −8 and −6-positions relative to Tyr497 in the I4R motif (Leu495 and Ile491) make contacts with residues in the PTB domain of IRS1.

In addition to IRS family members, IL-4 also induces the α-chain; JAK, Janus family kinase; STAT, signal transducer and activator of transcription; IRS, insulin receptor substrate; I4R motif, insulin IL-4 receptor motif; PTB, protein tyrosine binding; γc, γ-chain; huIL-4, human IL-4; huIL-4Rα, human IL-4Rα; mIL-4, murine IL-4; SH2, Src homology 2; FACS, fluorescence-activated cell sorting; WT, wild type.

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1 The abbreviations used are: IL, interleukin; IL-4Rα, IL-4 receptor α-chain; JAK, Janus family kinase; STAT, signal transducer and activator of transcription; IRS, insulin receptor substrate; I4R motif, insulin IL-4 receptor motif; PTB, protein tyrosine binding; γc, γ-chain; huIL-4, human IL-4; huIL-4Rα, human IL-4Rα; mIL-4, murine IL-4; SH2, Src homology 2; FACS, fluorescence-activated cell sorting; WT, wild type.
tyrosine phosphorylation of a member of the signal transducer and activator of transcription (STAT) family, STAT6 (20–23). The general model is that after tyrosine phosphorylation, STAT6 dimerizes in the cytoplasm, translocates to the nucleus, and subsequently binds to consensus sequences (termed γ-activated sequences) found within the promoter regions of IL-4-inducible genes. Recent studies of mice with a targeted disruption of the STAT6 gene clearly demonstrate that STAT6 is necessary for the induction of genes (CD23, major histocompatibility complexes II and Iε, and IL-4Rα genes) in response to IL-4 (24–26). Several lines of evidence suggest that the IRS and STAT6 pathways are separate at the initiation phase of the signal. In 32D cells lacking IRS expression, IL-4 treatment was able to activate the DNA binding activity of STAT6 as well as in cells expressing IRS1 (27). On the other hand, IL-4 was able to stimulate the tyrosine phosphorylation of IRS2 in lymphocytes derived from mice deficient in STAT6 expression as well as in lymphocytes derived from normal mice (28).

A series of deletion, mutagenesis, and chimeric receptor studies of the huIL-4Rα (13, 29, 30) demonstrated that a region containing three tyrosine residues with a consensus sequence of Gly(Y/K)QxF 90 amino acids downstream of the I4R motif was necessary for maximal IL-4-induced activation of STAT6 DNA binding activity and CD23 induction in M12.4.1. They also indicated that STAT6 activation, in the absence of IRS activation, was sufficient to signal maximal CD23 induction in these cells. Furthermore, these same studies also suggested that Tyr497 in the I4R motif could signal partial STAT6 activation, implying that any one of the first four cytoplasmic tyrosine residues could potentially act as a docking site for the SH2 domain of STAT6. Interestingly, mutation of Tyr497 to Phe, known to consistently affect activation of the IRS pathway in response to IL-4 (13, 29), also affected activation of the STAT6 DNA binding activity in approximately half of expressing clones, while the others responded normally (27, 31).

To analyze the function of the I4R motif in regulating IL-4 signaling in detail, we changed conserved residues upstream and downstream of the central Tyr to Ala. We analyzed the ability of these constructs to signal the tyrosine phosphorylation of IRS2 and STAT6, the induction of DNA binding activity, and CD23 induction in response to huIL-4 in transfected M12.4.1 cells. The results presented herein indicate the important role of the I4R motif in regulating IRS recruitment and suggest that I4R docking to a PTB domain-containing protein(s) regulates activation of the STAT6 pathway.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents—**The murine B cell lymphoma M12.4.1 (obtained from Dr. Richard Assaf, NIAID, National Institutes of Health) was maintained in RPMI (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10−7 M 2-mercaptoethanol. Recombinant huIL-4 and recombinant huIL-4 were obtained from R & D Systems (Minneapolis, MN).

**Mutagenesis and Transfection—**For the mutagenesis, we cloned the huIL-4Rα cDNA (provided by Dr. Melanie Spriggs, Immunex, Seattle, WA) into the pAlter vector obtained from Promega (Madison, WI). Amino acid numbering begins with +1 as the initiator methionine of the signal peptide of the huIL-4Rα cDNA. There are 25 amino acids in the signal peptide. Oligonucleotide-directed mutagenesis was performed according to the manufacturer’s protocol using mutant oligonucleotides that would convert the wild type codons to A codon (or as indicated a G). For example, the oligonucleotide used for the L489A mutation was designed as follows: 5′-AACA GAG ACG CCC GCC GTC ATC GCA GGC-3′ (L489A). We prepared single-stranded DNA containing the huIL-4Rα using helper phage R408. The mutant oligonucleotides were annealed with aliquots of the single-stranded DNA along with an oligonucleotide that repairs the mutation in the ampicillin resistance gene. Subsequently, synthesis and ligation were performed to link them. The DNA was transformed into a repair minus strain of *E. coli* (BMH 71–18 mutS), and the cells were grown in the presence of ampicillin. A second round of transformation in JM109 and selection for ampicillin resistance was performed. Bacterial colonies containing the desired mutation were identified by sequence analysis of plasmid DNA. Mutant huIL-4Rs was then cloned into the EcoRI site of pME18s. M12.4.1 cells were washed in phosphate-buffered saline. For each transfection, 2 × 105 cells were mixed with 2 μg of vector carrying neomycin resistance and 20 μg of huIL-4Rα-PME18s and subjected to electroporation using a Bio-Rad gene pulser set on 200 V and 960 microfarads. After transfection, the cells were cultured overnight before selection with 800 μg/ml neomycin (Life Technologies, Gaithersburg, MD) for 6–8 days. Neomycin-resistant cells were tested for the expression of huIL-4Rα by FACS analysis using monoclonal antibodies, M8 and M10 (a generous gift from Dr. Melanie Spriggs, Immunex, Seattle, WA) as described previously (13).

**Immunoprecipitation and Immunoblotting—**Analysis of phosphotyrosine-containing proteins was performed as described previously (32). Briefly, cells were deprived of serum in RPMI for 2 h at 37 °C. After washing, 105 cells were resuspended in RPMI with 50 μM Na3VO4 and incubated in the presence or absence of murine or human IL-4 (10 ng/ml) for 10 min at room temperature. The reaction was terminated by 10-fold dilution in ice-cold phosphate-buffered saline containing 100 μM Na3VO4. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 50 mM NaCl, 0.5% Nonidet P-40, 1 mM Na3VO4, 10 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) and clarified. The soluble fraction was immunoprecipitated with a polyclonal rabbit anti-IRS (a generous gift of Drs. Ling-Mei Wang and Jaelyn Pierce, NCI, National Institutes of Health), anti-STAT6 (Santa Cruz, CA), or anti-JAK3 (Upstate Biotechnology, Inc., Lake Placid, NY). The precipitates were washed in lysis buffer and solubilized in SDS sample buffer. The samples were separated on 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride membrane. The membranes were then probed with a monoclonal anti-phosphotyrosine antibody, RC20-H (Transduction Laboratories, Lexington, KY). The bond antibody was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech). Where indicated, the blots were stripped and reprobed with control antibodies. Band intensities were analyzed using the public domain software NIH Image.

**Electrophoretic Mobility Shift Assay—**M12.4.1 cells expressing huIL-4Rα constructs were incubated with media, 10 ng/ml huIL-4, or 10 ng/ml huIL-4 as indicated for 30 min and washed with phosphate-buffered saline. Total cell extracts were prepared exactly as described (29) and stored at −70 until use. Extracts (4 μg) were incubated with 1 ng of labeled double-stranded oligonucleotide (5 × 105 cpm) corresponding to the N4 γ-activated sequence element found in the promoter of the Ce gene (5′-CAACCTTCCCCAAGAACAGCA) at room temperature for 20 min as described previously (29). Where indicated, unlabeled Ce probe, anti-STAT3, or anti-STAT6 (1 μg, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were included in the binding reaction. Protein-DNA samples were analyzed by electrophoresis on 4.5% polyacrylamide gels in 0.22× TBE followed by autoradiography.

**CD23 Induction Assay—**M12.4.1 cells expressing the various constructs of the huIL-4Rs were incubated at 1 × 105/ml in media in the presence or absence of murine or human IL-4 (10 ng/ml) for 48 h at 37 °C. Expression of murine CD23 was tested by FACS analysis using fluorescein isothiocyanate-B3B4 (anti-murine CD23), a generous gift of Dr. Daniel H. Conrad (MVC, Richmond, VA), in the presence of the anti-Fe receptor antibody 2.4D to block Fe binding before analysis on a FACS (Becton-Dickinson).

**Binding and Cross-linking of 125I-huIL-4—**For binding and cross-linking studies, 125I-huIL-4 was purchased from Amersham Pharmacia Biotech. Saturation binding analyses were done using 25 ng/ml 125I-huIL-4 essentially as described previously (13). 125I-huIL-4 was cross-linked to the IL-4 receptor complex using 5 mM bis(sulfosuccinimidyl) suberate (Pierce) as described previously (32).

**RESULTS**

Previous studies have shown that the central tyrosine in the I4R motif (Tyr497) of the huIL-4Rα is critical for the recruitment of IRS1 to the IL-4 receptor complex and the activation of the IRS pathway. These studies also suggested that Tyr497 has some capacity to recruit STAT6 and to influence the activation of the STAT6 pathway. Residues downstream of phosphotyrosines participate in the binding to SH2 domains, especially the +1 and +3 residues (33). It has recently become clear that residues upstream of phosphotyrosines participate in the bind-
I4R Motif Regulates IRS2 and STAT6 Activation

We first examined the ability of huIL-4 to activate the STAT6 pathway and CD23 induction in M12.4.1 cells expressing the huIL-4R mutants (Figs. 3 and 4). Treatment of cells expressing WT huIL-4R with human or mouse IL-4 induced a DNA binding activity specific for the Ce probe, indicating the activation of STAT6 (Fig. 3A). The IL-4-induced complex can often be resolved into two bands,2 where at other times the two bands are not distinguished. This DNA-binding complex was supershifted by anti-STAT6 antibody but not by anti-STAT3. Interestingly, when we analyzed the cells expressing the mutant constructs, we found that the pattern of responsiveness to huIL-4 was similar to the pattern we observed for IRS2 tyrosine phosphorylation (Fig. 3B); i.e. cells expressing the P488A mutant or the L-I-A mutant did not demonstrate STAT6 DNA binding activity in response to huIL-4, while they clearly responded to muIL-4. Cells expressing the P488G, L489A, I491A, and F500A mutants responded to huIL-4 by inducing STAT6 DNA binding activity. In keeping with these results, the pattern of CD23 induction in response to huIL-4 also corresponded to the pattern we observed for STAT6 DNA binding activity (Fig. 4). Treatment of cell lines expressing WT, P488G, L489A, I491A, and R498A mutants with huIL-4 resulted in CD23 induction to levels seen in cells treated with the muIL-4 control. However, treatment of cells expressing P488A or L-I-A with huIL-4 did not result in the induction of CD23, while treatment with muIL-4 was able to induce its expression.

These results indicate that residues within the I4R motif that are likely to be important for the interaction with PTB domains also participate in the regulation of the STAT6/gene induction pathway. It is possible that the I4R motif serves to regulate the activation of an additional signaling pathway, such as a serine kinase (34, 35), which would further modulate STAT6 DNA binding activity. On the other hand, the I4R motif could regulate the tyrosine phosphorylation of STAT6 itself. Therefore, we examined the ability of huIL-4 to induce the tyrosine phosphorylation of STAT6 (Figs. 5 and 6). Again, the pattern of responsiveness to huIL-4 corresponded to the pattern we observed for STAT6 DNA binding activity. Treatment of cell lines expressing WT, P488G, L489A, I491A, R498A, and F500A with huIL-4 resulted in CD23 induction to levels seen in cells treated with the muIL-4 control. However, treatment of cells expressing P488A or L-I-A with huIL-4 did not result in the induction of CD23 tyrosine phosphorylation, while treatment with muIL-4 was able to do so. To determine whether this difference was due to a change in the kinetics of STAT6 activation, we performed the analysis at various time points ranging from 1 to 60 min (Fig. 6). We observed that the induction of STAT6 tyrosine phosphorylation in response to huIL-4 was quite rapid in cells expressing WT, P488G, L489A, or R489A. Phosphorylation was easily detected within 1 min and reached plateau levels within 5 min, staying

2 H. Wang, J. Zamorano, and A. D. Keegan, unpublished observations.
elevated up to 60 min. Cells expressing P488A or L1-A mutant did not show induction of STAT6 tyrosine phosphorylation in response to huIL-4 at any of the time points. Consistent with previous studies with Y497F mutants (13, 27, 29, 31), these results demonstrate that mutations of the I4R motif of the huIL-4RA that affect IRS2 phosphorylation also affect STAT6 phosphorylation, DNA binding activity, and gene induction and suggest that PTB domain-containing proteins participate in the regulation of the STAT6 pathway.

To rule out the possibility that the P488A and L1-A mutations somehow disrupt dimerization with γc or JAK activation and therefore preclude any signaling whatsoever, we performed several control experiments (Fig. 7). We tested the ability of 125I-huIL-4 to bind and become cross-linked to the
characteristic components of the IL-4 receptor complex on cells expressing WT, P488A, and L,I-A constructs (Fig. 7A). All three cell lines showed cross-linking of 125I-huIL-4 to characteristic proteins of 140 and 70 kDa, representing the huIL-4Rα and γc, respectively (36), suggesting that these mutants are able to bind huIL-4 and induce dimerization of huIL-4Rα with the endogenous murine γc. In addition, cells expressing WT, P488A, and L,I-A constructs responded to huIL-4 with the tyrosine phosphorylation of JAK3. As has been observed in other murine B cell lines (37), we did not detect JAK1 phosphorylation in response to either murine or human IL-4 in these cells (data not shown). These results indicate that the P488A and L,I-A mutants are able to bind IL-4, dimerize with the γc, and signal the activation of the tyrosine kinase, JAK3.

**DISCUSSION**

Within the last few years, studies on the structure and signaling capacity of the IL-4 receptor have elucidated mechanisms by which IL-4 regulates its panoply of biological responses. However, a precise picture of how the IL-4 receptor recruits and activates its spectrum of signaling molecules that ultimately leads to a specific biological outcome has not been fully developed.

Previous studies defined distinct regions of the IL-4Rα that regulate growth and gene expression by using a series of deletion and point mutations of the huIL-4Rα and a chimeric receptor approach (2, 13, 27–30). Transfection of truncation mutants of the human IL-4Rα into 32D/IRS1 demonstrated that...
the region between amino acids 437 and 557 is important for human IL-4-induced IRS1 phosphorylation and growth in these cells (13). This interval contains the I4R motif. Mutation of the central tyrosine residue of the I4R motif to a phenylalanine in the huIL-4Rα impairs its ability to signal IRS1 phosphorylation and to induce a proliferative response upon treatment with IL-4. In addition, transfer of the region of the huIL-4Rα containing the I4R motif to a growth-impaired truncation mutant of IL-2Rα conveyed both IRS1 phosphorylation and sustained proliferation in response to IL-2 (30).

In addition to growth, the ability of the huIL-4Rα constructs to initiate gene expression and STAT-6 activation has been analyzed (27, 29). A construct terminating at amino acid 657 was fully capable of stimulating gene expression and STAT-6 DNA binding activity. However, a construct terminating at amino acid 557, one fully competent to signal proliferation, was fully capable of stimulating gene expression and STAT-6 DNA binding activity (27, 31). These results indicated that the I4R motif may participate in the regulation of STAT6. The huIL-4Rα construct terminating at 557, although greatly impaired at inducing STAT6 DNA binding activity, was still able to support modest STAT6 and CD23 induction, suggesting the hypothesis that Tyr497 might recruit STAT6 (27, 29). In addition, several cell lines expressing the Y497F mutant did not support the induction of IRS phosphorylation or the induction of STAT6 DNA binding activity (27, 31). These results lead us to analyze the contribution of the I4R motif to IL-4 signaling by using a mutagenesis approach.

Mutagenesis of residues downstream of Tyr497, such as Arg498 or Phe500 to Ala (in addition, Ser501, Ser502, and Asn502; data not shown) had no effect on any of the biochemical or biological responses analyzed. Residues at the +1 and +3-positions relative to the phosphorysine have been shown to be important for SH2 domain interactions in general (33), and residues in the +1 and +5-positions of the STAT1 docking site of the IFN receptor are critical for binding to the SH2 domain of STAT1 (38). The sequences surrounding the four cytoplasmic tyrosines in the huIL-4Rα, which have been shown to interact with (Ref. 22; GKAFSS, and GYKFQFD) or hypothesized to interact with STAT6 (Refs. 27 and 32; ARSFNSN and GYQEFVH) suggest that the +1 and +3 residues would be important, since these are fairly well conserved among these sites. These results presented herein suggest that the I4R motif may not play a critical role in activating important SH2 domain interactions with STAT6 or some unknown protein(s).

Several studies have not been able to demonstrate a direct interaction of STAT6 with the I4R motif. Hou et al. (22) showed that a phosphopeptide derived from the I4R motif could not block STAT6 dimer formation, while phosphopeptides derived from the downstream residues (Tyr603 and Tyr631) were able to do so (22). Interaction studies in the yeast two-hybrid system have also been unable to demonstrate a direct association between the I4R motif and STAT6 (27). Furthermore, we were unable to precipitate STAT6 with a phosphorylated I4R motif peptide coupled to agarose beads. Together these results suggest that the I4R motif is not a direct docking site for STAT6 and that its influence on STAT6 activation may be via an indirect mechanism (Fig. 8).

Interestingly, mutagenesis of Pro488 to Ala greatly diminished the tyrosine phosphorylation of IRS2 and STAT6 and abolished induction of CD23 and DNA binding activity in response to huIL-4. By contrast, a P488G mutant was competent to signal these responses to huIL-4. The observation that the Pro488 to Ala change was detrimental to function and that the Pro to Gly was not suggests that Pro488 regulates the availability of the I4R motif to PB domain-containing proteins. Mu
tagenesis of both Leu489 and Ile491 to Ala also greatly diminished the tyrosine phosphorylation of IRS2 and STAT6 and abolished induction of CD23 and DNA binding activity in response to huIL-4. Mutation of one of these residues to Ala was not enough to affect signaling function, indicating that potential interactions from either residue is sufficient to recruit and phosphorylate IRS2.

These findings are consistent with, but not identical to, studies on the interaction of isolated phosphopeptides derived from

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3. K. Nelms, personal communication.
4. A. D. Keegan, unpublished observation.
the I4R motif with the PTB domain of IRS1 (17). Wolf et al. (17) found that a phosphopeptide derived from the I4R motif of the IL-4Ra (LVIAGNPAPpYRS; where pY represents phosphotyrosine) would inhibit the binding of an iodinated phosphopeptide derived from the I4R motif of the insulin receptor (LYAASSNPpYLSASDV) to the PTB domain of IRS1 with an IC50 of 6.2 μM. This inhibition was dependent on the phosphotyrosine. Changing Arg226 or Ser249 to Ala had little effect on the IC50, phosphopeptide LVIAGNPApYR inserted in an extended complete IRS2 molecule to a complete huIL-4R in vitro measurements translate to the interaction of a composite PTB domain-containing protein. The PTB domain of IRS1 has been shown to interact with the PTB domain of IRS or other PTB domain-containing proteins. We found that a Gly residue is tolerated at this position (position −9 relative to the Tyr) in the huIL-4Ra protein. We found that a Gly residue is tolerated at this position (position −9 relative to the Tyr) in the huIL-4Ra protein. Interestingly, a Pro at the −9 position is conserved among the I4R motifs derived from the IL-4 and insulin receptors (13). In the insulin-like growth factor-I receptors, it is replaced with Val or Thr; however, a Gly is conserved at the −10 position. It will be of interest to determine whether the Gly at −10 or Pro at −9 in the insulin and insulin-like growth factor-I receptors is important for PTB domain docking.

An intriguing observation is that changing amino acids in the I4R motif affects not only the IRS pathway but also the STAT6 pathway. Similar results were variably observed for cells expressing the Y497F mutant (27, 31). One possible explanation for this finding is that by changing residues in the I4R motif we have disrupted the overall receptor structure and function. Based on the 125I-huIL-4 cross-linking data and the induction of JAK3 tyrosine phosphorylation (Fig. 7) the mutant receptors P488A and L,I-A are at least partially functional. Although we and others have demonstrated the importance of JAK1 in IL-4 signaling in human fibroblasts (28, 39), activation of JAK1 by IL-4 in murine cells of B lineage is not readily detected and could not be used as a criterion for receptor activation in these cells (37).

Another possibility is that the changes disrupt important protein structure in the STAT6 docking site −90 amino acids downstream of the I4R motif (Fig. 8, model II). Although we cannot formally rule this out without structural data on the IL-4Ra cytoplasmic domain, we do not favor this explanation. Only those mutations that affected IRS2 phosphorylation also affected STAT6 phosphorylation, DNA binding activity, and gene induction. These changes include the Y497F, P488A, and L,I-A mutations. No other changes significantly affected either IRS or STAT6 (including P488G, L489A, L489R, I491A, I491R, P495A, R498A, S499A, F500A, and S501A; this report and data not shown). The greater number of mutations of the I4R motif that had no effect on receptor signaling seems to make a structural effect on the downstream STAT6 domain less likely. Rather, we favor a third possibility, that the I4R motif recruits some PTB domain-containing protein(s) that participates in the recruitment of STAT6 to the receptor complex and/or tyrosine phosphorylation of STAT6 (Fig. 8, model I). In this regard, we have previously shown that the region of the receptor containing the I4R motif precipitates tyrosine kinase activity (13).

If model I proposed in Fig. 8 is correct, we should be able to identify a PTB domain-containing signaling molecule whose
expression is required for the IL-4-induced STAT6 tyrosine phosphorylation in murine B cells expressing physiologic levels of receptors. Previous studies in 32D cells demonstrated that IRS expression was not essential for STAT6 activation by IL-4, seemingly ruling out IRS1 and IRS2 as potential candidates (27). However, the specific role of IRS2 in regulating STAT6 in B cells has not been examined. There are a number of other proteins characterized to date with PTB domains including IRS3, She, FRIP-1, p62DOK, X11, and FE65 (15, 16, 40–42), the latter two bind an NPXY sequence in amyloid precursor protein without a strict requirement for a phosphoryrosine residue in their docking site. It remains to be determined whether one of these proteins or a novel protein will be important for the IL-4-induced activation of STAT6.

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