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# THE IL-4 RECEPTOR: Signaling Mechanisms and Biologic Functions

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ABSTRACT

Interleukin-4 is a multifunctional cytokine that plays a critical role in the regulation of immune responses. Its effects depend upon binding to and signaling through a receptor complex consisting of the IL-4R$\alpha$ chain and the common gamma chain ($\gamma$c), resulting in a series of phosphorylation events mediated by receptor-associated kinases. In turn, these cause the recruitment of mediators of cell growth, of resistance to apoptosis, and of gene activation and differentiation. Here we describe our current understanding of the organization of the IL-4 receptor, of the signaling pathways that are induced as a result of receptor occupancy, and of the various mechanisms through which receptor function is modulated. We particularly emphasize the modular nature of the receptor and the specialization of different receptor regions for distinct functions, most notably the independent regulation of cell growth and gene activation.

## INTRODUCTION

Interleukin-4 is a pleiotropic type I cytokine produced by a subset of CD4+ T cells, designated TH2 cells, and by basophils and mast cells, in response to

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receptor-mediated activation events (1). IL-4 is also produced by a specialized subset of T cells (2), some of which express NK1.1 and appear to be specific for CD-1 (NK T cells) (3). $\gamma/\delta$ T cells have been reported to produce IL-4 (4), and mice lacking these cells fail to develop IL-4-dependent airway hypersensitivity upon immunization with ovalbumin in alum (5). Eosinophils have also been reported to be capable of producing IL-4 (6).

IL-4 plays a central role in regulating the differentiation of antigen-stimulated naive T cells. IL-4 causes such cells to develop into cells capable of producing IL-4 and a series of other cytokines including IL-5, IL-10 and IL-13 (i.e. TH2-like cells) (7, 8). It powerfully suppresses the appearance of IFN$\gamma$-producing CD4+ T cells. A second function of major physiologic importance is IL-4's control of the specificity of immunoglobulin class switching. IL-4 determines that human B cells switch to the expression of IgE and IgG4 (9) and mouse B cells to IgE and IgG1(10, 11). Indeed, in IL-4 (12) and IL-4 receptor (13) knockout mice as well as in mice that lack a principal substrate of the IL-4 receptor, Stat-6 (14–16), IgE production is diminished by a factor of 100-fold or more. IL-4 receptor knockout mice (13) and Stat-6 knockout mice (16) are also deficient in the development of IL-4-producing T cells in mice infected with the helminthic parasite *Nippostrongylus brasiliensis*. These physiologic functions of IL-4 give it a preeminent role in the regulation of allergic conditions; it also plays a major role in the development of protective immune responses to helminths and other extracellular parasites. In experimental and clinical situations, it appears to be capable of ameliorating the effects of tissue-damaging autoimmunity (17).

IL-4 has a variety of other effects in hematopoietic tissues. It increases the expression of class II MHC molecules in B cells (18), enhances expression of CD23 (19), upregulates the expression of the IL-4 receptor (20), and, in association with lipopolysaccharide, allows B cells to express Thy 1 (21). It also acts as a co-mitogen for B cell growth (22). Although not a growth factor by itself for resting lymphocytes, it can substantially prolong the lives of T and B lymphocytes in culture (23) and can prevent apoptosis by factor-dependent myeloid lines that express IL-4 receptors (24–28).

IL-4 also has an important role in tissue adhesion and inflammation. It acts with TNF to induce expression of vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells (29), and it downregulates the expression of E-selectin (30). This shift in balance of expression of adhesion molecules by IL-4 is thought to favor the recruitment of T cells and eosinophils, rather than granulocytes, into a site of inflammation.

An understanding of how IL-4 mediates this wide range of effects requires an analysis of the function of the IL-4 receptor. Here we review many aspects of the structure and function of the receptor, with particular emphasis on the

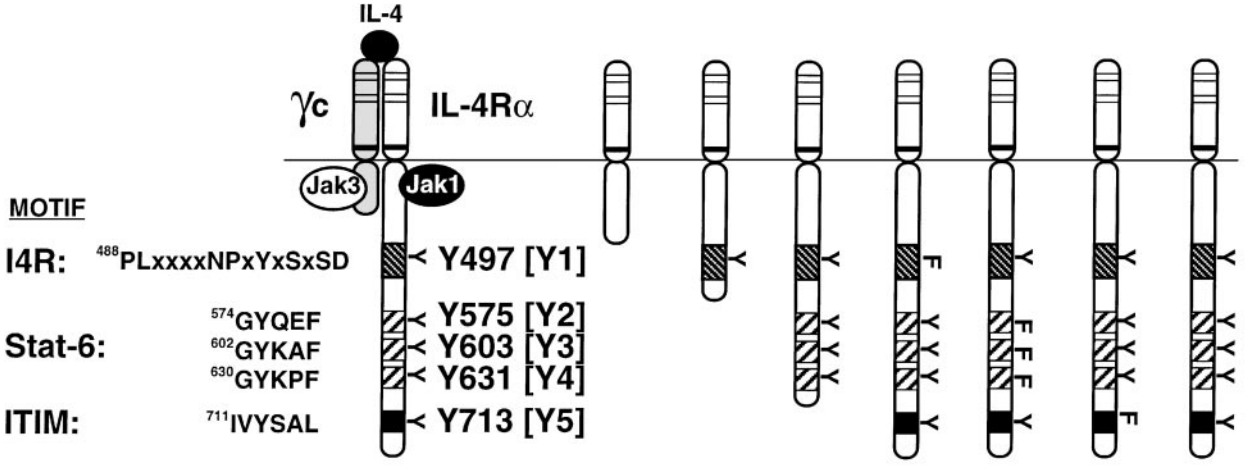
biochemical mechanisms through which it transmits signals. Such an analysis not only will be of relevance to the understanding of IL-4 receptor function but should also help to illuminate functions of other type I cytokine receptors and the receptors for other families of ligands.

## THE IL-4 RECEPTOR COMPLEX

IL-4 receptors are present in hematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte and brain tissues and are usually expressed at 100 to 5000 sites per cell (31, 32), in keeping with the broad range of action of this cytokine. The receptor consists of a 140-kDa IL-4R$\alpha$ chain (Figure 1) that binds IL-4 with high affinity ($K_d$ 20 to 300 pM). Although artificial homodimerization of the IL-4R$\alpha$ chain can result in the generation of biochemical signals within the cell (33–35), physiologic signaling depends upon IL-4-mediated heterodimerization of the IL-4R$\alpha$ chain with a second chain. The gamma common chain ($\gamma$c), first identified as a component of the IL-2 receptor (36–38), appears to be the dominant chain involved in this heterodimerization in many cell types (Figure 1). Molecular binding studies have indicated that the $\gamma$c chain recognizes a complex of IL-4 and the IL-4R$\alpha$ chain (39). Although the $\gamma$c chain only modestly increases the observed affinity of the IL-4R complex for IL-4, it is required for the activation of signaling pathways after binding of IL-4 (36).

The IL-4R$\alpha$ chain also functions as a component of the IL-13 receptor (IL-13R) (40–43). IL-13 appears not to utilize the $\gamma$c chain; rather, its receptor employs other cell surface polypeptides, the IL-13R$\alpha$ and IL-13R$\alpha'$ chains (42–45), presumably in place of $\gamma$c. A number of cell lines lacking $\gamma$c are IL-4 responsive (40, 46), raising the possibility that IL-13R$\alpha$ and/or IL-13R$\alpha'$, which are expressed in these lines, may function, with the IL-4R$\alpha$ chain, as components of the IL-4R complex. Indeed, recent studies indicate that the IL-13R$\alpha'$ is the predominant accessory chain of the IL-4R complex in nonhematopoietic cells (43).

IL-4R$\alpha$ is a member of the hematopoietin receptor superfamily. Among the defining features of the members of this superfamily of receptors are shared structural motifs in the extracellular region, which consists of type III fibronectin domains (47). These motifs include conserved paired Cys residues and, in the membrane proximal region, a WSXWS motif. The latter has been proposed to be required for maintaining the receptor in a conformation favorable to cytokine binding (48). Structural alterations in the IL-4R$\alpha$ extracellular region may result in altered receptor signaling capabilities. Indeed, a variant of the human IL-4R$\alpha$ chain containing a Ile50Val substitution was isolated from atopic individuals and has been shown to enhance signal transduction resulting in the increased production of IgE (49).




| | *Δ437* | *Δ557* | *Δ657* | *Y1F* | *Y2,3,4F* | *Y5F* | *WT* |
|---|---|---|---|---|---|---|---|
| *IRS-1/2 phosphorylation* | - | + | + | - | + | + | + |
| *Shc phosphorylation* | - | + | + | - | nd | + | + |
| *FRIP phosphorylation* | nd | nd | nd | - | nd | nd | + |
| *Cellular proliferation* | - | + | + | +/- | nd | + | + |
| *Protection from apoptosis* | - | + | - | - | nd | + | + |
| *Stat-6 phosphorylation* | - | +/- | + | +* | - | + | + |
| *Gene activation* | - | +/- | + | +* | - | nd | + |
| *SHIP phosphorylation* | - | nd | + | + | nd | + | + |

The murine IL-4R$\alpha$ is 785 amino acids long with a 553 amino acid cytoplasmic region (50). The cytoplasmic region contains sequences found in other members of the hematopoietin receptor family as well as residues that are highly conserved between IL-4R$\alpha$ chains of different species. In particular, there are five tyrosine residues within the IL-4R$\alpha$ cytoplasmic region whose position and surrounding sequences are highly conserved, suggesting that these sequences are functionally important (Figure 1). A short proline-rich sequence in the membrane proximal region of the IL-4R$\alpha$, termed a "box1 motif," is found in a number of hematopoietin receptor family members. A mutational analysis of the gp130 chain of the IL-6 receptor demonstrated the importance of this sequence for the function of the receptor (51). An acidic region adjacent to the box1 motif is similar to a region of the IL-2 receptor $\beta$ that has been shown to interact with Src-family kinases (52).

## *Activation of Signal Transduction by the IL-4R*

Insight into the initiation of signal transduction by hematopoietin receptors has come from elegant structural studies of the growth hormone (GH) receptor (53, 54). These studies demonstrated that a single GH molecule cross-links two GH receptor molecules, resulting in the cross-activation of kinases associated with the cytoplasmic domain of the GH receptor. The erythropoietin (EPO) receptor and c-Kit, like the GH receptor, are homodimerized by their respective cytokine ligands. Studies of chimeric models made with the cytoplasmic domain of the IL-4R$\alpha$ and the extracellular domains of the EPO or c-Kit receptors have also indicated that stimulation of cells expressing EPO- or c-Kit-IL-4R$\alpha$ chimeras with their respective ligands induces IL-4R signaling pathways in cells. Thus, IL-4R$\alpha$ cross-linkage appears capable of initiating signal transduction (33–35). As noted above, IL-4, rather than homodimerizing the IL-4R$\alpha$

←

*Figure 1* IL-4R structure and function. The IL-4R complex is composed of the IL-4R$\alpha$ and $\gamma$c receptor subunits that associate with the Jak1 and Jak3 kinases, respectively. The cytoplasmic domain of the human IL-4R$\alpha$ contains five conserved Tyr residues. The most membrane proximal, Y497, is within the I4R motif that is critical for generating proliferative signals. The second, third, and fourth Tyr residues (Y575, Y603, and Y631) are within a highly conserved sequence motif critical for the activation of Stat-6. The C-terminal Tyr, Y713, is within a ITIM motif that may serve as a docking site for different phosphatases. The ability or inability (−) of deletion (d437, d557, and d657) and point (Y497F, Y2,3,4F and Y5F) mutants of the IL-4R$\alpha$ to fully activate (+) or to partially activate (+/−) cellular proliferation, gene expression, protection from apoptosis, or the IRS-1/2, Shc, FRIP, Stat-6 and SHIP signaling pathways is summarized. In two situations, Stat-6 phosphorylation and gene activation in response to occupancy of Y1F mutants, there is heterogeneity among stably transfected cell lines, with some lines displaying full activation and others displaying virtually none. Such differences are not determined by numbers of receptors and remain to be explained. These results are designated as $+^{*}$.

chain, causes hetereodimerization of this chain with the $\gamma$c chain leading to the activation of IL-4R signaling pathways (55).

Ligand induced dimerization (or multimerization) of cytokine receptors results in the activation of tyrosine kinases that phosphorylate cellular substrates and initiate signaling cascades (47). Neither the IL-4R$\alpha$ or the $\gamma$c chain has endogenous kinase activity; rather the IL-4R (and all members of the hematopoietin receptor family) require receptor-associated kinases for the initiation of signal transduction. The Janus-family (Jak) tyrosine kinases are critical in the initiation of signaling by hematopoietin receptors (56, 57).

Three members of the Janus kinase family, Jak-1, Jak-2, and Jak-3, have been demonstrated to be activated in response to IL-4R engagement and to associate with components of the IL-4R complex (58–60). Jak-1 has been proposed to associate with the IL-4R$\alpha$ chain while Jak-3 associates with the $\gamma$c chain (Figure 1) (61, 62). In certain cell lines, Jak-2 has also been demonstrated to associate with the IL-4R$\alpha$ (60). IL-4 engagement of the IL-4R$\alpha$ chain results in tyrosine phosphorylation of Jak-1 and Jak-3. Analysis of members of the Ba/F3 pro-B cell line expressing mutant human IL-4R$\alpha$ chains suggests that the membrane proximal region containing the box1 motif and the acidic region may be required for IL-4-mediated responses (63). The importance of this region may reflect the fact that it is a potential site of interaction with Jak1. In addition to these Jak-family kinases, the Src-family kinase Fes has also been reported to associate with the IL-4R$\alpha$ and to be activated in response to IL-4 stimulation (64).

Activation of IL-4R-associated kinases leads to the tyrosine phosphorylation of the IL-4R$\alpha$ chain itself, a process that occurs rapidly after IL-4R engagement (65). The five conserved Tyr residues in the cytoplasmic region of the IL-4R$\alpha$ are potential sites of phosphorylation and of subsequent interaction with downstream signaling proteins through Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains within these molecules. A critical point that remains to be clarified is which kinases actually catalyze the IL-4-induced phosphorylation of receptor tyrosines and of tyrosines on substrates that dock to the receptor.

The identification of tyrosine residues critical for activation of signaling pathways and the subsequent analysis of molecules that interact with these residues have led to the biochemical characterization of pathways activated by IL-4R engagement. Independent studies in which truncation and deletion mutants of the human IL-4R$\alpha$ chain were expressed in different hematopoietic cells showed that the region between residues 437 and 557, containing one conserved Tyr residue (Y497) (numbering according to 66), was required for IL-4-mediated activation of proliferation (Figure 1) (63, 67–69). Mutant receptors lacking this region were unable to transmit signals that normally result in phosphorylation of a set of key PTB-domain-containing substrates (see

below). In most instances, IL-4-mediated proliferative responses did not occur in factor-dependent myeloid progenitor cell lines (32D.IRS-1) expressing these mutant receptors; such responses were greatly diminished in Ba/F3 cells expressing these receptors.

Although IL-4R$\alpha$ sequences C-terminal to residue 557 do not appear to be essential for IL-4-stimulated proliferative responses, further analyses of deletion mutants have indicated that these sequences are important in induction of IL-4-responsive genes (Figure 1). In particular, the three conserved Tyr residues, Y575, Y603, and Y631, that lie within this region are critical for transducing signals that result in activation of a series of IL-4-induced genes (35, 70).

Thus, on a first level of analysis, the IL-4R$\alpha$ chain cytoplasmic region appears to have three functionally distinct domains, one that acts as an interaction site for the Janus kinase, one required for activation of proliferative pathways, and a third involved in the activation of pathways leading to induction of gene expression. Whether this represents an evolutionary process in which distinct segments mediating distinct functions are independently acquired is uncertain. The segregation of functions into distinct regions of the receptor would be consistent with this view. However, the regions of the receptor responsible for stimulation of proliferation and for gene activation are encoded in a single exon, which might suggest that the receptor achieved its current form long ago.

In the next section, we discuss in more detail the signaling pathways initiated by IL-4 receptor engagement.

## IL-4R SIGNALING PATHWAYS

Initial experiments directed at characterizing the signaling pathways activated by IL-4R engagement compared the pattern of cellular proteins phosphorylated in response to IL-4 and IL-3 in hematopoietic cell lines (71). Strikingly, a 170-kDa phosphoprotein was uniquely phosphorylated in response to IL-4. In further studies this protein, initially termed the IL-4 phosphorylation substrate (4PS), was shown to be related to insulin receptor substrate-1 (IRS-1), the primary substrate phosphorylated in response to treatment of nonhematopoietic cells with insulin or IGF-1 (72, 73). The gene encoding the 4PS phosphoprotein has a high degree of homology to IRS-1; accordingly, IRS-2 was adopted as the formal designation for 4PS (74).

### *The IRS-1/2 Signaling Pathway*

The importance of IRS-1 and IRS-2 in responses to IL-4 was demonstrated using the factor-dependent myeloid progenitor cell line 32D; 32D cells do not express detectable levels of IRS-1 or IRS-2 (73, 74). Whereas other myeloid cell lines that expressed IRS-2 proliferated in response to IL-4 stimulation, 32D cell lines did not, suggesting a possible role for IRS-1/2 in IL-4-mediated

proliferation. Stable transfectants of 32D cells were prepared that expressed IRS-1 or IRS-2; these transfectants phosphorylated the IRS substrate they expressed when stimulated with IL-4 and showed IL-4-dependent cell growth. These observations led to the conclusion that IRS-1/2 molecules link the IL-4R to signaling pathways involved in cellular proliferation.

INTERACTION OF IRS-1/2 WITH THE I4R MOTIF OF IL-4R$\alpha$ How is IRS-1/2 activated in response to IL-4? The truncation mutants indicated the importance of the sequence between amino acids 437 and 557 of the IL-4R$\alpha$ in this process. The sequence surrounding the single Tyr in this interval is $^{488}$PL-$(x)_4$-NPxYxSxSD$^{502}$, which is highly homologous to sequences in the cytoplasmic regions of the insulin and IGF-1 receptors that also activate the IRS-1/2 signaling pathways (Figure 1) (67). Mutation of the central Tyr residue, Y497, of this motif, to Phe greatly diminished the ability of mutant receptors to signal proliferation in response to IL-4 and blocked IRS-1/2 phosphorylation (Figure 1) (67). Additionally, chimeric receptors consisting of a truncated IL-2 receptor $\beta$ (IL-2R$\beta$) molecule linked to a protein segment containing the IL-4R$\alpha$ I4R motif were able to activate IRS-1/2 phosphorylation on IL-2 stimulation, while the truncated IL-2R$\beta$ alone did not, nor did a chimeric receptor expressing an I4R motif with a Y497F mutation (75). It had been previously demonstrated that mutation of the homologous Tyr (Y960) in the insulin receptor diminished insulin-directed cell activation (76). Thus, this sequence, being critical for transducing signals through the insulin receptor and the IL-4R$\alpha$, was termed the insulin IL-4 receptor, or I4R, motif. Moreover, the importance of this central Tyr suggested that the I4R motif, once phosphorylated at Y497, is a site of interaction with IRS-1/2.

Direct evidence for an interaction between the phosphorylated I4R motif and IRS-1/2 molecules came from co-precipitation experiments and analysis of this interaction in the yeast two-hybrid system. Immunoprecipitation of the IL-4R$\alpha$ from FDC-P1 hematopoietic cells transfected with IRS-1 co-precipitated IRS-1 after IL-4 stimulation (67). Similarly a GST-fusion protein that expressed a 368 amino acid region of the IL-4R$\alpha$ containing the I4R motif was capable of precipitating phosphorylated IRS-1 from IL-4-stimulated cell extracts (67). However, this GST-fusion protein precipitated IRS-1 less efficiently than did the full receptor, possibly due to inefficient phosphorylation of the fusion protein in cell extracts.

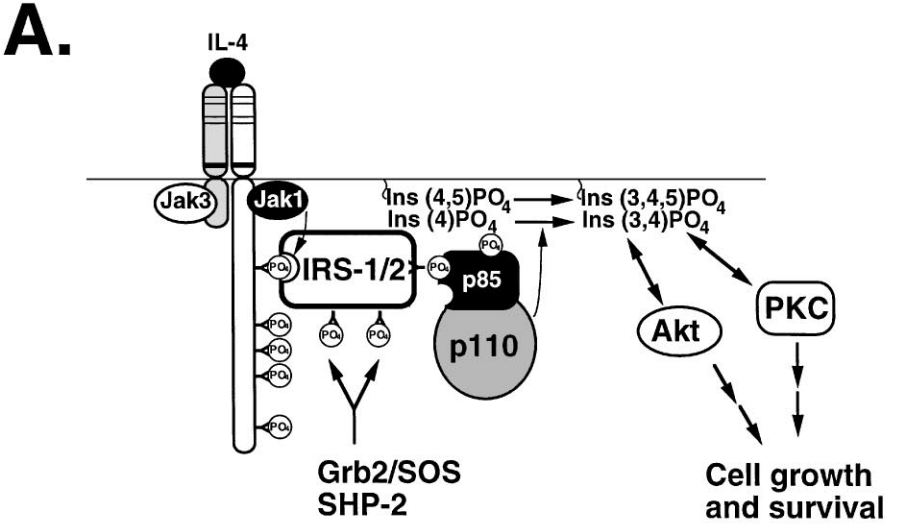
Studies by Gustafson and colleagues utilizing a modified yeast-two hybrid system, in which the inclusion of the insulin receptor kinase in the bait allowed phosphorylation of the central tyrosine of the I4R motif, indicated that the IRS-1/2 molecules bind to phosphorylated I4R motifs through an N-terminal PTB domain (Figure 2A) (77, 78). This PTB domain has a three-dimensional

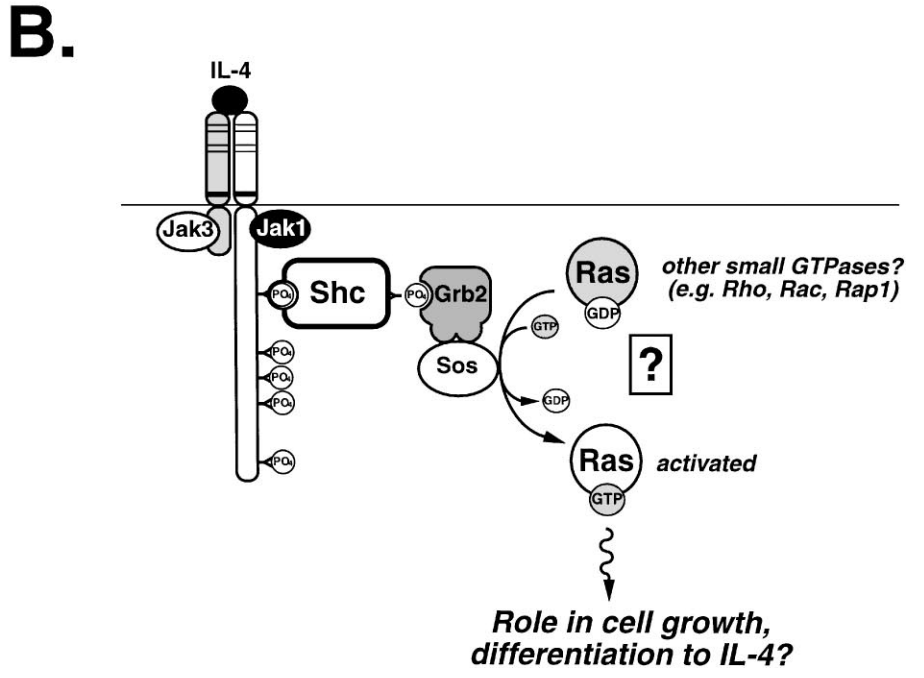
structure composed of a $\beta$–sandwich capped by a C-terminal $\alpha$ helix, similar to the PTB domain first described in the adapter molecule Shc (79–82). PTB domains bind to phosphopeptides with a core NxxY sequence, similar to that found in the I4R motif (83). Phosphopeptides derived from the I4R-motif of the IL-4R$\alpha$ (LVIAGNPApYRS) inhibited the binding of a phosphopeptide derived from the I4R-motif of the insulin receptor (LYASSNPApYLSASDV) to the PTB domain of IRS-1 (84).

The structural basis for the interaction between a phosphopeptide derived from the I4R-motif of the IL-4R$\alpha$ and the IRS-1 PTB domain has been determined by nuclear magnetic resonance spectroscopy (81). This analysis showed that a cleft formed between the $\beta$–sandwich and the C-terminal $\alpha$-helix of the IRS-1 PTB-domain served as the binding site for the phosphopeptide. The phosphopeptide (LVIAGNPApYR) inserts in the cleft rather like a shepherd's crook. The NPApY sequence forms a Type I $\beta$ turn making up the hook, and the amino terminal hydrophobic residues make up the extended cane that lies in a groove parallel to the $\alpha$-helix. Amino acid residues at the $-8$ (L) and $-6$ (I) positions relative to Y497 in the I4R-motif make contact with residues in the PTB domain of IRS-1.

A detailed mutational analysis of the I4R-motif in the human IL-4R$\alpha$ confirmed the critical nature of L489 and I491 and identified additional residues necessary for regulating IL-4R signaling (85). Cell lines expressing the Y497F mutant consistently failed to activate the IRS-1/2 pathway and did not proliferate in response to IL-4, reflecting the requirement for I4R motif phosphorylation in PTB binding. Mutagenesis of P488 to A also greatly diminished the tyrosine phosphorylation of IRS-2 in response to IL-4 while mutation of a P488 to G resulted in a receptor competent to signal IRS-2 phosphorylation. The tolerance of the P to G change suggests that P488 controls the availability of the I4R-motif to PTB-domain containing proteins since this residue would lie just outside the binding cleft described by NMR. As predicted from the NMR structural data, mutation of *both* L489 and I491 to A also greatly diminished the tyrosine phosphorylation of IRS-2 to IL-4. However, mutation of only one of these residues to A did not affect signaling function, indicating that potential interactions from either the Leu or Ile is sufficient to make the receptor competent to recruit and phosphorylate IRS2 in these cells. These results indicate the important role of P488, L489, I491, and Y497 of the I4R-motif in regulating IRS-recruitment/activation.

In contrast to the N-terminal residues of the I4R motif, mutagenesis of residues downstream of Y497 such as R498 or F500 to A had no effect on IL-4-induced biochemical or biological responses, although, as described below, these sequences may be important in the weak Stat-6 activating capacity of the I4R motif of the IL-4R.



The N-terminal region of IRS-1/2 also contains a pleckstrin homology (PH) domain, similar to those found in a large number of signaling molecules. These PH domains have tertiary structures quite similar to that of PTB domains (80). Although the function of the PH domain is still being defined, it likely plays a role in localizing proteins to the plasma membrane by interacting directly with phosphatidylinositol membrane lipids that are generated through the activation of the phosphoinositide-3-kinase signaling pathway (discussed below) (86).

IRS1/2 becomes phosphorylated as a result of interaction with phosphorylated IL-4R$\alpha$, presumably through the action of receptor-associated kinases (Figure 2). Indeed, in vitro experiments have indicated that Jak1, Jak2, and Jak3 are capable of directly phosphorylating IRS-1 (87). IRS-1/2 molecules are multiply phosphorylated in response to stimulation by a number of cytokines in addition to IL-4, including IL-2, IL-7, IL-9, and IL-15, indicating the presence of IRS-1/2 docking sites within these receptor complexes as well as the ability of different kinases to mediate IRS-1/2 phosphorylation (88). Analysis of cell lines lacking specific Jaks have indicated that Jak1 is critical for IL-4-stimulated induction of IRS-1 phosphorylation (89–91). This likely occurs through the direct action of Jak1 on IRS-1; however, this has not been proven directly. Jak2 and Tyk2 also mediate IRS-1 phosphorylation in certain cell lines (91).

IRS-1/2 molecules each have approximately 20 potential sites for tyrosine phosphorylation (74, 92). A number of these sites are bound by specific SH2 domains indicating that IRS-1/2 act as cytosolic docking proteins capable of linking a variety of SH2 domain signaling molecules to phosphorylated receptors (74, 92, 93). Among the molecules that interact with phosphorylated IRS-1/2 molecules are the regulatory subunit of phosphoinositide-3-kinase (PI-3-K) and the adapter molecule, Grb-2 (Figure 2). These interactions lead to the activation of the PI-3-K and Ras/MAPK signaling pathways, respectively.

THE PHOSPHOINOSITIDE-3-KINASE PATHWAY Several biochemically distinct forms of PI-3-K have been characterized, but the primary form activated by IL-4 is a complex of two subunits, a 85-kDa regulatory (p85) and a 110-kDa

*Figure 2* Activation of signaling pathways through the I4R motif of the IL-4R$\alpha$. A. The PI-3-kinase pathway can be activated through the interaction of IRS-1/2 molecules with the phosphorylated I4R motif of the IL-4R$\alpha$. This interaction leads to the phosphorylation of IRS-1/2 molecules by IL-4R associated kinases, the interaction of the p85 subunit of PI-3-kinase with IRS-1/2, the production of phosphoinositides, and the activation of downstream effectors (e.g. Akt, PKC). B. The phosphorylated I4R motif can act as a docking site for the adapter Shc. This may lead to the activation of small GTPases such as Ras in certain cell types. The activation of Ras by IL-4 is not seen consistently in all cell types and the importance of Shc and Ras activation in IL-4 proliferative responses remains to be fully delineated.

catalytic (p110) subunit (Figure 2A). The p85 subunit contains tandem SH2 domains in the C-terminus and an N-terminal SH3 domain (94, 95). The SH2 domains of the p85 subunit flank a 104 amino acid sequence that mediates the interaction with the p110 catalytic subunit (96, 97). The p85 subunit thus acts as an adapter molecule linking the p110 subunit to tyrosine phosphorylated molecules. IL-4 stimulation leads to the binding of the p85 subunit of PI-3-K to phosphorylated IRS-1/2 molecules (Figure 2A) (74, 93). IRS-1 and IRS-2 have four and ten potential sites of p85 subunit binding, respectively.

Interaction of the p85 subunit with phosphorylated IRS-1/2 molecules results in a conformational change in the PI-3-K complex leading to the activation of the p110 catalytic subunit (98). The PI-3-K complex also interacts with Fes kinase after IL-4R engagement (99). Once activated, the p110 catalytic subunit is capable of phosphorylating membrane lipids as well as Ser/Thr residues of proteins (98). The lipid kinase activity mediates the transfer of phosphate from ATP to the D3 position of inositol in phosphotidylinositol in the cellular membrane (Figure 2A) (100, 101). Several forms of phosphorylated phosphotidylinositol have been identified, but the most important biologically appear to be phosphotidylinositol-(3,4,5)-triphosphate and phosphotidylinositol-(3,4)-bisphosphate. These are produced within seconds of stimulation (100, 102). Their rapid production led to the hypothesis that these molecules act as second messenger molecules for IL-4 function. Indeed, phosphoinositides have since been implicated in the activation of a number of downstream kinases including different forms of protein kinase C ($\delta$, $\varepsilon$, and $\eta$ isozymes) and the Akt kinase (also known as protein kinase B) that play a key role in cell survival (Figure 2A) (103, 104). IL-4 has been demonstrated to enhance the survival of hematopoietic cells (24–26). Thus, it could be hypothesized that activation of the PI-3-K pathway by IL-4 may enhance cell survival through the production of phosphoinositides and the subsequent activation of kinases critical for cell survival. This hypothesis is supported by the finding that inhibitors of PI-3-K, such as Wortmannin, block the ability of IL-4 to prevent apoptosis in hematopoietic cells (26).

In contrast to the lipid kinase activity of the p110 catalytic subunit of PI-3-K, the importance of the Ser/Thr kinase activity has not yet been fully defined. However, PI-3-K has been shown to catalyze the Ser/Thr phosphorylation of IRS- 1 (105). Since the Ser/Thr phosphorylation of IRS-1 has been suggested to diminish the interaction of IRS-1/2 with the I4R motif of the insulin receptor (106), it is possible that IRS-1/2 activation of PI-3-K may result in the Ser/Thr phosphorylation of IRS-1/2 by p110 and the inhibition of further IRS-1/2 activation. Thus, activation of the PI-3-K Ser/Thr kinase activity may result in a negative feedback loop that contributes to the regulation of the IRS-1/2 signaling pathway.

THE Ras/MAPK PATHWAY Activation of the IRS-1/2 signaling pathway is associated with the activation the Ras/MAPK pathway in response to a number of cytokines, including insulin; IL-4 activation of the Ras/MAPK pathway is not consistently observed (107, 108). Phosphorylated IRS-1/2 has been proposed to interact with the SH2 domain of the adapter Grb2 (Figure 2A) (93). Grb2 is constitutively complexed to the guanine nucleotide exchange protein Sos (109). The primary function of Sos is to catalyze the exchange of GDP in inactive Ras for GTP, producing the active GTP-bound form of Ras (109, 110). IRS-1/2 interaction with phosphorylated receptors results in the accumulation of phosphorylated IRS-1/2 molecules at the cellular membrane where Ras is located. The subsequent interaction of phosphorylated IRS-/2 with Grb2/Sos also increases the concentration of Sos at the membrane, leading to activation of Ras.

The MAPK pathway is initiated by the Ser/Thr kinase Raf following its activation by Ras-GTP. Although the mechanism of Raf activation is not yet understood, active Raf initiates a cascade of kinase activation events that ultimately result in the phosphorylation and activation of the mitogen activated protein kinases ERK-1 and ERK-2 (111, 112). Active ERK-1/2 translocates to the nucleus and activates the expression of genes such as c-*fos* by phosphorylating specific transactivating factors (113). Distinct kinases with functions similar to the ERKs, such as the Jun nuclear kinase (JNK), can be activated through kinase cascades initiated by Ras as well as by other small GTPases related to Ras (111). Activation of these kinases results in the nuclear phosphorylation and activation of c-Jun as well as of other transcription factors.

As noted above, although IL-4 dramatically activates IRS-1/2 phosphorylation, IL-4 activation of the Ras/MAPK pathway is not consistently observed. In particular, stimulation of a number of hematopoietic cell lines with IL-4 failed to result in detectable activation of components of the Ras/MAPK pathway (108, 114, 115). Additionally, expression of the IL-4R in an L6 myoblast line enabled IRS-1 to be phosphorylated on stimulation with IL-4 and led to its association of Grb2/Sos (116). Nonetheless, MAPK and cellular proliferation were not activated in response to IL-4 in these myoblast lines, while insulin stimulation resulted in Grb2/Sos association with IRS-1, MAPK activation and proliferation. In these myoblast lines, phosphorylation of Shc, which also acts as an adapter molecule between receptors and Grb2/Sos, did correlate with the activation of the Ras/MAPK pathway. Insulin induced Shc phosphorylation whereas IL-4 did not. Thus, IRS-1 phosphorylation and association with Grb2/Sos is not sufficient for the activation of cellular proliferation by IL-4 in certain cells; activation of the Ras/MAPK pathway may require the activation of other signaling molecules such as Shc.

Other studies have shown that IL-4 stimulation does lead to activation of the Ras/MAPK pathway and to Shc phosphorylation in certain cell types including

B cells and keratinocytes (Figure 2B) (117, 118). In addition, we have observed activation of Shc in the myeloid progenitor cell line 32D (119) and in the IL-4 responsive cell line CT.4R (K Nelms, unpublished data). Thus, activation of the Ras/MAPK pathway by IL-4 may critically depend on cell type and more specifically on the array of signaling molecules expressed in these cells.

Recently, a homolog of the PTB-domain protein $p62^{dok}$ has been cloned using the yeast two-hybrid system with a bait consisting of the I4R motif from the IL-4R$\alpha$ chain linked to the insulin receptor kinase (120). This molecule, designated the IL-four receptor interacting protein (FRIP), is rapidly phosphorylated in T cells and some myeloid cells stimulated with IL-4. FRIP, like $p62^{dok}$, binds to the N-terminal SH2-domain of Ras-GAP. FRIP may thus link activated IL-4R$\alpha$ molecules with RasGAP, leading to the activation of the endogenous GTPase activity of Ras and to the inactivation of the Ras pathway. FRIP is discussed in greater detail below.

## *Other Adapter Molecules*

As discussed above, Shc may play a pivotal role in the ability of certain cells to activate the Ras/MAPK pathway in response to IL-4. Shc shares some structural and functional characteristics with IRS-1/2. Shc contains two distinct domains capable of binding tyrosine-phosphorylated receptor sequences. The C-terminal region of the Shc protein contains an SH2 domain while the N-terminal domain contains a PTB domain (121, 122). The PTB and SH2 domains of Shc mediate its interaction with phosphorylated receptor molecules. The PTB domain of Shc is very similar in structure to the PTB domain of IRS-1/2 that mediates its interaction with the I4R motif of the IL-4R$\alpha$ (82). Once this interaction occurs, Shc itself is phosphorylated at Tyr317, which then serves as a docking site for the SH2 domain of Grb2 (123, 124). In this way, Shc may link the Grb2/Sos complex to phosphorylated receptors and thus catalyze Ras activation (Figure 2B). In addition to the IL-4-mediated induction of Shc phosphorylation in certain cell types, a number of other cytokines and growth factors have been demonstrated to activate Shc phosphorylation (108, 117, 118).

The adapter molecule Cbl, encoded by the proto-oncogene c-*Cbl* , may also play a role in the activation of signaling pathways by IL-4. Like IRS-1/2 and Shc, Cbl is phosphorylated in response to IL-4 (125) and cytokine-induced phosphorylation of Cbl has been demonstrated to link Grb2/Sos to receptor complexes and thus may play a role in activation of the Ras/MAPK pathway (125–127). IL-4-induced phosphorylation of Cbl also leads to its association with the p85 subunit of PI-3-K. Cbl has an N-terminal PTB domain that mediates its interaction with the ZAP-70 tyrosine kinase (128). Cbl also contains a proline-rich sequence that can interact with SH3 domains of different molecules (126, 127, 129).

The relative importance of the activation of Cbl phosphorylation in response to IL-4R engagement has not yet been determined. Likewise, the roles of newly identified members of the IRS signaling molecule family, IRS-3 and IRS-4, in IL-4R signaling processes have not yet been defined (130, 131). Because of the structural similarity and likely functional redundancy of IRS proteins, it will be of interest to evaluate the function of IRS-3 and IRS-4 in cells lacking the IRS-1 and IRS-2 molecules.

### *The Stat-6 Activation Pathway*

While the I4R motif region of the IL-4R$\alpha$ is critical for activating pathways involved in regulating the proliferation of cells to IL-4, additional regions of the cytoplasmic tail are required for activation of IL-4-induced gene expression. Indeed, analyses of IL-4R$\alpha$ deletion mutants have indicated that the region between residues 557 and 657 of the human IL-4R$\alpha$ is critical for the induction of signaling pathways leading to the expression of IL-4-responsive genes (Figure 1) (70).

M12.4.1 mouse B lymphoma cells expressing a truncated human IL-4R$\alpha$ that terminates at residue 657 are as active as cells expressing the full-length receptor in IL-4-induced expression of CD23, class II MHC, or germline $\varepsilon$ Ig H chain mRNA (I$\varepsilon$) (70). However, receptors truncated at residue 557 ($\Delta$557) are greatly diminished in their ability to induce expression of these genes in response to challenge with human IL-4. These studies have been further supported by the demonstration that a chimeric receptor consisting of truncated IL-2R$\beta$ coupled to IL-4R$\alpha$ sequences from residues 557 to 657 induces CD23 expression in response to IL-2 (75). Thus, we have termed the IL-4R$\alpha$ region between residues 557–657 the gene regulation domain.

The gene regulation domain contains three conserved Tyr residues (Y575, Y603, and Y631), which represent potential sites of phosphorylation and subsequent association of SH2-containing proteins (Figure 1). Studies of IL-4R$\alpha$ receptors specifically mutated at these Tyr residues have indicated that any one or any two can be mutated to Phe without ablating the capacity of the receptor to fully induce gene expression in response to IL-4; however, it is generally necessary to express substantially more mutant receptors than wild-type receptors to make M12.4.1 cells competent to optimally express IL-4-inducible genes. Mutation of all three Tyr residues results in a receptor with a very limited capability to activate gene expression (Figure 1). Thus, the gene regulation domain requires at least one functional Tyr residue for activity.

An important development in understanding the mechanism by which IL-4 and other cytokines rapidly activate gene expression has been the identification and characterization of molecules termed signal transducers and activators of transcription, or Stats. One or more Stat molecules are activated by each

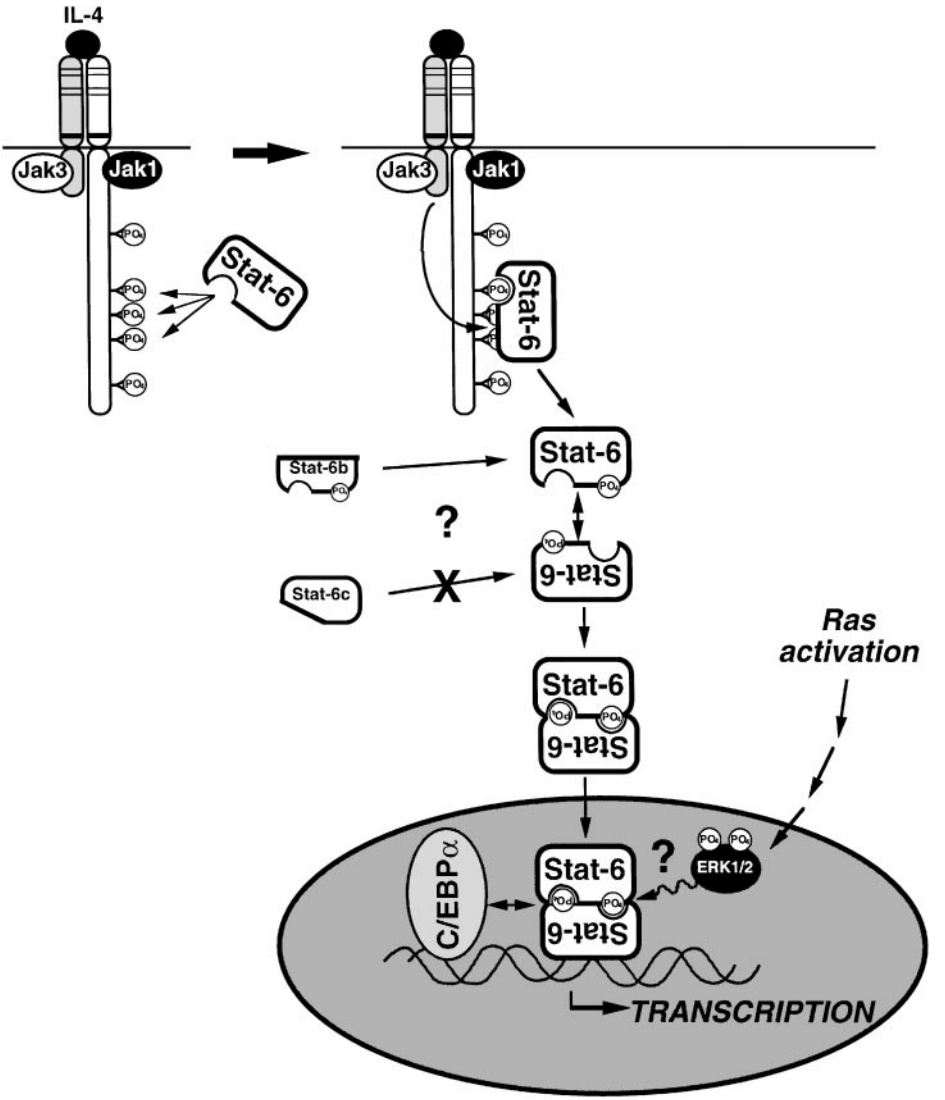
member of the hematopoeitin receptor superfamily and the related set of receptors for interferon-related molecules (132). Elegant experiments utilizing mutant cell lines that lack specific Jak kinases have shown that Jak activation is required for Stat activation (133). Thus, the Stat activation pathway is often referred to as the Jak-Stat pathway.

Stat-6 is the primary Stat activated in response to IL-4 stimulation. It is critical in the activation or enhanced expression of many IL-4-responsive genes, including those for class II major histocompatibility molecules, CD23, germline immunoglobulin $\varepsilon$ and $\gamma 1$, and IL-4R$\alpha$ chain (14, 15, 35, 70, 134, 135).

Stats act as direct connections between the cytokine receptor and the transcription apparatus. The mechanism of Stat-6 activation reflects the general model proposed for all Stat activation events (Figure 3). IL-4R engagement results in the activation of Jak1 and Jak3 and phosphorylation of specific tyrosine residues in the receptor cytoplasmic region. Stat-6 then binds to the phosphorylated receptor through a highly conserved SH2 domain, enabling the activated kinases to phosphorylate Stat-6 at a C-terminal tyrosine residue (132, 136). Once phosphorylated, the Stat-6 molecule disengages from the receptor and forms homodimers through interaction of its SH2 domain with the C-terminal phosphotyrosine residue of a second Stat-6 molecule. The dimerized Stat-6 complexes translocate to the nucleus where they bind to specific DNA motifs in the promoter of responsive genes. The DNA motifs bound by different STATs bear remarkable similarity to each other and reflect a dyad symmetry. Stat-6 appears to bind in particular to the sequence TTC-$N_4$-GAA (137, 138).

The importance of the Stat-6 activation pathway in the expression of IL-4-responsive genes was examined using IL-4R$\alpha$ mutants deficient in their ability to activate IL-4-responsive genes. The Stat-6 activation capability of the different IL-4R$\alpha$ mutants matched the ability of these receptors to stimulate IL-4-responsive gene expression (Figure 1). In particular IL-4R$\alpha$ mutants that lacked the gene regulation domain or had Tyr to Phe mutations at each of the Tyr residues in this region (Y575F, Y603F, and Y631F) induced little or no Stat-6 phosphorylation and DNA binding (70). This suggested that phosphorylation of Tyr residues in the gene regulation domain is critical for Stat-6 activation and

*Figure 3* The Stat-6 activation pathway. After IL-4R engagement and phosphorylation, Stat-6 binds to phosphotyrosine residues in the gene regulation domain, becomes phosphorylated, disengages from the IL-4R$\alpha$ cytoplasmic tail, dimerizes and translocates to the nucleus. Activation of gene transcription by Stat-6 may require cooperative interactions with additional transcription factors (e.g. C/EBP$\alpha$) or phosphorylation by kinases activated in the Ras/MAP kinase cascade (e.g. ERK1/2). Alternately spliced forms of Stat-6 have deletions in the N-terminal (Stat-6b) or SH2 (Stat-6c) regions and may play a role in Stat-6 regulation. Stat-6c can act as a dominant negative form of Stat-6.



that activation of Stat-6 is a critical step leading to expression of IL-4-responsive genes.

Each of the three Tyr residues in the gene regulation domain are equidistantly spaced 27 amino acids from one another. This spacing is conserved in the rat and mouse IL-4R$\alpha$ chains, while the spacing between other Tyr residues is not (139). The residues immediately surrounding each Tyr possess a GYK/QXF sequence, even though there is little sequence conservation in the remainder of the gene regulation domain (Figure 1). The three conserved Tyr motifs in the gene regulation domain have been proposed to be docking sites for the SH2 domain of Stat-6.

The central Tyr of the I4R motif is within a sequence, AYRSF, that is similar to those in the gene regulation domain. Indeed, this I4R motif sequence appears to be a weak Stat-6 binding site. The $\Delta$557 truncation mutant that lacks the gene activation domain nonetheless can signal weak Stat-6 and IL-4-responsive gene activation (Figure 1) (70). Replacing the AYRSF sequence in the $\Delta$557 truncation mutant with the sequence EYLSA, drawn from the insulin receptor I4R motif, results in the loss of the weak gene activating function of the mutant receptor.

The requirements for forming a Stat-6 binding site were examined by replacing the NPAYRSF sequence surrounding Y497 in the $\Delta$557 truncation mutant with sequences derived from those surrounding Y575, Y603, and Y631; as shown above, each of these Ys is part of a sequence that conveys Stat-6 activation function to the IL-4R$\alpha$ chain. Substituting the juxta-Y575 sequence (EAGYKAF) into the $\Delta$557 truncation mutant resulted in a receptor that had strikingly enhanced capacity to induce Stat-6 DNA-binding activity, CD23 expression, and class II MHC upregulation when expressed in M12.4.1 cells (140). This response, however, was significantly reduced in comparison to the response obtained with the full-length IL-4R$\alpha$. Furthermore, transfer of shorter sequences, such as GYKAF, did not confer Stat-6 activating potential, even though this "core" sequence contains those residues in which the three Stat-6 sites are homologous. These results suggest that the overall structure of the IL-4R$\alpha$ chain or the presence of multiple Stat-6 binding sites within the gene activation domain is required for the full Stat-6-activating function. Indeed, transfer of this domain, containing all three Stat-6 sites in the appropriate context and spacing, to a truncated IL-2R$\beta$ chain resulted in maximal activation of Stat-6 and CD23 in response to IL-2 (75) (AD Keegan, unpublished observations). The superiority of the longer sequence has not been fully explained.

These studies thus indicate that the division of the IL-4R$\alpha$ chain into "domains" that principally regulate growth (residues 437 to 557) and gene activation (residues 557 to 657), respectively, while largely correct is imperfect. Indeed, the IRS1/2 pathway leads to phosphorylation of the DNA-binding

protein HMG-I (Y) that participates in the regulation of I$\varepsilon$ expression in response to IL-4 (141, 142). An additional complexity is the observation (85) that altering certain residues in the I4R motif (within the "growth domain") affected not only the IRS-1/2 pathway, but also the Stat-6 activation pathway (largely a function of the "gene activation domain"). In particular, mutation of P488 to A in the full-length human IL-4R$\alpha$ greatly diminished the tyrosine phosphorylation of Stat-6, as well as that of IRS-2, and abolished the induction of CD23 and Stat-6 DNA-binding activity in response to IL-4. In contrast, a P488G mutant was competent to signal these responses to IL-4. Mutating both L489 and I491 to A also diminished the tyrosine phosphorylation of Stat-6 and abolished induction of CD23 and of Stat-6 DNA-binding activity in response to human IL-4.

These observations have not yet been completely explained. It is possible that changing residues in the I4R-motif disrupts the overall receptor structure. However, $^{125}$I-huIL-4 cross-linking data and Jak3 tyrosine phosphorylation studies show no gross alterations in the capacity of the receptor to bind ligand or to activate Jak3, making this possibility unlikely. Another possibility is that the changes in the I4R-motif disrupt important protein structures in the gene activation domain or make the Ys in this region unavailable to kinases. A third possibility is that the structure of the I4R-motif must be maintained to recruit PTB-domain containing proteins that participate in the recruitment and/or tyrosine phosphorylation of Stat-6.

Deletion and mutational analyses of the Stat-6 protein itself have defined domains and residues within Stat-6 that are required for DNA binding and transcriptional activation. Deletions in the C-terminus of Stat-6 blocked its ability to activate transcription (136). Similar deletions in Stat-1 abrogate its ability to activate transcription (143). The effect of these C-terminal deletions may reflect the importance of this region for transcriptional activation or the presence of critical residues. Indeed, mutation of a Tyr residue (Y641) in this C-terminal region whose position is conserved between different STAT molecules also blocked Stat-6 function and DNA binding (136). This C-terminal Tyr residue in Stat-6 is thus predicted to be the site of Jak phosphorylation and the target of SH2 domains of other Stat-6 molecules.

In addition to the C-terminal deletions, amino acid substitutions in the DNA-binding domain that blocked binding activity inhibited Stat-6 transcriptional activation (136). Similarly, mutation of a conserved Arg residue in the SH2 domain (R562), predicted to be critical for phosphotyrosine binding, also abolished activation of transcription and DNA binding by Stat-6 (136). This mutation likely prevents receptor interaction and dimerization of the mutated Stat-6 molecules.

Naturally occurring deletion mutants of Stat-6, termed Stat-6b and Stat-6c, that result from alternate splicing have also been characterized (144). Stat-6b

contains an N-terminal truncation that attenuates but does not block its function, in agreement with in vitro deletion studies (136). Co-expression of Stat-6b with full-length Stat-6 did not alter the activation or function of the full-length Stat-6 molecule (144) as expected from other studies performed with N-terminal deletion mutants of Stat-6 (Figure 3) (136). In contrast to Stat-6b, Stat-6c contains a deletion in the SH2 domain and does not become phosphorylated in response to IL-4 stimulation. Nonetheless, transfection of Stat-6c prevented FDC-P2 cells from expressing I-$A^d$, CD16/CD32 and CD23 in response to IL-4 and diminished IL-4-mediated cellular proliferation. Stat-6c appeared to mediate these inhibitory functions by preventing the dimerization of full-length Stat-6 (Figure 3) (144). This is in contrast to studies done with a Stat-6 mutant containing a point mutation in the SH2 domain whose overexpression did not alter the activation of endogenous Stat-6 molecules (136). The mechanism by which Stat-6c inhibits Stat-6 activation as well as the importance of the Stat-6 splice variants in vivo remain to be elucidated.

The exact mechanism by which Stats activate transcription is still being determined. It is likely that Stat molecules themselves activate the basic transcriptional machinery, but Stat molecules form complexes with other well-characterized transcription factors such as c-Jun and SP1 and thus may activate transcription through cooperative interaction with these factors (145, 146). Cooperative action with the transcription factor C/EBP$\alpha$ and NF-$\kappa$B appears to be particularly important in the transcriptional activation of the immunoglobulin $\varepsilon$ gene by Stat-6 (136, 147, 148).

Additional signaling pathways may also contribute to general activation of Stat function as indicated by the observation that activation of the Ras/MAPK pathway is required for the full function of some Stat molecules (149, 150). In particular, a serine residue in the C-terminal region of Stat-1$\alpha$ was shown to be phosphorylated in response to activation of the Ras/MAPK pathway, presumably through the action of MAPKs such as ERK1/2 (149). Mutation of this serine resulted in a threefold reduction in Stat-1$\alpha$-induced transcriptional activation. Serine phosphorylation of Stat-3 was required for optimal DNA binding (151). This is in contrast to the findings with Stat-1$\alpha$, in which serine phosphorylation did not seem to alter DNA binding. Phosphorylation of Stat-6 on Ser/Thr residues has not yet been shown to play a role in Stat-6 function. However, it is likely that Ser/Thr phosphorylation is a general phenomenon in regulating Stat function, so its role in the regulation of Stat-6 should be carefully examined.

The critical importance of Stat-6 activation in vivo has been demonstrated in Stat-6 knockout mice. These mice have undetectable serum levels of IgE and respond to infection with *N. brasiliensis* or injection of anti-IgD with increases in IgE that are less than 1% of wild-type mice. They fail to develop CD4+ T cells

of the TH2 type in response to *N. brasiliensis* infection (135). Furthermore, they fail to expel the parasite (152). In each respect, they resemble IL-4R$\alpha$ chain knockout mice (13).

When lymphocytes from Stat-6 knockout mice are studied in vitro, they fail to show switching to IgE and IgG1 in response to LPS plus IL-4; they also fail to show IL-4-mediated enhancement in expression of CD23, class II MHC molecules and IL-4 receptors. Furthermore, their CD4+ T cells fail to respond to immobilized anti-CD3 and IL-4 with the development of TH2 cells and IL-4 fails to prevent the differentiation of naive CD4+ T cells into IFN$\gamma$-producing cells.

The specific immunodeficiency of Stat-6−/− mice likely results both from a block in IL-4-dependent Th2 cell development and from an inability of B cells to target the C$\varepsilon$ gene for class switching both because IL-4 is not being produced and because Stat-6−/− cells would be insensitive to the switch-stimulating effects of IL-4 even if it were produced.

Analyses of cells from Stat-6 deficient mice have shown that IL-4's action as a co-mitogen for B and T cells is not ablated. IL-4 is able to protect B and T lymphocytes from Stat-6−/− mice from spontaneous apoptosis (153) (J Zamorano, J Austrian, H-Y Wang, AD Keegan, submitted for publication). However, depending upon the circumstances of the stimulation, lymphocytes from Stat-6 knockout mice can display a moderate or even striking diminution in IL-4-dependent DNA synthesis. The impairment of IL-4-mediated growth effects in cells from Stat-6 knockout mice may be a consequence of reduced expression of factors in Stat-6−/− cells required for IL-4-induced proliferation. Indeed, expression of the IL-4R$\alpha$ chain (15) and IRS-2 (154) are diminished in Stat-6−/− cells. The altered expression of other factors in Stat-6−/− cells may also diminish the ability of these cells to proliferate to IL-4. In particular, IL-4 results in the accumulation of the cyclin-dependent kinase $p27^{Kip1}$ in Con A-stimulated Stat-6−/− cells when compared to control cells (155). Increased levels of $p27^{Kip1}$ lead to a decrease in cdk2-associated kinase activity and thus inhibit the progression of cells from the G1 to S phases of the cell cycle (155). In contrast to IL-4 stimulation, Stat-6−/− and control T cells proliferated similarly in response to IL-2 stimulation, indicating that levels of $p27^{Kip1}$ are not elevated by all cytokines in Stat-6−/− cells. It is likely that the altered expression of these and other proteins in Stat-6−/− cells all contribute to the reduced level of proliferation of these cells to IL-4.

A polymorphism of the human IL-4R$\alpha$ chain, Q576R, in the core Stat-6-binding sequence surrounding Y575, has recently been reported and found in 3 of 3 patients with the hyper-IgE syndrome and 4 of 7 patients with severe atopic dermatitis (156). Among 50 adults, it was present in 13 of 20 subjects with atopy and only 5 of 30 without atopy. Cells from individuals expressing

the R576 allele responded to IL-4 with higher levels of expression of CD23 than did cells from individuals homozygous for Q576.

The association of the R576 allele with increased IgE production and enhanced induction of CD23 implies that the IL-4R$\alpha$ chain containing R576 signals more vigorously upon IL-4 engagement. It might have been anticipated that a substitution of R for Q at 576 would enhance the activity of the sequence as a Stat-6 docking site. This expectation is based on the fact that the sequences surrounding Y603 and Y631 are GYKXF; these two sites were identified by Hou et al (157) in their studies of peptide inhibition of Stat-6 dimerization. Large peptides containing these core sequences inhibited Stat-6 dimerization, leading the authors to identify those sites as Stat-6 docking sites. They failed to detect the sequence surrounding Y575, which has a GYQXF core; Ryan et al were able to demonstrate that the Y575 site was a Stat-6 docking site by mutational analysis. Thus, the presence of a K (or the closely related residue R) rather than a Q in the Y+1 position might be anticipated to improve the Stat-6 activation function of the receptor. However, the authors of the report showed that the substitution of R for Q at this site diminished the affinity of the peptide for SHP-1, suggesting that the R575 form of the receptor might be less subject to the action of phosphatases and thus might signal more vigorously. Somewhat surprisingly, unpublished observations involving both R576Q and Y575F mutant receptors transfected into M12 lymphoma cells revealed no differences in their capacity to induce CD23 expression in response to IL-4 (AD Keegan, JJ Ryan, unpublished observations).

## MODULATION OF IL-4 RECEPTOR SIGNALING PATHWAYS

Recent evidence has emphasized the importance of regulatory pathways that function to modulate intracellular signals initiated by IL-4 and other cytokines. Just as the IRS-1/2 and Jak/Stat signaling pathways are activated through several different cytokine and growth factor receptors, certain negative regulatory pathways also appear to be involved in the regulation of signaling by different cytokine receptors.

### *General Signal Modulation: The Role of Phosphotyrosine Phosphatases*

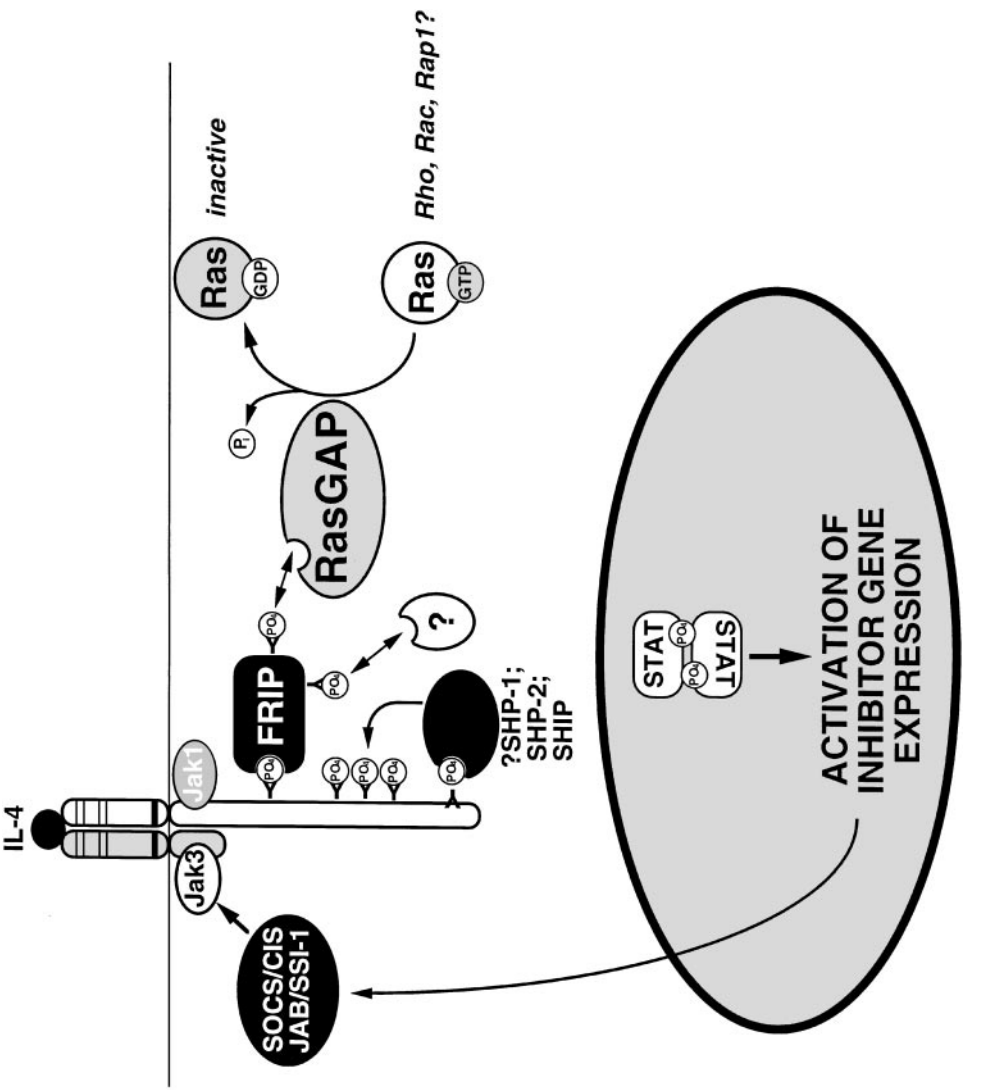
The tyrosine phosphorylation and interaction of signaling proteins represent the foundation of many signaling pathways. General control of tyrosine phosphorylation of signaling molecules is accomplished through the action of phosphotyrosine phosphatases (PTP). The SH2-containing phosphatases SHP-1 and SHP-2 and the SH2-containing inositol-5-phosphatase (SHIP) have been recognized

to be critical modulators of cytokine signaling (158). Although specific roles played by SHP-1/2 and SHIP in IL-4 signaling have yet to be fully delineated, inhibition of phosphatase activity can result in Jak1 and Stat-6 activation, suggesting a modulatory role of these enzymes in IL-4R signaling (159).

SHP-1 and SHP-2 are highly related and share a number of structural characteristics including tandem SH2 domains located in the N-terminal region. These SH2 domains are thought to be critical for linking SHP-1/2 to phosphorylated receptors and proteins, leading to their dephosphorylation. In particular, a specific sequence motif (ΦxYxxL), termed an immunoregulatory tyrosine-based inhibitory motif or ITIM, is found in the cytoplasmic domains of the Fc$\gamma$RIIb1 immunoglobulin receptor, KIR and CTLA-4 molecules and functions in the generation of negative regulatory signals by these receptors (158, 160–163). When phosphorylated, ITIMs serve as docking sites for the SH2 domains of SHP-1/2 and SHIP. The IL-4R also has a sequence related to the ITIM motif in its C-terminus that may play a role in regulating IL-4-stimulated signal transduction by interacting with SHP-1/2 or SHIP (Figure 4) (discussed below). Indeed, Marsh et al have reported that SHP-1 is associated with the IL-4R$\alpha$ chain in unstimulated cells (164). SHP-2 has also been shown to constitutively associate with Jak1 and Jak3 and to co-precipitate with IRS-1, Grb2 and the p85 subunit of PI-3-kinase after cytokine stimulation (165, 166). However, SHP-2 did not associate with IRS-1 or IRS-2 after IL-4 treatment (167).

Recent crystallographic evidence has indicated that the tandem SH2 domains of SHP-1/2 play a pivotal role in the regulation of SHP-1/2 activity. The structure of SHP-2 indicates that its N-terminal SH2 domain binds to the catalytic domain and in so doing blocks both the phosphatase active site and the phosphopeptide recognition site (168); the C-terminal SH2 domain is still capable of binding to phosphopeptides. Thus, the activation of SHP-2 has been proposed to require the sequential interaction of its SH2 domains with phosphoproteins. SHP-2 binding would be initiated through the interaction of the C-terminal SH2 domain with appropriate target sequences, localizing the N-terminal SH2 domain in the proximity of other tyrosine phosphorylated sequences. These phosphorylated sequences would then achieve a high enough local concentration to compete for binding to the N-terminal SH2, thus freeing the phosphatase active site and activating phosphatase activity.

While SHP-2 is expressed in many tissues, SHP-1 is expressed primarily in hematopoietic tissue. The importance of SHP-1 in cytokine signaling has been indicated from analysis of mice homozygous for the *motheaten* allele that present marked hyperproliferation of hematopoietic cells (169). The *motheaten* phenotype results from point mutations in the SHP-1 gene that cause aberrant splicing of the SHP-1 transcript. Activation of macrophages from motheaten mice by interferon-$\alpha$ results in a dramatic increase in Jak1 phosphorylation



leading to the suggestion that SHP-1 acts directly on this kinase (170). In contrast, the activity of a second Jak kinase activated by interferon-$\alpha$, Tyk2, is not elevated in macrophages from motheaten mice. This demonstrates that SHP-1 is critical to the regulation of Jak-1 activity and suggests that SHP-1 may modulate the activity of Jak-1 that is induced by heterodimerization of the IL-4R$\alpha$ chain and $\gamma$c as a result of binding of IL-4.

In contrast to SHP-1/2 that act on phosphoproteins, SHIP acts on the 5′ phosphates of PtdIns(3,4,5)$P_3$ and thus appears to regulate the PI-3-kinase pathway by dephosphorylating the products of this enzyme (158). This phosphatase activity, however, does not necessarily result in the negative regulation of the PI-3-kinase pathway. In particular, the formation of PtdIns(3,4)$P_2$, a critical activator of the anti-apoptotic kinase Akt, results from the dephosphorylation of PtdIns(3,4,5)$P_3$ by SHIP (103). SHIP can interact with phosphorylated ITIM motifs such as that in the C-terminus of the IL-4R$\alpha$ (158, 171). IL-4 will stimulate the tyrosine phosphorylation of SHIP (119). However, the Y713F mutant of the human IL-4R$\alpha$ chain expresses the capacity to signal SHIP phosphorylation. IL-4 can also induce the association of SHIP with Shc as has been observed with other cytokines (172, 173). Therefore, SHIP could potentially be recruited to the IL-4 receptor complex by at least two different mechanisms, by direct docking to the ITIM site at Y713 or by indirect recruitment through binding to Shc at the I4R-motif (Y497).

The function of the C-terminal ITIM of the IL-4R$\alpha$ and, more generally, the role of phosphatases in IL-4 signaling pathways have yet to be delineated. Initial deletion studies indicated that the C-terminal region containing the ITIM motif was dispensable for short-term proliferation, gene induction, and tyrosine phosphorylation of Stat-6 and IRS-1/2 in response to IL-4 (67, 70). In contrast, more recent studies implicate the C-terminal region in certain aspects of IL-4 signaling. In particular, SHP-1 interacts with the IL-4R$\alpha$ after IL-4 treatment and promotes the dephosphorylation of the p85 subunit of PI-3 kinase (164). Phosphopeptide pull-down experiments have indicated that the SHP-1 interaction with the IL-4R$\alpha$ may involve Y575 in the gene regulation domain as well as Y713 in the C-terminal ITIM (156) (P Rothman, personal communication).



*Figure 4* Modulation of IL-4R signaling pathways. General modulation of IL-4R signaling pathways may result from the activation of phosphatases such as SHP-1, SHP-2, and SHIP that interact with phosphorylated Tyr residues of the IL-4R$\alpha$. These phosphatases may attenuate signals by dephosphorylating proteins such as the IL-4R$\alpha$. Activation of Stat-6 results in the expression of the SOCS/CIS/JAB/SSI-1 family of inhibitor proteins that attenuate the Jak-Stat pathway. Activation of small GTPases such as Ras can be modulated through the action of RasGAP, which is recruited to phosphorylated IL-4R$\alpha$ through its interaction with the phosphorylated adapter FRIP.

In spite of these interaction data, receptors with Y575F or Y713F mutations do not show enhanced tyrosine phosphorylation of IRS-1/2 or Stat-6, as would be expected if SHP-1 docking to these sites was blocked (67, 70, 119). Additionally, Y713 and its surrounding amino acid sequence play a positive role in signaling the protection of cells from apoptosis by IL-4 (119). Thus, a precise delineation of the roles of phosphatases in regulating IL-4 signaling pathways and the mechanism of their activation by the IL-4R will require further investigation.

## *Negative Regulation of the Jak-Stat Pathway*

A second regulatory pathway has been described that plays a specific role in the modulation of the Jak-Stat pathway. The regulatory components of this pathway are a series of related SH2-domain proteins whose expression is induced in response to cytokine-induced Stat activation. These molecules, termed CIS (for cytokine-induced SH2), SOCS-1, 2, 3 (for suppressors of cytokine signaling), JAB (for Jak binding), and SSI-1 (for Stat-induced Stat-inhibitor), are expressed within 1 h of cytokine stimulation although the kinetics differed somewhat between the different genes (174–176). The level of expression of each molecule appears to differ depending on the activating cytokine. IL-4 in particular increases CIS and SOCS-3 expression predominantly in bone marrow–derived cells but also stimulates SOCS-1 and SOCS-2 expression to a lesser extent (174). SSI-1 was also induced by IL-4 in the CT.4S cell line (175).

The mechanism by which the CIS/SOCS/JAB/SSI molecules act remains to be elucidated, but they appear to interact directly with and inhibit active Jaks (Figure 4). JAB in particular was cloned based on its capacity to bind the phosphorylated kinase domain of Jak2 (176). This interaction is likely to occur through the SH2 domain. The action of CIS/SOCS/JAB/SSI molecules appears to be specific for Janus kinases as evidenced by the fact that JAB expression diminished Jak1, Jak2, and Jak3 kinase activity while SOCS-1 and SSI-1 specifically abrogated the activation of Stat-3 and gp130 phosphorylation in response to IL-6 (174–176). In contrast, the overall induction of phosphorylation of cellular substrates was unaffected by CIS/SOCS/JAB/SSI expression, indicating that not all tyrosine kinases were inhibited. Since the expression of CIS/SOCS/JAB/SSI is dependent on Stat activation and results in the inactivation of Jaks, the CIS/SOCS/JAB/SSI pathway represents a classical negative feedback loop that specifically modulates the Jak-Stat activation pathway.

It is has not yet been determined if CIS/SOCS/JAB/SSI proteins play a role in the regulation of IL-4R signaling pathways other than the Stat-6 activation pathway. Due to their Jak-inhibitory activity, CIS/SOCS/JAB/SSI expression might be hypothesized to result in a general downregulation of the IRS-1/2 and other pathways through inhibition of Jak-1/3. Indeed, it has been shown that

cell lines expressing a Jak-1 mutation fail to phosphorylate IRS-1 in response to IL-4 stimulation (89, 90). However, the contribution of other IL-4R-associated kinases such as Fes in the activation the IRS-1/2 and other pathways has not yet been elucidated. Thus, if Fes or other non-Jak kinases can function to initiate these IL-4R signaling pathways under certain circumstances or in certain cell types, expression of CIS/SOCS/JAB/SSI may not fully inhibit IL-4R signaling.

### *Negative Regulation of the Ras/MAP Kinase Pathway*

The importance of the Ras/MAPK pathway in the response of cells to IL-4 has not yet been fully resolved. Both IL-4 and insulin stimulation result in the phosphorylation of IRS-2 and its interaction with the adapter molecule Grb2, which in turn can provide a link to the Ras pathway through its potential association with SOS. IL-4 activation of Ras appears to occur in a cell type–dependent manner. By contrast, insulin activation of Ras appears to be more general. It has not yet been determined whether IL-4 stimulates the activation of other small GTPases related to Ras such as Rho, Rac, and Rap1.

A central protein involved in the regulation of small GTPases is the 120 kDa Ras GTPase activating protein (RasGAP) that binds to the active, GTP-bound form of Ras and activates its GTPase activity, catalyzing the formation of inactive Ras-GDP (177, 178). Because of its GTPase activating function, RasGAP is thought to function primarily as a negative regulator of Ras activation (110). An understanding of the processes that lead to RasGAP action on Ras has been enhanced by the identification of two molecules, $p62^{dok}$ and FRIP, that interact with RasGAP.

The $p62^{dok}$ (downstream of kinases) molecule has long been observed as a 62-kDa phosphoprotein that co-precipitates with RasGAP. The *Dok* gene was cloned after purification of the $p62^{dok}$ protein from both Abelson murine leukemia virus (AbMuLV)-transformed cell lines and human chronic myelogenous leukemia cells (179, 180). $p62^{dok}$ has domains common to a number of adapter molecules such as N-terminal PH and PTB domains. In addition, it has a C-terminal region that contains consensus binding sites for the N-terminal SH2 domain of RasGAP (179, 180). The specific function of $p62^{dok}$ remains to be determined, but it has been shown to be phosphorylated in response to stimulation by cytokines including stem cell factor, IL-3 and IL-4 (120, 179).

A second molecule highly homologous to $p62^{dok}$ was cloned based on its ability to bind to the phosphorylated I4R motif of the IL-4R in the yeast two-hybrid system. This molecule, termed FRIP (interleukin-Four Receptor Interacting Protein) has been demonstrated to be phosphorylated in response to different cytokines including IL-4, IL-3, IL-2, and insulin (120). FRIP is highly homologous to Dok, with a 35% overall amino acid identity and 48% identity in the PTB domain. This molecule was also cloned independently from

Bcr-Abl-transformed cells based on its interaction with RasGAP and was termed $p56^{dok\text{-}2}$ (181). In contrast to $p62^{dok}$, which is expressed in a wide variety of tissues, FRIP expression is limited to hematopoietic cells. It is expressed at particularly high levels in T cells and has not been detected in B cells. IL-4-stimulation induced the tyrosine phosphorylation of FRIP; in extracts from IL-4-treated 32D myeloid progenitor cells, a GST-fusion protein containing the N-terminal SH2 domain of RasGAP precipitated phosphorylated FRIP, indicating that such stimulation could increase the interaction of FRIP with RasGAP. Activation of other cytokine receptors such as the IL-2, IL-3, and insulin receptors has also been demonstrated to activate FRIP phosphorylation, presumably due to the presence of NxxY PTB-domain docking motifs in the cytoplasmic domains of these receptors. Thus, FRIP may play a role in the regulation of signaling by a number of cytokines in addition to IL-4.

Mutation of the central Tyr of the I4R motif in the IL-4R to Phe inhibited the IL-4-stimulated phosphorylation of FRIP. The mutation of two Arg residues in the PTB domain of FRIP also dramatically diminished FRIP phosphorylation and prevented its cytokine-induced interaction with RasGAP (K Nelms, unpublished observations). These two Arg residues are homologous to such residues in the PTB domain of IRS-1 that interact directly with the phosphotyrosine residue of the I4R motif of the IL-4R.

Based on these results, we postulate that upon IL-4 stimulation, FRIP interacts with the phosphorylated I4R motif of IL-4R$\alpha$, becomes phosphorylated by receptor-associated tyrosine kinases, and is then bound by the N-terminal SH2 domain of RasGAP (Figure 4). In this way, FRIP can link RasGAP to activated receptor complexes. Such FRIP/RasGAP complexes may function by interacting locally with Ras-GTP and increasing its hydrolysis, thus inactivating the Ras/MAPK pathway. It remains to be determined whether these complexes function with FRIP still bound to the IL-4R$\alpha$ chain or whether the complex diffuses away from the receptor and perhaps remains locally concentrated as a result of the binding of its PH domain to phosphoinositides deposited in the cell membrane as a result of the activation of PI-3 kinase by the receptor.

Evidence supporting this model has come from the observation that the *Frip* gene is linked to the *hairless* locus on mouse chromosome 14 (120). T cells isolated from mice homozygous for the *hairless* allele (*hr/hr*) express three- to fivefold lower levels of FRIP mRNA and protein compared to control T cells. *hr/hr* mice develop splenomegaly, lymphadenopathy and leukemia (182). Their purified T cells respond to anti-CD3 plus IL-2 or IL-4 with a three to fivefold higher level of cytokine-induced proliferation in comparison to control (*+/hr*) T cells. Additionally, overexpression of FRIP in 32D and A.E7 cells decreased MAPK activation and Ras/MAPK pathway-dependent AP-1 transactivation in response to IL-2, respectively (120). Together, these observations strongly

suggest that FRIP plays an important role in negatively regulating cytokine-induced activation of the Ras/MAPK pathway. The contribution of FRIP to the regulation of other pathways regulated by other small GTPases remains to be determined. It also remains to be determined whether FRIP and $p62^{dok}$ function similarly or if the significant sequence differences in the C-terminal regions of these proteins lead to different functionalities.

Additional mechanisms may be involved in the negative regulation of Ras activation in response to IL-4 and other cytokines. Evidence for one such mechanism has come from the study of a state of T cell nonresponsiveness, termed anergy, that is induced through stimulation of the T cell receptor in the absence of co-stimulation. Inhibition of Ras activation is important in maintaining T cell anergy and may result in part from the hyperphosphorylation of the Cbl adapter (183). Cbl hyperphosphorylation leads to its interaction with the Crk/C3G guanine-nucleotide exchange complex that activates the Ras-related protein Rap1 but not Ras. Like Ras, Rap1 is a small GTPase but is not associated with the plasma membrane. Rap1 inhibits activation of the Ras/MAPK pathway probably by acting as a cytoplasmic competitor for Ras effectors such as Raf. Thus, Cbl hyperphosphorylation can result in Rap1 hyperactivation and thus prevent cell proliferation by blocking the activation of the Ras/MAPK pathway. Since IL-4 also induces the phosphorylation of Cbl, it is possible that Cbl phosphorylation may contribute to the regulation of the Ras/MAPK pathway activated by IL-4.

It is interesting to note that FRIP, like Cbl, is hyperphosphorylated in anergic T cells (K Nelms, J Powell, WE Paul, RH Schwartz, unpublished observations). Cbl hyperphosphorylation can account for inhibition of downstream activation of the Ras/MAPK pathway, but it does not account for the inability of anergic T cells to induce Ras activation as measured by accumulation of active Ras-GTP. Thus, hyperphosphorylation of FRIP in anergic T cells could result in heightened levels of membrane-associated RasGAP and specifically could inhibit Ras-GTP accumulation in anergic T cells.

## CONCLUSION

The recent and dramatic expansion in our knowledge of the mechanisms underlying cytokine signaling pathways has led to a better understanding of how cytokines elicit their diverse biological effects. The signaling pathways that are activated by IL-4R engagement, such as the IRS-1/2 and Jak-Stat pathways, mirror those activated by a number of other cytokines. Nevertheless, the activation of these pathways results in a unique array of cellular responses to IL-4. In the case of IL-4, specificity is in part achieved through the activation of Stat-6, an event that, among type I cytokine receptors, has been demonstrated

to occur only through engagement of the IL-4R$\alpha$. An important challenge for the future will be to determine how the activation of similar signaling pathways by different cytokines results in varied biological responses. Specific cellular responses to IL-4 may also result from the unique character of the IL-4R. Indeed, the IL-4R$\alpha$ appears to have a distinct domain structure that results in the activation of a specific array of signaling pathways. Functionally distinct domains of the IL-4R$\alpha$ are required for IL-4 binding, the activation of receptor associated kinases, proliferative pathways, and gene expression. The association of particular functions with particular regions of the receptor suggests that the receptor may have acquired different functions evolutionarily by adding segments with particular functions. Genetic polymorphisms in two of the functional domains have been identified that result in heightened responsiveness to IL-4 and a susceptibility to atopy (49, 156). This emphasizes that gaining a fuller understanding of the signaling processes initiated by IL-4 can make a important contribution to determining the pathogenesis of allergic, anti-parasitic, and autoimmune diseases and may suggest potential opportunities for therapy.





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