Interleukin 4 signals through two related pathways

(cytokine/Janus kinases/signal transducers and activators of transcription)

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ABSTRACT   The interleukin 4 (IL-4) signaling pathway involves activation, by tyrosine phosphorylation, of two distinct substrates, a signal-transducing factor (STF-IL4) and the IL-4-induced phosphotyrosine substrate (4PS). It is not known whether the IL-4-mediated activation of these substrates occurs via related or distinct signaling pathways. We report that 32D cells, an IL-3-dependent myeloid progenitor cell line in which no phosphorylated 4PS is found, activate high levels of STF-IL4 in response to IL-4. Consistent with the known requirement for 4PS or insulin receptor substrate 1 (IRS-1) in IL-4-mediated mitogenesis, activation of STF-IL4 in 32D cells is not sufficient for IL-4-inducible c-*myc* expression. In addition, we have examined the ability of 32D cells transfected with different truncation mutants of the human IL-4 receptor to activate Jak-3 kinase and STF-IL4 in response to human IL-4. As in the case of 4PS/IRS-1, we have found that activation of both Jak-3 and STF-IL4 requires the presence of the IL-4 receptor region comprising aa 437–557. The finding that the same region of the IL-4 receptor is required for the induction of both 4PS/IRS-1 and STF-IL4 suggests that the IL-4-stimulated activation of these two substrates might involve common factors.

Interleukin 4 (IL-4) is a cytokine produced predominantly by T cells, mast cells, and basophils. It stimulates proliferation of T cells, B cells, and mast cells and exerts distinctive biologic effects on a variety of cells (1). The biologic functions of IL-4 are mediated via its binding to a specific cell surface receptor. This receptor, which is widely distributed, has been found to consist of two chains that are members of the type I cytokine receptor superfamily (2), a ligand-binding chain (IL-4R) and a chain, the γ common (γ_c) chain, that is shared with the IL-2, IL-7, IL-9, and IL-15 receptors (3–8, 28).

The ability of IL-4 to stimulate cellular proliferation of the IL-3-dependent myeloid progenitor cell line 32D is dependent on the expression of either IL-4-induced phosphotyrosine substrate [4PS, also termed insulin receptor substrate 2 (IRS-2)] or the antigenically related IRS-1 (a substrate of the insulin receptor kinase) (9, 10). Studies with truncation mutants of the human IL-4R (hIL-4R) have revealed that the region spanning hIL-4R aa 437–557 is essential for IRS-1 phosphorylation and cell proliferation (11). This region contains an insulin receptor motif which includes a tyrosine at position 497. Mutation of tyrosine to phenylalanine (Y497F) results in the inability to adequately phosphorylate IRS-1 and, in most cell lines, to proliferate in response to IL-4. Interestingly, in addition to interacting with IRS-1, the region from the murine IL-4R (mIL-4R) containing aa 437–557 has been shown to coprecipitate an unidentified tyrosine kinase activity (11).

In addition to 4PS phosphorylation, IL-4 can stimulate, presumably via its activation of Jak-1 and Jak-3 kinases (12, 13), the rapid tyrosine phosphorylation of another substrate, a latent cytoplasmic factor, Stat6 (also termed IL-4 Stat) (14). Phosphorylation allows Stat6 to dimerize, forming a signal-transducing factor (STF-IL4) which can then undergo nuclear translocation. Once in the nucleus, STF-IL4 binds to specific DNA sequences related to the interferon (IFN) γ activation site (GAS) (15–17). IL-4 response elements containing these sequences have been identified in the promoters of a variety of genes—e.g., the immunoglobulin heavy-chain germline ε promoter and CD23—whose transcriptional activation is stimulated by IL-4. Recently, a 27-bp segment from the immunoglobulin heavy-chain germline ε promoter which contains the STF-IL4 binding site has been shown to confer IL-4 inducibility on a minimal c-*fos* promoter (18).

The ability of IL-4 to stimulate the activation of distinct substrates raises the question of how these signaling pathways are related. One possibility is that the 4PS/IRS-1 pathway, which has been shown to be critical for IL-4-induced proliferation in one system, may be independent of the Janus kinase (JAK)–signal transducers and activators of transcription (STAT) pathway. Alternatively, the activation of STF-IL4 might be integrally related to the activation of 4PS/IRS-1. Therefore, we have characterized the relationship between 4PS and STF-IL4 by assessing the ability of 32D cells, in which 4PS phosphorylation does not occur, to activate STF-IL4. We found that STF-IL4 induction was not affected by the absence of 4PS/IRS-1. Moreover, the presence of STF-IL4 was not sufficient to turn on c-*myc* expression. To determine whether, as already described for the IL-2 receptor β chain (19), different regions of the IL-4R chain might be linked to the activation of distinct signaling cascades, we investigated the ability of 32D cells transfected with IL-4R truncation mutants to activate STF-IL4. Surprisingly, induction of STF-IL4, as well as phosphorylation of Jak-3, required the presence of the hIL-4R region aa 437–557, which has been shown to be critical for 4PS/IRS-1 phosphorylation and cell proliferation. Thus, although STF-IL4 induction can occur without 4PS/IRS-1 phosphorylation, the activation of these molecules requires similar regions within IL-4R, suggesting that their activation might require common factors.

MATERIALS AND METHODS

Cells and Reagents. The 32D cell line was cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum, 50

μM 2-mercaptoethanol, 2 mM L-glutamine, and 5% WEHI-3B conditioned medium (9). Derivation and culture conditions of 32D cells overexpressing mIL-4R in the presence (32D.IL4R.IRS-1) or absence (32D.IL4R) of rat IRS-1 have been described (10). 32D cells expressing the wild-type hIL-4R or various deletion (d557 and d437) or substitution (Y497F) mutants were prepared and maintained as described (11). Cytokine treatments were as follows. 32D cells overexpressing mIL-4R with or without IRS-1 were either stimulated with murine IL-4 (400 units/ml; DNAX) or resuspended in RPMI 1640 medium containing WEHI conditioned medium for 15 min at 37°C for electrophoretic mobility-shift assay (EMSA) or for 30 min at 37°C for Northern blot analysis. 32D cells carrying wild-type or mutant hIL-4R were stimulated with either recombinant murine IL-4 (400 units/ml; DNAX) or recombinant human IL-4 (100 units/ml; Schering–Plough) for 15 min at 37°C for EMSA or for 30 min to 4 hr at 37°C for Northern analysis. Prior to cytokine treatment, all cells were deprived of IL-3 for 2 hr.

EMSAs and Cell Extracts. The preparation and use of oligodeoxynucleotide probes for EMSAs have been described (15, 20). The IRF-1 GAS probe employed in these studies was 5'-gatcGATTTCCCGAAAT-3'. Whole cell extracts were prepared as described (15, 20).

Immunoprecipitations. Extracts were immunoprecipitated with a Jak-3 antiserum (9, 12). The precipitates were fractionated by SDS/7% PAGE prior to immunoblotting with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). Bands were detected by enhanced chemiluminescence (ECL; Amersham).

Northern Analysis. Total RNA was extracted by lithium chloride. Northern blot analysis was performed with 10 μg of total RNA according to standard protocols. The blot was probed with either a murine c-*myc* cDNA or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA labeled by random hexamer priming.

RESULTS

Presence of 4PS/IRS-1 Is Not Required for STF-IL4 Activation. To determine whether STF-IL4 activation can occur independently from that of 4PS/IRS-1, we stimulated 32D cells with IL-3 or IL-4 for 15 min. We then prepared whole cell extracts and assayed them for the induction of DNA-binding activity as detected by EMSA using the GAS element from the IRF-1 gene as a probe (Fig. 1). Previous work has shown that both STF-IL3 (the IL-3-inducible signal-transducing factor) and STF-IL4 bind this element (20). Extract from untreated 32D cells did not contain any DNA-binding activity. However,
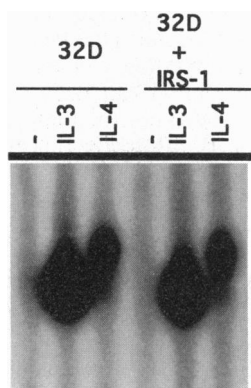

FIG. 1. Induction of STF-IL4 is not affected by the absence of 4PS/IRS-1. Wild-type 32D cells or 32D cells expressing rat IRS-1 (32D + IRS-1) were cultured for 15 min at 37°C in the absence or presence of murine IL-3 or IL-4. Whole cell extracts were examined by EMSA with an IRF-1 GAS probe.

extract from IL-4-stimulated cells revealed a GAS binding activity. IL-3, which is required for the growth of these cells (10), induced a complex whose mobility was clearly distinct from that of STF-IL4. Interestingly, extract from IL-4-stimulated 32D cells which had been transfected with mIL-4R (32D.IL-4R), IRS-1 (32D.IRS-1), or both (32D.IL4R.IRS-1) (10) exhibited an STF-IL4 complex of intensity similar to that of the parent 32D cell line (Fig. 1 and data not shown). Although the STF-IL4 complex detected in 32D cells exhibited a mobility slightly different from that of the STF-IL4 activated by a B-cell line (M12) (15), the 32D STF-IL4 complex was recognized by an antiserum directed against the Src homology domain 2 (SH2) domain of Stat6 (data not shown). In addition, formation of the labeled 32D STF-IL4 complex was blocked in a manner similar to the STF-IL4 complex in B cells by unlabeled GAS-related competitor oligonucleotides and by phosphopeptides derived from IL-4R (14, 21) (data not shown). Thus, the IL-4-inducible GAS binding activity in 32D cells appears to contain Stat6. These data indicate that STF-IL4 activation does not require the presence and/or activation of 4PS/IRS-1. Moreover, the presence of 4PS/IRS-1 does not appear to influence the degree of GAS binding activity induced by IL-4.

STF-IL4 Activation Is Not Sufficient for c-*myc* Expression. 32D cells do not proliferate optimally in response to IL-4 unless they express IRS-1 or 4PS (ref. 10 and J.H.P., unpublished results). Thus, 4PS/IRS-1 appears to be a critical mediator in the IL-4 mitogenic response in at least some cell types. The proliferative effect of IL-4, as well as that of other cytokines, is believed to involve induction of the c-*myc* protooncogene (22). We therefore examined whether IL-4-inducible c-*myc* expression was detectable in cells which activate only STF-IL4 but not 4PS/IRS-1. We performed Northern analysis on RNA extracted from 32D and 32D.IRS-1 cells which had been cultured with either IL-3 or IL-4 for 30 min or 4 hr (Fig. 2 and data not shown). While IL-3 stimulation of cells led to the appearance of a c-*myc*-hybridizing RNA in both 32D and 32D.IRS-1 cells, culture with IL-4 led to significant c-*myc* expression only in 32D.IRS-1 cells. These results indicate that activation of STF-IL4 by itself is not sufficient to induce c-*myc* expression and suggest that optimal c-*myc* inducibility might be a critical feature of 4PS/IRS-1-mediated proliferation.

STF-IL4 Activation Requires IL-4R aa 437–557. To further define the activation requirements for STF-IL4, we investigated the ability of 32D.IRS-1 cells stably transfected with various mutants of hIL-4R (11) to induce the appearance of STF-IL4. Since human and murine IL-4 do not crossreact, the endogenous IL-4R will not interfere with the analysis of the effects of various hIL-4R mutations on STF-IL4 activation. We cultured cells expressing the wild-type or different truncation mutants of hIL-4R with either murine or human IL-4 for 15
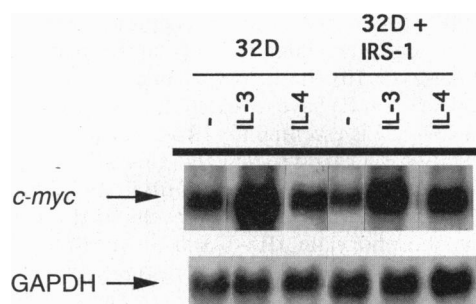

FIG. 2. Presence of STF-IL4 is not sufficient for IL-4-mediated c-*myc* induction. Wild-type 32D cells or 32D cells expressing rat IRS-1 (32D + IRS1) were either unstimulated or stimulated with murine IL-3 or IL-4. Total RNA was extracted for Northern blot analysis with a c-*myc* cDNA (*Upper*) or GAPDH cDNA (*Lower*) probe labeled with ^{32}P by random hexamer priming.
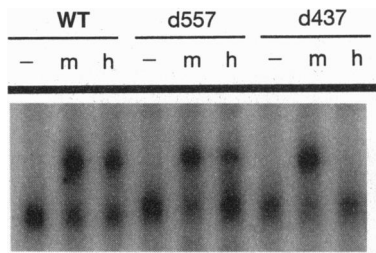
FIG. 3. Effects of hIL-4R deletion mutations on STF-IL4 activation. Transfectants expressing the wild-type hIL-4R (WT) or truncations of this chain to aa 557 (d557) or to aa 437 (d437) were cultured for 15 min at 37°C without cytokine (−) or with either murine IL-4 (m) or human IL-4 (h). Whole cell extracts were examined by EMSA with an IRF-1 GAS probe.

min. Whole cell extracts were then tested in EMSAs (Fig. 3). When the transfectant carrying the wild-type hIL-4R was stimulated with either murine or human IL-4, a GAS binding activity of equivalent intensity was detected. In contrast, when the d557 mutant was cultured with human IL-4, lower levels of STF-IL4 were induced than upon stimulation of these cells with murine IL-4. The d437 transfectant was unable to activate STF-IL4 in response to human IL-4. These same cells were able to activate STF-IL4 in response to murine IL-4. These data indicate that the hIL-4R region between aa 437 and 557 is important in STF-IL4 activation. Maximal induction of STF-IL4, however, requires additional receptor regions.

Optimal STF-IL4 Activation Requires a Tyrosine at Position 497 of hIL-4R. The hIL-4R region from aa 437 to aa 557 contains a sequence motif which is also found in members of the insulin receptor family (11). Within this consensus sequence there is a highly conserved tyrosine residue (at position 497) whose replacement with a phenylalanine residue profoundly affects 4PS/IRS-1 phosphorylation and, in most cases, IL-4-mediated proliferation. To determine whether this tyrosine is critical for STF-IL4 induction, we investigated the ability of Y497F mutants to activate STF-IL4. Cells from various Y497F transfectants were cultured with either murine or human IL-4 as described above. Whole cell extracts were then assayed by EMSA (Fig. 4). Upon treatment with human IL-4, STF-IL4 was not detected in 8-5B6 and 8-4G6, two of the Y497F mutants which do not phosphorylate 4PS/IRS-1 and do not proliferate in response to IL-4. These cells, as expected, activated STF-IL4 in response to murine IL-4. Interestingly, another transfectant, 8-5D3, which proliferates in response to IL-4 in spite of its inability to phosphorylate IRS-1 was able to respond to human IL-4 by activating STF-IL4. The level of STF-IL4 activation was, however, lower than that detected when 8-5D3 was stimulated with murine IL-4. These data

indicate that the tyrosine-497 is important, although not absolutely necessary, for induction of STF-IL4.

IL-4R Region from aa 437 to aa 557 Is Necessary for Jak-3 Phosphorylation. IL-4 stimulation leads to the activation, via tyrosine phosphorylation, of two distinct members of the JAK family of kinases, Jak-1 and Jak-3 (12, 13). As has been shown for other cytokines (23, 24), these two kinases are believed to associate with IL-4R and to be responsible for the phosphorylation, and subsequent complex formation, of STAT proteins—e.g., Stat6—which are specifically recruited to the IL-4R. To evaluate whether the inability to detect STF-IL4 in the d437 transfectant correlates with the inability to activate the JAK kinases, we stimulated cells from the different truncation mutants with either murine or human IL-4 as described above. Extracts from these cells were then immunoprecipitated with an antiserum directed against Jak-3. The precipitated proteins were separated by SDS/PAGE for immunoblot analysis with an anti-phosphotyrosine antibody (Fig. 5). As already observed for STF-IL4 activation, the transfectant carrying wild-type hIL-4R was able to respond to human IL-4 by inducing phosphorylation of Jak-3 in an amount similar to that detected in response to murine IL-4. The d557 mutant, instead, exhibited a lower level of Jak-3 phosphorylation in response to human IL-4 than in response to murine IL-4. The d437 transfectant was unable to respond to human IL-4 with detectable levels of Jak-3 phosphorylation but retained its ability to phosphorylate Jak-3 upon stimulation with murine IL-4. Reprobing this filter with a Jak-3 antiserum confirmed that cells from the different transfectants contained equivalent amounts of Jak-3 (data not shown). Thus, Jak-3 phosphorylation is impaired when the IL-4R region from aa 437 to aa 557 is missing. In parallel to the results obtained for STF-IL4, optimal Jak-3 activation requires regions of the IL-4R in addition to aa 437–557.

IL-4-Mediated Proliferation Correlates with IL-4 Induction of c-*myc* Expression. All of the Y497F transfectants have lost their ability to phosphorylate 4PS/IRS-1 (11). One of the Y497F mutant lines (8-5D3), however, still proliferates in response to IL-4 (11). To determine whether IL-4-mediated proliferation in this cell line correlates with IL-4-induced c-*myc* expression, we carried out Northern blot analysis of RNA hIL-4R mutants which had been cultured with murine or human IL-4 (Fig. 6A). In the Y497F mutants, induction of c-*myc* in response to IL-4 was seen only in the mutant (8-5D3) which is still able to proliferate in response to IL-4. Interest-
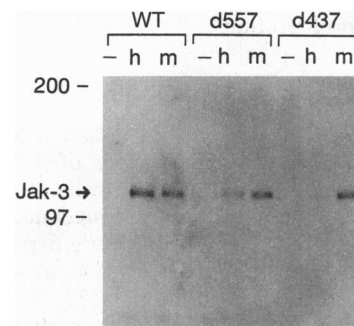
FIG. 5. Effects of hIL-4R deletion mutations on Jak-3 activation. Transfectants expressing the wild-type hIL-4R (WT) or transfectants expressing truncations of this chain to aa 557 (d557) or to aa 437 (d437) were cultured for 15 min without cytokine (−) or with either human IL-4 (h) or murine IL-4 (m). Extracts were prepared from cells as described in Fig. 2. Extracts were then immunoprecipitated with a Jak-3 antiserum. The proteins were separated by SDS/7% PAGE and analyzed by Western blotting using an anti-phosphotyrosine antibody. The blot was subsequently stripped and reprobed with a Jak-3 antiserum, which revealed equivalent levels of Jak-3 among the different extracts (data not shown). Positions of 200-kDa and 97-kDa size markers are shown.
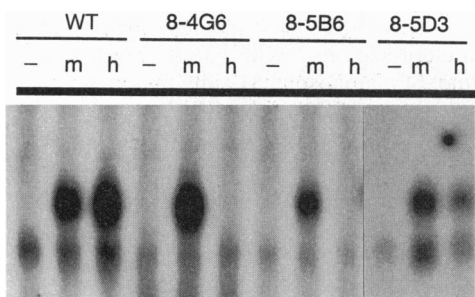

FIG. 4. Effects of Y497F substitution on STF-IL4 activation. Transfectants expressing the wild-type hIL-4R (WT) or three different transfectants expressing Y497F substitution mutant (8-4G6, 8-5B6, and 8-5D3) were cultured for 15 min at 37°C without cytokine (−) or with either murine IL-4 (m) or human IL-4 (h). Whole cell extracts were examined by EMSA with an IRS-1 GAS probe.
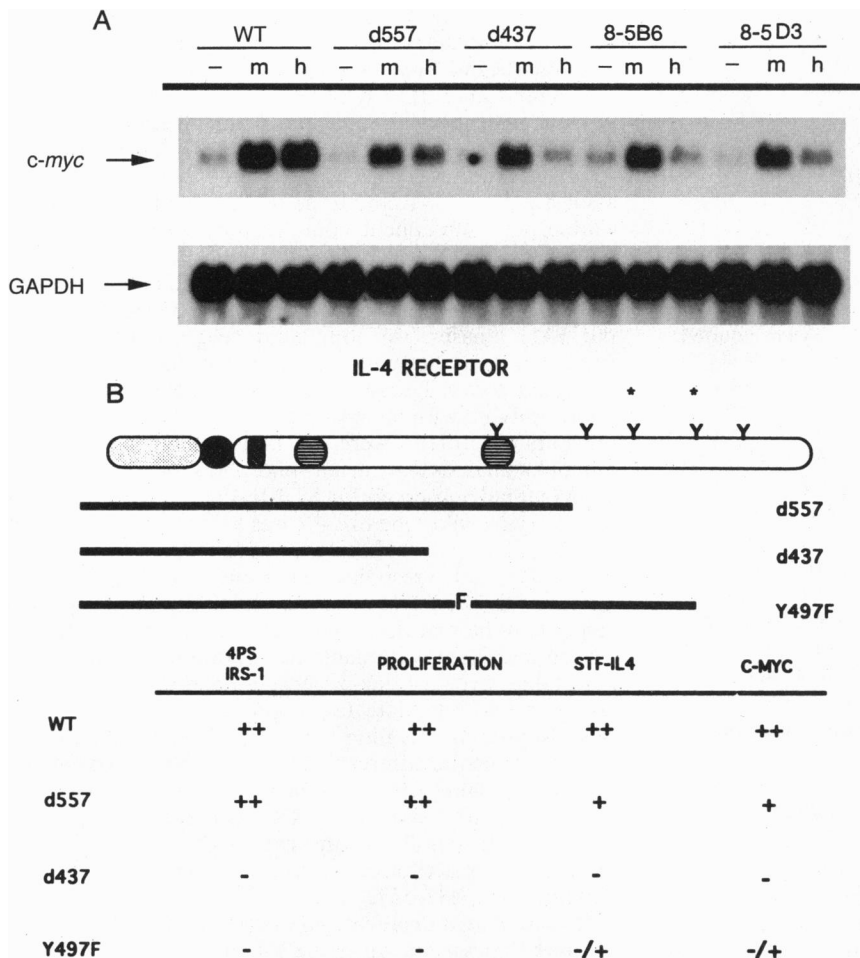
FIG. 6. (*A*) Effects of various hIL-4R mutations on IL-4-mediated c-*myc* induction. Transfectants expressing the wild-type hIL-4R (WT) or truncations to aa 557 (d557) or to aa 437 (d437), Y497 mutant (transfectants 8-5B6 and 8-5D3) were unstimulated (−) or stimulated for 15 min with either murine IL-4 (m) or human IL-4 (h). Total RNA was then extracted for Northern blot analysis with a c-*myc* cDNA (*Upper*) or a GAPDH cDNA (*Lower*) probe labeled by random hexamer priming. (*B*) Schematic representation of the intracytoplasmic regions of the hIL-4R. Tyrosine residues (Y) and acidic domains (stripped circles) within the cytoplasmic tail are indicated.

ingly, this is the only Y497F mutant which can activate STF-IL4. In addition, consistent with its proliferation in response to IL-4, the d557 mutant responded to human IL-4 by activating c-*myc* expression, although at levels lower than that seen in response to murine IL-4 or in the wild-type receptor transfectant. The d437 mutant, which cannot proliferate in response to IL-4, has lost its ability to respond to IL-4 by activating c-*myc*. These data suggest that there is a strict correlation between IL-4 inducibility of c-*myc* expression and IL-4-mediated proliferation.

DISCUSSION

Our studies indicate that IL-4 can induce the activation of STF-IL4 in 32D cells in the absence of 4PS/IRS-1. The presence of STF-IL4 alone in these cells, however, is not sufficient for c-*myc* induction or IL-4-mediated mitogenesis. Interestingly, in a series of transfectants carrying different hIL-4R mutants, induction of STF-IL4, as well as Jak-3 phosphorylation, maps to IL-4R aa 437–557. This region is also known to be important for 4PS/IRS-1 phosphorylation and for IL-4-induced proliferation. In addition, IL-4-mediated proliferation appears to correlate with its ability to induce c-*myc* expression (Fig. 6*B*).

Our data point to a complex relationship between 4PS/IRS-1 and STF-IL4. Indeed, in 32D cells, STF-IL4 activation does not require 4PS/IRS-1. However, our studies with transfectants expressing hIL-4R mutants demonstrate that activation of STF-IL4 and 4PS/IRS-1, as well as Jak-3 phosphorylation, maps to the same region of hIL-4R. Moreover, recent work has shown that IRS-1 coprecipitates with Jak-1 (25), whose presence is essential for STF-IL4 induction (A.P.,

unpublished results). Thus activation of these two IL-4 substrates may share common features. We speculate that phosphorylation by Jak-1 might be the common link in the IL-4-mediated activation of STF-IL4 and 4PS. Although the JAKs have been shown to bind to the proximal cytoplasmic region (box 1 with or without box 2) of cytokine receptors (24), IL-4R aa 437–557 may be essential for optimal Jak-1 activation—e.g., by allowing specific conformational changes or effective Jak-1/Jak-3 cross-phosphorylation.

The increasing inability to activate STF-IL4 by transfectants carrying progressively larger deletions of the intracytoplasmic region of hIL-4R is in accordance with recent studies showing that Stat3 activation by the IL-6 family of cytokines depends on specific modular tyrosine-based motifs (26). Thus the d557 mutant, which retains one of the five conserved tyrosines, is still able to activate STF-IL4, albeit at lower levels. Surprisingly, immunoprecipitation with a Stat6 antiserum has failed to demonstrate Stat6 phosphorylation in the d557 mutant in response to human IL-4 (27). This discrepancy might be explained by a greater sensitivity of EMSA, as compared with immunoprecipitation, to detect STF complexes. The d437 transfectant, on the other hand, which does not contain any of the conserved tyrosines, has lost the ability to activate STF-IL4. The inability of the d557 mutant to achieve full activation of STF-IL4 is consistent with at least two interpretations: (*i*) the presence of additional tyrosines might be able to recruit more Stat6 molecules to the receptor and therefore increase the substrate for the JAK-associated phosphorylation or (*ii*) receptor regions distal to aa 557 may possess tyrosine-containing motifs which, when phosphorylated, bind Stat6 with a higher affinity than those which are membrane-proximal to aa 557. This is consistent with the differential

ability of various phosphopeptides derived from hIL-4R to disrupt the formation of Stat6 dimers *in vitro* (14) Although the phosphopeptide derived from the sequence surrounding tyrosine-497 was unable to prevent Stat6 homodimer formation *in vitro*, our data indicate that the mutant hIL-4R in which the other conserved tyrosines have been deleted (d557) can activate STF-IL4 in response to IL-4. This suggests that this receptor motif may function *in vivo* as a docking site for Stat6.

Substitution of phenylalanine for the conserved tyrosine-497 appears to abolish the ability of the transfectant to activate 4PS and, in most cases, STF-IL4. Functionally, these changes are reflected in lack of IL-4-mediated c-*myc* induction and proliferation. The inability of most Y497F mutants to activate STF-IL4 is again consistent with the requirement of specific phosphotyrosine motifs for STAT activation as described above. One of the Y497F transfectants (8-5D3) exhibits an unusual phenotype. This transfectant has previously been shown to proliferate in response to IL-4 in spite of having lost the ability to phosphorylate IRS-1 (11). Here, we show that this same transfectant, unlike the other Y497F mutants, can activate STF-IL4 and turn on c-*myc* transcription in response to IL-4. The reason for this difference among the Y497F transfectants is unclear. Our results reveal that although the induction of STF-IL4 does not require the presence of IRS-1/4PS, the activation of these two substrates requires similar regions of hIL-4R. It is likely that the activation of these two signaling substrates will require a common factor, perhaps one or both of the IL-4-activated JAK kinases. Alternatively, activation of STF-IL4 may precede that of 4PS/IRS-1 in the IL-4-mediated signaling cascade. Thus, 4PS/IRS-1 phosphorylation might be dependent on STF-IL4 activation. It remains to be established whether STF-IL4 and 4PS activate distinct or overlapping downstream targets.

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