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A Single Tyrosine of the Interleukin-9 (IL-9) Receptor Is Required for STAT Activation, Antiapoptotic Activity, and Growth Regulation by IL-9

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Interleukin-9 (IL-9), a T-cell-derived cytokine, interacts with a specific receptor associated with the IL-2 receptor γ chain. In this report, we analyze the functional domains of the human IL-9 receptor transfected into mouse lymphoid cell lines. Three different functions were examined: growth stimulation in factor-dependent pro-B Ba/F3 cells, protection against dexamethasone-induced apoptosis, and Ly-6A2 induction in BW5147 lymphoma cells. The results indicated that a single tyrosine, at position 116 in the cytoplasmic domain, was required for all three activities. In addition, we observed that human IL-9 reduced the proliferation rate of transfected BW5147 cells, an effect also dependent on the same tyrosine. This amino acid was necessary for IL-9-mediated tyrosine phosphorylation of the receptor and for STAT activation but not for IRS-2/4PS activation or for JAK1 phosphorylation, which depended on a domain closer to the plasma membrane. We also showed that JAK1 was constitutively associated with the IL-9 receptor. Activated STAT complexes induced by IL-9 were found to contain STAT1, STAT3, and STAT5 transcription factors. Moreover, sequence homologies between human IL-9 receptor tyrosine 116 and tyrosines of other receptors activating STAT3 and STAT5 were observed. Taken together, these data indicate that a single tyrosine of the IL-9 receptor, required for activation of three different STAT proteins, is necessary for distinct activities of this cytokine, including proliferative responses.

Interleukin-9 (IL-9) is a multifunctional cytokine secreted by activated T cells (35). Biological targets for IL-9 include mast cells, B lymphocytes, T-cell clones, hematopoietic progenitors, and immature neuronal cell lines (27, 28). In addition, IL-9 seems to be involved in both human and murine tumorigenesis. In vitro, IL-9 induces proliferation of T-cell lymphomas and protects them against dexamethasone-induced apoptosis (30). In vivo, IL-9 transgenic mice spontaneously develop lymphomas and are highly susceptible to chemical mutagenesis (29). Finally, the existence of an IL-9-mediated autocrine loop in Hodgkin's disease has been suggested (9, 21).

IL-9 activities are mediated through a receptor that belongs to the hematopoietin receptor superfamily (26). Like other members of this family, the cytoplasmic part of the human IL-9 receptor (hIL-9R) does not bear any consensus sequence for tyrosine kinase domains and was only characterized by a high percentage of proline and serine residues. Several observations have stressed similarities between IL-9R and IL-2R. The intracellular juxtamembrane region of hIL-9R, which contains the box 1 consensus sequence (24), shows a significant homology with the IL-2 receptor β chain (IL-2Rβ). Moreover, although hIL-9R is sufficient for high-affinity binding of IL-9, it was recently shown to interact with the IL-2R γ chain (γ_c) (17, 31). On the basis of this observation, IL-9 was associated with the IL-2 cytokine family, which includes IL-2, IL-4, IL-7, and IL-15.

These cytokines share common signal transduction pathways. They indeed involve the same JAK tyrosine kinases, namely, JAK3, which was shown to associate with γ_c, and JAK1, which interacts with IL-2Rβ and IL-4R (30, 37). Furthermore, IL-2, IL-7, and IL-15 activate a similar set of STAT transcription factors, including STAT5 (12, 20), whereas IL-4 activates STAT6 (11). Depending on the cell lines used, distinct STAT proteins, including STAT1 (39), STAT3 (38), and STAT5 (18), have been shown to be activated in response to IL-9. In addition, both IL-9 and IL-4 were found to activate an insulin receptor substrate 1 (IRS-1)-related protein (38).

In the present work, we analyzed the structure-function relationship of the hIL-9R by using a set of mutated human receptors. We demonstrated that a single tyrosine was required for IL-9-mediated Ly-6A2 gene induction and antiapoptotic activity as well as both positive and negative growth regulation. We showed that this tyrosine was phosphorylated after IL-9 binding and mediated STAT1, STAT3, and STAT5 activation but not IRS-2/4PS phosphorylation. Finally, we showed that JAK1 was constitutively associated with the IL-9R and that its activation depended on a region more proximal to the plasma membrane. In contrast with previous reports with IL-2Rβ and IL-4R (7, 10, 11, 34), our data suggest that a STAT-binding tyrosine of the hIL-9R is necessary for growth regulation and protection against apoptosis.

MATERIALS AND METHODS

Cells and cell culture. BW5147, a murine lymphoma cell line, was cultured in Iscove-Dulbecco's medium supplemented with 10% fetal calf serum, 0.24 mM asparagine, 1.5 mM glutamine, 0.55 mM arginine, and 50 μM 2-mercaptoethanol. Ba/F3 is a pro-B cell line (kindly donated by R. Palacios, Basel Institute for Immunology, Basel, Switzerland [25]) maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and IL-3 (250 U/ml, produced in CHO transfected cells; kindly donated by A. Burgess, Ludwig Institute, Melbourne, Australia). The human macrophage cell line THP1 was cultured in RPMI medium supplemented with 10% fetal calf serum. Recombinant hIL-9, mouse IL-4 (mIL-4), mIL-6, and mIL-9 were produced in the baculovirus system and purified as previously described (6, 13). Recombinant mouse gamma interferon (IFN-γ)

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and hIL-4 were kindly provided by W. Fiers (University of Ghent, Ghent, Belgium) and J. Banchereau (Schering-Plough, Dardilly, France), respectively.

Production of anti-hIL-9R antibodies. P815 mastocytoma cells transfected with hIL-9R cDNA in the pEF-BOS plasmid were injected into DBA/2 mice. These cells initially formed a tumor that was ultimately rejected. The sera of these mice had high titers of anti-hIL-9R antibodies and were used in immunoblot experiments. Several hybridomas specific for the hIL-9R were derived from these immunized animals. Two such hybridomas, AH9R1 [immunoglobulin E(κ)] and AH9R2 [immunoglobulin G2a(κ)], were used in the present experiments.

Plasmid constructions, DNA transfection, and analysis of transfected cells. For stable transfections, the hIL-9R cDNA h9RA6 was subcloned in the pEF-BOS plasmid (22), in which a puromycin resistance gene for the selection of transfectants has been inserted. Mutagenesis was performed by PCR as described previously (1), and the coding sequence of each mutant was checked with a Δ*Taq* sequencing kit (Amersham, Arlington Heights, Ill.). Plasmid DNA was prepared with a Nucleobond kit (Macherey-Nagel).

BW5147 and Ba/F3 were transfected by electroporation (1,200 μF, 74 Ω, and 260 V and 1,500 μF, 74 Ω, and 300 V, respectively) with 50 μg of sterile DNA. Clones and pools of transfected cells were selected with puromycin (1.5 and 3 μg/ml for BW5147 and Ba/F3, respectively), and hIL-9R expression was checked by fluorescence-activated cell sorter (FACS) analysis with biotinylated anti-hIL-9R antibody AH9R1 followed by phycoerythrin-conjugated streptavidin (Becton Dickinson). Pools corresponding to ~100 transfectants were used for most experiments described here. For BW-h9R and BW-phe116 transfectants (BW5147 cells expressing the wild-type hIL-9R and the hIL-9R with Tyr-116 mutated to Phe, respectively), pools and four independent clones were used with similar results in every bioassay and further analysis was performed with clones.

Tritiated-thymidine incorporation, propidium iodide staining, and hexoseaminidase assays were performed as previously described (30). Ly-6A2 induction was monitored after a 24-h stimulation by FACS analysis with biotinylated anti-Ly-6A/E antibodies (Pharmingen, San Diego, Calif.) followed by phycoerythrin-conjugated streptavidin.

Immunoprecipitations and immunoblot analysis. For JAK1 phosphorylation analysis, cells (5×10^7) were stimulated with 1,000 U of IL-9 per ml for 5 min in 25 ml of medium. JAK1 was then immunoprecipitated according to the antibody manufacturer's recommendations (Upstate Biotechnology Inc., Lake Placid, N.Y.). Briefly, cells were lysed in 1 ml of modified ice-cold RIPA buffer (1% Nonidet P-40, 0.25% deoxycholate, 50 mM Tris [pH 8], 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin [both 10 μg/ml]). Cell debris was removed by centrifugation, and supernatants were incubated for 1 h with 4 μl of anti-JAK1 antiserum. Protein A-linked agarose (30 μl; Gibco-BRL, Gaithersburg, Md.) was added to the lysate before overnight incubation at 4°C. After extensive washing of the beads, proteins were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 3 min, separated on a precast SDS-PAGE (6% polyacrylamide) gel (Novex) and transferred to Hybond-polyvinylidene difluoride (Amersham). The membranes were first immunoblotted with antiphosphotyrosine antibody 4G10 (1 μg/ml; Upstate Biotechnology Inc.) and then reprobed with anti-JAK1 antiserum (1/1,000) under incubation conditions recommended by Amersham for visualization by enhanced chemiluminescence detection (ECL kit; Amersham). IRS-2/4PS tyrosine phosphorylation in transfected Ba/F3 cells was analyzed by a similar protocol. These cells were starved for 6 h in Dulbecco's modified Eagle medium containing bovine serum albumin (5 mg/ml) before stimulation with hIL-9 (1,000 U/ml). Anti-IRS-2 antiserum (Upstate Biotechnology Inc.) was used at 4 μg/ml for immunoprecipitation and 1 μg/ml for immunodetection.

In coimmunoprecipitation experiments, 2×10^8 stimulated cells were lysed in the same buffer as that used for JAK1 immunoprecipitation, except that deoxycholate was replaced by 0.8% Brij 96 (Sigma) and the Nonidet P-40 concentration was reduced to 0.2%. Cell lysates were first treated for 1 h with 50 μl of protein A-agarose (Gibco-BRL) and centrifuged. Supernatants were incubated for 2 h with 10 μg of anti-hIL-9R antibody AH9R2 and for 2 additional h with 30 μl of protein A-agarose. Immunoblotting was performed with anti-JAK1 antiserum, and membranes were reprobed with anti-hIL-9R antiserum (1/500) as a control.

In order to check for IL-9R phosphorylation, we incubated the cells (10^8) for 5 min with or without hIL-9 (1,000 U/ml) at 37°C and then for 1 h at 4°C with 5 μg of AH9R2 monoclonal antibodies in 10 ml of buffer (Hanks balanced salt solution, 3% fetal calf serum, 0.02% sodium azide). Subsequently, cells were washed and processed as described for JAK1 phosphorylation analysis. Immunoprecipitation was controlled by reprobing the membrane with anti-IL-9R antiserum (1/500).

Electrophoretic mobility shift assay. Nuclear extracts were obtained as described previously (32), with minor modifications. Briefly, cells (1.5×10^7) were stimulated for the indicated period with IL-9, IFN-γ, IL-6, or IL-4 (1,000 U/ml except for IL-6 [50,000 U/ml] and hIL-4 [200 U/ml]), washed with phosphate-buffered saline (PBS), and resuspended in 1 ml of ice-cold hypotonic buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer [pH 7.5] containing 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, aprotinin [10 μg/ml], 1 mM Na_3VO_4, and 5 mM NaF) for 15 min. The cells were lysed by adding 65 μl of 10% Nonidet P-40 and vortexing. Nuclei were pelleted (30 s at 20,000 × *g*) and extracted over 30 min in 100 μl of hypertonic buffer B (buffer A supplemented with 20 mM HEPES, 20% glycerol, and 420 mM NaCl). Nuclear debris was removed by a 15-min centrifugation. Analysis of DNA binding activity was performed as described previously (5) with a ^{32}P-labeled oligonucleotide probe based on the 3′ 18 bp of the gamma response region (GRR) of the FcγRI gene (8): upper strand, 5′-ATGTATTTCCCAGAAA-3′; lower strand, 5′-CCTTTTC TGGGAAATAC-3′. In brief, 5-μl volumes of nuclear extracts were preincubated in binding buffer [12 mM HEPES (pH 7.6), 10 mM KCl, 0.5 mM EDTA, 2.5% glycerol, 0.1 mg of poly(dI-dC) · poly(dI-dC) (Sigma) per ml] for 5 min. Radiolabeled GRR probe (10^5 cpm; ~0.5 ng) was added, and the incubation was continued for 25 min before loading on a 5% polyacrylamide gel. For gel shift inhibition, phosphopeptide TLAY^{P}LPQEDWA (superscript P indicates that Y is phosphorylated) and the unphosphorylated control peptide were synthesized, according to the 9-fluorenylmethoxycarbonyl method, and added to the nuclear extract 30 min before incubation with the oligonucleotide as described previously (11).

Analysis of GRR-binding activity by immunoblotting. Cells (10^8) were stimulated by cytokines for 15 min as described above, washed, and incubated for 10 min on ice in 1 ml of hypotonic buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM NaF). After Dounce homogenization, nuclei were pelleted (15 min, 3,300 × *g*) and incubated for 30 min in 500 μl of extraction buffer (20 mM HEPES [pH 7.8], 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM NaF). Nuclear extracts were clarified by centrifugation, diluted with 1 ml of electrophoretic mobility shift assay binding buffer, and incubated first for 1 h with agarose-linked streptavidin (Pierce) to remove nonspecific material and then overnight with immobilized GRR oligonucleotide (obtained by incubating 40 μl of agarose-linked streptavidin with 0.2 μg of double-stranded GRR oligonucleotide biotinylated at the 5′ end of the upper strand [Eurogentec, Liège, Belgium]). Agarose-bound materials were washed and immunoblotted as described above with antiphosphotyrosine 4G10, anti-STAT1α (Santa Cruz Biotechnology, Santa Cruz, Calif.), anti-STAT3, anti-STAT4, anti-STAT5 or anti-STAT6 monoclonal antibodies (Transduction Laboratories, Lexington, Ky.), all at 1 μg/ml in PBS.

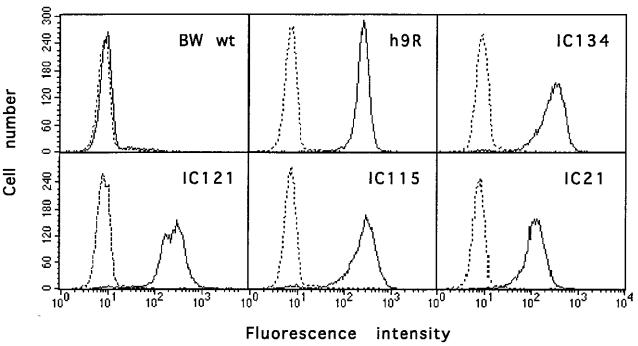

FIG. 1. hIL-9R expression in transfected cells. Cells were incubated sequentially with biotinylated AH9R1 antibody and phycoerythrin-labeled streptavidin before FACS analysis. For autofluorescence controls, the antibody was omitted (dashed line). wt, wild type.

RESULTS

A single tyrosine of the hIL-9R is involved in protection against apoptosis and Ly-6A2 induction. In a previous report, we showed that transfection of the hIL-9R cDNA into mouse cells conferred responsiveness to hIL-9 (26). To dissect the functional domains of the cytoplasmic part of the hIL-9R, we chose to use a similar approach with mouse T-lymphoma BW5147, whose response to mIL-9 includes Ly-6A2 up-regulation (20a) and protection against dexamethasone-induced apoptosis (30). BW5147 cells were stably transfected with the wild-type hIL-9R and with a series of C-terminally truncated receptors including IC134, IC121, IC115, and IC21, which retain 134, 121, 115, and 21 of the 231 cytoplasmic hIL-9R amino acids, respectively. hIL-9R expression was monitored by FACS analysis with biotinylated monoclonal anti-IL-9R antibody AH9R1, and similar expression levels were found in each transfectant tested (Fig. 1).

Next, we assessed whether these cells could respond to hIL-9. We observed (Fig. 2) that hIL-9, like its murine ho-

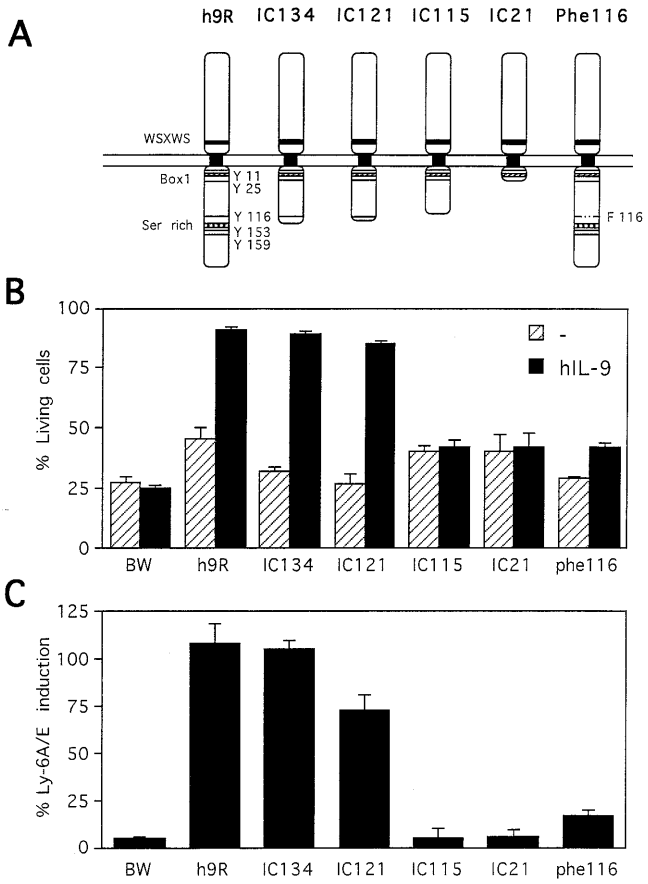

FIG. 2. IL-9-induced antiapoptotic activity and Ly-6A2 expression in BW5147 cells expressing wild-type or mutant hIL-9R. (A) Schematic representation of the wild-type and mutated hIL-9Rs transfected into BW5147 cells. Numbers refer to the amino acids remaining in the intracytoplasmic domain (IC) of the truncated receptors. The tryptophan-serine motif (WSXWS) and the transmembrane domain are depicted by solid boxes. The serine-rich domain and box 1 are indicated by shaded boxes. All five tyrosines (Y) located in the cytoplasmic tail of the receptor and the phenylalanine (F) mutation are indicated. hIL-9R expression in transfected cells is shown in Fig. 1, except for BW-phe116, which expressed a similar level of hIL-9R (data not shown). (B) BW5147 and derived transfected cells (30,000 cells per well) were incubated for 24 h in the presence of dexamethasone (100 ng/ml) and cyclosporine (500 ng/ml) (which enhances apoptosis [30]) with or without (−) hIL-9 (500 U/ml). Cell viability was measured by FACS analysis after staining with propidium iodide. One significant experiment out of three is shown (error bars, standard deviations measured from triplicate cultures). (C) Ly-6A2 induction was assessed by FACS analysis after a 24-h incubation with IL-9. A ratio (expressed as a percentage) was obtained by dividing the mean channel increase in response to hIL-9 (500 U/ml) by the mean channel increase in response to mIL-9 (500 U/ml). Typical Ly-6A2 induction in BW-h9R by mIL-9 was 2.2-fold ± 0.2-fold. Data presented here correspond to the means of at least three independent experiments ± standard errors of the means (error bars).

molog, conferred protection against dexamethasone and upregulated Ly-6A2 gene transcription in cells expressing the wild-type hIL-9R (called BW-h9R). The 97 C-terminal amino acids, including the serine-rich motif, could be deleted without affecting hIL-9 activity (mutant IC134 in Fig. 2). When the receptor was further truncated (IC121), removing a proline-rich sequence (Pro-124...Pro-130–Ala–Pro–Pro), IL-9-mediated Ly-6A2 induction was slightly diminished but the antiapoptotic effect was not modified. Finally, cells transfected with shorter receptors such as IC115 and IC21 no longer responded to hIL-9 (Fig. 2), thereby underlining the importance of residues 116 to 121 (Tyr-116–Leu–Pro–Gln–Glu–Asp).

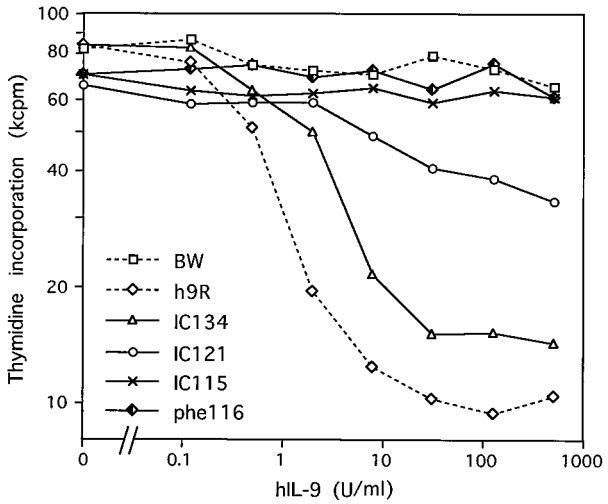

FIG. 3. Growth inhibition of BW5147 transfectants by hIL-9. Cells were seeded in microtiter plates (1,000 cells per well) in the presence of the indicated concentrations of hIL-9. Cell proliferation was assessed by measuring [^{3}H]thymidine incorporation into DNA after 4 days of culture. Results are representative of three independent experiments. Standard deviations were calculated from triplicate cultures and were less than 10%. Typically, addition of hIL-9 on BW-h9R resulted in an increase in doubling time from 12 to 24 h (data not shown).

Since phosphorylated tyrosines are known to play a key role in cytokine receptor signal transduction, we mutated tyrosine 116 into phenylalanine. The response to hIL-9 in cells transfected with this modified receptor (BW-phe116) was greatly diminished (Fig. 2), demonstrating the importance of this single tyrosine in these two bioassays.

Both growth inhibition and stimulation by IL-9 are dependent on the same tyrosine. BW5147 lymphoma cells spontaneously proliferate in culture, irrespective of any cytokine addition. Unexpectedly, we observed that the BW-h9R proliferation rate diminished in the presence of hIL-9. Furthermore, when our series of mutant hIL-9Rs were tested in this assay, we found that the effect was dependent on the presence of tyrosine 116 in the transfected receptor (Fig. 3). However, we observed that IC121 receptor was less efficient, suggesting that the proline-rich sequence deleted in this receptor may play a significant role in this process.

Inhibition of BW-h9R proliferation by hIL-9 contrasted with the previously described growth-promoting activity of IL-9 for a number of factor-dependent cell lines (28, 35, 36). To determine whether transfection of the hIL-9R could also confer IL-9-dependent proliferation, we transfected the IL-3-dependent pro-B cell line Ba/F3, which has been successfully used by several groups for similar receptor studies (4, 7, 24, 34). As shown in Fig. 4, hIL-9 could indeed replace IL-3 when Ba/F3 expressed the wild-type hIL-9R, IC134, or IC121, although IC121 conferred a weaker IL-9 responsiveness. By contrast, in cells transfected with IC115, IC21, or phe116 receptor, the response to IL-9 was very low or absent (Fig. 4). Taken together, these experiments demonstrated that tyrosine 116 was not only important for IL-9-mediated gene induction and protection against apoptosis but also paradoxically required for both positive and negative regulation of growth by hIL-9.

IL-9R tyrosine 116 is required for receptor phosphorylation. The requirement of a tyrosine for receptor function suggested that this residue could be phosphorylated in response to IL-9. To test this hypothesis, we immunoprecipitated the hIL-9R with a specific monoclonal antibody, AH9R2, and subsequently performed an immunoblot assay with an antiphospho-

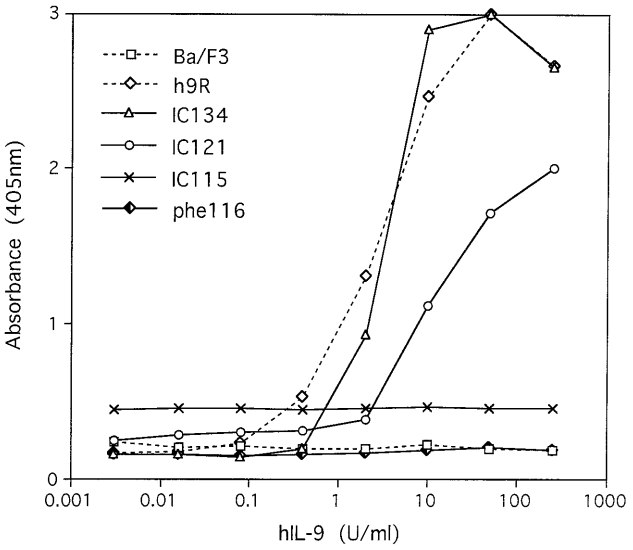

FIG. 4. Proliferative responses of Ba/F3 cells expressing wild-type or mutant IL-9R. Cells were seeded in microtiter plates (3,000 cells per well) in the presence of the indicated concentrations of hIL-9. Cell numbers were evaluated after 3 days by measuring hexoseaminidase activity. Shown are the means of duplicate cultures, which varied by less than 10%. All transfectants responded equally to IL-3 (data not shown). Results are representative of three independently performed experiments.

tyrosine antibody (Fig. 5). In the absence of ligand, no tyrosine-phosphorylated receptor was detected in BW-h9R cells. Following the addition of IL-9, an early phosphorylation of the receptor was observed. By contrast, we did not detect any phosphorylated receptor in BW-phe116 cells, suggesting that tyrosine 116 is the only phosphorylated tyrosine of the hIL-9R.

JAK1 is activated by IL-9 and constitutively associated with IL-9R. The IL-9R does not contain any tyrosine kinase consensus sequence, but IL-9 was recently shown to activate JAK1 and JAK3 tyrosine kinases (31, 39). In the IL-2R and IL-4R complexes, JAK3 is associated with γ_c, a common component also interacting with hIL-9R. On the other hand, JAK1 has been shown to associate with IL-4R (39) and IL-2Rβ (31),

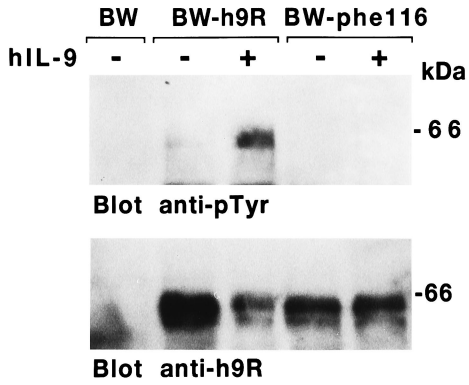

FIG. 5. Tyrosine 116 is necessary for IL-9R phosphorylation. Cells were incubated for 3 min with 1,000 U of IL-9 per ml. hIL-9R was immunoprecipitated as described under Materials and Methods, analyzed by SDS-PAGE, and subjected to Western blotting with 4G10 monoclonal antiphosphotyrosine antibody. The membrane was reprobed with anti-IL-9R antiserum. A control experiment was performed with nontransfected BW5147 cells (BW), demonstrating the specificity of anti-hIL-9R antibodies. Similar results were obtained after up to 30 min of IL-9 stimulation (data not shown).

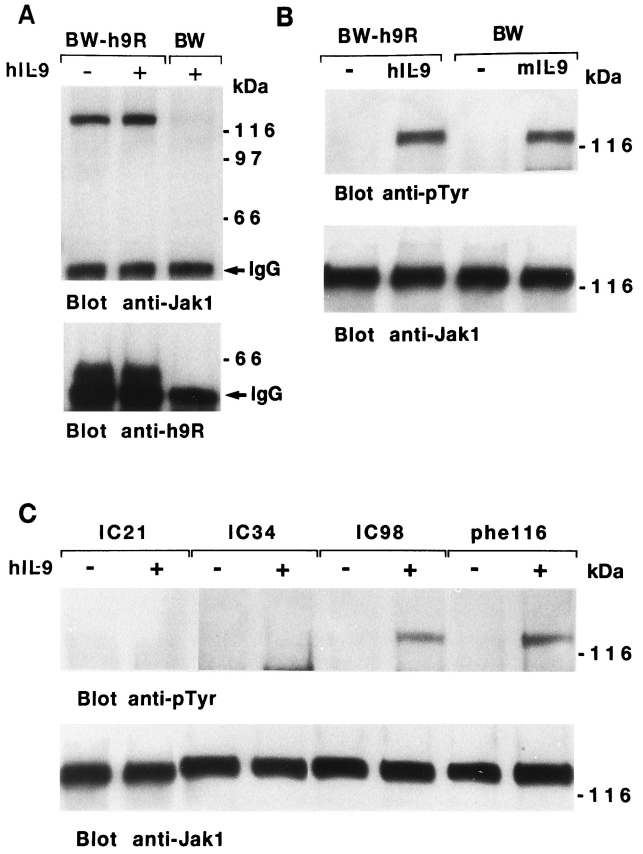

FIG. 6. JAK1 kinase phosphorylation in response to IL-9 and association with hIL-9R. BW5147 cells not transfected or expressing the indicated receptor were stimulated for 5 min with 1,000 U of IL-9 per ml. (A) hIL-9R was immunoprecipitated as described under Materials and Methods. Immunoblots were performed sequentially with anti-JAK1 and with anti-hIL-9R antisera. (B and C) Jak1 was immunoprecipitated from stimulated cells and sequentially subjected to Western blotting with antiphosphotyrosine and with anti-JAK1 antiserum.

which have partial homology with hIL-9R. We therefore tested whether JAK1 could coimmunoprecipitate with hIL-9R. As shown in Fig. 6A in BW-h9R, JAK1 was detected after anti-IL-9R immunoprecipitation, independently of IL-9 stimulation, thereby demonstrating its constitutive association with the IL-9R. JAK1 activation, however, required IL-9 binding to the receptor (Fig. 6B). To determine which region of the hIL-9R was responsible for interaction with JAK1, we tested receptors with deletions, including two additional truncated forms, IC34 and IC98 (which were not functional in all tests described above [data not shown]), for JAK1 activation. As illustrated in Fig. 6C, hIL-9 induced phosphorylation of JAK1 neither in BW-IC21, which includes the box1 homology sequence, nor in BW-IC34, which contains the domain homologous to IL-2Rβ. However, JAK1 was activated in BW-IC98 and BW-phe116. In conclusion, the mutation of tyrosine 116 did not affect JAK1 activation, which was dependent on amino acids 34 to 98 of the hIL-9R cytoplasmic domain.

IL-9-induced GRR-binding activity depends on tyrosine 116 and contains STAT1, STAT3, and STAT5. It is well established that phosphotyrosines constitute docking sites for SH2 domain-containing proteins, including the STAT transcription factors, which are substrates for JAK kinases (8, 14). In this study, we analyzed the IL-9-induced STAT activation by electrophoretic mobility shift assays using a ^{32}P-labeled oligonucleotide derived from the GRR of the FcγRI gene promoter. This motif is known to avidly bind STAT factors, including

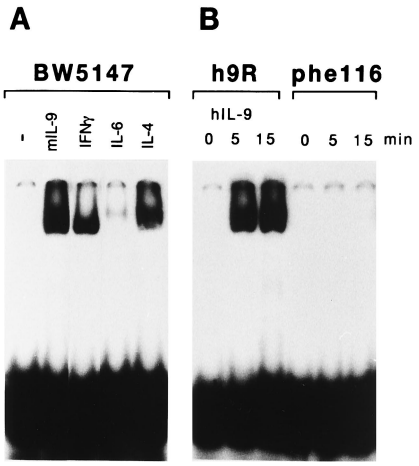

FIG. 7. IL-9-induced GRR-binding activity in BW5147, BW-h9R, and BW-phe116. (A) Gel shift assays were performed with nuclear extracts prepared from wild-type BW5147 cells treated or not with mIL-9 or IFN-γ (both treated for 30 min) or IL-6 or IL-4 (both treated for 15 min). IL-9-induced DNA-binding complex was selectively inhibited by a cold competitor excess (data not shown). (B) The same experiment was performed with BW5147 cells expressing wild-type hIL-9R or phe116 mutant receptor after stimulation with hIL-9.

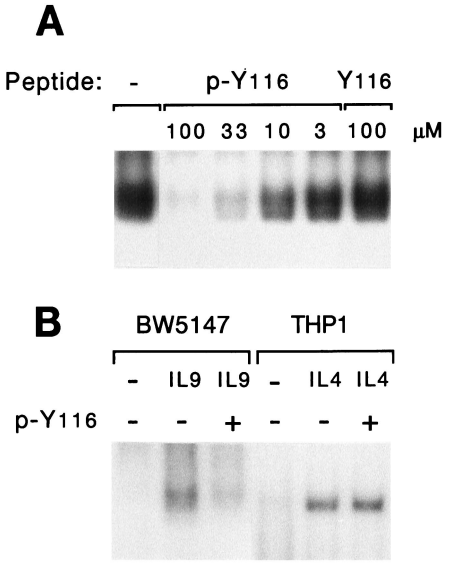

FIG. 8. A phosphopeptide corresponding to IL-9R tyrosine 116 inhibits IL-9-induced GRR-binding activity. (A) Nuclear extracts from IL-9-treated BW5147 cells (1,000 U of mIL-9 per ml, treated for 30 min) were incubated with the indicated concentrations of the phosphopeptide corresponding to hIL-9R tyrosine 116 (p-Y116) prior to the binding assay with a labeled GRR oligonucleotide. A control experiment was performed with the nonphosphorylated peptide (Y116). (B) The same experiment was performed with BW5147 cells stimulated by IL-9 and THP1 cells treated with IL-4. The phosphopeptide concentration was 33 μM.

STAT1, -3, -4, -5, and -6 (8, 11, 12, 15, 20). As illustrated in Fig. 7A, nuclear proteins from mIL-9-stimulated BW5147 cells were able to associate with the GRR oligonucleotide. Stimulation of BW5147 cells with IFN-γ, IL-6, or IL-4 resulted in slightly different band shifts (Fig. 7A). A GRR-binding activity was also observed in BW-h9R stimulated with hIL-9, but not in BW-phe116 (Fig. 7B), indicating that tyrosine 116 is involved in STAT activation. To further confirm this hypothesis, we tested the ability of a phosphorylated peptide corresponding to this region of the hIL-9R to disrupt activated STAT dimers and thereby GRR binding. As shown in Fig. 8A, the phosphopeptide completely abolished all IL-9-induced band shifts, while the control nonphosphorylated peptide had no effect. By contrast, the STAT6-mediated band shift induced by IL-4 in THP1 cells (11) was not affected by this phosphopeptide (Fig. 8B). Moreover, other phosphorylated peptides, corresponding to the other tyrosines of the IL-9R, did not affect IL-9-induced band shift (data not shown). These observations indicate that, upon phosphorylation, tyrosine 116 specifically interacts in vitro with IL-9-activated STAT proteins.

In order to determine further which STAT proteins were activated by IL-9 in BW-h9R cells, we used immobilized biotinylated GRR oligonucleotide, which allowed us to partially purify IL-9-induced GRR-binding complexes from nuclear extracts. We then tested the presence of particular STAT proteins in these complexes by Western blot (immunoblot) analysis. These experiments demonstrated the activation of STAT1α, STAT3, and STAT5 in response to IL-9 (Fig. 9). As expected, these factors are likely to be phosphorylated on tyrosine, as shown when antiphosphotyrosine 4G10 antibody is used (Fig. 9). We also noticed that STAT3 and STAT6 factors were weakly preactivated in BW-h9R cells. Taken together, these results indicate that tyrosine 116 was required for IL-9-induced GRR-binding activity, which contained STAT1α, STAT3, and STAT5.

IL-9-induced IRS-2/4PS activation does not depend on tyrosine 116. Previous reports indicate that both IL-4 and IL-9 induce the phosphorylation of IRS-2/4PS (39), an adapter protein reportedly involved in several activation pathways (16). Moreover, IRS-2/4PS has been shown to be a major mediator of the IL-4R signal transduction, particularly regarding proliferation (16). We therefore tested if tyrosine 116 of the hIL-9R plays any role in IRS-2/4PS activation. As shown in Fig. 10, a similar IRS-2/4PS phosphorylation was induced by IL-9 in Ba/F3 cells transfected either with the wild-type hIL-9R or with the mutated receptor lacking tyrosine 116. This experiment indicates that IRS-2/4PS activation is sufficient neither for inducing Ba/F3 cell proliferation nor for the other activities of IL-9 analyzed above.

DISCUSSION

The results presented in this work underscore the importance of hIL-9R tyrosine 116 in IL-9 signal transduction. Indeed, deletion or mutation of this amino acid suppressed receptor tyrosine phosphorylation, STAT activation, and all the IL-9 biological activities that were examined, namely, Ly-6A2 induction, antiapoptotic activity, and both negative and positive growth regulation. On the other hand, JAK1 phosphory-

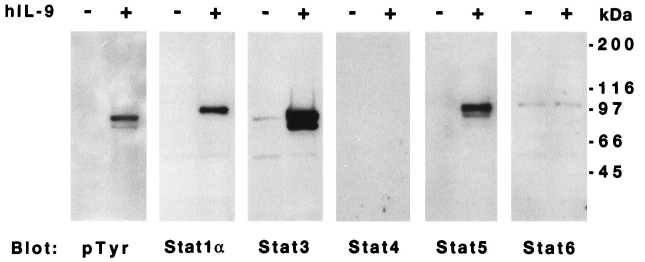

FIG. 9. STAT1, STAT3, and STAT5 are present in IL-9-induced GRR-binding proteins. GRR-binding complexes were isolated from nuclear extracts of 10^8 untreated or IL-9-stimulated BW-h9R cells, using immobilized GRR oligonucleotide. DNA-bound proteins were analyzed by Western blotting with antiphosphotyrosine-, anti-STAT1α-, anti-STAT3-, anti-STAT4-, anti-STAT5-, or anti-STAT6-specific monoclonal antibodies.

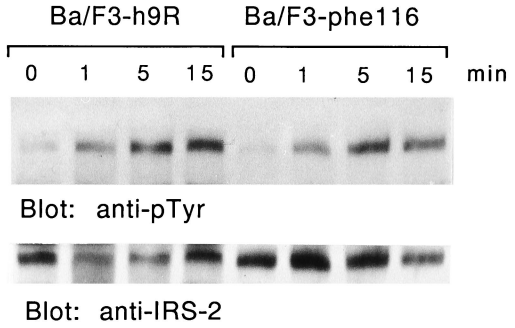

FIG. 10. IL-9 induces IRS-2/4PS phosphorylation independently of hIL-9R tyrosine 116. Ba/F3 cells expressing the indicated receptor were starved for 6 h before IL-9 stimulation (1,000 U/ml). IRS-2/4PS was immunoprecipitated with a specific antiserum and subjected to Western blot analysis sequentially with antiphosphotyrosine and anti-IRS-2/4PS antibodies.

lation did not depend on this tyrosine, which is compatible with a role of JAK1 kinase upstream of tyrosine 116 phosphorylation and STAT activation. Furthermore, we demonstrated the constitutive association of JAK1 with the IL-9R. A similar observation has been made with IL-2Rβ (31) but not with IL-4R, which fixes JAK1 only after ligand binding (38). We further analyzed requirements for JAK1 activation and showed that it depended on the first 98 amino acids of the cytoplasmic part, whereas the box 1 and the IL-2Rβ homology regions were not sufficient.

Accumulating data indicate that JAK kinases directly phosphorylate STAT transcription factors bound to phosphotyrosine residues of activated cytokine receptors (14). In contrast with that of the majority of the cytokine family receptors (8, 33), hIL-9R phosphorylation was found to be dependent on only one of its five cytoplasmic tyrosines. In addition, we showed that mutation of this single amino acid or in vitro competition with the corresponding phosphopeptide abolished IL-9-induced GRR-binding activity, which included STAT1, STAT3, and STAT5. This suggests that tyrosine 116, phosphorylated upon IL-9 binding, acts as a docking site for at least three distinct STAT proteins.

STAT5 activation in response to IL-9 is an additional feature shared by IL-9, IL-2, IL-7, and IL-15 in signal transduction. In line with this observation, we found a significant homology in the amino acids surrounding hIL-9R tyrosine 116 and other tyrosines involved in STAT5 activation by IL-2, IL-7, prolactin, and erythropoietin (Fig. 11A). This raises the possibility that the Ala-phosphotyrosine-Leu sequence, surrounded by negatively charged amino acids (Asp or Glu), could be important in STAT5 recognition. In addition, this region of the hIL-9R also fits the consensus sequence for STAT3 binding site YXPQ (33), deduced from IL-6R component gp130 and leukemia inhibitory factor receptor (Fig. 11B). Taken together, these observations suggest that positions −1 and +1 relative to the tyrosine, with negative charges in the vicinity, could determine STAT5 recruitment, whereas positions +2 and +3 would be important for STAT3.

Besides the key role of tyrosine 116, the downstream region could also cooperate in signal transduction. Although the truncated receptor IC121 retained tyrosine 116, IC121-transfected cells presented a response to hIL-9 slightly diminished relative to those of wild-type and IC134 receptors. This was particularly clear for Ba/F3 proliferation and BW5147 growth inhibition, but Ly-6A2 induction was also significantly lowered. The region from position 121 to 134 of the receptor cytoplasmic tail includes a proline-rich sequence flanked by an arginine (Arg-

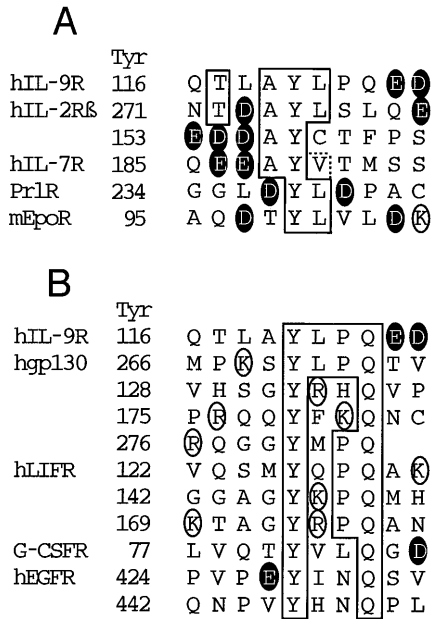

FIG. 11. Homology within amino acids surrounding hIL-9R tyrosine 116 and receptor tyrosines involved in STAT3 or STAT5 activation. Shown is the alignment of the hIL-9R sequence containing tyrosine 116 with the regions of cytokine receptors known to mediate STAT5 activation in IL-2Rβ (20), IL-7R (20), prolactin receptor (PrlR) (19, 37), and erythropoietin receptor (mEpoR) (4) (A) and with tyrosines involved in STAT3 activation (2, 33) (B). The numbers refer to the positions of the critical tyrosines in the cytoplasmic domain of the receptors. Boxes denote the conserved amino acids. Positive and negative residues are represented in open and solid circles, respectively. Abbreviations for tyrosines: hgp130, human glycoprotein 130; hLIFR, human leukemia inhibitory factor receptor; G-CSFR, granulocyte colony-stimulating factor receptor; hEGFR, human epidermal growth factor receptor.

128–Pro–Ala–Pro–Pro), a feature shared by SH3-binding domains (3). Since the STAT proteins contain a putative SH3 domain (14), it is possible that this proline-rich region of hIL-9R stabilizes the interaction with STAT proteins, thus enhancing signal transduction.

We observed that STAT activation correlates with IL-9-mediated proliferation, protection against apoptosis, and Ly-6A2 induction, suggesting that STAT factors could be involved in these effects. This has been demonstrated for Ly-6A2 induction. Indeed, STAT proteins bind to the IFN-γ activation site element, which was found to be necessary for the IL-9-dependent activation of the promoter of this gene (20a). The role of STAT proteins in the regulation of proliferation by cytokines is still ill-defined. Mutagenesis of IL-2Rβ, IL-4R, and other cytokine receptors has shown that STAT-binding tyrosines could be deleted without affecting growth-promoting activity, at least in Ba/F3 or related models (7, 10, 11, 17). By contrast, recent reports have shown that expression of dominant negative STAT5 can partially inhibit IL-3-dependent Ba/F3 growth (23) and that erythropoietin receptor tyrosine 343 is involved in both STAT5 activation and proliferation at low erythropoietin concentrations (4). Our data indicate that STAT proteins may play a key role in IL-9-induced proliferation of Ba/F3 cells, although we do not rule out the possibility that other pathways might cooperate with STAT factors. In this respect, the role of IRS-2/4PS activation in response to IL-9 remains to be determined. Here, we show that IRS-2/4PS phosphorylation is not, by itself, sufficient for inducing proliferation and does not depend on hIL-9R tyrosine phosphorylation. The latter finding indicates that the IL-9R and IL-4R activate IRS-2/4PS through distinct mechanisms since IRS-2/4PS is known to associate

with IL-4R through a tyrosine-phosphorylated motif called I4R (16).

Finally, we showed that IL-9 could induce opposite proliferative responses through the same phosphorylated tyrosine of the hIL-9R. This observation suggests an indirect regulation of cell growth by IL-9 and is in line with previous reports indicating that thymocytes, mast cells, and erythroid progenitors only proliferate in response to IL-9 in synergy with other cytokines (27) and that IL-9 is much more efficient as an antiapoptotic factor than as a growth-promoting agent for thymic lymphomas (30). Moreover, the observation that hIL-9 simultaneously protected BW-h9R cells against dexamethasone-induced apoptosis and down-regulated its proliferation demonstrated that the antiapoptotic activity of this cytokine is completely independent from any growth-promoting effect. This experimental model thereby provides us with an attractive tool to analyze the mechanisms by which cytokines regulate apoptosis independently from their proliferative activity.

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REFERENCES

1. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley and Sons, New York.
2. **Coffer, P., and W. Kruijer.** 1995. EGF receptor deletions define a region specifically mediating STAT transcription factor activation. Biochem. Biophys. Res. Commun. **210:**74–81.
3. **Cohen, B., R. Ren, and D. Baltimore.** 1995. Modular binding domains in signal transduction proteins. Cell **80:**237–248.
4. **Damen, J., H. Wakao, A. Miyajima, J. Krosl, R. K. Humphries, R. Cutler, and G. Krystal.** 1995. Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and STAT5 activation. EMBO J. **14:**5557–5568.
5. **de Smet, C., S. Courtois, I. Faraoni, C. Lurquin, J.-P. Szikora, O. De Backer, and T. Boon.** 1995. Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics **42:**282–290.
6. **Druez, C., P. Coulie, C. Uyttenhove, and J. Van Snick.** 1990. Functional and biochemical characterization of mouse P40/IL-9 receptors. J. Immunol. **145:** 2494–2499.
7. **Fujii, H., Y. Nakagawa, U. Schindler, A. Kawahara, H. Mori, F. Gouilleux, B. Groner, J. Ihle, Y. Minami, T. Miyazaki, and T. Taniguchi.** 1995. Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor β chain but is not essential for the proliferative signal transmission. Proc. Natl. Acad. Sci. USA **92:**5482–5486.
8. **Greenlund, A. C., M. Farrar, B. L. Viviano, and R. D. Schreiber.** 1994. Ligand-induced IFNγ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J. **13:**1591–1600.
9. **Gruss, H.-J., M. Brach, H.-G. Drexler, K. Bross, and F. Herrmann.** 1992. Interleukin 9 is expressed by primary and cultured Hodgkin and Reed-Sternberg cells. Cancer Res. **52:**1026–1031.
10. **Harada, N., G. Yang, A. Miyajima, and M. Howard.** 1992. Identification of an essential region for growth signal transduction in the cytoplasmic domain of the human interleukin-4 receptor. J. Biol. Chem. **267:**22752–22758.
11. **Hou, J., U. Schindler, W. Henzel, T. C. Ho, M. Brasseur, and S. McKnight.** 1994. An interleukin-4-induced transcription factor: IL-4 Stat. Science **265:** 1701–1706.
12. **Hou, J., U. Schindler, W. Henzel, S. Wong, and S. McKnight.** 1995. Identification and purification of human Stat proteins activated in response to interleukin-2. Immunity **2:**321–329.
13. **Houssiau, F., J.-C. Renauld, M. Stevens, F. Lehmann, B. Lethé, P. G. Coulie, and J. Van Snick.** 1993. Human T cell lines and clones respond to IL-9. J. Immunol. **150:**2634–2640.
14. **Ihle, J., and I. Kerr.** 1995. Jaks and Stats in signalling by the cytokine receptor superfamily. Trends Genet. **11:**69–74.
15. **Jacobson, N. G., S. J. Szabo, R. M. Weber-Nordt, Z. Zhong, R. D. Schreiber, J. E. Darnell, and K. M. Murphy.** 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. J. Exp. Med. **181:**1755–1762.
16. **Keegan, A., K. Nelms, L. M. Wang, J. Pierce, and W. Paul.** 1994. Interleukin 4 receptor: signaling mechanisms. Immunol. Today **15:**423–432.
17. **Kimura, Y., T. Takeshita, M. Kondo, N. Ishii, M. Nakamura, J. Van Snick, and K. Sugamura.** 1995. Sharing of the IL-2 receptor γ chain with the functional IL-9 receptor complex. Int. Immunol. **7:**115–120.
18. **Kirken, R., H. Rui, G. Malabarba, Z. Howard, M. Kawamura, J. O'Shea, and W. Farrar.** 1995. Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and Stat5 recruitment by a COOH-terminal region of the IL-2 receptor β-chain. Cytokine **7:**689–700.
19. **Lebrun, J., S. Ali, V. Goffin, A. Ullrich, and P. Kelly.** 1995. A single phosphotyrosine residue of the prolactin receptor is responsible for activation of gene transcription. Proc. Natl. Acad. Sci. USA **92:**4031–4035.
20. **Lin, J.-X., T.-S. Migone, M. Tsang, M. Friedmann, J. Weatherbee, L. Zhou, A. Yamauchi, E. Bloom, J. Mietz, S. John, and W. Leonard.** 1995. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity **2:**331–339.

20a. **Maisin, D., et al.** Unpublished data.

21. **Merz, H., F. A. Houssiau, K. Orscheschek, J.-C. Renauld, A. Fliedner, M. Herin, H. Noel, M. Kadin, H. K. Mueller-Hermelink, J. Van Snick, and A. C. Feller.** 1991. IL-9 expression in human malignant lymphomas: unique association with Hodgkin's disease and large cell anaplastic lymphoma. Blood **78:**1311–1317.
22. **Mizushima, S., and S. Nagata.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. **18:**5322.
23. **Mui, A., H. Wakao, T. Kinoshita, T. Kitamura, and A. Miyajima.** 1996. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J. **15:**2425–2433.
24. **Murakami, M., M. Narazaki, M. Hibi, H. Yamata, K. Yasukawa, M. Hamaguchi, T. Taga, and T. Kishimoto.** 1991. Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA **88:**11349–11353.
25. **Palacios, R., and M. Steinmetz.** 1985. IL3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration and generate B lymphocytes in vivo. Cell **41:**727–734.
26. **Renauld, J.-C., C. Druez, A. Kermouni, F. Houssiau, C. Uyttenhove, E. Van Roost, and J. Van Snick.** 1992. Expression cloning of the murine and human interleukin 9 receptor cDNAs. Proc. Natl. Acad. Sci. USA **89:**5690–5694.
27. **Renauld, J.-C., F. Houssiau, J. Louahed, A. Vink, J. Van Snick, and C. Uyttenhove.** 1993. Interleukin 9. Adv. Immunol. **54:**79–97.
28. **Renauld, J.-C., A. Kermouni, A. Vink, J. Louahed, and J. Van Snick.** 1995. Interleukin 9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. J. Leukocyte Biol. **57:**353–360.
29. **Renauld, J.-C., N. van der Lugt, A. Vink, M. van Roon, C. Godfraind, G. Warnier, H. Merz, A. Feller, A. Berns, and J. Van Snick.** 1994. Thymic lymphomas in interleukin 9 transgenic mice. Oncogene **9:**1327–1332.
30. **Renauld, J.-C., A. Vink, J. Louahed, and J. Van Snick.** 1995. IL-9 is a major anti-apoptotic factor for thymic lymphomas. Blood **85:**1300–1305.
31. **Russel, S., J. Johnston, M. Noguchi, M. Kawamura, C. Bacon, M. Friedmann, M. Berg, D. McVicar, B. Witthuhn, O. Silvennoinen, A. Goldman, F. Schmalstieg, J. Ihle, J. O'Shea, and W. Leonard.** 1994. Interaction of IL-2Rβ and γc chains with Jak1 and Jak3: implications for XSCID and XCID. Science **266:**1042–1045.
32. **Schreiber, E., P. Matthias, M. Müller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
33. **Stahl, N., T. Farruggella, T. Boulton, Z. Zhong, J. Darnell, and G. Yancopoulos.** 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science **267:**1349–1353.
34. **Tanigushi, T.** 1995. Cytokine signaling through nonreceptor protein tyrosine kinases. Science **268:**251–255.
35. **Uyttenhove, C., R. Simpson, and J. Van Snick.** 1988. Functional and structural characterization of P40, a mouse glycoprotein with T cell growth factor activity. Proc. Natl. Acad. Sci. USA **85:**6934–6938.
36. **Vink, A., J.-C. Renauld, G. Warnier, and J. Van Snick.** 1993. Interleukin-9 stimulates in vitro growth of mouse thymic lymphomas. Eur. J. Immunol. **23:**1134–1138.
37. **Wakao, H., F. Gouilleux, and B. Groner.** 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J. **13:**2182–2191.
38. **Yin, T., S. Keller, F. Quelle, B. Witthuhn, M. L.-S. Tsang, G. Lienhard, J. Ihle, and Y.-C. Yang.** 1995. Interleukin-9 induces tyrosine phosphorylation of insulin receptor substrate-1 via JAK tyrosine kinases. J. Biol. Chem. **270:** 20497–20502.
39. **Yin, T., M. L.-S. Tsang, and Y.-C. Yang.** 1994. JAK1 kinase forms complexes with interleukin-4 receptor and 4PS/insulin receptor substrate-1-like protein and is activated by interleukin-4 and interleukin-9 in T lymphocytes. J. Biol. Chem. **269:**26614–26617.