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Protein-tyrosine Kinase-dependent Activation of STAT Transcription Factors in Interleukin-2- or Interleukin-4-stimulated T Lymphocytes*

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The proliferation of activated T lymphocytes is critically dependent on the binding of the T-cell growth factors, interleukin (IL)-2 and IL-4, to distinct but evolutionarily related cell surface receptors. Previous results suggest that the IL-2 receptor (IL-2R) and IL-4R are coupled to both overlapping and distinct intracellular signaling pathways in T lymphocytes. In this study, we demonstrate that activation of Janus tyrosine kinases (JAKs) and STAT transcription factors is rapidly induced by exposure of factor-dependent murine T-cell lines to IL-2 or IL-4. Both IL-2 and IL-4 stimulated the rapid activation of JAK1 and JAK3, whereas JAK2 activity was unaffected by either cytokine. These responses were accompanied by the appearance in cell nuclei of 3 DNA binding activities that recognized a high-affinity binding site for STAT factors. In transient transfection assays, this STAT factor target sequence conferred IL-2 and IL-4 inducibility on a synthetic luciferase reporter gene. Antibody supershifting experiments indicated that IL-2 induces the formation of STAT dimers containing STAT3 and STAT1α. Although IL-4 also activated STAT1α, the major IL4-induced STAT factor is not STAT3 and remains undefined. Pretreatment of the T-cells with the protein-tyrosine kinase inhibitor herbimycin A blocked both the nuclear translocation of STAT factors and STAT-dependent reporter gene transcription. Immunoblot analyses confirmed that cytoplasmic STAT3 was heavily phosphorylated on tyrosine in IL-2-stimulated cells, and that phosphorylated STAT3 appeared in the nuclei of these cells. These results indicate that identical JAKs and partially overlapping sets of STATs are activated by IL-2 and IL-4 in T lymphocytes.

Antigenic stimulation renders quiescent T-cells competent to proliferate by inducing the expression of high-affinity surface receptors for T-cell growth factors. The principal growth factors for activated T-cells are interleukin 2 (IL-2)[1] and IL-4. Whereas activated T-cells uniformly respond to IL-2, a more restricted subpopulation of T-helper cells acquires the ability to use IL-4 as an alternative growth factor (1, 2). In addition to their growth-promoting activities, both IL-2 and IL-4 exert pleiotropic stimulatory effects on the survival, differentiation, and effector functions of immature and post-thymic T-cells (3).

The cellular responses to IL-2 and IL-4 are initiated by the interaction of each cytokine with its cognate surface receptor. The high-affinity IL-2 receptor (IL-2R) is a heterotrimeric complex composed of α, β, and γ_c subunits (4, 5). Although expression of all three subunits is required for high-affinity binding to IL-2, the signaling function of this receptor complex resides entirely within the cytoplasmic domains of the β and γ_c polypeptides (5). The high-affinity IL-4R contains a single ligand-binding subunit, which, like the IL-2R β and γ_c chains, shares conserved sequence motifs with members of the cytokine receptor superfamily (6). In the presence of IL-4, the ligand-bound receptor forms functional dimers with the above-described γ_c subunit (7, 8). The presence of a common γ_c cytoplasmic domain in the IL-2R and IL-4R suggests that these receptors recruit at least partially overlapping sets of signal-transducing proteins during stimulation by their respective ligands.

A notable characteristic of the IL-2R and IL-4R, as well as other cytokine receptors, is the absence of consensus sequences indicative of a catalytic function, such as protein kinase activity. Nonetheless, IL-2R occupancy triggers a rapid increase in protein tyrosine phosphorylation in activated T-cells, indicating that this receptor regulates the activities of one or more non-receptor protein-tyrosine kinases (9–11). Although the *src*-related protein-tyrosine kinases, Lck and Fyn, participate in IL-2R signaling (12, 13), other evidence suggests that activation of these protein-tyrosine kinases is not sufficient for the transmission of mitogenic signals from the βγ heterodimer (5). Furthermore, many other cytokines, including IL-4, activate protein-tyrosine kinase-dependent signaling pathways in their target cells, without detectable involvement of members of the Src family (14, 15).

Recent reports have defined a novel pathway for the transmission of regulatory signals from cytokine receptors to the nucleus. Studies of gene activation in interferon (IFN)-treated cells (16–19) revealed that members of a novel protein-tyrosine kinase family, the Janus kinases (JAKs), served as proximal transducers of nuclear regulatory signals from both IFN-α and IFN-γ receptors. The JAK family currently consists of 4 members (JAK1, JAK2, JAK3, and TYK2). Subsequent reports indicated that various JAKs were physically and functionally linked to many members of the cytokine receptor superfamily, including the IL-2R and IL-4R (20–23). A compelling body of data indicates that JAK activation is a crucial event for signal initiation from most if not all members of this receptor family.

Studies of the more distal components of IFN signaling pathways also yielded a novel mechanism for the regulation of gene transcription by hormonal stimuli. Cellular stimulation with either IFN-α or IFN-γ induced the tyrosine phosphorylation of members of a family of latent cytoplasmic transcription factors termed Signal Transducers and Activators of Transcription

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[1] The abbreviations used are: IL, interleukin; IL-2R, interleukin-2 receptor; IL-4R, interleukin-4 receptor; IFN, interferon; SIE, *sis*-inducible element; PAGE, polyacrylamide gel electrophoresis; SH, Src homology; pY, phosphotyrosine.

(STAT) (24). Genetic studies indicated that coupling of the IFN receptors to their cognate JAKs was required for STAT protein phosphorylation (16, 17). The known STAT proteins (at least 6 family members have been described) contain SH2 and SH3 domains, which are commonly found in cytoplasmic signaling proteins, but not in DNA-binding proteins that regulate specific gene transcription. Tyrosine phosphorylation of STATs induces the SH2 domain-dependent dimerization, nuclear translocation, and sequence-specific DNA binding activities of these proteins (24, 25). Because many of the cytokine receptors are coupled to JAKs, it was predicted that these receptors might also regulate gene expression, in part, through STAT protein activation. Subsequently, the receptors for a variety of polypeptide ligands, including epidermal growth factor, IL-3, IL-4, and IL-6, were shown induce the appearance of STAT factors in the nuclei of their host cells (24–30).

We report here that both IL-2 and IL-4 stimulate activation of the JAK-1 and JAK-3 kinases, together with the nuclear translocation of specific STAT family members, in growth factor-dependent T-cell lines. The major STAT protein activated by IL-2 is STAT3, whereas the major species present in nuclei from IL-4-stimulated T-cells may be identical with the IL-4 STAT recently isolated from monocytic cells (31). Finally, IL-2 or IL-4-stimulation induced the transcriptional activation of a STAT-dependent reporter gene in T-cells. These results suggest that the JAK-STAT pathway participates in the regulation of both overlapping and distinct sets of genes by T-cell growth factors.

MATERIALS AND METHODS

Reagents and Antibodies—Recombinant human IL-2 was generously provided by Hoffmann-LaRoche Inc. Recombinant murine IL-4 was supplied by Dr. D. McKean (Mayo Clinic). Rabbit polyclonal antibodies specific for STAT3 and STAT4 were generous gifts of Dr. J. Darnell (Rockefeller University). Rabbit polyclonal anti-JAK-1 (UBI Catalog No. 06-272), -JAK-2 (UBI Catalog No. 06-255), and -JAK-3 (UBI Catalog No. 06-342) antisera, and monoclonal anti-phosphotyrosine (pY) antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal anti-STAT1α (p91) antibody was from Transduction Laboratories (Lexington, KY). Although this antibody exhibits weak cross-reactivity with the related STAT1β (p84) protein, immunoblot analyses indicated that the antibody detected only the STAT1α isoform in the T-cell lines used in this study (results not shown).

Cell Culture, Cytokine Induction, and Preparation of Nuclear Extracts—The CTLL-2 cell line was obtained from American Type Culture Collection. Both HT-2 and CTLL-2 cells were maintained in IL-2-containing culture medium as described previously (32). Cells were deprived of cytokines in serum-free arrest medium (RPMI 1640 medium containing 100 μg/ml bovine serum albumin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10 mM HEPES, pH 7.2) for 4 h prior to restimulation with IL-2 or IL-4. Where indicated, the cells were pretreated with 9 μM herbimycin A or drug vehicle (dimethyl sulfoxide) during the 4-h period of growth factor deprivation.

To prepare nuclear extracts, the cells (1×10^7 cells/sample) were harvested by centrifugation, washed in phosphate-buffered saline, and were lysed for 5 min on ice in 100 μl of lysis buffer (10 mM HEPES, 30 mM NaCl, 20 mM NaF, 1 mM EDTA, pH 8.0, 1 mM dithiothreitol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM sodium orthovanadate, 120 nM okadaic acid, 5 μg/ml each of leupeptin, pepstatin, and aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) containing 500 mM sucrose and 0.2% Triton X-100. All remaining steps were performed at 4 °C or on ice. Nuclei were pelleted for 3 min at 6000 × *g* and were washed twice with 300 μl of wash buffer (lysis buffer containing 25% (v/v) glycerol). The postnuclear supernatants from the initial cell lysate were combined with the nuclear washes to prepare cytosolic extracts. Nuclear proteins were extracted for 20 min in 25 μl of nuclear extract buffer (wash buffer containing 330 mM NaCl). The nuclear extracts were cleared of insoluble material by centrifugation for 10 min at 12,000 × *g*, and the concentration of extracted protein was quantitated using a protein assay kit from Bio-Rad.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed according to a modification of a previously described procedure (33). Briefly, nuclear extract (10 μg of protein) was preincubated for 15 min on ice in poly(dI-dC)-containing binding buffer (see below). Where indicated, cold competitive oligonucleotides or antibodies were included during the preincubation period. The ^{32}P-labeled SIE m67 (27, 34) oligonucleotide probe (2.5×10^4 cpm or approximately 2.5 fmol/reaction) was added, and the reactions were incubated for 15 min at 25 °C. The final binding reactions (20 μl) contained 12.5 mM HEPES, 87.5 mM NaCl, 1 mM dithiothreitol, 0.15 mM EDTA, 0.02% Nonidet P-40, 12.5% glycerol, and 100 μg/ml poly(dI-dC). The samples were electrophoresed through 4% polyacrylamide gels (25:1, acrylamide:bisacrylamide) in 45 mM Tris borate buffer containing 1 mM EDTA, pH 8.0. Gels were dried under vacuum, and radiolabeled species were detected by autoradiography at −70 °C.

Immunoprecipitation, Immunoblotting, and Kinase Assays—For JAK kinase activation assays, factor-deprived cells (5×10^7 cells per sample) were stimulated with cytokines as described above. Cells were harvested by centrifugation, washed in phosphate-buffered saline, and suspended in 1 ml of TNET buffer (20 mM Tris, 40 mM NaCl, 5 mM EDTA, 30 mM $Na_4P_2O_7$, 50 mM NaF, pH 7.4), containing 1% Triton X-100, 0.1% bovine serum albumin, 1 mM sodium orthovanadate, 20 mM *p*-nitrophenyl phosphate, and the protease inhibitor mixture described above. After 10 min on ice, lysates were cleared of insoluble material by centrifugation for 10 min at 12,000 × *g*. The cleared extracts were immunoprecipitated for 1 h with the indicated antisera and protein A-coupled Sepharose beads. The immune complexes were washed twice with TNET buffer and twice with kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, and protease inhibitors). The washed immunoprecipitates were incubated for 30 min at 30 °C in 40 μl of kinase buffer containing 5 mM $MnCl_2$ and 0.5 mCi/ml [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol). After two washes with phosphate-buffered saline containing 5 mM EDTA, the precipitated proteins were eluted with 2 × reducing sample buffer (35), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to Immobilon-P membranes. The membranes were blocked with 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.2% (w/v) Tween 20 (TBST) supplemented with 2% bovine serum albumin. The membranes were subsequently probed with the following antisera, diluted in TBST as indicated in parentheses: anti-pY (100 ng/ml), anti-JAK-1 (1:1000), anti-JAK-2 (1:1000), or anti-JAK-3 (1–1000). Immunoreactive proteins were detected with protein A coupled to horseradish peroxidase and the enhanced chemiluminescence detection (ECL) reagent (Amersham). After immunoblotting, the membrane was treated with 1 M KOH for 2 h at 55 °C, and radiolabeled proteins were detected by autoradiography at −70 °C.

For STAT3 activation assays, 4×10^7 growth factor-deprived cells were stimulated with 50 units/ml IL-2 and harvested by centrifugation. Nuclear and cytosolic extracts were prepared as described above, except that 200 μl of lysis buffer and 0.5 ml of nuclear extraction buffer were used in the preparation of cell extracts. Nuclear extracts were diluted to 1 ml with dilution buffer (10 mM HEPES, pH 8.0, 20 mM NaF, 0.1 mM EDTA, 1 mM dithiothreitol, 40 mM *p*-nitrophenyl phosphate, 1 mM sodium orthovanadate, 1% Triton X-100, and protease inhibitors), while cytosolic extracts were diluted to 1 ml with dilution buffer containing 300 mM NaCl. Extracts were immunoprecipitated for 2 h with STAT3-specific antisera and protein A-Sepharose beads and then washed three times with isotonic wash buffer (dilution buffer containing 150 mM NaCl). Immunoprecipitated proteins were eluted with SDS sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes, and membranes were blocked as described above. The membrane was probed with anti-pY antibody (500 ng/ml) and anti-STAT3 antisera (1:1000), and immunoreactive proteins were detected with the ECL system as described above.

Transcriptional Activation Assays—Complementary oligonucleotides containing three tandem SIE m67 sequences (5′-CATTTCCCGTAAATC-3′) or three SIE m56 (34) sequences (5′-CAGTTCCCTTCAATC-3′) were synthesized with *Bgl*II and *Kpn*I cohesive termini at the 5′ and 3′ ends, respectively. After annealing, the double-stranded oligonucleotides were ligated into the *Bgl*II- and *Kpn*I-digested pGL2pro luciferase reporter plasmid (Promega) immediately upstream of the SV40 promoter. For transient transfections, CTLL-2 cells or HT-2 cells were precultured for 2 h in serum-free arrest medium. The factor-deprived cells (1.5×10^7 cells/sample) were suspended in 0.85 ml of serum-free arrest medium, transferred to a 4-mm cuvette, and transfected by electroporation with 6 μg of empty vector or SIE-containing plasmid. Electroporation was performed with a BTX electroporation system (San Diego, CA) at instrument settings of 400 V, 400 microfarads, and 13 ohms. The transfected cells were divided into three equal aliquots, diluted to 5×10^5 cells per ml, and cultured for 2 h. Herbimycin A was added to one aliquot from each transfection during the 2-h culture period. IL-2 (50 units/ml) or IL-4 (10 units/ml) was added to the indi-

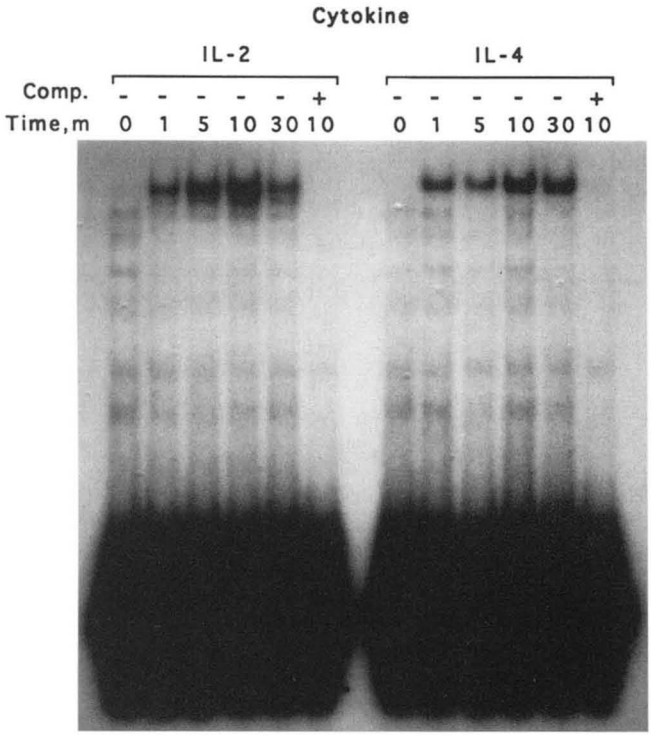

FIG. 1. **Cytokine-induced STAT factor activation.** Factor-deprived CTLL-2 cells were stimulated for the indicated times with 50 units/ml IL-2 or 10 units/ml IL-4. Nuclear extracts were prepared, and DNA binding activities were measured by gel mobility shift assay using a ^{32}P-labeled SIE m67 oligonucleotide probe. Where indicated (+), binding reactions were performed in the presence of a 100-fold molar excess of unlabeled SIE m67 probe (*Comp.*).

cated samples from each transfection, and the cells were cultured for an additional 4 h. Cell lysates were prepared for measurement of luciferase activity as recommended by the manufacturer (Promega).

RESULTS

Induction of SIE Binding Activities by IL-2 and IL-4—The murine cytotoxic T-cell line, CTLL-2, expresses high-affinity surface receptors for IL-2 and IL-4. In serum-containing culture medium, IL-2 functions as both a growth and viability maintenance factor for CTLL-2 cells. IL-4 prolongs the survival but does not support the proliferation of this cell line.[2] In contrast, the murine helper T-cell line, HT-2, exhibits a strong mitogenic response to both cytokines (36). In initial studies, we examined the effects of IL-2 and IL-4 on the activation of STAT proteins in CTLL-2 and HT-2 cells.

The induction of DNA binding activities in the nuclei of cytokine-stimulated T-cells was determined in gel mobility shift assays with an oligonucleotide probe containing a high-affinity target sequence for STAT factors. This m67 target sequence was derived from the *sis*-inducible element (SIE) of the human c-*fos* promoter (27, 34). Nuclear extracts were prepared from CTLL-2 cells at various times after stimulation with IL-2 or IL-4, and the extracts were incubated with the double-stranded, ^{32}P-labeled SIE m67 oligonucleotide probe (Fig. 1). Both IL-2 and IL-4 induced the rapid assembly of SIE m67-binding complexes in CTLL-2 cell nuclei. These complexes were observed within 1 min of cytokine addition and reached maximal levels after 10 min. The putative STAT factor-DNA complexes were effectively competed by a 100-fold molar excess of the unlabeled SIE m67 oligonucleotide, but not of the SIE m56 oligonucleotide (data not shown), which contains a mutated, nonfunctional STAT factor target sequence (34).

[2] G. Brunn and R. T. Abraham, unpublished observations.

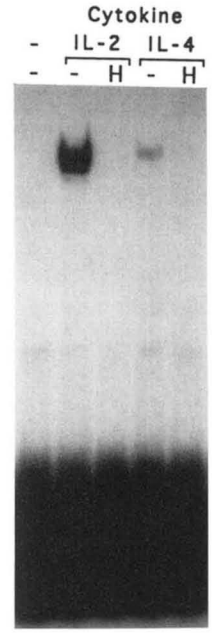

FIG. 2. **Effect of herbimycin A on STAT factor activation.** Factor-deprived CTLL-2 cells were treated with 9 μM herbimycin A (*H*) or drug vehicle (−) during the period of cytokine deprivation. Cells were stimulated with IL-2 or IL-4 for 10 min, and nuclear extracts were assayed for SIE m67 DNA binding activities by gel mobility shift assay.

A comparison of the SIE m67 binding activities stimulated by IL-2 and IL-4 in CTLL-2 cells revealed some apparent differences. Whereas IL-2 induced the formation of three readily discernible DNA·protein complexes, a single complex was observed in nuclear extracts from IL-4-stimulated cells. However, longer periods of autoradiographic exposure revealed the presence of two faster-migrating complexes in these samples (data not shown). Moreover, IL-4 stimulation clearly induced the formation of 3 SIE m67-binding complexes in the T-helper cell line, HT-2 (see Fig. 5). These results indicate that both IL-2 and IL-4 activate multiple SIE m67 binding complexes in factor-dependent T-cells.

Dependence of STAT Factor Activation on Protein-tyrosine Kinase Activity—Earlier reports demonstrated that the nuclear translocation of STAT proteins requires the activation of receptor-associated protein-tyrosine kinases (16, 18, 24, 27–30, 37, 39). To determine whether a cytoplasmic protein-tyrosine kinase(s) participates in the coupling of the IL-2R or IL-4R to STAT proteins, CTLL-2 cells were treated with the protein-tyrosine kinase inhibitor, herbimycin A, prior to cytokine stimulation (Fig. 2). The appearance of SIE m67 binding complexes in nuclear extracts from both IL-2- and IL-4-stimulated cells was abolished by herbimycin A pretreatment. Thus, the activation of herbimycin A-sensitive protein-tyrosine kinases is a requisite intermediate event for the nuclear translocation of SIE m67 binding factors induced by both cytokines.

Phosphorylation of STAT1α on a single tyrosine residue triggers the dimerization and nuclear translocation of this protein in cytokine-stimulated cells (24, 25). To determine whether the IL-2- or IL-4-induced SIE m67 binding activities in CTLL-2 cells were dependent on tyrosine phosphorylation, nuclear extracts were incubated with a monoclonal anti-pY antibody prior to addition of the SIE m67 probe (Fig. 3). The anti-pY antibody inhibited, in a concentration-dependent fashion, the DNA binding activities stimulated by either cytokine. The disruptive effect of the anti-pY antibody on DNA binding was reversed in the presence of excess phosphotyrosine, indicating that this effect was due to an interaction with a phosphotyrosyl-containing component(s) of the SIE m67 binding complexes.

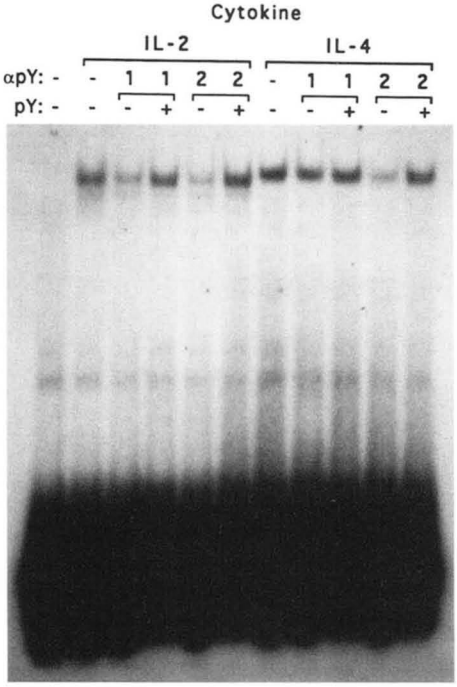

FIG. 3. **Effect of anti-pY antibody on cytokine-induced DNA binding activities.** Factor-deprived CTLL-2 cells were stimulated for 10 min with vehicle only or with the indicated cytokine. Nuclear extracts were incubated with the indicated amount (μg) of anti-phosphotyrosine (αpY) monoclonal antibody in the absence (−) or presence (+) of 1 mM phosphotyrosine (pY). SIE m67 binding activities were analyzed by gel mobility shift assay.

Composition of Cytokine-inducible STAT Factor Complexes—The appearance of three SIE m67 binding activities in nuclei from IL-2- or IL-4-stimulated T-cells suggested that both cytokines induced the dimerization and nuclear translocation of at least two STAT proteins. In an effort to identify these STAT family members, nuclear extracts from cytokine-stimulated CTLL-2 cells were incubated with labeled SIE m67 oligonucleotide in the absence or presence of antibodies specific for STAT1α, STAT3, or STAT4 (Fig. 4, *upper panel*). The STAT3-specific antibody (40) completely inhibited the major DNA binding activity induced by IL-2, but not by IL-4. Although not as apparent in this autoradiograph, the formation of the middle SIE m67 binding complex in IL-2-stimulated nuclear extracts was also blocked by anti-STAT3 antibodies. Identical results were obtained with nuclear extracts from IL-2- or IL-4-stimulated HT-2 cells (Fig. 4, *lower panel*). Antibodies to the STAT4 protein (41) had no effect on the STAT factor complexes induced by either IL-2 or IL-4. These results suggest that the major STAT protein activated in response to IL-2 is STAT3, whereas the principal STAT protein activated by IL-4 is not STAT1α, STAT3, or STAT4.

The antibody supershifting experiments further demonstrated that both the middle and lower complexes induced by IL-2 or IL-4 stimulation contained STAT1α or an immunologically related STAT protein. Again, the relatively low levels of these complexes in the nuclei of CTLL-2 cells hindered the interpretation of antibody supershifting experiments. However, the autoradiographic exposure shown in Fig. 5 clearly demonstrates that the anti-STAT1α antibody disrupts the middle and lower SIE m67 binding complexes presently observed in nuclear extracts from IL-2- or IL-4-stimulated HT-2 cells (Fig. 5). Thus, we conclude that both IL-2 and IL-4 activate STAT1α in these T-cell lines.

To confirm that STAT3 undergoes tyrosine phosphorylation in response to IL-2, CTLL-2 cells were stimulated for 10 min with medium only or with IL-2, and cytosolic and nuclear extracts from these cells were immunoprecipitated with a STAT3-specific antiserum. The immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-pY antibodies (Fig. 6). IL-2 stimulation induced the appearance of a pY-containing protein that co-migrated with STAT3 in CTLL-2 cell cytoplasmic extracts. Moreover, immunoblotting of the same membrane with anti-STAT3 antibodies revealed that the electrophoretic mobility of cytoplasmic STAT3 was slightly reduced after IL-2 stimulation. Similar alterations in electrophoretic behavior have been reported for STAT1α following cellular stimulation with IFN-γ (42).

Immunoblot analyses of anti-STAT3 immunoprecipitates of nuclear extracts from IL-2-starved or -stimulated CTLL-2 cells revealed some unexpected results. In the absence of IL-2, the nuclear extracts contained an anti-pY antibody reactive band that comigrated with the nonphosphorylated STAT3 found in the cytoplasm of these cells (Fig. 6, *upper panel*). In nuclear extracts from IL-2-stimulated cells, this immunoreactive band was completely replaced by a faint band with an electrophoretic mobility identical with that of tyrosine-phosphorylated STAT3 observed in the cytoplasm of these cells. These alterations fully paralleled the changes in the electrophoretic behavior of intranuclear STAT3 from control *versus* IL-2-stimulated cells (Fig. 6, *bottom panel*). Again, the latter results are highly reminiscent of those observed in STAT1α immunoprecipitates of nuclear extracts from control *versus* IFN-γ-stimulated fibroblasts (42). The apparent presence of tyrosine-phosphorylated STAT3 in the nuclei of IL-2-starved cells was unexpected, given that no detectable SIE m67 binding activity is observed in these nuclear extracts (see Fig. 1). Although the actual mechanism remains unclear, it is possible that multisite phosphorylation of STAT3 is required for both the retarded electrophoretic mobility and functional activation of this protein. In this case, STAT3 might be inactivated (in terms of SIE m67 binding) by partial dephosphorylation, with a fraction of this partially dephosphorylated form persisting in the nucleus during IL-2 starvation.

Cytokine-induced Activation of JAK Kinases—Somatic cell genetic experiments have implicated members of the JAK family of protein-tyrosine kinases (JAK-1, JAK-2, and TYK-2) in the coupling of IFN receptor stimulation to the tyrosine phosphorylation of STAT factors (16–18, 39). Other studies suggest that JAK kinase activation may be a widespread response to cytokine receptor stimulation (21, 22, 43–46). As a first step toward understanding the linkage between IL-2R or IL-4R ligation and the activation of STAT proteins, we examined the effects of cytokine stimulation on JAK-1, JAK-2, and JAK-3 phosphorylation and catalytic activities.

Factor-deprived CTLL-2 cells were stimulated for 10 min with IL-2 or IL-4, and detergent extracts were immunoprecipitated with the indicated anti-JAK antiserum (Fig. 7). The immune complexes were incubated with [γ-^{32}P]ATP under phosphorylating conditions, and the bound proteins were separated by SDS-PAGE. The phosphorylation state and kinase activity of the immunoprecipitated JAK isoform was examined by anti-pY antibody immunoblotting and by autoradiographic detection of radiolabeled protein, respectively. In CTLL-2 cells, IL-2 and, to a lesser extent, IL-4 stimulation increased both the tyrosine phosphorylation and *in vitro* kinase activities of JAK-1 and JAK-3 (Fig. 7, *A* and *C*). The anti-JAK-1 antibody immunoprecipitates contained an additional, lower molecular mass species that may represent a 110-kDa JAK-1 degradation product that reacts with the anti-JAK-1 antiserum used in this study (see manufacturer's product description). In contrast to the results obtained with JAK-1 and JAK-3, stimulation of CTLL-2 cells with IL-2 or IL-4 had only marginal effects on JAK-2 (Fig. 7*B*).

JAK1 and JAK3 were also activated by both IL-2 and IL-4 in

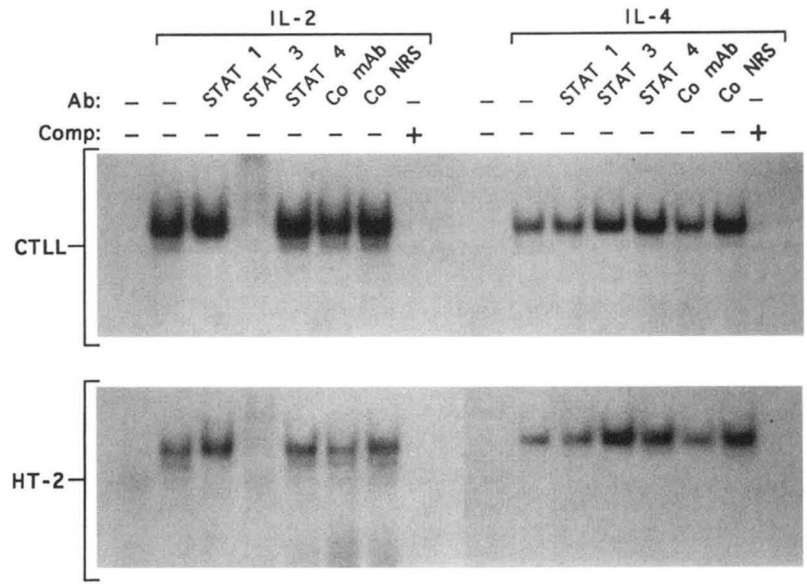

FIG. 4. **Effect of anti-STAT factor antibodies on cytokine-induced DNA binding activities.** Cells were stimulated with cytokines as described in the Fig. 3 legend. Nuclear extracts were incubated with ^{32}P-labeled SIE m67 oligonucleotide in the presence of anti-STAT1α (0.5 μg), or an isotype-matched control monoclonal antibody (*Co mAb*), anti-STAT3 (0.5 μl), anti-STAT4 (0.5 μl), or control nonreactive antiserum (*Co NRS*). SIE m67 binding activities were analyzed by gel mobility shift assay.

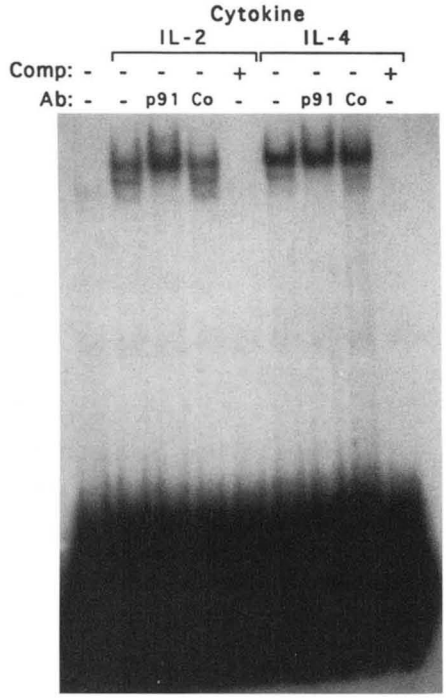

FIG. 5. **Effect of anti-STAT1α antibody on cytokine-induced DNA binding activities.** Nuclear extracts from unstimulated or IL-2- or IL-4-stimulated HT-2 cells were incubated with 0.5 μg of anti-STAT1α (*p91*) or isotype-matched control (*Co*) monoclonal antibody. SIE m67 binding activities were analyzed by gel mobility shift assay.

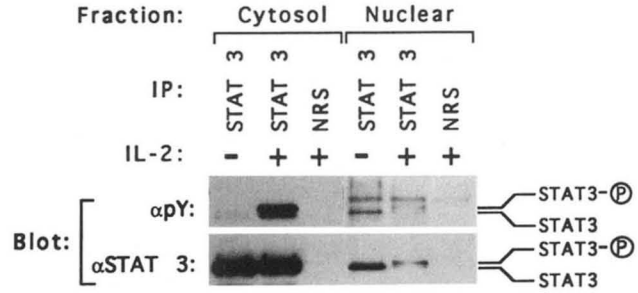

FIG. 6. **Cytokine-induced STAT3 phosphorylation.** Factor-deprived CTLL-2 cells were stimulated for 10 min with vehicle only or with IL-2. Cytosolic or nuclear fractions were immunoprecipitated with anti-STAT3 antiserum (*STAT 3*) or with nonreactive serum (*NRS*), and the bound proteins were resolved by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted sequentially with αpY and anti-STAT3 antibodies (α*STAT 3*). The more slowly migrating form of STAT3 induced by IL-2 stimulation is designated *STAT 3Ⓟ*.

HT-2 cells (Fig. 7*D*). However, in this cell line, the increases in JAK3 phosphorylation and catalytic activity induced by IL-4 were similar in magnitude to those stimulated by IL-2. These results correlate with the ability of IL-4 to induce a mitogenic response from HT-cells, whereas CTLL-2 cell growth is not supported by this cytokine.

The cytokine-dependent modifications of both JAK-1 and JAK-3 were abolished by pretreatment of the CTLL-2 cells with herbimycin A. In contrast, the protein-tyrosine kinase inhibitor had little effect on the tyrosine phosphorylation or autokinase activity of JAK-2. Interestingly, herbimycin A pretreatment resulted in a moderate reduction in the level of JAK-1 protein and induced a near-complete loss of JAK-3 protein (Fig. 7, *A* and *C*, *lower panels*). The decreased expression of JAK-1 and JAK-3 was not readily explained by a nonspecific effect of the drug on cellular metabolism, as the levels of JAK-2 protein were not reduced by herbimycin A exposure.

To examine the time course of JAK kinase activation, JAK-1 immunoprecipitates were immunoblotted directly with anti-pY antibodies at various times after addition of IL-2 to factor-deprived CTLL-2 cells. The IL-2-dependent modification of JAK-1 was extremely rapid, with maximal tyrosine phosphorylation of the enzyme observed within 1 min of cytokine addition to CTLL-2 cells (results not shown). The phosphotyrosine content of JAK-1 began to decline after 30 min of IL-2 stimulation. Thus, the time courses of JAK-1 activation and the appearance of intranuclear SIE m67 binding activities were closely correlated in the IL-2-stimulated T cells.

Transcriptional Activation by SIE m67 Binding Activities—Previous studies have shown that STAT factors regulate gene transcription by binding to specific enhancers, including the GAS element of the IRF-1 gene and the SIE element of the c-*fos* gene (16, 38, 47–49). As a preliminary step toward understanding the role of the STAT pathway in T-cell growth factor-induced gene expression, transient transfection assays were performed with a STAT factor-dependent reporter gene. The reporter construct (SIE-Luc) contained three tandem SIE m67 sequences situated upstream of an SV40 minimal promoter and the luciferase coding sequence. Growth factor-deprived CTLL-2 cells were transfected with the SIE-Luc vector, or with control plasmids containing either no enhancer elements

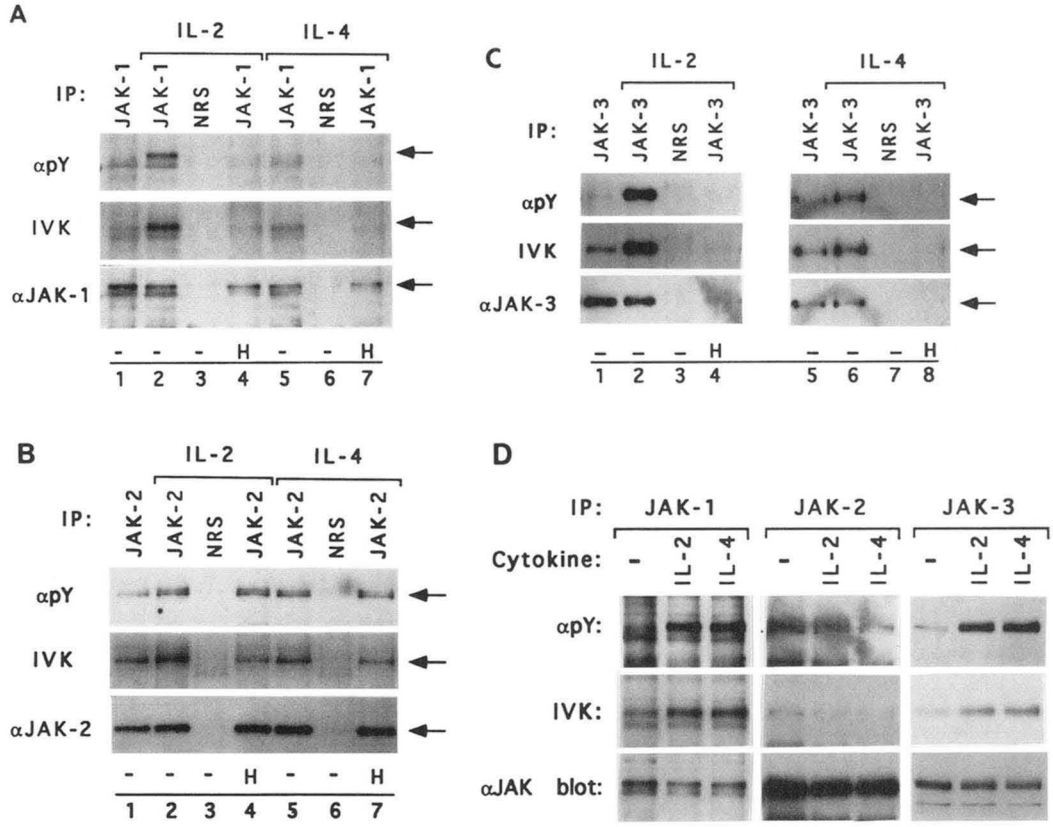

FIG. 7. **Cytokine-induced JAK kinase activation.** *A*, factor-deprived CTLL-2 cells were pretreated for 4 h with drug vehicle only (−) or with herbimycin A (*H*). Cells were stimulated for 10 min with no cytokine (*lane 1*), IL-2 (*lanes 2–4*), or IL-4 (*lanes 5–7*). Detergent extracts were immunoprecipitated (*IP*) with anti-JAK-1 antiserum or nonreactive serum (*NRS*), and the washed immunoprecipitates were subjected to *in vitro* kinase assays (*IVK*). Proteins were resolved by SDS-PAGE and were immunoblotted sequentially with αpY and anti-JAK-1 (*αJAK-1*) antibodies. The membrane-bound proteins were treated with KOH, and radiolabeled species were detected by autoradiography (*in vitro* kinase activity). *B*, CTLL-2 cells were pretreated with drug vehicle only (−) or with herbimycin A (*H*). Detergent-soluble proteins were immunoprecipitated with anti-JAK-2 antibodies, and the samples were analyzed as described in *A*. *C*, CTLL-2 cells were pretreated with drug vehicle only (−) or with herbimycin A (*H*). Detergent-soluble proteins were immunoprecipitated with anti-JAK-3 antibodies, and the samples were analyzed as described in *A*. *D*, factor-deprived HT-2 cells were stimulated with IL-2 or IL-4 for 10 min, and detergent extracts were immunoprecipitated with the indicated JAK antiserum. Protein kinase assays and immunoblot analysis were performed as described in *A*.

(pGL2-pro) or three copies of the mutated, nonfunctional SIE m56 sequence (mSIE-Luc). CTLL-2 cells transfected with SIE-Luc displayed a 3.9 ± 0.7-fold (mean ± S.D., $n = 3$) increase in luciferase activity in response to IL-2 (Fig. 8*A*). IL-2 stimulation had little or no effect on luciferase expression in cells transfected with either pGL2-pro or mSIE-Luc, indicating that the observed increase in transcriptional activity was dependent on the presence of the SIE m67 target sequences. Pretreatment of the transfected cells with herbimycin A abolished the IL-2-induced increase in luciferase gene expression. The inhibitory effect of herbimycin A was probably not due to a nonspecific drug effect on gene transcription or translation, because luciferase expression from a reporter vector containing a constitutively active cytomegalovirus promoter was unaffected by this protein-tyrosine kinase inhibitor (data not shown).

In parallel experiments, IL-4 failed to induce a reproducible increase in luciferase activity in SIE-Luc-transfected CTLL-2 cells (data not shown). We had previously noted that IL-4 was a relatively weak inducer of STAT protein activation in CTLL-2 cells (see Fig. 1). Transient transfections were therefore performed with the HT-2 cell line, which, unlike the CTLL-2 line, displays a robust mitogenic response to IL-4 (Fig. 8*B*). In HT-2 cells, IL-2 and IL-4 stimulated comparable increases in luciferase gene expression from the SIE-Luc construct. Once again, these cytokine-induced responses were blocked by cellular pretreatment with herbimycin A.

DISCUSSION

The present results demonstrate that both the IL-2R and IL-4R couple to the JAK-STAT signaling pathway in activated T-cells. Stimulation of factor-dependent murine T-cell lines with IL-2 or IL-4 induced rapid increases in the catalytic activities of two Janus family protein-tyrosine kinases, JAK-1 and JAK-3. These responses were temporally correlated with the nuclear translocation and functional activation of distinct arrays of STAT proteins in IL-2- or IL-4-stimulated cells. Finally, the insertion of a STAT factor target sequence upstream of a luciferase reporter cDNA conferred IL-2 and IL-4 inducibility on this synthetic gene. Pretreatment of the T cells with the protein-tyrosine kinase inhibitor, herbimycin A, blocked the cytokine-dependent activations of both JAK family members and STAT proteins. The latter results were consistent with earlier genetic data, which indicated that JAK kinase activation provides a requisite proximal signal for STAT factor assembly and translocation in other cytokine receptor systems.

The cytoplasmic signaling events that couple growth factor receptor occupancy to the transcription of immediate early genes in activated T-cells remain poorly understood. Ligation of the IL-2R activates the Ras-dependent signaling cascade (50), which transmits mitogenic signals to the nucleus via the induction of sequence-specific DNA-binding proteins, including the AP-1 transcription factor (51). The underlying rationale for the present study stems from earlier observations suggesting

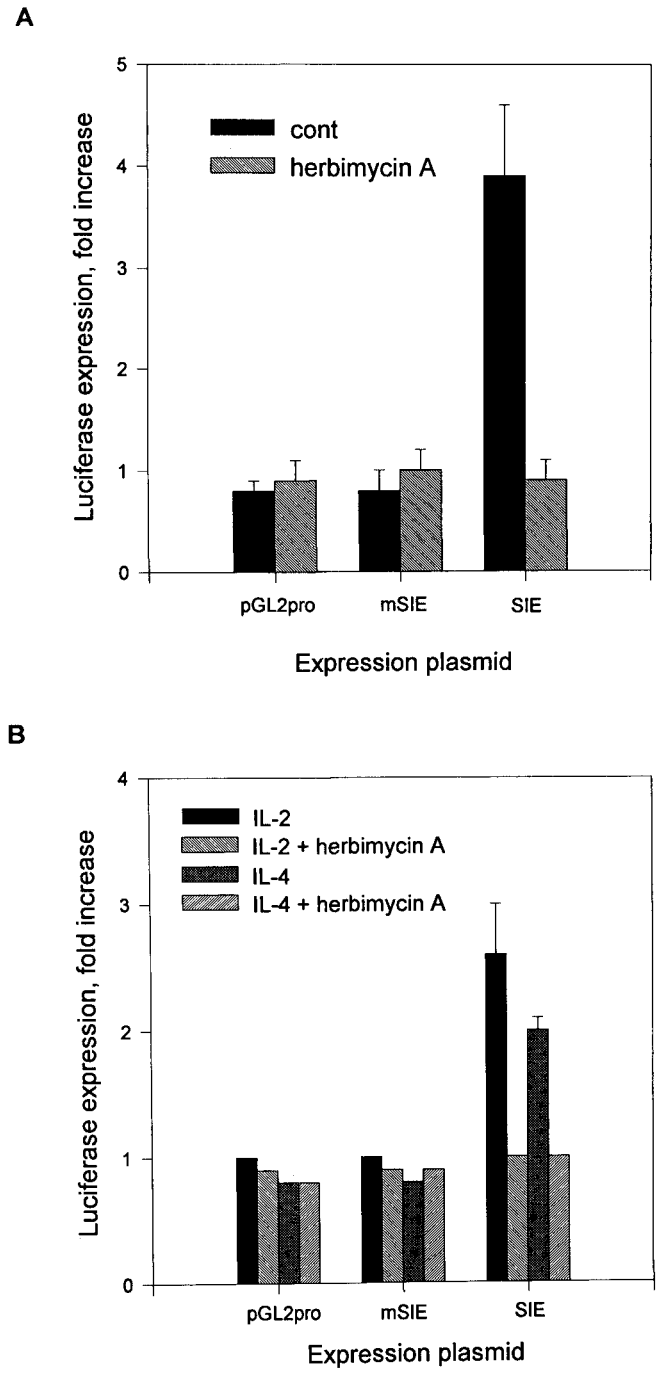

FIG. 8. **STAT factor-dependent luciferase expression.** CTLL-2 (*A*) or HT-2 (*B*) cells were deprived of growth factors for 2 h and then transfected with a luciferase reporter plasmid only (pGL2-pro) or with the same plasmid containing three copies of the STAT factor binding site (SIE) or three copies of the m56 site, which does not bind STAT factors (mSIE). The transfected cells were treated for 2 h with drug vehicle only or with herbimycin A. CTLL-2 cells (*A*) were then stimulated for 4 h with IL-2. HT-2 cells (*B*) were stimulated for the same time period with IL-2 or IL-4 as indicated. Luciferase activities were assayed, and values were normalized to those obtained with unstimulated cells from the same transfection. Data are presented as mean ± S.D. from three (*A*) or 2 (*B*) independent experiments.

that proliferative signal transduction from T-cell growth receptors involves both Ras-dependent and independent pathways (5). The JAK-STAT pathway is a reasonable candidate for the Ras-independent mechanism of nuclear signaling, as previous studies have shown that a dominant-negative Ras mutant does not suppress epidermal growth factor-dependent STAT protein activation in A431 carcinoma cells (29).

Gel mobility shift analyses revealed that both IL-2 and IL-4 induce the formation of three distinct SIE m67 binding complexes in the nuclei of CTLL-2 and HT-2 cells. In the case of IL-2, the appearance of these complexes likely reflects the formation of homodimers and heterodimers containing STAT3 and STAT1α. The dimerization of STAT1α, and presumably other STAT proteins, is dependent on the phosphorylation of a single tyrosyl residue located immediately C-terminal to the SH2 domain (24). The formation of STAT protein homodimers and heterodimers is essential for both the nuclear translocation and the sequence-specific DNA binding activities of STAT factors (25). We have shown that cytoplasmic STAT3 is rapidly phosphorylated on tyrosine in IL-2-stimulated CTLL-2 cells. Our antibody supershifting data support the conclusion that the STAT3 homodimer is the principal IL-2-inducible STAT factor in T cells. These analyses further suggest that the less abundant middle and lower complexes are composed of STAT3-STAT1α heterodimers and STAT1α-STAT1α homodimers, respectively.

The STAT1α protein is also a target for a protein-tyrosine kinase activated in response to IL-4 receptor occupancy. However, the principal STAT protein activated by IL-4 is clearly not STAT3 and may be identical with the IL-4 STAT protein recently cloned from cytokine-stimulated monocytic cells (31). The plasticity of the JAK-STAT signaling pathway is revealed by the observation that IL-2 and IL-4 induce only partially overlapping sets of STAT factors, in spite of the fact that these cytokines activate identical JAK family members in T-cells. The coupling of both the IL-2R and IL-4R to STAT1α may involve the shared γ_c subunit, whereas the distinct cytoplasmic domains of the IL-2R β subunit and the IL-4R may mediate the differential interactions of these receptors with STAT3 and the IL-4-inducible STAT protein in cytokine-stimulated cells.

The induction of distinct arrays of STAT factors by IL-2 and IL-4 provides a mechanism for cytokine-specific gene expression in T lymphocytes. The effects of IL-2 on STAT3 and STAT1α activation in CTLL-2 cells are reminiscent of those reported previously in epidermal growth factor- and IL-6-stimulated cells (40). During the preparation of this report for publication, Raz *et al.* (52) reported that STAT3 was not activated in an IL-2-stimulated CTLL-2 cell line. The reason for this discrepancy remains unclear, although one possibility is that the profiles and relative abundances of STAT proteins expressed in a different T-cell lines or subsets may also influence the types of STAT factors assembled in response to IL-2.

Studies of IFN-nonresponsive somatic cell mutants predicted a general role for members of the JAK protein-tyrosine kinase family in the coupling of cytokine receptors to cytoplasmic STAT proteins (16, 17, 38, 39). The intricacy of this regulatory mechanism is suggested by findings that single receptors often couple to more than one JAK family member. For example, IFN-α activates JAK-1 and TYK-2, whereas IFN-γ stimulates JAK-1 and JAK-2 kinase activities (16, 19). In the present studies, we report that both IL-2 and IL-4 induce the tyrosine phosphorylation and catalytic activation of JAK-1 and JAK-3, with only marginal effects on JAK-2. While the present studies were in progress, two independent reports documented the stimulatory effect of IL-2 on JAK-3 activity in T-cells (21, 22). Although IL-2 stimulation has a greater impact on JAK-3 catalytic activity, it is likely that both JAK1 and JAK3 participate in signal transduction through the IL-2R $\beta\gamma_c$ heterodimer. An earlier study reported a strong correlation between cytokine-stimulated JAK-1 activation and the tyrosine phosphorylation of STAT1α, suggesting that the two events might be causally related (19). If JAK-1 serves as the STAT1α kinase in cytokine-stimulated T cells, then the present results imply that stimulation of JAK-3 catalytic activity is critical for the coupling of

the IL-2R to the STAT3 protein. The absence of a phosphotyrosine-containing target sequence for the SH2 domain of STAT3 in the IL-4R cytoplasmic domain might explain the failure of JAK3 to activate STAT3 in IL-4-stimulated cells.

The catalytic activities of JAK-1, JAK-2, and JAK-3 were differentially affected by exposure of intact T-cells to herbimycin A. Pretreatment of CTLL-2 cells with herbimycin A partially or completely reduced the expression of JAK-1 or JAK-3, respectively, whereas JAK-2 expression and catalytic activity were only slightly affected by this drug. The differential sensitivities of the JAK family members to herbimycin A might reflect a high level of inhibitory selectivity toward the different JAKs. Alternatively, it is conceivable that JAK-1 and JAK-3 expression are controlled by an upstream, herbimycin A-sensitive protein-tyrosine kinase(s) in IL-2-dependent T-cells. Obvious candidates are Lck and Fyn, which are known targets for herbimycin A in lymphoid cells (53). Earlier studies have shown that herbimycin A pretreatment blocks IL-2-induced Ras activation and S-phase entry in T-cells (54). These results supported a model which positioned the Src family members, Lck or Fyn, as proximal transducers of IL-2-dependent mitogenic signals. However, the observation that herbimycin A simultaneously disrupts the linkages between T-cell growth factor receptors and JAK kinases indicates that the effects of this drug on mitogenic signaling in T-cells are more complex than was originally suspected.

The idea that protein tyrosine phosphorylation represents an obligatory proximal event in the propagation of growth factor receptor-mediated signals has become a central paradigm in the field of cell biology. The discovery of a direct link between receptor-regulated protein-tyrosine kinases and a family of SH2 domain-containing transcription factors adds further credence to this concept. One of the main goals for the immediate future will be to determine whether STAT proteins actually regulate the expression of genes involved in cell cycle progression in growth factor-stimulated T-cells. The c-*fos* gene is under the control of both STAT- and Ras-responsive enhancer elements (34, 48), which suggests the possibility for interplay between these two signaling pathways during the expression of mitogen-inducible genes. Interestingly, STAT1α contains a leucine zipper-like motif (24), which supports the idea that STAT proteins may interact with other transcription factors, including Fos and Jun. Finally, mutagenesis studies have delineated a membrane-proximal, serine-rich domain of the IL-2R β subunit which apparently delivers a Ras-independent signal for c-*myc* gene expression and mitogenesis (4). A homologous region of the erythropoietin receptor cytoplasmic domain is required for ligand-induced JAK-2 activation (43). It will be interesting to determine whether the membrane-proximal region of the IL-2R β subunit stimulates c-*myc* expression and T-cell cycle progression via activation of the JAK-STAT pathway in T-cells.

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