Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8429–8433, September 1993
Medical Sciences

Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction

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Communicated by George R. Stark, May 18, 1993

ABSTRACT **Interleukin 3 (IL-3) regulates the proliferation and differentiation of hematopoietic cells. Although the IL-3 receptor chains lack kinase catalytic domains, IL-3 induces tyrosine phosphorylation of cellular proteins. To investigate the potential role of the JAK family of protein-tyrosine kinases in IL-3 signal transduction, we have obtained full-length cDNA clones for murine Jak1 and Jak2 protein-tyrosine kinases and prepared antiserum against the predicted proteins. Using antisera against Jak2, we demonstrate that IL-3 stimulation results in the rapid and specific tyrosine phosphorylation of Jak2 and activates its *in vitro* kinase activity.**

Hematopoiesis is regulated through the interaction of growth factors with their cognate receptors (1, 2). Interleukin 3 (IL-3) supports the proliferation and differentiation of early progenitors and cells committed to several myeloid lineages (3). The receptor for IL-3 is composed of an α subunit of 60–70 kDa and a β subunit of 130–140 kDa (4). Both subunits contain the extracellular conserved motifs found in the cytokine receptor superfamily. The cytoplasmic domains share limited similarity with other cytokine receptors and lack detectable catalytic domains. However, considerable evidence suggests that signal transduction involves tyrosine phosphorylation (1, 4). Thus there has been considerable interest in identifying a protein-tyrosine kinase that associates with the receptor and is activated by IL-3 binding.

Polymerase chain reactions (PCRs) have been done with degenerative oligonucleotides to conserved tyrosine kinase domains to identify potential kinases in myeloid cells (5). By using this approach and Northern blot analysis, IL-3-dependent cells express (6) the genes for a limited number of tyrosine kinases including *Lyn*, *Tec*, c-*Fes*, *Jak1*, and *Jak2*. Lyn kinase has been implicated in IL-3 signaling (7). However, we have not detected an effect of IL-3 on Lyn kinase activity or on its tyrosine phosphorylation (unpublished data). We have also not detected any tyrosine phosphorylation or activation of Tec or c-Fes kinase activity (unpublished data). Therefore, we focused on assessing the role of murine *Jak1* and *Jak2* genes in IL-3 signal transduction.

The JAK (Janus kinase; alternatively referred to as *j*ust *a*nother *k*inase) family of kinases was detected in PCR amplification of tyrosine kinase domains in hematopoietic cells (8). The complete structure of the human *JAK1* gene has been reported (9) and, recently, a partial sequence of the murine *Jak2* gene has been reported (10). Independently, a third member (*tyk2*) was isolated by homology screening (11). The family is characterized by two kinase domains. The C-terminal domain has all the hallmarks of protein kinases and the second domain has the hallmarks of a protein kinase but differs significantly from both the protein-tyrosine and -serine/threonine kinases. There are no SH2 and SH3 domains that characterize many tyrosine kinases. There is extensive similarity in the N-terminal region among the JAK family members (10).

A role for the *tyk2* gene in signal transduction was established in studies examining interferon α (IFN-α) (12). By using a genetic approach, the *tyk2* gene was cloned by its ability to reconstitute the cellular response to IFN-α in mutant cells. It is hypothesized that the kinase activity of Tyk2 is activated after IFN-α binding and is responsible for the phosphorylation of the 113- and 91/84-kDa proteins of the IFN-stimulated gene factor 3α complex (13, 14). This complex associates with the IFN-stimulated gene factor 3γ protein and activates gene expression by binding to the interferon-stimulated response element.

Jak2 is involved in the response to erythropoietin (EPO) (15). EPO stimulation induces tyrosine phosphorylation of Jak2 and activates its *in vitro* autophosphorylation activity. Using a series of EPO receptor mutants, the induction of Jak2 tyrosine phosphorylation was found to correlate with the induction of biological responses. Jak2 was also shown to physically associate with the membrane-proximal cytoplasmic region of the EPO receptor that is required for biological activity. Here we report the complete structure of the murine *Jak2* gene.¶ We demonstrate that Jak2 is rapidly tyrosine phosphorylated and its kinase activity is activated in response to IL-3.

MATERIALS AND METHODS

Isolation of Murine Jak2 Clones. PCR, with degenerative oligonucleotides corresponding to the conserved protein-tyrosine kinase domain, was used to amplify cDNAs from murine bone-marrow-derived monocytes (16). The Jak2 cDNA clone was used to screen myeloid cDNA libraries (17–19). The longest cDNAs were subcloned and the nucleotide sequence was determined (20).

Cells and Cell Culture. The cell lines used have been described (21) and were maintained in IL-3 (25 units/ml). Mouse bone-marrow-derived monocytes were grown as described (17).

Generation of Antibodies. Synthetic peptides corresponding to the hinge region between domains 1 and 2 (aa 758–776) were coupled to keyhole limpet hemocyanin by gluteraldehyde and used for immunization of rabbits. A synthetic peptide to Jak1 (aa 785–804) was used for competition studies.

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Abbreviations: IL-3, interleukin 3; IFN-α, interferon α; EPO, erythropoietin.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L16956).

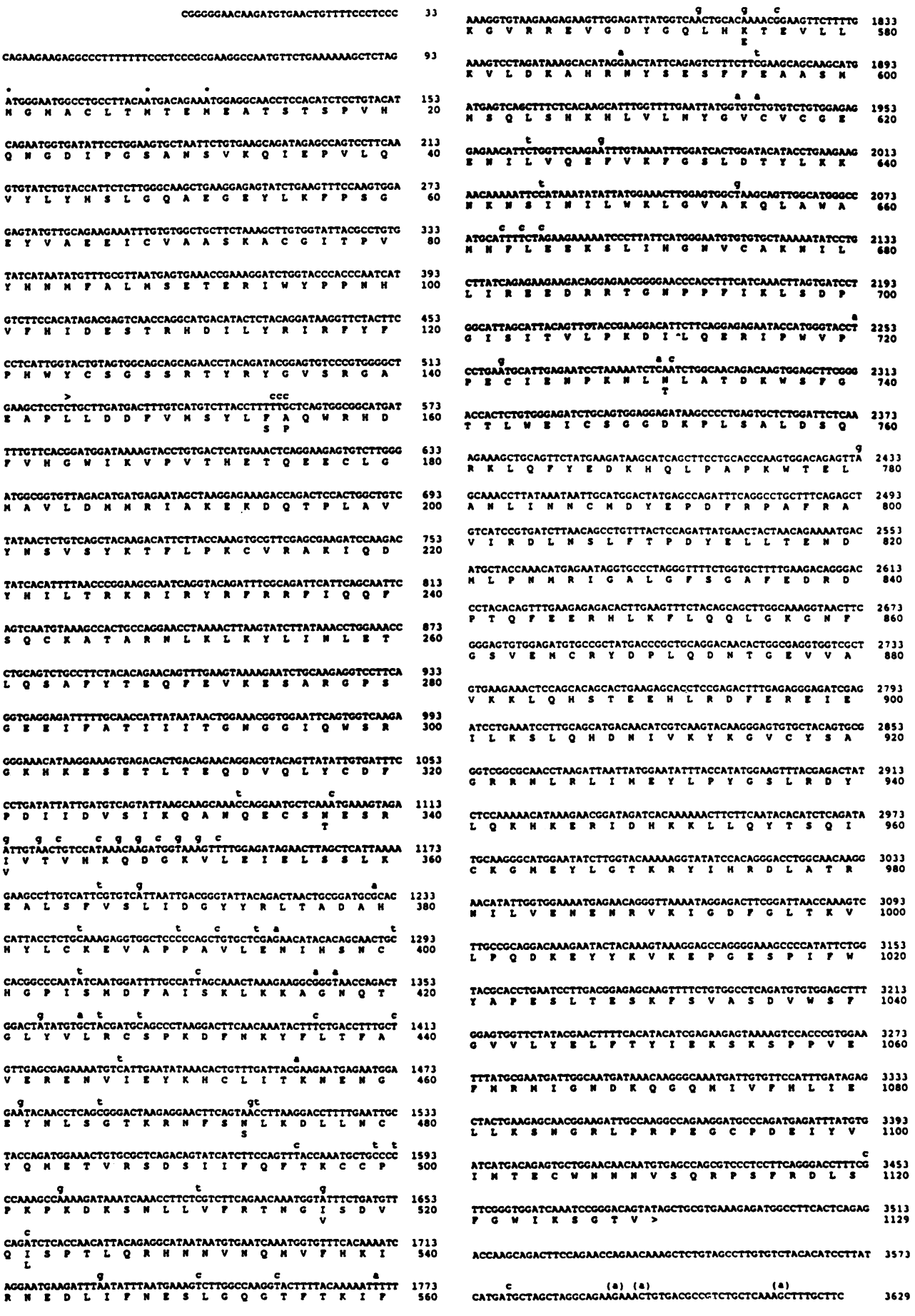

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

***In Vitro* Translation and Transcription.** Full-length Jak1 or Jak2 cDNAs were inserted into pBSK (Stratagene) to make transcripts with T3 RNA polymerase. Approximately 3 μg of RNA was used in translation reaction mixtures (Stratagene) in the presence of ^{35}S translabel (NEN). The products were divided equally and either subjected to SDS/PAGE directly or immunoprecipitated with Jak1 or Jak2 antisera. Competitions were preformed by incubating peptides (100 μg/ml) with antisera for 1 h at 4°C prior to immunoprecipitation.

***In Vitro* Kinase Assays.** Immunoprecipitated proteins on protein A-Sepharose (Pharmacia) were washed with kinase buffer (50 mM NaCl/5 mM $MgCl_2$/5 mM $MnCl_2$/0.1 mM Na_3VO_4/10 mM Hepes, pH 7.4) and incubated for 30 min at room temperature with an equal volume of kinase buffer containing [γ-^{32}P]ATP at 0.25 mCi/ml. After extensive washing, proteins were eluted with sample buffer for SDS/PAGE and separated on 7% gels. ^{32}P-containing proteins were visualized by autoradiography. *In vitro*-phosphorylated Jak2 was isolated from gel slices and the phosphoamino acid content was determined (22).

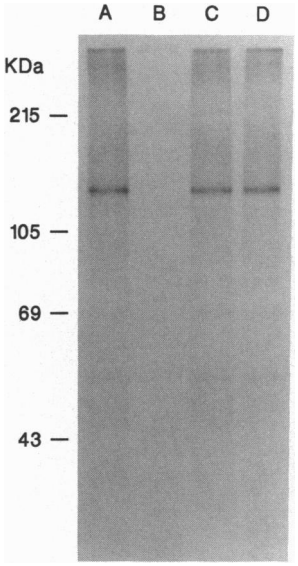

FIG. 2. *In vitro* translation and immunoprecipitation of Jak2. Capped Jak2 mRNA was used in *in vitro* translation reaction mixtures and products labeled with [^{35}S]methionine. After the reactions, Jak2 was immunoprecipitated from reaction mixtures in the absence of competing peptides (lane A) or in the presence of the peptide to which the antiserum was raised (lane B), to an irrelevant peptide (lane C), or to a peptide corresponding to the same region of Jak1. The immunoprecipitates were resolved by SDS/PAGE.

RESULTS

The tyrosine kinases expressed in IL-3-dependent cells were identified by PCR using degenerative oligonucleotides corresponding to the conserved regions of the tyrosine kinase domain (5). One of the most frequently isolated cDNA clones was identical to Jak2 (16). Expression analysis indicated that *Jak2* was widely expressed and prompted us to obtain full-length cDNA clones. Screening of myeloid cDNA libraries allowed the isolation of several overlapping clones, the longest (4 kb) of which contained the entire coding region of *Jak2*. The nucleotide sequence of *Jak2* contains an open reading frame of 3387 bp and the 5′ end has three stop codons before the first ATG (Fig. 1). Although the first ATG does not fulfill the Kozak consensus flanking sequences, it is immediately followed by an ATG codon in the typical translation initiation environment (23). The 5′ end does not contain an obvious signal peptide. The compiled size of the 3′ untranslated region of the *Jak2* clones is 0.9 kb, which would correspond to a 4.4-kb transcript. The *Jak2* open reading frame encodes 1129 aa with a calculated molecular mass of 130 kDa. Hydrophilicity analysis failed to identify transmembrane regions. During our studies, a partial sequence of *Jak2* was published (10), which lacked the first 143 aa. A comparison of the sequences indicates 71 nt differences in the coding region, resulting in 9 aa changes (Fig. 1). Our cDNA clones did not contain a 7-aa insert at position 711 found in a cDNA clone described by Harpur *et al.* (10).

The murine *Jak2* gene is closely related to the human *tyk2* and *JAK1* genes (42% and 43% identities, respectively). We have also obtained full-length cDNA clones for the murine *Jak1* gene (data not shown), which has 45.5% identity to *Jak2* at the nucleotide level in the coding region. The Jak2 protein has a 600-aa N terminus that lacks obvious SH2 or SH3 domains. After this is a kinase-related domain (domain 2) and a C-terminal kinase domain (domain 1). The C-terminal kinase domain contains the conserved residues in subdomains VI–VIII that are characteristically found in tyrosine kinases (24). Subdomain VIII, which may contribute to substrate recognition, contains a unique FWY amino acid motif found in all Jak family members. Domain 2 begins at aa 543 and the structural subdomains can be identified. However, clear differences in the amino acid composition and spacing in critical kinase subdomains I, II, VI, and VIII (24) raise the possibility that this domain may have a regulatory function or an unknown substrate specificity.

Although the N terminus of the JAK family proteins is less homologous than the kinase domains (36–39% vs. 49–56%), there are several stretches of homology. Database searches with the N-terminal sequence did not show homology with other proteins. Close comparisons of the region immediately N-terminal to the kinase-like domain reveal some similarity to SH2 domains.

To biochemically characterize the Jak2 protein, anti-peptide antisera were prepared against a region that was unique for Jak2 (aa 758–776). To assess the reactivity, immunoprecipitations were done with *in vitro*-synthesized Jak2 (Fig. 2). *In vitro* translation of Jak2 RNA yielded a 130-kDa protein (data not shown). This protein was immunoprecipitated by the Jak2 anti-peptide antiserum (Fig. 2, lane A) but not by an irrelevant antiserum (data not shown). Immunoprecipitation was competed by the homologous peptide (lane B) but not by an irrelevant peptide (lane C) or by a peptide that is the homologous region of Jak1 (lane D). The Jak2 antiserum did not immunoprecipitate *in vitro*-synthesized Jak1 (data not shown). Lastly, the Jak2 antiserum immunoprecipitated a 130-kDa protein from *in vivo*-methionine-labeled cells, which was competed by the homologous peptide (data not shown).

IL-3 stimulation induces tyrosine phosphorylation of several cellular substrates including the IL-3 receptor β subunit (3, 25). We therefore determined whether Jak2 was a substrate (Fig. 3). Western blot analysis of cell lysates with a monoclonal antibody against phosphotyrosine (4G10) detected the appearance of several proteins after IL-3 stimulation including a broad band at 130–140 kDa, a minor band at 70 kDa, and major bands at 55 kDa, 50 kDa, and 38 kDa. When extracts were immunoprecipitated with Jak2 antiserum, a 130-kDa protein was readily detected in stimulated cells. Also of note are proteins of 110 kDa, 70 kDa, and 60 kDa that coimmunoprecipitated with Jak2. These have been consistently seen in Jak2 immunoprecipitations. Immunoprecipitation with Jak1 antiserum consistently detected a weak

FIG. 1 (*on opposite page*). Sequence of the murine Jak2 cDNA. The sequence of the *Jak2* open reading frame and noncoding regions is shown. Nucleotide and amino acid differences from the published partial Jak2 cDNA sequences (10) are shown. The ATG codons are indicated (*). The symbol > above nt 522 designates the 5′ end of the reported Jak2 sequence. The symbol ∧ at nt 2226 indicates the location of the 7-aa insert as detected (10). The nucleotides shown in parentheses in the 3′ noncoding region were present in the previous studies (10) but not detected in our studies.

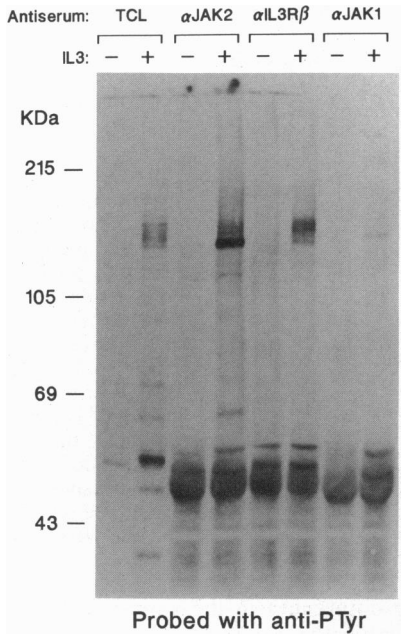

FIG. 3. IL-3 stimulation of the tyrosine phosphorylation of Jak2. DA-3 cells were deprived of growth factors for ≈14 hr, either unstimulated (−) or stimulated with IL-3 (10 units/ml) (+), and lysed, and extracts were incubated with the indicated antisera. The immunoprecipitates or total cell lysates (lanes TCL) were resolved by SDS/PAGE. The proteins were transferred to nitrocellulose and the blots were probed with the 4G10 monoclonal antibody against phosphotyrosine. The positions of the migration of standards are indicated on the left. Antisera: αJAK2, anti-JAK2; αIL3Rβ, anti-IL-3 receptor β chain; αJAK1, anti-JAK1.

band at 130 kDa, indicating that Jak1 may also be a substrate. The inducible tyrosine phosphorylation of the IL-3β chain, which migrates slightly above Jak2, is shown in Fig. 3. Thus the broad band seen in total cell lysates consists of both Jak2 and the IL-3β chain.

To further establish Jak2 phosphorylation, cells were stimulated with IL-3 and the phosphotyrosine-containing fraction was isolated on Sepharose beads containing anti-phosphotyrosine monoclonal antibody 1G2. Jak2 was readily detected on Western blots of the IG2-isolated proteins (Fig. 4*A*) from

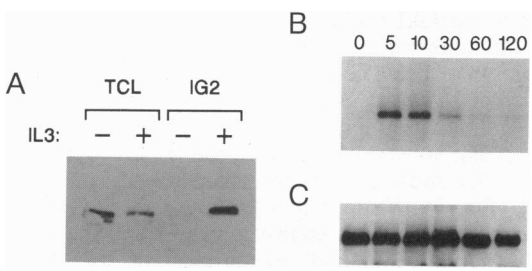

FIG. 4. Specificity and kinetics of Jak2 tyrosine phosphorylation after IL-3 stimulation. Cell extracts were prepared from unstimulated cells (lanes − and 0) or from cells stimulated with IL-3 at 10 units/ml for 10 min (+) or stimulated for the indicated times. (*A*) The phosphotyrosine-containing protein fraction was isolated by binding to and elution from 1G2-coupled Sepharose beads; the 1G2 monoclonal antibody against phosphotyrosine is as described (26, 33). The proteins were resolved by SDS/PAGE and transferred to nitrocellulose, and the blots were probed with the Jak2 antiserum. Alternatively, the total cell lysate (TCL) was electrophoresed and transferred, and the blots were probed with the Jak2 antiserum. (*B* and *C*) The cell extracts were immunoprecipitated with the Jak2 antiserum, the precipitates were resolved by SDS/PAGE and transferred to nitrocellulose, and the blots were probed with the 4G10 monoclonal antibody (*B*) or the Jak2 antiserum (*C*).

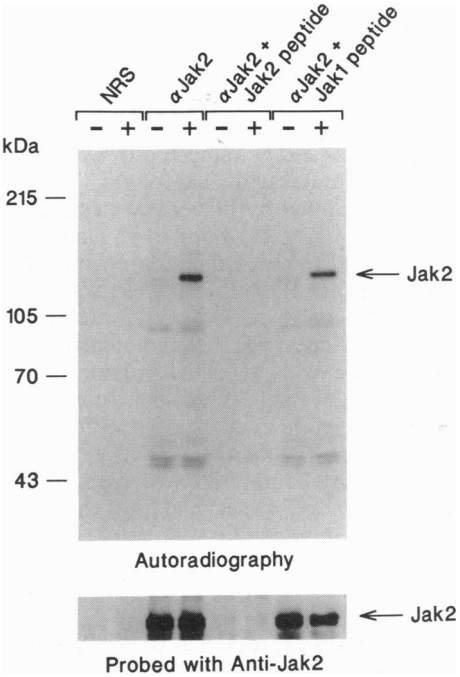

FIG. 5. IL-3 stimulation activates Jak2 *in vitro* kinase activity in immunoprecipitates. DA3 cells were removed from growth factors and were either unstimulated (−) or stimulated with IL-3 for 10 min (+). Extracts were immunoprecipitated with normal rabbit serum (NRS) or Jak2 antiserum in the absence of competing peptide (αJak2) or in the presence of the peptide (30 μg/ml) to which the antiserum was raised (αJak2 + Jak2 peptide) or in the presence of an equivalent amount of the peptide that corresponds to the comparable region of Jak1 (αJak2 + Jak1 peptide). The immunoprecipitates were used for *in vitro* kinase assays and the products were resolved by SDS/PAGE, transferred to nitrocellulose, and detected by autoradiography (*Upper*). The blots were subsequently probed with the Jak2 antiserum (*Lower*).

stimulated but not unstimulated cells. The kinetics are illustrated in Fig. 4*B*. Jak2 tyrosine phosphorylation was maximal at 5 min after IL-3 stimulation and, subsequently, decreased. These kinetics are comparable to the general pattern of tyrosine phosphorylation (26). During this period, there was no change in the levels of Jak2 as assessed by Western blot analysis with Jak2 antiserum (Fig. 4*B*).

To assess the effect on Jak2 kinase activity, cells were stimulated with IL-3 for 10 min, Jak2 was immunoprecipitated and *in vitro* kinase assays were performed. No *in vitro* kinase activity was detected in immunoprecipitates with normal serum from unstimulated or stimulated cells (Fig. 5). However, immunoprecipitates with Jak2 antiserum contained kinase activity and resulted in the phosphorylation of a protein of 130 kDa that comigrated with Jak2. In contrast, the 130-kDa band was not detected with unstimulated cells. Phosphoamino acid analysis of the 130-kDa band demonstrated the presence of predominantly phosphotyrosine (data not shown). Interestingly, there were no other major protein bands phosphorylated in these *in vitro* reactions. The Jak2 peptide blocked precipitation of kinase activity, whereas a peptide to the corresponding region of Jak1 had no effect.

DISCUSSION

Our studies provide the complete sequence of the murine *Jak2* as indicated by three criteria. (*i*) The coding sequence initiates at the same site as the published sequences of human *tyk2* and *JAK1*. (*ii*) The first ATG is preceded by stop codons in all reading frames. (*iii*) The size of the compiled cDNAs is consistent with the observed transcript. Our sequence varies

from the published partial sequence (10). Since a portion of the previous sequence was obtained from PCR-amplified DNAs, some differences may be PCR artifacts.

IL-3 stimulation induces tyrosine phosphorylation (3, 27). One of the substrates is Jak2 (3). Among the tyrosine kinases in IL-3-dependent cells, there was a remarkable specificity for Jak2. In particular, no changes in phosphorylation of Lyn, Tec, or c-Fes have been seen (data not shown). However, we consistently see a low level of Jak1 phosphorylation after IL-3 stimulation. Therefore, Jak1 may share sufficient similarity to Jak2 to weakly associate with the IL-3 receptor complex. Alternatively, since there is considerable sequence homology between Jak1 and Jak2 at the potential autophosphorylation site, Jak1 may be a substrate for Jak2. However, we have not detected activation of Jak1 *in vitro* kinase activity (data not shown).

IL-3 stimulation results in the activation of Jak2 *in vitro* kinase activity. The C-terminal protein-tyrosine kinase domain of Jak2 contains the characteristic autophosphorylation site of tyrosine kinases (24).

The requirement for IL-3 binding for kinase activity indicates that Jak2 is highly regulated in cells, consistent with a major role in growth regulation. The primary substrate of the *in vitro* kinase reactions was Jak2. In particular, there was no detectable phosphorylation of immunoglobulins nor is enolase a substrate for Jak2 (data not shown), indicating that Jak2 may have a strict substrate specificity. The requirement for receptor activation and the substrate specificity may account for the inability to demonstrate Jak1 protein-tyrosine kinase activity in previous studies (9).

We have shown (15) that Jak2 is tyrosine-phosphorylated and activated after EPO stimulation. Moreover, these studies demonstrated that Jak2 physically associates with a membrane-proximal region of the cytoplasmic domain of the EPO receptor that is essential for function. However, like the EPO receptor, the β subunit of the IL-3 receptor is rapidly tyrosine-phosphorylated and it can be hypothesized that this phosphorylation is mediated by Jak2. For the EPO receptor, tyrosine phosphorylation occurs at sites in the cytoplasmic C-terminal end and this region is not required for mitogenesis.

The ability of IL-3 and EPO to activate Jak2 suggests the possibility that Jak2 may be a component in the signal transducing pathways of several cytokine receptors. In preliminary experiments, granulocyte–macrophage and granulocyte colony-stimulating factors were found to induce the tyrosine phosphorylation of Jak2. This is consistent with studies showing that these factors induce comparable patterns of tyrosine phosphorylation (3). We have also observed tyrosine phosphorylation of Jak2 in response to IFN-γ in a macrophage cell line (unpublished data).

The hematopoietic growth factor receptors are members of a receptor superfamily that includes growth hormone receptor, the prolactin receptor, ciliary neurotropic factor receptor, and others (28). Moreover, the receptors for IFN have been speculated to have evolved from the same progenitor. The recent studies implicating Tyk2 in IFN-α signaling (12) and Jak2 in signaling by IL-3, EPO (15), granulocyte and granulocyte–macrophage colony-stimulating factor, IFN-γ, and growth hormone (29) suggest that the JAK family kinases are involved in the signal-transducing pathways utilized by the cytokine/IFN superfamily of receptors. If correct, it is further tempting to speculate that the JAK family of kinases may regulate gene expression through comparable pathways involving family members related to the IFN-stimulated gene factor 3α proteins (30, 31) and the IFN-stimulated gene factor 3γ-related DNA binding proteins including ICSBP, IRF1, IRF2, and possibly Myb (32).

We thank Linda Snyder, Brenda Simmons, and Cynthia Miller for excellent technical assistance in these studies. This work was supported in part by the National Cancer Institute Cancer Center Support (CORE) Grant P30 CA21765, by Grants RO1 DK42932 (J.N.I.) and DK44158 (J.L.C.) from the National Institute of Diabetes and Digestive and Kidney Diseases and by the American Lebanese Syrian Associated Charities (ALSAC).

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