Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6374–6378, July 1994
Immunology

Molecular cloning of L-JAK, a Janus family protein-tyrosine kinase expressed in natural killer cells and activated leukocytes

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Communicated by Richard D. Klausner, March 10, 1994

ABSTRACT **Protein-tyrosine kinases (PTKs) are critical enzymes for receptor-mediated signaling in lymphocytes. Because natural killer (NK) cells are large granular lymphocytes with specialized effector function, we set out to identify PTKs preferentially expressed in these cells. One such PTK was identified and molecularly cloned. The predicted amino acid sequence shows that this kinase lacks SH2 or SH3 domains typical of src family kinases but has tandem nonidentical catalytic domains, indicating that it is a member of the Janus family of PTKs. Immunoprecipitation using antiserum generated against a peptide corresponding to the deduced amino acid sequence of this gene revealed a kinase with a molecular weight of ≈125,000. The pattern of expression of this kinase contrasted sharply with that of other Janus kinases, which are ubiquitously expressed. The kinase described in the present study was found to be more limited in its expression; expression was found in NK cells and an NK-like cell line but not in resting T cells or in other tissues. In contrast, stimulated and transformed T cells expressed the gene, suggesting a role in lymphoid activation. Because of its homology and tissue expression, we have tentatively termed this PTK gene L-JAK for leukocyte Janus kinase.**

Protein-tyrosine phosphorylation is an early and requisite event in lymphocyte receptor-mediated signaling (reviewed in ref. 1) for both multichain immune recognition receptors such as the T-cell antigen receptor (TCR) (2–4) and cytokine receptors (5–7). Unlike growth factor receptors, neither of these types of receptors has intrinsic protein-tyrosine kinase (PTK) activity. Rather, they are coupled to nonreceptor tyrosine kinases. For example, there is considerable evidence indicating a role for the src family PTKs Lck and Fyn in TCR-mediated signaling (2, 3, 8–17) and the non-src family PTK Zap-70 has been shown to associate with the TCR upon activation (18–21). Additionally, the src family PTKs, Lck, Fyn, and Lyn have been implicated in interleukin 2 receptor-mediated signaling (5, 6, 22–25). Recently, an additional family of PTKs, the Janus family of kinases (JAKs), has been described. These kinases, JAK1, JAK2, and Tyk2, are structurally quite distinct in that they possess tandem nonidentical catalytic domains (26–29). These PTKs have also been shown to be involved in signaling by a number of cytokine and hormone receptors (30–36). These family members are also of interest in that they appear to exert their effect through tyrosine-phosphorylated transcription factors (37, 38). Thus there is now abundant evidence implicating a variety of PTKs in lymphocyte activation.

Natural killer (NK) cells are a distinct lymphocyte subset that do not undergo rearrangement of antigen receptor chain genes but otherwise shares a number of similarities with T lymphocytes (reviewed in refs. 39–41). NK cells and T cells are developmentally related and express similar surface molecules (42, 43). Nonetheless, they are functionally quite different. NK cells can be thought of as primed effector cells capable of killing susceptible targets without additional activation. In contrast, T cells generally do not lyse targets in the absence of prior stimulation. To begin to understand the molecular underpinning of this difference and because of the critical role of PTKs in NK cell function (44–47), we compared the status of tyrosine-phosphorylated substrates in these two lymphocyte subsets (unpublished results). We demonstrated that NK cells have high levels of tyrosine phosphorylation of a variety of substrates relative to T cells. This was not due to differential expression or activity of Lck or differences in protein-tyrosine phosphatase activity. We hypothesized, therefore, that NK cells might express PTKs not expressed in resting T cells, and to this end used a PCR-based strategy to identify such kinases. In the present study we report the molecular cloning of one such PTK¶ that is a member of the Janus family of PTKs. However, unlike other JAKs, which are widely expressed, the expression of the NK-derived kinase is much more limited; it is expressed only in NK cells and other activated leukocytes. We have, therefore, termed this kinase gene L-JAK for leukocyte JAK.

MATERIALS AND METHODS

T Cells and NK Cell PTK. A modification of the strategy described by Wilks *et al.* (28) was used to clone PTK expressed in NK cells (48). The forward primer used was 5′-CCAGCGGCCGCGT(G/A/T/C)CA(C/T)CG(G/A/T/C)GA(C/T)CT(G/A/T/C)GC-3′ and the reverse primer was 5′-CCAGCGGCCGCCC(G/A)AA(G/A/T/C/)(G/C)(A/T)CCA(G/A/T/C)AC(G/A)TC-3′. The resulting products were digested with *Not* I, subcloned, and sequenced. The PCR fragment corresponding to one novel kinase was isolated, labeled, and used to screen several libraries including λgt11, oligo(dT)-primed cDNA libraries derived from phytohemagglutinin (PHA)-stimulated peripheral blood T cells and the HUT-78 T-cell line (Clontech), a λZAP YT library (provided by Warren Leonard, National Heart, Lung, and Blood Institute), and a λ ZAP library from PHA-activated T cells (provided by K. Kelly, National Cancer Institute). Purified phage DNA was digested and subcloned into pBluescript for sequencing. Sequence data were manipulated and analyzed using the programs of the Genetics Computer Group of the University of Wisconsin and the BLAST program of the National Center for Biotechnology Information.

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Abbreviations: JAK, Janus kinase; JH, JAK homology; L-JAK, leukocyte JAK; NK, natural killer; PHA, phytohemagglutinin; PTK, protein-tyrosine kinase; TCR, T-cell antigen receptor.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09607).

For Northern analysis, total RNA from various human tissues was purchased (Clontech) or prepared from NK cells and T cells.

Immunoprecipitation and Immunoblotting. A peptide corresponding to the deduced C terminus of the L-JAK gene (see Fig. 1; amino acids 1104–1124) was synthesized (Multiple Peptide Systems, San Diego) coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) and used as an immunogen in rabbits. Cells (10^7 cells per point) were labeled with [^{35}S]methionine (0.5 mCi/ml; 1 Ci = 37 GBq) for 2 hr, washed with phosphate-buffered saline, and lysed in buffer containing 1% Triton X-100 (lysis buffer). Postnuclear supernatants were immunoprecipitated with 10 μl of antiserum prebound to protein A-Sepharose, washed in buffer containing 0.1% Triton X-100 (wash buffer), eluted, and electrophoresed in 8% polyacrylamide gels that were subsequently fixed, rinsed in Fluoro-Hance (Research Products International), and dried for autoradiography.

Kinase assays were performed as described (33, 34) by solubilizing cells in lysis buffer supplemented with 1 mM Na_3VO_4 and 1 mM EDTA and immunoprecipitating with the antipeptide antiserum. The washed immunoprecipitates were incubated in 50 μl of buffer containing 20 mM Tris, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 μM ATP, and [γ-^{32}P]ATP (Amersham) at 200 μCi/ml. The reaction was carried out for 15 min at 25°C and was terminated by the addition of ice-cold wash buffer. After washing the beads again, the reaction products were eluted and electrophoresed.

For immunoblot analysis, cells were solubilized in lysis buffer, and postnuclear supernatants (≈100 μg of protein) from the indicated cells were electrophoresed, transferred to nitrocellulose, and immunoblotted. Filters were blocked, incubated with antiserum (1:1000), washed, and incubated with peroxidase-conjugated goat anti-rabbit IgG. Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham).

RESULTS

Cloning of an NK Cell-Derived Janus Family PTK. To detect previously unknown PTKs expressed in NK cells, we employed PCR, an approach used successfully by others (28, 48). We prepared cDNA from NK cell mRNA using reverse transcriptase and then performed PCR with degenerate oligonucleotide primers corresponding to conserved motifs in the catalytic domains of PTKs. The forward primers were designed to correspond to residues in subdomain VI and were designed to exclude src family PTKs (48). The reverse primer corresponded to the reverse complement of the DVWSFG motif (subdomain IX) conserved in a large number of PTKs. Out of ≈200 clones, seven previously unknown putative kinases were identified. One of these genes was found by Northern analysis to be expressed preferentially in NK and activated T cells (see below). This PCR-generated fragment was, therefore, used to screen libraries derived from the NK-like cell line, YT, PHA-activated T cells, and HUT-78 cells. Approximately 5×10^5 plaques from each library were screened to obtain multiple overlapping clones that generated sequence corresponding to a single large open reading frame. Fig. 1 depicts the deduced amino acid sequence of the NK cell-derived gene compared to closely related PTKs.

The deduced polypeptide encoded by this gene exhibits features typical of a PTK (49). Like other PTKs, a catalytic domain is present in the C-terminal portion of the molecule (subdomains I–XI). The domain begins with a typical ATP-binding motif at residues 829–834 (subdomain I) in which the canonical GXGXXG motif is evident (Fig. 1, shaded) that is followed by a critical lysine residue in subdomain II (residue 855). Just C-terminal to subdomain VII is a pair of tyrosine residues following an acidic residue that likely represents the autophosphorylation site. In subdomain VIII, phenylalanine and tyrosine residues surround the invariant tryptophan residue. This atypical motif contrasts with the motifs seen in src- and abl-related proteins and growth factor receptors. Notably though, this motif (FWYAPE; Fig. 1, shaded) is present in the Janus family of kinases. The entire catalytic domain, termed the JH1 domain, is composed of 273 amino acids (residues 822–1095) and is followed by a unique C terminus.

In addition to a kinase catalytic domain, the NK-derived gene has a region N-terminal to the PTK catalytic domain that also has elements typical of a protein kinase catalytic domain (Fig. 1, Ia–XIa). This tandem kinase-like (JH2) domain is a characteristic feature of the JAKs (28–31). However, like other family members, this domain in the NK cell-derived gene lacks some standard features of a PTK catalytic domain such as an autophosphorylation site in subdomain VII.

The known JAK family PTKs have large extracatalytic segments (JH3–7 domains) N-terminal to the kinase (JH1) and kinase-like (JH2) domains. While motifs corresponding to SH2 or SH3 domains are lacking, there is a motif that has been suggested to be SH2-like (30), which (Fig. 1, shaded) is conserved in three of the four family members, including the NK cell-derived gene. Immediately N-terminal to this motif is a highly conserved motif that is a potential tyrosine phosphorylation site (VDGYFRL). Other areas of striking homology between the NK-derived gene and other JAKs are evident in the remaining domains (JH5–7). Structurally then, it appears that the NK-derived gene has all of the characteristics of a JAK family PTK. The overall homology of the NK PTK to the most closely related Janus family member, JAK2, is ≈68% identity. A hydrophilicity plot did not show the presence of a hydrophobic domain, suggesting that the NK-derived gene encodes a nonreceptor type of PTK like other Janus proteins.

Using the initiation site indicated, the open reading frame of this gene encompasses 3372 nucleotides and is predicted to encode a polypeptide of 1124 residues. This predicts a molecular weight of 125,014, roughly equivalent to, but slightly smaller than, other JAKs. This predicted molecular weight is consistent with that of the polypeptide identified using the antiserum prepared against this kinase (see below), supporting the contention that this is the correct initiation site.

Expression of the NK-Derived Janus Family PTK. Other members of the Janus family of PTKs (JAK1, JAK2, and Tyk2) are present in a variety of tissues (27–29). We sought, therefore, to determine if the NK-derived Janus PTK also was widely expressed or if its expression was more limited. As shown in Fig. 2*A*, unlike other family members, this PTK gene was found to have restricted tissue distribution. In the absence of stimulation, the gene was found to be expressed solely in NK cells. This filter was also probed with a JAK1 cDNA and, as previously reported, ubiquitous tissue expression was noted with the exception of small intestine (data not shown). Equivalent loading of RNA was confirmed by ethidium bromide staining and by hybridization with a probe against ribosomal RNA (not shown). It should be noted that the signal observed to the left of the liver lane is artifactual and represents binding of the probe to the edge of the filter and not to RNA. Interestingly, while the NK PTK gene was expressed at very low levels in resting T cells, we observed that following activation this gene was induced whereas activation of NK cells did not alter the level of expression. Because the time course of induction of this gene might begin to provide clues to its function, this was more carefully analyzed in the experiment shown in Fig. 2*B*. The expression of this gene in T cells was found to peak 16–24 hr after stimulation with PHA. We next analyzed the expression of

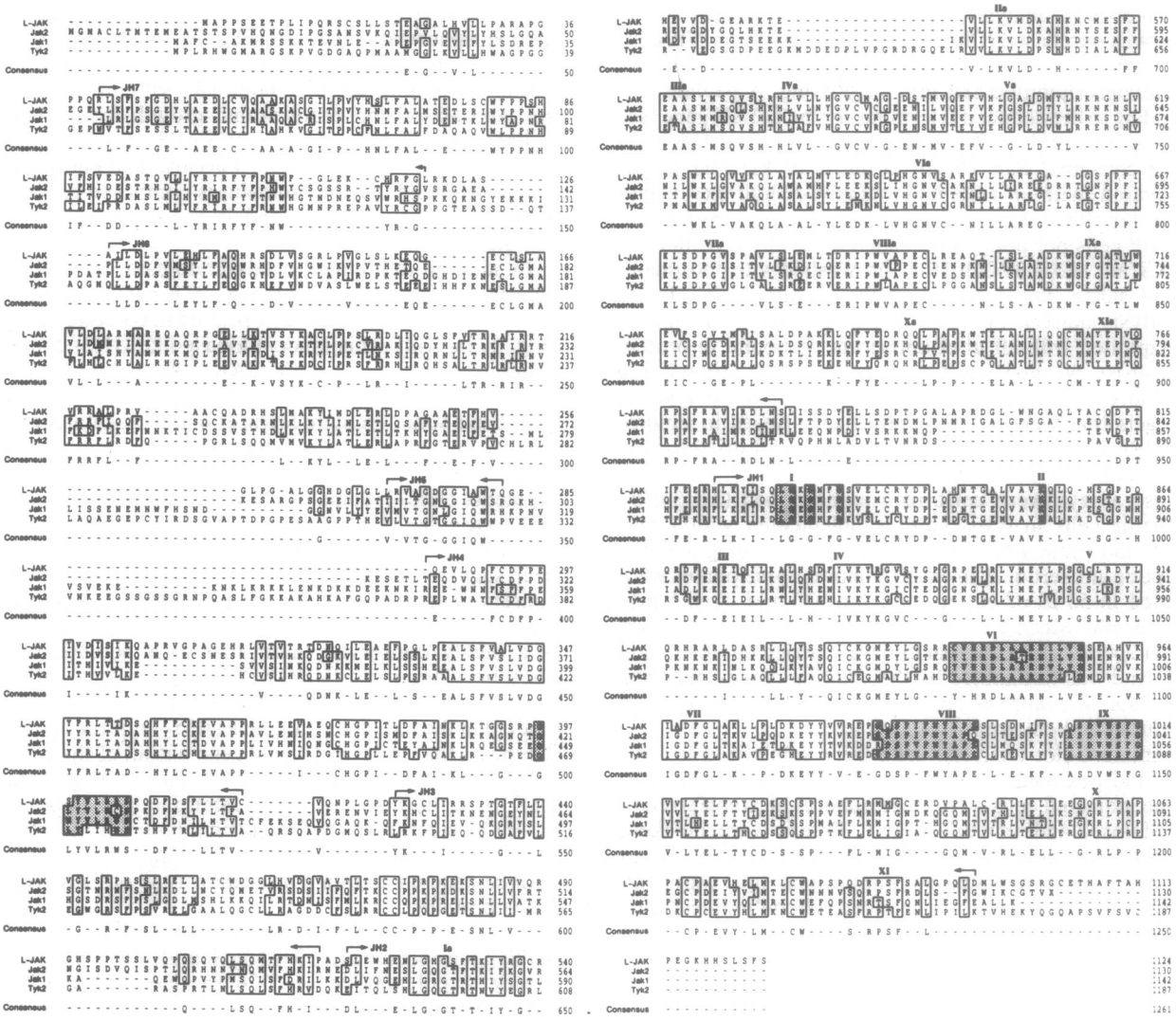

FIG. 1. Predicted amino acid sequence of the L-JAK gene and alignment to the known members of the Janus family of PTKs. The deduced primary structure of the L-JAK gene is depicted in the single-letter amino acid code, and residues that are identical among JAKs are boxed. The subdomains of the PTK domain are indicated with Roman numerals. The subdomains of the kinase-like domain are indicated by Roman numerals followed by the letter a (e.g., Ia). The boundaries of JAK homology (JH) domains are denoted by arrows. Motifs of particular interest are shaded.

this gene in cell lines (Fig. 2*C*). The YT cell line has characteristics of NK cells and, as expected, this gene was found to be present constitutively in these cells. Also as predicted by studies with peripheral blood T cells, the gene was found not to be expressed in the Jurkat T-cell line but was inducible upon activation. HUT-78 cells are a transformed T-cell line, and, interestingly, expression of the gene was found to be constitutive in this cell line. No expression of this gene was detected in a variety of other cell lines, including the erythroleukemia cell line K562. Moreover, it was not inducible in this cell line. Because of the homology to other JAKs and the pattern of tissue expression of the NK-derived PTK gene, we have termed the gene L-JAK for leukocyte JAK.

The deduced C terminus of L-JAK was found to be unique, so a synthetic peptide was generated corresponding to this portion of this gene product (Fig. 1, amino acids 1104–1124) and used as an immunogen. In good agreement with the molecular weight predicted by the deduced primary structure, analysis of metabolically labeled HUT-78 cells showed specific immunoprecipitation of a polypeptide with a M_r of ≈125,000 (Fig. 3*A*). Immunoblot analysis of these cells also showed reactivity of the antibody with a protein of approximately the same mobility in HUT-78 cells. In contrast, Jurkat T cells expressed minimal levels of this protein. In additional experiments (data not shown), the expression of the L-JAK-encoded polypeptide was found to parallel the expression seen by analysis of mRNA. Expression of the protein was detected in NK cells, activated T cells, and in some transformed leukocyte cell lines. Immunoblotting with preimmune serum or antiserum competed with cognate peptide versus irrelevant peptide confirmed the specificity of this reactivity (data not shown).

To ascertain that the L-JAK-encoded kinase had enzymatic activity, *in vitro* kinase assays were performed. A phosphorylated polypeptide with the expected M_r was evident in immunoprecipitates from NK cells (Fig. 3*C*, lane 1) but not in resting T cells (lane 4) or in control immunoprecipitates (lanes 2, 3, and 5). The phosphorylated residues were resistant to KOH, consistent with tyrosine phosphorylation. This likely represents autophosphorylation of L-JAK.

DISCUSSION

The Janus family comprises PTKs that have tandem nonidentical catalytic domains and a large extracatalytic segment (27–29). The structural features of the NK-derived PTK, L-JAK, clearly argues for membership in the Janus family of PTKs. However, unlike the three other members of this

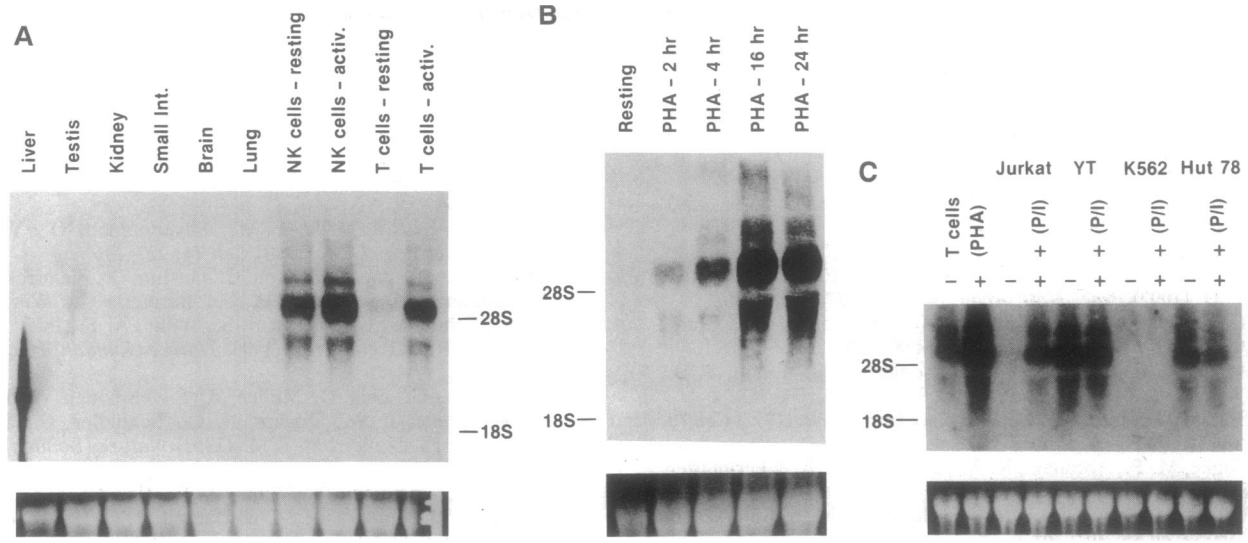

FIG. 2. Expression of L-JAK. (*A*) Tissue-specific expression. Total RNA (20 μg) from various human tissues (purchased from Clontech), NK cells, and T cells was electrophoresed in formaldehyde/agarose gels, transferred to membranes, and probed with a cDNA that corresponds to the JH1 and JH2 domains of L-JAK. T cells were activated (activ.) with PHA for 24 hr. NK cells were activated with interleukin 2 (1000 units/ml) for 24 hr. (*B*) Expression of L-JAK in activated T cells. T cells were stimulated with PHA (2 μg/ml) for the indicated periods prior to preparation of RNA. (*C*) Expression of L-JAK in cell lines. T cells were activated with PHA for 24 hr prior to analysis. Cell lines were grown in complete medium, stimulated or not with phorbol myristate acetate (P; 10 ng/ml) and ionomycin (I; 1 μg/ml) for 24 hr prior to preparing RNA. Equivalent RNA loading was ascertained by ethidium bromide staining and hybridization with a probe corresponding to ribosomal RNA.

family, L-JAK was detected in a narrow spectrum of cells. Among the src family of PTKs, there exist members that are ubiquitous such as Src itself and Yes, whereas others such as Lck, Blk, Fgr, and Hck have restricted tissue distribution (50). Analogously, whereas JAK1, JAK2, and Tyk2 are widely expressed, L-JAK appears to be the first Janus family member with limited tissue expression. Compelling evidence now supports the functional role of Janus family PTKs in cytokine receptor-mediated signaling. We presently do not understand the function of the L-JAK-encoded kinase in NK cells and activated leukocytes. However, it is tempting to speculate that like other family members the L-JAK-encoded kinase will be linked to a cytokine receptor. It will be important to determine how its function relates to the function of other JAKs and if it too works in concert with, or independently of, other family members since a degree of interdependence appears to exist among these kinases.

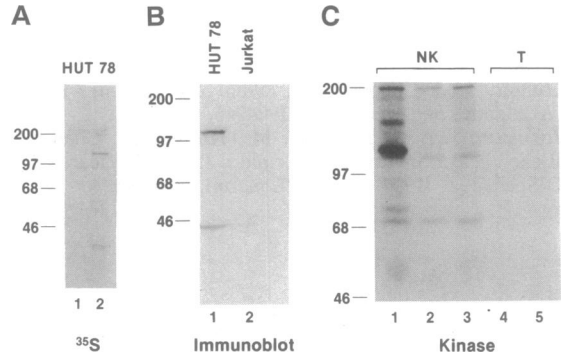

FIG. 3. Expression of the L-JAK-encoded polypeptide. (*A*) Immunoprecipitation. HUT-78 cells were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with anti-peptide antiserum (lane 2) versus irrelevant antiserum (lane 1). (*B*) Immunoblot analysis. Lysates (100 μg) from HUT-78 (lane 1) and Jurkat cells (lane 2) were electrophoresed, transferred to nitrocellulose, immunoblotted with anti-peptide antiserum followed by peroxidase-conjugated anti-rabbit IgG and developed by enhanced chemiluminescence. (*C*) Kinase assay. NK (lanes 1–3) and T (lanes 4 and 5) cell lysates were immunoprecipitated with anti-peptide antiserum (lanes 1, 2, and 4) or preimmune serum (lanes 3 and 5). Lane 2 depicts immunoprecipitation in the presence of cognate peptide. The immunoprecipitates were incubated with [γ-^{32}P]ATP, washed, and electrophoresed.

We previously observed that NK cells have much higher basal protein-tyrosine phosphorylation than T cells despite the many similarities of these two subsets. We speculated that one explanation for this finding might be the existence of PTKs in NK cells that are not expressed in resting T cells. Herein we provide evidence of one such PTK that is preferentially expressed in NK cells. It will be important to relate how expression of L-JAK influences specialized NK functions. Notably, L-JAK is also inducible upon activation in T cells. Upon activation, T cells acquire a variety of new functions, such as cytotoxicity, that are constitutive in NK cells. It will be important to determine if and how expression of the L-JAK kinase and perhaps other PTKs influence the functions acquired by activated lymphocytes. Based on the time course of induction of this L-JAK, it is unlikely that it functions in the early events of TCR-mediated activation in T cells. More likely, because of its differential expression, it might be argued that L-JAK kinase is coupled to a receptor that is constitutively functional in NK cells and inducible in T cells. Defining what factors regulate the enzymatic activity of L-JAK kinase may help to elucidate the mechanisms by which lymphoid receptors exert their effects. Additionally though, dissecting the regulation of expression of L-JAK also may provide clues into how the differentiated states of NK cells and activated leukocytes are maintained. It is anticipated that understanding the regulation of enzymatic activity and expression of L-JAK in NK cells and activated leukocytes will provide important insights into the molecular basis of lymphoid function.

Note Added in Proof. The L-JAK kinase has been renamed JAK3 (51).

We thank Drs. Warren Leonard and Kathy Kelly for providing cDNA libraries. We thank Dr. Joyce Frey for assistance in the generation of the anti-peptide antiserum, Drs. Steven Anderson, Stephanie Simek, and Howard Young for helpful technical suggestions, and Joyce Vincent for editing assistance.

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