Brief Definitive Report

Interleukin 12 (IL-12) Induces Tyrosine Phosphorylation of JAK2 and TYK2: Differential Use of Janus Family Tyrosine Kinases by IL-2 and IL-12

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Summary

Interleukin (IL-12) has many effects on the function of natural killer and T cells, and is important in the control of cell-mediated immunity. IL-2 and IL-12 display many similar activities, yet each also induces a distinct set of responses. A human IL-12 receptor subunit has recently been cloned and, like the IL-2Rβ and IL-2Rγ, is a member of the hematopoietic receptor superfamily; however, the molecular mechanisms of IL-12 action are unknown. In this report we show that IL-12 and IL-2 induce tyrosine phosphorylation of distinct members of the Janus (JAK) family of protein tyrosine kinases in human T lymphocytes. IL-12, but not IL-2, stimulates the tyrosine phosphorylation of TYK2 and JAK2, whereas JAK1 and JAK3, which are phosphorylated in response to IL-2, are not phosphorylated after IL-12 treatment. The use of distinct but related JAK family tyrosine kinases by IL-12 and IL-2 may provide a biochemical basis for their different biological activities.

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IL-12 is a recently characterized cytokine important in the control of cell-mediated immunity (1, 2). Produced by monocytes, macrophages, and possibly other accessory cells (e.g., B cells), in response to bacteria, bacterial products, or intracellular parasites (3, 4), IL-12 has multiple effects on both NK and T cells. Among these, IL-12 induces cytokine production (most notably IFN-γ), enhances cytotoxicity, and stimulates proliferation in combination with other activators such as IL-2 (1, 2, 5–8). The functional relationship between IL-2 and IL-12 is complex; while the two cytokines share many biological effects, each also induces a distinct set of responses. For example, while both IL-2 and IL-12 directly enhance cytotoxic activity and cellular adhesion molecule expression by NK cells (7), only IL-2 is mitogenic when used alone (7). Similarly, both IL-2 and IL-12 induce IFN-γ gene expression and secretion by NK and T cells (6), yet only IL-12 can promote differentiation of $CD4^+$ T cells to the Th1 phenotype (9).

The molecular mechanisms of IL-12 action are poorly understood. The recently cloned low-affinity IL-12 receptor subunit is a member of the hematopoietin receptor superfamily, closely related to gp130 (10). This family also includes the β and γ subunits of the IL-2 receptor (11). Although these receptors lack intrinsic tyrosine kinase activity, rapid tyrosine phosphorylation of multiple cellular substrates is a common and requisite intracellular event in their signaling pathways (12). Recent studies have suggested that this phosphorylation may be mediated in large part by members of a newly described family of cytoplasmic protein tyrosine kinases (PTKs), the Janus (JAK) kinases (13, 14). To date, four JAK family members have been identified: JAK1 (15), JAK2 (16), JAK3 (17), and TYK2 (18). Genetic complementation experiments have demonstrated the requirement for both JAK1 and TYK2 in IFN-α signal transduction (19, 20), and for both JAK1 and JAK2 in the IFN-γ pathway (19, 21); and biochemical studies have shown that JAK1 and JAK2 are coupled to many of the hematopoietin receptors (22, 23). Furthermore, we and others have recently demonstrated that IL-2 stimulation of T and NK cells results in tyrosine phosphorylation of JAK1 and JAK3, elevation of JAK3 phosphotransferase activity, and association of JAK3 with IL-2R (24, 25).

It was recently reported that IL-12 can induce protein tyrosine phosphorylation in human T cells (26); however, the role of JAK family kinases in IL-12 signaling remains unknown. In this study we show tyrosine phosphorylation of multiple substrates after IL-12 stimulation of human lymphocytes, describe differences in the patterns of phosphorylation induced by IL-12 and IL-2, and show that while IL-2 induced phosphorylation of JAK1 and JAK3, IL-12 induced

tyrosine phosphorylation of TYK2 and JAK2, but not JAK1 and JAK3. These data implicate TYK2 and JAK2 in the IL-12 signaling pathway and suggest a biochemical basis for the different functions of IL-2 and IL-12.

Materials and Methods

Reagents. Recombinant human IL-12 (sp act 4.5 × 10^6 U/mg) was the generous gift of Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). Recombinant human IL-2 (18 × 10^6 IU/ml) was kindly provided by Cetus Oncology Corporation (Emeryville, CA). Recombinant human IFN-γ (2.02 × 10^7 IU/mg) was kindly provided by Dr. H. Michael Shepard (Genetech Laboratories, San Francisco, CA). Recombinant human IFN-α (2.4 × 10^8 U/mg) was generously provided by Hoffmann-LaRoche, Inc. (Nutley, NJ). Polyclonal rabbit antisera against JAK1, JAK2, and TYK2, and monoclonal anti-phosphotyrosine antibody 4G10 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit antiserum against JAK3 has been described (17).

Cell Culture and Activation. Human T cells (94 ± 5% $CD3^+$) were isolated from peripheral blood of normal healthy consenting donors by Percoll gradient centrifugation as previously described (27) and cultured for 3 d with 2 μg/ml PHA (Sigma Chemical Co., St. Louis, MO). The IL-12–responsive human NK cell line NK3.3 (28; Young, H. A., manuscript in preparation) was generously provided by Dr. J. Kornbluth (Arkansas Cancer Research Center, Little Rock, AR) and grown in RPMI medium with 15% FCS further supplemented with 10% Lymphocult-T (Biotest Diagnostics Corp., Denville, NJ) and 20 U/ml IL-2. Before stimulation, cells were washed in acidified RPMI (pH 6.4) and rested for 24 h in RPMI with 2% FCS.

Immunoprecipitation and Immunoblotting. Stimulated cells were washed before being lysed in buffer containing 0.5% Triton X-100 as previously described (24, 29). Clarified lysates were immunoprecipitated with anti-JAK1, -JAK2, -TYK2, or -JAK3 antisera conjugated to protein A–coupled Sepharose beads, or with anti-phosphotyrosine mAb conjugated to protein G–coupled Sepharose beads. The immunoprecipitates were resolved on 8% SDS–polyacrylamide gels and transferred to Immobilon (Millipore Corp., Bedord, MA) (29).

For immunoblotting with antiphosphotyrosine, membranes were blocked in Tris-buffered saline (TBS) containing 1% fish gelatin, 2% goat serum, 0.1% BSA, and 0.5% Tween 20 and sequentially incubated with antiphosphotyrosine antibody, biotinylated goat anti–mouse IgG (Oncogene Science, Inc., Cambridge, MA), and horseradish peroxidase–conjugated streptavidin (Oncogene Science, Inc.). Detection was performed by use of enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

For immunoblotting with antibodies to JAK family kinases, membranes were blocked in TBS containing 0.1% Tween and 5% nonfat dried milk, incubated sequentially with primary antibody and horseradish peroxidase–conjugated goat anti–rabbit IgG (Boehringer Mannheim, Indianapolis, IN), and detected with ECL. When a membrane was reprobed, it was first treated with 15% H_2O_2.

Results

IL-12, but not IL-2, Induces Tyrosine Phosphorylation of the JAK Family Kinases TYK2 and JAK2 in Human T Cells. IL-12 and IL-2 have similar yet distinct biological effects on human T and NK cells. To investigate the biochemical events induced in human T cells by stimulation with IL-12 or IL-2, lysates were immunoprecipitated with antibody to phosphotyrosine and analyzed by antiphosphotyrosine immunoblotting. As shown in Fig. 1, stimulation with IL-12 (lanes *2–5*) caused increased tyrosine phosphorylation of a number of substrates compared with untreated control cells (lane *1*). These substrates were each phosphorylated with similar kinetics: maximal phosphorylation was observed within 5 min, and this was maintained for 30 min of stimulation. While many proteins phosphorylated in response to IL-12 were also phosphorylated in response to IL-2 (Fig. 1, lane *6*), differences were observed in the tyrosine phosphorylation of proteins with apparent molecular masses of 120–140 kD. One of the most prominent substrates, a phosphoprotein of ∼135 kD that was not detectable by antiphosphotyrosine immunoblotting in unstimulated cells, became strongly phosphorylated after stimulation with IL-12, but not IL-2.

The JAK family of cytoplasmic PTKs, which have been implicated in signaling by the hematopoietin family of receptors, have molecular masses between 120 and 140 kD. Moreover, we recently have shown that IL-2 causes tyrosine phosphorylation of JAK3 and JAK1 (24). We therefore sought to establish whether JAK family proteins were also phosphorylated in response to IL-12. Since the 135-kD substrate was of the approximate molecular mass of TYK2, the antiphosphotyrosine immunoprecipitates shown in Fig. 1 were reprobed with antiserum to TYK2 (Fig. 2 *A*). A single band corresponding to the 135-kD phosphoprotein was de-

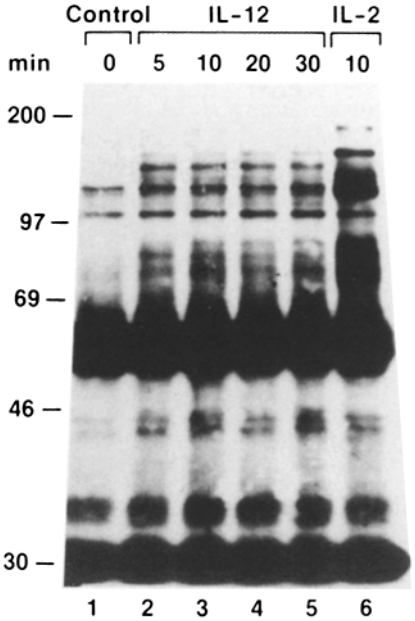

Figure 1. IL-12 and IL-2 induce distinct patterns of tyrosine phosphorylation in human T cells. PHA-activated human T cells (2 × 10^7) were incubated for the indicated times in medium alone (lane *1*), with 100 U/ml IL-12 (lanes *2–5*), or with 1,000 U/ml IL-2 (lane *6*) and lysates immunoprecipitated with antiphosphotyrosine mAb. The immunoprecipitates were resolved by SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting.

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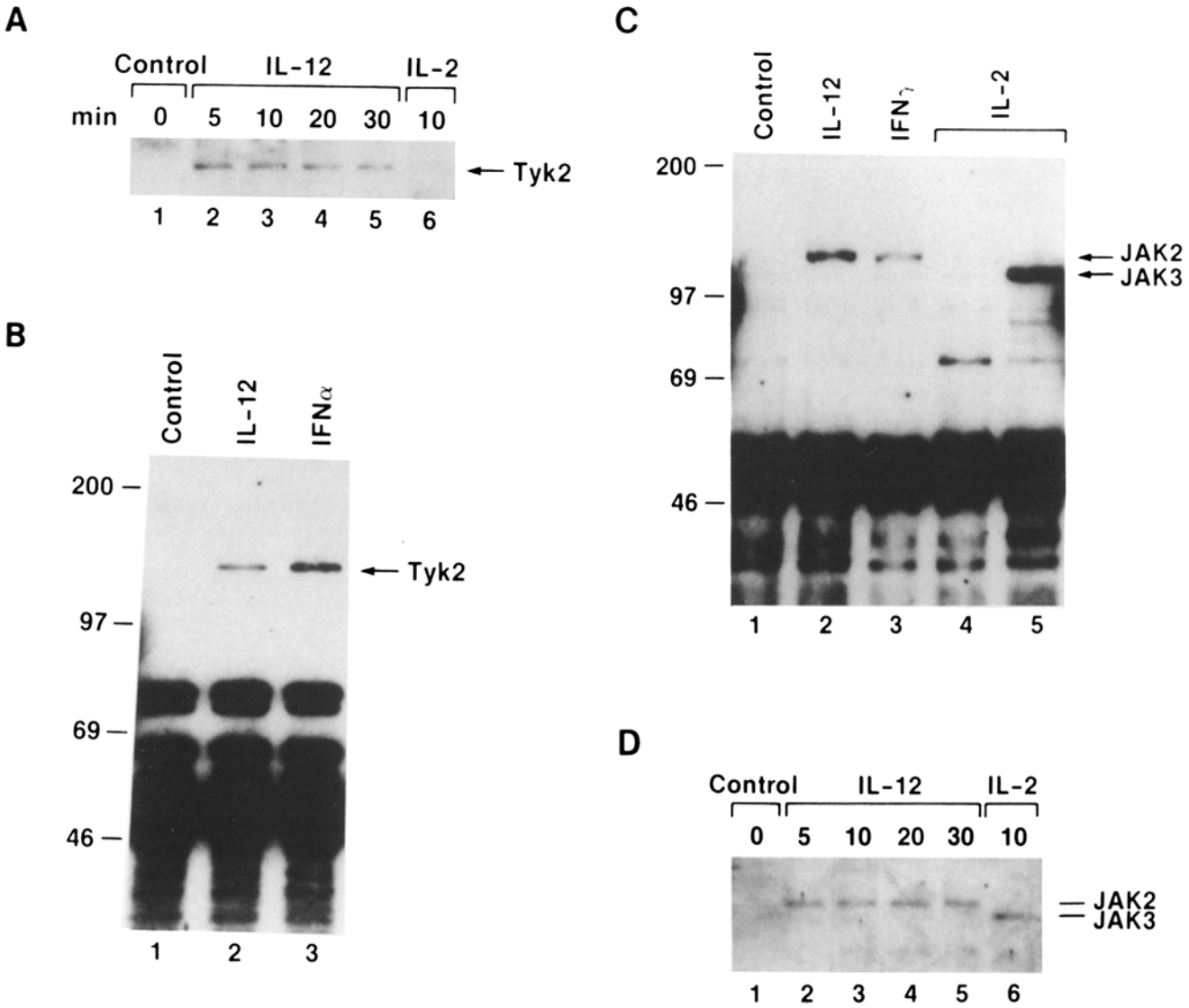

Figure 2. IL-12 induces phosphorylation of JAK family kinases JAK2 and TYK2. (*A*) Anti-TYK2 immunoblotting: the blot of antiphosphotyrosine precipitates from IL-12– (lanes *2–5*) and IL-2– (lane *6*) activated T cells shown in Fig. 1 was reprobed with antiserum to TYK2. (*B*) Antiphosphotyrosine immunoblotting of anti-TYK2 immunoprecipitates: PHA-activated human T cells (2×10^7) were incubated for 15 min in medium alone (lane *1*), with 100 U/ml IL-12 (lane *2*), or with 1,000 U/ml IFN-α (lane *3*), and lysates immunoprecipitated with anti-TYK2 antiserum. The immunoprecipitates were resolved by SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting. (*C*) Antiphosphotyrosine immunoblotting of anti-JAK2 immunoprecipitates: PHA-activated human T cells (2×10^7) were incubated for 15 min in medium alone (lane *1*), with 100 U/ml IL-12 (lane *2*), with 1,000 U/ml IFN-α (lane *3*), or with 1,000 U/ml IL-2 (lanes *4* and *5*). Lysates were precleared with a control antiserum (lanes *1–3* and *5*) or with antiserum to JAK3 (lane *4*) and then immunoprecipitated with antiserum to JAK2. The immunoprecipitates were resolved by SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting. (*D*) Anti-JAK2 immunoblotting of antiphosphotyrosine immunoprecipitates: the blot of antiphosphotyrosine precipitates from IL-12– (lanes *2–5*) and IL-2– (lane *6*) activated T cells shown in Fig. 1 was reprobed with antiserum to JAK2.

tected only in antiphosphotyrosine immunoprecipitates from cells stimulated with IL-12 (Fig. 2 *A*, lanes *2–5*) and not from unstimulated cells (lane *1*) or cells stimulated with IL-2 (lane *6*).

To further confirm the 135-kD phosphoprotein as TYK2, the converse approach was used. Lysates of unstimulated or cytokine-stimulated T cells were immunoprecipitated with antibody against TYK2 followed by antiphosphotyrosine immunoblotting. As shown in Fig. 2 *B*, TYK2 kinase from IL-12–stimulated T cells (lane *2*) was phosphorylated on tyrosine residues, whereas that from unstimulated cells (lane *1*) was not. In agreement with previous reports (19, 30), tyrosine phosphorylation of TYK2 was also observed in these cells in response to IFN-α (Fig. 2 *B*, lane *3*). However, consistent with our previous findings (24) and with the data presented in Fig. 2 *A*, TYK2 immunoprecipitated from IL-2–stimulated T cells was not tyrosine phosphorylated (data not shown).

Several cytokines (including IL-2, IL-6, IFN-α, and IFN-γ) have been shown to be linked to more than one JAK family kinase in the same cell (19, 22, 24). We thus examined whether other JAK family kinases were phosphorylated in T cells in

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response to IL-12. As shown in Fig. 2 *C*, the 130-kD JAK2 kinase was tyrosine phosphorylated in immunoprecipitates from IL-12–treated cells (lane *2*) but not from unstimulated cells (lane *1*). IFN-γ similarly induced tyrosine phosphorylation of JAK2 (lane *3*). The response to IL-2 is more complicated as the commercial anti-JAK2 antiserum cross-reacts with JAK3. Nevertheless, the two kinases can be distinguished by their molecular weights (25). Immunoprecipitation of lysates from IL-2–stimulated cells with this antiserum thus revealed a tyrosine phosphoprotein of 125-kD, JAK3, but showed no phosphorylation of JAK2 itself (Fig. 2 *C*, lane *5*). Preclearing of lysate from IL-2–treated cells with antiserum to JAK3 before immunoprecipitation with anti-JAK2 confirmed the identity of the 125-kD band as JAK3 and not JAK2 (lane *4*).

To verify that the 130-kD protein phosphorylated in response to IL-12 (Fig. 1) was JAK2, the same blot shown in this figure was stripped a second time and reprobed with antiserum to JAK2 (Fig. 2 *D*). A single band migrating exactly with the 130-kD protein was detected in the antiphosphotyrosine immunoprecipitates from IL-12–activated cells (Fig. 2 *D*, lanes *2–5*) but not from unstimulated cells (lane *1*) or IL-2–stimulated cells (lane *6*), confirming that this protein is indeed JAK2. Again, a 125-kD band was detected in antiphosphotyrosine immunoprecipitates from IL-2–stimulated cells (Fig. 2 *D*, lane *6*) representing cross-reactive phosphorylated JAK3 protein as discussed above. These data indicate that IL-12, but not IL-2, is able to induce rapid tyrosine phosphorylation of the Janus family PTKs, TYK2 and JAK2.

In Contrast to IL-2, IL-12 Does Not Induce Tyrosine Phosphorylation of the JAK Family Kinases JAK3 and JAK1 in Human T Cells. We recently have reported that IL-2 stimulates tyrosine phosphorylation of the 125-kD JAK family kinase JAK3 and the 130-kD JAK1 (24). We therefore investigated directly whether IL-12 would stimulate tyrosine phosphorylation of these kinases. Immunoprecipitation with polyclonal antiserum against JAK3, followed by antiphosphotyrosine blotting, confirmed that this kinase was phosphorylated on tyrosine in response to IL-2 (Fig. 3 *A*, lane *2*) but clearly not in response to IL-12 (lane *3*). Similarly, antiphosphotyrosine immunoblotting of JAK1 immunoprecipitates showed that tyrosine phosphorylation of JAK1 was enhanced by stimulation of T cells with IL-2 (Fig. 3 *B*, lane *2*) but not by IL-12 (lane *3*).

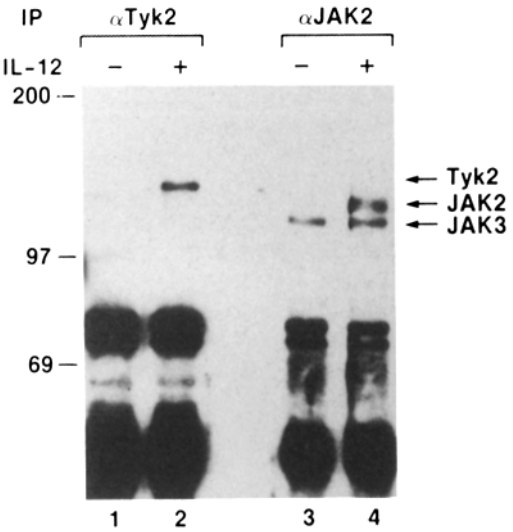

Figure 4. IL-12 induces phosphorylation of TYK2 and JAK2 in the NK3.3 cell line. NK3.3 cells (10^7) were incubated for 15 min in medium alone (lanes *1* and *3*) or with 100 U/ml IL-12 (lanes *2* and *4*). Lysates were immunoprecipitated with antiserum to either TYK2 (lanes *1* and *2*) or JAK2 (lanes *3* and *4*). Immunoprecipitates were resolved by SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting.

IL-12 Induces Tyrosine Phosphorylation of TYK2 and JAK2 in the Human NK Cell Line NK3.3. Individual cytokines might induce phosphorylation of different JAK family kinases in different cell types (22). Because IL-12 has potent effects on NK cells, we investigated the phosphorylation of TYK2 and JAK2 after IL-12 stimulation of the human NK cell line NK3.3. Lysates of unstimulated or IL-12–stimulated NK3.3 cells were immunoprecipitated with antisera against JAK2 or TYK2 and immunoblotted with antiphosphotyrosine. Fig. 4 shows that in NK3.3 cells, like T cells, IL-12

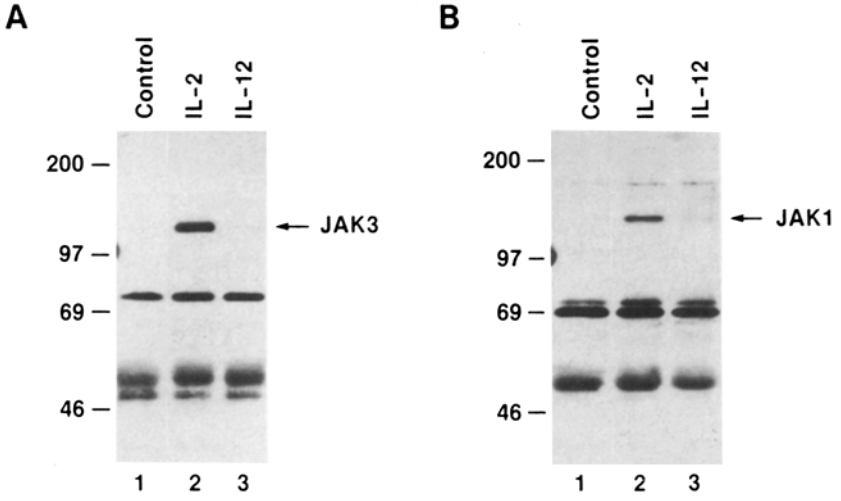

Figure 3. IL-2, but not IL-12, induces tyrosine phosphorylation of JAK1 and JAK3. PHA-activated human T cells (2×10^7) were incubated for 15 min in medium alone (lanes *1*), with 1,000 U/ml IL-2 (lanes *2*), or with 100 U/ml IL-12 (lanes *3*). Lysates were immunoprecipitated with antiserum to either JAK3 (*A*) or JAK1 (*B*). Immunoprecipitates were resolved by SDS-PAGE and analyzed by antiphosphotyrosine immunoblotting.

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treatment leads to tyrosine phosphorylation of both TYK2 (lanes *1* and *2*) and JAK2 (lanes *3* and *4*). Since NK3.3 cells are routinely cultured in the presence of IL-2, and thus exhibit background levels of JAK3 phosphorylation, phosphorylated JAK3 was immunoprecipitated in these experiments by the cross-reactive JAK2 antiserum. JAK3, however, migrated slightly faster than JAK2, and its phosphorylation was clearly not altered by IL-12 treatment of the cells. Stimulation of NK3.3 cells by IL-2 was unable to induce tyrosine phosphorylation of TYK2 or JAK2 (data not shown). Additionally, IL-2 stimulation of NK3.3 cells led to tyrosine phosphorylation of JAK1 and JAK3, whereas stimulation with IL-12 did not (data not shown) (24).

Discussion

In this report, we demonstrate that IL-12 stimulation of human lymphocytes results in the rapid tyrosine phosphorylation of a number of cellular substrates, including the JAK family kinases TYK2 and JAK2. In contrast, we show that IL-2 induces tyrosine phosphorylation of the kinases JAK1 and JAK3 but not TYK2 or JAK2.

Accumulating evidence supports a model of cytokine signaling whereby ligand binding induces homo- or heterodimerization of receptor chains, leading to the formation of a multiprotein signaling complex in which receptor-associated JAK kinases are tyrosine phosphorylated and activated (13, 31). Our finding that IL-12 treatment of human lymphocytes leads to rapid tyrosine phosphorylation of TYK2 and JAK2 implicates these kinases in the immediate intracellular response to IL-12. Both TYK2 and JAK2 have been shown to be involved in the signal transduction pathways of other cytokine receptors, including the IFN (20, 21), IL-6 (22), and IL-3/IL-5/GM-CSF (23, 32) cytokine families. However, in contrast to their apparently promiscuous use by many cytokines, the tyrosine phosphorylation of both JAK2 and TYK2 by a single cytokine in multiple cell types thus far is unique to IL-12. Perhaps the combinatorial use of JAKs is one means that different receptors use to achieve specificity in signaling. It will be important to determine the relative requirements for TYK2 and JAK2 in IL-12 signaling.

As described above, while IL-12 and IL-2 have many similar functions, each also induces a variety of distinct response (5–8). The molecular basis for such functional redundancy and specificity between cytokines is unclear. IL-12 and IL-2 cause tyrosine phosphorylation of related but distinct JAK kinases, the substrate specificities of which have not yet been determined. However, since these kinases are highly homologous (16), it is likely that some cell-signaling proteins will be substrates for all JAKs, while others will be phosphorylated only by certain family members. The similar biological functions of IL-2 and IL-12 might result from activation of common signaling pathways, while specific effects might be produced by stimulation of unique pathways by the distinct combination of JAKs used by each cytokine. The ability of different cytokine receptor chains to bind cell signaling proteins and recruit them to a functional receptor-signaling complex where they may serve as substrates for JAK family kinases is also likely to be important in determining specificity of response from receptors that share signaling components.

A likely set of substrates for the JAKs is the family of latent cytoplasmic transcription factors termed STATs (signal transducers and activators of transcription) (31). Ligand binding to many cytokine receptors (such as gp130 and the IFN receptors) induces tyrosine phosphorylation of STAT family members that subsequently translocate to the nucleus, bind to related DNA sequences, and promote transcription (13, 31). It will be interesting to determine whether IL-12 and IL-2 differentially regulate unique or known STAT family members.

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