Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2

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INTERLEUKIN-2 is an autocrine growth factor for T cells[1,2] which also activates other cells including B cells[3] and natural killer cells[4]. The subunits of the interleukin-2 receptor (IL-2R) lack intrinsic enzymatic activity, but protein tyrosine phosphorylation is a critical event following ligand binding and *src* family kinases, such as Lck, are known to be activated by IL-2 (refs 5–9). However, IL-2 signalling can occur in the absence of receptor interaction with Lck, suggesting that other protein tyrosine kinases might be important[10]. Here we report that a new member of the Janus family of kinases (Jak-3) is coupled to the IL-2R in human peripheral blood T cells and natural killer cells.

Jak-3 (previously termed L-JAK) is a unique kinase of relative molecular mass 125,000 (M_r 125K) which we have cloned from natural killer (NK) cells[11]. It differs in expression from other Jaks as measured both by northern analysis[11] and western blotting using a Jak-3 peptide antiserum which does not crossreact with other Jaks (Fig. 1). Expression was detected in NK and T cells but not in other cells examined, whereas the other Jaks were more widely expressed (data not shown). Additionally, Jak-3 was expressed at low levels in resting peripheral blood T cells, but the abundance was greatly increased after activation by either phytohaemagglutinin (PHA) (Fig. 1*a*, *b*) or anti-CD3 (data not shown), the maximal expression being seen at 24–48 h. Although Jak-3 can be induced in other cells, such as monocytes, given the appropriate stimulus (T. Musso *et al.*, manuscript in preparation), Jak-3 induction on T-cell activation suggested that this kinase may be functionally important in these cells.

Because the Jaks have been shown to be important in haematopoietic receptor family signalling[12–14] and because the induction of the IL-2 and the IL-2R α-chain are critical events in the activation of T cells[15,16], we next examined whether Jak-3 might be coupled to this receptor. We observed little basal tyrosine phosphorylation of Jak-3 in YT, NK 3.3, NK and HUT-78 cells (Fig. 2*a*, lanes 1, 3, 5; *b*, lane 1). However, after IL-2 treatment, marked phosphorylation was evident (Fig. 2*a*, lanes 2, 4, 6; *b*, lanes 2, 3, 5) but was not detectable in control immunoprecipitates (Fig. 2*b*, lane 4).

Cytokine stimulation of cells results in increased phosphotransferase activity of the Jak family kinases[17]. Jak-3 immunoprecipitated from YT cells (Fig. 2*c*, lanes 3 and 4), NK cells (lane 7) and Hut 78 cells (lane 9) stimulated with IL-2 also displayed elevated enzymatic activity, as measured by *in vitro* autophosphorylation, which peaked at about 10 min (Fig. 2*c*, lane 3). The kinase activity was not precipitated in the presence of competing Jak-3 peptide (Fig. 2*c*, lanes 2 and 4). This was exclusively tyrosyl phosphorylation, as determined by resistance to potassium hydroxide and by phosphoamino acid analysis (data not shown). Although this measure of activity may not necessarily reflect activity towards other substrates, it suggests enzymatic activation of Jak-3 by IL-2.

To link more firmly Jak-3 to the IL-2R signalling pathway, we next sought to determine if Jak-3 became associated with the IL-2R complex after ligand binding. Although Jak-3 was minimally, but detectably, associated with IL-2Rβ in untreated YT cells (Fig. 2*d*, lane 1), 15 and 30 min after IL-2 stimulation, it was readily apparent in the receptor complex (lanes 2, 3), but not in control immunoprecipitates (lane 4). Thus, although Jak-3 kinase activity was reduced by 30 min, it remains in the receptor complex; the significance of this is unclear.

IL-2 stimulation induces tyrosyl phosphorylation of a variety of substrates[7,18] and in YT cells the most prominent substrate was a 125K polypeptide (Fig. 3*a*, lane 2) which comigrated with Jak-3. To determine the identity of this substrate, lysates from IL-2-treated cells were first precleared with rabbit polyclonal antisera (Fig. 3*a*, lanes 1 and 2), anti-Jak-3 (lanes 3 and 4) or anti-Jak-1 (lanes 5 and 6), before immunoprecipitation with antiphosphotyrosine. Depletion using anti-Jak-3 antiserum specifically removed the 125K tyrosine-phosphorylated protein, indicating that Jak-3 was one of the most prominent phosphoproteins detected in response to IL-2 in these cells (Fig. 3*a*).

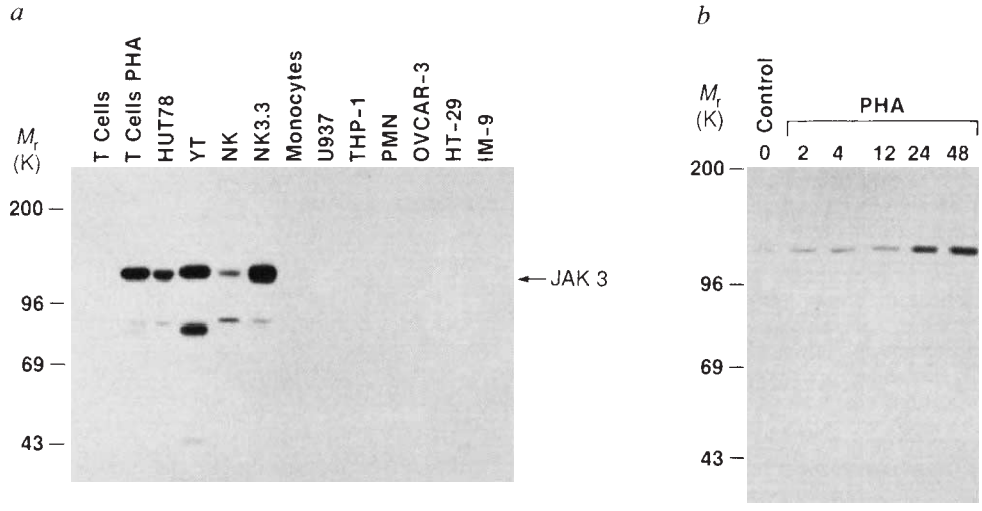

FIG. 1 Expression of Jak-3 in activated T cells and NK cells. *a*, Whole-cell lysates from human peripheral blood T cells (unstimulated or stimulated for 48 h with PHA), the transformed T cells lines Hut 78 and YT, peripheral blood NK cells, the NK 3.3 cell line, human peripheral blood monocytes, the myelomonocytic cell lines U937 and THP-1, and the tumour cell lines OVCAR-3, HT-29 and IM-9 were subjected to SDS–PAGE, transferred to Immobilon and probed with antisera to Jak-3. *b*, Jak-3 expression in human T lymphocytes activated with PHA for 0–48 h.
METHODS. Human peripheral blood T lymphocytes, NK cells and monocytes (>97% pure) were obtained by leukophoresis from donors. T lymphocytes were either untreated or treated with PHA (10 μg ml^{-1}) for 0–48 h. The procedures used for lysis, electrophoresis and blotting were described previously[11]. The anti-Jak-3 antiserum was raised against a synthetic peptide corresponding to the C-terminal region of the Jak-3 protein (amino acids 1,104–1,124)[11].

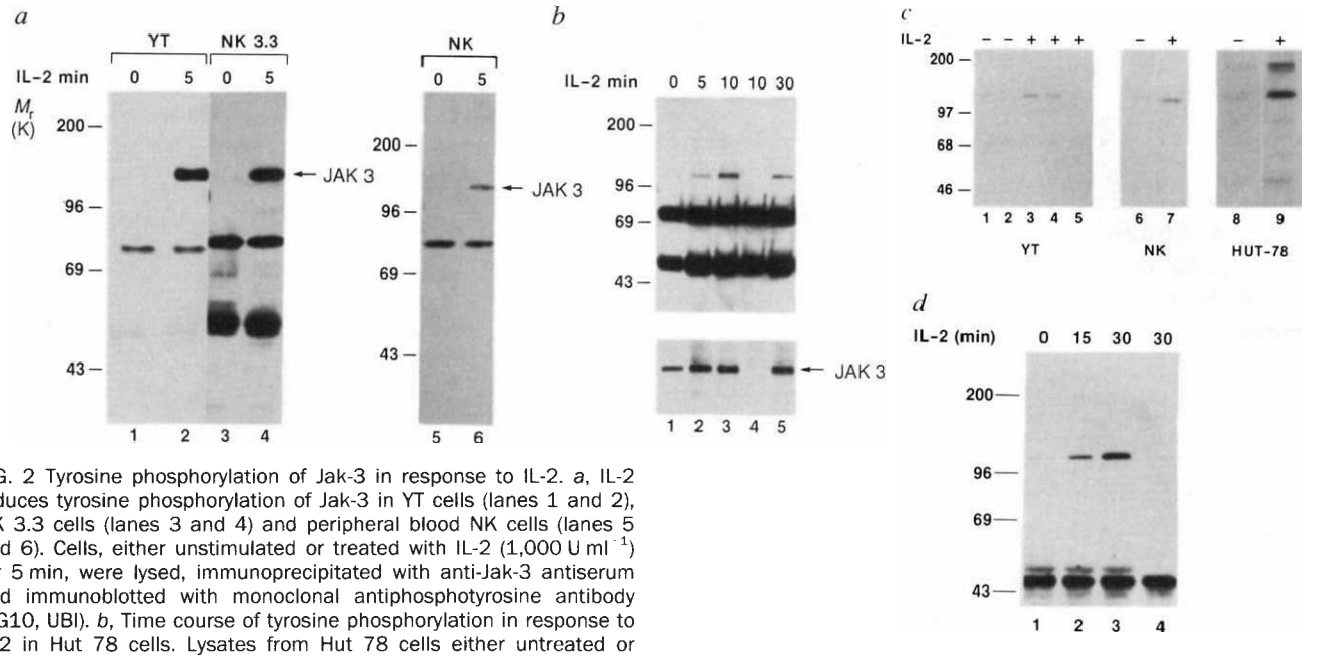

FIG. 2 Tyrosine phosphorylation of Jak-3 in response to IL-2. *a*, IL-2 induces tyrosine phosphorylation of Jak-3 in YT cells (lanes 1 and 2), NK 3.3 cells (lanes 3 and 4) and peripheral blood NK cells (lanes 5 and 6). Cells, either unstimulated or treated with IL-2 (1,000 U ml^{-1}) for 5 min, were lysed, immunoprecipitated with anti-Jak-3 antiserum and immunoblotted with monoclonal antiphosphotyrosine antibody (4G10, UBI). *b*, Time course of tyrosine phosphorylation in response to IL-2 in Hut 78 cells. Lysates from Hut 78 cells either untreated or treated with IL-2 for 5, 10 or 30 min, were immunoprecipitated with anti-Jak-3 and blotted with antiphosphotyrosine antibody and with anti-Jak-3 (lower panel). Lane 4 shows competition with Jak-3-specific peptide. *c*, IL-2 stimulates Jak-3 *in vitro* kinase activity. YT cells were treated with IL-2 (1,000 U ml^{-1}) for 0 (lanes 1 and 2), 10 (lane 3) or 20 (lanes 4 and 5) min. *In vitro* kinase activity was measured in anti-Jak-3 immunoprecipitates. Lanes 2 and 5 represent competition with Jak-3-specific peptide. Jak-3 *in vitro* kinase activity in response to IL-2 stimulation (15 min) was measured in peripheral blood NK cells (lanes 6 and 7) and Hut 78 cells (lanes 8 and 9). *d*, Coimmunoprecipitation of Jak-3 with IL-2Rβ YT cells were treated with IL-2 for 0, 15 and 30 min lysed in 0.1% Triton, 0.75% Brij 96 and the lysate immunoprecipitated with anti-IL-2Rβ chain (lanes 1, 2, 3) or an isotyped matched irrelevant antibody and blotted with anti-Jak-3.

In Fig. 3*b*, immunoblots from a separate experiment were deliberately overexposed to reveal additional substrates. We observed a tyrosyl phosphoprotein induced in response to IL-2 (Fig. 3*b*, lanes 2 and 3) which was larger than Jak-3 (lanes 5 and 6). Immunoblotting confirmed that this protein was Jak-1 (Fig. 3*b*, lanes 8 and 9). Although both Tyk-2 and Jak-2 could be detected in YT cells, no tyrosyl phosphorylation of either protein was observed in response to IL-2. This was determined both by immunoprecipitating with antiphosphotyrosine antibodies and blotting with anti-Jak antibodies and the converse (not shown).

To determine the specificity of Jak-3 phosphorylation, we next examined the response to a number of other cytokines. In YT cells, Jak-3 was intensely tyrosine phosphorylated in response to IL-2, but only minimal alterations of phosphorylation were seen in response to growth hormone[19] interferon-α (IFN-α) or -γ (Fig. 4*a*), whereas in these cells we could detect tyrosine phosphorylation of Jak-2 in response to IFN-γ[20] (Fig. 4*b*). In addition, Jak-3 was not phosphorylated in response to IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF) or erythropoietin (data not shown) stimuli that have previously been shown to activate Jak-2[21,22].

Recently the IL-4 receptor has been shown to comprise the common γ-chain of the IL-2 receptor (γ_c)[23,24], suggesting that common signalling pathways may also be used by these cytokines. This prompted us to examine whether Jak-3 was phos-

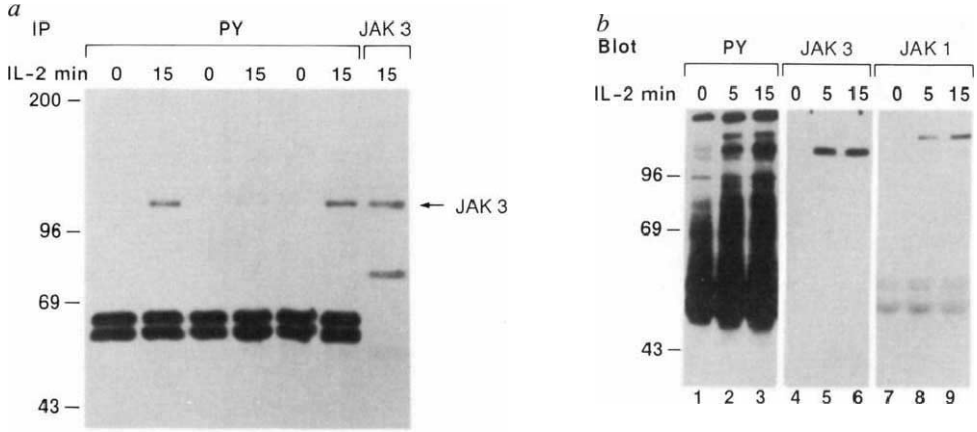

FIG. 3 IL-2-induced tyrosyl phosphorylation of Jak family kinases. *a*, Jak-3 is the most prominent tyrosine phosphorylated protein in response to IL-2 in YT cells. Lysates from YT cells treated with IL-2 (1,000 U ml^{-1}) for 0 or 15 min were precleared with either rabbit polyclonal antiserum (lanes 1 and 2), anti-Jak-3 (lanes 3 and 4) or anti-Jak-1 (lanes 5 and 6) before immunoprecipitation and immunoblotting with anti-phosphotyrosine. Lane 7 represents immunoprecipitation with anti-Jak-3 and immunoblotting with antiphosphotyrosine. *b*, Jak-3 and Jak-1 are phosphorylated in response to IL-2. YT cells treated with IL-2 (1,000 U ml^{-1}) for 0, 5 or 15 min, immunoprecipitated with antiphosphotyrosine and immunoblotted with antiphosphotyrosine (lanes 1–3), anti-Jak-3 (lanes 4–6) or anti-Jak-1 (lanes 7–9).

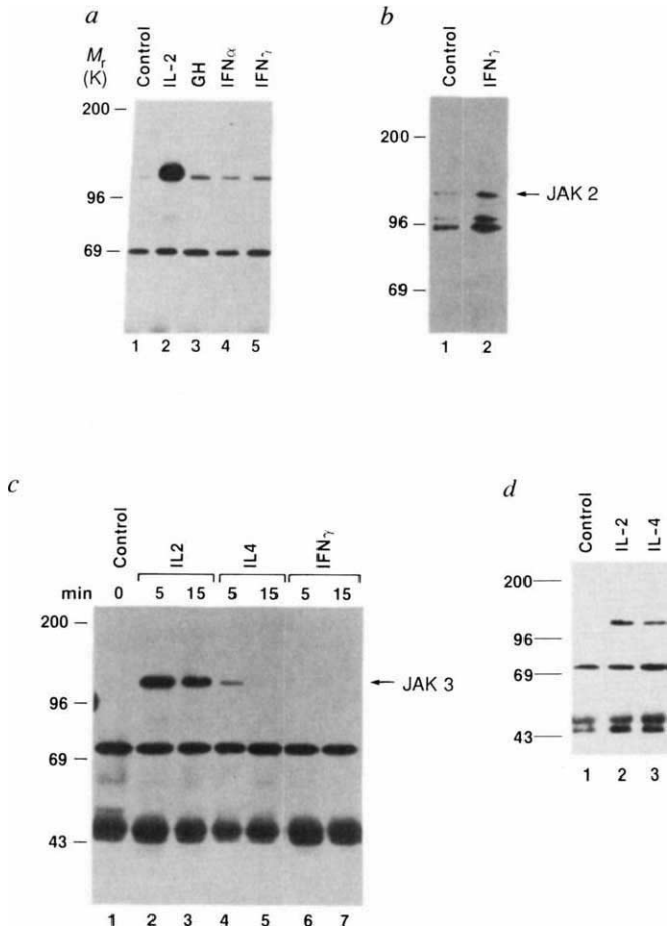

FIG. 4 Phosphorylation of Jak-3 in response to IL-4 but not other cytokines. *a*, YT cells were treated with IL-2 (1,000 U ml^{-1}), growth hormone (GH; 50 ng ml^{-1}), IFN-α (1,000 U ml^{-1}) or IFN-γ (500 U ml^{-1}) for 15 min and the lysates immunoprecipitated with anti-Jak-3 and blotted with antiphosphotyrosine. *b*, YT cells were either unstimulated (lane 1) or treated with IFN-γ for 15 min (lane 2), immunoprecipitated with antiphosphotyrosine and blotted with Jak-2. *c*, NK 3.3 cells rested overnight in 2% serum were challenged with IL-2 (1,000 U ml^{-1}; lanes 2 and 3), IL-4 (100 U ml^{-1}; lanes 4 and 5) or IFN-γ (500 U ml^{-1}; lanes 6 and 7) for 5 or 15 min and the lysates were immunoprecipitated with anti-Jak-3 and blotted with antiphosphotyrosine. *d*, Similarly, PHA-activated (72 h) peripheral blood human T cells were rested overnight in 5% serum before challenge with IL-2 (lane 2) or IL-4 (lane 3) as above.

phorylated in response to IL-4. Consistent with this hypothesis, we found that Jak-3 was tyrosine phosphorylated in response to IL-4 as well as IL-2 in NK 3.3 cells (Fig. 4*c*), and human peripheral blood T cells (Fig. 4*d*). Additionally, IL-4 stimulation induced tyrosine phosphorylation of Jak-1 (data not shown).

Two of the IL-2 receptor chains ($\beta + \gamma$) are members of the haematopoietic receptor family[25], many other members of which have been shown to couple to Jak family kinases[26,27].The data here support the contention that both Jak-3 and Jak-1 probably play important roles in lymphoid activation through the IL-2 and IL-4 receptors. Our findings are in agreement with those of Witthuhn *et al.* (accompanying paper)[28]. It will be important to determine precisely how these kinases interact with receptor subunits and *src* family protein tyrosine kinases. Additionally, it will be important to determine if Jak-3 and Jak-1 are necessary intermediates in IL-2 signalling and if an IL-2-dependent protein related to signal transduction and activation of transcription exists. Ultimately, IL-2 receptor Jak interactions may become a useful target for pharmacological intervention. □

Received 24 March; accepted 31 May 1994.

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ACKNOWLEDGEMENTS. We thank D. Longo, D. Ferris and F. Ruscetti for reading the manuscript; N. Rice, T. Copeland and L. Cromwell for assistance on antibody production; R. Robb for providing the IL-2β monoclonal antibody; D. Blair for advice; and S. Charbonneau and J. Vincent for typing and editing the manuscript.

Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells

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MANY cytokines function through interaction with receptors of the cytokine receptor superfamily. Although lacking catalytic domains, cytokine receptors couple ligand binding to induction of protein tyrosine phosphorylation. Recent studies[1–10] have shown that one or more of the Janus kinase family members (Jaks) associate with cytokine receptors and are tyrosine phosphorylated and activated following ligand binding. Here we describe a new Jak family kinase, Jak-3, and demonstrate that Jak-3, and to a lesser extent Jak-1, are tyrosine phosphorylated and Jak-3 is activated in the responses to interleukin-2 and interleukin-4 in T cells and myeloid cells. Jak-3 activation requires the serine-rich, membrane-proximal domain of the interleukin-2 receptor β-chain, but does not require the acidic domain that is required for association and activation of Src family kinases.

Polymerase chain reaction (PCR) experiments identified a complementary DNA fragment encoding a novel Jak in breast cancer cells[11] and, more recently, rat hippocampal neurons[12]. Using the initial fragment, cDNAs were obtained from a murine

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