Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 1878–1883, March 1997
Immunology

Requirement for an initial signal from the membrane-proximal region of the interleukin 2 receptor γ_c chain for Janus kinase activation leading to T cell proliferation

BRAD H. NELSON*, BRYAN C. MCINTOSH, LORI L. ROSENCRANS, AND PHILIP D. GREENBERG

Fred Hutchinson Cancer Research Center, Seattle WA, 98104; and Departments of Immunology and Medicine, University of Washington, Seattle, WA 98195

Communicated by Richard Palmiter, University of Washington, Seattle, WA, December 16, 1996 (received for review August 16, 1996)

ABSTRACT **The interleukin 2 receptor (IL-2R) generates proliferative signals in T lymphocytes by ligand-induced heterodimerization of two chains, IL-2Rβ and γ_c, which associate with the tyrosine kinases Jak1 and Jak3, respectively. Genetic and molecular studies have demonstrated that Jak3 is essential for mitogenic signaling by the γ_c chain; because it is also the only molecule known to associate with γ_c, we speculated that Jak3 might be sufficient for signaling by this chain. Therefore, fusion proteins were constructed in which all or part of the cytoplasmic domain of γ_c was replaced by Jak3. Signaling was evaluated in the IL-2-dependent T cell line CTLL-2 using chimeric IL-2Rβ and γ_c chains that bind and are activated by the cytokine granulocyte–macrophage colony-stimulating factor. Chimeric γ_c chains containing only Jak3 in the cytoplasmic domain failed to mediate proliferation of CTLL-2 cells, but addition of a conserved membrane-proximal (PROX) domain of γ_c in tandem with Jak3 fully reconstituted γ_c function. The requirement for the PROX domain reflected an essential role in the activation of Jak3 *in vivo*. Despite lacking defined catalytic motifs, PROX induced an early Jak-independent signal, including tyrosine phosphorylation of IL-2Rβ and the tyrosine phosphatase SHP-2. The results define the minimal signaling components of γ_c and suggest a new mechanism by which the IL-2R initiates signaling in response to ligand.**

A common theme for signaling by growth factor receptors involves ligand-induced dimerization of receptor chains followed by the rapid induction of intracellular tyrosine kinase activity, mediated either by kinase domains intrinsic to the receptor chains or by cytoplasmic kinases that associate in a noncovalent manner (1). The interleukin 2 receptor (IL-2R) is a member of the hematopoietic receptor superfamily and regulates the proliferation of T lymphocytes (2). The high affinity receptor consists of three distinct chains (α, β, and γ_c) and, upon binding IL-2, generates intracellular signals through heterodimerization of the cytoplasmic domains of IL-2Rβ and γ_c (3, 4). Like other hematopoietic receptors, the IL-2R has no intrinsic catalytic activity but instead induces the activation of Janus tyrosine kinases (Jaks) that associate with the receptor chains: Jak1 with IL-2Rβ and Jak3 with γ_c (5–8). The β chain is also reported to associate constitutively with the tyrosine kinases Syk (9) and Lck (10) and inducibly with the adapter molecule Shc (11–14) and the transcription factor Stat5 (15–18). By contrast, studies of γ_c have yet to identify molecules other than Jak3 that associate in the presence or absence of ligand. Moreover, Jak3 appears to associate exclusively with γ_c and hence is activated only by those hematopoietic receptors that use this chain, including the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (5, 19–24).

The exclusive nature of the γ_c/Jak3 interaction suggests that Jak3 might be the sole mediator of signals originating from the γ_c chain. Indeed, the phenotype of Jak3-null mice is virtually identical to that of γ_c-null animals, involving severe defects in the development of T and B cells (25–29). Likewise, human patients with genetic lesions at the Jak3 or γ_c loci suffer from severe combined immune deficiency (30–33). These phenotypes likely reflect an essential role for Jak3 and γ_c in IL-7R signaling during early lymphocyte development because they are recapitulated in mice with targeted disruption of the IL-7 or IL-7R α chain genes (34, 35). An essential role for Jak3 in IL-2R signaling has been demonstrated by overexpressing a kinase-deleted form of the molecule in the pro-B cell line BAF3 together with a reconstituted IL-2R complex. This protein functioned as a dominant-negative inhibitor of endogenous Jak3 catalytic activity, which resulted in severe impairment of the IL-2R-mediated proliferative response (36). In T cells, mutational analysis of the cytoplasmic domain of γ_c by our group and others (37–39) has led to the identification of two cytoplasmic regions that are critical for activation of Jak3 and induction of the proliferative response: (*i*) a membrane-proximal (PROX) domain that has partial SH2 homology though lacks many of the residues critical for binding phosphotyrosine and (*ii*) an adjacent, 14-residue segment with Box 2 homology. Both regions are required to bind Jak3 (6, 7, 40) and together are sufficient to mediate the mitogenic signaling functions of γ_c (37).

These results suggested a simple mechanism for IL-2R signaling in which the sole function of the γ_c chain is to recruit Jak3 to the receptor complex. We reasoned that ligand-induced dimerization of γ_c with IL-2Rβ might activate Jak3 by bringing the kinase in close proximity to Jak1, allowing cross-phosphorylation and trans-activation of both molecules, a mechanism that has been proposed to account for activation of Jak kinases in general (2, 41, 42). This model was evaluated in the present work by constructing chimeric receptor chains in which all or part of the cytoplasmic domain of γ_c was replaced by Jak3. Contrary to expectations, Jak3 alone was unable to mediate the mitogenic signaling functions of γ_c in T cells. Full γ_c function was reconstituted, however, when Jak3 was linked in tandem with the PROX domain of γ_c. PROX was shown to generate a novel signal that preceded, and appeared essential for, activation of Jak3 in response to ligand, thus suggesting a new model for the initiation of the IL-2R signal.

MATERIALS AND METHODS

Receptor Chain Construction and Expression. Expression vectors encoding the chimeric chains $\beta\beta$, $\alpha\gamma$, $\alpha\gamma$-PROX, and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Copyright © 1997 by THE NATIONAL ACADEMY OF SCIENCES OF THE USA
0027-8424/97/941878-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: IL-2R, interleukin 2 receptor; PROX, membrane-proximal; GM-CSF, granulocyte–macrophage colony-stimulating factor.
*To whom reprint requests should be addressed at: Virginia Mason Research Center, 1000 Seneca Street, Seattle, WA 98101.

$\alpha\gamma$-ΔPROX (formerly denoted GMβ/2β, GMα/γ, ΔT39, and ΔPROX, respectively) have been described (4, 37). Fusions and other modifications of $\alpha\gamma$ and Jak3 were accomplished using site-directed, mutagenic oligonucleotides and PCR. The sequences of all mutagenized DNA fragments were confirmed by standard methods. CTLL-2 cells were maintained, transfected, and selected for resistance to G418 or hygromycin as described (4, 37). To achieve equivalent expression of $\beta\beta$, all cells were derived from a single subclone stably expressing this chain, except those denoted $\alpha\gamma$PROX-J3 + ΔS, which expressed a derivative of $\beta\beta$ lacking the serine-rich cytoplasmic domain of IL-2Rβ (residues Ser^{267}–Ser^{322}) (10). After introduction of an $\alpha\gamma$-derived chain, cells were subcloned and screened for surface expression of both chimeric chains with antibodies to human granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor β or human GM-CSF receptor α (Santa Cruz Biotechnology). All receptor combinations demonstrated equivalent binding of phycoerythrin-conjugated human GM-CSF over a three-log unit range of concentrations [by a previously described assay (37); data not shown]. Transfectants were maintained on IL-2 and were not exposed to GM-CSF before analysis of signaling.

Proliferative Assays. Methods to evaluate thymidine incorporation, cell growth, and nuclear expression of c-Myc and c-Fos proteins have been described (37).

Immunoprecipitation and Immunoblot Analysis. Aliquots of 1.5×10^7 cells were stimulated with media alone, human IL-2 (100 units/ml), or human GM-CSF (100 ng/ml), washed once with cold PBS, and then lysed at 5×10^7 cells/ml in lysis buffer (50 mM Tris, pH 7.4/0.5% Nonidet P-40/150 mM NaCl/5 mM EDTA/50 mM NaF/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/10 μg/ml leupeptin) on ice for 20 minutes. Cytoplasmic proteins were immunoprecipitated with the indicated antibodies and agarose-conjugated protein A or G (Santa Cruz Biotechnology); antibodies were purchased from Santa Cruz Biotechnology (Jak1, Stat5, SHP-2, human IL-2Rβ, murine IL-2Rβ, and human GM-CSF receptor α), Upstate Biotechnology (Jak3 and anti-phosphotyrosine), and Transduction Laboratories (Lexington, KY; Stat5 and Shc). Agarose-bound immune complexes were washed twice with lysis buffer and either boiled in sample buffer for immunoblotting (37) or subjected to an *in vitro* kinase assay (below). For immunoprecipitations using anti-phosphotyrosine antibody, the possibility that the protein of interest (IL-2Rβ or SHP-2) was precipitating indirectly because of an interaction with a phosphotyrosine-containing intermediary protein was precluded by (*i*) washing immune complexes twice with radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0/150 mM NaCl/0.1% SDS/1% Nonidet P-40/0.5% sodium deoxycholate/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride), which contains two ionic detergents that generally disrupt protein–protein interactions, and (*ii*) reprobing each blot with anti-phosphotyrosine antibody to confirm the presence of a tyrosine-phosphorylated protein at the same position on the gel as seen after probing with antibodies to IL-2Rβ or SHP-2.

***In Vitro* Kinase Assays.** Anti-GM-CSF receptor α immune complexes on protein G–agarose or anti-Jak3 immune complexes on protein A–agarose were washed three times with cold kinase buffer (25 mM Hepes, pH 7.4/0.1% Nonidet P-40/10 mM $MgCl_2$/3 mM $MnCl_2$/30 μM Na_3VO_4) and then incubated at room temperature for 15 minutes in kinase buffer containing 0.25 mCi/ml (1 Ci = 37 GBq) [γ-^{33}P]ATP (DuPont/NEN). Complexes were washed once with cold lysis buffer and processed for autoradiography as described (37).

Time Course Analysis. Aliquots of 2×10^7 cytokine-deprived CTLL-2 cells in 4 ml of complete media were stimulated with IL-2 or GM-CSF at room temperature; at 37°C, signaling events proceeded too rapidly to distinguish the temporal order of substrate phosphorylation. After cytokine stimulation, cell cultures were added directly to 2 ml of 3-fold concentrated lysis buffer on ice. Postnuclear fractions were divided into two aliquots for immunoprecipitation with antibodies to Jak3 or phosphotyrosine followed by immunoblotting as described above.

RESULTS

Proliferative Signaling in T Cells After Replacement of the γ_c Cytoplasmic Domain with Jak3. The signaling functions of γ_c and Jak3 were studied in the physiological context of nontransformed, IL-2-dependent T cells expressing a functional endogenous IL-2R by coexpressing two chimeric receptor chains, $\alpha\gamma$ and $\beta\beta$ (Fig. 1). These chains bind GM-CSF through extracellular domains derived from the GM-CSF receptor α and β chains but generate an authentic IL-2R signal after the resultant heterodimerization of cytoplasmic domains derived from γ_c and IL-2Rβ (4, 37) (Figs. 2 and 3). To evaluate the signaling capacity of Jak3 independent of any other potential signals from γ_c, we replaced the cytoplasmic domain of $\alpha\gamma$, except for the PROX three residues, with the entire Jak3 polypeptide by way of a tetra-glycine linker ($\alpha\gamma$3–J3; Fig. 1 *B* and *C*). When coexpressed on the cell surface with $\beta\beta$, $\alpha\gamma$3–J3 bound GM-CSF [as per Nelson *et al.* (37); data not shown] but failed to induce cell proliferation or expression of the proto-oncogene products c-Myc and c-Fos (Fig. 2). To relieve Jak3 of potential steric or conformational hindrances related to the chimeric construct, a longer, flexible linker consisting of glycine and serine residues was inserted at the γ_c–Jak3 fusion site, but this chain also failed to generate a proliferative signal ($\alpha\gamma$3GS–J3; Figs. 1 and 2).

One explanation for the inability of chains containing Jak3 alone to signal was that one or more cytoplasmic regions of γ_c

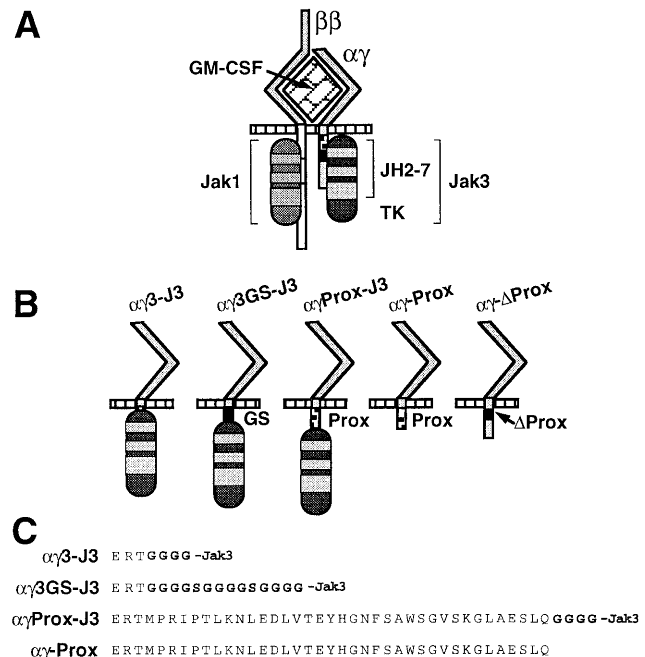

FIG. 1. Description of chimeric receptor chains. (*A*) The chimeric GM-CSF/IL-2 receptor consists of two chains, $\beta\beta$ and $\alpha\gamma$, that bind human GM-CSF but generate an IL-2 signal through cytoplasmic domains derived from human IL-2Rβ and γ_c (4, 37). The chains associate noncovalently with the kinases Jak1 and Jak3, respectively (6–8, 40), each of which contains a C-terminal tyrosine kinase (TK) domain and six other conserved regions (JH2–7). (*B*) Derivatives of $\alpha\gamma$ containing an attached murine Jak3 molecule in the cytoplasmic domain (46), the PROX domain alone ($\alpha\gamma$-PROX), or the C-terminal region of γ_c alone (37) ($\alpha\gamma$-ΔPROX). (*C*) Amino acid sequence of the cytoplasmic domains of $\alpha\gamma$-derived receptor chains beginning with the first cytoplasmic residue (19). Linker sequences are in bold type.

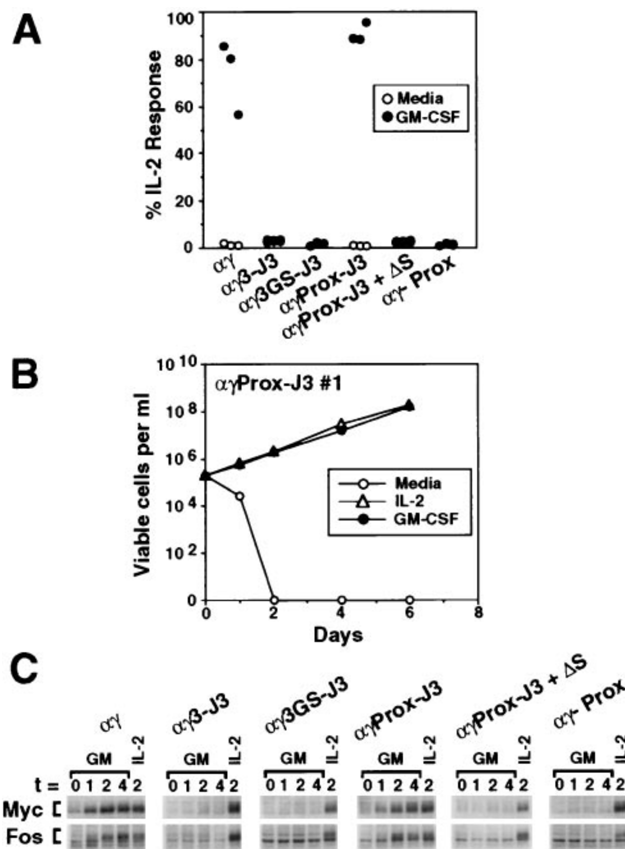

FIG. 2. Proliferative responses of CTLL-2 cells expressing chimeric receptor chains derived from $\alpha\gamma$ and Jak3. (*A*) S phase entry assessed by incorporation of tritiated thymidine in response to human GM-CSF (10 ng/ml) or media alone plotted as a percentage of the response to human IL-2 (20 units/ml), which ranged from $3–6 \times 10^4$ cpm. All cells also expressed an intact version of $\beta\beta$, except those denoted $\alpha\gamma$PROX–J3 + ΔS, which expressed the deletion mutant $\beta\beta$–ΔS. For each receptor combination, the responses of three independent CTLL-2 subclones are shown. Similar relative responses were observed over a range of GM-CSF concentrations from 0.1 to 100 ng/ml (data not shown). (*B*) Growth of a subclone of CTLL-2 cells coexpressing $\alpha\gamma$PROX–J3 and $\beta\beta$ and cultured with GM-CSF (100 ng/ml), IL-2 (100 units/ml), or media alone. Identical results were obtained with three other independent subclones. (*C*) Western blot analyses to detect nuclear expression of the proto-oncogene products p67$^{c\text{-}myc}$ and p62$^{c\text{-}fos}$ after stimulation with GM-CSF (GM; 100 ng/ml) or IL-2 (100 units/ml) for 1–4 h.

was required in addition to the kinase. The only known function of the PROX 39 residues of γ_c (referred to as the PROX domain) is to participate in binding of Jak3 (40). However, we reasoned that this region might serve additional functions because many residues are conserved among a subset of cytokine receptor chains (IL-3Rα, IL-5Rα, and GM-CSF receptor α) that do not appear to bind Jak kinases (43, 44). A truncated $\alpha\gamma$ chain containing only the PROX domain, coexpressed with $\beta\beta$, failed to induce proliferation (37, 38) ($\alpha\gamma$–PROX; Figs. 1 and 2), but a chain modified to contain PROX in tandem with Jak3 induced a proliferative response equivalent to that mediated by the endogenous IL-2R ($\alpha\gamma$PROX–J3; Figs. 1 and 2). Like wild-type γ_c, $\alpha\gamma$PROX–J3 required interaction with the cytoplasmic domain of the β chain for signaling because deletion of the S region (10) of $\beta\beta$, which contains the binding site for Jak1 (6, 45), abrogated the proliferative response to GM-CSF ($\alpha\gamma$PROX–J3 plus ΔS; Fig. 2).

Intracellular Tyrosine Phosphorylation Mediated by the PROX Domain and Jak3. Biochemical analyses revealed that the $\alpha\gamma$3–J3 chain, when coexpressed with $\beta\beta$, failed to become phosphorylated on tyrosine in response to GM-CSF or mediate detectable phosphorylation of Jak1, Shc, or Stat5, substrates associated with IL-2R signaling (Fig. 3*A*). Similarly, $\alpha\gamma$-PROX, which contains PROX alone in the cytoplasmic domain, failed to induce phosphorylation of these substrates or of endogenous Jak3 (Fig. 3*A*) (37). By contrast, the chimeric chain containing PROX in tandem with Jak3 mediated tyrosine phosphorylation and catalytic activation of Jak1 and phosphorylation of Shc and Stat5 in response to GM-CSF ($\alpha\gamma$PROX–J3; Fig. 3*A*). Indeed, with respect to all of the events analyzed, the signal mediated by $\alpha\gamma$PROX–J3 and $\beta\beta$ was indistinguishable from that of the endogenous IL-2R but for the expected difference that GM-CSF induced tyrosine phosphorylation of the Jak3 component of the $\alpha\gamma$PROX–J3 chain whereas IL-2 induced phosphorylation and catalytic activation of endogenous Jak3 (Fig. 3 *A* and *B*).

We considered the possibility that the Jak3 component of $\alpha\gamma$3–J3 might not be competent to signal because of conformational or steric hindrances resulting from covalent attachment to the receptor chain. Wild-type Jak3, when overexpressed in lymphocytes, has been reported to exhibit ligand-independent catalytic activity *in vitro* (36); therefore, we evaluated $\alpha\gamma$3–J3 and $\alpha\gamma$PROX–J3 by this assay to confirm that each had a functional Jak3 catalytic domain. Both chains, after immunoprecipitation from CTLL-2 cells, demonstrated constitutive *in vitro* kinase activity (Fig. 3*B*). Thus, the PROX domain does not appear necessary for Jak3 catalytic activity but rather for receptor-mediated activation of the kinase *in vivo*. This latter function could not be restored by simply providing a longer linker between the receptor chain and Jak3 because $\alpha\gamma$3GS–J3 also failed to undergo tyrosine phosphorylation or to mediate substrate phosphorylation in response to GM-CSF (data not shown). Indeed, with $\alpha\gamma$3GS–J3 or a similar chain containing a 22-residue linker, the chimeric Jak3 molecule was tyrosine phosphorylated in response to IL-2 rather than GM-CSF (data not shown), suggesting that these longer linkers provided sufficient flexibility for Jak3 to interact with, and become activated by, the endogenous, wild-type IL-2R chains but nevertheless failed to induce activation of Jak3 after GM-CSF-mediated interaction with the chimeric $\beta\beta$ chain.

Jak3-Independent Signaling by the PROX Domain of γ_c. The fact that PROX can promote activation of Jak3 despite the fixed, artificial linkage imposed by $\alpha\gamma$PROX–J3 suggested that PROX might operate through biochemical intermediates rather than a direct structural interaction with Jak3. The S region of IL-2Rβ is essential for activation of Jak3 (Fig. 3) (46), so we reasoned that PROX might generate an activation signal for the kinase through an interaction with the β chain or an associated protein. IL-2 induces tyrosine phosphorylation of the IL-2Rβ chain (47, 48), and, similarly, GM-CSF induces tyrosine phosphorylation of $\beta\beta$ when the chain is coexpressed with $\alpha\gamma$ or $\alpha\gamma$PROX–J3 (Fig. 3*D*). The truncated mutant of $\alpha\gamma$ containing only the PROX domain ($\alpha\gamma$–PROX), although unable to bind (40) or activate Jak3 (37) (Fig. 3 *A* and *B*), did induce detectable tyrosine phosphorylation of $\beta\beta$ in response to GM-CSF (Fig. 3*D*). Phosphorylation of the β chain specifically required the PROX domain; it was not observed with a deletion mutant of $\alpha\gamma$ lacking PROX ($\alpha\gamma$–ΔPROX; Fig. 1*B*) (37) nor with $\alpha\gamma$3–J3, which contains Jak3 but not PROX (Fig. 3*D*). Thus, the PROX domain alone can mediate a Jak-independent signal involving phosphorylation of the β chain. The magnitude of β-chain phosphorylation induced by $\alpha\gamma$-PROX was less than that mediated by full length γ_c or $\alpha\gamma$PROX–J3, indicating that additional phosphorylation of the chain occurs subsequent to the activation of Jak3.

Time Course of Tyrosine Phosphorylation of the β Chain and Jak3. To determine whether the endogenous IL-2R generates a signal involving tyrosine phosphorylation of the β chain and occurring before the phosphorylation of Jak3, parental CTLL-2 cells were stimulated with IL-2 at room

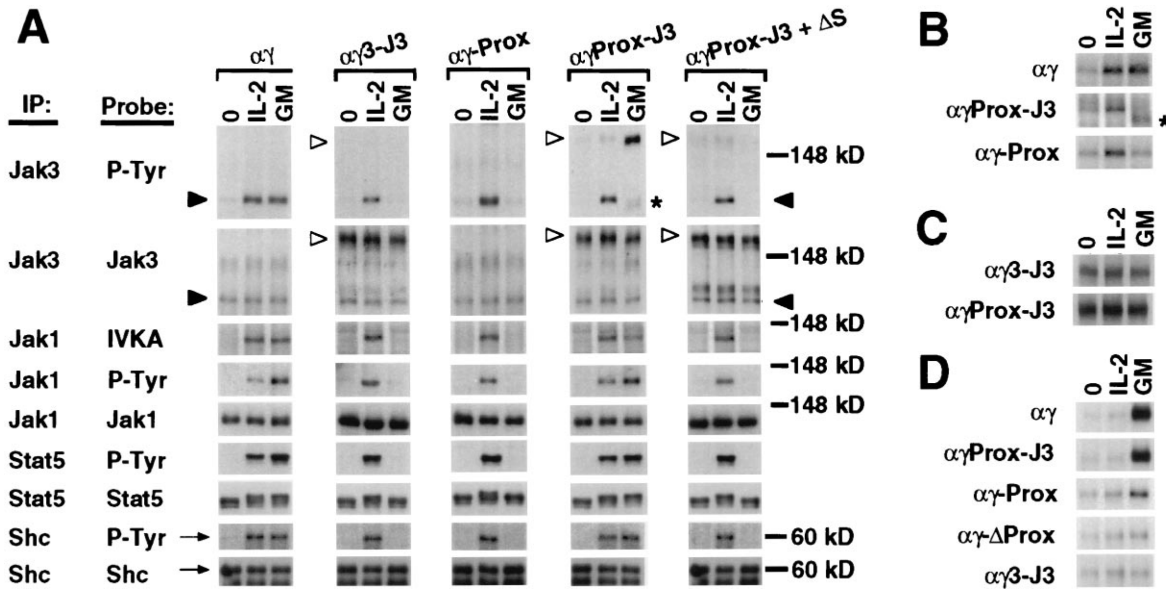

FIG. 3. Tyrosine phosphorylation and Jak kinase activation mediated by chimeric receptor chains or the endogenous IL-2R in the T cell line CTLL-2. Cells coexpressing $\beta\beta$ and the indicated derivative of $\alpha\gamma$ or coexpressing $\beta\beta$–ΔS and $\alpha\gamma$PROX–J3 were deprived of IL-2 for 4 h and then stimulated with media alone (0), human IL-2 (100 units/ml), or human GM-CSF (GM; 100 ng/ml) for 10 minutes at 37°C. Cells were lysed, and cytoplasmic fractions were subjected to immunoprecipitation (IP) with antibodies to the indicated proteins. Immunoprecipitated proteins were either probed by Western blot analysis with the indicated antibodies (*A* and *D*) or subjected to an *in vitro* kinase assay (IVKA) and visualized by autoradiography (*A-C*). The asterisks in *A* and *B* indicate a band in the GM lane of $\alpha\gamma$PROX–J3 corresponding to phosphorylated $\beta\beta$, which coprecipitates with $\alpha\gamma$PROX–J3 in the presence of GM-CSF. (*A*) Tyrosine phosphorylation of Jak3, Jak1, Stat5, and Shc [assessed by probing with anti-phosphotyrosine antibody (P-Tyr)] and catalytic activation of Jak1 (assessed by IVKA). Endogenous Jak3 (closed arrowheads) can be distinguished from the chimeric forms of Jak3 (open arrowheads) by molecular mass. (*B*) Autoradiogram showing *in vitro* kinase activity of endogenous Jak3 immunoprecipitated with anti-Jak3 antiserum from cells coexpressing $\beta\beta$ and the indicated derivative of $\alpha\gamma$. (*C*) Autoradiogram showing *in vitro* kinase activity of the Jak3 component of the chimeric chains $\alpha\gamma$3–J3 and $\alpha\gamma$PROX–J3 immunoprecipitated with an mAb to the extracellular domain of human GM-CSFRα. (*D*) Tyrosine phosphorylation of the chimeric $\beta\beta$ chain in cells coexpressing the indicated derivative of $\alpha\gamma$. Phosphorylation of $\beta\beta$ was assessed by immunoprecipitating with an antibody to phosphotyrosine and probing with antiserum to the cytoplasmic domain of human IL-2Rβ, rather than the converse procedure, because this allowed better visualization of low level phosphorylation events. This antiserum does not recognize endogenous murine IL-2Rβ by immunoblot. The phosphotyrosine content of $\beta\beta$ was confirmed upon reprobing with antibody to phosphotyrosine; the presence of constitutively phosphorylated proteins on these second blots allowed confirmation of equivalent loading of samples (data not shown).

temperature, which allowed the temporal ordering of events to be distinguished. After intervals ranging from 20 s to 8 minutes, cells were lysed instantly on ice, and cytoplasmic fractions were subjected to immunoprecipitation and immunoblotting. Phosphorylation of endogenous IL-2Rβ was detected within the 1st minute of stimulation and peaked by 4 minutes whereas the level of Jak3 phosphorylation did not change before 2 minutes and was still increasing at 8 minutes (Fig. 4*A*). Stimulation of cells coexpressing $\beta\beta$ and $\alpha\gamma$-PROX with GM-CSF demonstrated that the PROX domain alone, although unable to induce Jak3 phosphorylation, induced phosphorylation of $\beta\beta$ with similar kinetics as seen with the endogenous IL-2R (Fig. 4*A*). Thus, tyrosine phosphorylation of the IL-2Rβ chain normally begins before the phosphorylation of Jak3 and is mediated by a PROX-dependent kinase.

Other PROX-Mediated Signaling Events. The full range of events induced by the PROX domain before activation of Jak3 has yet to be defined; however, immunoprecipitation and blotting with antibody to phosphotyrosine revealed that $\alpha\gamma$-PROX and $\beta\beta$ can induce tyrosine phosphorylation of a second protein of ≈70 kDa. This substrate, phosphorylated within 1–2 minutes of cytokine stimulation via the chimeric or endogenous IL-2R chains, was identified as the protein tyrosine phosphatase SHP-2 (49) (Fig. 4*A*). Although not previously implicated in IL-2R signaling, SHP-2 is a positive regulator of multiple hematopoietic and growth factor receptor pathways (41, 50). As with phosphorylation of the β chain, phosphorylation of SHP-2 specifically required PROX; it was mediated by $\alpha\gamma$-PROX and $\alpha\gamma$PROX–J3 but not by $\alpha\gamma$3–J3 or $\alpha\gamma$-ΔPROX (Fig. 4; data not shown). Moreover, like the β chain, SHP-2 was phosphorylated to a greater extent in settings in which Jak3 activity was also induced, suggesting that additional phosphorylation of both IL-2Rβ and SHP-2 is mediated by Jak3 or a kinase downstream of Jak3. Thus, the results demonstrate that the PROX domain induces an initial, Jak-independent signal involving, at a minimum, tyrosine phosphorylation of the β chain and the phosphatase SHP-2.

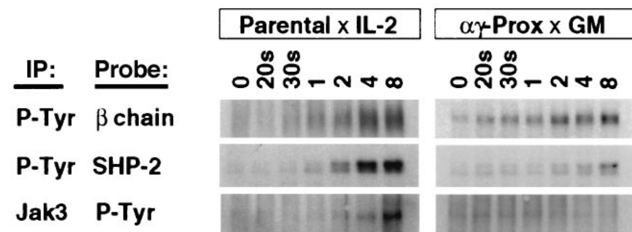

FIG. 4. Time course of receptor-mediated tyrosine phosphorylation of the β chain, SHP-2, and Jak3. Cytokine-deprived parental CTLL-2 cells or cells coexpressing the chimeric receptor chains $\alpha\gamma$-PROX + $\beta\beta$ were stimulated at room temperature with either IL-2 (200 units/ml) or GM-CSF (200 ng/ml). At the indicated time points [expressed in seconds (s) or minutes], cells were added directly to lysis buffer and subjected to immunoprecipitation (IP) followed by immunoblotting with antibodies to murine IL-2Rβ (*Left*), human IL-2Rβ (*Right*), SHP-2, or phosphotyrosine. Equivalent loading of samples across lanes was confirmed by reprobing with the same antibody used for immunoprecipitation (data not shown). These second blots also were used to confirm the phosphotyrosine content of murine IL-2Rβ, chimeric $\beta\beta$, and SHP-2.

DISCUSSION

Current models for Janus kinase activation by cytokine receptors originate from observations that receptor chains undergo ligand-induced dimerization and that the catalytic activity of Jak molecules is density-dependent. Thus, it has been proposed (2, 41, 42) that receptor chain oligomerization after ligand binding induces cross-phosphorylation and transactivation of associated Jak molecules, resulting in the initiation of an intracellular signal. The IL-2R signals through heterodimerization of two chains, IL-2Rβ and γ_c (3, 4), associated with the kinases Jak1 and Jak3, respectively (5–8). We reasoned that, if Jak3 were activated as a simple consequence of receptor-mediated heterodimerization with Jak1 or another kinase associated with IL-2Rβ, replacement of the cytoplasmic domain of γ_c with the Jak3 polypeptide should permit activation of the kinase and, potentially, mitogenic signaling in response to ligand. However, hybrid γ_c chains containing Jak3 alone in the cytoplasmic domain showed no evidence of functional activation in response to GM-CSF, irrespective of the length of the linker between the receptor and Jak3. By contrast, hybrid chains containing the PROX domain of γ_c in tandem with Jak3 not only mediated substrate phosphorylation *in vivo* but completely reconstituted the mitogenic signaling function of the wild-type γ_c chain. Therefore, these studies identify a novel role for the PROX domain in the activation of Jak3 and define PROX and Jak3 as necessary and sufficient for γ_c-mediated proliferative signaling. Although residues C-terminal to PROX are required for binding of Jak3 (6, 7, 40) and hence for signaling by the wild-type chain (37, 38, 51), they are clearly dispensable when this requirement is fulfilled by covalent attachment of the kinase.

There are several possible mechanisms by which the PROX domain could promote activation of Jak3 in response to ligand. In principle, PROX could play an obligate structural role by placing Jak3 in the correct orientation or conformation for interaction with substrates after ligand binding. However, it seems unlikely that such a structure-based mechanism would operate normally in the context of the $\alpha\gamma$PROX–J3 chain, given the arbitrary and fixed linkage of Jak3 and PROX via their N and C termini, respectively. An alternative, but not mutually exclusive, possibility is that PROX might activate Jak3 through a biochemical intermediate. Indeed, a truncated γ_c chain containing PROX alone, although devoid of catalytic motifs, was found to induce Jak-independent tyrosine phosphorylation of the β chain and the tyrosine phosphatase SHP-2. Derivatives of γ_c lacking the PROX domain failed to induce these events. Moreover, phosphorylation of the β chain, mediated either by endogenous γ_c or by the truncation mutant containing PROX alone, occurred before the phosphorylation of Jak3. This demonstrates that the PROX domain can rapidly induce, potentially through steric interaction, the activity of a receptor-associated tyrosine kinase that is independent of Jak3. This initial signal appears to be amplified with subsequent phosphorylation and activation of Jak3, suggesting that the receptor complex may achieve full catalytic activity through cooperative interactions between kinases.

Several tyrosine kinases are known to associate with the β chain and could therefore mediate the initial PROX-dependent signal. Jak1 associates with the S region of IL-2Rβ (6, 45), which is essential for activation of Jak3 (46); however, we failed to detect induction of Jak1 activity by the truncated γ_c chain containing PROX alone. Moreover, previous experiments in which the catalytic activity of Jak3 was inhibited by overexpression of a dominant-negative form of the enzyme demonstrated that catalytic activation of Jak1 is dependent on Jak3 (36). Finally, in a recent report, a mutated form of IL-2Rβ that is unable to bind or activate Jak1 was shown to still mediate activation of Jak3 (45). The tyrosine kinase Syk can also associate with the S region of IL-2Rβ and become catalytically active in response to IL-2 in some cell types (9); however, in CTLL-2 cells, we have been unable to detect binding or activation of Syk by the IL-2R (B.H.N., unpublished results). The tyrosine kinase Lck also has been reported to associate with IL-2Rβ (52) but, like Jak1, does not appear to be activated by the PROX domain (B.H.N., unpublished results). Therefore, the identity of the kinase responsible for PROX-dependent tyrosine phosphorylation of IL-2Rβ and SHP-2 is currently unknown but is under investigation.

Attempts thus far to reconstitute cytokine receptor signaling by covalent attachment of Jak molecules in place of the cytoplasmic domain have yielded mixed results. For the interferon-γ receptor, which generates a nonmitogenic signal, Jak2 alone can functionally replace the cytoplasmic domain of the β chain to induce Stat1-dependent cellular responses (53). However, analogous chimeric constructs involving mitogenic cytokine receptors have been reported to generate only partial signals. For example, receptor constructs containing only Jak2 or the Jak2 catalytic region in place of the cytoplasmic domain failed to mediate normal proliferative signaling after ligand-induced homodimerization (54–56), consistent with a requirement for additional signals normally provided by the PROX region of the cytokine receptor. In one setting, a full mitogenic signal was obtained from a chimeric IL-2R in which the cytoplasmic domain of γ_c was replaced by a truncated erythropoietin receptor containing a PROX region and an associated Jak2 molecule (57). Based on our results, this receptor might function because the PROX region of the erythropoietin receptor, which shares homology with the PROX domain of γ_c, cannot only bind Jak2 but can also interact with the β chain to induce an initial activation signal.

The ability of the chimeric chain $\alpha\gamma$PROX–J3 to mediate a normal proliferative response in T cells demonstrates that any essential mitogenic signals from γ_c must originate from either the PROX domain or Jak3. Although the results demonstrate that one function of PROX is to induce activation of Jak3 in response to ligand, an event that is critical for proliferation (36) (B.H.N., unpublished observations), it remains possible that PROX also performs other Jak3-independent signaling functions that were not detected in these experiments. For example, there is evidence that the *bcl-2* gene is regulated by a kinase-independent pathway from γ_c (36). It also is possible that the noncatalytic domains of Jak3, which are extensive and highly conserved, mediate such kinase-independent signals. The strategy described here of covalently linking Jak3 to γ_c makes the receptor chain and Jak3 fully accessible to mutational analysis in T cells and should ultimately allow the signaling functions of each to be precisely defined.

We thank B. Witthuhn and J. Ihle for the cDNA encoding Jak3 and James Lord for providing the ΔS mutant of IL-2Rβ. This work was supported by grants from the National Institutes of Health/National Institute of Allergy and Infectious Diseases (AI 36613) and the National Institutes of Health/National Cancer Institute (CA 18029 and CA 33084).

1. Heldin, C. H. (1995) *Cell* **80,** 213–223.
2. Taniguchi, T. (1995) *Science* **268,** 251–255.
3. Nakamura, Y., Russell, S., Mess, S., Friedmann, M., Erdos, M., Francois, C., Jacques, Y., Adelstein, S. & Leonard, W. (1994) *Nature (London)* **369,** 330–333.
4. Nelson, B. H., Lord, J. D. & Greenberg, P. D. (1994) *Nature (London)* **369,** 333–336.
5. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedmann, M., Berg, M., McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J. & Leonard, W. J. (1994) *Science* **266,** 1042–1045.
6. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z., Oishi, I., Silvennoinen, O., Witthuhn, B. & Ihle, J. (1994) *Science* **266,** 1045–1047.

7. Boussiotis, V. A., Barber, D. L., Nakarai, T., Freeman, G. J., Gribben, J. G., Bernstein, G. M., D'Andrea, A. D., Ritz, J. & Nadler, L. M. (1994) *Science* **266,** 1039–1042.
8. Tanaka, N., Asao, H., Ohbo, K., Ishii, N., Takeshita, T., Nakamura, M., Sasaki, H. & Sugamura, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7271–7275.
9. Minami, Y., Nakagawa, Y., Kawahara, A., Miyazaki, T., Sada, K., Yamamura, H. & Taniguchi, T. (1995) *Immunity* **2,** 89–100.
10. Hatakeyama, M., Mori, H., Doi, T. & Taniguchi, T. (1989) *Cell* **59,** 837–845.
11. Friedmann, M. C., Migone, T.-S., Russell, S. M. & Leonard, W. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2077–2082.
12. Ravichandran, K. S., Igras, V., Shoelson, S. E., Fesik, S. W. & Burakoff, S. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5275–5280.
13. Ravichandran, K. S. & Burakoff, S. J. (1994) *J. Biol. Chem.* **269,** 1599–1602.
14. Evans, G. A., Goldsmith, M. A., Johnston, J. A., Xu, W., Weiler, S. R., Erwin, R., Howard, O. M. Z., Abraham, R. T., O'Shea, J. J., Greene, W. C. & Farrar, W. L. (1995) *J. Biol. Chem.* **270,** 28858–28863.
15. Frank, D. A., Robertson, M. J., Bonni, A., Ritz, J. & Greenberg, M. E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7779–7783.
16. Gaffen, S. L., Lai, S. Y., Xu, W., Gouilleux, F., Groner, B., Goldsmith, M. A. & Greene, W. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7192–7196.
17. Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J., Minami, Y., Miyazaki, T. & Taniguchi, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5482–5486.
18. Lin, J., Migone, T., Tsang, M., Friedmann, M., Wetherbee, J. A., Zhou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S. & Leonard, W. J. (1995) *Immunity* **2,** 331–339.
19. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M. & Sugamura, K. (1992) *Science* **257,** 379–382.
20. Giri, J. G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D. & Anderson, K. (1994) *EMBO. J.* **13,** 2822–2830.
21. Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S. & Sugamura, K. (1994) *Science* **263,** 1453–1454.
22. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K. & Sugamura, K. (1993) *Science* **262,** 1874–1877.
23. Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X. & Leonard, W. J. (1993) *Science* **262,** 1877–80.
24. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E. & Leonard, W. E. (1993) *Science* **262,** 1880–1883.
25. Cao, X., Shores, E. W., Hu-Li, J., Anver, M. R., Kelsall, B. L., Russell, S. M., Drago, J., Noguchi, M., Grinberg, A., Bloom, E. T., Paul, W. E., Katz, S. I., Love, P. E. & Leonard, W. J. (1995) *Immunity* **2,** 223–238.
26. DiSanto, J. P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 377–381.
27. Nosaka, T., van Deursen, J. M., Tripp, R. A., Thierfelder, W. E., Witthuhn, B. A., McMickle, A. P., Doherty, P. C., Grosveld, G. C. & Ihle, J. N. (1995) *Science* **270,** 800–802.
28. Park, S. Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T. & Saito, T. (1995) *Immunity* **3,** 771–782.
29. Thomis, D. C., Gurniak, C. B., Tivol, E., Sharpe, A. H. & Berg, L. J. (1995) *Science* **270,** 794–797.
30. Puck, J. M., Deschenes, S. M., Porter, J. C., Dutra, A. S., Brown, C. J., Willard, H. F. & Henthorn, P. S. (1993) *Hum. Mol. Genet.* **2,** 1099–1104.
31. Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W. & Leonard, W. J. (1993) *Cell* **73,** 147–157.
32. Macchi, P., Villa, A., Gillani, S., Sacco, M. G., Frattini, A., Porta, F., Ugazio, A. G., Johnston, J. A., Candotti, F., O'Shea, J. J., Vezzoni, P. & Notarangelo, D. (1995) *Nature (London)* **377,** 65–68.
33. Russell, S. M., Tayebi, N., Nakajima, H., Riedy, M. C., Roberts, J. L., Aman, M. J., Migone, T. S., Noguchi, M., Markert, M. L., Buckley, R. H., O'Shea, J. J. & Leonard, W. J. (1995) *Science* **270,** 797–800.
34. Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., Meyer, J. D. & Davison, B. L. (1994) *J. Exp. Med.* **180,** 1955–1960.
35. von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. & Murray, R. (1995) *J. Exp. Med.* **181,** 1519–1526.
36. Kawahara, A., Minami, Y., Miyazaki, T., Ihle, J. N. & Taniguchi, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8724–8728.
37. Nelson, B. H., Lord, J. D. & Greenberg, P. D. (1996) *Mol. Cell. Biol.* **16,** 309–317.
38. Goldsmith, M. A., Lai, S. Y., Xu, W., Amaral, M. C., Kuczek, E. S., Parent, L. J., Mills, G. B., Tarr, K. L., Longmore, G. D. & Greene, W. C. (1995) *J. Biol. Chem.* **270,** 21729–21737.
39. Asao, H., Tanaka, N., Ishii, N., Higuchi, M., Takeshita, T., Nakamura, M., Shirasawa, T. & Sugamura, K. (1994) *FEBS Lett.* **351,** 201–206.
40. Russell, S., Johnston, J., Noguchi, M., Kawamura, M., Bacon, C., Friedmann, M., Berg, M., McVicar, D., Witthuhn, B. & Silvennoinen, O. (1994) *Science* **266,** 1042–1045.
41. Ihle, J. N. (1995) *Nature* **377,** *(London)* 591–594.
42. Taga, T. & Kishimoto, T. (1995) *Curr. Opin. Immunol.* **7,** 17–23.
43. Cornelis, S., Fache, I., Heyden, J. V. d., Guisez, Y., Tavernier, J., Devos, R., Fiers, W. & Plaetinck, G. (1995) *Eur. J. Immunol.* **25,** 1857–1864.
44. Quelle, F. W., Sato, N., Witthuhn, B. A., Inhorn, R. C., Eder, M., Miyajima, A., Griffin, J. D. & Ihle, J. N. (1994) *Mol. Cell. Biol.* **14,** 4335–4341.
45. Higuchi, M., Asao, H., Tanaka, N., Oda, K., Takeshita, T., Nakamura, M., Snick, J. V. & Sugamura, K. (1996) *Eur. J. Immunol.* **26,** 1322–1327.
46. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T. & Ihle, J. N. (1994) *Nature (London)* **370,** 153–157.
47. Asao, H., Takeshita, T., Nakamura, M., Nagata, K. & Sugamura, K. (1990) *J. Exp. Med.* **171,** 637–644.
48. Sugamura, K., Takeshita, T., Asao, H., Kumaki, S., Ohbo, K., Ohtani, K. & Nakamura, M. (1990) *Lymphokine Res.* **9,** 539–542.
49. Adachi, M., Fischer, E. H., Ihle, J., Imai, K., Jirik, F., Neel, B., Pawson, T., Shen, S., Thomas, M., Ullrich, A. & Zhao, Z. (1996) *Cell* **85,** 15.
50. Ali, S., Chen, Z., Lebrun, J. J., Vogel, W., Kharitonenkov, A., Kelly, P. A. & Ullrich, A. (1996) *EMBO J.* **15,** 135–142.
51. Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M. & Sugamura, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4127–4131.
52. Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S. D., Perlmutter, R. M. & Taniguchi, T. (1991) *Science* **252,** 1523–1528.
53. Bach, E. A., Tanner, J. W., Marsters, S., Ashkenazi, A., Aguet, M., Shaw, A. S. & Schreiber, R. D. (1996) *Mol. Cell. Biol.* **16,** 3214–3221.
54. Sakai, I., Nabell, L. & Kraft, A. S. (1995) *J. Biol. Chem.* **270,** 18420–18427.
55. Nakamura, N., Chin, H., Miyasaka, N. & Miura, O. (1996) *J. Biol. Chem.* **271,** 19483–19488.
56. Frank, S. J., Yi, W., Zhao, Y., Goldsmith, J. F., Gilliland, G., Jiang, J., Sakai, I. & Kraft, A. S. (1995) *J. Biol. Chem.* **270,** 14776–14785.
57. Lai, S. Y., Xu, W., Gaffen, S. L., Liu, K. D., Longmore, G. D., Greene, W. C. & Goldsmith, M. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 231–235.