in medium containing 10% fetal calf serum for 1 hour.
26. NK3.3 cells were used in Fig. 1, C and D, but IL-2 also failed to induce tyrosine phosphorylation of Tyk2 or Jak2 in PHA-activated T cells (S. M. Russell *et al.*, unpublished observations).
27. B. A. Wittuhn *et al.*, *Cell* **74**, 227 (1993).
28. We thank M. Tsang and R&D Systems for recombinant IL-9 and MO7E cells; J. Yodoi for YT cells; J. Kornbluth for NK3.3 cells; Cetus and G. Ju and J. Hakimi for recombinant IL-2; C. Reynolds for IL-2, IL-4, IL-7, and IL-9; M. Tsudo and J. Hakimi for mAb Mikβ1; K. Sugamura for mAb TU11 to IL-2Rβ and mAb TUGm2 to murine γ_c; J. Ritz and T. Nakarai for the mAb 3B5 to human γ_c; and T. A. Waldmann for anti-Tac mAb to IL-2Rα. Supported in part by National Cancer Institute Center Support (CORE) grant P30 CA21765 (J.N.I.), grant RO1 DK42932 from the National Institute of Diabetes and Digestive and Kidney Diseases (J.N.I.), the American Lebanese-Syrian Associated Charities (J.N.I.), a Postgraduate Research Grant, University of Sheffield (C.M.B.), and Fulbright Cancer Studentship (C.M.B.). We thank J.-L. Boulay and W. E. Paul for suggesting that the IL-9 receptor might contain γ_c (*8*). We thank W. E. Paul, M. Tsang, and J. Ritz for valuable discussions.

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Functional Activation of Jak1 and Jak3 by Selective Association with IL-2 Receptor Subunits

Tadaaki Miyazaki,* Atsuo Kawahara,* Hodaka Fujii, Yoko Nakagawa, Yasuhiro Minami, Zhao-Jun Liu, Isao Oishi, Olli Silvennoinen, Bruce A. Witthuhn, James N. Ihle, Tadatsugu Taniguchi

The interleukin-2 receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγ chains, the last of which is also used in the receptors for IL-4, IL-7, and IL-9. Stimulation with IL-2 induces the tyrosine phosphorylation and activation of the Janus kinases Jak1 and Jak3. Jak1 and Jak3 were found to be selectively associated with the "serine-rich" region of IL-2Rβ and the carboxyl-terminal region of IL-2Rγ, respectively. Both regions were necessary for IL-2 signaling. Furthermore, Jak3-negative fibroblasts expressing reconstituted IL-2R became responsive to IL-2 after the additional expression of Jak3 complementary DNA. Thus, activation of Jak1 and Jak3 may be a key event in IL-2 signaling.

IL-2 plays a pivotal role in immune responses by inducing the proliferation and differentiation of lymphocytes (*1*). The IL-2Rβ and IL-2Rγ subunits, but not the IL-2Rα subunit, are members of the cytokine receptor superfamily (*2*). A membrane-proximal cytoplasmic region of IL-2Rβ, termed the "serine-rich" region (S region) (*3*), and the cytoplasmic domain of IL-2Rγ are critical for IL-2 signaling (*4*, *5*). Mutations of IL-2Rγ are also associated with X-linked severe combined immunodeficiency (XSCID) (*6*). Although lacking intrinsic kinase activity, IL-2R couples ligand binding to induction of tyrosine phosphorylation of cellular substrates, including IL-2Rβ and IL-2Rγ (*7*). IL-2R is also linked to the serine-threonine kinase Raf-1 and phosphatidylinositol-3 kinase and to the hydrolysis of glycosyl-phosphatidylinositol (*1*). IL-2Rβ is physically associated with Src-family protein tyrosine kinases (PTKs) and Syk PTK (*8*, *9*). Both IL-2 and IL-4 induce the tyrosine phosphorylation and activation of the Janus kinases Jak1 and Jak3 (*10*, *11*), which suggests that these kinases might also associate with IL-2R and participate in IL-2 signaling.

To determine if Jaks associate with the IL-2R subunits, we used CD4 chimeric receptors that could be detected with the same monoclonal antibody to human CD4 (anti-CD4) (Fig. 1A). Extracts of COS cells cotransfected with each chimeric receptor complementary DNA (cDNA) and one of the Jak cDNAs were immunoprecipitated with anti-CD4 and subjected either to immunoblot analysis with antisera to the respective Jaks (Fig. 1B) or to in vitro kinase assay (*12*). Expression levels of the respective Jaks and chimeric receptors did not vary among cotransfected COS cells (*13*). Both assays showed that Jak1 could be coimmunoprecipitated with CD4β, but not with CD4γ or CD4γM1, whereas Jak3 could be coprecipitated with CD4γ, but not with CD4β or CD4γM1 (Fig. 1B) (*12*). In contrast, the association of Jak2 with any of the CD4–IL-2R chimeras was marginal (Fig. 1B) (*12*). Thus, the cytoplasmic domains of IL-2Rβ and IL-2Rγ possess regions capable of selectively associating with Jak1 and Jak3, and additional lymphoid-specific proteins are not required. Similar results were obtained in the IL-3–dependent cell line BAF-B03 that stably expresses each of the chimeric receptor cDNAs (*14*).

We examined the association sites within the respective cytoplasmic domains of the IL-2R subunits with the respective Jaks, using cDNAs encoding CD4 chimeras that contained deletions in their cytoplasmic β or γ domains (CD4βS, CD4βA, and CD4γM2; Fig. 1A). CD4γM1 and CD4γM2 bear the cytoplasmic domains of IL-2Rγ with COOH-terminal truncations of 79 and 48 amino acids, respectively. Jak1 can be detected, both by immunoblotting (Fig. 1C) and in vitro kinase assays (*12*), in anti-CD4 immunoprecipitates from COS cells cotransfected with the Jak1 cDNA and the cDNA for CD4β or CD4βA, but not CD4βS. A similar analysis was done for Jak3, which could associate with CD4γ but not with CD4γM1 or CD4γM2 (Fig. 1C) (*12*). Protein expression levels of the two Jaks, as well as of the different chimeric receptors, did not vary among the cotransfected COS cells (*13*). Thus, the cytoplasmic S region of IL-2Rβ is required for the association of IL-2Rβ with Jak1, whereas the COOH-terminal 48 amino acids of IL-2Rγ are necessary for the association of IL-2Rγ with Jak3. These two regions are known to participate in IL-2–induced cell proliferation (*3*, *5*, *15*). It is also worth pointing out that the COOH-terminal region of IL-2Rγ is deleted by nonsense mutations in many XSCID patients (*6*). The utilization of IL-2Rγ by a number of receptors (*16*) explains the activation of Jak3 by IL-4, IL-7, and IL-9 and predicts the activation of Jak3 in response to IL-13 or IL-15 (*17*). Jak1 associated with a region of IL-2Rβ that has structural similarity with the corresponding regions of other cytokine receptors (*3*, *18*). A comparable region in the erythropoietin receptor binds Jak2 (*19*); hence, the membrane proximal region can discriminate among Jaks.

We investigated the importance of these associations in the activation of Jaks by IL-2. We examined whether Jaks were activated after IL-2 stimulation in a BAF-B03–derived cell line, FWT-2, which expresses both human IL-2Rβ and human IL-2Rγ (*5*). Stimulation of FWT-2 cells by IL-2 induced tyrosine phosphorylation of endogenous Jak1 and Jak3, but not of Jak2 (*15*). The respective anti-Jak immunoprecipitates were tested for kinase activity in an in vitro kinase assay (Fig. 2A). Jak1

T. Miyazaki, A. Kawahara, H. Fujii, Y. Nakagawa, Y. Minami, Z.-J. Liu, I. Oishi, T. Taniguchi, Institute for Molecular and Cellular Biology, Osaka University, Suita-shi, Osaka 565, Japan.
O. Silvennoinen, B. A. Witthuhn, J. N. Ihle, St. Jude Children's Research Hospital, Department of Biochemistry, Memphis, TN 38101, USA.
*These authors, to whom correspondence should be addressed, contributed equally to this work.

and Jak3, but not Jak2, were activated after IL-2 stimulation. Immunoblot analysis revealed the expression of the three Jak proteins in FWT-2 cells (Fig. 2B). Thus, IL-2 induced the selective activation of Jak1 and Jak3, and this activation correlated with the ability of the Jaks to associate with IL-2R.

We have previously shown that reconstitution of IL-2R in NIH 3T3 fibroblasts (3T3αβγ cells) does not allow fibroblasts to respond to IL-2 stimulation (*20*). Because fibroblasts express Jak1 and Jak2, but not Jak3 (*15*) (see Fig. 3 inset), we could test the role of Jak3 in response to IL-2 stimulation. When the Jak3 cDNA was stably expressed in the 3T3αβγ cells, it was found that three independent Jak3-expressing clones (*21*) all responded to IL-2 at levels that were comparable to serum stimulation (Fig. 3).

Stimulation with IL-2 induced the association of the cytoplasmic domains of IL-2Rβ and IL-2Rγ, an event that is required for biological responses (*4*, *5*). Our results suggest that this step may be necessary to bring Jak1 and Jak3 into close proximity and hence promote cross-activation. Consistent with this view, Jak3 is not activated in a receptor complex containing an IL-2Rβ chain lacking the S region that is required for binding to Jak1 (*10*). It has been reported that the IL-2Rβ is associated with Jak3 (*11*). Because this previous experiment was done under conditions in which IL-2Rγ would be coimmunoprecipitated with IL-2Rβ, it is most likely that Jak3 was coimmunoprecipitated through its association with IL-2Rγ. Alternatively, Jak3 may associate with IL-2Rβ following ligand-induced receptor aggregation. The association of IL-2Rγ with Jak2 has been reported by others (*22*), but this is probably a result of the cross-reactivity of the Jak2 antiserum with human Jak3 (*23*).

Our results highlight the complex nature of IL-2 signaling. In addition to Jak1, IL-2Rβ can recruit multiple PTKs, such as

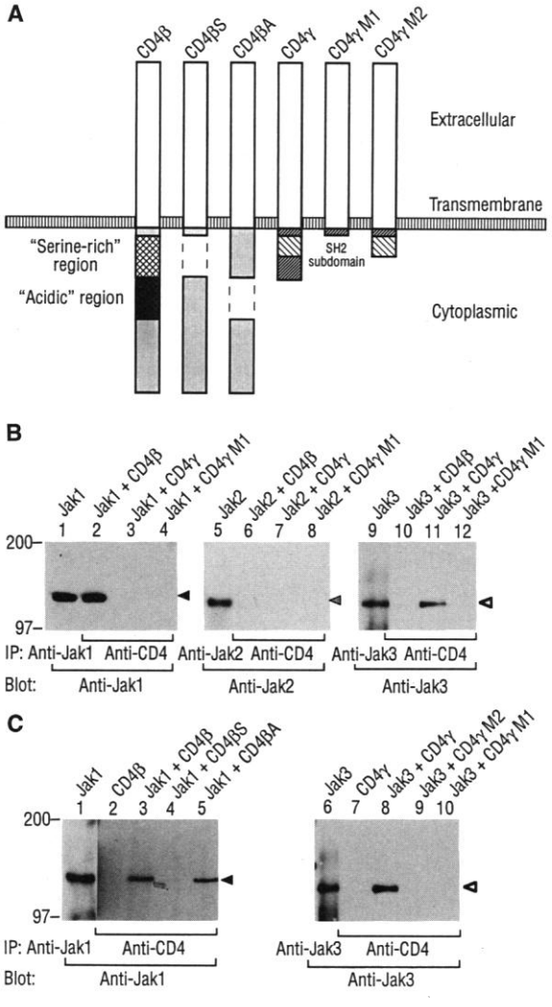

Fig. 1. Selective association of Jak1 and Jak3 with the restricted cytoplasmic regions of IL-2Rβ and IL-2Rγ, respectively. (**A**) A schematic representation of the CD4–IL-2R chimeric receptors. The chimeric constructs consist of the extracellular and transmembrane domain of the human CD4 molecule fused to the cytoplasmic domains of human IL-2Rβ and IL-2Rγ (the wild-type or the cytoplasmic deletion mutants) (*3*, *5*). CD4βS and CD4βA bear the cytoplasmic domain of IL-2Rβ lacking the membrane proximal "serine-rich" region (S region) (56 amino acids) and the "acidic" region (A region) (70 amino acids) distal to the S region, respectively. CD4γM1 and CD4γM2 bear the cytoplasmic domains of IL-2Rγ with COOH-terminal truncations of 79 and 48 amino acids, respectively. (**B**) Selective association of Jak1 and Jak3 with the cytoplasmic domains of IL-2Rβ and IL-2Rγ. COS cells were transfected by the calcium phosphate method (*24*) with the indicated cDNAs (*10*, *25*). These cell lysates were immunoprecipitated (IP) with anti-CD4 (lanes 2 to 4, 6 to 8, and 10 to 12), anti-Jak1 (lane 1), anti-Jak2 (lane 5), or anti-Jak3 (lane 9) (*26*). Immunoprecipitates were separated by 7.5% SDS-PAGE and subsequently immunoblotted (Blot) with anti-Jak1 (lanes 1 to 4), anti-Jak2 (lanes 5 to 8), or anti-Jak3 (lanes 9 to 12) (*27*). (**C**) Physical association of Jak1 and Jak3 with the restricted cytoplasmic regions of IL-2Rβ and IL-2Rγ. COS cells were transfected with cDNAs as indicated. Cell lysates were immunoprecipitated with anti-CD4 (lanes 2 to 5 and 7 to 10), anti-Jak1 (lane 1), or anti-Jak3 (lane 6). Immunoprecipitates were separated by 7.5% SDS-PAGE and subsequently immunoblotted with anti-Jak1 (lanes 1 to 5) or anti-Jak3 (lanes 6 to 10). The positions of Jaks are shown by the arrowheads. Molecular sizes are indicated on the left (in kilodaltons).

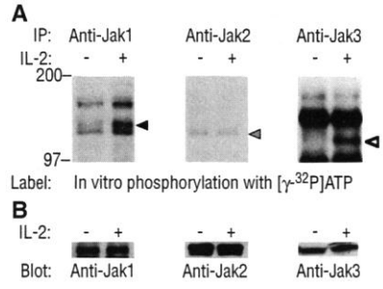

Fig. 2. Activation of Jak1 and Jak3 after IL-2 stimulation. Factor-starved FWT-2 cells (1×10^8) were stimulated (+) or not stimulated (−) with 2 nM human IL-2 for 5 min. The cell lysates from these FWT-2 cells were subjected to immunoprecipitation with anti-Jak1, anti-Jak2, or anti-Jak3. (**A**) Immunoprecipitates were subjected to in vitro kinase assay (Label) (*12*) and separated by 7.5% SDS-PAGE. Gels were alkaline-treated and autoradiographed. The positions of Jaks are shown by the arrowheads. Molecular sizes are indicated on the left (in kilodaltons). (**B**) Immunoblot of the respective Jaks after immunoprecipitation. Immunoprecipitates were separated by 7.5% SDS-PAGE and immunoblotted with the respective anti-Jaks.

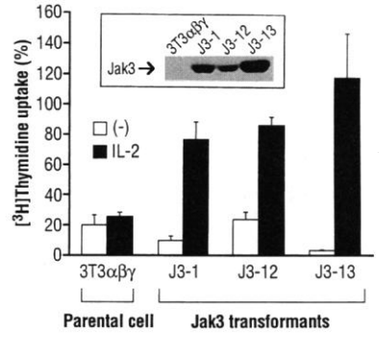

Fig. 3. Functional role of Jak3 in IL-2 growth signaling. After FCS starvation of 3T3αβγ, J3-1, J3-12, and J3-13 cells (*21*) for 16 hours, either IL-2 (5 nM) or FCS (10%) was added and the cells were incubated for an additional 12 hours. [^{3}H]thymidine uptake was determined as described (*20*). The results are represented as the percentage of [^{3}H]thymidine uptake observed with 10% FCS in the respective cells (3T3αβγ, 47,751 ± 4014 cpm; J3-1, 32,403 ± 4342 cpm; J3-12, 24,184 ± 1220 cpm; J3-13, 33,888 ± 4869 cpm). The error bar represents the standard deviation of four determinations. Essentially identical results were obtained in the three independent experiments. (**Inset**) Expression of Jak3 in Jak3-transfected NIH 3T3αβγ cells. Cell lysates of these cells were separated by 7.5% SDS-PAGE and immunoblotted with anti-Jak3. The activation of Jak3 by IL-2 was also observed in these clones (*15*).

Src-family PTKs (8) and Syk PTK (9). Thus, IL-2R components recruit one or more PTKs, all of which may be required for maximum signaling. Finally, our experiments with reconstituted IL-2R provide functional evidence for the involvement of Jaks in the growth signal transmission by cytokines.

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12. Anti-Jak1, anti-Jak2, anti-Jak3, or anti-CD4 immunoprecipitates were resuspended in 30 μl of the kinase buffer [20 mM Hepes (pH 7.2), 10 mM $MnCl_2$, 30 μM Na_3VO_4, 0.1% (v/v) Brij 96] containing 10 μCi of [γ-^{32}P]ATP (adenosine triphosphate) (5000 Ci/mmol; Amersham) and incubated for 15 min at 25°C. The reaction was terminated by the addition of Laemmli buffer. Subsequently, samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (7.5%) under reducing conditions and were transferred to polyvinylidene difluoride membrane filters (Immobilon, Millipore). Subsequently, membrane filters were treated with 1 N NaOH for 1 hour at 65°C, fixed, and subjected to autoradiography. Similar to the results obtained by immunoblot analysis, in vitro kinase assays revealed that the cytoplasmic S region of IL-2Rβ and the COOH-terminal 48 amino acids within IL-2Rγ are required for association with Jak1 and Jak3, respectively.
13. Metabolic labeling experiments of COS cells with [^{35}S]Met and [^{35}S]Cys were done as described (*24*). Expression of the CD4–IL-2R chimeras in COS cells was found to be comparable when the radioactivity of the band corresponding to respective chimeras was normalized by methionine-cysteine content (quantitated with a Fujix imaging analyzer, BAS 2000). Expression of the respective Jaks in transfected COS cells was also comparable in the results of immunoblot analysis of these cell lysates with antisera against the respective Jaks.
14. The chimeric receptors CD4β, CD4γ, and CD4γM1 were stably transfected into an IL-3–dependent cell line, BAF-B03, and clonal lines expressing comparable levels of chimeric proteins were used. When cell lysates from these lines were immunoprecipitated with anti-CD4 and subjected to immunoblotting with anti-Jak1, anti-Jak2, or anti-Jak3, selective coimmunoprecipitation of Jak1 with CD4β, and Jak3 with CD4γ, but not CD4γM1, was observed. In contrast, Jak2 was coimmunoprecipitated with neither of these chimeric receptors.
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21. Jak3 cDNA (*10*) was inserted into pEF expression vector (*5*). This expression vector and neomycin-resistance gene (pST neoB) were cotransfected into 3T3αβγ cells by the calcium phosphate method (*20*). Selection was initiated 48 hours after the DNA transfection, using neomycin (700 μg/ml) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS). 3T3αβγ-derived transformants, J3-1, J3-12, and J3-13 clones, which express Jak3, were obtained.
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26. COS cells and BAF-B03–derived cells were solubilized with lysis buffer [10 mM tris-HCl (pH 7.5), 150 mM NaCl, 2 mM Na_3VO_4, 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (pAPMSF), aprotinin (10 μg/ml), 1% (v/v) Brij 96] for 30 min at 4°C. The lysates were centrifuged to remove insoluble materials and the resultant supernatants were then precleared by incubation for 1 hour at 4°C with protein A–Sepharose (Pharmacia). The precleared supernatants were then immunoprecipitated with the respective antibodies (OKT4, anti-Jak1, anti-Jak2, and anti-Jak3) and protein A–Sepharose for 2 hours at 4°C.
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Role of TCR ζ Chain in T Cell Development and Selection

Elizabeth W. Shores,* Kun Huang, Tom Tran, Eric Lee, Alexander Grinberg, Paul E. Love

Signals mediated by the T cell receptor (TCR) are required for thymocyte maturation and selection. To examine the role of TCR ζchain signals in development, TCR expression was restored in ζ-deficient mice with transgenic ζ chains that partially or completely lacked sequences required for signal transduction. The ζ chain played a role in thymic development by promoting TCR surface expression, but ζ-mediated signals were not essential because TCRs that contained signaling-deficient ζ chains promoted T cell maturation and transduced signals associated with thymic selection.

Differentiation of precursor thymocytes into mature, functional T cells is a multistep process controlled by signals delivered through the TCR (*1*). The TCR is composed of at least six different subunits that function either in antigen recognition or in signal transduction (*2*). The clonotypic TCRαβ (or TCRγδ) chains are responsible for ligand specificity, lack inherent signaling activity, and associate noncovalently with multiple signal-transducing subunits: the CD3γ, CD3δ, and CD3ε components and a dimer composed of one or more members of the ζ family of proteins [ζ, η, or the γ chain of the type I immunoglobulin (Ig) E Fc receptor, FcεR1γ] (*3*). The CD3 and ζ family proteins contain partially conserved sequences in their cytoplasmic domains, called tyrosine-based activation motifs (TAMs), that couple the TCR to intracellular signal transduction pathways (*4*). These motifs are not identical, and it has been suggested that they may recruit distinct signal transduction molecules (*4*, *5*). The CD3 chains each contain a single TAM, whereas ζ contains three TAMs and is thought to represent the predominant TCR signaling structure.

Thymocyte development is severely affected in ζ-deficient ($\zeta^{-/-}$) mice: TCR surface expression is barely detectable, $CD4^+CD8^-$ and $CD4^-CD8^+$ [single-positive (SP)] thymocytes are markedly decreased (<1% of control), and few T cells are found in the periphery (*6*, *7*). However, because ζ is required for efficient surface expression of the other TCR subunits including the CD3 signaling molecules, the role of ζ-mediated signals in development could not be directly assessed in $\zeta^{-/-}$ mice. To determine the importance of

E. W. Shores and T. Tran, Division of Hematologic Products, Center for Biologics, Food and Drug Administration, Bethesda, MD 20892, USA.
K. Huang, E. Lee, A. Grinberg, P. E. Love, Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed.