Delineation of the Regions of Interleukin-2 (IL-2) Receptor β Chain Important for Association of Jak1 and Jak3

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Interleukin-2 (IL-2) is the principal growth factor for T lymphocytes and is responsible for regulating the magnitude and duration of the T cell immune response following antigen encounter (1–4). Three classes of IL-2 receptors exist, binding IL-2 with low ($K_d = 10^{-8}$ M), intermediate ($K_d = 10^{-9}$ M), and high ($K_d = 10^{-11}$ M) affinity. The low affinity receptors contain only the IL-2 receptor α chain (IL-2Rα); intermediate affinity receptors contain IL-2Rβ and the common cytokine receptor γ chain, γc; and high affinity receptors contain all three chains (3, 4). The intermediate and high affinity receptors are the functional forms, and heterodimerization of the IL-2Rβ and γc cytoplasmic domains is necessary and sufficient for IL-2 signaling (5–7). The highly inducible α chain has a very short cytoplasmic domain (8, 9) and presumably mainly functions to increase the affinity for IL-2, allowing cellular responsiveness to the low levels of IL-2 that are physiologically present in vivo. In contrast, IL-2Rβ and γc have longer cytoplasmic domains that can associate with a number of signaling molecules, allowing the activation of signaling pathways (2–4). Stimulation of lymphocytes with IL-2 results in the rapid activation of the Janus family tyrosine kinases, Jak1 and Jak3 (10–14). Activated Jaks are critical for inducing rapid tyrosine phosphorylation of downstream substrates, including STATs (signal transducers and activators of transcription), which then dimerize, translocate into the nucleus, and regulate the transcription of target genes (4, 13–15).

It has been reported that IL-2Rβ and γc constitutively associate with two of the four Jak family kinases in a selective manner, IL-2Rβ with Jak1 and Jak3 (10, 16, 17). The S region (amino acids 267–322) of IL-2Rβ has been shown to be important for Jak1 association (17). In addition to its ability to constitutively interact with Jak1, although it is not well appreciated, IL-2Rβ can also associate with Jak3 following IL-2 stimulation of lymphoid cells (10), but the regions of interaction between IL-2Rβ and Jak3 have not previously been investigated.

A number of membrane proximal cytoplasmic point mutants of IL-2Rβ that diminish IL-2-induced proliferation have been identified (18–20). We found that these mutants also diminish IL-2-induced STAT protein activation and the association of both Jak1 and Jak3 with IL-2Rβ. This led us to further characterize the regions of IL-2Rβ required for the binding of Jak1 and Jak3, and we demonstrate that membrane distal as well as membrane proximal regions of IL-2Rβ are vital for Jak kinase interaction. Moreover, the association between Jak3 and IL-2Rβ is Jak1-independent and both Jak3 and Jak1 can be coprecipitated only in the presence of IL-2Rβ. Finally, we provide evidence indicating that the association between IL-2Rβ and Jak3 is important for potent Stat5 activation in response to IL-2 and, thus, that more than one IL-2Rβ-Jak kinase interaction is involved in IL-2 signaling.

MATERIALS AND METHODS

Constructs and in Vitro Mutagenesis—The IL-2Rβ constructs with point mutations in the cytoplasmic chain were prepared using an in vitro mutagenesis kit (5 Prime-3 Prime, Inc.) and wild-type IL-2Rβ as the template. The oligonucleotides used for mutagenesis were as follows (mutant nucleotides are underlined): 5'-AAGTTGTAACACTCTCGAGC-3' (for P257S); 5'-GTACACGACCCAGGCCCCCTGAGGTTTC-3' (for D258A); 5'-GACGAGTTAGGGGCTGTCTTCG-3' (for W277G); and 5'-CTGGCACTCTGATGTGCGACAGTAGGTTAGGAG-3' (for L299A). Successful mutagenesis was confirmed by DNA sequencing.

Wild-type IL-2Rβ or IL-2Rβ constructs containing internal deletion

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or truncation mutations in its cytoplasmic domain (see Fig. 3A), were cloned in the expression vector, pME18S, in which transcription is directed by the SRα promoter. pME18S also contains the SV40 origin of replication and is expressed at high copy number in either 293T or COS-7 cells, both of which express the SV40 large T antigen. Internal deletion mutants of IL-2Rβ (β3A, β3S) were generated by loop-out mutagenesis using single-stranded M13 bacteriophage as a template. IL-2Rβ truncation mutants (β3T, β3T1, β3S2, β3S5, β3S3, β3S13, β3S30, β3S90, and β3S290) were prepared using the polymerase chain reaction to amplify BclI to Xbal fragments of IL-2Rβ with premature termination codons, followed by subcloning into pME18S-IL-2Rβ in which the BclI to Xbal fragment was excised. The IL-2Rβ construct with four tyrosines changed to phenylalanines (β3FSFSY) was described previously (21).

The murine Jak1 cDNA in pMLCMV was provided by Dr. J. Ihle; the Jak1-independent Functional Recruitment of Jak3 to IL-2Rβ

**Reagents and Antibodies**—Anti-IL-2Rβ hMikβ1 (humanized Mikβ1; Ref. 25) or anti-Jak3 antibody (provided by J. O’Shea) and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) were used for immunoprecipitation. Immunoblots were performed for experiments with fluorescein isothiocyanate-conjugated anti-p75 (IL-2Rβ) monoclonal antibody (Endogen) and analyzed on a FACSort (Becton-Dickinson).

**Cell Lines and Transfections**—COS-7 cells (ATCC), 293T cells (provided by Dr. N. Rice, National Cancer Institute), and E1C3 cells (Jak1-deficient HeLa cells, provided by Dr. R. Flavell, Yale University) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml each of penicillin and streptomycin. Transient transfections were performed using either DEAE-dextran (for COS-7 cells) or calcium phosphate (for 293T and E1C3 cells) methods. For immunoprecipitation experiments, cells were transfected in 150-mm dishes using 2–3 μg of each plasmid. Transfectants were harvested 36–48 h later. For experiments in which IL-2-induced STAT binding activity was reconstituted, cells were transfected in 100-mm dishes using 1–2 μg of each plasmid, and nuclear extracts were made 36–48 h later.

32D cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5% from WEHI-3B cells conditioned medium (WEHI-CM) as a source of IL-3, 2 mM glutamine, and 100 units/ml each of penicillin and streptomycin. Stably transfectants expressing wild-type or mutant forms of IL-2Rβ were created by electroporation using a Gene Pulser (Bio-Rad) at 300 V and 960 microfarads. Cells (5 × 10⁶/condition) were cotransfected with linearized IL-2Rβ constructs and pcDNA3neo. 24 h after electroporation, cells were aliquoted into 24-well plates using medium containing 1 mg/ml G418 (Life Technologies, Inc.). Resistant clones were stained for IL-2Rβ expression with fluorescein isothiocyanate-conjugated anti-p75 (IL-2Rβ) monoclonal antibody (Endogen) and analyzed on a FACSort (Becton-Dickinson).
IL-2β-Jak3 or Jak3-IL-2β-Jak1 associations were assessed by immunoprecipitation with hMik1 or anti-Jak3 at 4 °C for 1-2 h. Samples were washed four times with lysis buffer, analyzed on 8 or 10% SDS-polyacrylamide gel electrophoresis (NOVEX), transferred to Immobilon-P membranes (Millipore Corp.), immunoblotted with different antibodies, and developed with ECL (Amersham Pharmacia biotech or Pierce).

Preparation of Nuclear Extracts and Electrophoresis Mobility Shift Assays (EMSA)—Extracts were prepared from 293T or COS-7 transfected cells (cells from one 100-mm culture dish) or from 32D cells (1 × 10^7 cells) that were starved of growth factor for 4 h in RPMI 1640 medium and treated with 2 μM IL-2 for 30 min at 37 °C. Cells were washed with ice-cold phosphate-buffered saline, nuclear extracts were prepared as described previously (26), and 1 μg of protein from 293T or COS-7 transfecteds or 5-10 μg of protein from 32D cells were used in EMSAs. For EMSAs, 1 μg of poly (dl-c) was used as a nonspecific competitor and 15,000 cpm of 32P-labeled double-stranded oligonucleotide containing a trimer of the GAS sequence (5’-AGATTTCTAGGAATTC-3’) from the β-casein promoter (a motif capable of binding IL-2-activated STAT proteins) was used as the probe. The reactions were separated on 6% polyacrylamide gels in 0.5 × Tris borate-EDTA and autoradiographed.

Thymidine Incorporation Assays—32D cells were washed and started with growth factor for 4 h in RPMI medium. Cells were aliquoted at 2–4 × 10^5 cells/well in a 96-well plate in triplicate in 200 μl of medium or medium containing 2 μM IL-2 or 5% WEHI-CM. After 20 h of incubation at 37 °C, 1 μCi of [H]-labeled thymidine (NEN Life Science Products) was added, and the cells were incubated at 37 °C for 4 h. Cells were harvested using a cell harvester (Tom Tec), and thymidine incorporation was assayed using a Betaplate 1205 counter (Wallac). For each transfected, at least three clones with similar IL-2Rb expression were assayed.

RESULTS

IL-2Rβ Point Mutants That Affect Proliferation Diminish IL-2-induced Stat5 DNA Binding Activity—Four IL-2Rβ mutants, including P257S (proline 257 replaced by serine), D258A, W277G, and L299A, have been reported to impair IL-2-induced proliferation in Ba/F3 or MOLT4 cells (18-20) even though they exhibit similar surface expression and IL-2 binding affinities (19). We sought to investigate the basis for the decreased proliferation of these mutants. We first made stable transfectants of each of these mutants in 32D cells and confirmed similar cell surface expression by flow cytometry (Fig. 1A). As expected, we confirmed that these mutants mediated greatly diminished proliferation, as compared with wild-type IL-2Rβ in 32D cells, which lack IL-2Rβ but can proliferate in response to IL-2 after IL-2Rβ is transfected and expressed (Refs. 21 and 27; Fig. 1B). Moreover, each of these mutations also diminished IL-2-induced STAT binding activity in transfected 32D cells (Fig. 1C) as well as in transiently transfected COS-7 cells (Fig. 1D). In 32D cells, previous studies indicate that the IL-2-induced STAT binding activity is due to Stat5 rather than Stat3 (28). For the COS-7 cell experiments, cells were transfected with γc, Jak1, Stat5α, Stat5β, and the different IL-2Rβ constructs using a system previously shown to reconstitute IL-2-induced Stat5 DNA binding activity with wild-type IL-2Rβ (24).

IL-2Rβ Point Mutants Also Exhibit Diminished Association with Both Jak1 and Jak3—Given the diminished STAT activation and that each of these mutations are contained in a region of IL-2Rβ where Jak kinase interactions might be affected (Box 1/Box 2 region, see Refs. 29-32), we tested if these mutations diminished the association of Jak1 or Jak3 as a possible explanation for the decreased IL-2 signaling. Because the IL-2Rβ-Jak3 interaction is only well seen in T cells following IL-2 stimulation, we used an overexpression system to map the regions of IL-2Rβ that mediate association with Jak1 and Jak3. COS-7 cells were transfected with Jak1 or Jak3 and IL-2Rβ mutants, cells were lysed, and lysates were immunoprecipitated with hMik1 antibody to IL-2Rβ and then blotted with antibodies to IL-2Rβ (Fig. 2A), Jak1 (Fig. 2B), or Jak3 (Fig. 2C). Jak1 and Jak3 each exhibited less binding to each of the IL-2Rβ mutants than to wild-type IL-2Rβ (Fig. 2, B and C). The Regions of IL-2Rβ Required for Jak1 and Jak3 Binding Partially Overlap—Because each of the IL-2Rβ point mutations interfered with the association of both Jak1 and Jak3, we hypothesized that the regions of IL-2Rβ that were important for Jak kinase interaction might be similar. To investigate this possibility and to map the regions of IL-2Rβ involved in the binding of both Jak kinases, COS-7 cells were transiently transfected with Jak1, Jak3, and either mutant or wild-type IL-2Rβ, were immunoprecipitated with hMik1, and then were Western blotted with anti-Jak1 (top panel). Lysates were Western blotted with anti-Jak1 to confirm the expression of Jak1 in different transfecants (bottom panel). C, the P257S, D258A, W277G, and L299A point mutations in IL-2Rβ also resulted in decreased association of Jak3. The blots in panel C were stripped and reblotted with anti-Jak3.
but no detectable coprecipitation of Jak3 with β330 even at longer exposure times (Fig. 3D and data not shown). Wild-type IL-2Rβ and IL-2Rβ mutants retaining the first 362, 371, or 379 amino acids could associate with both Jak1 and Jak3. These results in COS-7 cells were confirmed using 293T cells (data not shown). Thus, the 300–350 and 330–362 regions of IL-2Rβ are important for Jak1 and Jak3 binding, respectively (summarized below in Fig. 8).

We next tested the effect of internal deletions of the S region (residues 267 to 325) or the A region (residues 313 to 382) on the binding of Jak1 (Fig. 4A) and Jak3 (Fig. 4B). Deletion of the S region (β3S) resulted in a dramatic decrease in IL-2Rβ association with Jak1, consistent with previously reported results (17), whereas deletion of the A region only modestly decreased Jak1 association (Fig. 4A). In contrast to the findings for Jak1, deletion of the A region had a much greater effect on the association of Jak3 than did deletion of the S region (Fig. 4B).

Thus, the A region of IL-2Rβ is more important for Jak3 association, whereas the S region is more important for Jak1 association. Consistent with the data in Fig. 3, these data indicate that Jak3 binding extends to a more distal region of the IL-2Rβ cytoplasmic domain than does Jak1. Therefore, the data contained in Figs. 3 and 4 demonstrate that Jak1 and Jak3 interact with different, albeit overlapping regions of IL-2Rβ.

As the A region contains four tyrosines (Tyr-338, Tyr-355, Tyr-356, and Tyr-361), we evaluated the ability of Jak3 to associate with IL-2Rβ containing mutations in these tyrosines (IL-2RβFFFFFY). As shown in Fig. 4D, Jak3 efficiently associated with this mutant, indicating that the interaction does not depend on phosphorylated tyrosine residues.

Jak3 Can Bind to IL-2Rβ in Jak1-deficient HeLa Cells—Given that Jak1 is ubiquitously expressed, it was possible that the interaction of Jak3 with IL-2Rβ required Jak1. To investigate this possibility, we transfected Jak1-deficient HeLa cells (E1C3 cells) with Jak3 + wild-type IL-2Rβ + Jak1. Transfected cells were lysed and immunoprecipitated with hMikβ1, followed by blotting with an antiserum to Jak3. We found that IL-2Rβ and Jak3 could interact even in the absence of Jak1, and the presence of Jak1 did not enhance this interaction (Fig. 5A, first two lanes). The uniformity of expression of Jak3, Jak1, and IL-2Rβ was verified by immunoblotting with appropriate antibodies (Fig. 5B). We also used E1C3 cells to map the region of IL-2Rβ required for its interaction with Jak3, and confirmed
shown that disruption of the Jak1-IL-2R
It has previously been
induced Stat5 DNA Binding Activity—
with IL-2R
binding activity could be reconstituted following transfection
of Jak3 and Jak1 required IL-2R
cDNAs.

HeLa cells lacking Jak1 (E1C3 cells) were transfected with Jak1, Jak3, and either pME18S,
lysates of the E1C3 transfectants were Western blotted with anti-Jak3,
anti-Jak1, or anti-IL-2R to confirm the expression levels of transfected cDNAs.
the findings reported above in Figs. 3 and 4 (data not shown).

Jak1 and Jak3 Can Only Be Coprecipitated in the Presence of IL-2Rβ—Because the association between Jak3 and IL-2Rβ was Jak1-independent, and Jak1 could be coprecipitated with IL-2Rβ in the absence of Jak3, we next investigated whether Jak1 and Jak3 could be coprecipitated through IL-2Rβ. COS-7 cells were transfected with Jak1, Jak3, and either pME18S, wild-type IL-2Rβ, or IL-2Rβ deletion constructs (βΔA, βΔS, and βΔ350) that were missing regions important for the interaction of either Jak1 and/or Jak3 (see Figs. 3 and 4). Coprecipitation of Jak3 and Jak1 required IL-2Rβ (Fig. 6A, lane 2 versus lane 1); this association was markedly decreased when the βΔA, βΔS, or βΔ350 mutants were used instead of wild type IL-2Rβ (lanes 3–5), further confirming that the association between Jak1 and Jak3 is dependent on the presence of IL-2Rβ.

Association between IL-2Rβ and Jak3 Is Required for IL-2-induced Stat5 DNA Binding Activity—It has previously been shown that disruption of the Jak1-IL-2Rβ interaction diminished IL-2 signaling (2). To investigate the functional significance of the association between IL-2Rβ and Jak3, we used Jak3-deficient 293T™ cells in which IL-2-induced Stat5 DNA binding activity could be reconstituted following transfection with IL-2Rβ, γc, Jak3, Stat5a, and Stat5b (Fig. 7A, lanes 5 and 6; Fig. 7B, lanes 1 and 2). Previous studies indicate the vital role of Jak3 for IL-2-induced STAT activation (24). Both IL-2Rβ and γc were required since little, if any, IL-2-induced Stat5 DNA binding activity was seen in the absence of either γc (Fig. 7A, lanes 1 and 2) or IL-2Rβ (Fig. 7A, lanes 3 and 4). However, a truncated form of γc (γcΔCT) that is missing 80 of 86 amino acids of the γc cytoplasmic domain and contributes to IL-2 binding (22, 23) but does not interact with Jak3 (10) still allowed partial IL-2-induced DNA binding activity (Fig. 7B, lanes 3 and 4). This activity was diminished when Jak3 (Fig. 7B, lanes 5 and 6) was deleted, implicating the IL-2Rβ-Jak3 interaction as being important for STAT activation.

**DISCUSSION**

IL-2 signaling requires the dimerization of both IL-2Rβ and γc. As Jak1 has been shown to associate with IL-2Rβ and Jak3 with γc, an attractive model has been that each receptor chain associates with a different Jak family kinase in a selective manner and that IL-2-mediated activation of Jak1 and Jak3 initiates a signaling cascade(s). It is well established that the γc-Jak3 interaction (10) and Jak3 activation (33, 34) are vital for signaling. We now provide evidence that Jak3 and IL-2Rβ can associate with each other in a Jak1-independent fashion. The fact that IL-2Rβ provides interaction sites for Jak3 as well as Jak1 (see Figs. 8 and 9) suggests that a function of γc might be not only to recruit Jak3 but also to facilitate the “delivery” of Jak3 to IL-2Rβ. Moreover, the ability of Jak3 to associate with both IL-2Rβ and γc suggests that Jak3 might stabilize the receptor complex and promote downstream signaling. Our studies on the reconstitution of IL-2-induced Stat5 activation in 293T™ cells provide evidence that the full activation of Stat5 requires IL-2Rβ association with both Jak1 and Jak3, and that the heretofore poorly appreciated IL-2Rβ-Jak3 association has physiological significance.

We have now delineated regions on IL-2Rβ that are impor-
tant for the interaction of Jak1 and Jak3. We show that four point mutations in the Box 1/Box 2 region of IL-2Rβ that diminished proliferation also decreased the binding of both Jak1 and Jak3. This is consistent with the important role of this region of a number of type I cytokine receptors for Jak interaction (14, 29–32). Interestingly, however, analysis of a series of deletion and truncation mutants not only demonstrated differences in the regions of IL-2Rβ that mediate recruitment of Jak1 versus Jak3, but unexpectedly also provided evidence that regions more distal than previously suspected play major roles in the recruitment of the Jak kinases (see Fig. 8). To our knowledge, these data represent the most detailed mapping on a cytokine receptor of the region/residues involved in Jak kinase association. Previously, for all cytokine receptors studied, including IL-2Rβ, only the membrane proximal and Box1/Box2 regions have been shown to be important for the association of Jak kinases; thus, our findings have implications regarding the interaction sites of Jak kinases for other type I cytokine receptors as well. Although some receptor chains, such as γc, appear to be uniquely associated with a single Jak, the gp130 signal transducing receptor component that is shared by the receptors for IL-6, IL-11, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and cardiotrophin-1, can associate with more than one Jak. gp130 has been reported to associate with Jak1, Jak2, and Tyk2 (35, 36), but it remains unknown whether these three Jak family kinases serve completely distinctive roles and how they associate with gp130. Our data therefore provide the first example wherein more than one Jak (Jak1 and Jak3) can independently interact with a single receptor molecule (IL-2Rβ) via overlapping but non-identical regions.
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