Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor β chain but is not essential for the proliferative signal transmission

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ABSTRACT The high-affinity interleukin 2 (IL-2) receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγc chains. Two members of the Janus kinase family, Jak1 and Jak3, are associated with IL-2Rβc and IL-2Rγc, respectively, and they are activated upon IL-2 stimulation. The cytokine-mediated Jak kinase activation usually results in the activation of a family of latent transcription factors termed Stats (signal transducers and activators of transcription) proteins. Recently, the IL-2-induced Stat protein was purified from human lymphocytes and found to be the homologue of sheep Stat5/mammary gland factor. We demonstrate that the human Stat5 is activated by IL-2 and that Jak3 is required for the efficient activation. The cytoplasmic region of the IL-2Rβc chain required for activation of Stat5 is mapped within the carboxyl-terminal 147 amino acids. On the other hand, this region is not essential for IL-2-induced cell proliferation.

Interleukin 2 (IL-2) plays a critical role in immune responses by inducing the proliferation and differentiation of lymphocytes (1–6). The functional high-affinity IL-2 receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγc chains (7–12). The latter two subunits also function as common subunits of other cytokine receptors—i.e., IL-2Rβc for IL-15R and IL-2Rγc for IL-4R, IL-7R, IL-9R, and IL-15R (13–18). A membrane-proximal cytoplasmic region, termed the “serine-rich” region (the S-region), of IL-2Rβc and the cytoplasmic domain of IL-2Rγc are both critical for IL-2 signaling (19–23). Although lacking tyrosine kinase activity, the IL-2Rγc coupled-ligand binding to induction of tyrosine phosphorylation of cellular substrates, including IL-2Rβc and IL-2Rγc (24, 25). The IL-2R couples with members of at least three families of the non-receptor-type protein tyrosine kinases (PTKs): the Jak1 and Jak3 of the Janus PTK family (26, 27), Src-family PTKs (28–30), and Syk PTK (31). In fact, stimulation with IL-2 induces tyrosine phosphorylation and activation of Jak1 and Jak3 but not Jak2. Jak1 and Jak3 were found to be selectively associated with the S-region of IL-2Rβc and the carboxyl-terminal region of IL-2Rγc, respectively (32–34). Both regions were necessary for the IL-2-induced transcription of mitogenic signal(s) (19, 23). Furthermore, Jak3-negative fibroblasts expressing reconstituted IL-2R became responsive to IL-2 after the additional expression of Jak3 cDNA (34). Thus, activation of Jak1 and Jak3 may be a key event in IL-2 signaling.

Despite mounting evidence supporting the role of Jak kinases in IL-2 and other cytokine signaling, little is known about the mechanisms by which these PTKs invoke the downstream pathways for signal transmission. The best-characterized molecular event after activation of Jak PTKs thus far is the activation of a series of family of latent transcription factors, termed Stats (signal transducers and activators of transcription). The Stat proteins usually reside within the cytoplasm, and they become activated upon selective phosphorylation of tyrosine residues, presumably directly by Jak PTKs, and form homo- or heterodimers to become an active complex as a transcription factor (35–37). Evidence has also been provided that the Src homology 2 domain that is embedded in each Stats plays a critical role in both the specific recruitment of a given Stat factor to the respective receptors and subsequent homo- or heterodimerization of the factor(s) to become a functionally competent transcription factor (38). IL-2 has also been observed to activate an otherwise latent DNA-binding activity bearing properties related to Stat proteins (39, 40). However, the role of Stats in cytokine signaling, in particular proliferative signaling, has not yet been assessed.

Recently, one of our groups has purified the IL-2-activated Stat protein(s) from human peripheral blood lymphocytes and molecularly characterized by cDNA cloning (J. Hou, U.S., W. J. Henzel, S. C. Wong and S. L. McKnight, unpublished work). The IL-2-activated Stat protein appeared to be highly homologous to sheep mammary gland factor or Stat5 (42) and, hence, is designated hStat5. In addition, IL-2-induced activation of Stat3 in human T cell lines has been reported (43). In the present study, we first examined the functional relationship between IL-2-induced activation of Jak1/Jak3 and that of Stat5 and have adduced evidence that the cDNA-encoded hStat5 becomes activated by IL-2 in IL-2R-reconstituted fibroblast cell lines. Next, we confirmed the activation of endogenous Stat5 by IL-2 in a hematopoietic cell line expressing the reconstituted high-affinity IL-2R. We also provide evidence that the carboxyl-terminal region of the IL-2Rβc chain is essential for the IL-2-induced Stat5 activation. However, this region is not required for the IL-2-induced Jak1/Jak3 activation or cell proliferation in the hematopoietic BAF-B03-derived cells. These results are discussed in the light of the role of Jak PTKs and Stats in cytokine signaling.

MATERIALS AND METHODS

Cell Culture. NIH 3T3-derived cells were maintained in Dulbecco’s modified Eagle’s medium/10% (vol/vol) fetal calf serum. BAF-B03 cell is a subclone of BA/F3, a mouse

Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; PTK, protein tyrosine kinase; hStat5, human Stat5; EMSA, electrophoretic mobility shift assay; IFN, interferon; IRF-1, IFN regulatory factor 1; GAS, IFN-γ activated site; CRE, serum response element.
IL-3-dependent hematopoietic cell line (19). BAF-B03-derived cells were maintained as described (23).

**Plasmid Construction and DNA Transfection.** For the construction of pEF-hStat5, a CDNA expression vector for human Stat5, a full-length hStat5 cDNA was excised from plasmid TPU403 by XbaI and XhoI and, after filling in both ends with T4 DNA polymerase, was inserted into EcoRI-cleave pEF expression vector (23) whose cleavage sites were filled in similarly. For the transient DNA transfection assay into NIH 3T3-derived cell lines, the cells were transfected with 10 µg of the expression vector pEF-hStat5 or control pEF vector by the calcium phosphate method (44) and incubated for 48 hr. The cells were starved for 8 hr and then stimulated by IL-2 (5 nM) or mock-stimulated.

F-7 is a BAF-B03-derived transformant clone expressing the wild-type IL-2Rβc, H-4, ST-14, A-15, and S-25 are transformants expressing the H-mutant, ST-mutant, A-mutant, and S-mutant of IL-2Rβc, respectively (19). FM1-3 is a F-7-derived transformant expressing mutant human IL-2Rγc (23). For the construction of plasmid pLCKRβ-SS, pLCKRβ (19) was first digested with SphI, AflII, and StuI. SphI-AflII fragment (7.2 kb), AflII-StuI fragment (90 bp), and StuI-SphI fragment (1.2 kb) were recovered, then ligated with an XbaI linker (pTGCTCTAGAGCA, New England Biolabs) to make an in-frame nonsense codon after the StuI site. The SS-1 cell line stably expressing pLCKRβ-SS was obtained as described (19).

**Electrophoretic Mobility Shift Assay (EMSA).** Preparation of whole-cell extract, binding reaction, and gel electrophoresis were done as described (45). The DNA probes for the mouse interferon (IFN) regulatory factor 1 (IRF-1) IFN-γ activated site (GAS) (5'-GCCGTATTTCCCGAATGATGA-3') (46) and the serum response element (SRE) of the mouse c-fos promoter (5'-CAGGATGTCATATTTAGGACATCTGCGTCA-3') (47) were chemically synthesized. For competition assays, unlabeled oligonucleotides were included in the reaction mixture. For supershifts, control antibody or antibody against sheep Stat5 N-terminal (amino acid 6-131) fusion protein was preincubated with cell extracts for 1 hr at 4°C before probe addition. Band intensity was quantitated with a Fuji imaging analyzer, BAS 2000.

**Immunoprecipitation and Immunoblot.** Immunoprecipitation and immunoblot analyses were done as described (34). Anti-Jak1 antibody (aJak1) (34) and anti-Jak3 antibody (aJak3) (Upstate Biotechnology, Lake Placid, NY) were used for immunoprecipitation; anti-phosphotyrosine (aPY) (4G10; Upstate Biotechnology), aJak1, and aJak3 (34) were used for immunoblot analyses.

**Measurement of [3H]Thymidine Uptake Levels.** Cells (5 x 10^4) were cultured with or without recombinant human IL-2 (2 nM) or mouse IL-3 (10% WEHI-3B conditioned medium) in RPMI 1640 medium/10% fetal calf serum in a 96-well microtiter plate for 24 hr. Cells were pulsed with 1 µCi of [3H]thymidine (1 Ci = 37 GBq) for 4 hr before harvest. [3H]Thymidine uptake was determined as described (23).

**RESULTS**

**IL-2-Induced Activation of Stat5.** Previously, IL-2-induced DNA binding activity was purified from human peripheral blood lymphocytes and from human natural killer cell line YT. Peptide sequencing of the purified factor and subsequent cDNA cloning have revealed that the IL-2-activated human Stat is highly homologous to sheep Stat5/mammary gland factor (42), hence it was designated hStat5 (J. Hou, U.S., W. J. Henzel, S. C. Wong, and S. L. McKnight, unpublished work). We first examined activation of the cDNA-encoded hStat5 by IL-2 by a transient assay. When the hStat5 cDNA in an expression vector (pEF-hStat5) was transfected into 3T3aβγ, a mouse NIH 3T3-derived cell line expressing the reconstituted high-affinity IL-2R (44), IL-2 stimulation resulted in a weak DNA-binding activity with the IRF-1 GAS probe (Fig. 1A, lane 4); however, this activity became ~10 times higher in cell line J3-12 (lane 8), which is a 3T3aβγ-derived cell line stably expressing the transfected Jak3 cDNA (34). The Stat activation still remained ligand-dependent. These results suggest the role of Jak3 in the IL-2-induced Stat5 activation. The IL-2 induced complex was almost completely eliminated by an excess of unlabeled IRF-1 GAS oligonucleotide (Fig. 1B, lane 2); yet it was insensitive to competition by an unrelated SRE oligonucleotide (lane 3). As shown in Fig. 1B, lane 4, the DNA-binding complex was supershifted by an antibody against sheep Stat5 (αStat5) but was not shifted by the control antibody (lane 5). A monoclonal antibody against Stat1 (Transduction Laboratories, Lexington, KY), antiserum against Stat2 (Upstate Biotechnology), or antisera against Stat3 (provided by X.-Y. Fu, Yale University) did not affect the IL-2-DNA complex either (results not shown). These observations confirm that Stat5 is activated by IL-2 stimulation and indicate the critical role of Jak3 in the IL-2-induced Stat5 activation.

**Activation of Stat5 in a Hematopoietic BAF-B03-Derived Cell Line.** We next analyzed Stat protein activation by IL-2 and other cytokines in an IL-3-dependent BAF-B03-derived cell line F-7, which expresses the functional IL-2R by supplementing the human IL-2Rβc expression (19). As shown in Fig. 2A, lane 2, IL-2 stimulation resulted in the DNA-binding activities on IRF-1 GAS probe. In fact, EMSA revealed that the IL-2-induced Stat can bind to other probes—including SIE, Fcγ receptor GAS, and β-casein GAS, consistent with the previous reports by others on the IL-2-induced Stat activity in T cells (39, 40). The affinities to IRF-1 GAS and β-casein GAS probes were higher than to other probes (data not shown). As shown in Fig. 2B, both IL-2- and IL-3-induced Stat activities, but not the interferon (IFN)γ-induced Stat activity (which is known as Stat1 homodimer; ref. 38) reacted with αStat5. Interestingly, in a F7-derived line FM1-3, in which a mutant form of human IL-2Rγc chain lacking the intracellular region (i.e., the Jak3-binding region) was overexpressed (23), IL-2 failed to activate Stat5 (Fig. 2A, lane 5), whereas there was no effect on IL-3-induced DNA-binding activity (lane 6), which was reported to contain Stat5 (ref. 48; M. Azam, H. Erdjument-Bromage, B. Kreider, X. Min, F. W. Quelle, R. Bazu, C. Saris, P. Tempst, J.N.I., and C. Schindler, unpublished work).
FIG. 2. Activation of Stat5 in a BAF-B03-derived cell line. (A) F-7 cells or FM1-3 cells were untreated or stimulated with 2 nM IL-2 or IL-3 (10% WEHI-conditioned medium) for 10 min. Whole-cell extracts were prepared and incubated with 32P-labeled IRF-1 GAS probe. Complexes were resolved by separation on 4% acrylamide gels and detected by autoradiography. Arrow marks the position of the IL-2- and IL-3-induced complex. (B) IL-2-induced DNA-binding complexes contain Stat5. F-7 cells were stimulated with IFN-γ at 25 units per ml, 2 nM IL-2, or IL-3 (10% WEHI-conditioned medium). J3-12 cells were stimulated with 5 nM IL-2. Before incubation with 32P-labeled IRF-1 GAS probes, extracts were incubated for 1 hr at 4°C with nonimmune serum (NIS) or antibody specific to sheep Stat5 (αStat5). It is reported that two highly related genes encoding Stat5 isoforms are present in mice (ref. 48; M. Azam, H. Erdjument-Bromage, B. Kreider, X. Min, F. W. Quelle, R. Basu, C. Saris, P. Tempst, J.N.I, and C. Schidler, unpublished work). The upper arrow marks the IL-2- and IL-3-induced complex. The lower arrow marks the Stat1 homodimer.

Requirement of the Carboxyl-Terminal Region of IL-2Rβc for the IL-2-Induced Stat5 Activation but Not for Jak1/Jak3 Activation and Subsequent Cell Proliferation. To delineate the critical region of IL-2Rβc within the cytoplasmic domain for Stat5 activation, we performed EMSA, using the BAF-B03-derived transformant clones expressing the respective IL-2Rβc chain mutants [Fig. 3A, (19)]. As shown in Fig. 3B, Stat5 activation was observed in cells expressing the wild-type IL-2Rβc (F-7) and the IL-2Rβc A-mutant (A-15) lacking the association site for Src-family PTKs (28), and these cells proliferated in response to IL-2 (Fig. 4; ref. 19). On the other hand, both Stat5 activation and IL-2-induced cell proliferation were abolished in cells expressing the IL-2Rβc ST- or S-mutant (ST-14 and S-25 cells, respectively). Interestingly, cells expressing the H-mutant of IL-2Rβc lacking the 147 carboxyl-terminal amino acids failed to activate Stat5. However, the

FIG. 4. Proliferation response of BAF-B03 transformants. Cells (5 × 10^6) were cultured with or without recombinant human IL-2 (2 nM) or mouse IL-3 (10% WEHI-conditioned medium) in RPMI 1640 medium/10% fetal calf serum in a 96-well microtiter plate for 24 hr. Cells were treated with 1 μCi of [3H]thymidine for 4 hr before harvest. [3H]Thymidine uptake was determined as described (23). Results are represented as the percentage of [3H]thymidine uptake observed with IL-3 in the respective cells (F-7, 324,842 ± 47,321 cpm; H-4, 403,263 ± 13,213 cpm; SS-1, 218,141 ± 5,727 cpm; ST-14, 374,093 ± 6,647 cpm; A-15, 273,088 ± 13,408 cpm; S-25, 395,377 ± 7,428 cpm). The error bar represents the SD of four determinations. The IL-2-induced long-term proliferation has been confirmed with F-7, H-4, SS-1, A-15 but not with ST-14 and S-25 cells ([19]; with SS-1 (H.F., Y.N., and T.T., unpublished observation)].

FIG. 3. The carboxyl-terminal region of IL-2Rβc chain is required for IL-2-induced Stat5 activation but not for Jak1/Jak3 activation and subsequent cellular proliferation. (A) Schematic view of the mutant IL-2Rβc molecules. Positions of tyrosine residues are indicated as Y510, etc. H-mutant, SS-mutant, and ST-mutant retain 140, 113, and 27 amino acids of the cytoplasmic domain, respectively. A- and S-mutants are internal deletion mutants 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. Proliferation response and activation of Stat5 were summarized as Yes or No. (B) Transformant clones each expressing the IL-2Rβc chain mutant were mock-stimulated or stimulated with 2 nM IL-2 for 10 min. Whole-cell extracts were incubated with 32P-labeled IRF-1 GAS probe. Complexes were resolved on 4% acrylamide gels and detected by autoradiography. (C) Tyrosine phosphorylation of Jak1 and Jak3 in response to IL-2 in H-4 cells. H-4 cells were mock-stimulated or stimulated with 2 nM IL-2 for 10 min. Cells were lysed, and Jak1 or Jak3 was immunoprecipitated with specific antisera. Immunoprecipitants were separated by SDS-PAGE and subjected to immunoblot analysis with an anti-phosphotyrosine antibody (αPY) (Upper). Jak kinase protein levels were determined by immunoblot with the respective Jak antisera (Lower). Positions of Jakas are shown by the arrowheads. Molecular sizes are indicated at left (in kDa).
IL-2-induced tyrosine phosphorylation of Jak1 and Jak3 (Fig. 3C), as well as cell proliferation, still occurred (Fig. 4; see also ref. 19). In addition, another transformant clone SS-1, which expresses the SS-mutant lacking the 175 carboxyl-terminal amino acids of the IL-2Rβc chain (Fig. 3A), also responded to IL-2, although weakly, without Stat5 activation.

**DISCUSSION**

Recently, much attention has been focused on the Janus kinase (Jak) family PTKs in cytokine signaling (35-37). In addition to the type I and type II IFN systems (50-52), the critical role of the Jak family PTKs has also been demonstrated for growth-promoting cytokine receptors, including IL-2R. The Jak association sites for many cytokine receptors have been mapped to the region containing the box 1 motif (35), arguing for the functional role of this PTK family member in intracellular signaling. For IL-2R, it has been shown that the IL-2Rβc chain and IL-2Rγc chain selectively recruit Jak1 and Jak3, respectively (32-34). These interactions require the S-region of IL-2Rβc and carboxyl-terminal 48 amino acid residues of IL-2Rγc, both of which are indeed critical for the proliferative signal transmission (refs. 19 and 23; A.K., unpublished data). Recent results indicate that the Jak3 mutants lacking the JH3-JH7 region, but not the JH1 PTK domain, fail to interact with IL-2Rγc (A.K., unpublished data). It is likely that ligand-induced IL-2Rβc and IL-2Rγc heterodimerization brings about local aggregation of these molecules, resulting in the activation of Jak1 and Jak3 PTKs by cross-phosphorylation, a mechanism analogous to the well-established cross-activation of growth factor receptor PTKs upon ligand-induced receptor dimerization (53, 54).

In the present study, we first provided evidence that hStat5 is indeed activated by IL-2 by using a transient cDNA expression system in NIH 3T3-derived fibroblast cell lines expressing the reconstituted IL-2R. The hStat5 activation was dramatically augmented by the ectopic expression of Jak3 in the Jak3-negative fibroblasts (Fig. 1A). In addition, when a mutant form of the human IL-2Rγc chain lacking the intracellular region (i.e., the Jak3-binding region) was overexpressed in the BAF-B03-derived F-7 cell line expressing the IL-2R, IL-2 failed to activate endogenous Stat5 (Fig. 2A). These data indicate the critical role of Jak3 in the IL-2-induced Stat5 activation. In view of the fact that tyrosine phosphorylation of endogenous Jak1 becomes enhanced by ectopic expression of Jak3 in 3T3 fibroblast-derived cell lines (H.F., unpublished observation), it is likely that Jak1 also takes part in Stat5 activation by IL-2.

It has been reported that the specificity of Stat activation rests on coupling of the latent Stats to the intracellular domain of their cognate receptors, as originally proposed for type II IFN receptor and IL-4R (49, 55), which respectively activate Stat1 and IL-4 Stat (Stat6); phosphorylation of specific tyrosine residue(s) within the receptor by Jak5 results in the recruitment of the Stats for the activation. On the other hand, tyrosine phosphorylation of receptors may not be required for Stat activation for some cytokine receptors (37). In a BAF-B03-derived cell line, H-4, expressing the mutant IL-2Rβc, which lacks the carboxyl-terminal 147 amino acids, Stat5 activation by IL-2 is abrogated almost completely (Fig. 3B). The deleted region in the H-mutant contains two tyrosine residues, Y392 and Y510. It is noteworthy that receptor-derived peptide fragments harboring either Y392 or Y510 inhibit the Stat5 activation (S. L. McKnight, personal communication). Although these observations support the view that these two tyrosines of IL-2Rβc might be the docking sites for Stat5, further work [e.g., replacement of the tyrosine residues with other amino acid(s)] will be required to clarify this point. In addition, considering that the A-mutant cannot associate with and activate Src-family PTKs (28, 30), activation of Stat5 is thought to be independent of Src-family PTKs such as p56lck.

Although the critical role of Stat proteins in the IFN signaling has been well documented, it was not clear whether the Stat activation by cytokine receptors plays a role in the proliferative signal transmission. The observation that Stat5 activation is abrogated in the H-4 (and SS-1) cells raises the intriguing issue of whether Stats play a role of proliferative signal transmission by IL-2. In fact, H-4 cells respond well to IL-2 for proliferation, despite the apparent lack of Stat5 activation. The growth property of H-4 cells is comparable to that of F-7 cells expressing the wild-type IL-2Rβc in their dose dependency on recombinant IL-2 (19). In addition, the IL-2Rβc chain H-mutant can also deliver proliferative signal in primary T cells (presumably in the absence of Stat5 activation) (P. D. Greenberg and B. H. Nelson, personal communication). Transformants expressing the SS-mutant also responded to IL-2 without Stat5 activation, although proliferative response was poor compared with F-7 or H-4 cells. Higher concentration of IL-2 (1 nM) is required for maximal proliferative response of SS-1 (H.M., unpublished observation). This result may be presumably due to the fact that SS-mutant lacks about a half carboxyl-terminal portion of the A-region that is retained in the H-mutant. The results with SS-1 cells themselves are not conclusive but support the above conclusion drawn from the experiments with H-4 cells. Thus, although further work will be required for generalization, our results suggest that Stat activation participates in cytokine signaling in aspects other than cell proliferation. A similar observation has been made with Stats, the activation of which is not required for induction of mitogenesis after IL-3R or IL-4R stimulation (J.N.I., unpublished data).

Notwithstanding, the previous observation that ectopic expression of Jak3 cDNA in the IL-2R-expressing fibroblast line 3T3aβγ renders the cells to respond to IL-2 (34) indicates the critical role of the Jak PTKs in cell proliferation. In addition, when a mutant form of Jak3, which lacks the JH1 PTK domain but still binds to IL-2Rγc, was overexpressed in the BAF-B03-derived F-7 cell line expressing the IL-2R, the IL-2 response but not the IL-3 response was strongly inhibited (A.K., unpublished data). Taken together, these Jak family members clearly play critical roles in the IL-2-mediated proliferative signaling. We thus infer that one or more of the additional Jak-mediated pathway(s) that exist are linked to cell proliferation. In fact, cell proliferation is caused by the induction of a number of protooncogenes such as c-myc, bel-2, and c-fos/c-jun (refs. 41 and 56; T.M., Z.-J. Liu, A.K., Y.M., K. Yamada, Y. Tsujimoto, E. L. Barsoumian, R. M. Perlmutter, and T.T., unpublished work). Evidence has been provided for the critical role of these protooncogene inductions in IL-2 signaling, yet no data have been obtained for the participation of Stats in the induction of these genes.

One intriguing possibility is that Jak PTKs may participate in activating the other nonreceptor PTKs that are linked to protooncogene induction, such as Src-family PTKs and Syk PTK. Obviously, how these non-receptor-type PTKs interact with each other is a very important future issue. It will also be important to study the function of Stat5 in IL-2 signaling by identifying its target genes, as well as to elucidate the function of Jak PTKs in contexts other than that of Stat activation.

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