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c-*fos* gene induction by interleukin 2: Identification of the critical cytoplasmic regions within the interleukin 2 receptor β chain

(serum-responsive element/c-*fos* promoter)

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ABSTRACT **Interleukin 2 (IL-2) plays a critical role in the growth and differentiation of lymphoid cells. The IL-2 signal is delivered intracellularly by the IL-2 receptor β chain (IL-2Rβ); however, the mechanism by which the signal reaches the nucleus remains unclear. In this study, we demonstrate the rapid activation of c-*fos* protooncogene transcription by IL-2 and provide evidence that the serum-responsive element (SRE) within the c-*fos* promoter is responsible for the activation in a murine pro-B-cell line, BAF-B03, expressing the human IL-2Rβ cDNA. Interestingly, the same SRE is also responsible for c-*fos* gene activation by interleukin 3 or erythropoietin. Further, we show that the activation of c-*fos* by IL-2 requires defined cytoplasmic regions of IL-2Rβ—i.e., the "serine-rich" region, which is known to be essential for growth-signal transduction in BAF-B03 cells, and the "acidic region," which is located more distal to the cell membrane. These results indicate the functional importance of the two distinct regions within the IL-2Rβ cytoplasmic domain in IL-2-induced c-*fos* gene activation and point to a potential role of the acidic region in IL-2 signal transduction that could not be adequately assessed in a previous study.**

Interleukin 2 (IL-2), a lymphokine secreted by activated T lymphocytes (T cells), plays an essential role in the growth and differentiation of T cells, natural killer cells, and other cells involved in immune responses (1, 2). IL-2 elicits biological effects by binding to homologous cell membrane receptors (3). The functional high-affinity interleukin 2 receptor (IL-2R) consists of at least two distinct IL-2-binding molecules, the α chain (IL-2Rα) (4, 5) and the β chain (IL-2Rβ) (6). IL-2Rβ, but not IL-2Rα, transduces the IL-2 signal intracellularly (7, 8). A cytoplasmic region critical for growth-signal transduction, designated the "serine-rich" region, has been identified within the human IL-2Rβ (8). More recently, we have shown that IL-2Rβ interacts with a *src*-family protein tyrosine kinase, $p56^{lck}$ (9).

IL-2Rβ belongs to a cytokine receptor superfamily (10–12). Whereas the conserved structure in the receptor superfamily is rather restricted to the extracellular domain, several other members, such as the interleukin 3 (IL-3) receptor and the erythropoietin (EPO) receptor, share certain structural homologies in their cytoplasmic domain with IL-2Rβ (8, 10, 13). Therefore, it is possible that these receptors use common or closely related intracellular elements for ligand-mediated signal transduction. However, no catalytic function has been ascribed to IL-2Rβ and, hence, the molecular mechanism(s) by which IL-2Rβ transmits the intracellular signal(s) remains elusive.

In the present study, we have investigated the mechanism of IL-2-mediated activation of c-*fos* protooncogene transcription as an approach to explore the path of IL-2 signal(s) by which IL-2Rβ transmits activation signal(s) to the nucleus. The c-*fos* gene is a primary target for extracellular signals, and its transcription is rapidly stimulated in response to various stimuli, including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) as well as the immunoregulatory cytokines such as IL-2 (14–17); hence, c-*fos* may function in cell growth regulation (18). Expression of c-*fos* antisense RNA or microinjection of anti-Fos antibody can inhibit cell growth (19, 20).

The 5′ upstream region of c-*fos* contains multiple cis elements that act as inducible enhancers (for review, see ref. 21). The serum-responsive element (SRE), an inducible cis element that consists of 20 base pairs (bp) with a dyad symmetric structure, centered 310 bp upstream of the transcription initiation site, has been reported to be a major responsive element for some growth factors, such as EGF in nonlymphoid cells (22, 23).

Here we provide evidence that SRE is the primary target for c-*fos* induction by IL-2, IL-3, and EPO in a mouse pro-B-cell line, BAF-B03 (8). Further, we demonstrate that two distinct cytoplasmic regions in IL-2Rβ, the serine-rich and the acidic regions, are necessary for IL-2-induced c-*fos* activation. We discuss our findings in the light of the IL-2-induced cascade of signal transduction.

MATERIALS AND METHODS

Cell Cultures. The BAF-B03 cell line is a subclone of BA/F3, a mouse IL-3-dependent pro-B-cell line (8). F-7 and F-4 are BAF-B03-derived stable transfectants expressing the human IL-2Rβ cDNA (8). F-4 cells express the IL-2Rβ to a lesser extent than F-7 cells. A-15 and S-25 are BAF-B03-derived stable transfectants expressing the human IL-2Rβ mutants lacking the internal acidic region (amino acids 313–382) and the serine-rich region (amino acids 267–322), respectively (8). F7E2 is a double transfectant clone obtained by transfecting mouse EPO-R cDNA (24) (a gift from A. D. D'Andrea, Dana–Farber Cancer Institute, Boston) into F-7 cells, as described (25). Cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 10% (vol/vol) WEHI-3B-conditioned medium (WEHI supernatant) as a source of IL-3.

Plasmids. The c-*fos* promoter–chloramphenicol acetyltransferase (CAT) gene plasmids (FC3, FC5, FC10, FC11) were generous gifts from M. Fujii (Kanazawa University, Kanazawa, Japan) and I. M. Verma (Salk Institute, San Diego) and contain various lengths of c-*fos* promoter se-

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Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; IL-3, interleukin 3; EPO, erythropoietin; SRE, serum-responsive element; SIE, *sis* conditioned medium-inducible element; CRE, cAMP-responsive element; FBS, fetal bovine serum; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CAT, chloramphenicol acetyltransferase.

quence (26). The SRE–CAT and cAMP-responsive element (CRE)–CAT constructs were kindly provided by K. Kaibuchi (Kobe University, Kobe, Japan) (27). Point mutations were introduced into SRE sequences in the FC3 plasmid by site-directed oligonucleotides as described (28). The resultant plasmid, FC3(muSRE), has AAATATTATT instead of CCATATTAGG within the c-*fos* SRE sequence. Mutations were confirmed by nucleotide sequencing.

RNA Blotting. Total cytoplasmic RNA was isolated using the Triton X-100 extraction technique, separated by electrophoresis through a 1% agarose/formaldehyde gel, and transferred onto a nitrocellulose filter according to standard methods (29). Radiolabeling of the probes with [α-^{32}P]dCTP was performed with the use of random oligonucleotide primers.

Transfections and CAT Assays. DNA transfection was performed by the DEAE-dextran method (30). Cells (5×10^6) were incubated in 1 ml of 25 mM Tris, pH 7.4/137 mM NaCl/5 mM KCl/0.6 mM Na_2HPO_4/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$ containing 10 μg of plasmids and 500 μg of DEAE-dextran for 30 min at room temperature. The transfected cells were then cultured in RPMI-1640/10% FBS with 0.1% WEHI supernatant for 24 hr and stimulated with human IL-2 (2 nM recombinant IL-2), mouse IL-3 (2 nM recombinant IL-3 or 10% WEHI supernatant), or human EPO (2 nM recombinant EPO) for another 6 hr. Cell extracts were prepared and CAT assays were performed as described (30). Data were analyzed quantitatively with the use of the Betascope 603 image analyzer (Betagen, Waltham, MA).

Measurement of [^{3}H]Thymidine Incorporation. [^{3}H]Thymidine incorporation by cells following IL-2 or IL-3 stimulation was measured as described (8). For this, cells were stimulated with recombinant human IL-2 (2 nM) or WEHI supernatant (20%; source of IL-3) for 24 hr and then incubated with [^{3}H]thymidine for 4 hr prior to harvest.

RESULTS

Induction of c-*fos* mRNA by IL-2, IL-3, and EPO. The IL-3-dependent mouse pro-B-cell line BAF-B03 was found to acquire IL-2 or EPO responsiveness when transfected with IL-2Rβ cDNA or EPO-R cDNA, respectively (8, 25). Double transfection of IL-2Rβ and EPO-R cDNAs into BAF-B03 cells gives rise to the generation of transfectants that are capable of responding to IL-2 and EPO as well as IL-3. One such double transfectant clone, F7E2, was employed to examine c-*fos* activation by these cytokines. In this experiment, cells that had been passaged in RPMI-1640/10% FBS supplemented with 10% WEHI supernatant were washed and resuspended in RPMI-1640/10% FBS with 0.1% WEHI supernatant. The presence of a small amount of IL-3 allows the cells to survive but does not induce efficient cell proliferation. Twenty-four hours after the onset of IL-3 starvation (0.1% WEHI supernatant), cells (viability, >95%) were stimulated with saturating amounts of cytokines for various times. RNA blot analysis (Fig. 1) showed that IL-2, IL-3, and EPO were all capable of inducing c-*fos* mRNA in a rapid and transient manner. As reported previously (17), the induced mRNA was already detectable at 15 min following stimulation, peaked at 30 min, and decreased rapidly thereafter.

Activation of the c-*fos* Promoter by IL-2. The rapid induction of c-*fos* mRNA accumulation in the cytokine-treated BAF-B03 cells was most likely caused by the transcriptional activation of the c-*fos* promoter (26). Hence, activation of c-*fos* transcription by these cytokines was examined in a transient CAT assay system. A plasmid containing the c-*fos* promoter regulatory sequences (up to bp −712) connected upstream to the CAT coding sequences (FC3; ref. 26) was transfected into F-7 or F7E2 cells. The transfected cells were cultured in the presence of a suboptimal concentration of IL-3 (0.1% WEHI supernatant; IL-3, ≈5 units/ml). IL-3 at this concentration was neither sufficient to induce efficient cell growth nor able to induce c-*fos* gene expression (data not shown). After 24 hr of culture, the cells were stimulated with IL-2, IL-3, or EPO for 6 hr, and induction of CAT gene expression was examined. CAT activity from the c-*fos* promoter was markedly increased upon cytokine stimulation (Fig. 2*A*). With IL-2 stimulation, CAT activity was clearly detectable at 1 pM and saturated at 100 pM IL-2, indicating that the IL-2 signal was transmitted via the high-affinity receptor (Fig. 2*B*). The activation of the c-*fos* promoter was indeed mediated by the IL-2/IL-2R interaction, since removal of serum from the medium did not affect the above observations (Fig. 2*C*).

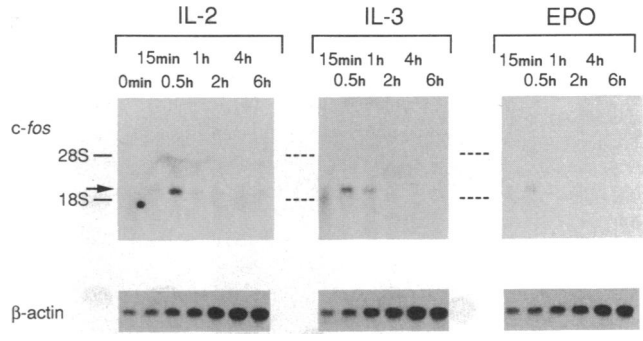

FIG. 1. Transient induction of endogenous c-*fos* mRNA by cytokines in F7E2 cells. Cells were cultured in RPMI-1640/10% FBS with 0.1% WEHI supernatant. After 24 hr of culture, they were stimulated with IL-2 (2 nM), IL-3 (10% WEHI supernatant), or EPO (2 nM) for various times up to 6 hr. Total RNA was extracted from the cytokine-treated cells and used for RNA blotting with a ^{32}P-labeled 0.4-kbp *Sac* I–*Stu* I fragment of the mouse c-*fos* gene as a probe. After dehybridization, the same filter was rehybridized with a β-actin probe. Positions of 28S and 18S rRNA are indicated at left.

Identification of the Cytokine-Responsive Element Within the c-*fos* Promoter. To determine the cis-acting responsive element required for IL-2-induced c-*fos* gene activation, a series of promoter deletion mutants, each linked to the CAT gene (FC3, FC5, FC10, FC11; ref. 26), were transfected into F7E2 cells, and the IL-2-induced CAT expression levels were examined. Deletion of the promoter region spanning bp −712 (FC3) to −307 (FC5) resulted in a dramatic reduction of CAT activity induction by IL-2, and only a weak CAT inducibility was noticeable with the promoter containing the further downstream region (Fig. 3). Essentially the same results were observed by stimulating the transfected F7E2 cells with IL-3 or EPO (data not shown). Since the identified c-*fos* promoter region contains two well-recognized regulatory elements, the SRE (22, 23) and the *sis* conditioned medium-inducible element (SIE) (31), we next examined which element was responsible for the cytokine responsiveness. To this end, point mutations were introduced by site-directed mutagenesis into SRE in FC3 plasmid (Fig. 4*A*). The mutant, FC3(muSRE), was transfected into F7E2 cells and CAT induction by IL-2, IL-3, or EPO was examined. Mutations in the SRE almost completely abolished CAT induction by cytokines. In contrast, introduction of point mutations into SIE did not alter the c-*fos* induction by these cytokines (data not shown). To confirm the role of SRE in c-*fos* gene activation by cytokines, a plasmid containing the SRE of c-*fos* promoter directly linked to the CAT structural gene (SRE–CAT) (27) was transfected into F7E2 cells. As shown in Fig. 4*B*, the SRE was functional in the activation of CAT gene by IL-2, IL-3, and EPO. In contrast, when CRE–CAT was transfected into F7E2 cells, CAT induction was not observed after IL-2, IL-3, and EPO treatment.

Distinct Cytoplasmic Regions of IL-2Rβ Are Involved in the Activation of c-*fos* by IL-2. We next addressed the question of

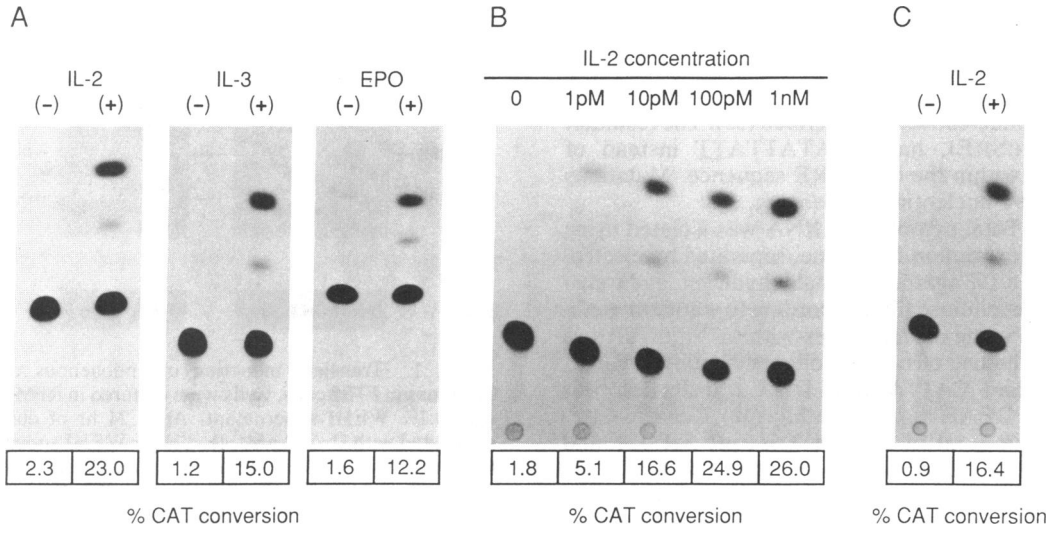

FIG. 2. Activation of c-*fos* promoter by cytokines. (*A*) F-7 cells (for IL-2 and IL-3 stimulations) or F7E2 cells (for EPO stimulation) were transfected with FC3. The transfected cells were stimulated with recombinant human IL-2, mouse IL-3, or human EPO at a final concentration of 2 nM for 6 hr. CAT activity was assayed as described in *Materials and Methods*. (*B*) F-7 cells transfected with FC3 were stimulated by various concentrations of recombinant human IL-2 for 6 hr. (*C*) F-7 cells transfected with FC3 plasmid were cultured for 24 hr in serum-free RPMI-1640 containing 0.1% WEHI supernatant and then were stimulated with 2 nM IL-2 for 6 hr.

which region(s) of IL-2Rβ is required for the IL-2 signal for c-*fos* gene activation. A previous study (8) showed that a defined cytoplasmic region of IL-2Rβ, designated the serine-rich region, was essential for IL-2-induced mitogenic signal transduction in BAF-B03 cells. We examined the effect of IL-2 on endogenous c-*fos* gene induction in BAF-B03 cells stably expressing wild-type or mutated IL-2Rβ molecules (F-7, F-4, A-15, and S-25 cells). As shown in Fig. 5*A*, BAF-B03 cells expressing the wild-type IL-2Rβ (F-7 and F-4 cells) transmitted the IL-2 signal for c-*fos* activation. In F-4 cells, which express IL-2Rβ at a lower level than F-7 cells, activation of the c-*fos* gene upon IL-2 stimulation was less than in F-7 cells. A-15 and S-25 cells express the mutant IL-2Rβ lacking the acidic region and the serine-rich region, respectively. In these cells, IL-2-induced activation of the c-*fos* gene was almost undetectable (Fig. 5*A*). Long exposure of the autoradiograph revealed that IL-2 stimulation of the A-15 cells resulted in activation of the c-*fos* gene to a very low extent; however, even such marginal activation was not detected in the S-25 cells under the same conditions (data not shown). Similar results were obtained when we measured the CAT activity of the above cells transfected with the CAT reporter gene (FC3) following IL-2 stimulation (data not shown). [^{3}H]Thymidine incorporation of A-15 cells after IL-2 stimulation was comparable to that of F-7 and F-4 cells, although this transfectant failed to transmit the IL-2 signal for c-*fos* promoter activation (Fig. 5*B*).

DISCUSSION

In the present study, we have provided evidence that IL-2 activates the SRE sequences to induce c-*fos* protooncogene expression. We have shown that the same SRE is responsible for activation by two other cytokines—i.e., IL-3 and EPO. The SRE has been known to be the primary element inducible by growth factors such as EGF, PDGF, and insulin (22, 23), and the present study further points to the general importance of SRE as a target of cell growth-signal transduction.

Although the precise mechanism by which the c-*fos* gene is activated through SRE upon various stimuli is not clearly understood, several distinct factors, such as the p62–67 serum-responsive factor (SRF) (22, 23, 32, 33), the SRF-associated tertiary complex factor p62^{TCF} (34) and the direct binding factor p62^{DBF} (35), have been shown to interact with SRE. In fact, we have identified a SRF-like factor in BAF-B03 cells by gel shift assay (data not shown). Accordingly, the molecular characterization of trans-acting factors that interact with SRE in IL-2-responsive cells will provide further insight into the mechanism(s) of IL-2-induced intracellular signal transduction and c-*fos* gene activation.

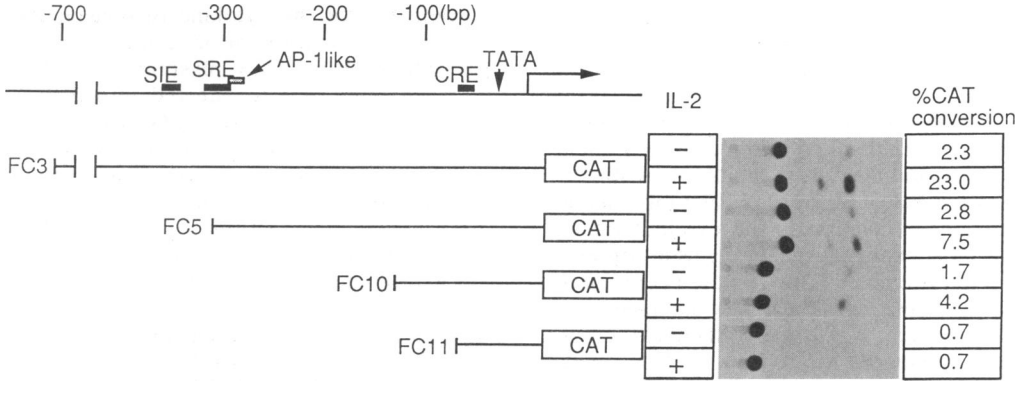

FIG. 3. Effect of 5′ deletions on the IL-2-induced expression of the c-*fos* promoter–CAT fusion gene. F-7 cells transfected with a series of c-*fos*–CAT fusion genes were either treated with 2 nM IL-2 (+) or mock-treated (−). CAT activities were determined 6 hr after the onset of IL-2 stimulation.

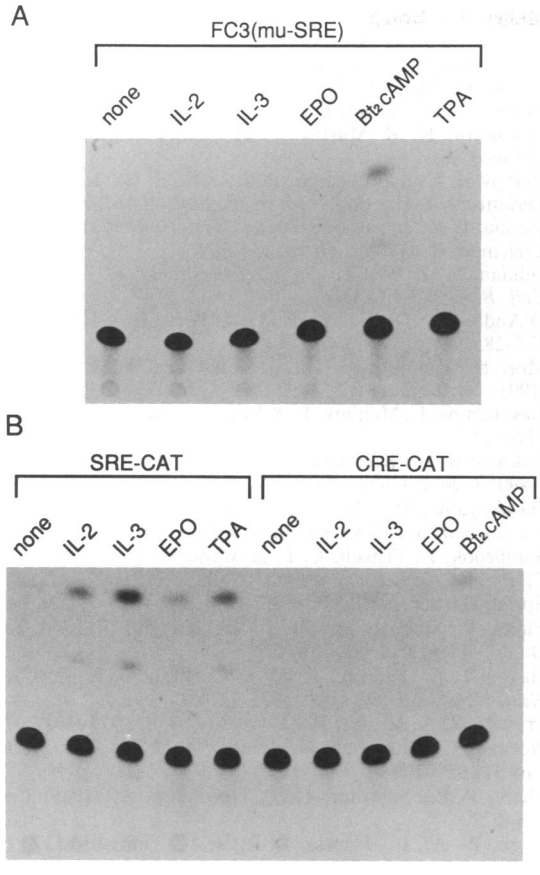

FIG. 4. Role of SRE in cytokine-induced c-*fos* activation. (*A*) Site-directed mutations were introduced into the SRE sequence in the FC3 plasmid to make FC3(muSRE) (see *Materials and Methods*). The FC3(muSRE) plasmid was transfected into F7E2 cells. The transfected cells were cultured for 24 hr and then stimulated with IL-2, IL-3, EPO, dibutyryl cAMP (Bt_2cAMP), or phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) at respective final concentrations of 2 nM, 2 nM, 2 nM, 2 μM, and 50 ng/ml. CAT activities were determined 6 hr after the treatments. (*B*) F7E2 cells were transfected with either SRE–CAT or CRE–CAT, cultured for 24 hr, and then stimulated with various reagents as described in *A*. Essentially identical results were obtained in three independent experiments.

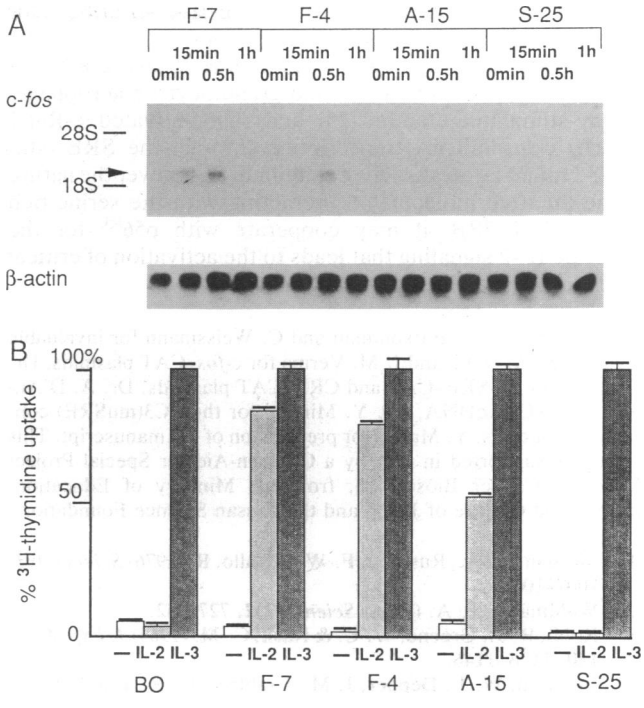

FIG. 5. Role of IL-2Rβ cytoplasmic domain in IL-2-mediated c-*fos* activation. (*A*) Transient induction of the endogenous c-*fos* mRNA in BAF-B03 transfectants expressing wild-type and mutant IL-2Rβ molecules following IL-2 stimulation. F-7, F-4, A-15, and S-25 cells were cultured in RPMI-1640/10% FBS with 0.1% WEHI supernatant. After 24 hr of culture, they were stimulated with 2 nM IL-2 for various times. RNA blotting analysis was performed as described in Fig. 1 legend. (*B*) [^{3}H]Thymidine incorporation of BAF-B03 cells expressing mutant IL-2Rβ following IL-2 or IL-3 stimulation. Data represent means ± SEM of triplicate experiments. Results are expressed as percentage of incorporation of [^{3}H]thymidine in the same cells incubated with WEHI supernatant.

The fact that abnormal expression of c-*fos* leads to cellular transformation (18), together with the findings that expression of c-*fos* anti-sense RNA or injection of anti-Fos antibody can block cell growth (19, 20), supports the idea that Fos protein is physiologically important for cell growth regulation in a wide range of cell types. This idea is also consistent with the finding that IL-2Rβ lacking the serine-rich region (S-mutant) neither transmits mitogenic signal (8) nor induces c-*fos* gene activation (present study) by IL-2. In addition, we found in this study that a distinct IL-2Rβ cytoplasmic region designated the acidic region (6, 8) is also involved in the c-*fos* activation. Internal deletion of this region (A-mutant) drastically reduced c-*fos* inducibility by IL-2, suggesting that the A-mutant IL-2Rβ is functionally impaired. Nonetheless, as shown in Fig. 5*B*, A-15 cells (BAF-B03 cells expressing the IL-2Rβ lacking the acidic region) are still capable of transmitting mitogenic signals. Are these observations reconcilable? The BAF-B03 cell line appears to be especially "permissive" for growth signals from various members of the cytokine receptor family, and hence it may proliferate readily in response to suboptimal stimuli (8, 25, 36, 37). This may explain why a very weak activation of the c-*fos* gene via the IL-2Rβ mutant lacking the acidic region may be sufficient for promoting the growth of BAF-B03 cells, whereas IL-2 signaling from both the serine-rich and acidic regions may be required in other cells, such as normal T cells. Alternatively, c-*fos* induction is not essential for BAF-B03 cell proliferation. Although further work will be required to assess to what extent c-*fos* induction is critical for the proliferation of BAF-B03 cells, our results with the c-*fos* gene as the target of IL-2-induced early cell response clearly point to the functional importance of the acidic region within IL-2Rβ in the IL-2 signaling process. Notably, this region is strongly conserved between the human and mouse IL-2Rβ molecules (38).

The cytoplasmic domains of receptor-type tyrosine kinases such as EGF receptor and PDGF receptor interact with various intracellular signal transducers such as phospholipase C-γ, phosphatidylinositol 3-kinase, c-Raf-1, GTPase-activating protein, and the *src*-family protein-tyrosine kinases (for review, see ref. 39). Whereas IL-2Rβ does not possess intrinsic tyrosine kinase activity, IL-2 can activate c-Raf-1 (40), the *src*-family protein-tyrosine kinase $p56^{lck}$ (41), $p21^{ras}$ (42), and hydrolysis of glycosylphosphatidylinositol (43, 44). Therefore, it is possible that IL-2Rβ may also interact physically with multiple intracellular signal transducers. We recently reported that the acidic region of the IL-2Rβ specifically interacts with $p56^{lck}$ (9). Furthermore, IL-2 promotes $p56^{lck}$ kinase activity in T cells (9). Hence, such an interaction (and subsequent IL-2-induced $p56^{lck}$ activation) may be critical for high-level induction of c-*fos*. Obviously, the functional role of complex formation between IL-2Rβ and a *src*-family protein-tyrosine kinase in c-*fos* gene induction requires further clarification. Moreover, it is not clear which molecule(s) associate and/or functionally couple

with another cytoplasmic region of the IL-2Rβ critical for signal transduction, the serine-rich region. In view of the reports that the protein-serine/threonine kinase c-Raf-1 is activated by IL-2 (40), IL-3, and granulocyte/macrophage-colony-stimulating factor (45) and that activated c-Raf-1 induces c-*fos* mRNA transcription through the SRE (46), c-Raf-1 might be an attractive candidate. Whatever the nature of the putative molecule(s) interacting with the serine-rich region of IL-2Rβ, it may cooperate with $p56^{lck}$ for the full-scale IL-2 signaling that leads to the activation of critical genes for cell proliferation such as c-*fos*.

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