Interaction of STAT5 Dimers on Two Low Affinity Binding Sites Mediates Interleukin 2 (IL-2) Stimulation of IL-2 Receptor α Gene Transcription*

(Received for publication, August 5, 1997, and in revised form, October 2, 1997)

Wolfram K.-H. Meyer‡, Patrick Reichenbach‡, Ulrike Schindler‡, Elisabeta Soldaini‡,§, and Markus Nabholz‡|

From the *Lymphocyte Biology Unit, Swiss Institute for Experimental Cancer Research (ISREC), 155 Chemin des Boveresses, CH-1066 Epalinges, Switzerland and §Tularik Incorporate, Two Corporate Drive, South San Francisco, California 94080

Stimulation of the interleukin 2 receptor α (IL-2Ra) gene by IL-2 is important for the proliferation of antigen-activated T lymphocytes. IL-2 regulates IL-2Ra transcription via a conserved 51-nucleotide IL-2 responsive enhancer. Mouse enhancer function depends on cooperative activity of three distinct sites. Two of these are weak binding sites for IL-2-activated STAT5 (signal transducer and activator of transcription) proteins, and mutational analysis indicates that binding of STAT5 to both sites is required for IL-2 responsiveness of the enhancer. The STAT5 dimers interact to form a STAT5 tetramer. The efficiency of tetramerization depends on the relative rotational orientation of the two STAT motifs on the DNA helix. STAT5 tetramerization on enhancer mutants correlates well with the IL-2 responsiveness of these mutants. This provides strong evidence that interactions between STAT dimers binding to a pair of weak binding sites play a biological role by controlling the activity of a well characterized, complex cytokine-responsive enhancer.

Interleukin-2 (IL-2)1 is a growth factor for antigen-activated lymphocytes (1, 2). It stimulates T cells through a high affinity cell surface receptor (IL-2R) composed of three transmembrane polypeptide chains designated IL-2Ra, IL-2Rβ, and IL-2Rγ or γc (3). The β and γ chains, which are shared with other interleukin receptors (4, 5), belong to the family of hematopoietic cytokine receptors. Resting T cells constitutively express the γ chain (6, 7) and basal levels of the β chain (8). IL-2Rβ expression increases upon antigen stimulation (9). IL-2Ra is not a member of this family and does not participate in the formation of other known receptors. IL-2Ra expression is undetectable on resting lymphocytes but is induced by signals from the antigen receptor (for review see Waldmann et al. (10)). The capacity of mature T cells to proliferate in response to IL-2 correlates with IL-2Ra expression (11). Mouse T cells that constitutively express the human IL-2Ra chain can respond to IL-2 in the absence of antigen stimulation (12). Thus, IL-2Ra expression controls, at least in part, IL-2 responsiveness.

IL-2Ra cell surface expression is regulated mainly through changes in IL-2Ra gene transcription (13–16). In mature T cells, antigen induces a transient wave of IL-2Ra transcription. Prolonged, maximal expression of IL-2Ra depends on stimulation by IL-2, which thus acts as a positive feedback regulator of its own high affinity receptor (17, 18).

We have shown that stimulation of the mouse IL-2Ra gene by IL-2 is controlled by an IL-2-responsive enhancer (IL-2rE) 1.3 kilobase pairs upstream of the major transcription start site (19). The IL-2rE maps to the same position as a DNase I hypersensitive site that appears in the chromatin of normal mouse T cells upon stimulation with concanavalin A and IL-2 (18). The enhancer is 51 nt long and contains three distinct elements (named sites I, II, and III, see Fig. 1), all of which are required for enhancer activity (19). As pointed out previously (19), sites I and II resemble binding sites for transcription factors of the STAT family (20–23). Site II also overlaps with a consensus binding site for GATA factors. Site III includes a consensus site for Ets proteins and contributes to IL-2rE activity by binding the constitutive Ets protein Elf-1 (24). The human homologue of the mouse IL-2rE has recently been identified approximately 4 kilobase pairs upstream of the transcription start site (25–27). The binding motifs in sites I, II, and III are conserved in the human IL-2rE (25, 26).

STAT proteins are latent transcription factors that dimerize in response to cytokine receptor activation. Dimerization results in activation of their specific DNA binding activity and accumulation in the nucleus (22). IL-2 predominantly activates STAT5α and STAT5b, two closely related proteins encoded by separate genes (28, 29). STAT5 is rapidly activated by IL-2 in antigen-stimulated but not in quiescent T cells (28, 30). The human IL-2rE site I does bind STAT5α and b (25, 26). Mutations in this site that abolish STAT5 binding also destroy IL-2-inducible IL-2rE activity in the human IL-2-dependent leukemia Kit225 cell line (25). Site II may also contribute to STAT5 binding (26). To what extent STAT5α and STAT5b functions overlap is not yet clear, but the phenotypes of STAT5 (31-) and STAT5b (32-) deficient mice show that they are not completely redundant.

Here we provide evidence that IL-2 responsiveness of the mouse IL-2rE depends on the binding of STAT5 to both sites I and II and that the synergistic effect of these two sites on enhancer activity is due to the interaction of the bound STAT5
dimers. Our data show that DNA-induced association of STAT dimers, which has been observed for STAT4 and STAT1 (33, 34), plays an important role in cytokine-regulation of gene expression.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Cytokine Stimulation—The PC60.21.14 cell line (referred to as PC60) is a hybrid between a mouse cytolytic T cell line and a rat thymic lymphoma. It has inherited from its lymphoma parent (35) the capacity to grow independently of IL-2 and a CD4-CD8- phenotype. Like normal CD4-CD8- thymocytes (36), it expresses IL-2Rα when stimulated with IL-1 and IL-2. IL-1 plays a similar role in these cells as antigen in mature T lymphocytes and induces PC60 cells to become IL-2-responsive. This effect has been described in detail in previous publications (16, 19, 35–37). The cells were cultured for 2–3 days in 1 ng/ml human IL-1β before stimulation with 100 units/ml human IL-2 in the continued presence of IL-1. Reconstitute interleukins were gifts of Glaxo Institute for Molecular Biology S.A. (Plan-les-Ouates, Geneva, Switzerland).

Probes—Oligonucleotides from MWG-Biotech (Duebendorf, Germany) or Microsynth (Balgach, Switzerland) were annealed, purified on acrylamide gels, quantified, and stored in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl. As a nonspecific competitor (NS) for bandshift assays, a double-stranded oligonucleotide with the sequence 5′-AGAGTTAGCTTGCGGTTCCCAGG-3′ was used.

PCR products were obtained either by annealing appropriate oligonucleotides (F+R+ single IL-2rE sites) or II, IL-2rE sites I and II, nonspecific probe NS (for sequences see Fig. 1) or by PCR amplification of different IL-2rE templates with primers spanning segments of the IL-2rE. For competition assays, probes extended from nt −1420 (primer A) to nt −1331 (primer B) of the IL-2rE 5′-flanking region, including sites I and II. For affinity precipitation and the bandshift experiment in Fig. 7C, probes extended from nt −1420 (primer A) to nt −1286 (primer C), including site III. To generate probes carrying a mutation in a single enhancer site, we amplified the corresponding reporter plasmid (M4 for site I, M9 for site II, and M12 for site III; see Fig. 1 and Sperisen et al. (19) for sequences). Probes with mutations in more than one site were obtained in three steps, essentially as described in Ho et al. (40) and explained here for a probe in which all three enhancer sites are destroyed. (a) The plasmid carrying the mutation in site I (M4) was amplified with oligonucleotide A and a 3′ oligonucleotide D′ covering site II, with changes destroying this site. Simultaneously the plasmid with a mutation in site III (M12) was amplified with a 5′ primer (D) complementary to oligonucleotide D′ and oligonucleotide C. (b) The resulting PCR products were gel-purified, denatured, annealed, and used as template for an extension reaction. The resulting full-length IL-2rE fragments were amplified with primers A and C. The final PCR products were quantified and analyzed by sequencing.

Recombinant Proteins—Expression, activation, and purification of recombinant STAT5a have been described previously (41, 42). The anti-STAT5 antibody purchased from Santa Cruz Biotechnology, Inc., STAT5a and b antibodies are from R & D Systems, and horseradish peroxidase-coupled goat-anti-rabbit antibodies are from Amersham.

Affinity Precipitation of IL-2rE-bound Proteins—Affinity precipitation of IL-2rE-bound proteins was performed as described in Ref. 39.

Electrophoretic Mobility Shift Assays (Bandshifts)—Nuclear extracts were prepared as described by Schreiber et al. (43). Lysis and extraction buffers contained protease (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM NaF, 1 mM Na3VO4). Binding reactions were performed in a final volume of 20 μl in binding buffer (10 mM Tris-HCl, pH 7.5, 60 mM KCl, 10% glycerol, 1 mM dithiothreitol, 1 mM dithiothreitol, 1 μg/ml bovine serum albumin, 1 μg of poly[dI-dC], 0.5 μg of sonicated salmon sperm DNA) containing 4 μg of cell nuclear extract or the indicated quantity of recombinant STAT5a protein and 2–3 × 106 cpm (30 fmol) of radiolabeled PCR fragments or oligonucleotides (see Fig. 1 for sequences).

Reactions were incubated on ice for 20 min and separated on 4% nondenaturing polyacrylamide gels in 0.3 × Tris/borate/EDTA. For competition experiments, unlabeled PCR fragments or oligonucleotides were included with labeled probe before addition of the proteins. When deoxycholate (DOC) was used, appropriate dilutions were added to the binding reaction at the same time as STAT5 and the probe. The mixture was incubated for 30 min at room temperature before loading on the gel.

Plasmids—The reference plasmid pGwACpGID and the plasmid pwt, in which the mouse IL-2rE 5′-flanking region and promoter has been joined to the rabbit β-globin gene, have been described previously (19, 44). Mutagenesis of pwt was performed as described by Ho et al. (40). For this purpose, an additional XhoI site was created by changing the adenine at position −1307 into a guanine. This substitution did not alter IL-2 responsiveness (data not shown). A unique BglII site and the new XhoI site were used to subclone PCR products of the site-directed mutagenesis. Positive clones were sequenced.

Reporter Gene Assays—IL-1-primed cells were transiently transfected using DEAE dextran according to Queen and Baltimore (45) and cultured with IL-1 only or with IL-1 and IL-2. Reporter gene expression was measured 48 h later, as described previously (44). Briefly, cells were cotransfected with a defined ratio of an IL-2rE rabbit β-globin gene together with the reference plasmid (pGwACpGID), which is constitutively expressed in PC60 cells and contains a 40-bp deletion in the second exon (46). Relative amounts of mRNA transcribed from the reference plasmid and the IL-2rE/β-globin construct were determined by PCR (see Refs. 19 and 44 for details).

Protocols and Amino Acid Sequence Analysis—20 μg of recombinant active STAT5a were digested with 3 μg of thermolysin (Boehringer Mannheim) in a total volume of 400 μl of digestion buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM dithiothreitol) at room temperature for 10 and 30 min. Thermolysin was inactivated by adding 4 μl of 500 mM EDTA, pH 8. Undigested protein was incubated in the same buffer without thermolysin. Electromobility shift assays were performed as described above, with 1 μl of the thermolysin reaction. The remaining reaction was dialyzed with SDS single buffer (47), and the fragments were separated on a 10% SDS gel. The proteins were blotted onto polyvinylidene difluoride membrane (Millipore) and visualized by Coomassie staining. N-terminal protein sequence analysis was performed by the protein facility at Tularik, Inc. using an AB477A protein sequencer and an ABI120A analyzer.

RESULTS

Sites I and II of the IL-2rE Are Weak Binding Sites for STAT5—Two of the functional sites in the IL-2rE (sites I and II) contain potential STAT binding motifs (see Fig. 1 and Sperisen et al. (19)). To determine whether the IL-2rE could bind IL-2-induced STAT5, we incubated PC60 extracts with a biotinylated, double-stranded oligonucleotide comprising the entire enhancer. Bound proteins were recovered by incubating the probe with streptavidin-coated beads and characterized by Western blotting. The complete IL-2rE binds proteins of about 90 kDa, which are present in extracts from IL-2-stimulated PC60 cells and react with anti-STAT5a and anti-STAT5b antisera (Fig. 2A) as well as with an anti-phosphotyrosine antibody (data not shown). No STAT5 proteins could be recovered from extracts of cells that had not been treated with IL-2. As expected, IL-2 induces rapid activation of STAT5. The amount of IL-2rE-bound STAT5 continues to increase and reaches a plateau only after 24–48 h of culture in IL-2 (Fig. 2B). This correlates with the kinetics of IL-2-induced accumulation of IL-2Ra message and IL-2rE-driven reporter gene expression (19). We did not detect any IL-2-inducible proteins that reacted with antisera against STAT1 and STAT3 (data not shown).

To determine which segment of the IL-2rE is responsible for STAT5 binding, we used biotinylated IL-2rE oligonucleotides that carry mutations in one or more of the sites required for enhancer function. Figure 2A shows that a probe containing an intact site I and mutations in the other sites bind STAT5a and b. Probes containing an intact site II and mutations in the other sites bind a trace of STAT5s, whereas probes with mutations in both sites I and II fail to bind STAT5s. Incubation of the same filters with an antibody against Elf-1 had shown that only oligonucleotides with an intact site III bind this transcription factor (24).

These results demonstrate that STAT5a and b can specifically bind to site I and, albeit very weakly, to site II. To confirm these data, we carried out bandshift experiments with recombinant STAT5a protein (Fig. 3A) and PC60 nuclear extracts (Fig. 3B). Figure 3 shows that both site I and site II can compete specifically for the binding of STAT5a to a probe that contains the STAT5 binding site of the FcyRI gene (see Fig. 1). Titration
of various competitors indicates that the affinities of sites I and II for STAT5a are, respectively, 10-fold and 50-fold lower than that of the FcγRI STAT binding site. Extracts from IL-2-stimulated but not from unstimulated PC60 cells form a complex with the FcγRI probe that is due to IL-2-induced STAT5, as shown by supershift experiments with STAT5-specific antibodies (data not shown). The competition experiment shown in Fig. 3 demonstrates that PC60 STAT5 and recombinant STAT5a have the same relative affinity for site I and site II. In certain experiments, IL-2 also stimulated the appearance of a small amount of STAT1 (visible as a faint specific band below the STAT5 complex in Fig. 3B), but it is unlikely that STAT1 plays a role in IL-2rE activity. Interferon-γ that induces STAT1 but not STAT5 does not stimulate IL-2Rα expression, nor does it affect the response to IL-2 (data not shown). Furthermore, IL-2-dependent IL2Rα expression in T lymphocytes from interferon-γ receptor-deficient mice is normal despite the fact that stimulation with concanavalin A and IL-2 fails to induce STAT1 activation.3

The IL-2rE STAT Motifs Induce Association of STAT5a Dimers—IL-2 responsiveness of the IL-2rE depends on synergy between the individual IL-2rE sites. This could reflect cooperative binding of the transcription factors controlling enhancer activity. Cooperative binding to pairs of weak binding sites has indeed been observed for STAT4 (33) and STAT1 (34). The experiments described so far were carried out with a large amount of STAT1 (visible as a faint specific band below the STAT5 complex in Fig. 3B), but it is unlikely that STAT1 plays a role in IL-2rE activity. Interferon-γ that induces STAT1 but not STAT5 does not stimulate IL-2rE expression, nor does it affect the response to IL-2 (data not shown). Furthermore, IL-2-dependent IL2Rα expression in T lymphocytes from interferon-γ receptor-deficient mice is normal despite the fact that stimulation with concanavalin A and IL-2 fails to induce STAT1 activation.3

The IL-2rE STAT Motifs Induce Association of STAT5a Dimers—IL-2 responsiveness of the IL-2rE depends on synergy between the individual IL-2rE sites. This could reflect cooperative binding of the transcription factors controlling enhancer activity. Cooperative binding to pairs of weak binding sites has indeed been observed for STAT4 (33) and STAT1 (34). The experiments described so far were carried out with a large amount of STAT1 (visible as a faint specific band below the STAT5 complex in Fig. 3B), but it is unlikely that STAT1 plays a role in IL-2rE activity. Interferon-γ that induces STAT1 but not STAT5 does not stimulate IL-2rE expression, nor does it affect the response to IL-2 (data not shown). Furthermore, IL-2-dependent IL2Rα expression in T lymphocytes from interferon-γ receptor-deficient mice is normal despite the fact that stimulation with concanavalin A and IL-2 fails to induce STAT1 activation.3

The IL-2rE STAT Motifs Induce Association of STAT5a Dimers—IL-2 responsiveness of the IL-2rE depends on synergy between the individual IL-2rE sites. This could reflect cooperative binding of the transcription factors controlling enhancer activity. Cooperative binding to pairs of weak binding sites has indeed been observed for STAT4 (33) and STAT1 (34). The experiments described so far were carried out with a large amount of STAT1 (visible as a faint specific band below the STAT5 complex in Fig. 3B), but it is unlikely that STAT1 plays a role in IL-2rE activity. Interferon-γ that induces STAT1 but not STAT5 does not stimulate IL-2rE expression, nor does it affect the response to IL-2 (data not shown). Furthermore, IL-2-dependent IL2Rα expression in T lymphocytes from interferon-γ receptor-deficient mice is normal despite the fact that stimulation with concanavalin A and IL-2 fails to induce STAT1 activation.3
IL-2Rα Regulation by STAT5

The interpretation is supported by experiments with a 39-bp probe in which site I was replaced by the high affinity FcyRI STAT consensus site I, and site II was inactivated (probe F/II). Fig. 4C shows that, at low protein concentrations, this probe forms a single complex C2 that comigrates with the complex formed by STAT5α binding to the short FcyRI probe (F) used in Fig. 3. At higher STAT5 concentrations the F/II probe, but not the short F probe, gives rise to an increasing proportion of C1 complexes. As expected, the ratio between C1 and C2 at a given STAT5 concentration is higher when the FcyRI STAT consensus site is combined with an intact site II (probe F/II). Thus, F/II, I/II, and I/II probes, but not the short F probe, induce STAT tetramerization, indicating that binding of STAT5 to a single specific binding site induces the formation of a STAT5 tetramer, provided that the probe contains sufficient flanking DNA.

STAT5 tetramers are expected to dissociate with a lower off-rate than dimers, as has indeed been demonstrated for STAT1 (34). This explains why the only complex detected in bandshifts with probes containing a single very weak binding site (−/II) is C1, whereas probes with a single high affinity binding site form predominantly complex C2. As expected, C1 complex formation is enhanced by the inclusion of two STAT5 binding sites in the same probe (I/II, F/II), resulting in a synergistic increase of STAT binding to such probes. Thus, in the presence of 1–2 μg of STAT5α, the total amount of STAT5α bound to probe I/II is 2.5–3 times higher than the sum of protein bound to probe I/− and to probe −/II.

Tetramer Formation Depends on Specific STAT5 Protein Segments—Formation of STAT4 and STAT1 tetramers depends on their N termini (33, 34). To determine whether STAT5 tetramer formation also requires interaction between specific protein domains, we investigated the effect of proteolytic clipping on the capacity of STAT5α to form tetrameric complexes. Treatment of STAT5α with thermolysin yields two proteolytic fragments with apparent molecular masses of about 75 and 60 kDa (Fig. 5A). Western blotting with an antibody against the C-terminal domain of STAT5α revealed that both proteolytic fragments lack the C terminus (data not shown). N-terminal amino acid sequencing of the 60-kDa fragment yielded the sequence ILVDAMSQK, indicating that this clipping product starts at amino acid 130. The molecular mass of this fragment is consistent with the absence of both N- and C-terminal domains of STAT5α. No N-terminal sequence could be obtained from the 75-kDa fragment. Its molecular mass suggests that this fragment still retains the N terminus, which is likely blocked.

We compared the complexes formed by full-length STAT5α and by the proteolytic digests with the IL-2Rα probe I/II and the short FcyRI probe F (Fig. 5B). As described above (Fig. 4A), intact STAT5α predominantly forms the slower migrating complex C1 with the probe I/II (in this experiment C2 is barely visible), whereas probe F gives rise to the faster migrating complex C2. The proteolytic fragments, on the other hand, form with either probe a complex C2* that migrates faster than C2. A small amount of an additional complex C1* is observed with probe I/II but not with probe F. These data suggest that complex C2* is formed by the 60-kDa fragment that lacks the domain necessary for protein-protein interaction, whereas C1* is due to the 75-kDa fragment that is still capable of forming tetramers. Thus, as observed for STAT1, proteolytic cleavage of STAT5 strongly reduced its propensity to form trimolecular complexes with a probe containing two weak binding sites, most likely because proteolysis has removed the N-terminal domain required for tetramerization. Alternatively, the small amount of the putative trimolecular complex C1* could be due
to simultaneous occupation of both STAT5 binding sites in probe I/II at high protein concentrations, even in the absence of protein-protein interactions. In either case, our data indicate that, as reported for STAT4 and STAT1, the capacity of STAT5 to cooperatively bind to a probe containing two binding sites depends on interactions involving modular protein domains.

We also investigated the effect of deoxycholate, which is known to disrupt protein-protein interactions (48), on the formation of C1 and C2 complexes. Fig. 6 shows that 0.25% DOC is required to substantially reduce formation of C2 complexes with the wild type IL-2rE probe as well as the high affinity, single site FcγRI probe. Formation of C1 complexes, on the other hand, is almost completely prevented by 0.06% DOC and severely reduced by 0.03% detergent (data not shown). This is further evidence that C1 complexes depend on an interaction between the bound STAT5 dimers and are not simply due to occupancy of both IL-2rE binding sites by independently binding STAT5 molecules.

**IL-2rE Activity Depends on Intact STAT5 Motifs in Sites I and II**—Previously we defined the limits of site I and of site II by substitution mutations. The finding that they coincided to within one nucleotide with the borders of STAT motifs provided the first evidence suggesting an involvement of STAT proteins in IL-2rE function (19). The binding site for STAT5 in site II overlaps with a binding motif for GATA factors. The previously described mutants did not exclude that the contribution of site
II to IL-2rE activity depends on a GATA protein. Fig. 7 shows that a mutation in site II that leaves the GATA binding motif intact but destroys the STAT site (mutant S-11.1) abolishes enhancer activity. This indicates that the binding of STAT5 to site II is required. In addition, a mutation that leaves the STAT motif intact and destroys the GATA motif (mutant S-11.2) conserves IL-2rE activity. In fact, this mutation, which transforms site II into a copy of site I, gives an enhancer that responds slightly better than the wild type IL-2rE. Probably this reflects the stronger affinity of STAT5 for site I compared with site II.

The inverse change, resulting in a replacement of site I with another copy of site II (S-1.1) abolishes IL-2 responsiveness and greatly diminishes STAT5 binding (data not shown), indicating that two copies of site II do not bind STAT5 with sufficient affinity to allow the enhancer to function.

The Topological Relationship between Sites I and II Affects STAT5 Binding and IL-2rE Function—The center-to-center distance between the STAT binding motifs in sites I and II is 20 bp or two turns of the DNA helix in both man and mouse (25, 26). Thus, the STAT molecules binding to the two sites face the same side of the helix, and it appeared likely that tetramer formation depends on this topological relationship between the interacting STAT5 molecules. To test this, we changed the spacing between the two sites by inserting 5 or 10 bp (Fig. 7A) and compared the complexes that such probes formed with STAT5 with those formed by the wild type probe. The addition of 5 bp or half a helical turn (mutant 1-5-II) clearly reduces the formation of complex C1 but has a lesser effect on complex C2 (Fig. 7B). PhosphorImager analysis showed that probe I-5-II binds approximately half the number of STAT5a molecules as the wild type probe. This mutation reduces the IL-2 response of the enhancer from 8- to 3.5-fold (Fig. 7C). Insertion of 10 bp (I-10-II) or one helical turn has no significant effect on either STAT5 complex formation or enhancer response. These results suggest that the rotational orientation of the STAT5 molecules

with regard to the helical axis of the DNA is important for an efficient association of the STAT5 dimers into tetramers and thereby affects IL-2rE enhancer activity.

DISCUSSION

Previously we showed that the response of the IL-2Ra gene to IL-2 is mediated by a 51-nt IL-2-responsive enhancer (IL-2rE). The IL-2rE consists of three sites, I, II, and III, that functionally cooperate to activate transcription (19). The function of site III most likely depends on Elf-1, constitutively present in PC60 cells and normal T lymphocytes (Serdobova et al. (24)). This paper provides strong evidence that cooperative binding of IL-2-activated STAT5 to sites I and II plays a crucial role in IL-2rE activity.

Stimulation of IL-2Ra Gene Transcription by IL-2 Depends on STAT5 Activation—IL-2 stimulates IL-2rE transcription via binding of induced STAT5 to the IL-2rE. The onset of STAT5 activation is as rapid as expected for STAT factors, but the DNA binding activity of STAT5 continues to increase for 48 h. This is parallel with increasing IL-2Ra expression and IL-2rE-driven reporter gene activity (19). IL-2rE sites I and II bind STAT5a as well as STAT5b with a weak affinity. Their relative affinity for STAT5 in nuclear extracts is the same as that for recombinant STAT5a, indicating that no accessory proteins are required for the binding of the IL-2rE sites to STAT5.

Previous mapping of site II had left open the question of whether the contribution of this site was due to a STAT motif or to an overlapping GATA binding site. The data presented here show that the STAT motif in site II is required for enhancer activity and argue against a role of GATA factors.

The affinity of site II for STAT5 is approximately 5 times lower than that of site I. A point mutation that changes the STAT motif in site I to that in site II abolishes the IL-2 response of the IL-2rE. Together these results support a predominant role of STAT5 in the regulation of IL-2rE activity. As has been pointed out by others (49, 50), STAT binding motifs in natural regulatory elements are often sites with relatively low
affinity. In contrast to high affinity binding sites, low affinity sites preferentially bind a subset of STAT factors. This may contribute to the selective responsiveness of a gene to particular STAT proteins and cytokines.

**Similarities and Differences between Human and Mouse IL-2 Responsive Enhancers**—The human homologue of the mouse IL-2rE is located approximately 4 kilobase pairs upstream of the transcription start site (25–27). The two enhancers share the consensus motifs first identified in the mouse gene (19), and the spacing between these motifs is conserved. Partial functional analysis of the human IL-2rE results in a picture that is similar but not identical to that of the mouse IL-2rE (25, 26). The most striking difference between the human and mouse IL-2rE is a base pair inversion, which creates an additional Ets motif that overlaps with the STAT motif in site I in the human IL-2rE. The effect of different mutations in this site as well as cotransfection experiments with Elf-1 expression vectors suggest that the Ets motif in the human site I acts as a negative regulator of IL-2rE activity in unstimulated cells (25, 26).

Since STAT5 binds more avidly to the human homologue, such an element may be required to prevent inappropriate IL-2Rα expression.

**STAT5 Controls IL-2rE Activity by Cooperative Binding to Two Adjacent Sites**—Our experiments indicate that the functional cooperativity between the two weak STAT5 binding sites in the mouse IL-2rE depends on the DNA-induced interaction between two STAT5 dimers. Efficient formation of STAT5 tetramers appears to require that both STAT5 molecules bind to the same side of the DNA helix, since insertion of a half-turn between the two binding sites reduces association, whereas insertion of a complete turn does not result in a significant change. The correlation of these effects with those on IL-2 responsiveness of the IL-2rE strongly indicates that the pair of STAT motifs in the IL-2rE affects enhancer activity by inducing STAT5 association. This is the first evidence that STAT tetramer formation plays an important role in the function of a well-characterized cytokine-responsive enhancer. Thus, our results demonstrate that the phenomenon of STAT tetramerization that has been described by others for STAT4 and STAT1 (33, 34) is indeed biologically important.

It is very likely that cooperative binding of STAT5 to the two IL-2rE sites reflects a reduction in the off-rate of the DNA from tetramers compared with that from dimers, as has been demonstrated for STAT1 (34). Our data suggest that, like STAT4 and STAT1, STAT5 requires the N-terminal domain for tetramerization. But our results also point to differences between the interactions of different STAT dimers. Unlike STAT4, STAT5 does not form tetramers with short (20 nt) probes. However, STAT5 tetramers are formed on a 39-nt probe that contains a single cognate binding site between nt 6 and 15. This suggests that tetramerization depends on simultaneous contacts of both participating dimers with the same DNA molecule.

**Fig. 7. Effect of IL-2rE mutations on IL-2 responsiveness and STAT5 binding.** The effects of different mutations (A) on STAT5 binding (B) and the IL-2 inducibility of the IL-2rE (C) were investigated. A, analysis of DNA binding complexes formed by different IL-2rE probes spanning the segment between nt −1226 and −1420. In the last two lanes a 100-fold excess of competitor was included in the reaction. Competitors were: F, FcgRII STAT motif; NS, nonspecific oligonucleotide. C, 2.5-kilobase pairs of the mouse IL-2rA gene 5′-flanking region containing either the wild type or the indicated mutant form of the IL-2rE were fused to the rabbit β-globin gene. These plasmids were transiently transfected into PC60 cells together with a reference plasmid. The transfected cells were cultured either with or without IL-2. Two days later, the relative amounts of reporter gene mRNA were measured, and the IL-2 response of each plasmid was determined (see "Experimental Procedures"). Transfections were repeated at least twice with all constructs. For each mutant, the mean response ± S.D. is given. Shading of the histogram bars distinguishes different classes of mutations: black, wild type (wt) IL-2rE; white, point mutations; hatched, insertions between sites I and II.

4 P. Lécine and J. Imbert, personal communication.
but that nonspecific interactions are sufficient to induce association.

Unlike that of STAT5, STAT1 tetramerization does not appear to be significantly affected by the rotational orientation of the two STAT motifs on the DNA (34). Regardless of these differences, our data indicate that tetramerization of STAT proteins (33, 34) is a general mechanism through which these factors control transcription via weak pairs of binding sites, which have been observed in several other putative regulatory elements (50–53).

Tetramer formation may result in functional cooperativity between the two weak STAT5 binding sites simply by increasing the level of occupancy of sites I and II to a threshold required for enhancer activity. But protein–protein interaction between STAT5 molecules may also lead to the formation of a new molecular surface that binds a coactivator required for IL-2RE activity. If tetramer formation is restricted to certain STAT combinations, it may contribute to cytokine response specificity. For example, STAT1, which also binds to site I and site II, does not contribute to IL-2RA regulation, perhaps because it does not form transactivating tetramers. If tetramerization is required for binding to coactivators or basic transcription factors, it may serve as a mechanism to select particular mediators of transactivation. We are planning to explore these questions, taking advantage of the IL-2RA as a good model for a natural STAT-controlled transcriptional enhancer.

Acknowledgments—We are grateful to Glaxo, Geneva, for generous gifts of interleukins. Dereen Cantrell and Carol Beadling of the Imperial Cancer Research Fund provided advice and help in setting up the system for affinity precipitation of STAT proteins. Claudeine Ravussin, Pierre Dubied, and Marcel Allegri helped with the preparation of the figures. We thank Dean Scott for amino acid sequence analysis as well as Sonia Barange and Corinne Rusterholz for letting us quote their unpublished results. Michel Aguet, Stefan Ryter, and Corinne Rusterholz made many helpful suggestions concerning the manuscript.

REFERENCES

1. Coffman, R. L., Lehman, D. A., and Shadrer, B. (1989) J. Exp. Med. 170, 1039–1044
2. Waldmann, T. A. (1996) Immunol. Today 14, 264–270
3. Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M., and Sugamura, K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4127–4131
4. Taga, T., and Kishimoto, T. (1995) Curr. Opin. Immunol. 7, 17–23
5. Thézé, J., Alzari, P. M., and Bertoglio, J. (1996) Immunol. Today 17, 481–486
6. Nakarai, T., Robertson, M. J., Streuli, M., Wu, Z., Ciardelli, T. L., Smith, K. A., and Nabholz, M. (1997) J. Exp. Med. 180, 241–251
7. Kondo, M., Ohashi, T., Tada, K., Nakamura, M., and Sugamura, K. (1994) Eur. J. Immunol. 24, 2026–2030
8. Takeshita, T., Ohtani, T., Asao, H., Kumaki, S., Nakamura, M., and Sugamura, K. (1992) J. Immunol. 148, 2154–2158
9. Hatakeyama, M., Tsuuo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaki, M., and Taniguchi, T. (1989) Science 244, 551–556
10. Waldmann, T. A., White, J. D., Goldman, C. K., Top, L., Grant, A., Bamford, R., Roessler, E., Horak, I. D., Zaknoen, S., Kasten-Sportes, C., England, R., Horak, I. D., Zaknoen, S., Kasten-Sportes, C., England, R., and Nossal, G. J. V. (1989) J. Immunol. 143, 931–939
11. Nishi, M., Ishida, Y., and Honjo, T. (1988) Nature 331, 267–269
12. Kronke, M., Leonard, W. J., Depper, J. M., and Greene, W. C. (1985) J. Exp. Med. 161, 1593–1598