

Distinct Tyrosine Residues within the Interleukin-2 Receptor β Chain Drive Signal Transduction Specificity, Redundancy, and Diversity*

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To explore the basis for interleukin (IL)-2 receptor (IL-2R) signaling specificity, the roles of tyrosine-based sequences located within the cytoplasmic tails of the β and γ_c chains were examined in the murine helper T cell line HT-2. Activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, cellular proliferation, and the induction of various genes were monitored. All four of the cytoplasmic tyrosine residues as well as the distal portion of the γ_c proved dispensable for the entire spectrum of IL-2R signaling responses studied. Conversely, select tyrosine residues within the β chain were essential and differentially required for various signaling events. Specifically, activation of *c-fos* gene expression was found to occur exclusively through the most membrane proximal tyrosine, Tyr-338, whereas proliferation and the activation of STAT-5 were induced either through Tyr-338 or through the two C-terminal tyrosine residues, Tyr-392 and Tyr-510. These tyrosine residues mediated the induction of two different STAT-5 isoforms, which were found to form heterodimers upon receptor activation. In contrast to the tyrosine dependence of *c-fos* and STAT-5 induction, *bcl-2* gene induction proceeded independently of all IL-2R β tyrosine residues. Thus, the tyrosine-based modules present within the IL-2R β cytoplasmic tail play a critical role in IL-2R signaling, mediating specificity, redundancy, and multifunctionality.

A select group of cytokines is responsible for coordinating a diverse array of biologic responses including hematopoiesis, neurological development, and control of the immune system. Understanding the molecular mechanisms by which such a limited number of cytokines regulate a variety of cellular responses has remained a central goal in the field of signal transduction (1). Cytokines act by binding to specific cell surface receptors, many of which are members of a single receptor

superfamily (2). One striking feature of this cytokine receptor superfamily is the shared use of common receptor subunits to generate combinatorial diversity. For example, the interleukin (IL)-2 receptor is composed of three subunits, α , β , and γ (reviewed in Ref. 3). The β and γ chains are shared by the IL-15 receptor, while the γ subunit also participates in the formation of the IL-4, -7, and -9 receptors (thus termed γ_c for “ γ common”) (4–7). Since most immune cells express receptors for multiple cytokines, signal integrity must somehow be preserved despite the use of overlapping signal transduction systems.

Considerable evidence has emerged supporting the view that cytokine receptors are composed of a series of functional signaling “modules.” At the level of the entire multimeric receptor, each individual subunit may be considered a distinct module, potentially serving a specialized function within the overall receptor complex. Moreover, individual receptor subunits contain within their cytoplasmic domains combinations of functional peptide sequences that link the receptor to a distinct array of intracellular signaling pathways. For example, the particular sequence surrounding receptor tyrosine residues may impart specificity by permitting the docking of particular SH2- or PTB-containing proteins (8, 9) such as “signal transducer and activator of transcription” (STAT) proteins (10). Ultimately, the combinatorial effects of these modular domains help to generate an integrated signal from the receptor that is unique to each cytokine.

The IL-2/IL-2 receptor (IL-2R) system exhibits the hallmark pleiotropy of the cytokine family. Studies dissecting the functional role of the IL-2R have yielded valuable information pertaining to the general principles underlying cytokine signaling. First, chimeric receptors have been employed to demonstrate that heterodimerization of receptor subunits is critical for signal transduction (11–13). For example, when the extracellular domains of the β and γ_c chains are replaced with the erythropoietin (EPO) receptor extracellular domain and expressed in IL-2-dependent T cells, co-expression of these chimeric receptors is necessary to recapitulate IL-2 signaling in response to EPO (11, 14). Similar results have been obtained with other chimeric signaling systems (12, 13). Moreover, since the IL-15 receptor also contains the IL-2R β and γ_c chains (7), these chimeras presumably also reflect signaling mechanisms induced by this cytokine.

A second principle that applies generally to cytokine recep-

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¹ The abbreviations used are: IL, interleukin; IL-2R, interleukin-2 receptor; EPO, erythropoietin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; EMSA, electrophoretic mobility shift assay; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; kb, kilobase pair(s).

tors is the physical association of the receptor subunits with members of the Janus family of tyrosine kinases (JAKs, reviewed in Ref. 15). The IL-2R β and γ_c subunits bind to JAK1 and JAK3, respectively (16–19); upon heterodimerization of the receptor by ligand, both the JAKs and the receptor subunits themselves become tyrosine-phosphorylated (14, 20, 21). JAK1 and JAK3 phosphorylation is associated with an increase in their enzymatic activities (15), while phosphorylation of the IL-2R subunits creates binding sites for cytoplasmic effector molecules. For example, the signaling adapter Shc has been shown to associate with the IL-2R β chain through a single tyrosine residue, Tyr-338 (22). In addition, phosphorylation and nuclear transport of the STAT factor STAT-5 is dependent upon tyrosine residues located in the IL-2R β chain (23–28).

Although heterodimerization of β and γ_c is necessary for IL-2R signaling, studies examining the relative contributions of the individual subunits have suggested that the γ_c subunit may play a more limited role in this signaling cascade. For example, all four of the tyrosine residues of γ_c are dispensable for both proliferation and STAT-5 activation (11, 23). Further recent studies have revealed that the entire γ_c chain and its associated JAK3 molecule can be replaced by a heterologous receptor that binds JAK2 without apparent disruption of IL-2-specific signaling (14). These observations suggest that β and γ_c contribute quite differently to the IL-2 signaling program, with γ_c acting mainly to trigger the signaling cascade, while β serves to drive signaling specificity. However, it is possible that some as yet unstudied downstream signaling events might be more dependent on the γ_c chain.

In the present report, the participation of the γ_c and β chains in determining specificity in both early and downstream IL-2 signaling pathways has been defined in detail. In particular, these studies assigned the molecular basis of signal transduction specificity largely to individual tyrosine-containing peptides within the cytoplasmic portion of the IL-2 receptor β chain. The findings strongly support a separation of function between IL-2R β and γ_c within the receptor complex, and reveal that the tyrosine residues of IL-2R β exhibit a high degree of specificity as well as multifunctionality in coupling to various signaling pathways.

MATERIALS AND METHODS

Cell Culture—The cell line HT-2, an IL-2-dependent murine helper T cell line (ATCC) was cultured in RPMI, 10% fetal bovine serum, antibiotics, and either 1 nM human IL-2 (Chiron) or 5 units/ml erythropoietin (EPO, Amgen) as described previously (11). HT-2EPO β -containing cell lines were generated by transfecting HT-2EPO γ cells by electroporation and selecting stable transfectants by limiting dilution either in hygromycin B (Boehringer Mannheim) and G418 (Life Technologies, Inc.) as described previously (29) (HT-2EPO β YF:1Y, HT-2EPO β YF:234Y, HT-2EPO β YF:5Y, HT-2EPO β YF:6Y cell lines) or in 5 units/ml EPO (HT-2EPO β YF:1Y, EPO β YF:5Y, EPO β YF:6Y, HT-2EPO β YF:56Y, HT-2EPO β YF:1234Y cell lines). No significant phenotypic differences were observed between cell lines generated by selection in G418/hygromycin B versus EPO.²

Cytokine Stimulations—For immunoprecipitations and nuclear extract preparation, cells were incubated for 2–4 h in RPMI with antibiotic and 1% bovine serum albumin (fraction V, Sigma) without growth factor as described previously (23). For RNA analyses, cells were incubated for 15 h in RPMI with antibiotic and 10% fetal bovine serum (Life Technologies, Inc.) in the absence of growth factor. Cells were stimulated for the indicated time intervals in 5–20 ml of the appropriate medium plus growth factor: IL-2 (10 nM), murine IL-4, (100 units/ml, Genzyme), human EPO (50–100 units/ml).

Plasmid Constructs—All receptor cDNAs were subcloned into the expression vectors pCMV4 or pCMV4neo (29). Tyrosine substitution mutants in the IL-2R β chain were created as described previously (29). These mutations were transferred into the pEPO β neo backbone by

replacing the *Afl*II/*Bam*HI fragment within the cytoplasmic tail of EPO β with an equivalent fragment containing the β YF:1Y, β YF:234Y, β YF:5Y or β YF:6Y, β YF:56Y, and β YF:1234Y (formerly β YF:56F) (11) mutations. Mutations were confirmed by sequencing. The pME18 s-STAT-5A and pME18s-STAT-5B constructs were generously provided by Dr. A. Mui (30).

RNA Preparation and Northern Blot Analysis—Cytoplasmic RNA was prepared from $1-2 \times 10^7$ cells using an RNeasy mRNA kit (Qiagen) and quantified by UV spectrophotometry. Denaturing gels were run with 10 μ g of RNA/lane and blotted to Zeta Probe membranes (Bio-Rad) as described elsewhere (31). Hybridization was at 42 °C for 12–16 h. Probes were random prime-labeled using a Megaprime labeling kit (Amersham Corp.) and [α -³²P]dATP and [α -³²P]dCTP (Amersham Corp.). The glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA was from ATCC (32), the *c-fos* cDNA was provided by Dr. I. Verma, and the *bcl-2* cDNA was provided by Dr. S. Korsmeyer. DNA fragments used to label probes were as follows: a 1.2-kb *Hind*III/*Kpn*I fragment of the extracellular murine EPO receptor, a 2.1-kb *Eco*RI murine *c-fos* cDNA fragment from pMc-fos (33), a 0.9-kb *Pst*I murine *bcl-2* fragment from pBS-bcl-2 (34), and a 1.0-kb *Eco*RI fragment from GAPD.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extractions and EMSA were performed using the FcyRI oligonucleotide as described previously (23, 35). Antibody supershifts were performed by preincubating nuclear extracts with 1 μ l of STAT-5A or STAT-5B antisera (see below), 3 μ g of 4G10 (anti-phosphotyrosine), or 3 μ g of MOPC195 (IgG2b control) antibodies for 30 min on ice prior to the binding reaction.

Protein Expression in COS-7 Cells—COS-7 cells (ATCC) were transfected with the indicated plasmids using Lipofectamine (Life Technologies, Inc.) per the manufacturer's instructions. Nuclear extracts were prepared from 1–2 million transfected cells as described previously (35).

Immunoprecipitations—Cells were lysed (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 8.0, 50 mM NaF, 100 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A) and immunoprecipitated with 1 μ l of anti-STAT-5A, anti-STAT-5B, or anti-JAK1 antiserum (Upstate Biotechnology, Inc.) per 20–30 million cells using protein A-agarose (Boehringer Mannheim). Immunoblotting studies were performed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) or anti-STAT-5A or anti-STAT-5B followed by enhanced chemiluminescence (ECL) (Amersham Corp.) signal development.

Proliferation Assays—Conventional 48-h [³H]thymidine incorporation assays and transient proliferation assays were performed as described previously (29). Data are expressed as a percentage of [³H]thymidine incorporation of cells treated with 10 nM IL-2.

Antibodies—Anti-STAT-5A and anti-STAT-5B antibodies were generated in rabbits against the C-terminal peptides specific for mouse STAT-5A (LDARLSPAGLFTSARSSLS) or mouse STAT-5B (MDSQWIPHAQS), and were used 1:50,000 in Western blotting and 1 μ l/500 μ g in immunoprecipitations.

RESULTS

Role of the IL-2R β and γ_c Tyrosine Residues in Downstream Signaling Events

Proliferation Signaling—All facets of IL-2R signaling examined thus far appear to require heterodimerization of the IL-2R β and γ_c chains; however, the relative contributions of these individual subunits to specific signaling processes may be distinct. In order to explore the functions of these chains in an IL-2-responsive cellular environment, this laboratory has developed a chimeric receptor system in which the extracellular domain of the EPO receptor (EPOR) is fused to the cytoplasmic domains of the IL-2R β and γ_c chains, thus forming EPO β and EPO γ (Fig. 1A). When expressed in IL-2-dependent helper HT-2 cells, EPO stimulation recapitulates all IL-2-mediated signaling events so far examined (11, 14, 23). In the present studies, a series of mutant tyrosine constructs were created to delineate the role of individual IL-2R β tyrosine residues in IL-2R signaling (Fig. 1A). These constructs were introduced into HT-2EPO γ cells (11), and receptor gene expression in the stable transfectants was assessed by RNA blotting analysis to identify positive clones for further study. Relative levels of EPO β and EPO γ mRNA were approximately equivalent in all clones, indicative of similar expression levels of receptors (data

² S. L. Gaffen, unpublished observations.

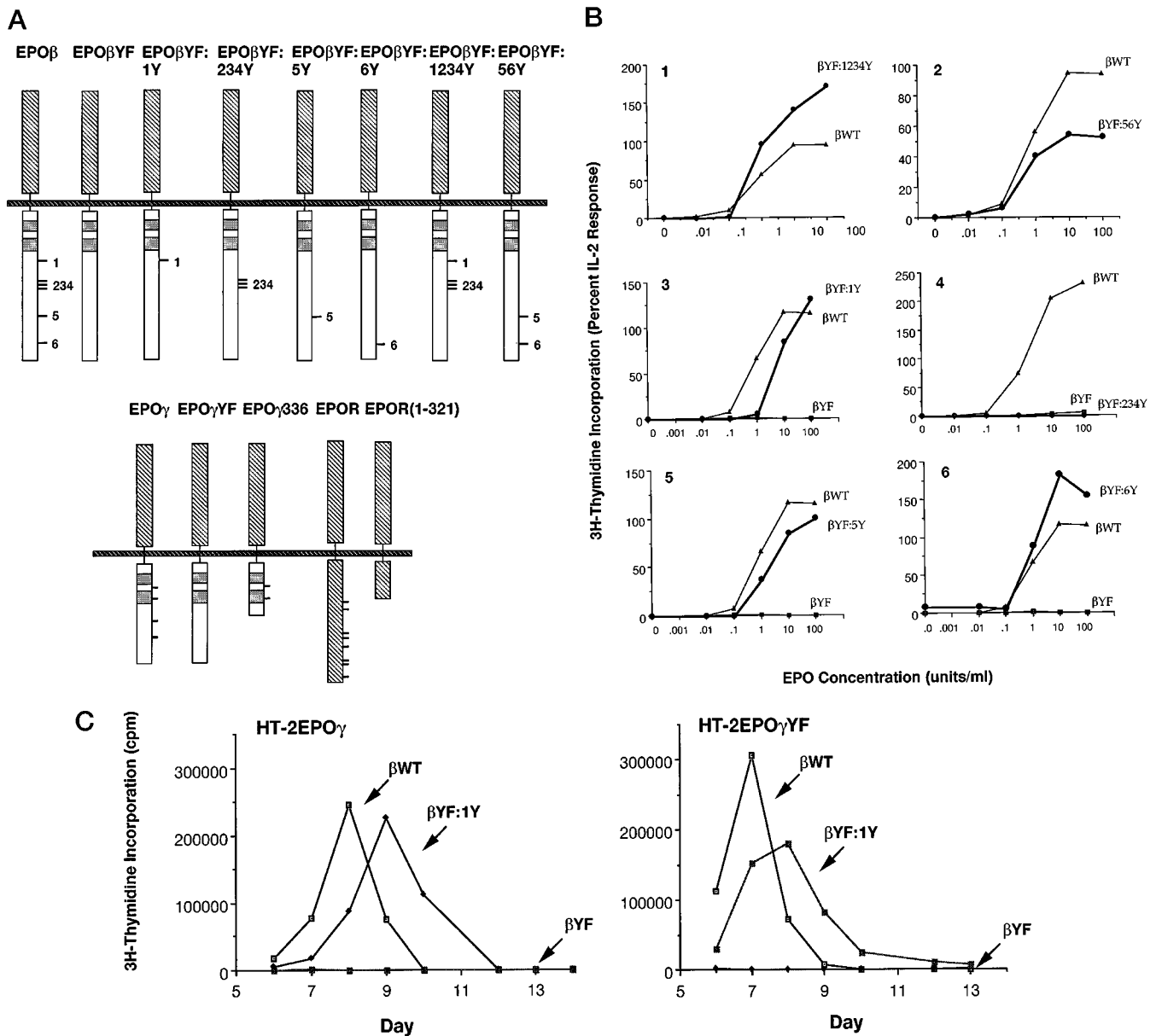


FIG. 1. *A*, diagrams of chimeric receptor constructs. *Hatched boxes* represent EPO receptor sequence, and *open boxes* represent IL-2R sequences. *Shaded boxes* within IL-2R sequences correspond to conserved Box1 and Box2 regions (53). Tyrosine residues are indicated by *horizontal bars* and are numbered as follows: Y1 = Tyr-338, Y2 = Tyr-355, Y3 = Tyr-358, Y4 = Tyr-361, Y5 = Tyr-392, and Y6 = Tyr-510. *B*, multiple tyrosine residues of the IL-2R β chain can support proliferation signaling. HT-2EPO β γ , HT-2EPO β YF/ γ , HT-2EPO β YF:1Y/ γ , HT-2EPO β YF:234Y/ γ , HT-2EPO β YF:5Y/ γ , HT-2EPO β YF:6Y/ γ , HT-2EPO β YF:1234Y/ γ , and HT-2EPO β YF:56Y/ γ cells were stimulated for 48 h with EPO at the indicated concentrations. Cells were pulsed with [3 H]thymidine for the final 4 h of the culture and harvested. Results are expressed relative to the level of incorporation occurring with stimulation in 10 nM IL-2 (100%). Each data point is the mean of triplicates, and is representative of three experiments. *C*, a single tyrosine residue within IL-2R β is sufficient for growth signaling in the absence of γ_c tyrosine residues. HT-2EPO γ (*left panel*) or HT-2EPO γ YF (*right panel*) cells were transiently transfected with the EPO β YF, EPO β YF:1Y, or EPO β (β WT) constructs as described previously (29), and [3 H]thymidine incorporation was measured at the indicated time points after transfection. Each data point is the mean of triplicates, and is representative of four experiments.

not shown). As observed previously (11), the wild type HT-2EPO β γ cells (β WT) exhibited a vigorous proliferative dose response to EPO (Fig. 1*B*, panels 1–6), whereas the HT-2EPO β YF/ γ cells failed to proliferate (Fig. 1*B*, panels 3–6). HT-2 cell lines expressing either the two most distal tyrosines of β (EPO β YF:56Y/ γ , panel 2) or the first four membrane-proximal tyrosines of β (EPO β YF:1234Y/ γ , panel 1) both showed a strong proliferative response to EPO and could support long term growth in EPO. Moreover, certain cell lines expressing a single β tyrosine residue, EPO β YF:1Y/ γ , EPO β YF:5Y/ γ , or EPO β YF:6Y/ γ , supported proliferation and long term growth comparably to those expressing wild type

EPO β γ (panels 3, 5, and 6). Finally, cells expressing EPO β YF:234Y/ γ did not proliferate significantly or support long term growth in EPO (Fig. 1*B*, panel 4). It is not clear whether or not the minor variations in proliferation among the EPO β γ , EPO β YF:1Y, EPO β YF:5Y, and EPO β YF:6Y cell lines represent important differences, and we do not attempt to draw quantitative conclusions from these data. In conclusion, these experiments indicate that HT-2 cellular proliferation depends on select tyrosines within the IL-2R β chain, namely 1Y, 5Y, or 6Y, which are functionally independent of one another. Moreover, the specific tyrosines capable of supporting proliferation only partially overlap with those found to be important in a pro-B

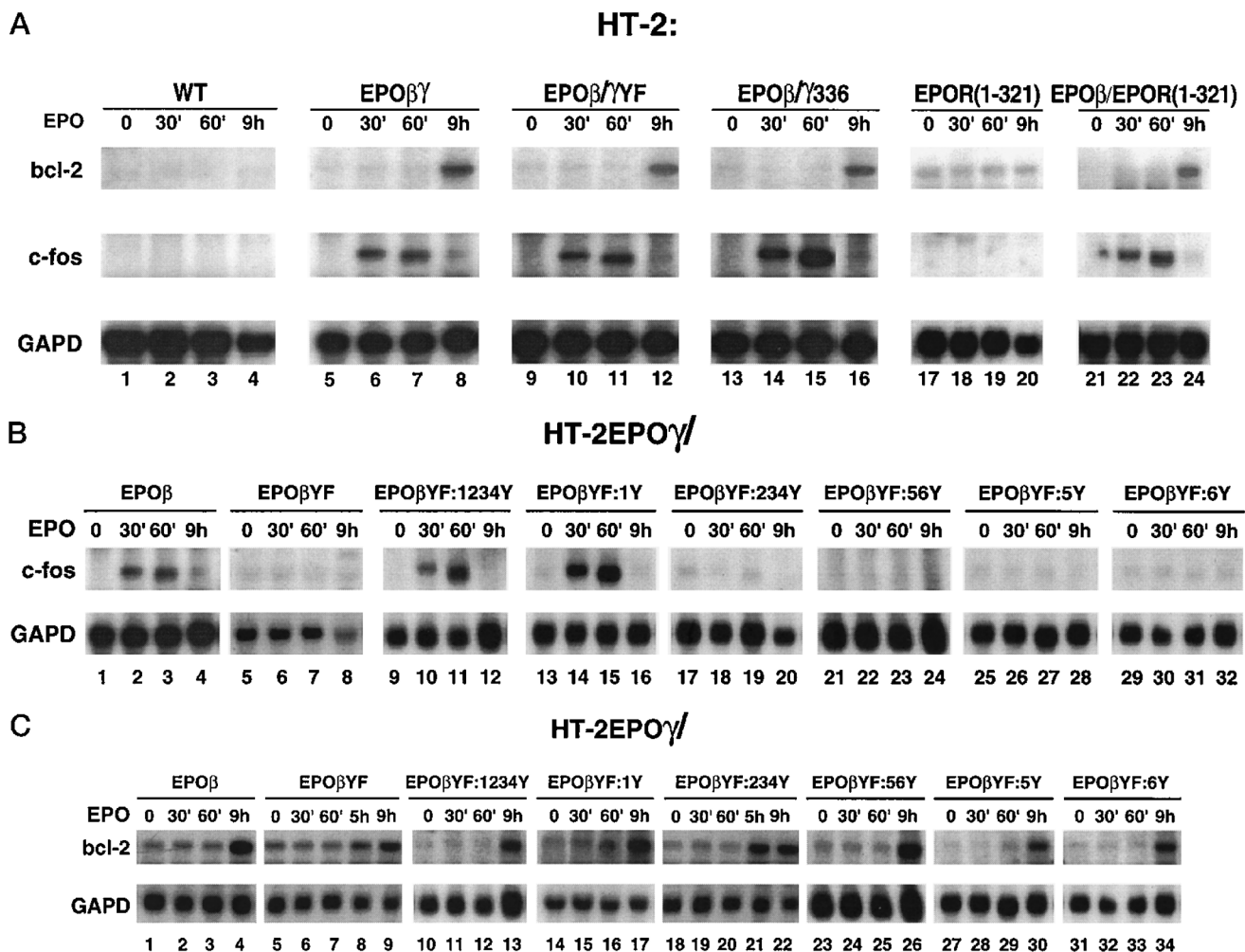


FIG. 2. A, the γ_c chain is not required to specify activation of the *bcl-2* and *c-fos* proto-oncogenes. HT-2, HT-2EPO $\beta\gamma$, HT-2EPO $\beta\gamma$ YF, HT-2EPO $\beta\gamma$ 336, HT-2EPOR(1-321), and HT-2EPO β /EPOR(1-321) cells were incubated in media without growth factor for 15 h and stimulated with 50 units/ml EPO for 0, 30, 60 min or 9 h, as indicated. Total cellular RNA was prepared, separated by Northern blotting, and probed with *bcl-2*, *c-fos* or GAPD cDNA probes. B, a single tyrosine residue of the IL-2R β chain is necessary and sufficient for induction of *c-fos* gene expression. HT-2EPO $\beta\gamma$, HT-2EPO β YF/ γ , HT-2EPO β YF:1234Y/ γ , HT-2EPO β YF:1Y/ γ , HT-2EPO β YF:234Y/ γ , HT-2EPO β YF:56Y/ γ , HT-2EPO β YF:5Y/ γ , and HT-2EPO β YF:6Y/ γ cells were stimulated with EPO as described in A. Total cellular RNA was separated by Northern blotting, and probed with *c-fos* and GAPD cDNA probes. C, induction of *bcl-2* gene expression occurs via a tyrosine-independent pathway. HT-2EPO $\beta\gamma$, HT-2EPO β YF/ γ , HT-2EPO β YF:1234Y/ γ , HT-2EPO β YF:1Y/ γ , HT-2EPO β YF:234Y/ γ , HT-2EPO β YF:56Y/ γ , HT-2EPO β YF:5Y/ γ , and HT-2EPO β YF:6Y/ γ cells were stimulated with EPO as described in A. Total cellular RNA was prepared, separated by Northern blotting, and probed with *bcl-2* and GAPD cDNA probes.

cell line, Ba/F3 (11), indicating that IL-2 signaling in T cells may differ in certain ways from that in pro-B cells (see "Discussion").

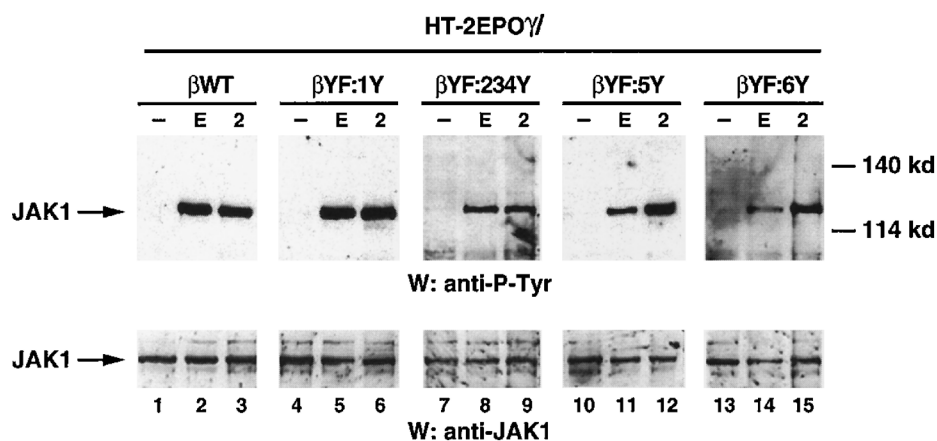
Prior studies have shown that the EPO β / γ YF-expressing cell lines proliferate in response to EPO equivalently to cells expressing wild type EPO $\beta\gamma$ receptors (11). Combined with the data presented above (Fig. 1B), it appeared that a single tyrosine residue of the IL-2R β chain was sufficient to support growth signaling. However, it remained formally possible that tyrosines of the γ_c chain could play a compensatory role in the context of a mutant β chain containing only a single tyrosine residue. Therefore, the ability of the IL-2R β tyrosine mutants to proliferate when paired with a γ_c chain lacking all of its cytoplasmic tyrosine residues (EPO γ YF) was assessed. As shown in Fig. 1C, both EPO β and EPO β YF:1Y induced proliferation equally well when transiently transfected into HT-2EPO γ cells and HT-2EPO γ YF cells, whereas EPO β YF failed to mediate the proliferative response in either case. Similar results were obtained for the EPO β YF:5Y and the EPO β YF:6Y (data not shown). Therefore, a single tyrosine residue in the β chain of the IL-2 receptor complex is both necessary and suffi-

cient to support proliferation signaling in HT-2 cells.

Induction of Gene Expression—A second important outcome of IL-2R signaling is the induced expression of a number of specific genes (36, 37). To assess the relative roles of the β and γ_c subunits in gene induction, cells expressing various combinations of mutant receptors were stimulated for varying lengths of time. Cytoplasmic RNA was analyzed by Northern blotting with probes to the *c-fos* and *bcl-2* proto-oncogenes (33, 34). As shown in Fig. 2A, EPO stimulation of HT-2EPO $\beta\gamma$ cells induced expression of these genes with reproducible and characteristic time courses (lanes 5–8), whereas EPO stimulation of HT-2 cells failed to induce these genes (lanes 1–4). To assess the role of the γ_c tyrosine residues and distal tail in induction of these genes, HT-2EPO β / γ YF or HT-2EPO β / γ 336 cells were examined. These cell lines were found to induce transcription of both *c-fos* and *bcl-2* identically to the wild type controls (Fig. 2A, lanes 9–16). Thus, neither the tyrosine residues nor the distal 35 amino acids of the γ_c chain appear necessary for the induction of *c-fos* or *bcl-2* in this cellular context.

To determine whether any other portion of the γ_c chain was required for the induction of these genes, EPO γ was replaced

FIG. 3. IL-2-induced JAK1 phosphorylation occurs independently of IL-2R β tyrosine residues. HT-2EPO β γ , HT-2EPO β YF:1Y/ γ , HT-2EPO β YF:234Y/ γ , HT-2EPO β YF:5Y/ γ , and HT-2EPO β YF:6Y/ γ cells were incubated in the absence of growth factor for 4 h and stimulated for 15 min with 50 units/ml EPO (E) or 10 nM IL-2 (2). JAK1 immunoprecipitates were prepared, immunoblotted with anti-phosphotyrosine (4G10) antibodies (*top*) or stripped and reprobed with anti-JAK1 antibodies (*bottom*), and visualized by ECL. Arrows indicate JAK1. Migration of molecular weight markers is indicated in *top panel*.



with a truncated version of the erythropoietin receptor, EPOR(1–321), that contains no tyrosine residues (14). Stimulation of cells expressing EPOR(1–321) alone failed to induce transcription of the *c-fos* or *bcl-2* genes (Fig. 2A, lanes 17–20). In contrast, co-expression of EPOR(1–321) with a wild type EPO β chain induced expression of these genes that was indistinguishable from cells expressing wild type EPO γ (lanes 21–24). Thus, γ_c can be replaced entirely with a heterologous receptor subunit with no apparent change in downstream activation of a variety of IL-2-induced genes. These data further indicate that JAK3, which binds to γ_c (16, 17), can be replaced by JAK2, which binds to the EPOR (38), without altering signaling specificity from the IL-2 receptor.

Since the tyrosine residues within the γ_c chain appeared to provide no detectable specificity to the IL-2 signaling program, the role of the six tyrosine residues within the cytoplasmic tail of the IL-2R β chain was examined. HT-2EPO γ cells expressing the EPO β tyrosine addback constructs (Fig. 1A) were stimulated, and Northern blots were prepared. As illustrated in Fig. 2B, *c-fos* induction was dependent on tyrosine-containing sequences in the β chain, since HT-2EPO β YF/ γ cells failed to induce these genes in response to receptor activation (Fig. 2B, lanes 5–8). Indeed, *c-fos* transcription was linked to a single tyrosine residue, as only EPO β constructs that retain the most membrane proximal tyrosine, Y1 (Tyr-338), supported its transcriptional induction (Fig. 2B, lanes 9–16). Thus, a single tyrosine residue within IL-2R β is critical for the induction of *c-fos*. These data further indicate that the mechanism of *c-fos* induction is likely to occur via pathways linked to Shc, an adapter molecule we have previously shown to be exclusively engaged by the first tyrosine (Tyr-338) of the IL-2R β chain (22).

In contrast to the regulation of *c-fos*, the induced expression of the *bcl-2* proto-oncogene was found to be independent of tyrosine residues. All β mutants examined, including a mutant lacking all six cytoplasmic tyrosine residues (HT-2EPO β YF), induced *bcl-2* transcription in response to ligand (Fig. 2C). Thus, at least one downstream signaling event is independent of tyrosine residues within the IL-2 receptor, while others are governed by individual tyrosines.

Role of the IL-2R β Tyrosine Residues in Proximal Signaling Events

JAK1 Activation—Proliferation and gene induction are both relatively late events in signaling through the IL-2R. One of the best characterized signaling pathways initiated rapidly upon receptor ligation is the phosphorylation of the Janus kinases JAK1 and JAK3 (18, 19), followed by the activation of STAT factors (23–28, 39). To assess the role of tyrosine residues within the IL-2R β chain in Janus kinase activation, cells expressing tyrosine mutant EPO β chains were stimulated with

EPO or IL-2 as a positive control, and JAK1 immunoprecipitates were prepared for immunoblotting with 4G10 anti-phosphotyrosine antibodies. As shown in Fig. 3, stimulation of all of the EPO β tyrosine mutant cell lines led to phosphorylation of JAK1 (*top panel*). The blots were stripped and reprobed with anti-JAK1 antibodies to ensure equivalent loading of the gels (*bottom panel*). These results were consistent with the previous demonstration that JAK1 and JAK3 activation is independent of IL-2R β tyrosine residues (data not shown) (11). The slight differences in relative JAK1 induction among cell lines may reflect variations in either endogenous JAK1 levels and/or receptor expression. However, it should be noted that these results serve as independent confirmation that each of the mutant tyrosine receptors is expressed at the cell surface and is capable of initiating signaling.

STAT-5 Activation—One consequence of Janus kinase activation in the IL-2 receptor system is the phosphorylation, nuclear import, and DNA binding activity of several STAT factors, notably factors related to STAT-5 (23–28, 40). In particular, stimulation of either native or chimeric IL-2 receptors expressed on HT-2 cells induces a nuclear DNA binding complex that is recognized by antibodies raised against sheep STAT-5/MGF (23). We examined the nature of the DNA binding complex in more detail than has been previously described. In particular, two murine isoforms of STAT-5 (STAT-5A and STAT-5B) have been identified recently that differ at their C termini from the originally identified sheep STAT-5/MGF (30, 41). Antibodies were raised against the unique, C-terminal portions of STAT-5A and STAT-5B. These antisera exhibited no detectable cross-reactivity by immunoblotting of cell lysates from COS-7 cells expressing either STAT-5A or STAT-5B alone (Fig. 4A). To evaluate the specificity of these antibodies in EMSA, either STAT-5A or STAT-5B was expressed with JAK1 in COS7 cells to permit constitutive, receptor-independent phosphorylation of the STATs in an overexpression system. Nuclear extracts were prepared from transfected COS7 cells, and an EMSA was performed using an oligonucleotide corresponding to the Fc γ RI STAT response element (39) as described previously (23). JAK1 overexpression in COS7 cells resulted in a DNA binding complex that represents endogenous STAT-1 (Fig. 4B, lane 4, and data not shown). However, when JAK1 was co-expressed with STAT-5A or STAT-5B, DNA binding complexes were observed that co-migrated with STAT-5. Importantly, addition of anti-STAT-5A and -5B antisera to the EMSA reaction caused selective supershifting of their respective DNA binding complexes, as demonstrated by the appearance of a slower migrating complex present at the top of the gel (Fig. 4B, lanes 9, 10, 15, 16, 19, and 20), and these supershifted bands were accompanied by a corresponding diminution of the original DNA binding complexes. The anti-STAT-5A antiserum

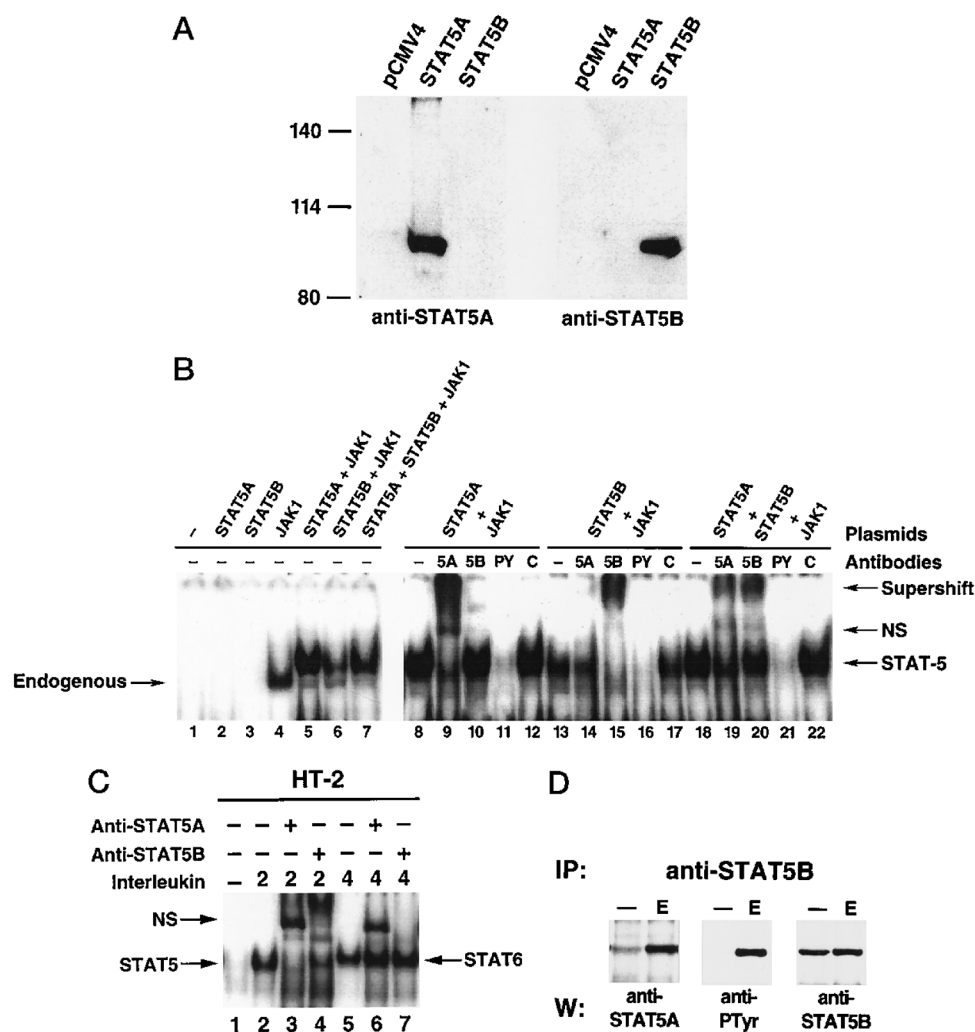


Fig. 4. *A*, anti-STAT-5A and anti-STAT-5B antibodies are specific in immunoblot analysis. Vectors containing STAT-5A, STAT-5B and a control vector (*pCMV4*) were transfected alone in COS7 cells, and cellular lysates prepared 48 h post-transfection. Lysates were separated by 8.75% SDS-polyacrylamide gel electrophoresis and immunoblotted with either STAT-5A or STAT-5B antisera, as indicated. Blots were visualized by ECL. Migration of molecular weight markers is shown. *B*, anti-STAT-5A and anti-STAT-5B antibodies exhibit specific supershifting in EMSA. COS-7 cells were transfected with the *pCMV4* (lane 1), *pCMV4*-JAK1 (lanes 4–22), *pME18s*-STAT-5A (lanes 2, 5, 7, 8–12, and 18–22) or *pME18s*-STAT-5B (lanes 3, 6, 7, and 13–22) vectors. Nuclear extracts were prepared 48 h post-transfection, and EMSA was performed as described under “Materials and Methods.” The specific probe employed was 32 P-end-labeled Fc γ RI STAT-response element (GTATTTCCAGAAAAAGGAC) (39). Nuclear extracts were incubated on ice with 1 μ l of anti-STAT-5A (5A, lanes 9, 14, and 19), 1 μ l of anti-STAT-5B (5B, lanes 10, 15, and 20), 3 μ g of 4G10 (PY, lanes 11, 16, and 21), or 3 μ g of MOPC195 IgG2b control antibodies (C, lanes 12, 17, and 22) for 30 min prior to EMSA. The top arrow indicates supershifted bands, and the bottom arrow indicates STAT-5 DNA binding complexes. Endogenous represents a STAT-1 DNA binding activity that appears constitutively in the presence of JAK1 overexpression (data not shown). *C*, IL-2R signaling induces both STAT-5A and STAT-5B in HT-2 cells. HT-2 cells were incubated in the absence of IL-2 for 2 h and stimulated for 15 min with 10 nM IL-2 (2) (lanes 2–4) or 100 u/ml murine IL-4 (4) (lanes 5–7), and nuclear extracts were prepared as described in *B*. Nuclear extracts were incubated with 1 μ l of anti-STAT-5A (lanes 3 and 6) or anti-STAT-5B (lanes 4 and 7) for 45 min on ice prior to EMSA. Arrows indicate STAT-5 and STAT-6; NS, nonspecific band. *D*, IL-2 induces heterodimerization of STAT-5A and STAT-5B in HT-2 cells. HT-2EPO β cells were stimulated with EPO as described in *C*, and cellular lysates were immunoprecipitated with 1 μ l anti-STAT-5B. Immunoprecipitates were separated by 8.75% polyacrylamide gel electrophoresis, immunoblotted with anti-STAT-5A, anti-phosphotyrosine (PTyr, 4G10), or anti-STAT-5B antibodies as indicated.

caused such a supershift only in extracts from STAT-5A transfectants, and the anti-STAT-5B antiserum caused a supershift only in extracts from STAT-5B transfectants. In addition, a nonspecific (NS) reactivity was present in the anti-STAT-5A and -5B antisera that was also found in preimmune sera (Fig. 5, lanes 5 and 6), but the bands representing specific supershifts are nonetheless diagnostic for the presence of STAT-5A or -5B.

In order to determine precisely which STAT-5 homologue(s) were induced by IL-2 in T cells, HT-2 cells were stimulated with IL-2 or IL-4 as a negative control and EMSA was performed as described in Fig. 4B. As shown in Fig. 4C, the IL-2-induced DNA binding complex reacted with both antisera specific for either STAT-5A or STAT-5B by causing a diminution in the DNA binding complex with a corresponding appear-

ance of a specific supershift at the top of the gel (Fig. 4C, lanes 3 and 4). These findings imply that the IL-2R mediates the induction of both STAT-5A and STAT-5B, consistent with previous observations in the IL-3R system (30). This reactivity was not observed with preimmune sera from the same animals (Fig. 5, lanes 5 and 6), nor were IL-4-induced DNA binding complexes containing STAT-6 affected in this way (Fig. 4C, lanes 6 and 7). The finding that virtually the entire DNA binding complex induced by IL-2 in HT-2 cells was competed with anti-STAT-5A antibodies suggested that all of the DNA binding complexes contained STAT-5A (Fig. 4C, lane 3). However, the fact that only a partial supershift of the complex was achieved with anti-STAT-5B (Fig. 4C, lane 4) argued that the nucleoprotein complexes represented a combination of STAT-5A homodimers and STAT-5A/STAT-5B heterodimers.

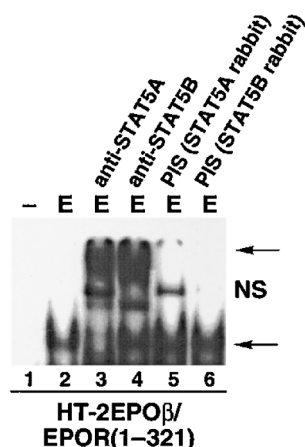


FIG. 5. The γ_c chain can be replaced with a heterologous receptor subunit in STAT-5A/B induction. HT-2EPO β /EPOR(1–321) cells were stimulated with EPO (E) and nuclear extracts subjected to EMSA as described in Fig. 4B. Nuclear extracts were also incubated with 1 μ l of specific preimmune serum (PIS) for 30 min on ice prior to EMSA (lanes 5 and 6). The top arrow indicates the supershifted band, and the bottom arrow indicates STAT-5. NS, nonspecific DNA binding complex.

To assess directly whether signaling through the IL-2R induces heterodimerization of STAT-5A and -5B, lysates from uninduced or EPO-induced HT-2EPO β γ cells were immunoprecipitated with anti-STAT-5B antibodies and immunoblotted with anti-STAT-5A antisera. As shown in Fig. 4D, the presence of STAT-5A was dramatically increased in anti-STAT-5B immunoprecipitates following induction of the IL-2R, indicating that STAT-5B and STAT-5A form heterodimers primarily after stimulation (panel 1). The low level of background bands seen in the uninduced cells may suggest that some STAT-5A and STAT-5B molecules are preassociated prior to receptor stimulation, but this association is significantly and reproducibly enhanced upon ligation of the receptor. Since dimerization of STAT factors is dependent on reciprocal SH2/phosphotyrosine interactions (39), the phosphorylation status of the STAT-5B immunoprecipitates was assessed by stripping the blot and re-probing with anti-phosphotyrosine antibodies. Indeed, STAT-5B was found to be phosphorylated only after EPO stimulation (Fig. 4D, panel 2). Finally, efficient immunoprecipitation of STAT-5B was confirmed by stripping and re-probing the blot with anti-STAT-5B (Fig. 4D, panel 3). Of course, these data do not rule out the possibility that STAT-5A and STAT-5B homodimers are also present in the DNA binding complexes in addition to the STAT-5A/B heterodimers. Nevertheless, IL-2R stimulation induces the DNA binding activities of both STAT-5A and STAT-5B, involving the formation of heterodimers (and possibly homodimers) *in vivo*.

Neither the Four Cytoplasmic Tyrosine Residues of γ_c nor JAK3 Are Required for STAT-5A/B Induction

Previous studies have demonstrated that a truncated EPO receptor (EPOR(1–321)) is capable of functionally substituting for EPO γ in the IL-2 receptor complex, even though it physically associates with and activates JAK2 rather than JAK3 (Fig. 2A) (14, 38). To determine whether the γ_c chain exerts any influence on the specific composition of the DNA binding complex induced by the IL-2R, supershift analyses were performed with nuclear extracts prepared from HT-2EPO β /EPOR(1–321) cells. Consistent with previous observations, STAT-5A and -5B were both significantly induced in HT-2EPO β /EPOR(1–321) cells (Fig. 5), indicating heterodimer formation. In addition, STAT-5A and STAT-5B heterodimers were also induced in HT-2EPO β / γ YF and HT-2EPO β / γ 336 cells (data not shown).

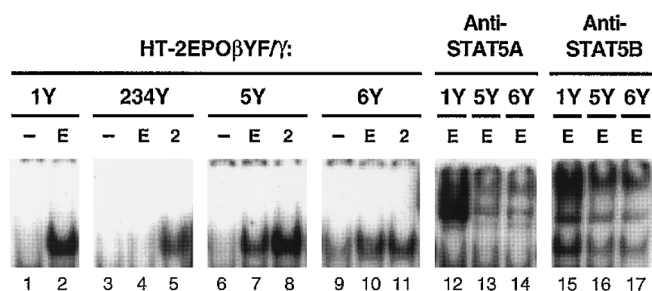


FIG. 6. Redundant tyrosine residues of the IL-2R β chain induce STAT-5A/B in HT-2 cells. HT-2EPO β YF:1Y/ γ , HT-2EPO β YF:234Y/ γ , HT-2EPO β YF:5Y/ γ , and HT-2EPO β YF:6Y/ γ cells were stimulated with 50 units/ml EPO (E) (lanes 2, 4, 7, 10, and 12–17) or 10 nM IL-2 (2) (lanes 5, 8, and 11) and nuclear extracts subjected to EMSA and antibody competitions performed as described in Fig. 4B.

These results confirmed that the γ_c chain plays no role in determining the specificity of the STAT-5 isoform induced following ligation of the IL-2R, and that JAK3 can be replaced with JAK2 in the receptor complex without adverse functional effects in the activation of select STAT factors.

Specific Tyrosine Residues of the IL-2R β Chain Regulate STAT-5A/B Induction

Since tyrosine residues within the γ_c subunit failed to specify STAT-5A/B induction (Fig. 5) (23), studies were performed to delineate the role of the IL-2R β chain in this process. Previous studies have established that one or more tyrosine residues of the IL-2R β are required for STAT-5 activation, because a mutant IL-2R β chain lacking all cytoplasmic tyrosine residues failed to induce STAT-5 DNA binding activity in HT-2 cells (23). The abilities of the EPO β tyrosine reconstitution mutants to activate STAT-5A and -5B were therefore tested in EMSA. Consistent with prior *in vitro* peptide competition data (23, 26), HT-2 cells expressing either of the two C-terminal tyrosine residues (EPO β YF:5Y/ γ or EPO β YF:6Y/ γ) each induced STAT-5A and STAT-5B in response to EPO (Fig. 6, lanes 7 and 10). Furthermore, supershift analyses suggested that typical STAT-5A/B heterodimers were assembled via these receptors (Fig. 6, lanes 12–17). In contrast, HT-2EPO β YF:234Y/ γ cells failed to induce significant STAT-5A/B DNA binding activity, despite the activation of Janus kinases in these cells (Fig. 3). Unexpectedly, HT-2 cells expressing EPO β YF:1Y/ γ also proved competent to activate STAT-5A/B (Fig. 6, lane 2). The relatively higher levels of STAT-5 induction by the EPO β YF:1Y cell line as compared to the EPO β YF:5Y and EPO β YF:6Y cell lines shown in this gel were not consistently observed; thus all three mutants appear to activate STAT-5A/B roughly equivalently. In addition, EPO β YF:1Y induced both STAT-5A/B in cells expressing EPO γ YF, indicating that γ_c tyrosine residues do not exhibit a compensatory function for absent IL-2R β residues (data not shown). These results underscore both redundancy and specificity within the IL-2R system, since STAT-5A/B can be activated through three different tyrosine residues but not by three other cytoplasmic tyrosines.

DISCUSSION

The IL-2 Receptor Is Composed of Distinct, Functional Modules—One of the paradoxes in cytokine receptor signal transduction is that a high degree of signaling specificity is achieved even though receptor subunits and intracellular signaling intermediates are shared among multiple receptor types. In this regard, the γ_c subunit corresponds to a relatively short chain that participates in the IL-2, -4, -7, -9, and -15 receptors (4–7). However, γ_c appears to confer no detectable specificity to the IL-2 signaling program. Indeed, none of the four cytoplasmic

tyrosine residues within γ_c nor distal sequences within the γ_c tail is required for effective signaling, including proliferation, induction of the *c-fos* and *bcl-2* proto-oncogenes, or STAT-5A and -5B activation (Figs. 1C, 2A, and 5) (14). Furthermore, while JAK3 is bound to proximal sequence elements in γ_c , this cytoplasmic domain and Janus kinase can be replaced by a truncated form of the erythropoietin receptor bound to JAK2 with no loss of signaling specificity (Figs. 2A and 5) (14). Nevertheless, γ_c -associated JAK activity appears to be necessary for native IL-2R signaling, since a dominant negative JAK3 mutant lacking intrinsic kinase activity effectively disrupts signal transduction (42). In addition, natural mutations of γ_c that abrogate JAK3 binding lead to clinically significant immunodeficiencies (43). Taken together, our present findings support the notion that γ_c plays an important role in the initiation of transmembrane signaling but is likely dispensable for subsequent execution and completion of specific signals. Accordingly, we have suggested that γ_c and its associated JAK3 function as a "trigger" chain in the IL-2R complex (14).

In contrast, the subunits that pair with γ_c in the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors have relatively long cytoplasmic domains that typically associate with multiple signaling intermediates. For example, while the IL-2R β chain binds specifically to such molecules as Shc and STAT-5 (22–24, 26–28), the IL-4R subunit binds to STAT-6 (44, 45), thus specifying a very different signaling program. The present findings provide further molecular evidence that these longer subunits play a major role in driving the specificity of the signaling program; thus, we have referred to this class of receptor subunits as "driver" chains (14).

Signaling Modules Are Specific, Redundant, and Multifunctional—The findings reported here illustrate several properties of the IL-2 receptor "driver" subunit, the IL-2R β chain, that characterize its mechanism of signaling. First, within the IL-2R β chain, there are multiple functional, tyrosine-based peptide modules that exhibit a high degree of specificity in coupling to signaling pathways (summarized in Fig. 7B). For example, the ability of an individual tyrosine residue to drive a specific signal is manifested in the activation of *c-fos* gene expression. Data presented here illustrate that Y1 (Tyr-338) is uniquely required for inducing *c-fos* mRNA levels (Fig. 2B), while another recent study has shown that Y1 (Tyr-338) is the only cytoplasmic tyrosine residue within IL-2R β that binds to the Shc adapter molecule after IL-2R β phosphorylation (22). Taken together, these observations suggest that *c-fos* is likely induced via the Ras-Raf pathway that is activated by Shc (reviewed in Ref. 46). These experiments have also shown that the *c-fos* and STAT-5 pathways appear to be distinct. STAT-5 can be activated through other tyrosine residues (Y5 (Tyr-392) and Y6 (Tyr-510)) that fail to induce *c-fos* or bind Shc (Figs. 2B and 6) (22). However, since both pathways are induced through an IL-2R β mutant that contains only Y1 (Tyr-338), these studies do not preclude the possibility that STAT-5 activation is necessary but not sufficient for AP-1 induction by this receptor (47).

Second, in contrast to the selective activation of *c-fos* by Y1 (Tyr-338), there is marked redundancy in the abilities of certain tyrosine residues within IL-2R β to mediate other signaling events. For example, Y1 (Tyr-338), Y5 (Tyr-392), and Y6 (Tyr-510) all promote activation of STAT-5 nuclear import and DNA binding activity in HT-2 cells (Fig. 6), despite clear sequence differences in the amino acid sequences flanking Tyr-338 and Tyr-392/Tyr-510 (Fig. 7A and see below). Importantly, efficient STAT5A/5B heterodimerization is induced via an IL-2R β mutant retaining a single STAT-binding motif, indicating that the receptor does not necessarily form a multivalent "platform" to

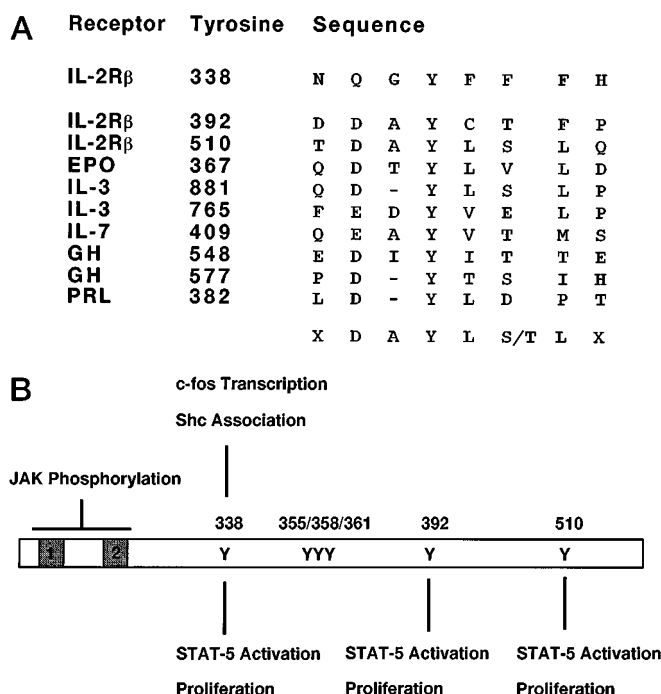


FIG. 7. A, amino acid motifs flanking tyrosine residues of STAT-5-inducing receptors. B, specific signaling events mediated by IL-2R β tyrosine residues located in the cytoplasmic tail. Conserved Box1 (1) and Box2 (2) motifs are shaded (53), and relative positions of the six tyrosine (Y) residues are indicated.

which multiple STATs are recruited simultaneously in order to direct heterodimer or homodimer formation. Similar redundancy among Y1 (Tyr-338), Y5 (Tyr-392), and Y6 (Tyr-510) is observed in the activation of cellular proliferation (Fig. 1) (11), although STAT-5 activation and proliferation are probably independent events (23, 24). The biological importance of such functional redundancy is unknown, but it may allow for amplification of signaling, as has been demonstrated for repetitive tyrosine-based motifs present in the T cell receptor complex (48).

Third, the studies performed here indicate that individual tyrosine-based motifs in the IL-2R β chain can serve multiple, apparently independent functions. For example, as described above, Y1 (Tyr-338) couples both to a Shc-dependent pathway as well as to the JAK-STAT pathway (Fig. 7B). Moreover, two isoforms of STAT-5 are induced through single tyrosine residues, indicating at least some potential for multifunctionality through these sequences. Since both STAT-1 and STAT-3 have been implicated in IL-2 signaling in other systems (26, 40), it is likely that more STAT factors may couple to some or all of the receptor tyrosine residues.

Interestingly, no obvious function for the Tyr-234 (Tyr-355/Tyr-358/Tyr-361) tyrosine cluster has yet been detected in HT-2 cells. Although it is unknown whether the restriction of function of Tyr-234 occurs at the level of phosphorylation or at the level of binding to particular signaling intermediates, it is nonetheless clear that there is strict selectivity in which tyrosines are capable of mediating such events.

Finally, at least some signaling pathways are coupled to the IL-2R through a tyrosine-independent mechanism, as represented by induction of the proto-oncogene *bcl-2*. It has been previously shown that *bcl-2* induction is independent of JAK3 activation (42), but the data presented here represent the first direct demonstration that *bcl-2* induction is also independent of tyrosine residues within the IL-2 receptor. Further studies are needed to determine how tyrosine-independent pathways are

initiated by cytokine receptors after receptor activation. Other studies with the growth hormone (GH) and erythropoietin receptor have suggested that receptor tyrosine residues are apparently not absolutely essential for activation of proliferation and the JAK-STAT pathway in these receptor systems (49, 50). It remains to be determined whether these observations represent a fundamental difference in the mechanism of signaling between the EPOR/GHR and the IL-2 receptors, or whether low levels of endogenous EPO and GH receptors might contribute to the observed functionality of the transfected receptor mutants.

Cell Context Specificity of STAT-binding Motifs—Among cytokine receptors it is often possible to identify putative functional “driver” modules based on sequence alone, such as IRS-1 or STAT-binding motifs (10, 44). The present studies reveal additional complexities underlying STAT-5-activation motifs and the role of additional cellular factors in permitting STAT activation. First, in HT-2 cells, we have shown that two isoforms of STAT-5 (STAT-5A and -5B) are induced by IL-2 stimulation through three different tyrosine residues within IL-2R β (Figs. 4 and 6). Surprisingly, however, although the sequences surrounding Y5 (Tyr-392) and Y6 (Tyr-510) are quite similar to tyrosine motifs found in other STAT-5-inducing receptors (D A Y L S/T L), the functionally redundant Y1 (Tyr-338) is notably divergent from these in primary sequence (N Q G Y F F F) (Fig. 7A). Moreover, the context of the prolactin receptor tyrosine residue that induces transcription through a STAT-response element differs somewhat from these motifs (LDYLDPT) (51). Therefore, STAT-5 appears to be activated through as many as three different tyrosine-based sequences.

In addition, there appears to be an influence of cellular environment on STAT-5 activation. First, in the pro-B/myeloid cell line Ba/F3, a truncated IL-2R β chain retaining Y1 (Tyr-338) fails to activate STAT-5 DNA binding, although it still maintains the ability to induce cellular proliferation (24). These observations contrast with the present observation that Y1 (Tyr-338) induces STAT-5A/B in IL-2-dependent T cells (Fig. 6). Second, the effects of point substitutions at Y1 (Tyr-338) on proliferation signaling appear to vary depending on cellular context (11). Moreover, the EPOR can induce STAT-5 only in certain cellular contexts³ (52). These data suggest that the type of cell chosen for receptor studies may be important in dissecting specific consequences of cytokine signaling. In summary, a full understanding of STAT-activation motifs and their functions in different cellular environments will require further study.

Modularity within the Cytokine Receptor Superfamily—In summary, the present studies reveal that tyrosine-based sequences located in the IL-2R β chain exhibit specificity, redundancy and multifunctionality in regulating signaling events initiated by IL-2. Since the signaling portion of IL-15 receptor contains both the IL-2R β and γ_c chains (7), these findings probably apply to this cytokine as well. It is likely that analogous modules in “driver” subunits within other receptor complexes also exhibit these characteristics, and that the combinatorial association of various modules within a receptor complex permits a diverse range of biologic consequences. For example, some domains may have synergistic or inhibitory influences on one another. A more complete understanding of such interactions is essential to developing strategies to manipulate these intracellular processes for clinical benefit.

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