Several distinct classes of cytokine receptors engage Jak kinases as primary effectors. Among type 1 receptors, Janus-activated kinase (Jak) recruitment is mediated by membrane-proximal cytoplasmic domains, which typically contain conserved box motifs. In the erythropoietin receptor (Epo-R), two such motifs (box1 and box2) have been suggested to be essential for the activation of Jak2 and mitogenesis. Presently, an Epo-R chimera containing the extracellular and box1 domains of the Epo-R (Jak2-associated receptor) and the box2 and carboxyl-terminal domains of the interleukin 2 β-receptor (IL2-βR; a Jak1-associated subunit) is shown to activate Jak2. Interestingly, Jak2 also was activated in FDC-P1 cells by a control Epo-R chimera containing the complete IL2-βR cytoplasmic domain, and mitogenesis was supported by each of these above chimeras. By comparison, in BaF3 cells expressing IL2 receptor α and γ subunits, an ectopically expressed IL2-βR chimera containing the box1 domain of the Epo-R, activated Jak2, and Jak3, and was as mitogenically active as the wild-type IL2-βR (Jak1 and Jak3 activation). Thus, the box1 domain of the Epo-R specifies Jak2 activation and functions efficiently within a heterologous IL2 receptor complex that normally activates Jak1 and Jak3.

Cytokine receptors of the type 1 subclass have emerged recently as a complex superfamily of single transmembrane proteins (1, 2). Homology among these receptors originally was recognized based on conserved coiled-coil domains, WSXWS motifs, and predicted paired 7-fold helical ligand binding regions within extracellular domains (1-4). Included within this family are the receptors for Epo, IL2, IL3, IL4, IL5, IL6, IL7, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, growth hormone, prolactin, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin, the env gene of mouse myeloproliferative leukemia virus, v-mpl, and its cellular homolog, c-mpl (1-5).

More recently, relatedness among these receptors has been extended based on their observed recruitment of common cytoplasmic signaling factors including: Jak kinases at membrane proximal domains (6, 7); signal transducers and activators of transcription factors at characterized phosphotyrosine motifs (8, 9); Shc (10) and Grb2 (11); p55/p105 phosphatidylinositol 3-kinase (12); HCP (13) and Syp (14) phosphatases at alternate phosphotyrosine motifs; and in at least certain receptor systems, members of the Src (1, 15) and Tec (16) families of tyrosine kinases. Correspondingly, homology also is discernible within the cytoplasmic subdomains of type 1 receptors, especially within membrane-proximal regions (1, 17-19). Specifically, an 8-amino acid motif containing two essentially invariant prolines (box1) commonly occur together with a closely appositioned 10-amino acid motif (box2), which commonly contains a conserved "LEVL" core. Based on deletional and site-directed mutagenesis, this region previously has been suggested by our laboratory (3) and others (21-26) to be essential for mitogenesis and for the recruitment and activation of Jak kinases. However, the specific functions exerted by distinct box domains (and intervening regions) are not well defined and may differ among receptor systems. In the growth hormone receptor, for example, carboxyl-terminal truncation mutants have been defined that essentially lack the box2 domain, yet efficiently activate mitogenesis and Jak2 kinase (20). In contrast, in the Epo-R and IL2-β-Rs, deletion or mutagenesis of the box2 domain is inactivating (21-24), with a strict requirement for the box1 domain within these receptors and essentially all type 1 receptors studied to date (17-26). Thus, the issue is raised as to whether the box2 domain in the Epo-R (and related systems) is involved in the recruitment and activation of Jak kinases or perhaps contributes to alternate signal transduction pathways.

To address this issue, we have constructed active chimeras in which the box1 subdomain (and flanking residues) of the Epo-R have been inserted at cognate domains of the structurally related IL2-β-R. Properties of these chimeras demonstrate that the specification, recruitment, and activation of Jak2 in the Epo-R system depends upon the box1 domain, and that this critical subdomain, in fact, functions efficiently when incorporated into an IL2-β-R chimera. Findings are discussed in the contexts of type 1 cytokine receptor structure, mitogenic signaling, and the significance of the selective activation of Jak2 and Jak3 kinases by the latter IL2-β-R/Epo-R chimera (versus Jak1 and Jak3 by wt IL2 receptor complexes).

MATERIALS AND METHODS

Cell Lines and Growth Factors—FDC-P1 (27), BaF3 (28), and derived cell lines were maintained in Opti-MEM medium (Life Technologies, Inc.) supplemented with 8% fetal bovine serum, 10-5 M 2-mercaptoethanol, and 4% conditioned medium from WEHI-3B cells (WEHI-3-CM) as
a source of IL3. Purified recombinant human erythropoietin was generously provided by Amgen (Thousand Oaks, CA). Recombinant human IL2 was from DNAX Inc. (Palo Alto, CA).

Epo-R and IL2β-R Constructs—For the expression of wt Epo-R and IL2β-R, a full-length Epo-R cDNA was cloned into a modified pXM expression vector (pXM190EXO) at 5′-KpnI and 3′-NotI sites (29), and a wt IL2β-R cDNA (30) was cloned into pMEneo at XhoI and NotI sites. For the construction of Epo-R/IL2β-R hybrids, EcoRV sites were created in both cDNAs at positions corresponding to extracellular, juxtamembrane sites (30). In pMEneo-Epo-R, the 5′-region of this Epo-R cDNA was ligated to the 3′-domain of the IL2β-R cDNA. In pMEneo-Eβ-R, XhoI and XbaI sites first were generated by PCR in the 3′-fragment of this Epo-R cDNA. The two resulting constructs (Epo-R/IL2β-R constructs) then were subcloned into pMEneo at 3′-NotI and 5′-KpnI sites (predicted products, 477 base pairs). For IL2β-R, primers used were 5′-GATGCGGGCTCAGGCACCTT-3′ and 5′-AGGACCTCTAGATGGGCAGG-3′, respectively. The primer used to create the XbaI site at the LEVL position of the IL2β-R cDNA was 5′-TCTCGGCTCTAGAAGTGCTGGA-3′.

Stable Transfection of FDC-P1 and BaF3 Cells—The constructs pMEnv-Eβj2β-R and pMEnv-Eβj2β-R (50 μg) were cotransfected with a zeocin-resistance plasmid (pZeolSV, 8 μg; Invitrogen) into FDC-P1 cells (10⁶ cells/ml in Opti-MEM medium) by electroporation (250V, 960 microfarads; GeneZapper; IBI, New Haven, CT). Transfected cells were then selected sequentially in 1 mg/ml zeocin, 1 mg/ml G418 (Life Technologies, Inc.), and 25 units/ml Epo. FDC-P1 cells expressing the wt murine Epo-R have been described previously (29). The constructs pMEnv-IL2β-R and pMEnv-Eβj2β-R were transfected into BaF3 cells (250V, 960 microfarads), and stably transfected lines were selected in 1 mg/ml G418 and 25 ng/ml purified human IL2.

Reverse Transcription and PCR—RNA from FDC- and BaF3-derived cell lines was isolated by the method of Chomczynski and Sacchi (31) (Trizol reagent; Life Technologies, Inc.). DNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega) and random primers. In PCR, primers used for Eβj2β-R and Eβj2β-R constructs were 3′-GAGTGGACGTGCTGGCAGGCAACCCG-3′ (Epo-R) and 5′-GATGGCGGCTCGAGCCACCTT-3′ (IL2β-R) (Operon; predicted products, 490 base pairs). For IL2β-R, primers used were 5′-TGGATCCCTGCGGTACAGCTGAACACCGAGG-3′ and 5′-GATGCGGGCTCAGGCACCTT-3′ and 5′-GATGCGGGCTCAGGCACCTT-3′ (predicted products, 477 base pairs).

Proliferation Assays—Cytokine-induced proliferation of FDC-P1, BaF3, and derived cell lines was assayed based on rates of reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2H-tetrazolium to formazan (Promega). Cells (3 × 10⁵ cells/ml, 50 μl/well, 96-well plates) were exposed to cytokines (50 μl) for the indicated intervals prior to the addition of 3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate. At 4 h of incubation, absorbance (A₄₉₀) was measured (microplate reader, model 3550; Bio-Rad).

Jak Kinase Activation Assays—FDC-P1 and BaF3-derived cell lines (8 × 10⁴ cells/ml, 40 ml) were washed and incubated at 37 °C and 5% CO₂ for 12 h in Opti-MEM medium containing 1% fetal bovine serum and 10⁻⁵ M dexamethasone. Cells were then stimulated with Epo (50 units/ml, 8 min) or IL2 (500 ng/ml, 9 min). Cells were collected, washed in Opti-MEM medium, and lysed in 0.3 ml of lysis buffer (1% Nonidet P-40, 50 mM dithiothreitol, 300 mM NaCl, 50 mM Tris, pH 7.5, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 50 μg/ml phenylmethylsulfonyl fluoride, and 2.2 μg/ml aprotinin). Cleared lysates were then incubated with Jαk1, Jαk2, or Jαk3 antisera (1.5-2 h at 4 °C; Upstate Biotechnology, Inc., Lake Placid, NY) and with protein A-Sepharose CL4B (90 min, 4 °C). Complexes were washed four times at 4 °C in lysis buffer, eluted with SDS-polyacrylamide gel electrophoresis sample buffer, electrophoresed (7% SDS-polyacrylamide gel electrophoresis), and transferred to Nitro Plus membranes (MSI, Westboro, MA). Membranes were blocked (3% skim milk in 0.05% Tween 20, 150 mM NaCl, and 10 mM Tris-Cl, pH 7.5) and probed with anti-phosphotyrosine antibody 4G10 (Upstate...
RESULTS

Signaling by Epo-R/IL2β-R Chimeras in FDC-P1 Cells—Primary studies aimed to define minimal cytoplasmic subdomains of the Epo-R that mediate Jak2 kinase activation, and mitogenesis. These studies were prompted, in part, by reports that mutations within the box2 domain of the Epo-R and at least certain additional type 1 cytokine receptors are inactivating (21–26), while in alternate related receptors (20) the deletion of corresponding cytoplasmic regions does not disrupt proliferative signaling.

To address this issue, chimeras were constructed in which the predicted cytoplasmic domain of the Epo-R either was replaced at the proximal boundary of the box2 motif with the box2 domain and carboxyl-terminal flanking region of the IL2β-R (Eββ-R chimera) or was replaced fully with the IL2β-R cytoplasmic domain (Eββ-R chimera; Fig. 1). These Epo-R/IL2β-R chimeras (and the wt Epo-R) were then expressed in IL3-dependent murine myeloid FDC-P1 cells, and the ability of derived cell lines (FDC-Eββ-R, FDC-Eββ-R, and FDC-ER cells, respectively) to mediate Epo-dependent activation of Jak2 kinases was assayed by immunoprecipitation with antibodies to Jak1 or Jak2 and Western blotting with an antibody to phosphotyrosine (monoclonal antibody 4G10). In these experiments, cells were exposed to Epo (25), lysed, and subdivided for parallel immunoprecipitation assays. The positions of Jak kinases (arrows) and molecular weight standards are indexed.

Biotechnology, Inc.). Complexes were detected by ECL (Amersham Corp.).

Mitogenic Activity of the above Hybrid Receptors was Supported by Epo-Induced Dimerization of each Chimera. Analyses of induced tyrosine phosphorylation of Jaks revealed that exposure of FDC-Eββ-R cells to Epo selectively activated Jak2 kinase, with no detectable activation of Jak1 (Fig. 3). In Eββ-R, the cytoplasmic domain is comprised of the box1 and flanking domains of the Epo-R, together with the box2 and carboxyl-terminal domains of the Jak1-specific IL2β-R. Thus, this result suggested that the Epo-R box1 region apparently specified Jak2. Interestingly, however, in control FDC-Eββ-R cells Epo likewise selectively activated Jak2 kinase with no detectable activation of Jak1. This latter finding was somewhat unexpected and suggests that Jak1 either may be incapable of functioning through a mechanism of direct homodimerization or may occur in FDC-Eββ-R cells at levels that are insufficient to efficiently support this event. This result also somewhat complicated interpretations of the possible selective role of the Epo-R box1 domain in Jak2 recruitment as studied using single chain Epo-R/IL2β receptor hybrids in FDC lines.

Signaling by an IL2β-R/ Epo-R box1 Chimera in BaF3 Cells—Based on the observed ability of Eββ-R and Eββ-R chimeras to efficiently mediate Epo-induced activation ofJak2 and mitogenesis, the prospect that the box1 and flanking residues of the Epo-R might function effectively (and selectively) within IL2β-R complexes was tested. Specifically, residues...
Jak2 Activation via an IL2β-R/Epo-R box1 Chimera

DISCUSSION

The present studies were performed to assess mechanisms by which Jak2 kinase is selectively activated in the Epo-R system and to address the extent to which the specific activation of select Jak kinases might be essential to the mitogenic activity of stimulated receptor complexes. As discussed above, type 1 cytokine receptor cytoplasmic subdomains that mediate Jak kinase activation have been mapped in several systems (via deletional and/or directed mutagenesis) to a membrane-proximal region of approximately 40 residues (1–3, 6–9). For certain receptors, including those for Epo and IL2 (β-chain), point or deletional mutations within box2 are inactivating, and this domain has been concluded to be important for Jak2 activation and mitogenesis (21–26, 38). In contrast, truncated forms of receptors for growth hormone (20) and prolactin (34) have been shown to lack box2 sequences and thereby efficiently mediate Jak2 activation. Thus, alternate bases for the observed inactivity of previously studied Epo-R box2 mutants may relate to nontypical effects, including the potential misfolding of the box1 domain and adjacent regions. In the present study, Epo-R box1 and box2 subdomains were replaced by residues Leu277-His329 of the Epo-R (including the Epo-R box1 motif and flanking residues; Fig. 4), and this chimeric IL2β-R/Epo-R subunit (Eβ-R) then was expressed stable in BaF3 cells expressing IL2 receptor α- and γ-subunits (28). As a control, BaF3 cells also were transfectioned stably with an expression vector encoding the wt IL2β-R (i.e., BaF3 wtβ-R cells). Expression of wt IL2β-R and ββ-receptor chimera was confirmed by reverse transcription-PCR, with no endogenous IL2β-R transcripts detected in parental BaF3 cells (Fig. 5A). As shown in Fig. 5B, the above ββ-R chimera, in fact, supported IL2-induced mitogenesis at rates somewhat greater than those observed for BaF3 cells transfected with the wt IL2β-R subunit.

Analyses of Jak kinase activation in BaF3 wtβ-R and BaF3-ββ-R cells next were performed. In BaF3 wtβ-R cells, ectopic expression of the wt β-receptor subunit detectably reconstituted IL2-dependent activation of Jak1 and Jak3 kinases (Fig. 6, left panel). Jak2 kinase phosphorylation were modest yet reproducibly detected (representative of three independent experiments). In BaF3-ββ-R cells, however, IL2 induced the rapid tyrosine phosphorylation of Jak2 and Jak3 (Fig. 6, right panel). These findings, therefore, clearly demonstrate the ability of the Epo-R box1 subdomain (and flanking residues) to specify the recruitment and activation of Jak2. Moreover, the observed co-activation of Jak3 in BaF3-ββ-R cells reveals that Jak2, in fact, can effectively and functionally substitute for Jak1 within the trimeric IL2 receptor system.

B. Joneja, A. Wrentmore, D. M. Wojchowski, manuscript in preparation.
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FDC cells by a control construct comprised of the Epo-R extra-cellular and transmembrane domains fused to the cytoplasmic region of the IL2β-R (Eββ) was unexpected since the IL2β-R normally activates Jak1 (39). However, whereas a significant number of type 1 receptors selectively activate Jak2, to date none have been identified that activate Jak1 without the necessary co-activation of an alternate Jak (Jak1 and Tyk2) in the IFN-α/β receptor system, for example; Refs. 36 and 37). Thus, activation of Jak1 may either depend upon heterodimerization and/or accessory factors, or Jak1 may be less active than Jak2 as a transducing kinase. Efficient activation of Jak2 (and of Jak1 may be less active than Jak2 and/or accessory factors, or Jak1 may be nonessential to Jak3 kinase activation and to functionally, using the presently developed chimeric receptor system, for example; Refs. 36 and 37). Thus, activation of Jak1 may either depend upon heterodimerization and/or accessory factors, or Jak1 may be nonessential to Jak3 kinase activation and to Jak3 kinase activation and to IL2-induced proliferation. The derived and novel implication that the specific activity of cytokine signaling in this system may not be impacted greatly by the recruitment of one versus another Jak kinase is an important notion that can be assessed further, and functionally, using the presently developed chimeric receptors and derived cell lines.

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