Anti-apoptotic Signaling by the Interleukin-2 Receptor Reveals a Function for Cytoplasmic Tyrosine Residues within the Common γ (γc) Receptor Subunit*

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The interleukin-2 receptor (IL-2R) is composed of one affinity-modulating subunit (IL-2Rα) and two essential signaling subunits (IL-2Rβ and γc). Although most known signaling events are mediated through tyrosine residues located within IL-2Rβ, no functions have yet been ascribed to γc tyrosine residues. In this study, we describe a role for γc tyrosines in anti-apoptotic signal transduction. We have shown previously that a tyrosine-deficient IL-2Rβ chain paired with wild type γc stimulates enhancement of bcl-2 mRNA in IL-2-dependent T cells, but it was not determined which region of the IL-2R or which pathway was activated to direct this signaling response. Here we show that up-regulation of Bcl-2 by an IL-2R lacking IL-2Rβ tyrosine residues leads to increased cell survival after cytokine deprivation; intriguingly, this survival signal does not occur in the absence of γc tyrosine residues. These γc-dependent signals are revealed only in the absence of IL-2Rβ tyrosines, indicating that the IL-2R engages at least two distinct signaling pathways to regulate apoptosis and Bcl-2 expression. Mechanistically, the γc-dependent signal requires activation of Janus kinases 1 and 3 and is sensitive to wortmannin, implicating phosphatidylinositol 3-kinase. Consistent with involvement of phosphatidylinositol 3-kinase, Akt can be activated via tyrosine residues on γc. Thus, γc mediates an anti-apoptotic signaling pathway through Akt which cooperates with signals from its partner chain, IL-2Rβ.

Defining the molecular mechanisms by which cytokines and their receptors trigger signal transduction has been the focus of intensive research. Indeed, a detailed understanding of cytokine receptor structure and dynamics at the molecular level has proven useful in several therapeutic modalities to treat cancer and autoimmune diseases (1, 2). This paper focuses on structure-function relationships in the interleukin (IL)-1/2 receptor (IL-2R), which is a prototypical member of the type I cytokine receptor superfamily (3). IL-2 is a crucial regulator of T cell proliferation, survival, and programmed cell death (apoptosis) (4) (for review, see Ref. 4). The IL-2R is a highly complex receptor that employs multiple subunits to activate a remarkable variety of cellular signaling cascades (4) (for review, see Ref. 5). Despite a wealth of information about the IL-2R signaling pathway gained in the last decade, it is still not fully understood how the various subunits within the IL-2R act in a coordinated fashion to trigger a coherent array of signaling pathways.

Most cytokine receptors are composed of multiple subunits, existing either as homomers of identical subunits (such as the erythropoietin (EPO) and tumor necrosis factor receptors) or as heteromers of distinct proteins (such as the IL-2 and interferon receptors). In addition, many cytokine receptor complexes share one or more subunits with other receptors; this is especially true of the type I family of cytokine receptors. The members of this family are characterized by several conserved structural features, including spaced cysteines and WSXWS motifs in their extracellular domains, and canonical box 1 and box 2 domains and tyrosine residues in their cytoplasmic tails. There are several subfamilies of the type I receptors, which are defined by the subunits that they share. For instance, the IL-3, -5, and granulocyte-macrophage colony-stimulating factor receptors employ an identical receptor subunit termed γc, and the IL-6 family of receptors shares the gp130 subunit (for review, see Ref. 6). Similarly, the IL-2 family of receptors is defined by their use of the common γc subunit. Originally identified as the IL-2Rγ chain (7), γc is now recognized to be an essential component of the IL-2R-4-7-9-15 and -21 receptors (8, 9). Humans with genetic deficiencies in γc suffer X-linked severe combined immunodeficiency syndrome caused by a phenotypic loss of these cytokines (9) (for review, see Refs. 8 and 10).

Upon interaction with their ligands, type I cytokine receptors activate numerous downstream signaling molecules, including Janus kinases (JAK), signal transducers and activators of transcription (STAT), mitogen-activated protein kinases (MAPK), phosphatidylinositol 3′-kinase (PI3K), and/or suppressors of cytokine signaling. Signaling pathways involving these molecules are initiated by recruiting signaling intermediates to the receptor complex, usually to phosphorylated tyrosines located on the receptor’s cytoplasmic tail. In some cases, signaling molecules bind directly to the receptor, via phosphotyrosine-binding motifs such as Src homology 2 or phosphotyrosine binding domains. Alternatively, sometimes one or more adapter molecules are used to bridge the signaling intermediate to the receptor. Therefore, tyrosines in cytokine receptor tails are crucial launching points for signaling pathways, and much

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The abbreviations used are: IL, interleukin; EPO, erythropoietin; EPOR, erythropoietin receptor; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IL-2R, interleukin-2 receptor; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; STAT, signal transducer and activator of transcription.
effort has been directed to mapping specific signaling pathways to individual tyrosine residues within cytokine receptors (e.g. Refs. 11 and 12).

At the level of the receptor, it is clear that signaling requires a cooperative interaction between receptor subunits. Indeed, for nearly all cytokines, dimerization or multimerization of receptor subunits is essential for signaling (13). In the case of receptors belonging to the type I family, mutational analyses have shown that both subunits in the complex must have functional JAK binding domains, which are located in the conserved box 1 and box 2 domains (14). Dimerization then permits trans-phosphorylation and subsequent activation of the associated JAKs (15, 16) followed by phosphorylation of the receptor tails on distal tyrosine residues.

Apart from JAK activation, are all subunits within a multimeric receptor complex necessary for signal transduction? In some circumstances, effective signaling is maintained even when large distal regions of the receptors are deleted by mutagenesis. For example, in the homodimeric erythropoietin receptor (EPOR), it has been shown that both JAK2 binding sites within the receptor tail must be present for STAT activation, but only one distal tail is required to be present (16). Frequently, multiple tyrosines within the cytoplasmic tail of a single receptor are able to activate identical pathways. For instance, in the EPOR, four different tyrosine residues are capable of activating the transcription factor STAT5 (17–19). Thus, homodimeric receptors apparently contain duplicated signaling domains, any one of which can activate downstream pathways such as STAT activation. Although these duplicated domains may serve to amplify or fine tune signaling responses (as has been demonstrated for tyrosine-containing motifs in the T cell receptor (20)), they may serve as redundant or “backup” mechanisms to ensure that essential pathways are activated.

Duplicated signaling domains also appear to exist in heteromeric receptors such as the IL-2R, although the receptor subunits contribute differentially to signaling (5, 12, 21–24). The IL-2R is composed of three subunits, IL-2Rα (CD25 or Tac), IL-2Rβ, and γc. The IL-2Rα is an affinity modulator that is dispensable for signaling, although it is essential in vivo for detection of physiological levels of IL-2. In contrast, IL-2Rβ and γc are necessary and sufficient for signaling. Both IL-2Rβ and γc are phosphorylated after receptor ligation (25, 26), and both subunits are highly conserved in mammalian evolution. The functional roles of tyrosine residues within the IL-2Rβ chain have been carefully examined, and several important signaling pathways have been found to be strictly dependent on tyrosines within this subunit. For example, Tyr-338 recruits the adaptor molecule She and activates the p38-MAPK pathway, leading to c-fos gene expression (27–29). Moreover, Tyr-338 is the only tyrosine within the IL-2R complex capable of activating this pathway. In contrast, three different tyrosine residues (Tyr-338, Tyr-392, and Tyr-510) all activate STAT5 and proliferation independently (11, 12). Although multiple IL-2Rβ tyrosines can activate these pathways, tyrosines in γc cannot do so. Both the MAPK and STAT5 activation proceed normally even in the absence of tyrosine residues on γc (12, 30). Conversely, tyrosines within γc cannot compensate for an absence of tyrosine residues on IL-2Rβ (11, 22, 23). Furthermore, replacing γc with a heterologous cytokine receptor (the EPOR) does not have a noticeably deleterious effect on these signals, as long as IL-2Rβ tyrosines are retained (21). These findings led to a model in which the only function of the γc cytoplasmic tail is to recruit JAK3 to the receptor complex, whereupon JAK3 is able to phosphorylate JAK1 and permit subsequent signaling via phosphorylated tyrosine residues within IL-2Rβ.

Despite the central role of IL-2Rβ tyrosine residues, a number of signals proceed in the absence of tyrosine residues on the IL-2Rβ chain. In particular, the gene encoding Bcl-2, an important anti-apoptotic effector, is still enhanced by a mutant IL-2Rβ that cannot be phosphorylated (12). Mutagenesis studies have indicated that γc appears to be crucial for regulating Bcl-2 in vivo even in the absence of its JAK3 binding domain (31). Moreover, the immune impairment observed in γc-deficient mice can be partially rescued by a bcl-2 transgene (32). Therefore, given the widespread use of γc as a signaling subunit and the evolutionary conservation of its cytoplasmic tyrosine residues, we hypothesized that γc may engage signaling pathways in addition to JAK3 activation, possibly through its tyrosine residues. Accordingly, we show here that tyrosines within the γc subunit mediate a signaling cascade that leads to up-regulation of Bcl-2 and inhibition of apoptosis, thus revealing a previously unrecognized function for the γc subunit in signal transduction.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cell Lines, and Cytokine Stimulation**—HT-2 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640, 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, penicillin/streptomycin, and 1 mM recombinant human IL-2 (generously provided by the Chiron Corporation, Emeryville, CA). HT-2.EPOγ and HT-2.EPOβ cells were maintained in this medium supplemented with 0.5 mg/ml G418 (Invitrogen). HT-2.EPOγYFγ, HT-2.EPOγYFβγ, HT-2.EPOβYFγ, and HT-2.EPOβD528αγ cells were maintained in this medium supplemented with 0.5 mg/ml G418 and 0.4 mg/ml hygromycin B (Invitrogen). HT-2.EPOβYF and HT-2.EPOγYF cells were maintained in this medium with recombinant human EPO (5 units/ml, a kind gift from A. A. Amgen, Thousand Oaks, CA) in place of IL-2. HT-2 transfectant cell lines were prepared as described previously (22). For stimulations, cells were washed twice in phosphate-buffered saline, stripped with 10 mM sodium citrate and 140 mM NaCl, and incubated in RPMI 1640 medium with 1% bovine serum albumin (Sigma) for 4 h. Stimulations were for indicated times with recombinant human IL-2 (5–10 nM) or EPO (50 units/ml unless otherwise indicated).

**Plasmids**—All EPO-IL-2R chimeras were expressed in the pCMV4 vector series (33), kindly provided by Dr. M. A. Goldsmith. Construction of EPOβ, EPOγF, EPOγ, and EPOγ-F is described elsewhere (22). EPOβD528αγ was made by subcloning a Bcl-2 domain fragment from IL-2Rβαβ (34) into corresponding sites within pCMV4.EPOγ.

**Proliferation Assays**—Conventional [3H]thymidine incorporation assays were performed as described previously (35). Briefly, 5 × 10^3 cells were washed twice in phosphate-buffered saline and incubated with EPO or IL-2 for 24 or 48 h in triplicate. Four h prior to harvesting, 1 μCi/well [3H]thymidine (PerkinElmer Life Sciences) was added. Cells were harvested on a Skatron microwell harvester (generously provided by Dr. W. C. Greene, Gladstone Institute of Virology and Immunology, San Francisco) and analyzed on a Wallac Microbeta counter (PerkinElmer Life Sciences).

**Northern Blotting**—Cytoplasmic RNA was prepared from 1–2 × 10^7 cells using an RNaseasy kit (Qiagen, Valencia, CA) and quantified by spectrophotometry. Denaturing 1.4% formaldehyde-agarose gels were prepared with 10 μg of RNA/lane, blotted to Zeta probe membranes (Bio-Rad), and hybridized with ^32P-labeled IL-2Rα, γc, or glyceralde- hyde-3-phosphate dehydrogenase cDNA probes.

**Immunoprecipitations and Western Blotting**—For immunoprecipitations, 2–3 × 10^7 cells were rested for 3–4 h in RPMI 1640 and 1% bovine serum albumin and stimulated with cytokines. Cells were lysed in buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.5 mg/ml sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM protease inhibitor mixture (0.5 mg/ml antipain, 0.5 mg/ml E. coliβ-glucuronidase, 0.75 mg/ml bestatin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin). Immunoprecipitations were performed with 1 μg of anti-JAK1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), 2.5 μg of anti-JAK3 antibodies (Upstate Biotechnology, Saranac NY), or 4 μl of anti-Akt antibodies (Cell Signaling Technology, Beverly, MA) and protein A- or protein G-agarose (Rocky Mountain Biochemicals). Western blots were probed with 4G10 anti-phosphotyrosine (Upstate Biotechnology), anti-phospho-Akt1 (Ser-473), anti-Akt, and Bcl-2 (Cell Signaling Technology), anti-JAK3, or anti-JAK1 (Santa Cruz Biotechnology).

**Flow Cytometry**—Apoptosis assays were performed as described pre-
chimeric receptors were rested for 4 h in serum-free medium and incubated without cytokines ( ), or 5 nM IL-2 ( ), 50 units/ml EPO ( ) ( ). Aspartic acid ( D ) within IL-2R is required for JAK1 activation, and the IL-2RΔABC mutant is truncated after amino acid 312 ( ). YF indicates a tyrosine-deficient subunit of either IL-2R or γc. Although these chimeric receptors do not incorporate IL-2Ra, it is well established that this subunit does not contribute to signal transduction by the IL-2R ( ). Note that similar IL-2R chimeric receptor systems have been described by others ( ). B, expression of EPO chimeras in HT-2 cells. Cytoplasmic mRNA was made from the indicated cell lines and separated on a 1.4% denaturing formaldehyde-agarose gel. The RNA was transferred to a nylon membrane and probed with 32P-labeled cDNAs corresponding to IL-2R, γc, and glyceraldehyde-3-phosphate dehydrogenase (GAPD). Arrows indicate endogenous IL-2Rβ and γc bands. The EPOγF construct migrates faster than the EPOγ construct because of differences in their 3′-untranslated regions. C, activation of JAK1 by chimeric IL-2 receptors. HT-2 cell lines stably transfected with the indicated pairs of IL-2Rc subunits, termed EPOγ, were fused into HT-2 cells and monoclonal cell lines derived by limiting dilution. All constructs were found to be highly expressed, especially compared with endogenous IL-2Rβ and γc levels, as assessed by Northern blotting ( ). To show that chimeric receptors composed of the extracellular domain of the EPO fused to the transmembrane and cytoplasmic tails of the IL-2Rβ and γc subunits, termed EPOγβ and EPOγγ, respectively ( ), most prior studies have confirmed that heterodimerization of the EPOγ and EPOγ cytoplasmic tails via EPO is both necessary and sufficient to direct signaling indistinguishable from the native IL-2R and that neither EPOγ nor EPOγ co-opts endogenous IL-2Rβ or γc subunits for signaling ( , ). Notably, the EPOγ/γ complex represents both IL-2R- and IL-15R-dependent signaling because these receptors both contain the IL-2Rβ and γc subunits ( , ). Henceforth we shall refer to our findings as IL-2-dependent, recognizing that these are presumably the same signals delivered by the IL-15R.

RESULTS

Cytoplasmic Tyrosine Residues within IL-2Rβ Are Not Required for Induction of Bcl-2 or Anti-apoptotic Signaling

To define regions within IL-2R subunits involved in engaging anti-apoptotic signal transduction pathways, we used a chimeric receptor system previously established in a murine, IL-2-dependent T cell line, HT-2 ( ). To bypass signaling by the endogenous IL-2R, HT-2 cells were stably transfected with chimeric receptors composed of the extracellular domain of the EPO fused to the transmembrane and cytoplasmic tails of the IL-2Rβ and γc subunits, termed EPOγβ and EPOγγ, respectively (Fig. 1A). Many prior studies have confirmed that heterodimerization of the EPOγ and EPOγ cytoplasmic tails via EPO is both necessary and sufficient to direct signaling indistinguishable from the native IL-2R and that neither EPOγ nor EPOγ co-opts endogenous IL-2Rβ or γc subunits for signaling ( , ), respectively. Notably, the EPOγ/γ complex represents both IL-2R- and IL-15R-dependent signaling because these receptors both contain the IL-2Rβ and γc subunits ( , ). Henceforth we shall refer to our findings as IL-2-dependent, recognizing that these are presumably the same signals delivered by the IL-15R.

Plasmids encoding the EPO chimeras were stably transfected into HT-2 cells and monoclonal cell lines derived by limiting dilution. All constructs were found to be highly expressed, especially compared with endogenous IL-2Rβ and γc levels, as assessed by Northern blotting ( ). To show that the chimeric receptors activated early responses equivalently, phosphorylation of JAK1 was monitored after treatment with...
EPO or IL-2 (Fig. 1C). Phosphorylation of the JAKs requires dimerization of the IL-2Rβ with γc cytoplasmic tails (15, 22); therefore, EPO-inducible phosphorylation of JAK1 indicates that both chimeric receptors are expressed productively on the cell surface. As shown, JAK1 was phosphorylated equivalently after IL-2 and EPO treatment in all lines tested except HT-2.EPOγ cells (where the mutation at Asp-258 disrupts JAK1 activation (35, 41)) (Fig. 1C). These data indicate that the EPO chimeras expressed in HT-2 cells are competent to deliver EPO-inducible signals essentially equivalent to those induced by the endogenous IL-2R.

IL-2 signaling is known to induce bcl-2 mRNA in hematopoietic cell lines (12, 42, 43), and both the JAK-STAT and MAPK pathways have been linked to control of bcl-2 expression (44–47). However, we found previously that an IL-2R composed of a tyrosine-deficient IL-2Rβ chain paired with a wild type γc receptor (i.e. the chimera EPOβγF/γ) is able to induce up-regulation of bcl-2 mRNA, thus implicating other signaling pathways in bcl-2 expression (44). Therefore, EPO-inducible phosphorylation of JAK1 indicates that signaling could proceed in the absence of γc tyrosines (Fig. 2D). Strikingly, in HT-2.EPOγYF/γ cells, EPO stimulation also caused significant up-regulation of bcl-2 (Fig. 2E). A similar pattern of bcl-2 mRNA induction was observed in HT-2.EPOβγF/γ, HT-2.EPOβ/γF, and HT-2.EPOβ/γ cells (data not shown), suggesting that Bcl-2 regulation occurs at the level of mRNA.

To ascertain whether Bcl-2 levels were associated with productive anti-apoptotic signals, cytokines were removed from the growth medium of HT-2.EPOβγ/γ or HT-2.EPOβγF/γ cells, and cell viability and progression to apoptosis were monitored by flow cytometry. Cells were treated with IL-2 or EPO and then stained with PI to test for viability and annexin V coupled to GFP (GFP-annexin V (37)) to test for apoptosis (Fig. 3). Note that stimulation of parental HT-2 cells with EPO does not trigger a detectable anti-apoptotic signal because these cells do not express endogenous EPO receptors (36). As expected, HT-2.EPOβγ/γ cells exhibited strong protection from apoptosis when incubated with EPO or IL-2. In addition, only a small percentage of the viable cells underwent apoptosis in EPO or IL-2, whereas most of the viable (PI-negative) cells were apoptotic in the absence of cytokines (Fig. 3A). In HT-2.EPOβγF/γ cells, EPO also induced significant protection from apoptosis (Fig. 3B). Similar to our observations with Bcl-2, the protection afforded by EPOβγF/γ was not as vigorous as that by the

**Fig. 2.** Tyrosines within the IL-2Rβ cytoplasmic tail are not required for up-regulation of Bcl-2. HT-2.EPOγ cells (A), HT-2.EPOβ cells (B), HT-2.EPOγF/γ cells (C), HT-2.EPOβγF/γ cells (D), and HT-2.EPOβγF/γ cells (E) were incubated without cytokines (Unstim.), filled histograms), 50 units/ml EPO (solid line), or 10 nM IL-2 (thin solid line) for 24 h. Cells were permeabilized, stained with anti-Bcl-2–FITC antibodies, and analyzed by flow cytometry. The thin dotted line (Control Ig) corresponds to background fluorescence in cells incubated in IL-2 and stained with isotype-matched control antibodies conjugated to FITC. Experiments were performed multiple times with comparable results. Boxed insets, the indicated cell lines were incubated without cytokines (U) or with EPO (E) or IL-2 (2) for 24 h as described above. Whole cell lysates were prepared from equivalent cell numbers, separated on SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to murine Bcl-2.

**Fig. 3.** A tyrosine-deficient IL-2Rβ chain paired with wild type γc protects HT-2 cells from apoptosis caused by IL-2 withdrawal. HT-2.EPOγF/γ cells (A) and HT-2.EPOβγF/γ cells (B) were incubated for 48 h without cytokines (Unstim.) or with 50 units/ml EPO or 10 nM IL-2, as indicated. Cells were stained with PI and GFP-annexin V (37) and analyzed by flow cytometry. The top row of histograms shows the viability of the entire population by PI, and the bottom row shows GFP-annexin V staining of PI-negative (boxed) populations. Data are representative of multiple experiments, and two independently derived cell lines were tested with similar results.
native IL-2R or the wild type EPOβ/γ chimeras. Part of the basis for the decreased survival is likely the reduced Bcl-2 in these cells (Fig. 2), but additional events may also be required to maintain long term survival (48). Together, these results agreed with previous findings that not all signals by the IL-2R are regulated through IL-2Rβ tyrosine residues (12). These data also raised the question of whether Bcl-2 induction and inhibition of apoptosis involve tyrosines on γc or could occur through pathways completely independent of cytoplasmic tyrosines.

γc Tyrosine Residues Are Required for Anti-apoptotic Signaling in the Absence of IL-2Rβ Tyrosines

The mechanism by which the IL-2R controls Bcl-2 expression is poorly defined. Most pathways implicated to date involve signals that depend on tyrosines within IL-2Rβ, such as p38-MAPK and STAT5 (44–47). To determine whether γc tyrosines can trigger additional pathways that regulate Bcl-2, we created cell lines expressing a truncated form of the IL-2Rβ chain (EPOβΔABC) paired with either a wild type γc tail (EPOγ) or a tyrosine-deficient γc tail (EPOγYF). The EPOβΔABC mutant retains the ability to bind and activate JAK1 (Fig. 1C) but does not contain cytoplasmic tyrosine residues or other distal sequences (34). First, we found that the HT-2.EPOβΔABC/γ cells behaved almost identically to the HT-2.EPOβ/YF cells with respect to Bcl-2 induction and survival signaling (Fig. 2 and Fig. 4, A and B). Thus, there do not appear to be “γc-independent” signaling motifs in the IL-2Rβ cytoplasmic tail required for promoting cell survival and Bcl-2 expression.

Strikingly, in the absence of IL-2Rβ tyrosines, γc tyrosines appear to be necessary to mediate up-regulation of Bcl-2. Specifically, treatment of HT-2.EPOβΔABC/γYF cells with EPO failed to up-regulate Bcl-2 detectably (Fig. 4A). This regulation occurred at the level of mRNA because treatment with EPO resulted in increased steady-state levels of bcl-2 message in HT-2.EPOβΔABC/γ cells but not in HT-2.EPOβΔABC/γYF cells (data not shown). These results revealed that although maximal regulation of Bcl-2 probably requires signals from IL-2Rβ tyrosines, signals are also relayed from γc tyrosine residues. Moreover, signals initiated directly from JAK1 and/or JAK3 are not sufficient for Bcl-2 up-regulation (as has been reported for PI3K activation (49)) because HT-2.EPOβΔABC/γYF cells activate these kinases efficiently.

Consistent with the involvement of γc tyrosine residues in up-regulating Bcl-2, we found that EPO stimulation failed to provide a survival signal to HT-2.EPOβΔABC/γYF cells, whereas EPO inhibited apoptosis in HT-2.EPOβΔABC/γ cells (Fig. 4B). As with Bcl-2 expression, the anti-apoptotic signal was reduced compared with that induced through the endogenous IL-2R or EPOβ/γ chimeras. The degree of survival was very similar to that induced in HT-2.EPOβ/YF cells (Fig. 3) and was consistent among independently derived lines. Because HT-2.EPOβ/γYF cells maintain the capacity to up-regulate Bcl-2 and inhibit apoptosis even in the absence of γc tyrosines (Figs. 2 and 4B), at least two distinct pathways are initiated by the IL-2R to regulate Bcl-2. Although IL-2Rβ-dependent anti-apoptotic pathways exist which proceed in the absence of γc tyrosines, at least one anti-apoptotic pathway is mediated through tyrosines within γc. These findings highlight

![Fig. 4. A. Tyrosine residues within γc mediate up-regulation of Bcl-2. HT-2.EPOβΔABC/γ cells (top panel) and HT-2.EPOβΔABC/γYF cells (bottom panel) were incubated without cytokines (Unstim) or with 50 units/ml EPO or 10 nM IL-2, and intracellular staining with anti-Bcl-2-FITC antibodies and Western blots were analyzed as described in Fig. 2. Data are representative of multiple experiments. B, tyrosine residues within γc contribute to anti-apoptotic signaling. The indicated cell lines were incubated without cytokines (Unstim, white bars) or with 50 units/ml EPO (hatched bars) or 10 nM IL-2 (black bars) and costained with PI and GFP-annexin V as described in Fig. 3. Percentages of live cells (% PI-negative) in the total cell population are shown in the top panel. Percentages of apoptotic cells (% GFP-Annexin V-positive cells within the PI-negative population) are shown in the bottom panel. Experiments were performed multiple times, and similar results were obtained in independently derived cell lines.](http://www.jbc.org/Downloadedfrom AT SEMMELWEIS UNIV OF MEDICINE on March 20, 2018)
Anti-apoptotic signaling through γc requires both JAK1 and JAK3. A, JAK1 activity is required for up-regulation of Bcl-2. HT-2.EPO∥D258A∥γ∥ cells were incubated without cytokines (Unstim) or with 50 units/ml EPO or 10 nM IL-2 for 24 h, and intracellular staining and Western blots were performed as described in Fig. 2. Data are representative of multiple experiments. B, JAK1 activity is required for anti-apoptotic signaling. HT-2.EPO∥D258A∥γ∥ and HT-2.EPO∥YF∥γ∥ cells were incubated without cytokines (Unstim, white bars) or with 50 units/ml EPO (hatched bars) or 10 nM IL-2 (black bars). Cells were costained with PI and GFP-annexin V and analyzed by flow cytometry, as described in Fig. 3. Data are representative of several experiments. C, the JAK3-inhibitor WHI-P154 blocks IL-2-dependent proliferation. HT-2.EPO∥β∥γ∥ cells were incubated without cytokines (Unstim, white bars; note that their values are too low to be observed in this graph) or with EPO (50 units/ml) or 5 nM IL-2 (black bars) in the presence of dimethyl sulfoxide (DMSO) or the indicated concentrations of WHI-P154. [3H]Thymidine incorporation was monitored after 48 h (similar results were obtained at 24 h; not shown). Experiments were performed in triplicate, and standard deviations are shown. D, WHI-P154 blocks JAK3 phosphorylation. HT-2 cells were incubated without cytokines (U) or 10 nM IL-2 (2) together with dimethyl sulfoxide or the indicated concentrations of WHI-P154 for 24 h. Cell lysates were immunoprecipitated (IP) with antibodies to JAK3, separated on SDS-PAGE, and transferred to nitrocellulose. Membranes were blotted with antibodies to anti-phosphotyrosine (P-Tyr, 4G10, top panels), then stripped and reprobed with antibodies to JAK3 (bottom panels). E, JAK3 activity is required for Bcl-2 up-regulation through the EPO∥ΔABC∥γ∥ chimera. HT-2.EPO∥ΔABC∥γ∥ cells were incubated without cytokines (Unstim) or with 50 units/ml EPO or 10 nM IL-2 in the presence of dimethyl sulfoxide (top panel) or 20 μg/ml WHI-P154 (bottom panel). After 24 h, Bcl-2 levels were analyzed by Western blotting and flow cytometry as
the complex interplay between the IL-2Rβ and γc chains in controlling cell survival.

**Mechanisms of Anti-apoptotic Signaling**

**JAK1 and JAK3 Activation**—Many signaling pathways have been linked to the IL-2R, all of which depend on activation of JAK1 (for review, see Ref. 50). To confirm that JAK1 was required for the anti-apoptotic signal mediated by γc, HT-2.EPOγ cells were stably transfected with a plasmid encoding EPOγD258A, which cannot activate JAK1 (and consequently JAK3) (15, 34, 41). EPO stimulation of HT-2.EPOγD258A/γ cells failed to induce Bcl-2 or protect cells from apoptosis after cytokine withdrawal (Fig. 5, A and B). In the γc-mediated pathway described here, JAK1 is presumably required for phosphorylation of JAK3 and subsequently EPOγ, thus initiating the anti-apoptotic cascade. In this regard, we have demonstrated that EPOγ is indeed phosphorylated on tyrosine(s) after stimulation of the receptor (data not shown).

In contrast to JAK1, JAK3 has been suggested to be at least partly dispensable for regulation of Bcl-2, both in cell lines and in vivo (31, 51, 52). Therefore, to examine whether JAK3 activity is required for survival signaling mediated by γc tyrosines, we took advantage of a pharmacological inhibitor of JAK3, WHI-P154 (53). First, we confirmed that WHI-P154 inhibits EPO- and IL-2-induced proliferation in HT-2.EPOβ/γ cells, a signal for which JAK3 is known to be crucial (15, 54). Proliferation was blocked completely at a concentration of 20 μg/ml WHI-P154, consistent with a published report (53) (Fig. 5C). Next, we showed that WHI-P154 at this concentration also blocked JAK3 phosphorylation (Fig. 5D). Finally, we found that WHI-P154 blocked Bcl-2 induction and inhibition of apoptosis through the EPOβABC/γ receptor (Fig. 5, E and F). The slight decrease of Bcl-2 in EPO-stimulated cells compared with unstimulated cells in the presence of WHI-P154 was not consistently observed. Based on these results, the anti-apoptotic signaling pathway mediated by γc tyrosines appears to be JAK3-dependent and supports a model in which the JAKs phosphorylate each other and then the γc tail, and γc subsequently recruits downstream anti-apoptotic signaling pathways.

Interestingly, although WHI-P154 completely blocked IL-2-dependent proliferation (Fig. 5C), it only partially blocked anti-apoptotic signals mediated by the endogenous IL-2R (Fig. 5, E and F). It is possible that these signals simply show different sensitivities to this compound. Alternatively, this finding provides some support for previous (albeit controversial) suggestions that a poorly defined, JAK3-independent signaling pathway regulates Bcl-2 (see Fig. 7A and Ref. 55) (31, 51, 52).

**Distal Signals**—Because tyrosines within IL-2Rβ are not involved in this anti-apoptotic signal, we inferred that neither the STAT5A/B nor p38-MAPK pathway was responsible. This hypothesis was confirmed in control experiments showing that HT-2.EPOβABC/γ cells did not activate STAT5 phosphorylation or nuclear import and that inhibitors of various MAPK pathways had no effect on the anti-apoptotic signal or Bcl-2 up-regulation (data not shown). In addition, we ruled out involvement of the nuclear factor-κB pathway because IL-2 signaling does not activate this transcription factor in HT-2 cells.2

A major pathway implicated in anti-apoptotic signaling involves the lipid kinase, PI3K (56, 57; for review, see Ref. 58), which activates the serine-threonine kinase Akt (also called protein kinase B). Because several studies have implicated Akt in anti-apoptotic signaling by IL-2 (59–62), we analyzed Akt activation in this system. HT-2.EPOβ/γ, HT-2.EPOβABC/γ, or HT-2.EPOβABC/γYF cells were stimulated with EPO or IL-2. Whole cell lysates were immunoprecipitated with antibodies specific for the phosphorylated form of Akt (Ser-473) (Fig. 6A). In all cells there was consistently a high background phosphorylation of Akt even after the starving period, a feature common to many T cell lines. As expected, stimulation of HT-2.EPOβ/γ cells with both EPO and IL-2 induced phosphorylation of Akt over basal levels. EPO stimulation of HT-2.EPOβABC/γ cells also triggered significant phosphorylation of Akt, demonstrating that this pathway proceeds without distal sequences and tyrosines within IL-2Rβ. In contrast, HT-2.EPOβABC/γYF cells did not trigger phosphorylation of Akt (Fig. 6B), confirming that tyrosines within γc are necessary for this signal. There was a more rapid decline in phosphorylation of Akt in HT-2.EPOβABC/γ cells compared with HT-2.EPOβ/γ cells, suggesting that tyrosines within IL-2Rβ may be needed to sustain optimal signaling. These data demonstrate that Akt can be at least partly activated independently of tyrosines within IL-2Rβ, and its activation correlates with the anti-apoptotic signaling cascade induced by γc.

Because several studies have shown that Akt contributes to anti-apoptotic signaling in T cells (60, 63), we expected that blocking this pathway would inhibit the anti-apoptotic pathway mediated by γc tyrosines. Indeed, treatment with the PI3K inhibitor wortmannin almost completely prevented induction of Bcl-2 and survival signaling through the EPOβABC/γ receptor (Fig. 6, C and D). However, wortmannin exerted only a mild (though reproducible) inhibitory effect on survival signals induced through the endogenous IL-2R or wild type EPOβ/γ chimeras. Comparable results were obtained using another PI3K inhibitor, Ly294002 (not shown). These findings suggest a requirement for the PI3K/Akt family in anti-apoptotic signaling mediated by γc. Furthermore, because signaling through the wild type receptor is only mildly impaired in the presence of PI3K inhibitors, additional anti-apoptotic pathways independent of PI3K apparently contribute to survival signaling in the context of the intact IL-2R (Fig. 7).

**DISCUSSION**

These data show for the first time that tyrosines within the common γ subunit (γc) play a role in mediating signal transduction, as revealed in the IL-2 receptor system. We have addressed the role of γc tyrosines in survival signaling using chimeric EPOR-IL-2Rα expressed in IL-2-dependent HT-2 cells. The initial impetus for this study was our previous report that a tyrosine-deficient IL-2Rα chain paired with a wild type γc subunit (EPOβYF/γ) stimulates significant enhancement of bcl-2 mRNA, indicating that some intracellular signals can be transmitted independently of IL-2Rβ tyrosines (12). However, that study did not define the region within the IL-2R which mediated this signal, leaving open the possibility that regions within IL-2Rβ or the JAKs might serve to recruit signaling effectors that lead to bcl-2 up-regulation. In the present study, we found that that this signal indeed requires the proximal, JAK1 binding region of IL-2Rβ and JAK1 activity but does not require the IL-2Rβ distal tail or cytoplasmic tyrosine residues. Instead, anti-apoptotic signaling (in the absence of IL-2Rβ described in Fig. 2, F) JAK3 activity is required for survival signaling through the EPOβABC/γ chimera. HT-2.EPOβABC/γ cells were incubated without cytokines (Untreated) or with 50 units/ml EPO or 10 ng IL-2 in the presence of dimethyl sulfoxide or 20 μg/ml WHI-P154. Viability was assessed after 90 h by measuring PI uptake by flow cytometry as described in Fig. 3. Data are representative of multiple experiments.

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S. L. Gaffen, unpublished observations.
tyrosines) depends on tyrosines within γc. Furthermore, the serine-threonine kinase Akt is induced by an IL-2R complex that lacks IL-2Rγ tyrosine residues, and this also requires γc. Consistent with a role for the Akt pathway, γc-mediated up-regulation of Bcl-2 and inhibition of apoptosis are sensitive to PI3K inhibitors. Thus, this work provides the first evidence that γc activates specific signals through its tyrosine residues.

Functions of γc—Surprisingly, very few specific signals to date have been linked directly to γc and none to its tyrosine residues. The primary recognized role of γc is to bind JAK3, enabling JAK3 to phosphorylate JAK1 and other substrates recruited to the receptor complex (for review, see Ref. 14). The biological importance of the γc-JAK3 association is underscored by the finding that γc mutations in many X-severe combined immunodeficiency syndrome patients lie in the JAK3 binding domain and also that JAK3-/- humans and mice exhibit an autosomal immunodeficiency syndrome very similar to X-severe combined immunodeficiency syndrome (for review, see Refs. 10 and 64). Although two tyrosine residues are present in

![Image of a graph showing anti-apoptotic signaling through γc involves the Akt signaling pathway.](http://www.jbc.org/)

**A.** HT-2.EPOβγ cells (lanes 1–4) or HT-2.EPOβABC/γ cells (lanes 5–8) were rested for 4 h in serum-free growth medium and then incubated without cytokines (U) or with 50 units/ml EPO or 10 nM IL-2 for the indicated time periods. Lysates were immunoprecipitated with anti-Akt antibodies, separated by SDS-PAGE on an 8% gel, transferred to nitrocellulose, and Western blots probed with antibodies to the phosphorylated form of Akt (Ser-473, top panel). Blots were stripped and reprobed with anti-Akt antibodies to confirm equivalent loading (center panel). Note that lanes 1–4 are derived from the same gel. To assess band intensities quantitatively, gels were scanned and analyzed using Quantity One software (Bio-Rad). The ratio of intensity of the P-Akt band to the Akt band is presented in graphical form in the bottom panel, with the unstimulated sample for each cell line assigned a value of 1.0. B, tyrosines within γc are required for activation of Akt. HT-2.EPOβABC/γ cells were incubated without cytokines (U) or with 50 units/ml EPO (E) or 5 nM IL-2 (2). Lysates were prepared, immunoprecipitated with antibodies to Akt, and Western blots analyzed as described for A. C, a pharmacological inhibitor of PI3K/Akt blocks up-regulation of Bcl-2 through γc. HT-2.EPOβABC/γ cells were incubated without cytokines (Unstim) or with 50 units/ml EPO or 10 nM IL-2 in the presence of dimethyl sulfoxide (DMSO; top panel) or 8 μM wortmannin (Wm; bottom panel). After 24 h, Bcl-2 levels were analyzed by Western blotting and flow cytometry as described in Fig. 2. Data are representative of multiple experiments. D, a pharmacological inhibitor of PI3K blocks survival signaling through γc. HT-2.EPOβγ or HT-2.EPOβABC/γ cells were incubated without cytokines (white bars), 50 units/ml EPO (hatched bars), or 10 nM IL-2 (black bars) in the presence of dimethyl sulfoxide or 8 μM wortmannin. Viability and apoptosis were assessed as in Fig. 3. Similar results were obtained using another PI3K inhibitor, Ly294002 (not shown). Data are representative of several experiments.
Anti-apoptotic Signals Mediated by γc

FIG. 7. A, model of IL-2 receptor signaling pathways that regulate Bcl-2. Based on our work and that of others, all IL-2-dependent signals appear to be downstream from JAK1 (51), whereas Bcl-2 may be at least partially induced by JAK3-independent pathways (Fig. 5 and Refs. 31, 51, and 52). Previous studies have shown that signals initiated through IL-2Rβ tyrosine residues such as STAT5 and p38-MAPK contribute to Bcl-2 expression (47, 75). This study demonstrates that in the absence of other known signals, activation of Akt can be induced via γc tyrosine residues, leading to Bcl-2 enhancement and prolonged cell survival. B, model of convergent anti-apoptotic signals derived from the IL-2 receptor.

The JAK3 binding region of γc (65), none of the four γc tyrosines is required for JAK3 activation (22, 23, 66, 67). Moreover, the cytoplasmic domain of γc can be functionally replaced with that of a heterologous receptor (the EPOR), which binds to a different Janus kinase (JAK2) with no apparent detriment to signaling (21). Thus, it has long been presumed that tyrosines within γc do not participate in signaling at all, even though γc is phosphorylated after receptor engagement, and its tyrosines and flanking regions are evolutionarily conserved (25). However, the present report reveals a role for γc tyrosines in anti-apoptotic signaling which was not identified previously, apparently because of redundant signals transmitted through IL-2Rβ tyrosines (Fig. 7).

Because γc is shared by multiple receptors, there may be a role for γc tyrosine residues in the context of other cytokine receptors. Similar to the IL-2/15R system, γc tyrosines are dispensable for STAT5 activation by both the IL-7R and IL-9R (66, 67). Interestingly, however, γc tyrosines may play a role in promoting cell growth by IL-9 because proliferation is reduced substantially in HT-2 cells expressing an EPO-IL-9R chimera (EPO9) together with EPOγYF compared with EPO9 paired with EPOγ (66). It will be interesting to determine whether close examination of other γc-containing receptors identifies an anti-apoptotic role for γc which was not recognized in previous analyses.

Convergent Survival Signals—The emerging model of signal transduction is that multiple, overlapping signals converge to create a coherent and integrated cellular response. Because the survival signal induced by γc is neither as potent nor as prolonged as that induced by the wild type IL-2R (Figs. 3 and 4), additional signals induced through IL-2Rβ tyrosines are apparently required for a complete signal. In support of this concept, it was recently shown that cytokines control apoptosis by stimulating glucose metabolism, which acts in concert with anti-apoptotic members of the Bcl-2 family to maintain cellular survival (48, 68, 69). Although ectopic Bcl-2 expression in vivo restores some aspects of T cell signaling when γc is absent (32), enforced Bcl-2 expression alone is not sufficient for all aspects of γc-mediated signaling (70, 71). Similarly, activation of the PI3K pathway by itself is not enough to drive proliferation in a model T cell line (72).

It is probable that at least one complementary signal derives from the p38-MAPK pathway. MAPK is activated through the most membrane-proximal tyrosine within IL-2Rβ (Tyr-338) (27, 29) and leads to transcription of the c-fos gene (11, 12). In turn, c-fos cooperates with STAT5, c-myc, and/or bcl-2 to promote proliferation by IL-2 (43, 72, 73). Similar functional cooperation among the STAT5, MAPK, and PI3K pathways is needed to achieve full oncogenic activity by the BCR/ABL kinase (74). Accordingly, although Bcl-2 is clearly an important target of cytokine-derived signals, it must act in concert with other events to regulate appropriate cellular outcomes.

Regulation of Bcl-2 expression by IL-2 is not well understood, and multiple signaling pathways have been implicated. For example, p38-MAPK regulates bcl-2 gene expression via the transcription factor Aiolos, which binds to specific sites in the bcl-2 promoter (75). STAT5 has also been shown to regulate Bcl-2 in response to IL-2 (47), IL-7, and IL-15 (46). However, because IL-2 can also enhance Bcl-2 expression without participation of the MAPK or STAT5 pathways (Figs. 2 and 4 and Refs. 12, 31, 51, 76), the IL-2R appears to activate a variety downstream signaling pathways that converge on regulation of Bcl-2 (Fig. 7A).

P13K Signaling by the IL-2R—It is still not entirely clear how the IL-2R activates PI3K, which is presumably upstream of Akt. The classic PI3K isomorph is composed of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85a), although several other isomorphs of PI3K exist. Several studies have indicated that p85α is recruited through She and Gab2, via Tyr-338 on IL-2Rβ (73, 77, 78). However, another report implicates IL-2Rβ-Tyr-392 in this process (56). Finally, a recent study suggests that p85α may bind directly to JAK1 (49). However, if there is constitutive association of p85α with JAK1 in HT-2 cells, it is not sufficient to mediate a detectable anti-apoptotic signal in the absence of γc tyrosine residues.
because the EPOΔABC/YF receptors fail to activate Akt phosphorylation and anti-apoptotic signaling (Figs. 4 and 6). Possibly γɛ serves as the first recruitment point for some member of PI3K family and then transfers it to JAK1 for subsequent activation, analogous to a model proposed for JAK3 (41). Other isoforms of PI3K have been identified which may perform equivalent functions to p85/p110. Our attempts to coimmunoprecipitate p85α with γɛ have been unsuccessful, suggesting that the anti-apoptotic signal induced by γɛ may be mediated by an alternative member of the PI3K family.

**Physiological Significance of γɛ-Mediated Signaling**—What is the role of the γɛ-tyrosine-mediated response in vivo? Although no studies have addressed this issue directly, several findings are consistent with this pathway being biologically significant. It is clear that IL-2 and IL-15 play important roles in maintaining immune homeostasis (79–81). IL-2Rβγɛ mice display severe dysregulation in immune homeostasis which results in fatal autoimmunity (82), and they fail to develop natural killer cells (83). Reconstitution studies in which deletion mutants of IL-2Rγɛ were expressed as transgenes in IL-2Rβγɛ–/– mice demonstrated that STAT5 was essential for natural killer cell development, but immune homeostasis was maintained even when receptors that cannot activate STAT5 or MAPK were expressed (84). Consistent with this observation, long term survival and homeostasis of T cells do not require STAT5 but instead correlate with Akt stimulation and up-regulation of Bcl-2 (76). Collectively, these studies imply a role for both γɛ and PI3K/Akt signaling in maintaining immune homeostasis.

IL-2R signaling has been proposed to regulate homeostasis by controlling the persistence of activated peripheral T cells via Fas and FasL expression, which is also regulated in part by STAT5 (80, 81, 85). However, peripheral expression of IL-2Rβγɛ may be dispensable for maintaining T cell homeostasis. When a wild type IL-2Rγɛ chain was expressed as a transgene in the thymus of IL-2Rβγɛ–/– mice, they exhibited little or no autoimmunity even in the absence of detectable peripheral IL-2Rβγɛ (86), suggesting that IL-2 and/or IL-15 plays a role in developing thymocytes to maintain immune homeostasis. In support of this model, IL-2 signaling may be directly involved in negative selection of major histocompatibility complex class II-restricted thymocytes (87). Putting these observations together, our findings provide a mechanism for how IL-2/15R-dependent signals contribute to immune balance. Namely, anti-apoptotic signaling through γɛ (mediated by Akt and Bcl-2) may confer prolonged survival on a subset of developing T cells (e.g. regulatory CD4^+^CD25^+^ T cells (88)), which ultimately restricts autoreactive peripheral T cells.

Another reason for the IL-2R to activate seemingly redundant pathways may be to allow for a continuum of Bcl-2 expression levels. It is clear that alterations in Bcl-2 levels exert potent effects on cellular survival; namely, Bcl-2 overexpression can be tumorigenic, and its underexpression can cause apoptosis (for review, see Ref. 89). In addition, several different signaling cascades influence Bcl-2 expression, such as MAPK (75), STAT5 (46, 47), γɛ-dependent pathways (Fig. 4), and JAK3-independent pathways (51, 52) (Fig. 7A). Perhaps each pathway contributes partially to the levels of Bcl-2, and all are regulated concurrently to ensure that appropriate expression is maintained. This model has precedence in antigen receptors such as the T cell receptor, which encodes multiple, redundant immunoreceptor tyrosine-based activation motifs (ITAMs) that recruit similar signaling intermediates and thereby amplify signaling responses appropriately (20). Thus, the contribution of γɛ pathways to regulation of Bcl-2 is likely to be biologically meaningful.

**Other Contributors to Anti-apoptotic Signaling**—There may be other anti-apoptotic events triggered by γɛ tyrosines which we have not yet identified. First, PI3K can lead to activation of the transcription factor c-myb, which mediates anti-apoptotic effects including regulation of Bcl-2 (46, 90). Second, the PI3K/Akt pathway prevents apoptosis through inhibitory phosphorylation of the forkhead transcription factor, which controls expression of pro-apoptotic factors such as Bim (91). Third, alterations in intracellular pH have been linked to increases in both pro-apoptotic and anti-apoptotic molecules such as Bcl-2 in the IL-7 system (92). Finally, in some cells IL-2 regulates secretion of extracellular apoptotic effector molecules, such as lipocalin (93). It will be interesting to determine whether any of these events is regulated by γɛ tyrosine residues.

In summary, the IL-2R activates numerous signaling pathways, several of which converge to maintain cell survival. We have shown that at least one of these anti-apoptotic signals is triggered through tyrosine residues within γɛ and is mediated by the PI3K/Akt cascade. Importantly, this is the first recognition of a specific signaling role for γɛ tyrosines, although it appears to play a subordinate role to the IL-2Rβγɛ chain in IL-2- and IL-15-dependent signaling. However, given the widespread use of γɛ among cytokine receptors, this pathway is likely to have implications for anti-apoptotic signaling in a variety of contexts.

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