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Regulation of Apoptosis by Tyrosine-Containing Domains of IL-4Rα: Y497 and Y713, But Not the STAT6-Docking Tyrosines, Signal Protection from Apoptosis

José Zamorano and Achsah D. Keegan

IL-4 is a cytokine with important antiapoptotic activity. We have analyzed the role that tyrosine-containing domains within the cytoplasmic tail of IL-4Rα play in IL-4-mediated protection from apoptosis. 32D cells expressing a wt huIL-4Rα or one truncated at aa 557 were protected by huIL-4 from apoptosis while cells expressing a receptor truncated at aa 657 were not, suggesting that the carboxyl-terminal domain signals protection from apoptosis. However, changing Y713 within this region to phenylalanine had no effect. To analyze the contribution of tyrosine-containing domains independently, we transplanted regions of the huIL-4Rα to a truncated form of the huIL-2Rβ that could not signal protection from apoptosis. Transplantation of the huIL-4Rα domains containing Y497 or Y713 partially prevented cell death and together signaled protection from apoptosis in response to IL-2 as well as the wt IL-2Rβ. Mutation of Y497 and Y713 to phenylalanine inhibited protection. In contrast, transplantation of the domain containing the potential STAT6-docking tyrosines alone had no effect, yet it inhibited the protection mediated by the other domains. Although IL-4Rα signals Shc and SH2-containing inositol phosphatase (SHIP) phosphorylation, we could not establish an association between their activation and protection from apoptosis. Taken together, this study suggests that the domains of the huIL-4Rα containing Y497 and Y713 positively regulate protection from apoptosis while the domain containing the STAT6 docking sites suppresses this protection, and that additional signaling molecules other than insulin receptor substrate-1 (IRS1), Shc, or SHIP may be involved in antiapoptotic signaling.


The cytokine IL-4 is produced by T cells, mast cells, and basophils and promotes multiple biologic responses involved in immune regulation (1). IL-4 regulates the expression of several genes (2–4) and the production of IgG1 (5) and IgE (6). In addition, the role of IL-4 in the regulation of apoptosis has been studied in a variety of systems. IL-4 acts as a viability factor and a growth cofactor for B cells, T cells, mast cells, and myeloid cells (1, 7–9). IL-4 has also been shown to decrease the spontaneous apoptosis of cultured B (10) and T cells (11). It also prevents apoptosis induced by anti-Ig treatment (12), Fas ligand (13), or glucocorticoids (11). Additionally, IL-4 is able to prevent cell death in some lymphomas and cell lines (14, 15).

IL-4 mediates its effects by binding to a cell surface receptor complex. This receptor complex consists of the IL-4-binding protein (IL-4Rα) and the γ-chain of the IL-2 receptor complex (16) or alternatively the low affinity IL-13 receptor (17). The IL-4R complex does not contain any consensus sequences encoding enzymatic activity; however, the binding of IL-4 to its receptor complex results in the activation of the Janus family tyrosine kinases Jak1 (18–20) and Jak-3 (18–20). In addition, IL-4 induces the tyrosine phosphorylation of its own receptor (21, 22), the insulin receptor substrates 1 and 2 (22, 23), the adapter protein Shc (24), and STAT6 (25, 26).

The IL-4Rα cytoplasmic domain contains five tyrosine residues and a surrounding amino acid sequence that are 100% conserved among the rat, mouse, and human IL-4Rα (27–30). These are Y497, Y575, Y603, Y631, and Y713 in the huIL-4Rα. We and others have previously defined two different functional tyrosine-containing domains in the huIL-4Rα, the I4R, or growth-promoting, and the STAT6, or gene-induction, domains (31–34). The domain of the huIL-4Rα between aa 437 and 557 (I4R domain) is important for IL-4-induced IRS phosphorylation, cell proliferation, and protection from apoptosis (31, 35). This domain contains Y497 surrounded by a sequence motif (14R-motif) that is homologous to sequences found in the insulin and insulin-like growth factor (IGF)-1 receptors. Mutation of Y497 within the I4R-motif of the IL-4Rα to phenylalanine (F) yields receptors that fail to signal IRS phosphorylation, proliferation, and protection from apoptosis in response to huIL-4 (31, 35). The STAT6 domain, between aa 558 and 657, includes Y575, Y603, and Y631. These tyrosines are STAT6 docking sites and have been implicated in gene regulation (32–34). In addition, the most carboxyl-terminal domain of the huIL-4Rα contains the conserved Y713 surrounded by a proline-rich sequence. However, the role that this domain plays in IL-4R complex signaling has not yet been defined.

We have recently demonstrated that the I4R domain plays a major role in IL-4-mediated protection from apoptosis in IL-3-dependent myeloid cells (35). However, those studies do not preclude a role for other tyrosine-containing domains of the huIL-4Rα in regulating this process. Herein, we investigated the role that these domains play in the signaling of protection from apoptosis in...
response to IL-4. We analyzed the ability of several huIL-4Rα deletion mutants to prevent cell death. To investigate the specific role of an individual domain, we have also transplanted specific tyrosine-containing domains from the huIL-2Rα to the cytoplasmic domain of a truncated form of the huIL-2Rβ that was not able to signal protection from apoptosis. We found that two huIL-4Rα-specific domains containing either Y497 or Y713 positively contribute to IL-4-mediated protection from apoptosis. In contrast, the domain containing the three STAT6-recruiting tyrosine residues would negatively regulate the protective signal mediated by the I4R or the carboxyl-terminal domains. Although we have observed Shc and SHIP phosphorylation in response to IL-4, we could not establish a relationship between the activation of these proteins and the ability of IL-4 to protect cells from apoptosis.

Materials and Methods

Cells and reagents

The IL-3-dependent myeloid cell line 32D expressing IRS1 as a result of transfection (32D/IRS1) was maintained in RPMI 1640 culture medium supplemented with glutamine, penicillin, streptomycin, 5% FCS, and 5% WEHI-3-conditioned medium. The 32D/IRS1 cells expressing the huIL-4Rα wt, and d557, d437, and Y497F mutants have been previously described (31). The 32D/IRS1 cells lacking or expressing the wt and the truncated huIL-2Rβ or the chimeric receptors chim1, chim2, and chim3 have been previously described (32). Recombinant muIL-4 expressed in baculovirus was affinity purified as described (36). Recombinant huIL-4 was purchased from R&D Systems (Minneapolis, MN). Recombinant huIL-2 was a gift from Dr. Steven Rosenberg (National Institutes of Health, Bethesda, MD).

Apoptosis assays

The percentage of apoptotic cells was determined by analyzing the nuclear DNA content by flow cytometry as indicated (10). After culture, 32D/IRS1 cells were resuspended in 0.25 ml of propidium iodide solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Nonidet P-40, and 50 μg/ml RNase (Sigma, St. Louis, MO) and incubated for 30 min at room temperature. DNA content was then analyzed by flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). The apoptotic cells were defined as those with less than 2 N DNA content.

Proliferation assays

The cells were incubated in 96-well plates at 20,000 cells in 0.2 ml of complete RPMI 1640 in the presence or absence of different concentrations of muIL-4 or huIL-4 for 24 h. Cells were pulsed with 1 μCi/well [3H]thymidine for the last 4 h of culture before harvesting using a Packard harvester and the Matrix 9600 direct β count system (Packard Instruments, Downers Grove, IL).

Construction of chimeric receptors and mutations

The truncated huIL-2Rβ containing aa 1 to 378 of the huIL-2Rβ and the chimeric receptors chim1, chim2, and chim3 have been previously described (32). The chimeric receptors chim4, chim5, chim6, and chim7 were made using the same strategy. In addition to the aa 1 to 378 of the huIL-2Rβ, the chim4 receptor contains the aa 466 to 768 of the huIL-4R chain. The chim5, chim6, and chim7 receptors were made combining the different domains of the huIL-4Rα containing chim1, chim2, and chim4, as shown in Figure 4A.

The Y713F mutation in the huIL-4Rα was accomplished using the Altered Sites In Vitro Mutagenesis System from Promega (Madison, WI) following the manufacturer’s protocol. To this end, the oligo 5'-AGTT GCAATGTTCT CCACGACC TTACC was used, in which the original TAC encoding Y713 was changed to TTC (underline) to obtain a phenylalanine. The correct introduction of the Y713F mutation in the huIL-4Rα was verified by the Sanger dyeoxy chain-termination method analyzed on an automated sequencing machine.

Transfections

Cells were washed and resuspended in PBS. For each transfection, 2 × 10⁶ cells were mixed with 2 μg of vector carrying neomycin resistance and 20 μg of chimeric receptor cDNA and subjected to electroporation using a Bio-Rad (Hercules, CA)gene-pulsar set on 200 V and 960 μF. After transfection, cells were cultured overnight in appropriate media before selection with G418 (Life technologies, Grand Island, NY). Neomycin-resistant lines were tested for expression of huIL-2Rβ by FACS analysis using biotin-anti-huIL-2Rβ (Endogen, Boston, MA) followed by streptavidin-phycocerythrin (Southern Biotechnological, Birmingham, AL). All clones used in this study demonstrated equivalent levels of huIL-2Rβ expression (Ref. 30 and data not shown).

Immunoprecipitation and immunoblotting

Analysis of phosphotyrosine-containing proteins was performed as previously described (31). Briefly, cells were starved in RPMI 1640 for 2 h at 37°C. After washing, 20 × 10⁶ cells were resuspended in media (RPMI 1640 plus 5% FCS). Where indicated, they were stimulated with muIL-4 (10 ng/ml), huIL-4 (10 ng/ml), or huIL-2 (200 U/ml) for 10 min. The reaction was terminated by 10-fold dilution in ice-cold PBS containing 100 μM Na3VO4. Cell pellets were lysed in HEPES lysis buffer (50 mM HEPES, 50 mM NaCl, 0.5% Nonidet P-40, 1 mM Na3VO4, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor mixture) and clarified. To detect Shc and SHIP phosphorylation, the soluble fraction was immunoprecipitated with a polyclonal anti-Shc (UBL, Lake Placid, NY) or anti-SHIP (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were suspended in lysis buffer and solubilized in SDS sample buffer. The samples were separated on a 7.5% SDS-polyacrylamide gels before transfer to a polyvinylidene difluoride (PVDF) membrane. The membranes were then probed with monoclonal antiphosphotyrosine Ab, RC20 (Transduction Laboratories, Lexington, KY). To detect IRS1 phosphorylation, the cell lysates corresponding to 3 × 10⁶ cells were separated by SDS-PAGE, and the transferred proteins were immunoblotted with the antiphosphotyrosine Ab. The bound Abs were detected using enhanced chemiluminescence (Amersham, Arlington, IL).

Results

Regulation of apoptosis by the tyrosine-containing domains of the huIL-4Rα

We have previously used the murine IL-3-dependent myeloid cell line 32D, which expresses IRS1 as a result of transfection (32D/IRS1), to analyze functionally important domains of the huIL-4Rα (31, 32, 35). We found that the Y497 in the cytoplasmic tail of the huIL-4Rα was important for signaling tyrosine phosphorylation of IRS1, proliferation, and protection from IL-3 withdrawal-induced apoptosis in response to huIL-4. In addition, we demonstrated that the activation of the IRS1 pathway was partially responsible for IL-4-mediated protection from apoptosis (35). These results established a role for the I4R domain and Y497 in this process. To investigate the role that other domains of the huIL-4Rα play in the protection from apoptosis mediated by IL-4, we cultured 32D/IRS1 cells expressing the wt huIL-4Rα or the deletion mutants d437, d557, or d657 (Fig. 1A) in the presence of media or huIL-4. As a control, muIL-4 was used since 32D cells express endogenous muIL-4 receptors that do not bind huIL-4. The percentage of apoptotic cells was analyzed by measuring nuclear DNA content with propidium iodide. Cells showing less than 2 N DNA content were scored as apoptotic. As we have previously shown (35), 32D/IRS1 cells expressing wt huIL-4Rα or the deletion mutant d557, which contains Y497, were protected by huIL-4 from the rapid onset of apoptosis induced by IL-3 withdrawal (Fig. 1B). Interestingly, those cells expressing the d657 form of the huIL-4Rα, which contains not only Y497 but also Y575, Y603, and Y631, were not protected from death by huIL-4. Cells expressing d657-huIL-4Rα cultured in the presence of huIL-4 had a higher percentage of apoptotic cells (47%) than those cells stimulated with muIL-4 (22%) and similar to levels seen in unstimulated cells (52%). In contrast, engagement of the wt, d557, and d657 receptors by huIL-4 induced comparable proliferative responses in 32D/IRS1 cells (Ref. 31; Fig. 1C). These effects were observed with various concentrations of huIL-4 (Refs. 31, 35; and data not shown). The huIL-4Rα truncated form d437 lacking all tyrosine...
residues signaled neither protection from apoptosis nor cell proliferation (Fig. 1, B and C). These data suggest that the carboxyl-terminal domain of the huIL-4Rα can positively regulate IL-4-induced protection from apoptosis and that the STAT6 domain diminished the protection from apoptosis mediated by the I4R domain in the absence of the carboxyl-terminal domain.

### Effect of Y713F mutation in IL-4 signaling

The carboxyl-terminal domain contains Y713, a conserved tyrosine residue. To investigate the specific role of this tyrosine, we changed Y713 to F and then analyzed the ability of huIL-4 to protect transfected 32D/IRS1 cells from apoptosis. We found that this mutation had no effect in huIL-4α signaling (Fig. 2). Treatment of Y713F-expressing cells with huIL-4 resulted in protection from apoptosis similar to levels of protection seen in muIL-4-treated cells (19% and 21%, respectively, Fig. 2A). Similarly, Y713F-expressing cells proliferated in response to both huIL-4 and muIL-4 (Fig. 2B). Moreover, the Y713F receptor was also able to signal IRS1 phosphorylation (Fig. 2C). By contrast, the Y497F receptor did not signal cell proliferation, protection from apoptosis, or IRS1 phosphorylation (Refs. 31, 35; Fig. 2). These results suggested that the regulation of apoptosis by the carboxyl-terminal domain is independent of the Y713 and IRS1.

### Analysis of Shc and SHIP phosphorylation by huIL-4α

As mentioned above, IL-4 was able to signal protection from apoptosis through IRS-dependent and -independent pathways, with both dependent on Y497. Therefore, it is possible that the regulation of apoptosis mediated by the carboxyl-terminal domain could be subordinate to a signaling mechanism activated through Y497. Since the mutant d657 signals IRS1 phosphorylation (31), but not protection from apoptosis (Fig. 1), other proteins could be involved in the protective effect mediated by the carboxyl-terminal domain. In addition to IRS1, Shc is able to bind the NPXY motif of the I4R domain (37, 38). Furthermore, the carboxyl-terminal domain contains an ITIM motif, IVYSAL, which could theoretically act as a docking site for the SH2 domains of phosphatases such as SHP1 and SHIP (39–43). Interestingly, SHIP has been shown to interact with Shc after cytokine stimulation (43). Therefore, we analyzed the ability of huIL-4Rα mutants to signal Shc and SHIP phosphorylation to determine whether their activation might account for the protection from apoptosis mediated by the carboxyl-terminal domain (Fig. 3). We observed that the wt and the mutants d657 and Y713F of the huIL-4Rα were able to signal Shc phosphorylation (Fig. 3A). By contrast, the mutant receptor Y497F did not promote Shc phosphorylation, as expected, since Shc requires this tyrosine to bind to IL-4Rα (Fig. 3A). These data correlate with the ability of huIL-4Rα to signal proliferation but not protection from apoptosis (see Figs. 1 and 2). On the other hand, all types of huIL-4Rα used, including Y497F and Y713F, signaled SHIP phosphorylation independently of their ability to signal protection from apoptosis (Fig. 3B). These data also indicate that additional sites instead of Y713 can recruit SHIP. Therefore, this analysis did not establish a correlation between the ability of

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**FIGURE 1.** Regulation of apoptosis by different huIL-4Rα constructs. A, Diagram showing the huIL-4Rα constructs. The arrows indicate at which aa the receptor construct ends. B, After IL-3 withdrawal, 32D/IRS1 cells expressing wt, d437, d557, or d657 huIL-4Rα were stimulated with media (squares), 10 ng/ml of muIL-4 (open circles), or 10 ng/ml of huIL-4 (closed circles) for 24 h. Cells were subsequently stained with propidium iodide, and the percentage of apoptotic cells was analyzed by cytometer. Apoptotic cells were defined as those with less than 2 N DNA content as explained in Materials and Methods. C, Cellular proliferation was analyzed in the same cell lines with the same stimulus as in (B). Cells were cultured for 28 h, and [3H]thymidine was added for the last 4 h of culture before harvesting. Results are representative of at least three cell lines expressing each type of receptor.
the carboxyl-terminal domain to signal protection from apoptosis and Shc and SHIP activation.

The I4R and the carboxyl-terminal domains, but not the STAT6 domain, signal protection from apoptosis in a chimeric IL-2/IL-4 receptor system

The inability of the Y713F mutation to affect IL-4 signaling may be due to the strong influence that Y497 has on the 32D cell response to IL-4. Moreover, the carboxyl-terminal domain of the huIL-4Rα contains, in addition to Y713, a proline-rich sequence that could dock potential cellular messengers (27–30). Therefore, we utilized an approach that allowed us to investigate the role that this carboxyl-terminal sequence plays in the protection from apoptosis away from the influence of the I4R domain. We have previously shown that IL-4-specific biologic functions can be transplanted to a truncated form of IL-2Rβ (32). Herein, we transplanted domains of the huIL-4Rα to the cytoplasmic domain of the truncated form of the huIL-2Rβ, and we then analyzed the ability of these chimeric receptors to signal protection from apoptosis (Fig. 4). We transplanted the I4R domain, aa 439 to 555 containing the I4R motif; the STAT6 domain, aa 558 to 657 containing the potential STAT6 tyrosine docking sites; the carboxyl-terminal domain, aa 666 to 768 containing Y713 and the surrounding proline-rich domain; or various combinations of them (Fig. 4A). These huIL-2Rβ/huIL-4Rα chimeric receptors were transfected into 32D/IRS1 cells, and positive clones were selected by FACS analysis using an anti-huIL2Rβ Ab (32). All clones expressed equivalent levels of huIL-2Rβ (Ref. 32; data not shown).

We took advantage of the fact that, although IL-2 was a survival factor for those cells bearing the wt huIL-2Rβ, it did not protect cells expressing the truncated huIL-2Rβ from apoptosis (Fig. 4B). Cells expressing the wt huIL-2Rβ grew equally well in IL-2 or IL-3. By comparison, cells expressing the truncated huIL-2Rβ could not be propagated in the presence of IL-2. They started to die after 24 h of culture, and, by 72 to 96 h of culture, most of the cells were dead (Fig. 4B; Ref. 47). Therefore, the ability of IL-2 to protect 32D/IRS1 cells expressing the chimeric receptors from apoptosis was examined after 86 h of culture (Fig. 4C). We observed that both chimeras 1 and 4, containing the domain surrounding Y497 or Y713, respectively, were partially protected from apoptosis in response to IL-2. On average, only 21% of cells expressing chim1 and 25% of cells expressing chim4 were dead after 86 h of culture in the presence of IL-2 (Fig. 4C-I). Interestingly, cells expressing chim2, containing the STAT6 domain, were not protected from apoptosis at this time by IL-2 even though we have previously observed that this chimera is competent to signal STAT6 activation and CD23 induction (32, 47). The results obtained with the chimeric receptors are consistent with the observations made with the huIL-4Rα deletion mutants (see Fig. 1). Therefore, not only the I4R domain, containing Y497, but also the carboxyl-terminal domain, containing Y713, promoted antiapoptotic signals.
Y497F and Y713F mutations inhibit protection from apoptosis
To analyze the specific role of Y713, we tested the ability of chim1 and chim4 with Y497 and Y713 changed to F (chim1F and chim4F, respectively) to signal protection from apoptosis in response to IL-2 (Fig. 4C-II). Both Y497F and Y713F mutations inhibited the ability of IL-2 to protect transfected cells from apoptosis. However, the effect in cells expressing chim1F was more dramatic. The percentage of apoptotic cells increased from 21% in chim1 to 78% in chim1F. In the case of chim4F, the percentage of apoptotic cells increased from 25% in wt chim4 to 56% in chim4F. These data suggest that, in addition to Y713, other sequences in the carboxyl-terminal domain may be involved in signaling protection from cell death.

The STAT6 domain inhibits the protection from apoptosis mediated by the I4R and the carboxyl-terminal domains
Since the results obtained with the d657 mutant of the huIL-4Rα suggested that the STAT6 domain could diminish the protection from apoptosis mediated by the I4R domain, we sought to determine whether the STAT6 domain could also modulate the protection from apoptosis mediated by the I4R and carboxyl-terminal domains in the chimeric receptor system. We used a chimeric receptor containing the I4R domain plus the STAT6 domain of the huIL-4Rα (chim3) or the STAT6 domain plus the carboxyl-terminal domain (chim5). Similar to the huIL-4Rα deletion mutant d657, the addition of the STAT6 domain inhibited the ability of the I4R domain (chim3) to protect cells from apoptosis (Fig. 4C-III) even though this chimera is perfectly competent to induce the tyrosine phosphorylation of IRS1 (32). On average, 72% of cells expressing chim3 stimulated with IL-2 were dead while only 21% of cells expressing chim1 were dead. The STAT6 domain could also inhibit partially the protection from apoptosis mediated by the carboxyl-terminal domain (chim5) stimulated by IL-2. These data indicate that the domain of the huIL-4Rα containing the STAT6 docking site can play a negative role in the protection from apoptosis mediated by IL-4.

The I4R and carboxyl-terminal domains cooperate in protecting from apoptosis
Since both domains containing Y497 and Y713 were able only partially to protect cells from apoptosis, we next analyzed whether they cooperated in this process. To this end, we made a chimera containing both the I4R domain and the carboxyl-terminal domains of the huIL-4Rα in the absence of STAT6 domain (chim6). This chimera-mediated protection from apoptosis in response to IL-2 to the same degree as wt huIL-4Rα or another chimera that contains all tyrosine residues (chim7) (Fig. 4C-IV). For both chim6 and chim7, the percentage of apoptotic cells was similar to the percentage observed in cultures stimulated with IL-3 with only ~5% of apoptotic cells. Therefore, the cooperation between the I4R and the carboxyl-terminal domains gives maximal protection from apoptosis.

Analysis of IRS1 phosphorylation in the chimeric system
Finally, we examined the correlation between the ability of the different chimeric receptors to signal IRS1 phosphorylation, which was associated with protection from apoptosis (35), and their ability to protect cells from death. In the chimeric system, we did not analyze Shc and SHIP since the truncated huIL-2Rβ was able to signal their phosphorylation as well as the wt huIL-2Rβ (J.Z., unpublished data). We have previously shown that transplantation of the I4R domain (chim1), but not the STAT6 domain (chim2), to the truncated huIL-2Rβ could signal IRS1 phosphorylation in response to IL-2 (32). Herein, we found that chim4 containing only Y713 was not able to signal IRS1 phosphorylation in response to IL-2 (Fig. 5). By contrast, all chimeric constructs containing Y497, chim1, chim6, chim7 (Fig. 5), and chim3 (32), could induce the tyrosine phosphorylation of IRS1. Therefore, while the ability of...
the I4R domain to protect from apoptosis is linked to its ability to activate IRS1, the protection from apoptosis mediated by the carboxyl-terminal domain is not related to the induction of IRS1 phosphorylation.

Discussion

IL-4 is able to protect a variety of cell types from apoptosis; however, the mechanisms that IL-4 uses to accomplish this important...
response are not completely defined. In a previous study, we reported that the I4R-motif plays a crucial role in IL-4-mediated protection from apoptosis (35). We demonstrated that IL-4 uses at least two different pathways to protect cells from apoptosis, and that one of them is mediated by IRS. Both pathways are dependent on Y497 within the I4R motif. However, these studies did not exclude a role for other domains of IL-4Rα in this process. In the present study, we provide evidence that the STAT6 and the carboxyl-terminal domains of the cytoplasmic tail of IL-4Rα are also involved in the regulation of protection from apoptosis mediated by IL-4.

The results presented in this study confirm the importance of Y497 in IL-4 signaling. As we have previously shown (35), either the huIL-4Rα deletion mutant lacking Y497 or the mutation Y497F completely eliminate the ability of huIL-4 to protect cells from apoptosis. Furthermore, we also show that the transplantation of the I4R-motif to the truncated huIL-2Rβ confers protection from apoptosis in response to IL-2 and that this protection is also dependent on Y497. The ability of this domain to prevent cell death is correlated with its ability to signal IRS1 activation. In addition, these data indicate that the protection from apoptosis mediated by the I4R domain is negatively regulated by the STAT6 domain and positively by the carboxyl-terminal domain of the huIL-4Rα.

These results suggest that the STAT6 domain of IL-4Rα can play a negative role in protecting from apoptosis. This effect was first manifested in the huIL-4Rα deletion mutants. The huIL-4Rα mutant d657, which contains both the I4R and the carboxyl-terminal domains, did not protect cells from apoptosis, while d557, which contains only the I4R domain, was able to do so. Although structural conformational changes in the huIL-4Rα constructs could be responsible for these differences, it is unlikely, since the d657 receptor was able to signal the phosphorylation of IRS1 (31), Shc, and SHIP (Fig. 3), induction of STAT6-DNA binding activity (34), and cell proliferation (31). In addition, the STAT6 domain blocked protection from apoptosis mediated by the I4R in the IL-2/IL-4 receptors chimeric system, and to a lesser extent, the protection mediated by the carboxyl-terminal domain. One possible explanation is that the STAT6 domain activates a pathway that negatively regulates protection from apoptosis (Fig. 6A). This pathway would be counteracted by signals delivered simultaneously through the I4R and carboxyl-terminal domains. The role that STAT6 plays in regulating apoptosis has not yet been defined; however, cell viability has not been reported to be affected in STAT6 knockout mice (44, 45), and we have observed that B lymphocytes and stable cell lines derived from these mice were...
well protected from apoptosis by IL-4 (I.Z., unpublished observation). In addition, chim2, which is able to induce STAT6 activation (47), was not able to signal protection from apoptosis. These data suggest that STAT6 does not mediate protection from apoptosis. Nevertheless, it is possible that additional proteins might interact with this domain of IL-4Rα and regulate apoptosis.

Our data also indicate that the carboxyl-terminal domain has a protective role in the regulation of apoptosis. First, huIL-4Rα lacking this domain (d657) did not signal protection from apoptosis. Second, transplantation of this domain to the truncated IL-2Rβ conferred protection from apoptosis. Although the Y713F mutation had no effect on protection from apoptosis in the context of the full length huIL-4Rα, such a mutation partially inhibited protection from apoptosis in the chimeric receptor system, indicating that Y713 can be involved in this process. In the full length huIL-4Rα, this mutation could still allow partial protein binding, perhaps through its proline-rich motifs, which could be enough to induce a positive response to IL-4. The importance of Y713 is suggested by data indicating that Y631, Y713, or Y821 may be the major phosphorylation sites on the huIL-4Rα (46). In addition, this tyrosine is surrounded by proline-rich residues, which suggests that they may be a docking site for intracellular proteins (27–30). Therefore, it would be of interest to determine whether this domain docks a protein that could be involved in apoptotic processes. Interesting candidates were SHIP and SHP-1, which could theoretically bind the ITIM motif, including Y713 (27–30, 37–41); however, we have detected SHIP phosphorylation in the absence of this domain, and we have not been able to detect SHP-1 regulation by IL-4 in these cells (J.Z. and A.D.K., unpublished observations).

The fact that Y497F mutation, but not Y713F, completely blocked protection from apoptosis and that the STAT6 domain inhibited the protection mediated by either the I4R or carboxyl-terminal domains suggests that the pathways activated through the domain containing Y713 may be subordinated to Y497. We have recently shown that the protection from apoptosis mediated by IL-4 is regulated by IRS1-dependent and -independent mechanisms (35). Since the mutant d657 signals IRS1 phosphorylation but not protection from apoptosis, additional protein(s) may be involved in the protection from death mediated by this domain. Y713 is included in the ITIM sequence IVYSAL, which could potentially dock SHIP, an inositol 5-phosphatase (39) containing two NPYX sequences (43) that could potentially be docking sites for the phosphoryrosyne-binding (PTB) domains of IRS1. In addition, SHIP has been shown to interact with Shc after cytokine stimulation (43). Moreover, Shc can bind through its phosphoryrosyne-binding domain to the I4R motif of IL-4Rα (37, 38), and thus, it could, at least theoretically, mediate the IRS1-independent pathway that IL-4 uses to protect cells from apoptosis. Therefore, a network could be established between IRS1, Shc, and SHIP after IL-4 stimulation (43). Moreover, Shc can bind through its phosphotyrosine motif docking sites for the phosphotyroline-binding (PTB) domains of IRS1. In addition, Shc has been shown to interact with Shc after cytokine stimulation (43). Moreover, Shc can bind through its phosphotyroline-binding domain to the I4R motif of IL-4Rα (37, 38), and thus, it could, at least theoretically, mediate the IRS1-independent pathway that IL-4 uses to protect cells from apoptosis. Therefore, a network could be established between IRS1, Shc, and SHIP after IL-4 stimulation (43). However, we have not found a direct correlation between Shc or SHIP phosphorylation and protection from apoptosis. Nevertheless, it appears that some cooperation between the I4R and the carboxyl-terminal domains is necessary to overcome the inhibition promoted by the STAT6 domain.

The data presented herein indicates that all tyrosine-containing domains of the cytoplasmic tail of IL-4Rα participate in the regulation of apoptosis by IL-4. In addition, they show that the signals promoted by each domain are influenced by the others, and the nature of the response depends on the balance between them. In Figure 6, we show two hypothetical models that illustrate this process. Signals delivered from Y497 activate IRS and an additional pathway, which could be through a molecule such as Shc, that mediate protection from apoptosis. Simultaneously, an unknown pathway is activated through the carboxyl-terminal domain, which also promotes cell survival. Finally, an inhibitory pathway is activated through the STAT6 domain that could block the protective signal mediated by the other domains. When the three domains are activated, the receptor transmits a protective signal; however, the STAT6 domain could block the protective effect when one of the positive signals is off (Fig. 6A). Alternatively, a novel protein involved in antiapoptotic signals could require both the I4R and the carboxyl-terminal domains to dock to the receptor and perhaps bridge Y497 and Y713 via a dual docking site (Fig. 6B). Since we have not found a relationship between the activation of known mediators other than IRS1 (35) and protection from apoptosis, it will be interesting to determine whether additional proteins are involved in IL-4R signaling protection from apoptosis.

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