98 to 1027) was synthesized by PCR (*23*) with primers that incorporated in-frame Nco 1 and Bam H1 sites at the 5′ and 3′ ends of the DNA, respectively. The DNA was inserted into the Nco 1 and Bam H1 sites of pET21d (Novagen, Inc.). The pET21d-IAP34 plasmid was transformed into *E. coli* BL21 (DE3) and expression was induced with IPTG as recommended by the suppliers. The inclusion body fraction containing IAP34 was purified from *E. coli* by the lysis and TX-100–washing method (*14*). IAP34 constituted approximately 50% of the total protein in the inclusion body fraction as estimated by Coomassie blue staining. The IAP34 was not further purified for the [α-^{32}P]GTP overlay assay.

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Prevention of T Cell Anergy by Signaling Through the γ_c Chain of the IL-2 Receptor

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When stimulated through their antigen receptor without requisite costimulation, T cells enter a state of antigen-specific unresponsiveness, termed anergy. In this study, signaling through the common γ chain of the interleukin-2 (IL-2), IL-4, and IL-7 receptors in the presence of antigen was found to be sufficient to prevent the induction of anergy. After culture with IL-2, IL-4, or IL-7, Jak3 kinase was tyrosine-phosphorylated, which correlated with the prevention of anergy. Therefore, a signal through the common γ chain may regulate the decision of T cells to either clonally expand or enter a state of anergy.

Although little is known about the molecular mechanisms responsible for the induction and maintenance of anergy (*1*), the critical costimulatory signal necessary to prevent the induction of anergy is probably mediated through the CD28 molecule on the T cell surface (*2–4*). After T cell receptor (TCR) signaling, ligation of CD28 by either of its natural ligands, B7-1 or B7-2, induces secretion of a number of cytokines, most notably IL-2, which results in T cell clonal proliferation and effector function (*5*). In the absence of CD28-mediated costimulation, addition of exogenous IL-2 during TCR activation can also prevent the induction of anergy (*6–8*). Therefore, the critical signal necessary to prevent anergy might not be mediated directly through CD28, but might be delivered by signaling through the IL-2 receptor (IL-2R). However, T cells from IL-2– and CD28-deficient mice are neither anergic nor severely defective in function (*9*), which suggests that additional signals, potentially delivered through one or more cytokine receptors, might also be capable of preventing the induction of anergy.

To address this issue, human T cell clones specific for the human leukocyte antigen class II molecule HLA-DR7 were stimulated with cell lines that expressed different costimulatory molecules (Fig. 1). All experiments were done with at least two T cell clones. T cells were stimulated with the Epstein-Barr virus (EBV)–transformed B lymphoblastoid cell line LBL-DR7, which is homozygous for HLA-DR7 and expresses B7-1, B7-2, LFA-1, LFA-3, and ICAM-1, with NIH 3T3 fibroblasts transfected with HLA-DR7 alone (t-DR7), or with NIH 3T3 fibroblasts transfected with both HLA-DR7 and the B7-1 costimulatory molecule (t-DR7.B7-1). HLA-DR7–specific alloreactive T cell clones stimulated with either LBL-DR7 or t-DR7.B7-1 cells proliferated in response to secondary rechallenge with LBL-DR7 cells. In contrast, when T cell clones were first cultured with either LBL-DR7 cells in the presence of α fusion protein consisting of the extracellular domain of CTLA4 and an immunoglobulin G (IgG) chain (CTLA4-Ig) (*3*) (to block B7 family–mediated costimulation) or with t-DR7 cells, they were anergized and did not respond when rechallenged with LBL-DR7 cells. Addition of IL-2, IL-4, or IL-7 to the primary culture of T cell clones with either LBL-DR7 cells plus CTLA4-Ig or with t-DR7 cells prevented the induction of anergy (Fig. 1, A and B). In contrast, addition of various concentrations of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), IL-6, IL-10, or IL-12 to the primary culture of T cell clones with either LBL-DR7 cells plus CTLA4-Ig or with t-DR7 cells alone did not prevent the induction of anergy. However, IFN-γ, IL-6, IL-10, and IL-12 each alone induced proliferation of the T cell clones (*10*), which shows that proliferation

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in the primary culture does not necessarily prevent anergy (*3*, 6, 8, *11*).

Because the human receptors for IL-2, IL-4, and IL-7 share a common γ chain (γ_c) (*12*), and mutations in γ_c result in human X-linked severe combined immunodeficiency disease (SCID) (*13*), we investigated whether γ_c signaling during primary culture is associated with the prevention of anergy. We used specific monoclonal antibodies (mAbs) to the α and β chains of the IL-2 receptor, to the conventional receptors for IL-4 and IL-7, and to γ_c (*14*). T cell clones were cultured with either LBL-DR7 cells plus CTLA4-Ig (Fig. 2A) or with t-DR7 cells (Fig. 2B) with or without mAbs that were cross-linked with rabbit antibodies to mouse Ig. Cross-linking of γ_c during the primary culture prevented the induction of anergy and resulted in both proliferation and IL-2 secretion during rechallenge, a response that was comparable to that observed with nonanergized control cells (Fig. 2, A and B). However, cross-linking of either IL-2Rα, IL-2Rβ, or conventional chains of IL-4R or IL-7R during the primary culture did not. Thus, cross-linking of γ_c in the presence of TCR signaling was sufficient to prevent the induction of anergy. These data support the hypothesis that the common effect of IL-2, IL-4, and IL-7 in preventing the induction of anergy is mediated through a γ_c signaling pathway.

To examine whether a common signaling pathway associated with γ_c could be identified after IL-2, IL-4, and IL-7 stimulation, T cell clones were cultured with media, IL-2, IL-4, or IL-7, and cell lysates were immunoprecipitated either with a mAb to γ_c or to IL-2Rβ or with an isotype-matched control mAb. After immunoprecipitations with a mAb to γ_c, a protein immunoblot with a mAb to phosphotyrosine revealed phosphorylation of both the 64-kD band of γ_c and a coprecipitated 116-kD band (Fig. 3A) in the presence of IL-2, IL-4, or IL-7. Thus, γ_c was physically associated with a 116-kD molecule that was cophosphorylated with γ_c by IL-2, IL-4, and IL-7 stimulation. In contrast, when a mAb to IL-2Rβ was used for immunoprecipitation, protein immunoblotting with antiphosphotyrosine showed that IL-2Rβ was associated with a 116-kD band that was phosphorylated in the presence of IL-2 and IL-4 (Fig. 3B).

The Jak family tyrosine kinases (*15*) associate with cytokine receptors (*16*). Signaling by IL-2 and IL-4 is associated with Jak1 and Jak3 (*16*–*19*). Because signaling through IL-2, IL-4, and IL-7 receptors resulted in the phosphorylation of the γ_c-associated 116-kD protein, we investigated whether this 116-kD phosphorylated substrate was a Jak kinase family member, using a polyclonal antiserum (R80) to the functional COOH-terminal kinase domain (JH1) of the Jak family members (*19*). Blotting with R80 after immunoprecipitation with the mAb to γ_c showed that the 116-kD band that was coprecipitated with the γ_c was recognized by R80 (Fig. 3A) and therefore was a member of the Jak kinase family. Reblotting of the immunoblot with peptide-specific antisera to Jak1 (Fig. 3A), Jak2 (*10*), or Jak3 (Fig. 3A) showed that the 116-kD band was recognized only by anti-Jak3. Blotting with antisera to common Jak (R80), Jak1, or Jak3, after immunoprecipitation with the mAb to IL-2Rβ, showed that IL-2Rβ was associated with Jak1, which was tyrosine-phosphorylated in the presence of IL-2 and IL-4; only in the presence of IL-2 did IL-2Rβ become associated with Jak3 (Fig. 3B). To determine whether Jak3 and γ_c were constitutively associated, T cell clones were treated with media, IL-2, IL-4, or IL-7 and then immunoprecipitated with either anti-γ_c or anti-Jak3 (Fig. 3C). Immunoblots with anti-Jak1 and anti-Jak3 showed that Jak3 but not Jak1 was coprecipitated with γ_c under all of the above culture conditions. However, immunoblots with a mAb to phosphotyrosine revealed that Jak3 was phosphorylated to a much greater extent in the presence of the cytokines.

Because γ_c signaling was associated with both phosphorylation of Jak3 kinase and the prevention of anergy, we examined whether Jak3 kinase was phosphorylated under various conditions that resulted in or prevented the induction of anergy. Culture of T cell clones with t-DR7.B7-1 cells but not t-DR7 cells resulted in phosphorylation of Jak3 (Fig. 4A). In contrast, culture of T cell clones with t-DR7 cells in the presence of IL-2, IL-4, or IL-7, but not TNF-α or IL-12, not only prevented the induction of anergy but also resulted in phosphorylation of Jak3 kinase (Fig. 4B). Immunoblotting with antiserum to Jak3 showed that Jak3 was constitutively expressed in equivalent amounts under all conditions (Fig. 4, A and B).

Although anergized cells cannot proliferate in response to TCR stimulation and

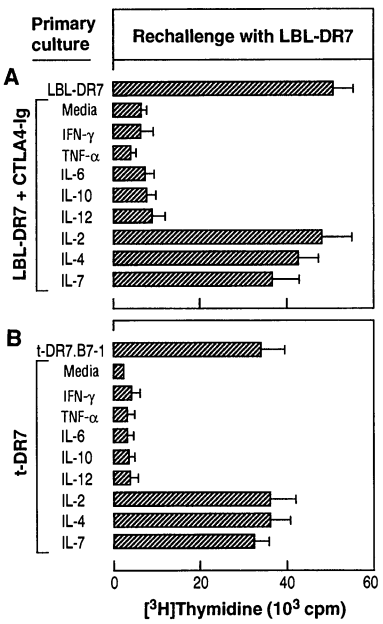

Fig. 1. Prevention of anergy induction by cytokines in T cell clones. HLA-DR7 alloantigen-specific T cell clones (*31*) TC-3 and TC-4 ($CD4^+$, $CD8^-$, $CD28^+$, and $B7^-$) were incubated in primary culture with (**A**) LBL-DR7 cells or LBL-DR7 cells plus CTLA4-Ig (10 μg/ml), either in media or in the presence of each of the indicated cytokines; or (**B**) t-DR7.B7-1 cells or t-DR7 cells either in media or in the presence of each of the indicated cytokines. Concentrations of cytokines: IL-2, 50 IU/ml (Genzyme); IL-4, 5 ng/ml (Genzyme); IL-6, 30 ng/ml (Genzyme); IL-7, 10 ng/ml (Genzyme); IL-12, 10 U/ml (Genetics Institute, Cambridge, Massachusetts); TNF-α, 500 U/ml (Genzyme); and IFN-γ, 500 IU/ml (Biogen, Cambridge, Massachusetts). Each population was subsequently rechallenged with LBL-DR7 stimulators in secondary culture (*31*). Samples were cultured and proliferation was measured by [^{3}H]thymidine (1 μCi) incorporation as previously described (*11*). Results, expressed as the means of triplicate cultures, represent response during rechallenge and are representative of five experiments with the same clone. The identical pattern of results was obtained with both TC-3 and TC-4 clones. Use of control Ig instead of CTLA4-Ig during primary culture did not result in anergy but in a competent immune response on rechallenge (*10*).

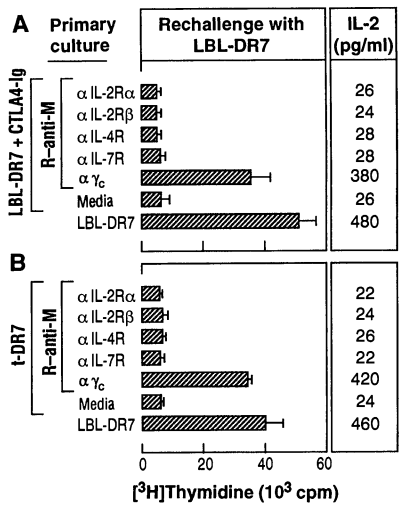

Fig. 2. Cross-linking of the common γ chain of the T cell clone in the presence of TCR signaling. During primary culture, the TCR signal was delivered by either (**A**) LBL-DR7 cells plus CTLA4-Ig or (**B**) t-DR7 cells. IL-2Rα, IL-2Rβ, private chains of IL-4R and IL-7R, and γ_c were cross-linked with specific mAbs and rabbit antibodies to mouse immunoglobulin (R–anti-M) (*25*). Primary culture and rechallenge were done as described (*31*). Results are representative of three experiments with specific mAbs and R–anti-M cross-linking and of two experiments with biotinylated mAb and streptavidin cross-linking, done with the same clone. An identical pattern of results was obtained with both the TC-3 and TC-4 clones.

costimulation provided by competent antigen-presenting cells, these cells can proliferate in response to exogenous IL-2; thus, their IL-2R signaling pathway is intact (*3*, *6*–*8*, *11*). In addition, our anergized T cell clones also proliferate in the presence of IL-4 and IL-7 (*10*). To determine whether γ_c signaling is intact in anergized cells, T cell clones anergized with t-DR7 were cultured with media, IL-2, IL-4, or IL-7 and then immunoprecipitated with antiserum to Jak3 and immunoblotted with mAb to phosphotyrosine (Fig. 4C). IL-2, IL-4, and IL-7 induced Jak3 tyrosine phosphorylation in anergized cells. Thus, γ_c signaling was intact in anergized cells.

Our data show that γ_c signaling correlates with prevention of anergy. Although γ_c signaling is associated with both prevention of anergy and phosphorylation of the Jak3 kinase, it is unknown whether other kinases are also important in γ_c signal transduction. Regardless of the distal signaling mechanisms, the functional outcome of γ_c cross-linking is distinct, because cross-linking of other receptor chains did not induce this functional effect. Nevertheless, this finding does not exclude a role for other γ_c-associated proteins in the cytoplasmic signaling process (*20*), because cross-linking of surface γ_c with specific monoclonal antibodies may also result in the cross-linking of associated structures.

These results and the previous observation that congenital mutations of human γ_c result in X-linked SCID (*13*) underscore the central role of γ_c in the regulation of T cell survival and function. Neonates with γ_c-deficient X-linked SCID lack T cells. Whether they are incapable of differentiating from hematopoietic precursors or are anergized and subsequently deleted by apoptosis (*3*) because of their inability to receive a γ_c signal is unknown. Participation of γ_c in maturation may also explain the observations that T cells isolated from IL-2–, IL-4–, and CD28-deficient mice (*9*, *21*) differentiate normally. Because CD28 costimulation both induces IL-2 accumulation and augments IL-2R expression, this pathway is highly efficient in preventing the induction of anergy. IL-4, IL-7, or other cytokines such as IL-13 (*22*) or IL-15 (*23*) that are capable of signaling through γ_c might be equally efficient at preventing the induction of anergy in other microenvironments.

After T cell receptor signaling, a critical signal necessary to prevent the induction of the anergic state is mediated through the γ_c chain of the IL-2, IL-4, and IL-7 receptors. Moreover, the γ_c chain is constitutively associated with Jak3 kinase, and phosphorylation of Jak3 is associated with the prevention of anergy. The importance of our findings is not that the common γ_c was associated with IL-2, IL-4, or IL-7 signaling, nor that these cytokines induced the phosphorylation of Jak3 kinase, but instead that the functional outcome of these events prevented T cells activated through their antigen receptor from entering an anergic state. Although the absence of Jak3 phosphorylation is associated with the induction of anergy, this is not the molecular defect responsible for the antigen-specific unresponsiveness. As has been previously suggested, the molecular defect responsible for the anergic state appears to be located along the TCR signaling pathway (*24*). Therefore, γ_c signaling may prevent the loss of a substrate critical for TCR signaling or it may dilute a negative factor responsible for the anergic state (*6*). Taken together, our results provide a function for γ_c and Jak3 and, more importantly, identify a signaling pathway that helps begin to decipher the molecular mechanism of T cell anergy.

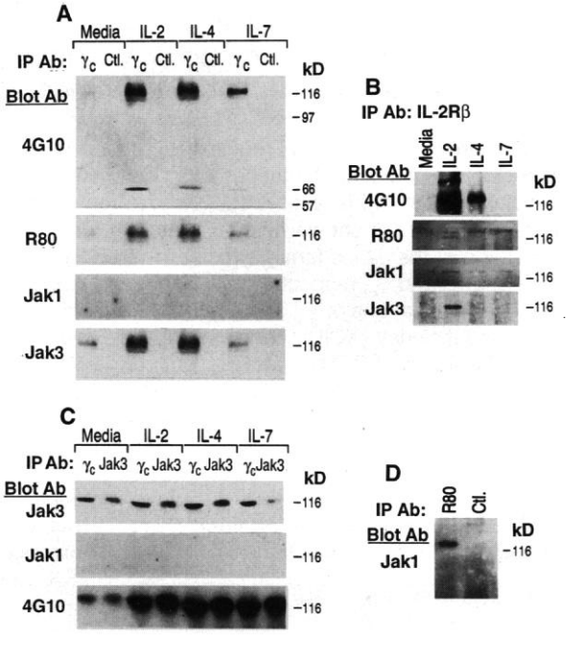

Fig. 3. (**A**) Jak3 kinase coprecipitation with γ_c and phosphorylation after incubation with IL-2, IL-4, or IL-7. Alloantigen-specific human helper T cell clones were incubated overnight in serum-free media without IL-2 and subsequently stimulated for 15 min with media, IL-2, IL-4, IL-7, TNF-α, or IL-12. Cells were lysed and immunoprecipitations (IP) were done (*32*) with mAb to γ_c or with isotype-matched control (Ctl.) mAb. Proteins transferred to nitrocellulose membranes (*32*), and the blots were incubated with antiphosphotyrosine (mAb 4G10 at 1:2000) (Upstate Biotechnology, Lake Placid, New York). Membranes were stripped (*33*), blocked, and reprobed with antiserum to common Jak (R80) (*19*) (at 1:1000), antiserum to Jak1 (Upstate Biotechnology), or antiserum to Jak3 (*18*) (at 1:2000). Detection was done with horseradish peroxidase–conjugated antibody to rabbit IgG (at 1:5000) (Amersham) and enhanced chemiluminescence (*32*). (**B**) Jak1 association with IL-2Rβ and Jak3 phosphorylation and association with IL-2Rβ in the presence of IL-2. T cell clones were stimulated with media, IL-2, IL-4, or IL-7 as above and subsequently lysed and immunoprecipitated with mAb to IL-2Rβ. Blotting of the membrane was done with mAb to phosphotyrosine (4G10). Immunoblots were stripped (*33*) and reprobed with R80, Jak1, or Jak3 antisera. (**C**) Jak3 kinase constitutive association with γ_c and induced phosphorylation by IL-2, IL-4, or IL-7. Alloantigen-specific human helper T cell clones were incubated overnight in serum-free media without IL-2 and subsequently stimulated for 15 min with media, IL-2, IL-4, or IL-7. Cells were lysed and then immunoprecipitated with mAb to γ_c or antiserum to Jak3. Immune complexes were resolved by SDS-PAGE, transferred on nitrocellulose membranes, and blotted with antisera to Jak3. Immunoblots were then stripped and reprobed with Jak1 antiserum or mAb to phosphotyrosine (4G10). Cell lysis, immunoprecipitation, blotting, immunodetection, stripping, and reprobing of the immunoblots were done as described (*32*, *33*). (**D**) Detection of Jak1 by blotting with Jak1-specific antiserum after immunoprecipitations with common Jak antiserum (R80) from lysates of the CTLL-2 cell line (used as a positive control for blotting with Jak1 antiserum).

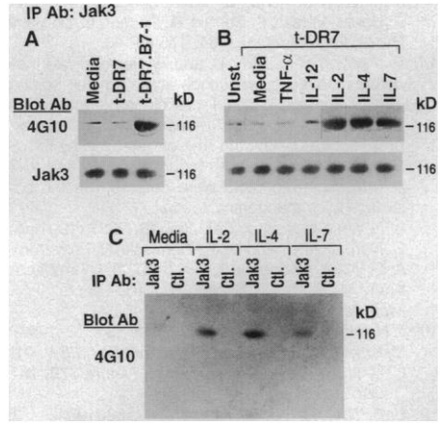

Fig. 4. Association of prevention of anergy with phosphorylation of the Jak3 kinase. Alloantigen-specific human helper T cell clones were cultured with (**A**) media, t-DR7 cells (anergizing conditions), or t-DR7.B7-1 cells (nonanergizing conditions), or with (**B**) t-DR7 cells in the presence of various cytokines, as indicated, for 24 hours. T cells were isolated from transfectants by Percoll gradient. Cell lysates were prepared, immunoprecipitated with the Jak3 antiserum, and analyzed by immunoblot with mAb to phosphotyrosine (4G10) as in Fig. 3. Immunoblots prepared under identical conditions were probed with antiserum to Jak3. (**C**) IL-2, IL-4, or IL-7 can induce Jak3 kinase phosphorylation in the anergized cells. Anergized alloantigen-specific human helper T cell clones were cultured for 15 min with media, IL-2, IL-4, or IL-7. Cells were lysed and then immunoprecipitated with antiserum to Jak3 or control antiserum. Immunoblot analysis with mAb to phosphotyrosine (4G10) was done as in Fig. 3.

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25. IL-2Rα (IgG2a; from Coulter, Hialeah, FL) (*26*), IL-2Rβ (IgG; from Coulter) (*27*), IL-4R (IgG1; from Genzyme, Cambridge, MA) (*28*), IL-7R (IgG1; from Genzyme) (*29*) or γ_c (IgG1) (*30*), and R–anti-M IgG (Jackson ImmunoResearch, West Grove, PA) were all used at the saturating concentration of 10 μg/ml. Identical results were obtained when biotinylated γ_c antibody was used and cross-linking was done with streptavidin (10 μg/ml), in biotin-free culture medium.
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31. HLA-DR7 alloantigen-specific T cell clones were generated according to standard methodology (*11*). Before each experiment, T cell clones were rested for 10 to 14 days in IL-2 without alloantigen restimulation. Before use, cells were cultured overnight in media. LBL-DR7 cells and NIH 3T3 transfectants were treated with mitomycin-C (*8*). T cell clones were cultured in a primary culture for 24 hours, separated from LBL-DR7 cells by Ficoll gradient centrifugation and from NIH 3T3 transfectants by Percoll gradient centrifugation, and cultured in media without IL-2 for 12 hours. Each population was subsequently rechallenged with LBL-DR7 stimulators in secondary culture.
32. Cells were lysed with lysis buffer containing 10 mM tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaFl, 1 mM sodium orthovanadate, aprotinin (5 μg/ml), pepstatin (1 μg/ml), soybean trypsin inhibitor (2 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.5% Brij 96 (Sigma). After immunoprecipitations, immune complexes were isolated on protein A–Sepharose, washed three times with lysis buffer, and analyzed on 6 to 12% gradient gels in SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose, and membranes were blocked for 1 hour at room temperature by being shaken in tris-buffered saline with Tween-20 (TBST) [20 mM tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween-20] containing 10% bovine serum albumin (BSA). For detection of phosphotyrosine proteins, the blots were incubated with antiphosphotyrosine (mAb 4G10 at 1:2000) for 60 min at room temperature. The blots were washed three times with wash buffer (TBST), then incubated for 60 min with horseradish peroxidase–conjugated antibody to mouse IgG (at 1:5000) (Amersham, Arlington Heights, IL). The blots were washed three times with wash buffer then incubated with the enhanced chemiluminescence substrate (Amersham), exposed to x-ray film, and developed.
33. Stripping of the immunoblot was done by incubation in 62.5 mM tris-HCl (pH 6.8), 3% w/v SDS, and 100 mM β-mercaptoethanol at 50°C for 1 hour. Subsequently, immunoblot was blocked for 1 hour at room temperature by being shaken in TBST containing 10% BSA, and it was reprobed with a different antibody.

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Interaction of IL-2Rβ and γ_c Chains with Jak1 and Jak3: Implications for XSCID and XCID

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Interleukin-2 (IL-2) signaling requires the dimerization of the IL-2 receptor β (IL-2Rβ) and common γ (γ_c) chains. Mutations of γ_c can result in X-linked severe combined immunodeficiency (XSCID). IL-2, IL-4, IL-7 (whose receptors are known to contain γ_c), and IL-9 (whose receptor is shown here to contain γ_c) induced the tyrosine phosphorylation and activation of the Janus family tyrosine kinases Jak1 and Jak3. Jak1 and Jak3 associated with IL-2Rβ and γ_c, respectively; IL-2 induced Jak3–IL-2Rβ and increased Jak3-γ_c associations. Truncations of γ_c, and a γ_c, point mutation causing moderate X-linked combined immunodeficiency (XCID), decreased γ_c-Jak3 association. Thus, γ_c mutations in at least some XSCID and XCID patients prevent normal Jak3 activation, suggesting that mutations of Jak3 may result in an XSCID-like phenotype.

The interaction of IL-2 with high-affinity IL-2 receptors regulates the magnitude and duration of the T cell immune response (*1*). High-affinity IL-2 receptors contain the IL-2 receptor α (*2*), β (*3*), and γ_c (*4*) chains. Intermediate-affinity receptors contain IL-2Rβ and γ_c and mediate IL-2 signals on natural killer (NK) and some resting T cells. Both IL-2Rβ and γ_c are members of the cytokine receptor superfamily (*5*), whereas IL-2Rα is not. The heterodimerization of β and γ_c chains is induced by IL-2 binding and is required for IL-2 signaling (*6*). Mutations of the γ_c gene can result in XSCID in humans (*7*); the severity of XSCID results from γ_c being a component of multiple cytokine receptors (*1*, *8*–*11*).

Interleukin-2 induces the tyrosine phosphorylation of multiple cellular substrates (*12*), including the Janus family kinases Jak1 and Jak3 (*13*, *14*) (Fig. 1, A and B),

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