The Common γ Chain (γc) Is a Required Signaling Component of the IL-21 Receptor and Supports IL-21-Induced Cell Proliferation via JAK3[†]

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ABSTRACT: The common cytokine receptor γ chain (γc), an essential component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, is critical for the development and function of lymphocytes. Recently, a novel lymphokine (IL-21) and its receptor (IL-21Rα) were described which profoundly affect the growth and activation state of B, T, and NK cells in concert with other lymphokines or stimuli [Parrish-Novak, J., et al. (2000) *Nature 408*, 57−63]. In this report, we show that γc is also a required signaling component of the IL-21 receptor (IL-21R) using the γc-deficient X-linked severe combined immunodeficiency (XSCID) lymphoblastoid cell line JT, and JT cells reconstituted with γc (JT/γc). Moreover, we demonstrate a functional requirement for both γc and the γc-associated Janus family tyrosine kinase 3 (JAK3) in IL-21-induced proliferation of pro-B-lymphoid cells engineered to express human IL-21Rα (BaF3/IL-21Rα). Retroviral-mediated transduction of wild-type γc into XSCID JT cells restored function to the IL-21R, as shown by IL-21-induced tyrosine phosphorylation of JAK1 and JAK3, and downstream activation of STAT5, in JT/γc cells as well as BaF3/IL-21Rα and primary splenic B cells. In contrast, IL-21 failed to activate the JAK-STAT pathway in nonreconstituted JT cells. Monoclonal antibodies specific for the γc chain effectively inhibited IL-21-induced growth of BaF3/IL-21Rα cells, supporting a functional role for this molecule in the IL-21R complex. In addition, the specific JAK3 tyrosine kinase inhibitor WHI-P131 significantly reduced IL-21-induced proliferation of BaF3/IL-21Rα cells. Taken together, these results definitively demonstrate that IL-21-mediated signaling requires the γc chain, and indicate that JAK3 is an essential transducer of γc-dependent survival and/or mitogenic signals induced by this cytokine.

The newly described lymphokines human and murine interleukin-21 (IL-21)[1] are 131 and 122 amino acid polypeptides produced by activated $CD4^+$ T-lymphocytes (*1*). Structurally, IL-21 is most closely related to IL-2, IL-4, and IL-15, members of the four-α-helix-bundle cytokine family that mediate pleiotropic biological functions within the immune system (*1, 2*). Preliminary biological characterization indicates that IL-21 regulates the proliferation of mature B and T cells in response to activating stimuli, and promotes the expansion and maturation of NK cells from bone marrow progenitors in vitro, in synergy with Flt-3 ligand and IL-15 (*1*). These biological effects are mediated through a class I cytokine receptor (IL-21Rα) that shares a number of structural features common to this receptor superfamily, including the Box1 and Box2 regions involved in Janus kinase (JAK) association (*1, 3, 4*). IL-21Rα has highest amino acid sequence homology with IL-2/15Rβ and IL-4Rα; thus, the complete IL-21 receptor complex likely shares a close relationship with the receptors for IL-2, IL-4, and IL-15 (IL-2R, IL-4R, and IL-15R), which utilize the γc chain as a signaling subunit (*1, 3, 5*).

Mutations in the human γc gene, located on the X chromosome, result in X-linked severe combined immunodeficiency (XSCID), a disease characterized by profoundly impaired T, B, and NK cell development (*6*). Various γc mutations have been characterized which disrupt cytokine receptor function at multiple levels; complete dysfunction of the γc subunit in ligand binding and/or signal transduction results in typical XSCID (*5, 6*). Analyses of γc-deficient mice revealed that the absence of γc-dependent signals differentially impairs the development and function of lymphoid sublineages, suggesting that although γc is shared, it mediates different biological functions in response to individual cytokines (*7*). Targeted disruption of these cytokines or their

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[1] Abbreviations: IL, interleukin; JAK, Janus kinase; γc, common γ chain; XSCID, X-linked severe combined immunodeficiency; STAT, signal transducers and activators of transcription; anti-P-Tyr, anti-phosphotyrosine; EBV, Epstein−Barr virus; LCL, lymphoblastoid cell line; FBS, fetal bovine serum; mIL-3, murine IL-3; FACS, fluorescence-activated cell sorting; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBST, Tris-buffered saline (pH 8.0) containing 0.05% Tween-20; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; HTLV-I, human T cell leukemia virus-I; mAbs, monoclonal antibodies.

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specific receptor subunits in mice subsequently demonstrated that certain γc-dependent cytokines are vital during the early stages of lymphocyte differentiation, while others appear critical for immune regulation and homeostasis (*5, 7*). Accordingly, IL-7 and IL-7Rα are essential for thymopoiesis and B cell development, IL-15-derived signals via the IL-15Rαβγc system are required during NK cell ontogeny, and IL-2/IL-2Rβγc and IL-4/IL-4Rαγc regulate the peripheral T and B cell compartments (*5, 7, 8*).

Among the most proximal biochemical signals triggered by ligand binding to γc-containing receptors is activation of the Janus tyrosine kinase family members JAK1 and JAK3 (*9*). JAK3 is known to physically associate with γc, and ligand-induced conformational changes of preformed receptor−JAK complexes are thought to allow the sequential transphosphorylation of JAK3, and then JAK1 (*10*−*13*). Once activated, the JAKs phosphorylate multiple substrates, most importantly the receptors themselves, creating docking sites for additional JAK substrates, adaptor proteins, and effector molecules (*14*). Among the numerous JAK substrates recruited to the receptor complex are the Signal Transducer and Activator of Transcription (STAT) proteins, a family of latent cytoplasmic transcription factors that play a crucial role in signal transduction pathways associated with the interleukins, interferons, and hematopoietic cytokines (*14*). In vivo functions that are dependent on STAT activation include cell proliferation, differentiation, survival, apoptosis, and oncogenic transformation (*14, 15*).

Mutations affecting either the expression of JAK3 or its association with γc result in a phenotypically similar immunodeficiency to XSCID, suggesting the important role of JAK3 in γc-dependent signaling in lymphocytes (*6, 16*−*18*). Recently, a comparative analysis of γc-, JAK3-, and γc/JAK3-double-deficient mice was undertaken to clarify whether γc-dependent cytokines can partially transduce their signals and support lymphocyte development in the absence of JAK3 (*18*). A similar reduction in the numbers of thymocytes and splenic T and B cells was found among γc −/−, JAK3 −/−, and doubly γc/JAK3 −/− mice, confirming that JAK3 is indispensable for γc-dependent T and B cell development in vivo (*18*). Moreover, IL-7-mediated Bcl-2 expression and prevention of apoptosis in thymocytes were entirely JAK3-dependent (*18*). Notably, NK cells are completely eliminated in mice deficient in γc or JAK3 (*7, 16, 18, 19*); however, deficiency of IL-15 leads to a significant reduction in the numbers of NK cells, but not their complete absence (*20*). These findings suggest that multiple γc-dependent cytokines may be required to fully support NK cell production, and together with the synergistic effects of IL-15 and IL-21 on NK differentiation (*1*), point to a potential functional role for γc and JAK3 in IL-21R signaling.

In the present study, we have examined whether the γc chain is a required signaling component of the IL-21R complex using a naturally occurring model provided by the γc-deficient XSCID B cell line JT, and JT cells reconstituted with γc (JT/γc). We provide biochemical, immunologic, and functional evidence demonstrating that IL-21, like its structural relatives IL-2, IL-4, and IL-15, is a γc-dependent cytokine. Furthermore, our study implicates JAK3 as an important mediator of γc-dependent proliferative signals transduced by IL-21.

MATERIALS AND METHODS

Reagents. Anti-JAK1 (06-272), anti-JAK3 (05-406), anti-STAT5A (06-553), anti-STAT5B (06-554), and anti-phosphotyrosine (anti-P-Tyr) (clone 4G10) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). A rabbit polyclonal antiserum to JAK3 was the kind gift of Dr. John O'Shea (National Institutes of Health, Bethesda, MD). Mouse monoclonal anti-JAK1 antiserum for immunoblotting was obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal phosphospecific antiserum that recognizes the tyrosine-phosphorylated form of STAT5 (P-STAT5-Tyr_{694}) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Purified rat anti-mouse γc chain blocking monoclonal antibodies (mAbs) 3E12, 4G3, and TUGm2, and the rat IgG_{2a} and IgG_{2b} monoclonal immunoglobulin isotype standards, were purchased from BD PharMingen (San Diego, CA). The specific JAK3 inhibitor WHI-P131 was generously provided by Dr. Fatih Uckun (Parker Hughes Institute, St. Paul, MN). Purified human IL-21 was provided by ZymoGenetics, Inc. (Seattle, WA). Recombinant human IL-2 was obtained from Chiron Corp. (Emeryville, CA).

Cells and Cell Culture. JT cells, an Epstein−Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) derived from a patient with X-linked SCID (*21*), were maintained in RPMI 1640 medium (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. To generate the LCL JT/γc, JT cells were transduced with the vector MND-γc which expresses the normal human γc cDNA under the control of the modified Moloney murine leukemia virus MND vector LTR (*22*). Fresh viral supernatants of the MND-γc vector pseudotyped in the gibbon-ape leukemia virus envelope-expressing PG13 amphotropic producer cell line were added to the JT cells 4 times within 48 h. After 1 week, the transduced JT cells were immunostained with a mAb specific for human γc (γc1, Biosource International, Camarillo, CA), and the γc-positive population was selected by fluorescence-activated cell sorting (FACS). After 2 sorts, all of the JT cells were positive for γc protein expression. The IL-3-dependent pro-B lymphoid cell line BaF3/IL-21Rα expressing human IL-21Rα was provided by ZymoGenetics, Inc., and maintaned in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL zeocin (Invitrogen, Carlsbad, CA) and supplemented with murine IL-3 (mIL-3, 0.1% v/v conditioned medium from baby hamster kidney cells engineered to secrete mIL-3). BaF3/IL-21Rα/IL-2Rβ cells coexpressing human IL-21Rα and human IL-2Rβ (hIL-2Rβ) were generated by electroporation of BaF3/IL-21Rα cells with the eukaryotic expression vector pHβAPr-1-neo encoding hIL-2Rβ, kindly provided by Dr. Brad Nelson (Virginia Mason Research Center, Seattle, WA), and with pMXpuro (a gift from Dr. Toshio Kitamura). Puromycin at a final concentration of 1 μg/mL was added 24 h after transfection. Surface expression of IL-2Rβ was determined by flow cytometry using a PE-conjugated mouse anti-human IL-2Rβ mAb (Pharmingen, San Diego, CA). Individual clones were isolated through limiting dilution in 96-well plates, and maintained in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL zeocin, 1 mg/mL G418 (Invitrogen,

Carlsbad, CA), and mIL-3. Murine splenic B lymphocytes were enriched from single-cell suspensions of total splenocytes by negative selection on magnetic beads coupled to CD43 (Miltenyi Biotec, Auburn, CA) following the manufacturers' instructions, leaving approximately 10^8 splenic B cells of 90% purity per 3−5 mice. Purity was assessed by flow cytometry using Cy-chrome-conjugated anti-CD45R, fluorescein isothiocyanate-conjugated anti-CD3, and phycoerythrin-conjugated anti-CD14 antibodies (BD Pharmingen). The recovered splenic B cells were rested in complete RPMI 1640 medium containing 10% FBS for 4 h prior to signaling studies.

Immunoprecipitation and Western Blot Analysis. Serum and cytokine-starved JT, JT/γc, and BaF3/IL-21Rα cells were stimulated with 50 ng/mL human IL-21 for 15 min, whereas freshly isolated primary murine splenic B cells were stimulated with 30 ng/mL murine IL-21 for 15 min. Cells were then washed twice in ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM $NaVO_4$, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of aprotinin and leupeptin. The protein concentration of the lysates was measured by Protein/DC assay (Bio-Rad, Hercules, CA). JAK1 and JAK3 proteins were immunoprecipitated from cell lysates by overnight incubation at 4 °C with the indicated anti-JAK antibodies. Protein A/G conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were then added and incubated for an additional 1−2 h at 4 °C. The pelleted beads were washed 3 times with lysis buffer, resuspended in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer, and boiled for 5 min. For P-STAT5-Tyr_{694} and anti-P-Tyr immunoblotting, equal amounts of whole cell lysates (50−75 μg for JT, JT/γc, and BaF3/IL-21Rα and 2×10^6 cell equiv per primary B cell sample) and anti-JAK immunoprecipitates were size-fractionated by electrophoresis in 7.5% SDS−polyacrylamide gels, as indicated. Protein was electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keane, NH) and blocked in Tris-buffered saline (pH 8.0) with 0.05% Tween-20 (TBST) and 5% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were further incubated in primary antibody at the recommended concentration by the supplier. After three 10 min washes with TBST, the appropriate secondary antibody (Bio-Rad goat anti-rabbit or goat anti-mouse coupled to horseradish peroxidase) was added in TBST/5% BSA, followed by three more washes with TBST and detection using a commercial chemiluminescence detection kit (LumiGLO; Cell Signaling Technology, Inc., Beverly, MA). The blots were then stripped of antibody by washing at 60 °C for 1 h in 62.5 mM Tris (pH 6.8), 2% $NaDodSO_4$, and 100 mM β-mercaptoethanol, followed by extensive washes with TBST, blocked, and then reprobed with the indicated antibody to verify equal protein loading.

MTT Proliferation Assays. To test the effect of γc-blocking antibodies on IL-2- and IL-21-induced proliferation, BaF3/IL-21Rα and BaF3/IL-21Rα/IL-2Rβ cells in logarithmic-phase growth were washed 3 times with serum and cytokine-free medium to remove mIL-3, resuspended in RPMI 1640 containing 10% FBS, and then plated in triplicate at 1×10^4 cells/well in 96-well plates. Cells were preincubated for 1 h with a mixture of 3E12, 4G3, and TUGm2 anti-γc mAbs, each at either 25 μg/mL or 50 μg/mL as indicated, prior to the addition of cytokine. To control for toxicity of the mAbs, cells were similarly preincubated with a 1:1 mixture of the IgG_{2a} and IgG_{2b} mAb isotype standards at the same final concentration. Serially increasing concentrations of IL-2 (0−100 units/mL), IL-21 (0−100 ng/mL), or mIL-3 (0.1% v/v) were subsequently added to either cells alone or cells preincubated with the indicated mAbs, and plates were further incubated for 72 h at 37 °C. The specific JAK3 inhibitor WHI-P131 was similarly tested by incubating BaF3/IL-21Rα or BaF3/IL-21Rα/IL-2Rβ cells in triplicate for 72 h with various concentrations of the indicated cytokine plus 10 μM final concentration of WHI-P131 dissolved in Me_2SO (Calbiochem, San Diego, CA) or plus an equivalent amount of vehicle (Me_2SO, 0.1% final concentration). To control for specificity of the JAK3 inhibitors, cells were also incubated in parallel with mIL-3 alone (0.1% v/v), mIL-3 plus 10 μM final concentration of WHI-P131, or mIL-3 plus an equivalent amount of Me_2SO (0.1% final concentration). The MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO] was then added at 1 mg/mL final concentration to each of the triplicate assays and incubation continued for 4−5 h. The formazan granules were solubilized by adding 100 μL of MTT lysis buffer (40% dimethylformamide/2% glacial acetic acid/20% $NaDodSO_4$/0.215% concentrated hydrochloric acid), and absorbance (546−630 nm) was determined 16−24 h later on an ELISA plate reader (Packard). The extent of MTT reduction measured in response to 0.1% mIL-3 is defined in each assay as maximal proliferation, or 100%. Proliferation was therefore expressed as a percentage of maximal proliferation induced by 0.1% mIL-3.

RESULTS

Previous reports indicate that IL-21Rα is most closely related to the IL-2Rβ subunit, a component of the IL-2 and IL-15 receptors, and IL-4Rα (*1*, *3*). These findings led us to hypothesize that IL-21 might utilize the γc chain for signaling. Cell lines derived from XSCID patients provide an optimal assay system for cytokine signal transduction potentially involving γc. The feasibility of this approach has previously been demonstrated (*21*, *23*). The EBV-transformed lymphoblastoid cell line, JT, contains a C→T transition mutation in the γc gene at base pair 660 which changes a glutamine in the extracellular domain of γc to a stop codon. Northern blot analysis and RT-PCR showed no endogenous 1.8 kb γc transcript (*21*), and γc protein expression was undetectable by FACS analyses and biochemical studies (data not shown). Genetic correction of XSCID by means of retroviral-mediated transduction of the γc-cDNA into JT cells (described under Materials and Methods) resulted in stable expression of the transgene and restoration of IL-2-mediated cellular functions in the γc-reconstituted cell line, JT/γc. In MTT proliferation assays, we found that IL-2 augmented proliferation of JT/γc cells in a dose-dependent fashion, inducing on average a 64% increase in the extent of proliferation (as measured by MTT reduction) relative to untreated JT/γc cells in response to the maximum dose of 10 000 units/mL of IL-2. In contrast, JT cells did not respond to any concentration of IL-2 tested.

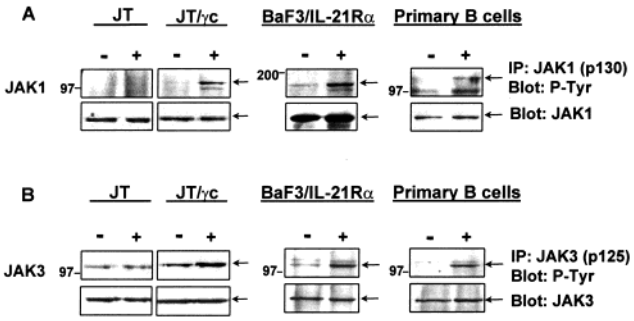

FIGURE 1: IL-21 induces tyrosine phosphorylation of JAK1 and JAK3 in JT/γc, BaF3/IL-21Rα, and primary splenic B cells, but not XSCID JT cells. JT, JT/γc, BaF3/IL-21Rα, and purified murine splenic B cells were left unstimulated (−) or stimulated (+) with IL-21 for 15 min. Cell lysates were immunoprecipitated with rabbit anti-JAK1 (A) or anti-JAK3 (B) polyclonal antibodies, separated by 7.5% SDS−PAGE, and probed for anti-P-Tyr. Blots were then stripped and reprobed with a mouse anti-JAK1 mAb (A, bottom panels) or rabbit anti-Jak3 polyclonal antibodies (B, bottom panels) to verify that equivalent total amounts of JAK1 or JAK3, respectively, were present in the immunoprecipitates from unstimulated and IL-21-stimulated cell lysates. The positions of each JAK are indicated with arrows. Similar results were obtained in two additional experiments.

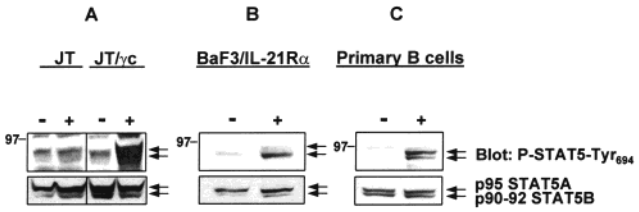

FIGURE 2: STAT5-Tyr_{694} is inducibly phosphorylated by IL-21 in JT/γc, BaF3/IL-21Rα, and primary B cells, but not XSCID JT cells. JT (A, left panels), JT/γc (A, right panels), BaF3/IL-21Rα (B), and primary B cells (C) were left unstimulated (−) or stimulated (+) with IL-21 for 15 min, and whole cell lysates (50 μg/lane for JT and JT/γc, 75 μg/lane for BaF3/IL-21Rα, and 2×10^6 cell equiv per lane for primary B cell lysates) were size-fractionated by 7.5% SDS−PAGE. Western analysis was carried out with anti-phospho-STAT5-Tyr_{694}, and blots were subsequently stripped and reprobed with rabbit anti-STAT5A and STAT5B polyclonal antibodies, simultaneously, to verify that similar total amounts of STAT5 were loaded in each lane. The positions of STAT5A and STAT5B are indicated with arrows. Blots shown are from a representative experiment of at least three performed.

The IL-21R is constitutively expressed in primary human and murine B lymphocytes and B cell lines (*1*); therefore, to determine the pattern of IL-21 signaling in these cells, we first identified the JAK proteins activated by IL-21 in JT, JT/γc, primary murine splenic B cells, and the pro-B lymphoid BaF3 cell line transfected with human IL-21Rα (BaF3/IL-21Rα). Cell lysates from unstimulated or IL-21-treated cells were immunoprecipitated with anti-JAK1 or anti-JAK3 antisera, then size fractionated and immunoblotted with an anti-P-Tyr (4G10) antibody. As shown in the upper panels of Figure 1A,B, JAK1 and JAK3 were inducibly tyrosine-phosphorylated following IL-21 stimulation of BaF3/IL-21Rα, JT/γc, and primary B cells, but not JT cells. The lower blots in Figure 1A,B indicate that equivalent total amounts of JAK1 and JAK3, respectively, were present in the immunoprecipitates from unstimulated and IL-21-stimulated cell lysates. Interestingly, we observed constitutive tyrosine phosphorylation of JAK3 in γc-deficient JT cells even after serum deprivation (Figure 1B), which appeared insufficient for detectable transphosphorylation of JAK1 (Figure 1A). Although the significance of this result is unclear, constitutive tyrosine phosphorylation of JAK3 has previously been reported with human T cell leukemia virus-I (HTLV-I)-transformed T cell lines (*24, 25*). It is therefore possible that EBV-immortalization of JT cells may similarly contribute to basal JAK3 activation in this cell line. Importantly, the γc-dependent enhancement of JAK1 and JAK3 tyrosine phosphorylation in IL-21-treated JT/γc cells, coupled with an identical profile of JAK activation in BaF3/IL-21Rα and primary B cells, strongly suggests that the γc chain is required for IL-21R signaling.

We next compared the capacity of IL-21 to stimulate STAT tyrosine phosphorylation in JT, JT/γc, BaF3/IL-21Rα, and primary B cells. Cells were stimulated with IL-21 for 15 min, and whole cell extracts were immunoblotted with a phosphospecific STAT5-Tyr_{694} antibody. STAT5 consists of STAT5A (p95) and STAT5B (p90−92), the products of two nearly identical but distinct genes (*26*), both of which appeared prominently tyrosine-phosphorylated in JT/γc and primary B cells after IL-21 stimulation (Figure 2A,C). In contrast, STAT5B was predominantly activated in BaF3/IL-21Rα cells (Figure 2B), and no phosphorylation of STAT5 on Tyr_{694} was detected after IL-21 stimulation of JT cells (Figure 2A). Collectively, these results clearly demonstrate that γc is indispensable for IL-21-induced activation of the JAK-STAT pathway. Moreover, our findings using primary splenic B cells recapitulated the signaling events observed in IL-21-treated JT/γc and BaF3/IL-21Rα cells, thus confirming their physiological relevance.

Previous studies have underscored the importance of γc-dependent signals in a number of crucial events in lymphocyte development and function, including the promotion of mitogenesis and induction of survival gene expression (*10, 18, 27*). To explore the functional role of the γc chain in IL-21R signaling, we conducted MTT proliferation assays with the IL-3-dependent BaF3/IL-21Rα cell line using blocking monoclonal antibodies (mAbs) specific for distinct epitopes of the mouse γc chain. The TUGm2, 3E12, and 4G3 mAbs have previously been used to demonstrate the involvement of the γc chain in functional high-affinity receptor complexes for IL-2, IL-4, and IL-7 (*28*−*30*). Preincubation of cells with either 75 or 150 μg/mL of the γc-blocking mAb mixture significantly inhibited the dose-dependent IL-21-induced proliferation of BaF3/IL-21Rα cells (Figure 3A). This effect was not caused by toxicity of the mAbs, as cells cultured in the same concentrations of IL-21 and isotype-matched control IgG proliferated almost as well as cells grown with IL-21 alone (Figure 3A). The inability of the γc-specific mAbs to completely abolish IL-21-induced growth of this cell line was likely due to either inherent limitations in their potential to effectively block IL-21 binding to the IL-21Rα/γc complex or the dissociation of one or more low-affinity γc mAbs during the course of the 72 h assay, allowing IL-21 to partially support proliferation. We also constructed BaF3/IL-21Rα cells expressing the human IL-2Rβ chain, providing an IL-3-dependent pro-B-lymphoid cell line that can respond to IL-2. As expected, preincubation of BaF3/IL-21Rα/IL-2Rβ cells with 75 μg/mL of the γc-specific mAbs efficiently blocked the dose-dependent IL-2-induced proliferation of this cell line (Figure 3B). Inclusion of an anti-IL-2Rβ blocking mAb (TMβ1, 25

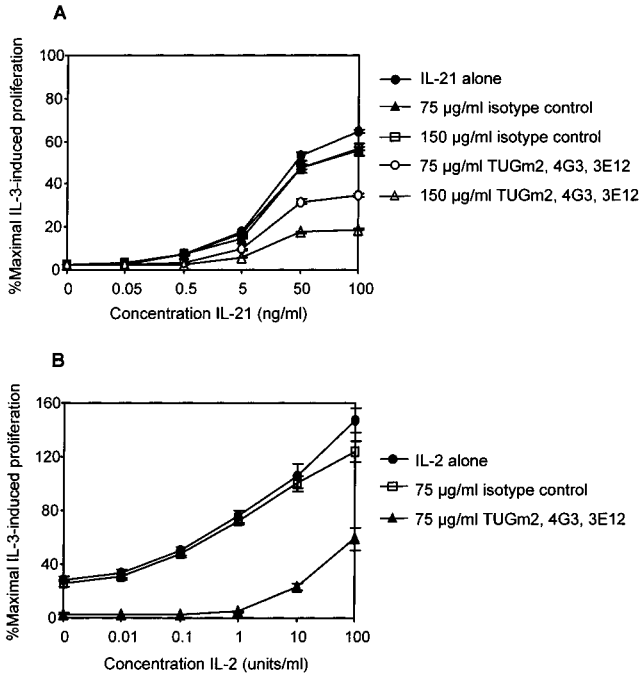

FIGURE 3: The γc chain is essential for transducing survival and/or mitogenic signals induced by IL-21. (A) MTT assay of IL-21-stimulated BaF3/IL-21Rα cells in the presence and absence of isotype control or γc-blocking mAbs. BaF3/IL-21Rα cells were washed and incubated with various concentrations of human IL-21 (0−100 ng/mL) alone or in the presence of the indicated final concentrations of isotype control (1:1 IgG2a and IgG2b) or anti-γc (1:1:1 TUGm2, 4G3, and 3E12) mAbs for 3 days. Values indicate extent of MTT reduction as a percentage of maximal proliferation induced by 0.1% mIL-3 (see Materials and Methods). Error bars represent 1 SD value of the mean for triplicate samples. Similar results were seen in two additional experiments. (B) MTT assay of IL-2-stimulated BaF3/IL-21Rα/IL-2Rβ cells in the presence and absence of isotype control or anti-γc mAbs. Cells were washed and incubated with serially increasing concentrations of human IL-2 (0−100 units/mL) alone or in the presence of 75 μg/mL isotype control (1:1 IgG2a and IgG2b) or anti-γc (1:1:1 TUGm2, 4G3, and 3E12) mAbs for 3 days. Values indicate extent of MTT reduction as a percentage of maximal proliferation induced by 0.1% mIL-3. Error bars represent 1 SD value of the mean for triplicate samples. Assay shown is a representative experiment of three performed. Another clone of BaF3/IL-2Rα/IL-2Rβ cells gave similar results.

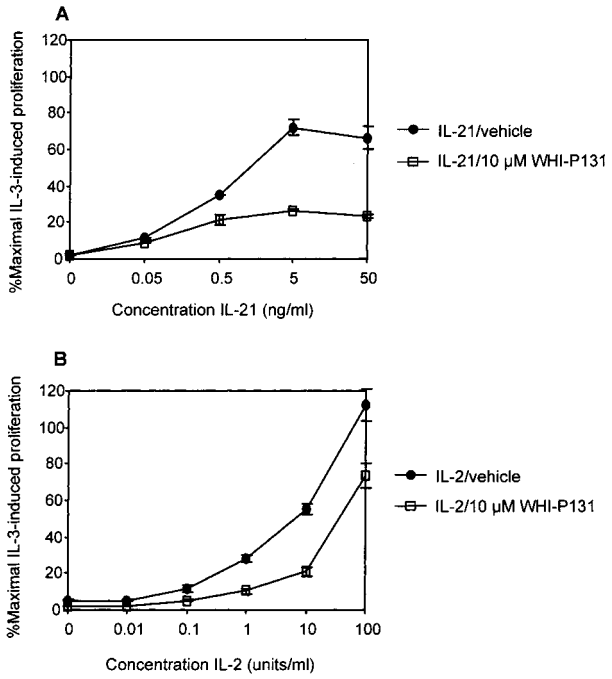

FIGURE 4: γc-associated JAK3 tyrosine kinase activity is essential for transducing survival and/or mitogenic signals induced by IL-21. (A) MTT assay of IL-21-stimulated BaF3/IL-21Rα cells in the presence and absence of the specific JAK3 inhibitor WHI-P131. Cells were washed and incubated with serially increasing concentrations of human IL-21 (0−50 ng/mL) in the presence of 10 μM WHI-P131, or an equivalent amount of Me_2SO (vehicle control), for 3 days. Values indicate extent of MTT reduction as a percentage of maximal proliferation induced by 0.1% mIL-3. Error bars represent 1 SD value of the mean for triplicate samples. Similar results were seen in two additional experiments. (B) MTT assay of IL-2-stimulated BaF3/IL-21Rα/IL-2Rβ cells in the presence and absence of WHI-P131. Cells were washed and incubated with serially increasing concentrations of human IL-2 (0−100 units/mL) in the presence of 10 μM WHI-P131, or an equivalent amount of Me_2SO diluent, for 3 days. Values indicate extent of MTT reduction as a percentage of maximal proliferation induced by 0.1% mIL-3. Error bars represent 1 SD value of the mean for triplicate samples. Similar results were seen in two additional experiments, including another independent clone of BaF3/IL-21Rα/IL-2Rβ.

μg/mL) with the anti-γc mixture enhanced this effect, resulting in complete inhibition of proliferation at all concentrations of IL-2 tested (not shown). To further examine the γc-dependence of the proliferative response to IL-21, we conducted MTT assays with the EBV-transformed JT/γc cells and found, on average, a 50% increase in the extent of proliferation (as measured by MTT reduction) relative to untreated JT/γc cells after a 5 day incubation with as little as 5 ng/mL IL-21 (data not shown). In contrast, JT cells did not respond to any concentration of IL-21 tested (data not shown). Thus, taken together with the ability of IL-21 to augment proliferation of JT/γc, but not JT cells, these experiments provide functional evidence confirming the involvement of the γc chain in IL-21 signaling.

Given the importance of the JAK3−γc interaction in mediating lymphoid cell growth and differentiation, we sought to determine whether IL-21 transduces proliferative signals via JAK3. To this end we used the potent and specific pharmacologic JAK3 inhibitor WHI-P131 (*31*) to investigate the relative contribution of JAK3 activity to IL-21-mediated proliferation of BaF3/IL-21Rα cells. As shown in Figure 4A, IL-21-induced proliferation of BaF3/IL-21Rα cells was markedly reduced by co-incubation with 10 μM WHI-P131. Importantly, at this dose, these compounds did not exhibit any detectable inhibitory activity against IL-3-induced proliferation of BaF3/IL-21Rα cells (to which JAK2 is critical), thus confirming their specificity for JAK3 (data not shown). Our results are consistent with the reported effects of WHI-P131 on IL-2- and IL-3-induced activation of the JAK-STAT pathway in 32Dc11/IL-2Rβ cells; WHI-P131 inhibited JAK3-mediated STAT activation in response to IL-2, whereas IL-3-induced JAK1/JAK2-dependent STAT activation was unaffected (*31*). Moreover, 10 μM WHI-P131 inhibited the dose-dependent IL-2-induced proliferation of BaF3/IL-21Rα/IL-2Rβ cells (Figure 4B), further demonstrating the sensitivity of γc-dependent cytokine receptors to this compound. Additional studies are in progress to determine if IL-21-induced activation of STAT proteins is inhibited by WHI-P131 in both BaF3/IL-21Rα cells and primary B cells. Nevertheless, our results indicate that JAK3 is an important mediator of γc-dependent proliferative and/or survival signals induced by IL-21, and raise the critical issue

of which factors downstream of JAK3 transmit these signals to the nucleus.

DISCUSSION

The IL-21 receptor (IL-21Rα), a novel member of the Class I cytokine receptor superfamily, shares a structural relationship with IL-2Rβ and IL-4Rα and contains a number of key intracellular signaling motifs characteristic of signaling subunits (*1, 3*). Previous reports indicate that IL-21Rα is capable of signal transduction through homodimerization, as is IL-4Rα (*1, 3*). However, these results were obtained using chimeric receptor systems in cells bearing the γc chain, and did not exclude the possibility that IL-21Rα may physiologically associate in a heteromeric receptor complex with the γc chain. In this report, we definitively establish that the γc chain, a shared component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, is a required signaling subunit of the IL-21 receptor complex. Moreover, our data support a functional role for JAK3-dependent γc signals in IL-21-mediated cell proliferation. Activation of JAK1 and JAK3, and downstream tyrosine phosphorylation of STAT5, was restored in IL-21-stimulated XSCID JT cells transduced with wild-type γc. These biochemical events correlated with a proliferative response of JT/γc cells to IL-21. Consistent with this result, specific γc-blocking mAbs effectively inhibited IL-21-induced proliferation of BaF3/IL-21Rα cells, further demonstrating the involvement of γc in IL-21 function. Of note, although we found a similar pattern of JAK-STAT protein activation in IL-21-stimulated JT/γc, BaF3/IL-21Rα, and primary splenic B cells, IL-21 alone cannot support the proliferation of primary B cells. Rather, IL-21 exhibits both enhancing and inhibitory effects on primary B cells depending upon the type of co-stimulation: In combination with CD40 engagement, IL-21 augments B cell proliferation, yet this cytokine profoundly inhibits the expansion of B cells stimulated with anti-IgM and IL-4 (*1*). Therefore, γc-derived IL-21R signals may be critical for modulating the coordinated action of different downstream signaling pathways upon co-stimulation of B cells, thus exerting these divergent effects.

Using the pharmacologic JAK3 inhibitor WHI-P131, we demonstrated that γc-associated JAK3 tyrosine kinase activity is indispensable for IL-21-induced proliferation of BaF3/IL-21Rα cells. The physical and functional coupling of γc and JAK3 has been well documented in a number of γc-dependent cytokine receptor systems (*16*). IL-2, IL-4, and IL-7 fail to mediate a proliferative signal in thymocytes from JAK3-deficient mice (*16*). Furthermore, overexpression of a catalytically inactive form of JAK3 in BaF3 cells was shown to inhibit IL-2R-mediated induction of c-fos, c-myc, and cell proliferation, yet bcl-2 gene induction was preserved (*32*). Interestingly, while JAK3 appears to be pivotal in promoting mitogenic signaling in response to a number of γc -dependent cytokines, recent studies using truncated versions of γc indicate that JAK3-independent signals emanating from the membrane-proximal region of the γc chain can mediate IL-2-induced activation of Jak1, STAT3/5, and phosphatidylinositol-3-kinase (*33*). In vivo, this membrane-proximal cytoplasmic region of γc, which lacks the JAK3 binding portion, was sufficient to support significant Bcl-2 protein expression and T lymphopoiesis when expressed on a γc-null background (*27*). Thus, these findings point to a differential requirement for JAK3 in γc-dependent lymphocyte development and function. The proliferation assays presented in Figure 4 represent overall IL-21-induced cell growth as assessed by MTT reduction; therefore, additional experiments are underway to dissect the proliferative capacity of IL-21 into cell cycle progression and cell survival, and determine the contribution of JAK3 activation to these events. Moreover, biochemical analysis of the effects of WHI-P131 on factors downstream of the IL-21R may help to elucidate the JAK3-dependent mitogenic signaling pathways induced by IL-21.

Finally, our results using the EBV-transformed XSCID B cell lines JT and JT/γc are in agreement with those of Asao and colleagues, who recently reported similar findings using the HTLV-I-transformed γc-deficient T cell line ED40515$^-$ and Edγ-16 expressing exogenous wild-type γc (*25*). We have extended our IL-21 signaling studies to primary murine splenic B cells to confirm the physiological relevance of the results obtained using cell lines. As shown by Asao and collagues, signals derived from the membrane-distal portion of γc containing the JAK3 binding region appear to be critical for IL-21-induced STAT activation, as a mutant form of γc lacking the C-terminal 50 amino acids did not support signal transduction when expressed in HTLV-I-transformed human T cells (*25*). However, further mechanistic studies will be required to assess how the γc chain contributes to IL-21-mediated lymphocyte proliferation and activation. More definitive confirmation of the roles played by IL-21 and IL-21Rα in lymphocyte development is likely to be forthcoming through knockout mouse studies.

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