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Overexpression of Interleukin-2 Receptor α in a Human Squamous Cell Carcinoma of the Head and Neck Cell Line Is Associated With Increased Proliferation, Drug Resistance, and Transforming Ability

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Abstract It has been previously demonstrated that human carcinomas express interleukin-2 receptor (IL-2R) α, β, and γ chains. The β and γ chains of IL-2R have intermediate binding affinity for IL-2 and are responsible for the intracellular signaling cascades after IL-2 stimulation. IL-2Rα lacks the cytoplasmic domain, but is essential for increasing the IL-2-binding affinity of other receptors. Overexpression of IL-2Rα in tumor cells is associated with tumor progression and a poor patient prognosis. To define molecular mechanisms responsible for the effects associated with IL-2Rα expression, ex vivo experiments were performed with the squamous cell carcinoma head-and-neck cancer line, PCI-13, which was genetically engineered to overexpress the IL-2Rα chain. While IL-2Rα-overexpressing PCI-13 cells were capable of forming colonies in soft agar, PCI-13 cells transfected with the control vector or those expressing IL-2Rγ did not. Consistently, IL-2Rα-expressing tumor cells proliferated more rapidly than the control or IL-2Rγ+ cells, associated with increased levels of cyclins A and D1 and cyclin-dependent kinase (cdk(s)) 2 and 4 proteins. In addition, IL-2Rα-expressing cells were significantly more resistant to apoptosis induction by a tripeptidyl proteasome inhibitor (ALLN) and two chemotherapeutic drugs (VP-16 and taxol) than the control or IL-2Rγ+ cells. Accompanying the drug resistance, high levels of anti-apoptotic Bcl-X_L and Bcl-2 proteins were found in the mitochondria-containing fraction of IL-2Rα-expressing tumor cells. Treatment of IL-2Rα-expressing cells with a specific Janus kinase 3 (Jak3) inhibitor decreased expression of cyclin A, cyclin D1, Bcl-X_L, and Bcl-2 proteins. Finally, high levels of ubiquitinated proteins were detected in the proliferating IL-2Rα-expressing cells. Our data suggest that increased proliferation rates and decreased drug sensitivity of IL-2Rα-expressing tumor cells are responsible for the enhanced tumor aggressiveness and poor clinical prognosis of patients whose tumors express IL-2Rα. J. Cell. Biochem. 89: 824–836, 2003.

Key words: IL-2 receptors; cell cycle; apoptosis; Bcl-2; Bcl-X_L; proteasome; Jak3

Interleukin-2 (IL-2) was first described as a cytokine capable of sustaining the proliferative potential of T-lymphocytes [Morgan et al., 1976; Gillis et al., 1979] and subsequently determined to be the primary growth factor for antigen-specific T-lymphocytes [Robb et al., 1984]. It has

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been shown that IL-2 is responsible for the G_1 to S transition of the cell cycle in T-lymphocytes [Stern and Smith, 1986]. In addition to being a growth factor for T-cells, IL-2 has also been shown to stimulate growth of natural killer (NK) cells and enhance their cytolytic functions [Whiteside and Herberman, 1995]. Later, IL-2 was found to also contribute to growth of human epithelial tumor cells [Reichert et al., 2000]. Thus, endogenous IL-2 was shown to play an important role in regulating the cyclin-dependent kinase (cdk) inhibitors p27 and p21 as well as cell cycle progression in human squamous cell carcinomas of the head and neck [Reichert et al., 2000].

IL-2 mediates its biologic effects via interleukin-2 receptor (IL-2R). IL-2R is comprised of three distinct subunits: α, β, and γ chains. IL-2Rβ and γ chains belong to the IL-2 receptor superfamily and bind ligand with intermediate affinity ($K_d = 10^{-9}$ M) [Taniguchi and Minami, 1993]. IL-2Rβ and γ both possess cytoplasmic domains necessary for signaling, while IL-2Rα has a short cytoplasmic tail (13-residues) that is incapable of signaling [Leonard et al., 1982]. Although, the α chain lacks the ability to transduce signals, its non-covalent binding with β and γ leads to an overall high binding affinity of the ligand ($K_d = 10^{-11}$ M) [Taniguchi and Minami, 1993].

Elevated levels of soluble IL-2R (sIL-2R) have been found in sera of patients with carcinomas, including lung, esophagus, and head and neck cancer [Ginns et al., 1990; Jablonska et al., 1994; Yasumura et al., 1994]. Most recently, IL-2Rα was found to be overexpressed in several types of cancers, including human lung adenocarcinomas, squamous cell carcinomas, leukemia, lymphomas, and ovarian tumors [Kaczmarski and Mufti, 1991; Barton et al., 1993; McDoniels-Silvers et al., 2002]. Moreover, IL-2Rα overexpression has been linked to a poor prognosis in cancer patients suffering from leukemia, lymphoma, nasopharyngeal carcinoma, or Langerhans cell histiocytosis [Kaczmarski and Mufti, 1991; Tsai et al., 2001; Ohno et al., 2002; Rosso et al., 2002].

IL-2R subunits lack intrinsic catalytic activity and therefore, must be phosphorylated prior to activating several key signal transduction pathways [Benedict et al., 1987]. One such mechanism involves the binding of the Janus kinase 3 (Jak3) to the γ chain after IL-2 stimulation [Boussiotis et al., 1994]. The γ subunit then phosphorylates the β subunit [Ellery and Nicholls, 2002], which acts as docking sites for the signal transducers and activators of transcription (STATs) 3 and 5. Previous studies have also shown that IL-2Rβ/γ subunits signal to activate Ras/Raf/Mek/Erk- and PI3K/Akt-mediated anti-apoptotic survival pathways [Miyazaki et al., 1995] and also mediate Jak1-/Jak3-independent Bcl-2 induction [Kawahara et al., 1995]. However, the role of IL-2Rα chain in these signal transduction pathways remains unclear.

The focus of our current study was to determine the molecular events that are associated with IL-2Rα expression in tumor cells. We report that transfection of human head-and-neck cancer PCI-13 cells with IL-2Rα cDNA produces a tumor cell line with increased transforming activity, as shown by colony formation in soft agar. IL-2Rα overexpression also leads to up-regulation of cyclin D, cyclin A, cdk2 and cdk 4 proteins, and is associated with decreased G_1 phase and increased G_2/M populations. Furthermore, overexpression of IL-2Rα in PCI-13 cells causes resistance to apoptosis induced by the proteasome inhibitor *N*-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN or MG-101) and the chemotherapeutic drugs VP-16 and taxol, which was associated with increased expression of mitochondrial Bcl-X_L and Bcl-2 proteins. The pharmacological inhibitor experiment suggests Jak3 as a mediator of IL-2-dependent signal pathways in IL-2Rα cells. Our results provide a molecular interpretation for the clinical reports of a poor prognosis for cancer patients whose tumors overexpress IL-2Rα.

MATERIALS AND METHODS

Materials and Reagents

Dulbecco's Modified Eagle Medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, ribonuclease RNase A, Hoechst 33342, ALLN, etoposide (VP-16), taxol, dimethyl sulfoxide (DMSO), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Bacto-Agar was purchased from Difco Laboratories (Detroit, MI). Monoclonal antibody to Bcl-2 (Ab-1) and polyclonal antibody to Bcl-X (Ab-1) were purchased from Oncogene Research Products (Boston, MA). Monoclonal

antibodies to Cyclin A (BF-683), Cyclin E (HE-111), Cyclin D1 (R-124), and Ubiquitin (P4D1) and polyclonal antibodies to Actin (C-11), Cyclin B1 (H-433) and pJak3 (Tyr 980) as well as anti-goat, anti-rabbit, and anti-mouse IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to proliferating cell nuclear antigen (PCNA; 32551A) and cdk2 (D-12) and polyclonal antibody to cdk4 (H-22) were purchased from BD Biosciences Pharmingen (San Diego, CA). Fluorogenic peptide substrates *Z*-IETD-AFC (specific for caspase-8 activity), Ac-DEVD-AMC (for caspase-3/-7 activities), Suc–Leu–Leu–Val–Tyr–AMC (for the proteasomal chymotrypsin-like activity), and the specific Jak3 inhibitor WHI-P131 were obtained from Calbiochem (San Diego, CA).

Cell Cultures

Human squamous cell carcinoma of the head-and-neck cell line, PCI-13 [Pittsburgh Cancer Institute-13; Heo et al., 1989], was stably transfected with the LacZ vector alone, IL-2Rα cDNA, or IL-2Rγ cDNA as previously described by Lin et al. [1993]. Briefly, the LacZ (pRcCMVLZ) plasmid was made by inserting a HindIII–BamHI *lacZ* segment into a pRcCMV vector that contains a cytomegalovirus (CMV) promoter and a neomycin-resistance marker gene. The human IL-2Rα chain plasmid provided by Dr. R.J. Robb contains a full-length IL-2Rα cDNA insert under the regulation of CMV promoter and the neomycin-resistance marker gene. The IL-2Rγ chain expression vector (pRSVIL-2Rγ) contains a 1.5 kb Xba I IL-2Rγ chain fragment provided by Dr. K. Sugamura inserted into pRcRSV plasmid under the regulation of Rous sarcoma virus promoter. The LacZ-transfected cells were used as a control for the studies reported here. All three transfected PCI-13 cell lines were cultured in DMEM, supplemented with heat inactivated 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in 5% CO_2 in air atmosphere at 37°C, and passed by trypsinization (0.05% Trypsin, 0.53 mM EDTA; Invitrogen, Carlsbad, CA).

Soft Agar Assay

DMEM, containing 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, was warmed to 48°C and diluted with Bacto-Agar to make a 0.6% agar solution. To each well of 6-well plates, 4 ml of 0.6% (w/v) agar solution was added and allowed to solidify for 10–15 min to provide the bottom feeder layer. The top layer was further diluted to 0.3% agar by adding an equal volume of warmed medium containing cells (20,000 cells/well). Two milliliters of the 0.3% agar/cell solution was then added to the wells. Cells were plated in triplicates and grown for 21 days in 5% CO_2 atmosphere at 37°C. MTT (1 mg/ml) in serum-free medium was then added to each well and incubated overnight to allow complete formation of purple formazan crystals. Plates were scanned on a Hewitt-Packard Scan Jet 4c and the contrast levels were adjusted utilizing Adobe Photoshop 5.0.2. Colony counting analysis was performed with Bio-Rad, Quantity One 4.0.3 and the means were determined by Microsoft Excel software.

Protein Extraction and Western Blot Analysis

At each time point, cells were harvested, washed with PBS, and homogenized with lysis buffer [50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride], followed by shaking for 30 min at 4°C. Lysates were then centrifuged at 13,200*g* for 15 min and supernatant was collected and quantified. Equal amounts of protein extract (50 μg or as indicated) were electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using Transblot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). Membrane was blocked in Tris-buffered solution [20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20] containing 5% non-fat dry milk for 1 h and probed with the indicated antibodies. Detection of protein was performed with enhanced chemiluminescence (ECL) and X-ray film. Quantitation of the Western blots was performed on ImageQuant 5.2. Briefly, intensities of interested protein bands were scanned and quantified by density plot. The normalized (to corresponding actin) intensity of each protein band was obtained and reported.

Preparation of Heavy Membrane and Cytosolic Fractions

Both heavy membrane (containing mainly mitochondrial membrane) and cytosolic fractions were isolated at 4°C using a previously described protocol with some modifications [Hockenbery et al., 1990; Gao and Dou, 2000].

Briefly, cells were suspended in a hypotonic buffer, incubated on ice for 30 min, and then broken by four passes through a 30 G ½ needle fitted on 1-ml syringe. The lysate was centrifuged at 2,000*g* for 10 min, and the resulting supernatant was then centrifuged at 10,000*g* for 15 min, followed by collection of both the supernatant and pellet fractions. The pellet was washed twice with a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl (pH 7.5), and 1 mM EDTA, and re-suspended in a lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.5% NP-40) as the mitochondria fraction. The supernatant was further centrifuged at 21,000*g* for 30 min and the resulting supernatant was collected as the cytosol fraction.

Cell Cycle Analysis

Asynchronous cells were collected independently from three dishes, washed twice with PBS, and fixed in 70% ethanol for 2 h at 4°C. The cells were then centrifuged, re-suspended in 1 ml of sample buffer containing propidium iodide [1× PBS, 50 μg propidium iodide, 1 mg/ml glucose, and 100 U/ml ribonuclease A] and incubated at room temperature for 30 min in the dark. Propidium iodide staining, indicative of G_0/G_1, S, and G_2/M phase distribution of the cell population, was visualized with FACScan (Becton Dickinson Immunocytometry, San Jose, CA) and cell cycle distribution was determined by ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

Fluorogenic Assays

To measure cell-free caspase activities, whole cell extracts (WCE) (20–30 μg) from untreated or treated PCI-13 cells were incubated with 20 μM of the fluorogenic substrates for caspase-8 (*Z*-IETD-AFC) or caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37°C in 100 μl of assay buffer (50 mM Tris, pH 8.0). Similarly, to measure the chymotrypsin-like activity of the proteasome, WCE (10 μg) were incubated with 20 μM of Suc–Leu–Leu–Val–Tyr–AMC. Measurement of the hydrolyzed AMC or AFC groups was performed on a VersaFluor™ Fluorometer (Bio-Rad) as described previously [Nam et al., 2001].

Nuclear Staining

After each drug treatment, both detached and attached PCI-13 cells were collected as described previously [An et al., 1998], washed twice in PBS, fixed in 70% ethanol for 2 h, and stained in 50 μM Hoechst 33342 for 30 min in the dark. Morphology was visualized on a fluorescent microscope (40×) (Leitz, Oberkochen, Germany). Digital Scientific obtained images with Smart Capture, v 1.4.

RESULTS

IL-2Rα Overexpression Increases the Transforming Activity of PCI-13 Cells

Because IL-2Rα overexpression has been reported to be associated with increased severity of cancer and decreased survival [Kaczmarski and Mufti, 1991; Tsai et al., 2001; McDoniels-Silvers et al., 2002; Ohno et al., 2002; Rosso et al., 2002], we determined the effects of IL-2Rα overexpression on transforming capabilities of the PCI-13 cells in a soft agar assay. The PCI-13 cells overexpressing the vector LacZ (as a control), IL-2Rα, and IL-2Rγ were plated in soft agar and then cultured for 3 weeks. The numbers of the formed colonies

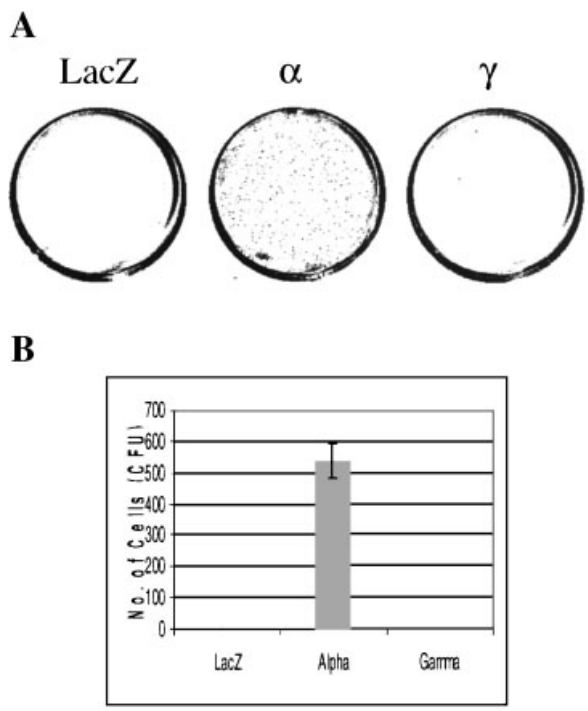

Fig. 1. Colony formation of PCI-13 cells overexpressing IL-2Rα. Squamous cell carcinoma head-and-neck cancer PCI-13 stable transfectants with IL-2Rα, IL-2Rγ, or the vector alone (LacZ) were plated in soft agar. Cells were cultured for 3 weeks then MTT (1 mg/ml) was added for determination of colony formation (**A**). Colonies were quantified with an automated counter and presented as mean values from triplicate experiments. Error bars denote standard deviation (**B**).

were determined (Fig. 1A). The IL-2Rα-overexpressing cells showed the significantly increased transforming ability: yielding 500–600 colonies per plate. In contrast, the control and the cells overexpressing IL-2Rγ showed of only 1–2 surviving colonies per plate (Fig. 1B). Therefore, overexpression of IL-2Rα greatly enhanced the transforming ability of PCI-13 cells.

IL-2Rα Overexpression Increases Cell Proliferation Rate Associated With Increasing Levels of Cell Cycle Regulatory Proteins

The increased transforming activity of IL-2Rα-overexpressing cells (Fig. 1) could be due to increased cell proliferation rate and/or decreased cell death rate. To investigate the first possibility, we determined whether the doubling time of PCI-13 cells was affected by IL-2Rα overexpression. The same number (1×10^5 per plate) of PCI-13 cells transfected with the vector LacZ, IL-2Rα or IL-2Rγ cDNA were plated on day 1, and the cell numbers were counted after growth for 24, 48, and 72 h. IL-2Rα cells grew 128% after 24 h over 0 h, compared to 48 and 64% for LacZ and IL-2Rγ cells, respectively (Fig. 2A). After 72 h, the IL-2Rα cell population expanded ~twofold, while the IL-2Rγ cells expanded only 1.2-fold, over the LacZ

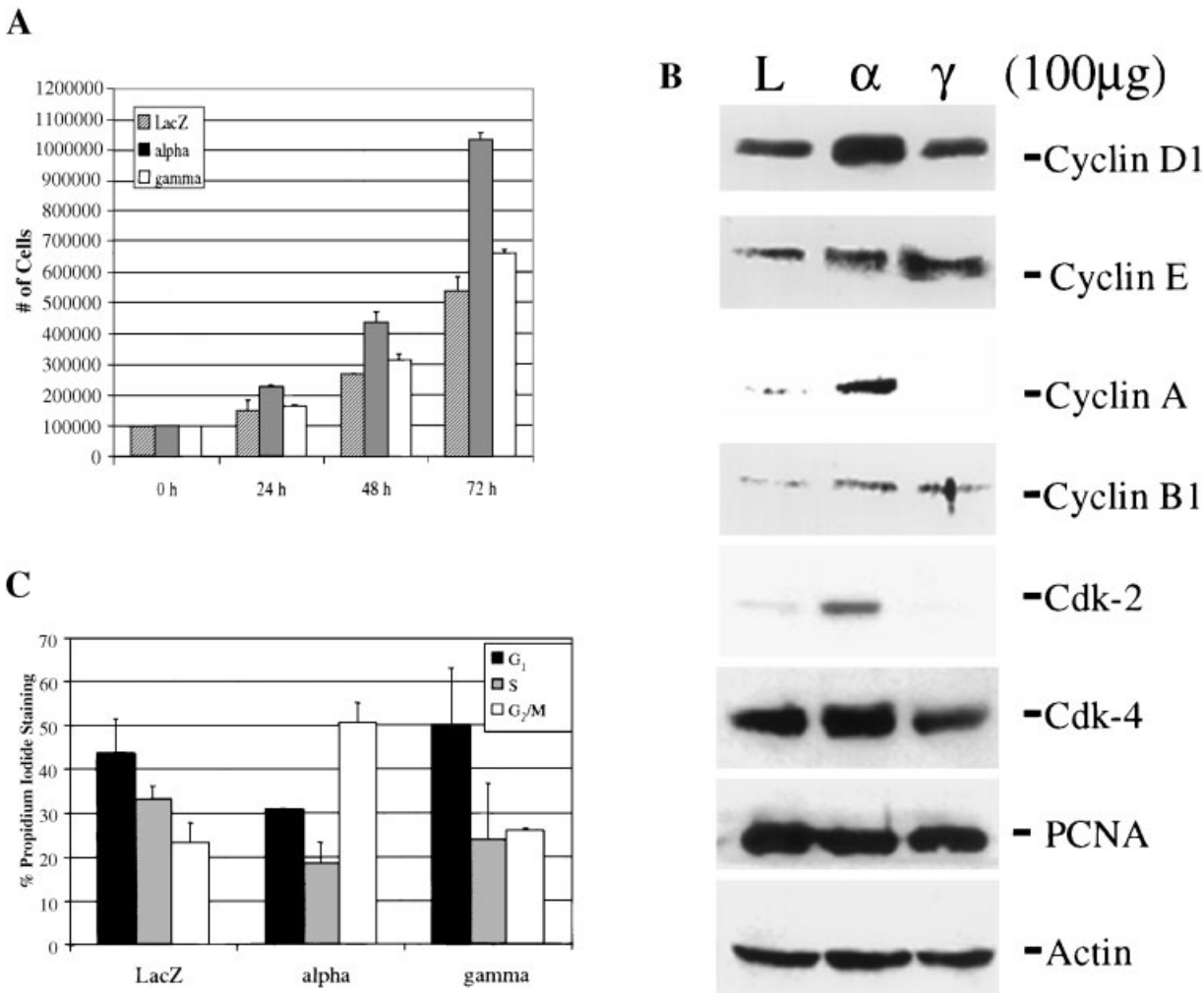

Fig. 2. IL-2Rα overexpressing increases cell proliferation rates and cell cycle progression. **A**: Equal numbers of LacZ, IL-2Rα, and IL-2Rγ cells were plated (10^5 cells/plate) and proliferation rates were determined at 24, 48, and 72 h by visual counting with a hemocytometer under a microscope (10×; Invertoscope Zeiss, OberKochen, Germany). Standard deviations are shown with error bars from a mean of at least three independent experiments. **B**: Western blot assay using antibodies specific to cyclins D1, E, A, and B1, cdks 2 and 4, PCNA or actin. LacZ cells are designated by L and are shown in **lane 1**. Similar results were obtained in three separate experiments. **C**: Asynchronous cells were analyzed by flow cytometry for cell cycle distribution of G_0/G_1, S, and G_2/M phases (cells in G_0/G_1, S, $G_2/M = 100\%$). Standard deviations are shown with error bars from a mean of three independent experiments.

cells (Fig. 2A). The doubling rates per 24 h of IL-2Rα, IL-2Rγ, and LacZ cells were 2.19, 1.89, and 1.76, respectively.

To investigate whether increased levels of cell cycle regulatory proteins in IL-2Rα-overexpressing PCI-13 cells accompany the increased growth rate, we determined expression of several cyclin and cdk proteins as well as PCNA in the three PCI-13 cell lines grown exponentially. We found that protein levels of cyclin D1 and cyclin A were much higher in IL-2Rα cells than those of LacZ and IL-2Rγ cells by threefold to fivefold (Fig. 2B). In contrast, levels of cyclins E and B1 were similar in all three PCI-13 cell lines (Fig. 2B). In addition, cdk2 protein was overexpressed by greater than tenfold in IL-2Rα cells versus LacZ and IL-2Rγ cells; levels of cdk4 protein were also higher in IL-2Rα cells than the other two PCI-13 cell lines (Fig. 2B). However, PCNA protein levels were similar in these three cell lines (Fig. 2B).

Increased levels of cyclin and cdk proteins might cause dysregulation of the cell cycle distribution in IL-2Rα-expressing cells [Sherr and Roberts, 1999]. To test this hypothesis, asynchronous LacZ, IL-2Rα, and IL-2Rγ cells were harvested and their cell cycle distribution was analyzed by flow cytometry. It was found that the G_1 population of IL-2Rα cells was lower than those of LacZ and IL-2Rγ cells (Fig. 2C). Associated with that, the G_2/M population of IL-2Rα cells was ~twofold higher than those of LacZ and IL-2Rγ cells (Fig. 2C). Therefore, overexpression of IL-2Rα in PCI-13 cells increases levels of cyclins D1 and A and cdks 2 and 4, which is probably responsible for the observed decreased G_1 population and accelerated doubling time.

IL-2Rα Cells Are Resistant to Apoptosis Induced by Multiple Stimuli

Increased transforming activity due to overexpression of IL-2Rα (Fig. 1) might also result from an inhibition of apoptotic death pathways [Reed, 1997]. To investigate this possibility, vector- or IL-2Rα-expressing cells were treated with various apoptosis stimuli, including the tripeptedyl proteasome inhibitor ALLN (at 10 μM), the topoisomerase II inhibitor VP-16 (at 25 μM), and the microtubule destabilization agent taxol (at 25 μM). It has been shown that proteasome inhibitors have the ability to induce tumor cell apoptosis and also to inhibit tumor growth in nude mice [An et al., 1998, Sun et al., 2001]. However, IL-2Rα cells were much more resistant to ALLN-induced apoptosis, as measured by activation of caspase-8 (Fig. 3A) and caspase-3/-7 (Fig. 3B), than the LacZ control cells. Only 1.5-fold increase in caspase-8 activity levels was detected in IL-2Rα cells, in contrast to a sixfold increase in the control cells after ALLN treatment (Fig. 3A). Similarly, only 0.5-fold increase in caspase-3/-7 activity was observed in IL-2Rα cells while a 2.5-fold increase found in the LacZ cells (Fig. 3B). Also, IL-2Rα cells also showed resistance to VP-16- and taxol-induced activation of caspase-8 and caspase-3/-7 (Fig. 3A,B). For example, taxol treatment increased caspase-8 activity by 2- and 12-fold, respectively, in the IL-2Rα and LacZ cells (Fig. 3A), and increased caspase-3/-7 activity only in LacZ, but not IL-2Rα cells (Fig. 3B). In contrast to IL-2Rα cells, IL-2Rγ-overexpressing cells were as sensitive as the control cells to the same drug treatment (see Fig. 3C).

To confirm the differential sensitivity of the three PCI-13 cell lines to various apoptosis stimuli, fluorescent microscopy was utilized to visualize the punctate nuclei, a characteristic of apoptotic cells [Kerr et al., 1972]. Hoechst dye staining revealed high levels of apoptosis in the LacZ and IL-2Rγ, but not IL-2Rα, cells after treatment with 10 μM ALLN for 24 h (Fig. 3C). Additionally, much more apoptotic population was also observed in the control and IL-2Rγ than IL-2Rα cells treated with 25 μM VP-16 for 24 h (Fig. 3C). These results suggest that overexpression of IL-2Rα in PCI-13 cells causes resistance to apoptosis.

Increased Levels of Anti-Apoptotic Bcl-2 Family Members in IL-2Rα Cells

It has been shown that overexpression of Bcl-2 or Bcl-X_L protein leads to drug resistance [Reed, 1997; Dalton and Jove, 1999]. To investigate the molecular basis for the increased drug resistance of IL-2Rα cells, we examined expression of several Bcl-2 family proteins in the three PCI-13 cell lines. Overexpression of IL-2Rα causes a dramatic increase in expression of Bcl-X_L protein, an anti-apoptotic Bcl-2 family member (Fig. 4A). In addition, Bcl-2 protein expression was also increased in the IL-2Rα cells, as compared with LacZ and IL-2Rγ cells (Fig. 4A). Interestingly, an abundant upper band of ~48 kDa (named p48, denoted with *), which was detected by the monoclonal Bcl-2

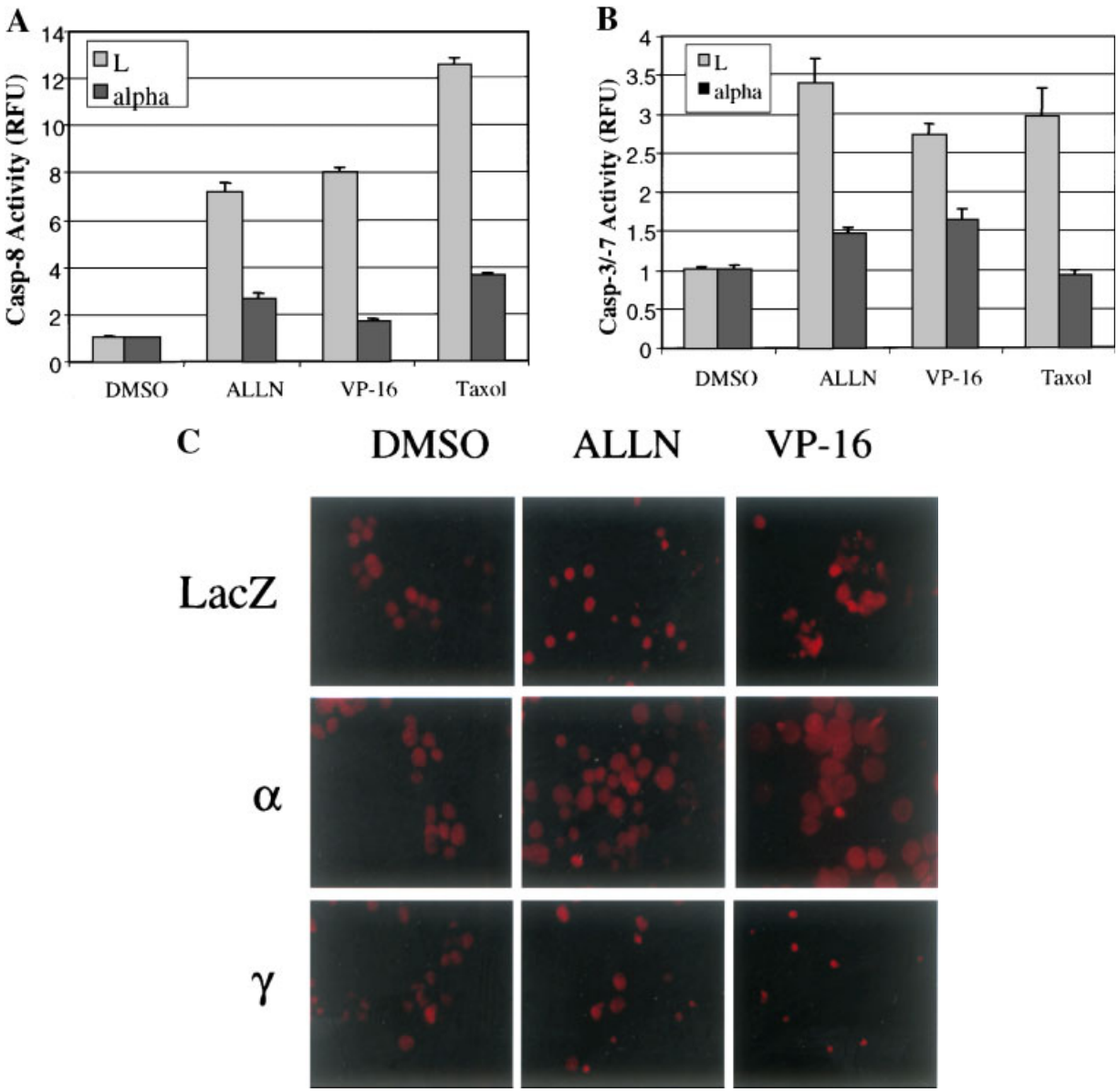

Fig. 3. IL-2Rα cells are resistant to apoptosis induced by ALLN, VP-16, and taxol. **A** and **B**: LacZ or IL-2Rα cells were treated with either DMSO, 10 μM ALLN, 25 μM VP-16, or 25 μM taxol for 24 h, followed by determination of cell-free caspase-8 (A) and caspases-3/-7 (B) activity assays. Standard deviations are shown with error bars from a mean of at least three independent experiments. **C**: LacZ, IL-2Rα, and IL-2Rγ cells were treated with either DMSO, 10 μM ALLN, or 25 μM VP-16 for 24 h, followed by nuclear staining assay with Hoechst 33342 dye. Apoptotic nuclei were visualized by fluorescent microscopy (40×). Data shown is representative of four independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

antibody, was only present in the LacZ control and the IL-2Rγ-overpressing cells, but absent in IL-2Rα cells (Fig. 4A). The p48 band was also detectable by two other monoclonal antibodies to Bcl-2 (data not shown). Similar levels of full-length Bid protein (23 kDa) were found in all the three PCI-13 cell lines (Fig. 4A).

We then examined localization of Bcl-X_L and Bcl-2 proteins in IL-2Rα-overexpressing cells by analyzing the cytoplasmic and heavy membrane fractions (HMF) using Western blot assay (Fig. 4B). WCE was used as a comparison. We found that Bcl-X_L was located in both the HMF and the cytoplasmic fraction of IL-2Rα cells and Bcl-2 protein was only in the HMF, but not the cytosol (Fig. 4B). The Bcl-2-related p48 band was also detected in the HMF (Fig. 4B). As a control, the mitochondria-specific cyclooxygenase-2

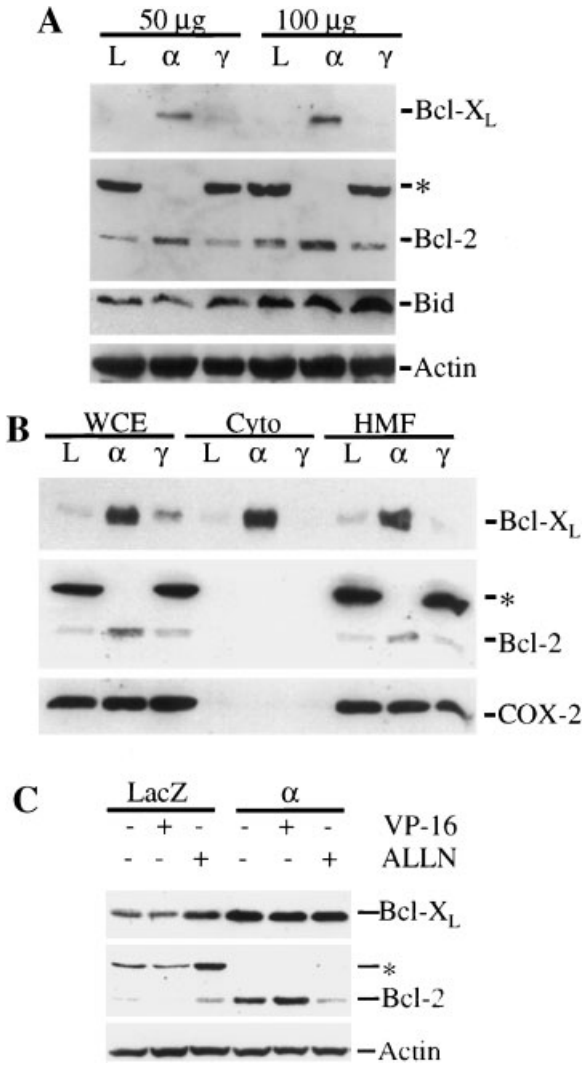

Fig. 4. Expression and localization of Bcl-2 family proteins in PCI-13 cell lines. **A**: Basal levels of Bcl-2 family proteins (Bcl-X_L, Bcl-2, Bid) were measured by Western blot assay using 50 or 100 μg of whole cells extracts prepared from PCI-13 cells expressing LacZ, IL-2Rα, or IL-2Rγ. Actin levels were determined as loading controls. Similar results were obtained it at least six separate experiments. **B**: Cellular localization of Bcl-X_L and Bcl-2 proteins. Whole cell extracts (WCE), cytoplasmic (Cyto), and heavy membrane fractions (HMF) were examined by Western blot assay using specific antibodies to Bcl-X_L, Bcl-2, and cyclooxygenase-2 (COX2). COX-2 is localized specifically to mitochondrial membranes and is used as a control. *, a p48 protein detected by the used monoclonal antibody to Bcl-2. Data shown is representative of at least three independent experiments. **C**: Levels of Bcl-X_L and Bcl-2 proteins during drug treatment. LacZ or IL-2Rα-overexpression cells were treated with 10 μM ALLN or 25 μM VP-16 for 24 h, followed by Western blot analysis using specific antibodies to Bcl-X_L, Bcl-2, or actin.

(COX-2) [Capaldi, 1990] was found only in the HMF, but not cytosol fraction (Fig. 4B).

To elucidate whether levels of Bcl-2 family proteins change during apoptosis process, the LacZ and IL-2Rα cells were treated with either 25 μM VP-16 or 10 μM ALLN for 24 h, followed by measurement of levels of Bcl-X_L and Bcl-2 proteins. ALLN, but not VP-16, treatment slightly increased levels of Bcl-X_L protein in LacZ cells, while neither treatment affected the high basal levels of Bcl-X_L in IL-2Rα cells (Fig. 4C). VP-16 treatment decreased Bcl-2 protein levels in LacZ cells, but slightly increased its expression in IL-2Rα cells (Fig. 4C). In contrast, ALLN treatment significantly decreased Bcl-2 expression in IL-2Rα (but not LacZ) cells (Fig. 4C). Interestingly, the p48 band related to Bcl-2 protein in LacZ cells was significantly increased by treatment with ALLN (but not VP-16) (Fig. 4C). We also found that p48 was not altered by λ-phosphatase treatment (data not shown) which suggests that it is probably not a hyperphosphorylated form of Bcl-2. Furthermore, stringent protocols such as additional heating time (10 min, 95°C), double amount of β-mercaptoethanol, double amount of SDS, or all three combined, did not affect levels of p48 (data not shown), indicating that it might not be a complex of Bcl-2 and another protein (also see Discussion).

Activation of Jak3 by IL-2Rα Overexpression Contributes to the Increased Levels of Cyclin D1, Cyclin A, Bcl-X_L, and Bcl-2 Proteins

Based on the results presented above (Figs. 1 to 4), we hypothesize that in the absence of exogenous IL-2, overexpression of the IL-2Rα chain would generate high affinity receptors that are able to bind to the low amounts of IL-2 produced by the tumor cells, activating the IL-2-dependent signal transduction pathway. Because Jak kinases are essential for IL-2-dependent pathways (see Introduction), we predicted that inhibition of Jak3 would affect expression of cell cycle and apoptosis regulatory proteins in IL-2Rα cells. To test this idea, we used a pharmacologic inhibitor, WHI-P131, that specifically inhibits activity of Jak3, but not Jak1 and Jak2. Treatment of IL-2Rα cells with 100 μM WHI-P131 for 24 h resulted in a twofold decrease in cyclin D1 protein expression and a complete loss of cyclin A protein (Fig. 5). Inhibition of Jak3 by this pharmacologic inhibitor in LacZ or IL-2Rγ cells gave similar results (Fig. 5). WHI-P131 treatment also partially inhibited expression of Bcl-X_L and Bcl-2 proteins in IL-2Rα cells (Fig. 5). Importantly, Jak3 inhibition by WHI-P131 in LacZ and IL-2Rγ

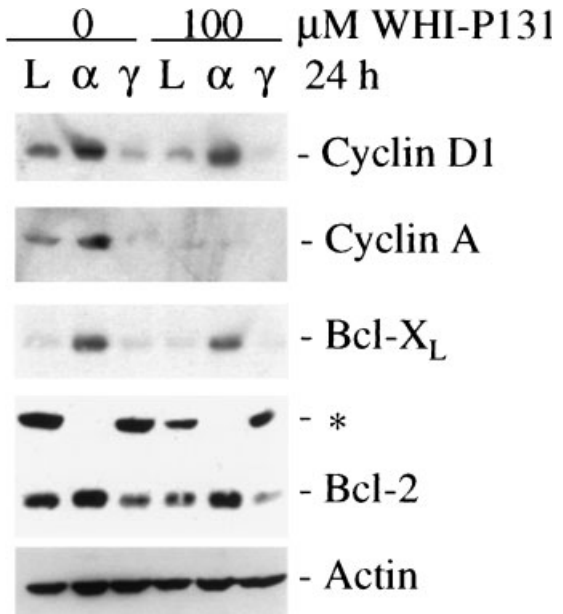

Fig. 5. Involvement of Jak3 kinase in IL-2Rα-mediated downstream events. Exponentially grown LacZ, IL-2Rα, and IL-2Rγ cells (**lanes 1–3**) were treated with 100 μM of the specific Jak3 kinase inhibitor WHI-P131 for 24 h (**lanes 4–6**), followed by Western blotting using specific antibodies to cyclin D1, cyclin A, Bcl-X_L, Bcl-2, and actin. The symbol '*' indicates the Bcl-2-related p48 protein (see Fig. 4).

cells decreased not only expression of Bcl-2 protein but also that of the Bcl-2-related p48 band (Fig. 5). To further investigate whether overexpression of IL-2Rα causes activation of Jak3, we measured levels of phosphorylated Jak3 (pJak3) in the three PCI-13 cell lines by Western blot assay using a polyclonal antibody specific to Tyr-980 pJak3. It has been shown that such a Jak3 phosphorylation is essential for its activation [Zhou et al., 1997 and also see Introduction]. We found a twofold increase in the pJak3 protein levels (~104 kDa) in IL-2Rα cells compared to LacZ and IL-2Rγ cells (data not shown). Interestingly, the same antibody also detected a protein band of ~38 kDa that was present in much higher levels in IL-2Rα cells than LacZ and IL-2Rγ cells (data not shown). It is possible that this band is either a pJak3-related protein or a proteolytic product of pJak-3. Taken together, these data are consistent with the conclusion that overexpression of IL-2Rα chain could activate autocrine and/or paracrine IL-2/IL-2R loops that trigger tyrosine kinases (including Jak3)-mediated signal transduction pathways, leading to increased transforming ability and malignancy in vivo.

Overexpression of IL-2Rα Causes a Decrease in the Proteasomal Chymotrypsin-Like Activity and an Increase in Ubiquitinated Proteins

The findings that expression of the Bcl-2-related p48 can be affected by a proteasome inhibitor (Fig. 4C) and is present only in the control and IL-2Rγ, but not IL-2Rα, cells (Fig. 4A) led us to determine levels of the proteasome activity in IL-2Rα-overexpressing cells. The basal levels of the proteasome activity were measured by a cell-free fluorogenic assay. IL-2Rα cells exhibited a lower amount of basal chymotrypsin-like activity than the control and IL-2Rγ cells (Fig. 6A). After the three cell lines were treated with 10 μM ALLN for 24 h, the chymotrypsin-like activity of the proteasome was inhibited by 35–55% in all the cell lines (Fig. 6A). We also determined the basal levels of ubiquitinated proteins in these three PCI-13 cell lines (Fig. 6B). Associated with low levels of proteasomal chymotrypsin-like activity in IL-2Rα cells (Fig. 6A), high levels of ubiquitinated proteins were observed in IL-2Rα, but not other two PCI-13 cell lines (Fig. 6B; lanes 4 vs. 1, 7). Treatment with 10 μM ALLN transiently increased in the control cells, but gradually increased in IL-2Rα cells, the ubiquitinated protein levels (Fig. 6B). However, no clear effects were observed in IL-2Rγ cells after ALLN treatment (Fig. 6B). These data suggest that overexpression of IL-2Rα affects the ubiquitin/proteasome-mediated protein degradation pathway.

DISCUSSION

Overexpression of IL-2Rα on tumor cells has been shown to be associated with an overall poor prognosis in human solid tumors and leukemias [Kaczmarski and Mufti, 1991; Barton et al., 1993; Tsai et al., 2001; McDoniels-Silvers et al., 2002; Ohno et al., 2002; Rosso et al., 2002]. In tumor biopsies overexpressing the α chain, elevated levels of IL-2Rα RNA were detected in squamous cell carcinoma or adenocarcinoma of the lung, for example, but not in the adjacent normal lung tissues [McDoniels-Silvers et al., 2002]. Additionally, serum concentrations of sIL-2Rα were up to 50-fold higher in patients with non-Hodgkin's lymphoma than in healthy individuals [Setoyama et al., 1994]. Patients with tumors not expressing IL-2Rα had a better prognosis and longer survival, an indication that in vivo the α chain may modulate tumor

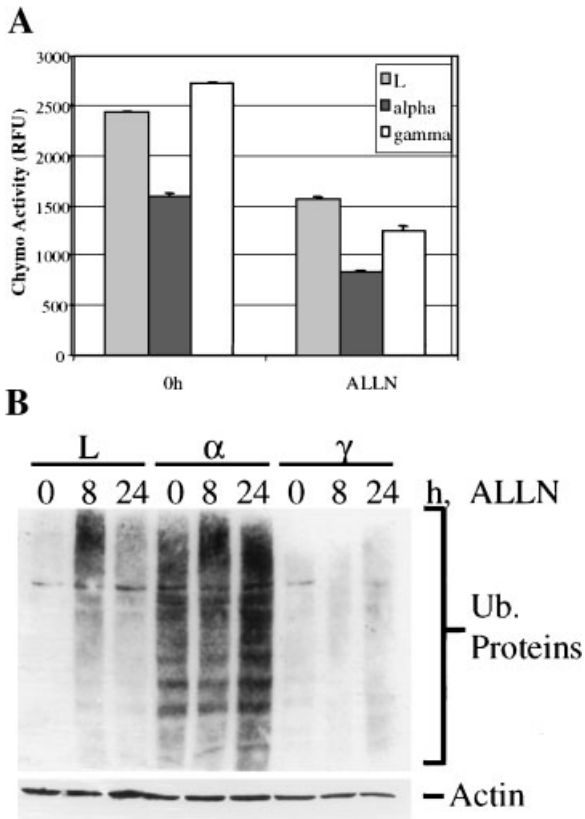

Fig. 6. Effects of IL-2Rα overexpression on levels of proteasomal chymotryptic activity and ubiquitinated proteins. **A**: PCI-13 cells expressing LacZ, IL-2Rα, or IL-2Rγ (0 h) were treated 10 μM ALLN for 24 h, followed by measurement of cell-free proteasomal chymotrypsin-like activity. **B**: PCI-13 cells expressing LacZ, IL-2Rα, or IL-2Rγ (0 h) were treated with 10 μM ALLN for 8 or 24 h, followed by Western blot analysis using antibodies to ubiquitin and actin. Ubiquitinated proteins are indicated.

growth. However, the involved molecular mechanisms are unknown. Towards the goal of understanding these important mechanisms, in the current study, we evaluated molecular changes in a squamous cell carcinoma of the head-and-neck cell line, PCI-13, transfected ex vivo with IL-2Rα cDNA. The transfected tumor cells expressed IL-2Rα on the cell surface, as measured by flow cytometry, in contrast to the parental or *LacZ* gene-transfected control cells, which expressed no detectable IL-2Rα [Lin et al., 1993]. In order to evaluate the contribution of the IL-2R to tumor cell growth, we also transfected PCI-13 with the γ chain gene, and showed that after selection, the transfected tumor cells expressed increased levels of mRNA for IL-2Rγ and also stained with anti-γ chain Abs after permeabilization [Lin et al., 1993]. Both the transfected and parental tumor cell lines expressed comparable levels of the β chain on the cell surface, as reported previously [Lin et al., 1993]. The transfected and parental cell lines were used in various functional and molecular assays to evaluate tumor cell characteristics associated with IL-2R overexpression.

Soft agar results clearly demonstrate that overexpression of IL-2Rα in PCI-13 cells is essential for transformation (Fig. 1). The transforming ability of PCI-13 cells was increased at least several 100-fold by overexpressing IL-2Rα (Fig. 1), which provides a possible molecular interpretation for the poor prognosis of IL-2Rα-associated tumors [Kopnin, 2000]. We then examined whether increased proliferation rates and/or decreased cell death rates are accompanied by the enhanced transforming activity in IL-2Rα cells.

It has been shown that IL-2 signaling is principally responsible for the G_1 to S phase transition in T-cells [Robb et al., 1981; Stern and Smith, 1986; Taniguchi and Minami, 1993]. Because binding of IL-2Rα to IL-2Rβ/γ increases these receptors' binding to IL-2 [Taniguchi and Minami, 1993], it is possible that overexpression of IL-2Rα could further accelerate the cell cycle progression. This hypothesis is supported by our results (Fig. 2). We discovered that IL-2Rα cells have an accelerated doubling time (~twofold faster than LacZ and IL-2Rγ cells; Fig. 2A), decreased G_1 population, and increased G_2/M population (Fig. 2C). To study the molecular players that are affected by IL-2Rα during the cell cycle, expression of several cyclins and cdks as well as PCNA was determined. We found that levels of cyclin D1 and cdk4 proteins were increased in IL-2Rα cells, which should be responsible for the decreased G_1 population (Fig. 2) [Meyerson et al., 1992; Imoto et al., 1997]. In addition, increased levels of cyclin A and cdk2 proteins in IL-2Rα cells might contribute to their increased G_2/M population (Fig. 2) [Yam et al., 2002]. The acceleration through G_1 phase might also account for the abnormally large population in G_2/M phase in IL-2Rα-overexpressing cells. These data suggest that IL-2Rα overexpression triggers signal transduction pathways driving the cancer cell cycle progression. Consistent with this argument, high levels of both cyclin D1 and cyclin A have been linked to high proliferation rates in several cancers [Imoto et al., 1997; Kim et al., 1998; Kushner et al., 1999; Allan et al., 2000].

Another mechanism for accumulation of tumor cells could be mediated by inhibition of their apoptosis [Reed, 1997; Dalton and Jove, 1999]. Apoptosis is the mechanism by which cells commit suicide in a highly regulated fashion [Wyllie et al., 1980]. Regulation of apoptosis involves the balancing of proapoptotic (e.g., Bad, Bax, Bid) and antiapoptotic (e.g., Bcl-2, Bcl-X_L) Bcl-2 family proteins. The caspases, a family of cysteine proteases, are highly conserved and are responsible for implementing the death program after a death signal is received by the cell. They can be divided into initiator (−8, −10) and effector (−3, −7) caspases [Hengartner, 2000; Zimmermann et al., 2001]. We found that IL-2Rα cells evaded apoptosis induced by ALLN, VP-16, and taxol, as demonstrated by low levels of caspase-8 and caspase-3/-7 activities and lack of apoptotic nuclei (Fig. 3). Because overexpression of Bcl-X_L or Bcl-2 can inhibit apoptosis [Reed, 1997; Dalton and Jove, 1999; Hengartner, 2000], we investigated whether several Bcl-2 family members were affected by IL-2Rα expression. High levels of Bcl-X_L and Bcl-2 proteins were found in IL-2Rα, but not in the control vector- or IL-2Rγ-transfected cells (Fig. 4A). It has been shown that the mitochondrial localization of Bcl-X_L and Bcl-2 is required for their anti-apoptotic functions [Cory, 1995; Green and Reed, 1998]. We found Bcl-X_L in both the mitochondrial membrane-containing and cytosolic fractions, while Bcl-2 was detected only in the mitochondrial membrane fraction (Fig. 4B). These data suggest that high levels of mitochondrial Bcl-X_L and Bcl-2 proteins might contribute to the observed drug resistance in IL-2Rα cells (Fig. 3). To ascertain whether stimulation with exogenous IL-2 had any effects on the Bcl-2 family proteins, we incubated the three PCI-13 cell lines with 5 ng/ml of recombinant IL-2 for various time periods. However, this treatment had no effect on the levels of Bcl-X_L and Bcl-2 proteins (data not shown).

If increased proliferation and decreased drug sensitivity of IL-2Rα cells are mediated through IL-2/IL-2R-dependent signal transduction pathway, then Jak and other tyrosine kinases should be involved. Indeed, inhibition of Jak3 kinase activity by the specific inhibitor WHI-P131 completely blocked expression of cyclin A protein in IL-2Rα cells (Fig. 5), demonstrating that Jak3 regulates cyclin A protein expression in IL-2Rα cells. The effects of WHI-P131 on cyclin D1, Bcl-X_L, and Bcl-2 proteins were not as significant as on cyclin A (Fig. 5). It remains to be determined whether Jak1 or other tyrosine kinases, acting downstream of IL-2Rα, regulate expression of these proteins. We also measured levels of phosphorylated and active Jak3 by Western blotting using a specific antibody to pJak3. Higher levels of pJak3 and a related protein were found in IL-2Rα cells than in LacZ and IL-2Rγ cells (data not shown).

Three specific monoclonal Bcl-2 antibodies detected a p48 band in the control and IL-2Rγ, but not IL-2Rα, cells (Fig. 4A and data not shown), suggesting that it is related to Bcl-2 protein. This p48 was detected only in the mitochondrial fraction, but not in cytosol, of control PCI-13 cells (Fig. 4B). These data raise an interesting hypothesis that the formation of p48 from Bcl-2 in the control cells is a potential mechanism contributing to inactivation of Bcl-2 and that IL-2Rα overexpression inhibits the conversion of Bcl-2 to p48, thereby restoring the anti-apoptotic function of Bcl-2 (see Fig. 3). We realize that further studies are needed to investigate the nature of p48 and, therefore, to examine the above hypothesis. However, our preliminary studies suggest that p48 might not be a phosphorylated form of Bcl-2, since its expression was not affected by phosphatase treatment (data not shown). The p48 might not even be a protein complex, since heating, β-mercaptoethanol, SDS, or combination of the above did not affect p48 levels (data not shown). Finally, it remains debatable whether p48 is an ubiquitinated form of Bcl-2. Although p48 levels could be increased by treatment with the proteasome inhibitor ALLN (Fig. 4C), p48 was not found in IL-2Rα cells that contain high levels of many ubiqitinated proteins (Figs. 4A vs. 6B). Further experiments are needed for a better characterization of p48.

To examine whether IL-2Rα overexpression might affect the ubiquitin/proteasome-mediated protein degradation pathway, we measured basal levels of the proteasomal chymotrypsin-like activity and ubiqutinated proteins (Fig. 6). Although previous experiments suggested that inhibition of the chymotrypsin-like activity of the proteasome is associated with induction of tumor cell apoptosis [Lopes et al., 1997; An et al., 1998], low levels of the proteasomal chymotrypsin-like activity were found in IL-2Rα cells (Fig. 6A), which were resistant to drug treatment (Fig. 3). One

interpretation is that Bcl-X_L and Bcl-2 function downstream of the proteasome and therefore, compromises the effects of the decreased chymotrypsin-like activity. Consistent with the low proteasomal chymotrypsin-like activity in IL-2Rα cells, a significant build-up of ubiquitinated proteins was observed in these cells (Fig. 6B). Therefore, IL-2Rα overexpression has indeed altered the ubiquitin/proteasome pathway, although the detailed molecular mechanisms remain to be determined.

In summary, IL-2Rα-overexpressing PCI-13 cells showed an enhanced transforming activity, associated with increased proliferation rates and drug resistance. These data are consistent with the clinical observation that many tumors and leukemias overexpressing IL-2Rα have a progressive phenotype and poor prognosis [Kaczmarski and Mufti, 1991; Barton et al., 1993; Tsai et al., 2001; McDoniels-Silvers et al., 2002; Ohno et al., 2002; Rosso et al., 2002]. Further studies on characterization of IL-2Rα- versus IL2Rβ/γ-mediated signal transduction pathways should help develop novel anticancer drugs selectively targeting cancers with overexpressed IL-2Rα.

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