# Characterization of the Interleukin 2 Receptors (IL-2R) Expressed on Human Natural Killer Cells Activated In Vivo by IL-2: Association of the p64 IL-2R γ Chain with the IL-2R β Chain in Functional Intermediate-Affinity IL-2R

By Stephan D. Voss,* Paul M. Sondel,*‡§ and Richard J. Robb‖

From the Departments of *Human Oncology, ‡Pediatrics, and §Genetics, the University of Wisconsin Clinical Sciences Center, Madison, Wisconsin 53792; and ‖DuPont Merck Pharmaceutical Co., Wilmington, Delaware 19880

## Summary

Interleukin 2 (IL-2) receptors expressed on the surface of activated T cells and natural killer (NK) cells exhibit a variety of affinity states depending on their subunit composition. Low-affinity binding is associated with a 55-kD α chain, intermediate-affinity binding with a 70–75-kD β chain, and high-affinity binding with a bimolecular complex of the α and β subunits. In a previous study of the IL-2 receptors expressed on NK cells obtained from cancer patients after in vivo IL-2 therapy, we documented a discrepancy between the level of β chain and the level of intermediate-affinity IL-2 binding sites expressed on the cell surface. Based on this result, we postulated that formation of intermediate-affinity receptor sites required a component in addition to the β chain, and that this component was present at limiting levels in the patient NK cells. In the present study we have examined the structure of the intermediate-affinity receptor complex using monoclonal antibodies that recognize the β chain, but that do not interfere with its ability to bind IL-2. Evidence is presented establishing the physical association of a novel protein of 64 kD with the β chain in intermediate-affinity IL-2 binding sites. This molecule, termed IL-2R γ chain, coprecipitated with β chains prepared from cells that had been incubated with IL-2, but was undetectable in immunoprecipitates prepared in the absence of IL-2. Examination of γ chain expression in post-IL-2 therapy NK cells, where only low levels of intermediate-affinity IL-2 binding were detectable, revealed that the γ chain was associated with, on average, only 10–12% of the β chains expressed on such cells. This contrasted with approximately equal levels of β and γ chain expression on YT cells, a cell line that has both high levels of cell surface β chain expression and high levels of IL-2 binding. Thus, the ratio of γ chain to β chain present in the immunoprecipitates roughly correlated with the proportion of β chain involved in intermediate-affinity receptor sites. This result suggests that the 64-kD γ chain is the component responsible for regulating the affinity of IL-2 association with the β subunit. By further defining the structural components necessary for IL-2 receptor formation, these studies provide additional insight into mechanisms whereby lymphocytes might regulate their responsiveness to IL-2.

IL-2 is a 15-kD glycoprotein produced by activated helper T lymphocytes that is responsible for activating and maintaining proliferative and cytolytic responses mediated by both T and NK cells (1–3). These cells are activated by soluble IL-2 after its interaction with specific cell surface receptors. The IL-2R is made up of at least two subunits, α (p55, Tac) and β (p70-75), that noncovalently associate on the cell surface to produce a high-affinity receptor complex capable of binding IL-2 with an equilibrium $K_d$ of ~5–20 pM (4–9). Each of the chains present in the IL-2R complex can bind IL-2 independently of the other, with either low ($\alpha$, $K_d$ =

5–20 nM) or intermediate ($\beta$, $K_d$ = 0.5–2.0 nM) affinity (2, 7–11). Low-affinity receptors bind IL-2 with both rapid association and dissociation rates, while intermediate-affinity receptors, in contrast, have slow association and dissociation rates (2, 10, 11). The interaction of the α and β chains allows the formation of a high-affinity receptor with kinetic and equilibrium binding properties that are a cooperative composite of its constituent chains, such that the high-affinity receptor combines the rapid rate of IL-2 association characteristic of low-affinity receptors with the slow rate of dissociation characteristic of intermediate-affinity receptors. This

results in a heterodimeric receptor complex that binds IL-2 with an equilibrium affinity one to three orders of magnitude higher than either the low- or intermediate affinity receptor. When expressed on lymphoid cells, both the intermediate and the high-affinity receptor can internalize IL-2 and mediate biological effects (12, 13).

In a previous study examining the IL-2 binding properties of the IL-2R expressed on human NK cells activated in vivo by systemic infusions of IL-2, we documented high levels of IL-2R $\beta$ chain expression on NK cells obtained after IL-2 therapy, a finding inconsistent with the low levels of intermediate-affinity IL-2 binding detected on these cells (14). No high-affinity IL-2R were detectable, and upon in vitro culture in IL-2, these cells responded exclusively through intermediate-affinity receptors. It was concluded from these studies that the regulation of functional IL-2R formation could not be determined solely by the level of $\beta$ chain expression at the cell surface, and that some other IL-2R subunit may be needed to allow the formation of a functional intermediate-affinity IL-2R (14). This conclusion was in agreement with other studies that had demonstrated a requirement for some other molecule(s) in addition to the p55 $\alpha$ and p70 $\beta$ chains for the formation of functional IL-2R (15–19). Based on quantitative kinetic studies, Ringheim et al. (19) proposed a role for a putative IL-2R $\gamma$ chain that influenced the equilibrium binding affinity of the receptor complex by controlling the rate of dissociation of bound IL-2. According to this hypothesis, cells (such as the leukemic cell line YT, or lymphoid cells transfected with the $\beta$ chain cDNA) that bind IL-2 with intermediate affinity express $\beta\gamma$ complexes. In contrast, isolated $\beta$ chains (18) or $\beta$ chains expressed on nonlymphoid cells (19) bind IL-2 with 50–100-fold lower affinity ($K_d$ = 70 vs. 0.5–2.0 nM for intermediate-affinity binding).

In attempts to identify additional components of the human IL-2R, a variety of receptor-associated proteins have been reported by a number of investigators (20–23), although none have received universal recognition as an IL-2R $\gamma$ chain. Takeshita et al. (24) have observed a 64-kD molecule in high-affinity IL-2R complexes that was only detectable in the presence of IL-2, and have proposed that this molecule be termed IL-2R $\gamma$ chain. The present study was undertaken to determine whether the presence of this additional receptor subunit could be linked to the formation of a functional intermediate-affinity IL-2R. In vivo activated NK cells provide a unique model system with which to pursue these studies, in that only a small fraction of the $\beta$ chains expressed on these cells are involved in intermediate-affinity IL-2 binding, while the majority of the $\beta$ chains present do not bind IL-2 with an appreciable affinity. Using two newly developed anti-IL-2R $\beta$ chain mAbs, the same 64-kD protein observed by Takeshita et al. (24) in high-affinity receptors was detected in intermediate-affinity IL-2R. In accordance with the proposal of Takeshita et al. (24), we have also termed this molecule IL-2R $\gamma$ chain. The p64 $\gamma$ chain physically associated with the $\beta$ chain in the presence of IL-2, while in the absence of IL-2 no association of $\gamma$ chain with $\beta$ chain was detected, confirming the absolute requirement for IL-2 bound in the receptor complex for the detection of $\gamma$ chain associated with $\beta$ chain.

Furthermore, the low level of IL-2R $\gamma$ chain that was associated with the $\beta$ chain in immunoprecipitates prepared from post-therapy NK cells corresponded to the low level of intermediate-affinity IL-2 binding detected on these cells. No IL-2R $\gamma$ was detected associated with the $\beta$ chain in fibroblasts transfected with the $\beta$ chain cDNA. These results suggest that the IL-2R $\gamma$ chain may play a crucial role in lymphoid cells, regulating IL-2R formation by modulating the ligand binding properties of the $\beta$ chain.

## Materials and Methods

*Patients.* All patients from whom samples were obtained were enrolled in phase I clinical trials of IL-2 currently underway at the University of Wisconsin Comprehensive Cancer Center, and had progressive metastatic renal cell carcinoma or malignant melanoma that was incurable by surgery. Patients received IL-2 at 1–3 × 10⁶ U/m²/d as a 96-h continuous infusion for a consecutive 2–4 wk, as described previously (25–27). All patients signed consent forms for in vivo and in vitro studies approved by the University of Wisconsin Committee for the Protection of Human Subjects. Patient PBMC (PBL) were obtained by Ficoll-Hypaque separation of heparinized blood specimens drawn 24 h after each 4-d cycle of IL-2, and were either used directly or cryopreserved by controlled rate freezing in 10% DMSO, followed by storage at −135°C until later use. For purification of NK cells from fresh peripheral blood, PBL were depleted of T cells by staining with biotinylated anti-CD5 and avidin-FITC, followed by sorting on a MACS magnetic cell separator (Miltenyl Biotec, Sunnyvale, CA) (28). Purification typically yielded NK cell populations that were 94–99% CD56⁺CD3⁻. For some patient specimens, the percentage of CD56⁺CD3⁻ NK cells in the peripheral blood obtained after in vivo IL-2 therapy exceeded 92%, and no further cell separation was performed.

*IL-2.* rIL-2 was generously provided by Hoffmann-La Roche (HLR), Inc. (Nutley, NJ) via the National Cancer Institute-Biological Response Modifiers Program (NCI-BRMP; Frederick, MD) for clinical treatment and in vitro studies. All IL-2 units are based on the NCI-BRMP standard for unit determination. The specific activity of HLR IL-2 is 1.5 × 10⁷ U/mg protein.

*Cell Lines.* The YT cell line (29) expresses a low level of IL-2R $\alpha$ chain and a relatively high number (~12,000–20,000/cell) of intermediate-affinity IL-2 binding sites (7, 8, and unpublished observations). Chinese hamster ovary (CHO) fibroblast cells transfected with either the cDNA for the IL-2R $\beta$ chain (CHO $\beta$) or both the IL-2R $\alpha$ and $\beta$ chain cDNAs (CHO $\alpha/\beta$) have been described previously (19).

*Production of mAb to the IL-2R $\beta$ Chain.* BALB/c mice were immunized (three times at 2–3-wk intervals) with a clone of CHO cells stably expressing 1–3 × 10⁶ copies per cell of a truncated version ($\beta_{261}$; see reference 19) of human rIL-2R $\beta$ chain. The cytoplasmic-deletion mutant of the $\beta$ chain was chosen over full-length $\beta$ because of its much higher (10–100×) expression on the surface of CHO cells (19). Splenocytes were harvested and fused with the nonproducing hybridoma line P3X63.653, followed by hybrid selection in HAT medium. Culture supernatants were tested for the presence of anti-$\beta$ antibodies by indirect fluorescence using CHO $\beta_{261}$ and wild-type CHO cells as targets and PE-conjugated goat anti–mouse IgG as a secondary reagent. Two hybrids (designated 341 [IgG1] and 561 [IgG2a]) were selected from the initial screen and subcloned by limiting dilution. Purified antibody was isolated from culture supernatants of the clones by affinity chromatography

on GammaBind Plus beads (Pharmacia LKB Biotechnology, Piscataway, NJ).

*Antibodies.* Mikβ1 and Mikβ3 (30) were kindly provided by M. Tsudo (Unitika Central Hospital, Kyoto, Japan). TU27 (31) was the generous gift of K. Sugamura (Tohoku University, Sendai, Japan). Control IgG1 (MOPC21) and IgG2a (UPC10) were purchased from Sigma Chemical Co. (St. Louis, MO).

*$^{125}I$-IL-2 Binding Assays.* Binding assays with $^{125}I$-IL-2 were performed as described previously (14, 19).

*FACS® Analyses.* FACS® staining (Becton Dickinson & Co., Mountain View, CA) was performed as described previously (14). For staining with mAbs 341 and 561, cells were incubated with purified mAb at 20 μg/ml, followed by staining with PE-conjugated, isotype-specific (i.e., GAM IgG1 for staining with 341 and GAM IgG2a for staining with 561) goat anti–mouse reagents (Southern Biotechnology Associates, Birmingham, AL).

*Iodination and Immunoprecipitation.* These were performed essentially as described previously (14), except that cells were radioiodinated with Iodogen (Pierce Chemical Co., Rockford, IL). Typically, $5 \times 10^7$ cells were resuspended at $2 \times 10^7$ cells/ml in HBSS. 5 mCi Na$^{125}$I (Amersham Corp., Arlington Heights, IL) were added to cells without carrier NaI. 500-μl aliquots of cells $+ ^{125}I$ were added to glass tubes coated with 150 μg Iodogen. After 30 min at room temperature, cells were washed and resuspended in binding buffer (RPMI 1640 with 25 mM Hepes, pH 7.4, and 2 mg/ml BSA). For experiments in which cells were incubated in IL-2, IL-2 was added to a final concentration of 50–80 nM, to insure complete saturation of intermediate-affinity IL-2 binding sites. After incubation for 60–90 min on ice, cells were pelleted, washed in ice cold HBSS, and lysed in NP-40 lysis buffer (0.5% NP-40, 140 mM NaCl, 5 mM EDTA, 100 μg/ml PMSF, and 10 μg/ml each leupeptin, aprotinin, and antipain). After 30 min on ice, lysates were cleared by centrifugation for 20 min at 14,000 rpm in a microfuge. Lysates were preincubated with nonspecific mouse IgG agarose (Sigma Chemical Co.), followed by incubation for 3–6 h with specific antibody coupled to Sepharose beads (Aminolink Sepharose; Pierce Chemical Co.) or with GammaBind Plus Protein G beads (Pharmacia LKB Biotechnology) that had been precoated with 25 μg of antibody. Immunoprecipitates were washed extensively, followed by addition of 7.5 μl of boiling buffer (20 mM Tris, pH 7.5, 5% β mercaptoethanol, 0.3% SDS) and heating at 95°C for 2–3 min. Precipitates were eluted from beads into 120 μl IEF sample buffer (9.5 M urea, 2% [wt/vol] NP-40, 1.6% pH 5–7 ampholines [Pharmacia LKB Biotechnology], 0.6% pH 3.5–10 ampholines, 5% β-mercaptoethanol) and analyzed by two-dimensional (2-D) IEF/SDS-PAGE. 5 μg of bovine muscle actin (Sigma Chemical Co.) was added to each sample as an internal IEF standard (pI = 5.4–5.5, molecular mass = 45 kD). Similar results were obtained when 10 mM dithiothreitol was used as the reducing agent.

*2-D IEF/SDS-PAGE.* This was performed according to the method of O'Farrell (32), with modifications by Jones (33). Samples were loaded on 15-cm IEF tube gels (9.2 M urea, 4% acrylamide, 2% [wt/vol] NP-40, 1.6% pH 5–7 ampholines, and 0.6% pH 3.5–10 ampholines) that had been prefocused at constant 2 mA until 800 V was reached. IEF was for 12,000–14,000 V-h at 800 V, followed by 2,000 V-h at 1,000 V. Tubes were extruded into SDS sample buffer (62.5 mM Tris, pH 6.8, 10% [wt/vol] glycerol, 2.3% SDS, 0.002% bromophenol blue) and stored at −20°C until second dimension gels were run. For second dimension, tubes were thawed quickly at 37°C, layered onto 10% polyacrylamide gels containing a 5-cm stacking gel, and subjected to electrophoresis at 30–40 mA constant current per gel. After electrophoresis, gels were stained

with coomassie blue, fixed, dried, and exposed to phosphor imaging plates (see below).

*Autoradiographic Detection by Storage Phosphor (Phosphorimager) Detection (34).* After staining, fixing, and drying, gels were exposed to photostimulable storage phosphor imaging plates (Molecular Dynamics, Sunnyvale, CA) for 3–5 d, followed by development and quantitation on a Phosphorimager (400A; Molecular Dynamics) using Image Quant software. Similar images to those presented were obtained on samples visualized by conventional autoradiographic exposure to x-ray film. However, the use of the phosphorimager provided ~100 times greater sensitivity in our hands than standard x-ray film. In addition, a linear dynamic range of magnitude and sensitivity for the storage phosphor imaging plates allowed accurate comparisons to be made between signals that varied significantly in intensity. Digitalized images were analyzed and printed on a LaserJet II printer (Hewlett-Packard Co., Palo Alto, CA).

## Results

*Isolation and Characterization of Anti-IL-2R β Chain mAbs 341 and 561.* mAbs recognizing the human IL-2R β chain were developed by immunizing BALB/c mice with CHO cells expressing recombinant human β chain (for details, see Materials and Methods). This procedure resulted in the identification of two unique mAbs, 341 (IgG1) and 561 (IgG2a). FACS® analysis of YT cells, which express the IL-2R β chain as predominantly intermediate-affinity IL-2 binding sites (7, 8, 14), and CHO cells transfected with either the full-length β chain cDNA or both the α and β chain cDNAs (19), confirmed that these mAbs recognize the β chain (Fig. 1). When these mAbs were characterized with respect to their effect on IL-2 binding, neither was found to inhibit IL-2 binding to high- or intermediate-affinity IL-2R. As shown in Fig. 2, mAb 341 had no effect on IL-2 binding to the high-affinity IL-2R expressed on HUT 102 cells (Fig. 2 *A*) or to the α/β complex expressed on CHO cells transfected with both α and β chain cDNAs (19) (Fig. 2 *B*). Similarly, mAb 341 had no inhibitory effect on IL-2 binding to the intermediate-affinity IL-2R expressed on YT cells (Fig. 2 *C*). Results similar to those shown for mAb 341 in Fig. 2 were obtained with mAb 561 (data not shown). In contrast, mAb TU27, known to inhibit the binding of IL-2 to the β chain (14, 31), blocked the binding of $^{125}I$-IL-2 to the intermediate-affinity sites expressed on YT cells (Fig. 2 *C*). TU27 similarly eliminated $^{125}I$-IL-2 binding to the high-affinity sites on HUT 102 cells (Fig. 2 *A*) and to the α/β complexes on transfected CHO cells (Fig. 2 *B*), reducing the IL-2 binding to these cells to an affinity characteristic of the low-affinity α chain. One-dimensional SDS-PAGE analysis of immunoprecipitates prepared from surface-iodinated YT cells confirmed that mAbs 341 and 561 recognized the same 70-kD protein that was precipitated by a panel of anti-β chain mAbs including the Endogen anti-β chain mAb, Mikβ1 (30), Mikβ3 (30), and TU27 (31) (data not shown). Thus, mAbs 341 and 561 are functionally comparable to Mikβ3 (30) and TU11 (35): these antibodies all bind to the IL-2R β chain, but do not competitively inhibit IL-2 binding to the β chain.

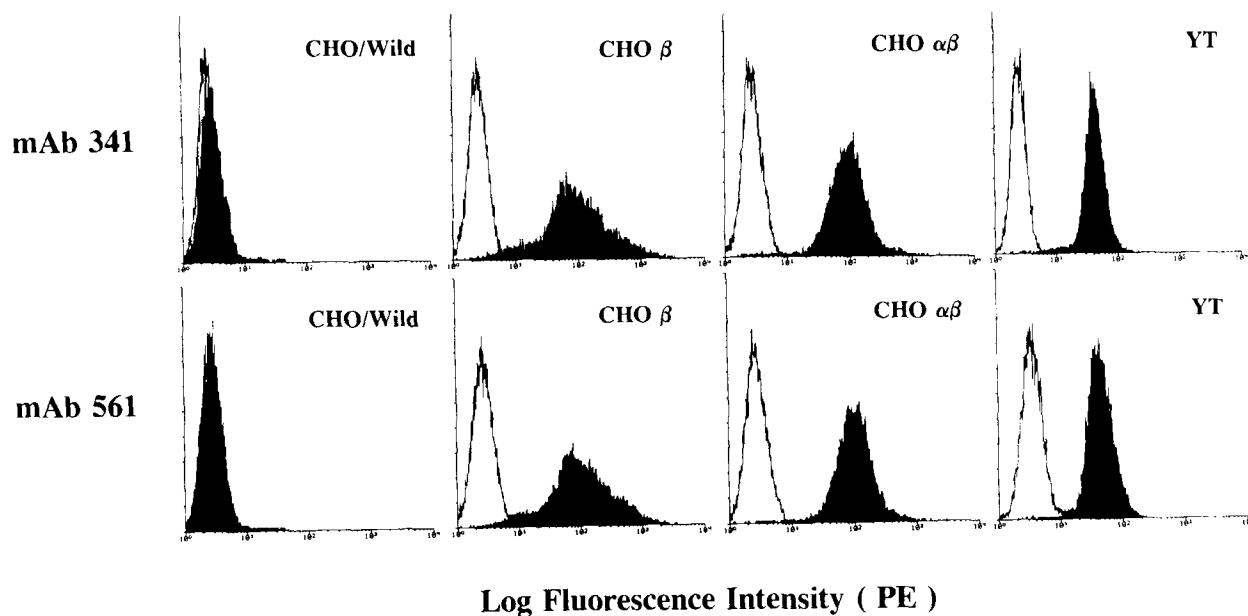Competitive binding assays (Table 1) showed that mAbs 341, 561, and Mikβ3 inhibited up to 100% of one another's

## Log Fluorescence Intensity ( PE )

**Figure 1.** Flow cytometric analysis of mAbs 341 and 561 staining on cells expressing the IL-2R β chain. CHO cells either untransfected (*CHO/Wild*), transfected with the full-length IL-2R β chain cDNA (CHO β), or transfected with both full-length α and β chain cDNAs (*CHO αβ*), as well as YT cells, were incubated with 20 μg/ml of control IgG1, control IgG2a, mAb 341, or mAb 561. After 30–60 min on ice, cells were washed twice and stained with the appropriate isotype-specific goat anti–mouse IgG-PE for 30 min. Positive staining is indicated by the solid histograms, while control staining is presented as an open histogram. As expected, CHO cells that had not been transfected with the β chain cDNA (*CHO/Wild*) had levels of staining with mAbs 341 and 561 that were indistinguishable from control staining, such that the overlaying of histograms as shown resulted in masking of the control histogram.

binding, while TU27 and Mikβ1 modulated the affinity of binding of mAbs 341 and 561 such that at the highest concentrations of TU27 or Mikβ1, the affinities of 341 and 561 were reduced approximately threefold. Similarly, in a reciprocal experiment, mAbs 341 and 561 reduced the affinity of TU27 binding approximately twofold. Competitive binding results with these same antibodies were confirmed by two-color FACS® analyses using isotype-specific secondary reagents (data

not shown). In these experiments, YT cells could be double stained with combinations of mAbs 341 and Mikβ1 (341/Mikβ1), or with 561/TU27, but not with 341/561. Furthermore, mAbs 341, 561, and Mikβ3, but not TU27 or Mikβ1, recognized a human/mouse chimeric β chain in which the NH₂-terminal 75 amino acids were murine and the remainder human (R. Robb, unpublished observations). These findings indicate that the epitopes on the human IL-2R

**Table 1.** *Summary of Results of Competitive Binding Assays with Anti-IL-2R β Chain mAb*

| mAb | $K_d$* | Effect on IL-2 binding | Competitive inhibition |
|-----|--------|------------------------|------------------------|
| | *nM* | | |
| 341 | 10.5 | Noninhibitory | Reciprocally inhibit the binding of one another to the β chain; reduce the affinity of binding |
| 561 | 6.7 | Noninhibitory | of TU27 approximately twofold. |
| TU27 | 4.9 | Inhibitory | |
| Mikβ1 | 13.0 | Inhibitory | Reduce the affinity of 341 and 561 binding by approximately threefold. |
| Mikβ3 | 1.1 | Noninhibitory | Inhibits 100% of binding of 341 and 561. |

* The $K_d$s of binding to CHO $β_{261}$ cells (19) for mAbs 341, 561, and TU27 were determined by competitive binding assays employing the corresponding Bolton Hunter reagent–radioiodinated mAb probes. The $K_d$ ($K_i$) of Mikβ1 binding was inferred from competition curves using $^{125}$I-TU27, titering Mikβ1 against a constant amount of $^{125}$I-TU27. Similarly, the $K_d$ ($K_i$) of binding for Mikβ3 was derived from competition curves, titering Mikβ3 against a constant amount of either $^{125}$I-341 or $^{125}$I-561.
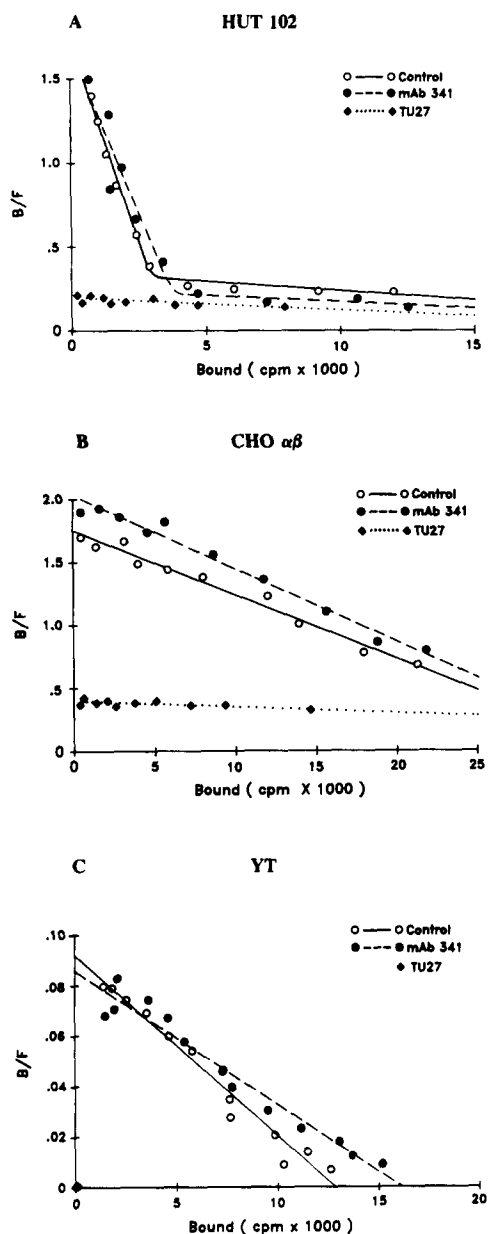
**Figure 2.** Scatchard analysis of the influence of mAbs 341 on $^{125}$I-IL-2 binding: 341 does not inhibit IL-2 binding. HUT 102 cells expressing both high-affinity and low-affinity IL-2R (*A*), CHO transfected with cDNAs, encoding the $\alpha$ and $\beta$ chains (*B*), and YT cells, which express predominantly intermediate affinity IL-2R (*C*), were incubated for 20–30 min on ice with 50 $\mu$g/ml of either control mAb (O—O), mAb 341 (●—●), or TU27 (◆···◆). Cells were then incubated with increasing concentrations of $^{125}$I-IL-2. Bound and free ligand were separated by centrifugation through silicone/paraffin oil. Results are presented as Scatchard plots of the binding data. Under the conditions used in the experiment shown in *B*, essentially all of the IL-2 binding to CHO $\alpha/\beta$ cells was to the $\alpha/\beta$ complex and was of a relatively high affinity ($K_d = 44$ pM), allowing evaluation of the influence of the antibodies on IL-2 binding to the $\alpha/\beta$ complex. In the experiment shown in *A*, the $K_d$ of high-affinity binding to HUT 102 cells was estimated at 5 pM, while the $K_d$ of intermediate-affinity binding to YT cells (*C*) was estimated at 1.3 nM.

$\beta$ chain recognized by mAbs 341 and 561 are similar and may lie within the COOH-terminal segment of the extracellular portion of the $\beta$ chain. They are distinct, however, from those recognized by anti-$\beta$ chain mAb that (unlike mAbs 341 and 561) interfere with IL-2 binding.

*Detection of p64-IL-2R $\gamma$ Chain in Intermediate-Affinity IL-2R.* Takeshita et al. (24) have reported that a 64-kD molecule is physically associated with the $\alpha$ and $\beta$ subunits in the high-affinity IL-2R complex. The presence of IL-2 bound to the high-affinity IL-2R complex was required for the detection of p64. Based on these observations, it was important to determine whether p64 was also present in the intermediate-affinity IL-2R complex found on the surface of YT cells (which expressed no detectable $\alpha$ chain), thus proving the association of this molecule with the $\beta$ chain. It was therefore essential to first establish whether mAbs 341 and 561, like TU11 (24, 35), were capable of recognizing and immunoprecipitating the putative IL-2R $\gamma$ chain physically complexed to the $\beta$ chain.

YT cells were radioiodinated and then detergent solubilized. Immunoprecipitates prepared with anti-$\beta$ mAb were analyzed by 2-D IEF/SDS-PAGE for the presence of the $\beta$ chain. As shown in Fig. 3 *A*, in the absence of IL-2 only a single specific spot was observed on the 2-D gel. A similar spot was observed with Mik$\beta$1 and TU27 (data not shown), consistent with its identification as the IL-2R $\beta$ chain. When iodinated YT cells were allowed to equilibrate with IL-2 before detergent solubilization, a protein of $\sim$64 kD formed a stable complex with the $\beta$ chain (Fig. 3 *B*). Both mAbs 341 and 561 were able to recognize this complex (Fig. 3 *B*), as was Mik$\beta$3 (data not shown). Under the reducing and denaturing conditions employed in the 2-D IEF/SDS-PAGE system, this 64-kD protein had a slightly more acidic pI than the larger $\beta$ chain, consistent with earlier work (24). Based on this finding we have termed the p64 molecule IL-2R $\gamma$ chain, as proposed by Takeshita et al. (24). The broad migration of the $\beta$ and $\gamma$ chains in both the IEF and molecular mass separations is likely due to variability in the degree of iodination on tyrosine (36). It is unlikely that these two species represent differentially glycosylated forms of the same protein (i.e., the $\beta$ chain) since increases in glycosylation would tend to make the larger protein more acidic, a result that has been directly demonstrated for the IL-2R $\beta$ chain (37). Peptide mapping experiments revealed that the $\beta$ and $\gamma$ chains had dramatically different sensitivities to V8 protease digestion (24 and our unpublished observations), and confirmed that the $\gamma$ chain is a unique chain and not simply a processed form of the $\beta$ chain.

The additional spot seen in Fig. 3 *B* in the 50–56-kD range has not been reproducibly observed, although it is noteworthy that others have described IL-2R-associated molecules in this size range (p56 [22] and p56$^{lck}$ [38]). This species is not the IL-2R $\alpha$ chain, since $\alpha$ chains migrate to a position even more acidic than the $\gamma$ chain (our unpublished observations).

The experiments shown in Fig. 3 were all performed in the absence of chemical crosslinkers, providing evidence for the stability of the $\beta\gamma$ complex when IL-2 is present. When crosslinking with the thiol-cleavable crosslinker, DSP, was performed on YT cells, association of the $\gamma$ chain with the

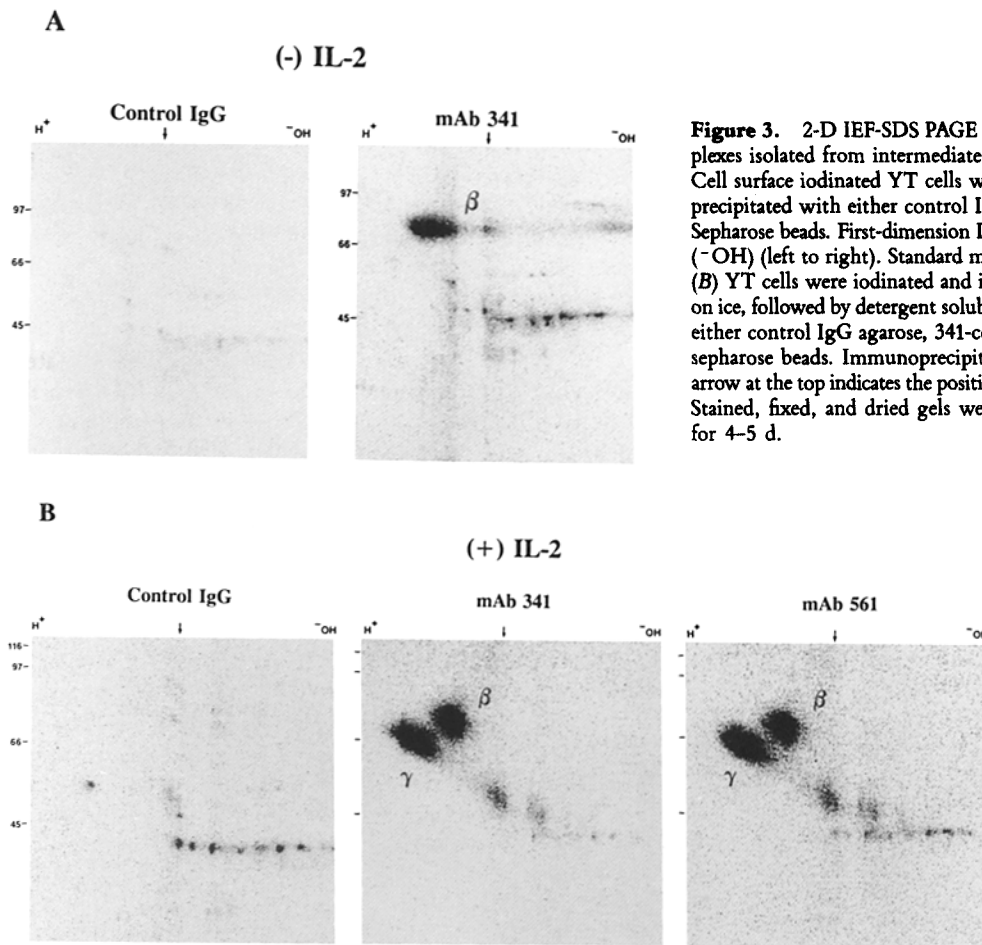**A**

(-) IL-2



**B**

(+) IL-2



Figure 3. 2-D IEF-SDS PAGE analysis of IL-2R $\beta$ and IL-2R $\beta\gamma$ complexes isolated from intermediate-affinity receptor-bearing YT cells. (*A*) Cell surface iodinated YT cells were detergent solubilized and immunoprecipitated with either control IgG agarose beads or mAb 341–coupled Sepharose beads. First-dimension IEF gels are oriented acidic (H⁺) to basic (⁻OH) (left to right). Standard molecular masses are indicated at the left. (*B*) YT cells were iodinated and incubated with 80 nM IL-2 for 120 min on ice, followed by detergent solubilization and immunoprecipitation with either control IgG agarose, 341-coupled Sepharose beads, or 561-coupled sepharose beads. Immunoprecipitates were analyzed as in *A*. The small arrow at the top indicates the position of bovine muscle actin (pI = 5.4–5.5). Stained, fixed, and dried gels were exposed to phophorimaging screens for 4–5 d.

$\beta$ chain was again observed only when IL-2 was present (Table 2, Exp. 5). This result indicates that IL-2 is required for the isolation of $\beta\gamma$ complexes and suggests that stable $\beta\gamma$ complexes are not preformed on the cell surface. We can not rule out the possibility, however, that in the absence of IL-2, the $\beta$ and $\gamma$ chains are positioned in a way that prevents them from being physically crosslinked. When 1% digitonin was used as the solubilizing detergent rather than 0.5% NP-40, no difference was seen in the relative abundances of $\beta$ and $\gamma$ on IL-2-treated YT cells (our unpublished observations). This finding supports the view that the IL-2-$\beta$/$\gamma$ complex is stable under mild nonionic lysis conditions and that its preservation is not improved by the use of the milder lysis conditions (i.e., 1% digitonin) that have been shown to result in improved stability of labile membrane complexes in other systems (39, 40).

*Association of IL-2R $\gamma$ Chain with Functional IL-2R Expression.* Our previous study characterizing the IL-2R expressed on NK cells that had been activated in vivo by continuous infusion IL-2 demonstrated that the majority of the $\beta$ chains expressed on those cells did not bind IL-2 with appreciable affinity (14). This suggested that some critical receptor component (such as the $\gamma$ chain described above) might be present

in limiting amounts on the surface of these cells. Based on the results shown in Fig. 3, it was of interest to determine whether the 64-kD $\gamma$ chain was a component of the IL-2R complexes isolated from post–IL-2 therapy NK cells and whether its abundance relative to that of the $\beta$ chain was consistent with a hypothetical role as a modulator of the ability of the $\beta$ chain to bind IL-2. NK cells obtained from patients after IL-2 therapy were examined for the expression of both $\beta$ and $\gamma$ chains. As shown in Fig. 4 only a small fraction of the $\beta$ chains immunoprecipitated from lysates prepared from surface-iodinated patient NK cells were complexed with the $\gamma$ chain. Consistent with our findings with YT cells (Fig. 3), preincubation of the cells with IL-2 was absolutely required for the detection of the $\gamma$ chain (compare – IL-2 and + IL-2 in Fig. 4). The ratio of $\gamma$ to $\beta$ in the patient samples sharply contrasted with the approximately equal levels of $\beta$ and $\gamma$ chain precipitated from YT cell lysates (Fig. 3 *B* and Fig. 4).

As noted, only a fraction of the $\beta$ chains expressed on the patient NK cells bound IL-2 with an intermediate affinity (14), while for YT cells the level of $\beta$ chain expression (41) roughly matched the number of intermediate-affinity binding sites (7, 8, 11–14). Thus, for both cell types the relative abun-

**Table 2.** *Quantitation of the Level of p64 IL-2R γ Chain Associated with p70 IL-2R β Chain on Patient Post-IL-2-therapy NK Cells and YT Cells*

| | | p64 (relative level*) | |
| --- | --- | --- | --- |
| | Patient | − IL-2 | + IL-2 |
| | 16 | 0.72 | 13.3 |
| | 19 | 0.65 | 11.0 |
| | 19‡ | 0.33 | 10.8 |
| | 25 | – | 25.4 |
| | 27 | 0.64 | 6.3 |
| | 28 | 0.28 | 7.0 |
| | 29 | 0.13 | 9.3 |
| | Exp. | | |
| | 1 | – | 111 |
| | 1‡ | – | 140 |
| YT | 2 | 0.9 | 121 |
| | 3 | – | 168 |
| | 4 | 0 | 112 |
| | 5§ | 2.8 | 60.4 |

* The level of p64 IL-2R γ chain is presented relative to the level of p70 β chain expression in the same gel. Presentation of the data as such depends on the assumption that the efficiency of iodination of β and γ chains is comparable in any given experiment. The level of β chain expression was arbitrarily assigned a value of 100, such that a value of p64 expression of 11 indicates the level of p64 was 11% that of p70. The observation that values of p64 relative to β varied ∼100% in the experiments with YT may reflect subtle differences in the degree of iodination of these two proteins or deviation from a 1:1 stoichiometry in a given experiment.

‡ Immunoprecipitations were performed with mAb 561. All others shown were with mAb 341.

§ Indicates experiment in which the thiol-cleavable crosslinker DSP was added to cells after incubation with or without IL-2.

dance of γ chain in the immunoprecipitates approximated the proportion of β chains present in an intermediate-affinity state on the cell surface. The low level of γ chain detected on patient NK cells was not due to failure to quantitatively immunoprecipitate the β/γ complex, since reprecipitation of lysates previously treated with mAb 341 revealed that >95% of the βγ complexes were cleared by the first treatment with antibody (data not shown). Results similar to that shown in Fig. 4 were obtained in five separate experiments in which six individual patients' NK cells were analyzed after in vivo IL-2 treatment. These results (Table 2) demonstrate that in every case only a small fraction of the β chains expressed on the surface of patient NK cells was physically associated with the γ chain. This observation, together with the low levels of IL-2 binding that we have detected on these cells, suggests that the γ chain is not only present as part of the IL-2R complex on the patient NK cells, but that it may be the component necessary for the β chain to assume

a conformation that allows a stable interaction with IL-2 to occur.

In a previous study (19) it had been shown that isolated β chains expressed on the surface of fibroblasts (CHO β cells) bound IL-2 with a very low affinity. Furthermore, once bound, IL-2 dissociated rapidly from the isolated β chains ($t_{1/2}$ = 1.6 min). This contrasted with β chains expressed as intermediate-affinity receptors, from which IL-2 dissociated more slowly ($t_{1/2}$ = 255 min). Similarly, α/β complexes expressed in fibroblasts bound IL-2 with an equilibrium $K_d$ approaching high affinity, but had relatively rapid rates of dissociation ($t_{1/2}$ = 18.5 min) (19). Based on these observations and the proposed role of the γ chain as a modulator of the ability of the β chain to bind IL-2, it was of interest to determine whether any γ chain could be detected in CHO cells expressing either the full-length β chain (CHO β) or both α and β chains (CHO α/β). CHO transfectants were iodinated and incubated with IL-2. After detergent solubilization, lysates were immunoprecipitated with mAb 341 and analyzed by 2-D IEF SDS-PAGE. As shown in Fig. 5, no γ chain was detected in fibroblasts expressing either the β chain alone or the α/β complex. Identical results were obtained in three separate experiments, in both the presence and absence of chemical crosslinking with DSP, arguing that the failure to detect γ chain was not due to a more rapid dissociation of IL-2 from the receptors.

## Discussion

Soon after the α and β chains of the IL-2R were cloned, sequenced, and reexpressed in nonlymphoid cell backgrounds, it became clear that a simple α/β structure could not account for the IL-2 binding and signal transducing characteristics of the IL-2R (15, 16, 19). The IL-2 binding sites created by expressing recombinant β chain in fibroblast cells were of a very low affinity ($K_d$ = 70 nM [19]), in sharp contrast to the intermediate-affinity binding ($K_d$ = 0.5–2 nM) usually associated with the β chain on lymphoid cells. Our studies of NK cells recovered from patients receiving IL-2 therapy proved that this was not simply the result of expressing the β chain in a nonlymphoid background, since the level of β chain expression on these NK cells greatly exceeded the level of intermediate-affinity IL-2 binding (14). Furthermore, although coexpression of α and β chains on fibroblasts gave rise to binding sites of relatively high affinity (15, 16, 19), such sites had a rapid dissociation rate when compared with high-affinity receptor sites on lymphoid cells (19) and were unable to transduce a signal (16). In addition to the discrepancies in binding affinity between recombinant β chain and β chain expressed on lymphoid cells, it was evident from the β chain amino acid sequence that it contained no consensus tyrosine kinase domains (15). Yet, a number of studies had demonstrated that the activation of tyrosine kinase activity was a rapid event that followed stimulation of cells with IL-2 (42–46). Thus, the evidence supports the notion that functional IL-2R contain, in addition to α and β chains, one or more lymphoid specific components necessary for modulating
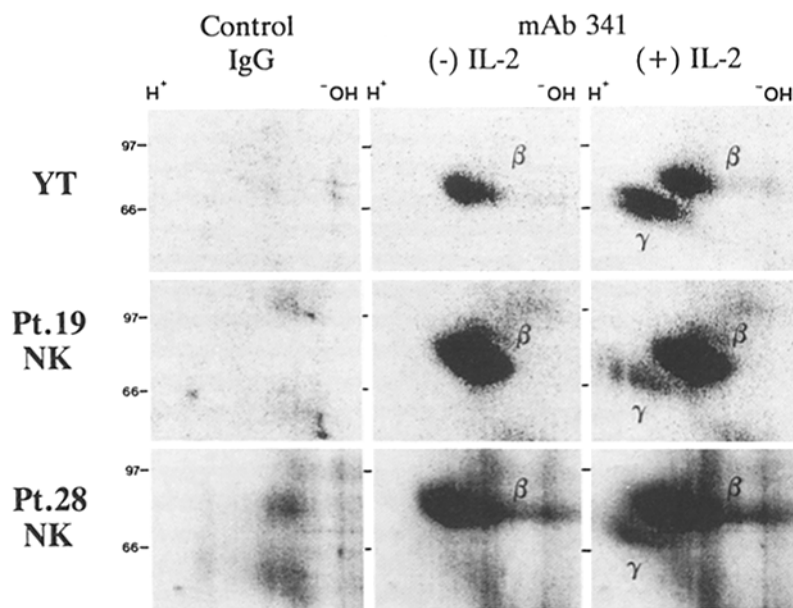
**Figure 4.** Analysis of IL-2R $\beta$ and $\gamma$ chain expression on NK cells obtained after in vivo IL-2 therapy: low level of p64 $\gamma$ chain expression correlates with low levels of IL-2 binding. YT cells and NK cells obtained from two representative patients who received in vivo continuous infusion IL-2 were surface iodinated with Iodogen as described in Materials and Methods. Pt. 19 posttherapy NK cells had been cryopreserved and were ~94–96% CD56+ CD3−. Pt. 28 NK cells were obtained by depletion of CD5+ T cells from freshly obtained posttherapy PBL using a MACS cell sorter (25). Purity of T cell–depleted NK cells was >98%. Iodinated cells were incubated in the presence (+) or absence (−) of 80 nM IL-2 for 2 h on ice, followed by solubilization in 0.5% NP-40 lysis buffer. After preclearing with nonspecific IgG agarose beads, lysates were immunoprecipitated with either control IgG agarose beads or mAb 341–coupled Sepharose beads. Immunoprecipitates were analyzed by 2-D IEF SDS-PAGE. First-dimension IEF gels are oriented acidic (H+) to basic (−OH) (left to right). Standard molecular masses are indicated at the left. Stained, fixed, and dried gels were exposed to phophorimaging screens for 4–5 d.

the affinity of IL-2 binding and transducing a signal from the receptor complex.

In the study presented here, we have focused on identifying a structural component of the functional IL-2R expressed on human NK cells (14, 47) that could confer on the $\beta$ chain the ability to bind IL-2 with intermediate affinity. Using two novel mAbs directed against the IL-2R $\beta$ chain, our results indicate that intermediate-affinity IL-2 binding sites on YT cells, and on NK cells obtained from patients after in vivo IL-2 therapy, are composed of a $\beta$ chain and an associated protein of 64 kD. This IL-2R-associated protein had been previously observed in high-affinity IL-2R complexes expressed on the surface of the leukemic cell line MT-2, and it was proposed that p64 be designated IL-2R $\gamma$ chain (24). Based on the finding that $\alpha$, $\beta$, and $\gamma$ chains could coprecipitate as a receptor complex, it was possible that the $\gamma$ chain was associated with either the $\alpha$ or $\beta$ chain, or both. Our studies of intermediate-affinity receptor-bearing cells extend these earlier observations, showing that the $\gamma$ chain is physically associated with the $\beta$ chain and can be detected in freshly obtained peripheral blood cells. The association of $\beta$ and $\gamma$ was only observed when IL-2 was bound in the receptor complex. No association of these two proteins was detected in the absence of IL-2, even when chemical crosslinkers were used to covalently couple cell surface molecules before lysis (Table 2).

Examination of patients' post–IL-2 therapy NK cells showed a low level of $\gamma$ chain expression relative to the high level of $\beta$ chain expression. The amount of $\gamma$ chain that was associated with the $\beta$ chain correlated roughly with the low level of intermediate-affinity IL-2 binding detected on these cells. We have never been able to detect more than ~1,500–2,000 intermediate-affinity IL-2 binding sites on post–IL-2 therapy NK cells, despite reproducibly observing levels of $\beta$ chain expression that exceeded that observed on YT cells,

which have 10-fold higher levels of intermediate-affinity IL-2 binding (14, and our unpublished observations). We were also unable to detect any $\gamma$ chain association with the $\beta$ chain in cDNA-transfected fibroblast cells that expressed the $\beta$ chain, a result in agreement with the fact that no intermediate-affinity IL-2 binding is detected on these cells. Similarly, no $\beta/\gamma$ association was detected on cDNA-transfected fibroblasts that expressed both $\alpha$ and $\beta$ chains. These cells bind IL-2 with a relatively high affinity but have a rapid rate of dissociation of IL-2 from the receptor complex (19). These findings support the notion that the p64 $\gamma$ chain may function to enhance the affinity with which the $\beta$ chain binds IL-2 mainly by slowing the rate with which IL-2 dissociates from the receptor.

Saito et al. (22), using $^{125}$I-IL-2 crosslinking to detergent solubilized lymphoid cells, have described a molecule, p56, that formed a stable complex with the $\beta$ chain in solution. It is possible that this molecule is the same as the $\gamma$ chain we have described here and that the difference in size estimates reflects differences in methodology. Indeed, in preliminary experiments we have evidence to suggest that it may be possible to crosslink $^{125}$I-IL-2 to the $\gamma$ chain, although this only demonstrates that the two molecules lie in close proximity (within 12 Å) to one another on the cell surface, and not that the $\gamma$ chain is an IL-2 binding protein. It is also possible that some other lymphoid cell–specific molecule associates with the $\beta$ chain, allowing the subsequent association of the $\gamma$ chain. However, under the iodination and immunoprecipitation conditions used, and given the sensitivity of detection of the phosphorimager used in these studies, this seems unlikely. If other molecules are required, then our data would indicate they may not have available residues exposed on the cell surface that can be iodinated.

These results point to the $\gamma$ chain as being involved in establishing the structural integrity of the IL-2R. Given the

**A**
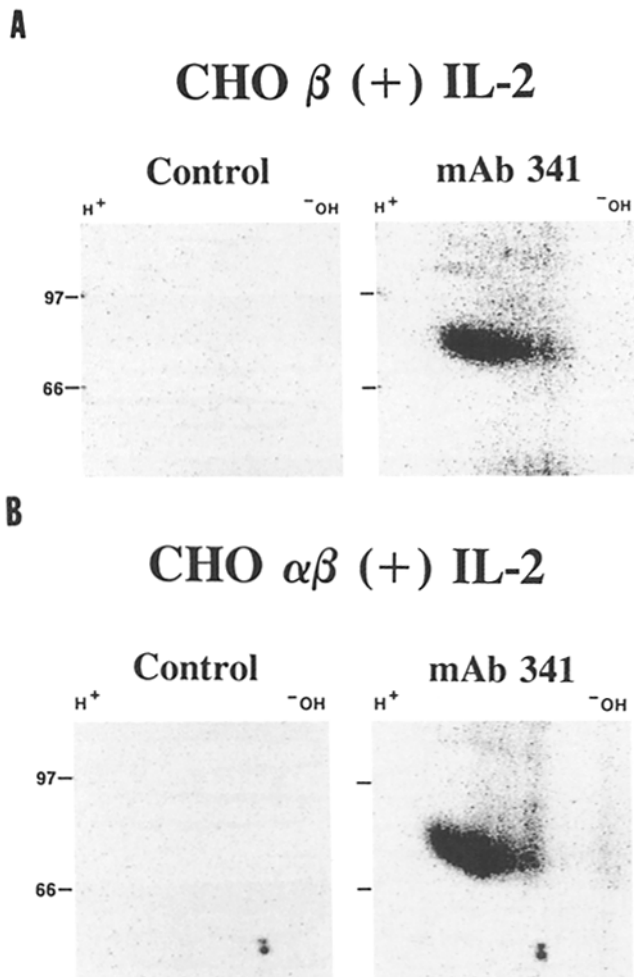
## CHO β (+) IL-2



**B**

## CHO αβ (+) IL-2



**Figure 5.** Analysis of IL-2R complexes isolated from cDNA-transfected fibroblast cells: nonlymphoid cells do not express the p64 IL-2R γ chain. CHO cells, expressing either the full-length IL-2R β chain cDNA (CHO β) (A), or both full-length α and β chain cDNAs (CHO α/β) (B) were surface iodinated with Iodogen. After incubation in IL-2, cells were lysed in 0.5% NP-40 lysis buffer. After preclearing, lysates were immunoprecipitated with either control IgG1 agarose beads or mAb 341–coupled Sepharose beads. Immunoprecipitates were analyzed by 2-D IEF SDS-PAGE. First-dimension IEF gels are oriented acidic (H+) to basic (−OH) (left to right). Standard molecular masses (kD) are indicated at the left. Stained, fixed, and dried gels were exposed to phophorimaging screens for 4 d. The experiments shown are representative of experiments performed on three separate occasions with different independently transfected CHO lines.

finding that tyrosine phosphorylation of a number of substrates, including the β chain, is observed after stimulation with IL-2 (42–46, 48), it would be attractive to speculate that the γ chain is also the IL-2R-associated tyrosine kinase. The absence of a consensus tyrosine kinase domain in the β chain (15, 49) suggests that the β chain, like many receptors not possessing intrinsic kinase activity, associates with and activates a kinase, and then in turn serves as a substrate for the associated kinase. The phosphorylated tyrosine residues on the β chain are not essential for signaling, however, since mutation of these residues had no effect on IL-2-mediated signaling through the receptor (49). Hatakeyama et al. (38) have

proposed that p56lck may be associated with the β chain and function as an IL-2R-associated kinase. Fully functional IL-2R complexes containing kinase activity, however, have been prepared from cells negative for p56lck expression (50). Furthermore, mapping of the site of interaction of p56lck with the β chain revealed that lck interacts with the β chain at residues shown to be dispensable for IL-2R signaling (38, 49), making it unclear what essential role p56lck has in IL-2R signaling. Sugamura et al. (51) have observed that the 64-kD IL-2R γ chain protein is rapidly phosphorylated on tyrosine after stimulation with IL-2 and have speculated that it may be an IL-2R-associated kinase. Michiel et al. (52), however, have evidence suggesting that another molecule, pp97, and not the 64-kD γ chain, is the tyrosine kinase physically associated with the IL-2R. Their studies using renaturation kinase assays (50) have further demonstrated that tyrosine kinase activity could be reconstituted from pp97 electrophoretically separated from IL-2R-containing immune complexes, a result consistent with the identification of pp97 as an IL-2R-associated tyrosine kinase.

From these studies it is evident that solving the structure of the IL-2R, both in terms of its IL-2 binding properties and its signaling requirements, is far more challenging than initially anticipated. The evidence presented here has opened up a new pathway through which IL-2/IL-2R interactions and cellular responses to IL-2 might be regulated. Such a model predicts that the level of p64 γ chain expression would regulate the affinity of both the β chain and α/β complexes by controlling the rate of dissociation of IL-2 from the receptor complex. Under this hypothesis, even the binding of IL-2 to α/β complexes (which occurs with relatively high affinity) would not lead to a functional cellular response due to the lack of sustained binding to the receptor. A similar role for an "affinity modulating" element associated with a ligand binding subunit has been described for the IL-6R (53), where GP130 serves to regulate both the level of high-affinity IL-6 binding and possibly signal transduction. It is not clear whether, even in the presence of the γ chain we describe, one could reconstitute fully functional IL-2R. There may be a further requirement for additional receptor-associated signaling molecules, such as pp97 (50, 52). The pursuit of future studies will greatly benefit from the cloning of the γ chain. Given the close homology between the IL-2R β chain and other members of the hematopoietin receptor superfamily (54), solving the structure of this receptor complex may also yield insight into the structural and functional requirements of other cytokine receptors.

*Final Note.* While this manuscript was in review, a report by Takeshita et al. (55) appeared that demonstrated, similar to the findings we have presented here, that the level of IL-2R γ chain associated with β chain in intermediate-affinity IL-2R correlated directly with the level of IL-2 binding. This same report also demonstrated, as have we, the absence of γ chain in fibroblasts transfected with the β chain. Thus, the results of these two independent studies are consistent with the IL-2R γ chain playing an important role in determining IL-2R affinity.

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