Expression and Ligand Binding Characterization of the β-Subunit (p75) Ectodomain of the Interleukin-2 Receptor†

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ABSTRACT: The baculovirus-mediated eukaryotic insect cell expression system was used to prepare large quantities of the β-subunit ectodomain of the high-affinity interleukin-2 receptor (IL-2Rβx). We describe the expression, purification, and biophysical characterization of this ligand binding domain. The human cDNA encoding IL-2Rβx was inserted into baculovirus transfer vectors. High titer recombinant baculovirus was produced in *Spodoptera frugiperda* (Sf9) insect cells, and the viral supernatants were subsequently used to infect monolayers of *Trichoplusia ni* (High Five) insect cells in serum-free culture. Maximal expression of the recombinant protein excreted into the cell culture supernatants was determined by SDS/PAGE analysis, where a band migrating with an apparent molecular mass of 31 kDa was identified by immunostaining. One-step purification was achieved by affinity chromatography on either a monoclonal antibody (TIC-1) column or an IL-2 column, with a final yield of approximately 5 mg/L of culture supernatant. Interestingly, partial purification was also demonstrated using metal chelate affinity chromatography. Amino-terminal sequence analysis of the protein matched the published sequence. Both equilibrium sedimentation analysis and gel filtration chromatography indicated that IL-2Rβx remains monomeric. Deconvolution of far-UV circular dichroism (CD) spectra indicated the predominant secondary structural element to be β-sheet, consistent with structural analysis and predictions for other members of the hematopoietic receptor family. A dissociation constant (K_d) for IL-2Rβx in solution of 5.3×10^{-7} M was calculated from competitive receptor binding assays. These results indicate that the IL-2 receptor β-subunit lacking both the transmembrane and cytoplasmic domains can bind IL-2 in solution with 1:1 stoichiometry.

Interleukin-2 (IL-2[1]) is one of several critical regulatory proteins that participate in the manifestation of a normal immune response. IL-2 was the first protein to be identified that was capable of mediating the proliferation of T-lymphocytes (Morgan, 1976; Smith, 1980). Resting T-cells are activated in response to stimulation by antigen or mitogen, leading to the synthesis and secretion of IL-2 and to the expression of high-affinity IL-2 receptors (IL-2R). The specific interaction of IL-2 with the IL-2R induces clonal proliferation of T-cells (Smith, 1988). In addition to its effects on T-cells, IL-2 causes both growth and differentiation of B-lymphocytes (Waldman, 1984) and stimulates natural killer (NK) cell activity (Henny, 1981). The IL-2R is composed of at least three cell-surface proteins, designated the α-, β-, and γ-subunits. These subunits can define a number of states of differing affinities for IL-2 at the cell surface (Taniguchi & Minami, 1993). The low-affinity site ($K_d \approx 1 \times 10^{-8}$ M) is composed of a single 55-kDa protein (IL-2Rα) (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al. 1984). The 75-kDa chain (IL-2Rβ) is essential for the formation of the intermediate-affinity ($K_d \approx 1 \times 10^{-9}$ M) and high-affinity ($K_d \approx 1 \times 10^{-11}$ M) sites (Hatakeyama et al., 1989). The recent cloning of the 64-kDa chain (IL-2Rγ) (Takeshita et al., 1992) demonstrated that, when transfected into fibroblasts, IL-2Rγ also participates in the formation of the intermediate- and high-affinity IL-2R (Asao et al., 1993; Matsuoka et al., 1993).

The β-subunit of the high-affinity IL-2R is a class 1 membrane-spanning protein that is a member of the hematopoietic receptor family (Bazan, 1989; D'Andrea et al., 1990). The 214 amino acid ectodomain (IL-2Rβx) contains the series of four conserved cysteines in the N-terminal region and the WSXWS amino acid motif proximal to the transmembrane domain that are characteristic of this class of cytokine receptors. Recently, structural details of a ligand/receptor interaction for this family of receptors were revealed by the solution of the crystal structure for the human growth hormone (HGH)/growth hormone receptor ectodomain (HGHRx) complex (De Vos et al., 1992). This structure indicated that one molecule of HGH bridges two identical HGHR subunits. Hence, the cross-linking of this receptor by HGH appears to be required for signaling. For other members of the cytokine family, signaling apparently involves the cross-linking of two different cell-surface proteins, with multiple ligands often sharing a common second receptor subunit. For example, the human granulocyte/macrophage colony-stimulating factor receptor and the IL-3 and IL-5 receptors all share a common subunit that, by itself, has little affinity for any of the three

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[1] Abbreviations: bp, base pair; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbant assay; Gdm·HCl, guanidinium chloride; HGH, human growth hormone; IDA, iminodiacetate; IL, interleukin; IL-2, interleukin-2; LIF, leukemia inhibitory factor; MOI, multiplicity of infection; NK, natural killer cells; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; R, receptor; SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda*; TFA, trifluoroacetic acid.

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ligands (Tavernier et al., 1991; Kitamura et al., 1991). More recently, it was shown that signaling by the IL-6 receptor (Murakami et al., 1993) and the leukemia inhibitory factor (LIF) receptor (Davis et al., 1993) involves the participation of a common subunit, gp130, in ligand-mediated receptor subunit association. The signaling complex of the IL-2R likely resembles the latter systems in which signal transmission is initiated by ligand-dependent cross-linking of the p75 β-subunit with the p64 γ-subunit, a protein subunit that is shared by other cytokine receptors (Russell et al., 1993; Kondo et al., 1993; Noguchi et al., 1993).

Previously, our laboratory has described the binding characteristics of a series of rationally designed IL-2 analogs on cell lines expressing the different affinity sites (Landgraf et al., 1992). These detailed equilibrium and kinetic binding studies suggested that receptor subunit cooperativity exists. In order to gain a clearer understanding of the nature of the IL-2/IL-2R interaction, we have produced large quantities of the receptor subunit ectodomains for biophysical, ligand binding, and structural studies. In this report, we describe the expression of the IL-2Rβ ectodomain in insect cells, a system that has previously been employed for the expression of a variety of recombinant proteins (Luckow & Summers, 1988; Luckow, 1991). *Trichoplusia ni* (High Five) insect cells were used for the expression of large quantities of soluble, correctly folded, and posttranslationally modified protein, enabling a comprehensive study of both the biophysical and IL-2 binding properties of IL-2Rβx in solution.

MATERIALS AND METHODS

Insect Cell and Viral Culture. Insect *Spodoptera frugiperda* (Sf9) cells were used for generating high titer recombinant virus and were cultured at 28 °C in 100-mL spinner flasks as previously described (Summers & Smith, 1987). Grace's Sf9 culture media and gentamicin were purchased from Gibco/BRL. Supplemental yeastolate and lactalbumin hydrolysate were obtained from Difco. *Trichoplusia ni* (High Five) cells (Invitrogen) were used to express the recombinant protein and were grown as monolayer cultures at 28 °C in serum-free EX-CELL 400 media (JRH Scientific). High Five cells were gently lifted by pipet action for routine passaging. After 30 passages, the cells were discarded and a new stock was started.

Preparation and Isolation of Recombinant Virus. Two recombinant baculovirus transfer vectors were constructed. The first transfer vector employed was pVL941 (Luckow & Smith, 1988). Initially, a hemagglutinin peptide tag epitope coding sequence (Huse et al., 1989) was added to the 3′-end of the human IL-2Rβx cDNA (642-bp *Eco*RI–*Bam*HI fragment) via PCR. This tag sequence encoded a decapeptide, enabling immunodetection of the recombinant protein with a tag-specific monoclonal antibody (gift of Dr. Ian Wilson, Scripps, La Jolla, CA). The modified cDNA was ligated into a *Bam*HI-linearized pVL941 transfer vector to generate pVLβxH. The correct orientation and sequence of the insert were verified by restriction enzyme digestion and by dideoxy sequencing. To generate recombinant virus, 2×10^6 Sf9 cells were cotransfected with 1 μg of *Autographa californica nuclear polyhedrosis* virus (AcNPV) DNA (a gift of Dr. Summers, College Station, TX) and 2 μg of recombinant plasmid pVLβxH. Putative recombinant plaques were visually identified under a phase contrast microscope 6 days post-transfection. Following five rounds of plaque purification, several putative recombinant plaques were isolated and judged to be free of wild-type virus contamination by PCR analysis (Webb et al., 1991). Recombinant viral clones were isolated and stored at −20 °C. Once expression in insect cells was verified with the epitope-tagged construction, a second expression vector, pBlueBac II (Invitrogen), was used to express a non-epitope-tagged IL-2Rβx cDNA. This vector contains the β-galactosidase gene, greatly facilitating the identification of occlusion-negative plaques.

Time Course Analysis of Protein Expression. Recombinant AcβxH virus was used to infect a monolayer of 2×10^6 High Five cells in T-25 culture flasks, with a multiplicity of infection (MOI) of 10. At specific intervals following infection, 1 mL of culture supernatant was collected, and the proteins were precipitated with 72% trichloroacetic acid and 0.15% sodium deoxycholate. After resuspension in 0.1 vol of sample buffer, SDS/PAGE (10% gel) (Laemmli, 1970) was performed, and the gel was either electroblotted onto Immobilon-P membranes (Millipore) for Western analysis of the tagged protein or directly stained with Coomassie Blue.

Large-Scale Production of IL-2Rβx. Milligram quantities of IL-2Rβx protein were expressed in High Five insect cells. Each large-scale infection was accomplished in 40 T-175 culture flasks containing $(2–3) \times 10^7$ High Five cells by infection with high titer recombinant virus stock (pfu 5×10^8/mL) at an MOI of 10. Cell-free supernatants were harvested 84 h postinfection by pelleting the cells at 600 rpm for 10 min at 4 °C. The cell-free supernatants were pooled, protease inhibitors were added (final concentrations of 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 2 μM PMSF), and the mixtures were then concentrated 20-fold at 4 °C in an Amicon P20 concentrator (M_r = 10 000 cutoff membrane). The concentrated supernatant was centrifuged at 6000 rpm (15 min at 4 °C) and then filtered through a 0.2-μm membrane prior to purification.

Metal Chelate Affinity Chromatography. Separations were performed as previously described (Hemdan et al., 1989). Briefly, a fast-flow, chelating Sepharose–copper(II) iminodiacetate (IDA) column (Sigma) was prepared by washing 5 mL of chelating Sepharose–IDA with 20 mL of 0.5 M sodium chloride/0.1 M sodium acetate buffer (pH 4.0). When the pH of the effluent was 4.0, the column was saturated with 25 mL of 5 mg/mL copper sulfate in the sodium acetate buffer and washed with an additional 25 mL of buffer to remove any loosely bound metal. Finally, the column was washed with 25 mL of loading buffer containing 1 mM imidazole in 20 mM sodium phosphate/0.5 M NaCl (pH 8.0). Concentrated culture supernatant (0.5 mL), 1 mM in imidazole and adjusted to pH 8.0, was applied to the column and allowed to enter the column bed. The column was capped and transferred to 4 °C for 30 min. Elution of the column was performed using a linear gradient of imidazole (from 1 to 30 mM) in 20 mM sodium phosphate containing 0.5 M sodium chloride, adjusted to pH 8.0. A flow rate of 0.5 mL/min was employed, and 1-mL fractions were collected. The fractions were monitored by their A_{280}/A_{320} absorbance ratios prior to SDS/PAGE and Western blot analyses. Recombinant protein samples were pooled and dialyzed against 25 mM sodium phosphate (pH 7.0) and 0.05% NaN_3.

Affinity Purification of IL-2Rβx. Affinity purification was achieved using IL-2Rβ monoclonal antibody (TIC-1) or IL-2 itself coupled to tresyl-activated agarose (Schleicher and Schuell) columns. Concentrated supernatants (5 mL) were loaded onto either of the affinity columns (2 mL of support) and incubated overnight at 4 °C. The immunoaffinity column was washed with 50 mL of PBS at 22 °C, and IL-2Rβx was eluted with 0.2 M acetic acid containing 0.2 M sodium chloride

in 1-mL fractions. The eluate was immediately neutralized with 2 N NH_4OH. The absorbance of the eluted fractions was monitored at 280 nm, and fractions containing protein were pooled, dialyzed against PBS (pH 7.4, 0.05% NaN_3), and concentrated in a Centricon-10 spin column. Alternatively, when an IL-2 ligand column was used for purification, washes were performed at 4 °C due to the rapid dissociation rate of the ligand. Elution of the column was achieved as before at 22 °C.

Protein Purity and Sequence Analysis. Protein purity was verified by reverse-phase HPLC on a Waters 850 system using a Dynamax 300A C-18 RP column (Rainin). Following quantitation by UV absorption spectroscopy (IL-2Rβx calculated molar extinction coefficient, 6.07×10^4 L mol^{-1} cm^{-1}, in 6 M Gdm·HCl; Johnson, 1988) 100 pmol of protein was submitted for N-terminal sequencing (Dartmouth Protein Core Facility).

Gel Filtration and Analytical Ultracentrifugation. Gel filtration HPLC was performed using a Hewlett-Packard 1090 system equipped with a diode array detector. A TSK-G3000SW$_{XL}$ size exclusion column (the Nest Group) calibrated with protein gel filtration calibration standards (MW-GF 200, Sigma) was employed for the separation of IL-2Rβx and IL-2 complexes. For determination of the size of the receptor/IL-2 complexes, mixtures of IL-2Rβx (20 μM) and IL-2 (1.5 nM or 8 μM containing trace [^{125}I]IL-2) were preincubated at room temperature for 1 h prior to injection. For elution, 25 mM $NaPO_4$ buffer (pH 7.0) containing 100 mM KCl was employed at a flow rate of 250 μL/min. Fractions were collected at 1-min intervals and counted individually by solid scintillation to determine relative IL-2 content.

Sedimentation equilibrium analysis was carried out at the Centrifugation Facility (University of New Hampshire) on a Beckman Model E analytical ultracentrifuge. Samples were dialyzed against reference phosphate buffer (25 mM, pH 7.0 containing 100 mM KCl) prior to analysis and run at 77, 33.5, and 15.4 μg/mL. Data were collected at 15 000, 20 000, 26 000, and 32 000 rpm after equilibrium was reached at each speed and fit to an ideal plot of radial distance squared versus log(concentration), as described (Laue et al., 1992).

Circular Dichroism. Far-ultraviolet CD spectra for IL-2Rβx were collected on a Jobin Yvon Mark V circular dichrograph. Measurements were obtained in a 0.1-cm cell at a protein concentration of 72 μg/mL in phosphate buffer (25 mM, pH 7.0 containing 100 mM KCl). An average of 30 scans was performed and a buffer blank was subtracted. Protein concentrations were determined from A_{280} values as described above. Deconvolution of the resulting spectrum was carried out according to Perczel et al., (1992).

Competitive Receptor Binding Assays. Competitive receptor binding assays were adapted from our competitive ligand binding assay (Landgraf et al., 1992). Briefly, the YT-2C2 cell line (Teshigawara et al., 1987) was used in a competition assay with IL-2Rβx and ^{125}I-recombinant IL-2. Competitor proteins (either an IL-2 control or IL2Rβx) were serially diluted in internalization inhibitor buffer (15 nM NaN_3, 50 nM 2-deoxyglucose, and 0.1% BSA in PBS, pH 7.4). [^{125}I]-IL-2 was added to a final concentration of 0.5 nM, and the solution was overlaid onto 0.2 mL silicone/paraffin oil (80:20) in 500 μL binding tubes. 100 μL of a cell suspension (2×10^7 cells/mL) in internalization inhibitor buffer was added to each tube. After 60 min at 37 °C, the tubes were centrifuged (10000*g*, 2 min) to separate free from cell-associated [^{125}I]-IL-2. The tubes were cut and the cell associated and free radioactivity were determined by solid scintillation counting. Nonspecific binding, determined in the presence of a 500-fold molar excess of unlabeled IL-2, was <3% of total ligand.

The solution K_d (K_{ds}) for IL-2Rβx was obtained from competitive binding data according to the following equation (Johnson et al., 1994):

$$K_s = \frac{1 - X}{R_{ts}X - C_tX(1 - X)}$$

where $X = 1/(2+K_cC_t)$, C_t is the total IL-2 concentration (molar, M), K_c is the equilibrium constant for IL-2 binding to the cell surface receptor (M^{-1}), R_{ts} is the concentration of soluble receptor at the IC_{50} (M), IC_{50} is the concentration of competitor protein causing a 50% inhibition in binding of labeled protein to the receptor (determined from the binding curves), K_s is the equilibrium constant for IL-2 binding to the solution receptor (M^{-1}), and $K_{ds} = 1/K_s$.

In this analysis, it is assumed that binding has reached equilibrium with negligible reduction in the free IL-2 concentration and that only a single cell-associated receptor population makes a significant binding contribution. The IL-2 concentration employed (C_t) was 0.5 nM, and the equilibrium constant for IL-2 (K_c) was 1.0×10^9 M^{-1} (Teshigawara et al., 1987).

RESULTS

Preparation of Recombinant Baculovirus in Sf9 Cells. The analysis of ligand binding and structural characteristics of IL-2Rβx requires milligram quantities of correctly folded and glycosylated recombinant protein. For this purpose, we chose baculovirus-mediated insect cell expression (Luckow & Summers, 1988). Since an antibody capable of visualizing IL-2Rβx by Western analysis was unavailable at the time we began this study, we modified the IL-2Rβx cDNA construct to encode a C-terminal hemagglutinin tag peptide identifiable by immunodetection. The modified IL-2RβxH cDNA was cloned into the baculovirus transfer vector pVL941. Following cotransfection of Sf9 cells with wild-type AcNPV DNA, several recombinant plaques were identified. Recombinant clones that were free of any traces of wild type were subsequently used to generate a high titer recombinant virus. Once it became clear, via Western blot analysis of the hemagglutinin-tagged protein, that expression of IL-2Rβx could be achieved efficiently in Sf9 cells, we switched to the then-available pBlueBac II baculovirus expression vector (Invitrogen) due to its ease of screening. The IL-2Rβx cDNA, lacking the hemagglutinin epitope tag, was inserted into this vector. After cotransfection of Sf9 cells, a simple visual color identification procedure was used to isolate blue recombinant plaques, from which a high titer recombinant virus was generated.

Expression of IL-2Rβ Ectodomains in Insect Cells. To verify the initial expression of IL-2RβxH, we collected Sf9 cell supernatants at specific intervals postinfection. After the proteins were separated by SDS/PAGE, the protein bands were immunoblotted with a monoclonal antibody to the hemagglutinin decapeptide. Receptor-specific bands were not present in either uninfected Sf9 cells or cells infected with wild-type virus, but a single band migrating with an apparent mass of 31 kDa was maximally expressed 72 h postinfection with the recombinant virus. Following the preparation of a second recombinant virus for the expression of the nontagged protein, a time course study of IL-2Rβx expression in High Five cells was also performed. Higher levels of expression allowed visualization by direct Coomassie Blue staining of

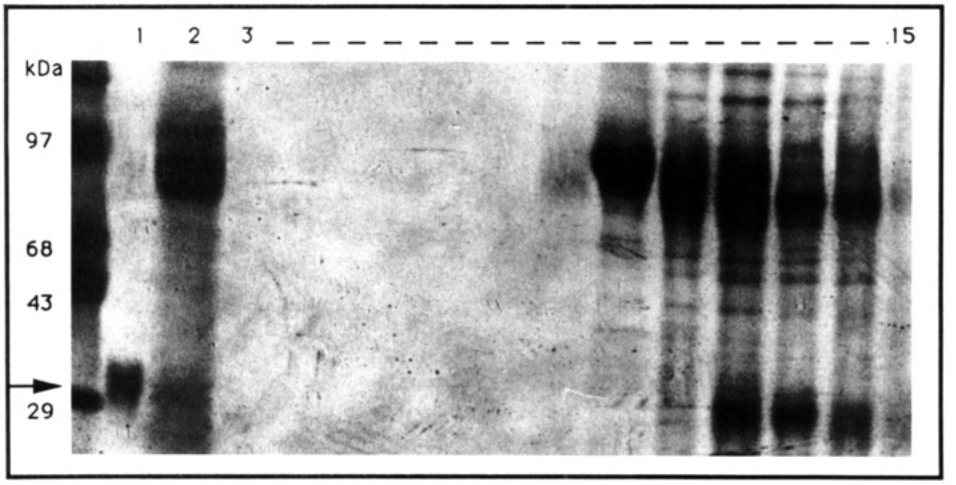

FIGURE 1: Immobilized metal affinity chromatography. Concentrated protein supernatants from High Five cells were applied to a 5-mL Cu(II) IDA column and eluted via a linear imidazole gradient as described under Materials and Methods. The collected protein fractions were resolved by SDS/PAGE (10% gel) and stained with Coomassie Blue: lane 1, 10 μg of IL-2Rβx standard; lane 2, unpurified supernatant concentrate; lanes 3–15, column eluate fractions. Molecular size markers are indicated at the left.

SDS/PAGE gels. A similar pattern of recombinant protein expression was observed in both Sf9 and High Five cells, although peak expression in High Five cells occurred as late as 84 h postinfection (data not shown).

Protein Purification and Analysis. Since the IL-2Rβ ectodomain specific TIC-1 antibody was initially unavailable for purification purposes, the utility of metal chelate affinity chromatography as an alternative means of purification was investigated. The IL-2Rβx protein contains six histidine residues that have the potential to bind transition metals such as copper and zinc immobilized on a chelating Sepharose column (Porath, 1992). Cellular supernatants from IL-2RβxH-expressing High Five cells were harvested, concentrated, and loaded onto a Cu(II) IDA column as outlined under Materials and Methods. Elution of the column was achieved with an increasing imidazole concentration gradient (1–30 mM), and fractions were collected and separated by gel electrophoresis. A band migrating with an apparent molecular mass of 31 kDa was detected by immunoblotting with a hemagglutinin tag epitope specific antibody in the 20–30 mM imidazole eluted fractions (data not shown). To determine the selectivity of this procedure in eluting the recombinant protein, the gel was stained with Coomassie Blue (Figure 1). The elution profile of the recombinant protein confirms a relatively strong affinity for the Cu(II) IDA column, while most of the higher molecular weight contaminating proteins eluted prior to IL-2Rβx. This demonstrated that partial purification of the IL-2Rβ ectodomain is possible using metal affinity chromatography. Since we subsequently determined that both immuno- and ligand-affinity chromatography were superior, we made no effort to optimize this procedure.

One-step affinity purification of IL-2Rβx was achieved with either a TIC-1 antibody or an IL-2 affinity column. Due to the lower affinity of IL-2Rβx for the IL-2 ligand column and a rapid dissociation rate (Arima et al., 1992), immunoaffinity purification was judged to be superior. Following elution from the columns, the purity of the protein was demonstrated by a single band corresponding to IL-2Rβx on SDS/PAGE (Figure 2) and by a single peak on reverse-phase HPLC chromatography (not shown). Gel filtration chromatography (Figure 3) indicated that, at micromolar concentrations, the protein eluted as a single species (apparent MW = 31 600). Furthermore, a predominant monomer was also observed when the protein was subjected to equilibrium sedimentation analysis

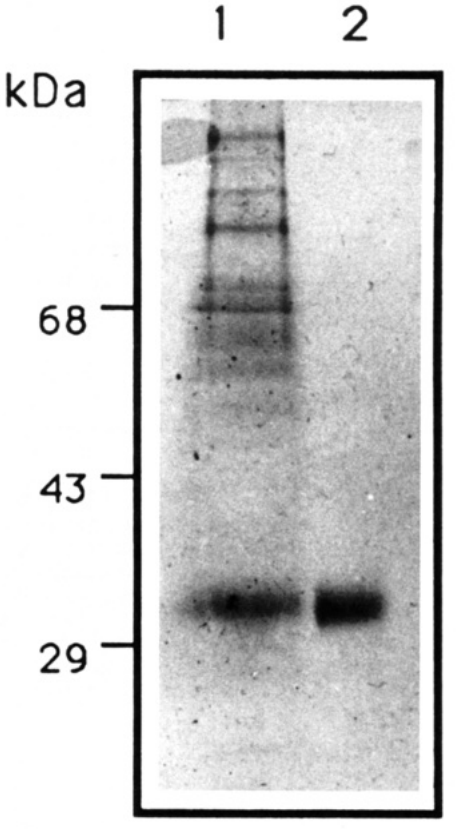

FIGURE 2: Coomassie-stained SDS/PAGE of (1) unpurified supernatant from Acβx-infected High Five insect cells and (2) TIC-1 column affinity-purified IL-2Rβx.

(MW = 30 120). When IL-2Rβx was incubated with IL-2 prior to gel filtration analysis, the receptor/ligand complex eluted with a size indicating a 1:1 stoichiometry of association (Figure 3).

Amino-terminal sequence analysis confirmed that the first nine amino acids matched those of the published sequence (Hatakeyama et al., 1989). Yields of up to 5 mg of IL-2Rβx per liter of supernatant were achieved.

Circular Dichroism. To evaluate the relative contributions of secondary structure in IL-2Rβx, far-UV circular dichroism spectra of the glycosylated protein were obtained (Figure 4). Deconvolution of the spectrum (Perczel et al., 1992) indicated relatively little contribution of α-helix, with large fractions of

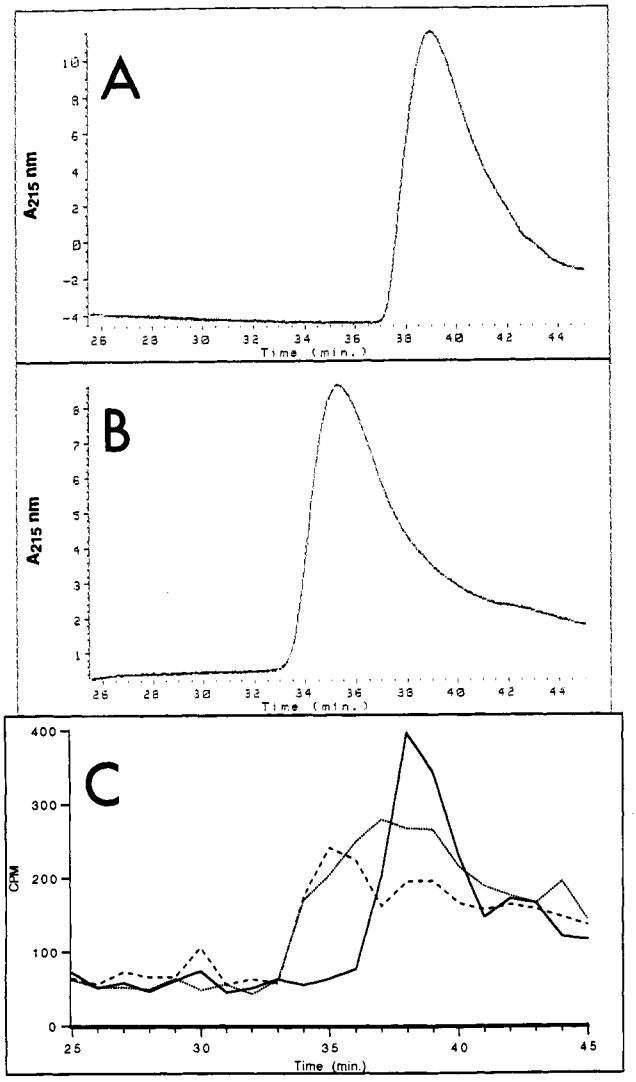

FIGURE 3: Gel filtration analysis of IL-2Rβx/IL-2 complex formation: (A) gel filtration chromatogram of recombinant IL-2 (containing trace [^{125}I]IL-2); (B) gel filtration chromatogram of affinity-purified IL-2Rβx (20 μM) preincubated with tracer containing IL-2 (1.5 nM). (C) plot of CPM vs time for fractions collected from chromatogram A (—) from chromatogram B (- - -), and from a similar chromatogram (• • •) of IL-2Rβx (20 μM) preincubated with tracer containing IL-2 (8 μM). Conditions were as described in Materials and Methods; absorbance was monitored at 215 nm.

β-sheet (30–35%) and other structures (turns and unordered, 60–65%).

Competition Ligand Binding by IL-2Rβx. Having purified IL-2Rβx to homogeneity from High Five cell supernatants, we performed receptor competition assays on a cell line (YT-2C2) expressing only the intermediate-affinity IL-2 receptor (Teshigawara et al., 1987). As depicted in Figure 5, the IL-2 control competition curve displays the expected IC_{50} of 1.5×10^{-9} M under the conditions of the experiment, resulting in $K_d = 1 \times 10^{-9}$ M. Purified IL-2Rβx was also capable of complete competition for IL-2 binding to the cell-associated receptors, with an IC_{50} of 8×10^{-7} M corresponding to $K_{ds} = 5.3 \times 10^{-7}$ M (see Materials and Methods).

DISCUSSION

In this study, we have described the expression of IL-2Rβx in the baculovirus/*Trichoplusia ni* (High Five) insect cell system. One version of the ectodomain incorporated a C-terminal hemagglutinin peptide epitope tag that facilitated the initial detection of protein expression by Western analysis.

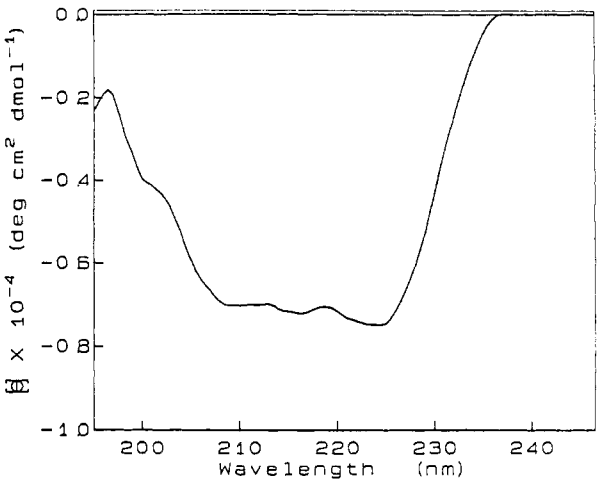

FIGURE 4: Far-UV circular dichroism spectrum of IL-2Rβx in 25 mM sodium phosphate and 100 mM KCl (pH 7.0). Spectra were recorded at 25 °C, and the results are an average of 35 scans with the buffer baseline subtracted.

For ligand binding analysis and structural characterization, a second version lacking the epitope tag was constructed and used for larger scale expression. *T. ni* cells were used for recombinant protein expression in serum-free culture, since the level of IL-2Rβx secreted by these cells was superior to that observed with Sf9 cells. Recombinant protein expression in serum-free culture subsequently simplified receptor purification by one-step affinity chromatography. Initially, however, a suitable antibody was unavailable for purification purposes, and metal chelate chromatography was investigated. The imidazole side chain of histidine may function as a ligand for transition metals (Sundberg & Martin, 1973), in particular for copper(II) complexes. Furthermore, there is often a correlation between the number of surface-accessible histidine residues in a protein and its affinity toward metals (Hemdan et al., 1989). It has previously been reported that the prolactin receptor, a member of the same hematopoietic receptor family, requires divalent metal cations for the binding of prolactin (Necessary et al., 1984). Therefore, we investigated whether a similar metal interaction could be exploited for the purification of IL-2Rβx by preparing a metal affinity column. The affinity of IL-2Rβx for a copper(II)-coupled IDA column was sufficient as to require a relatively high concentration of imidazole (20–30 mM) for the elution of protein. Presumably, this is primarily due to one or more of the six histidine residues located in the ectodomain. Although the utility of this technique for partial purification of this receptor was demonstrated (Figure 1), we made no attempt to optimize this procedure since other affinity techniques proved superior. Metal affinity chromatography could prove useful, however, in the purification of ectodomains of other members of the hematopoietic receptor family.

With respect to the physiological significance of metal binding, it is unlikely that metal chelation plays an important role in structural stabilization (Kellis et al., 1991) or in ligand binding, as was the case for the prolactin receptor, since preliminary results suggest that metals inhibit rather than facilitate IL-2 binding to its receptor (T. Sana, unpublished observations).

We subsequently determined that both immunoaffinity (TIC-1 anti-p75 monoclonal antibody) and ligand affinity (IL-2) columns could be employed in one-step purification procedures. Although it had been previously reported that the β-subunit was unable to interact with IL-2 by itself (Hatakeyama et al., 1989), its affinity for the IL-2 column

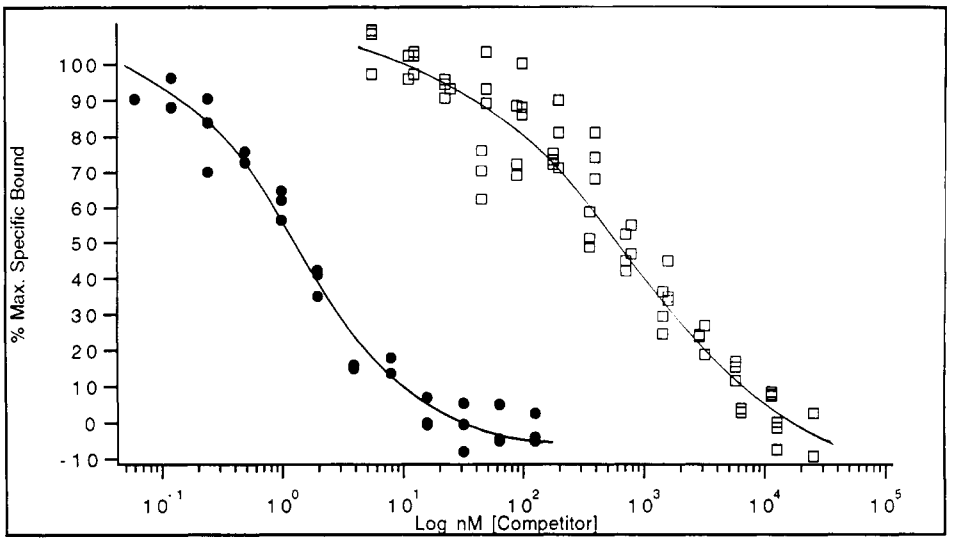

FIGURE 5: Competitive IL-2 receptor binding assay on the 2C2 cells expressing intermediate-affinity receptors. Both IL-2 (●) and IL-2Rβx (□) were serially diluted in internalization inhibition buffer and incubated with cells for 60 min at 37 °C as described under Materials and Methods. The data represent the result of two independent experiments employing triplicate determinations.

proved sufficient to allow purification. We observed that the TIC-1 column yielded significantly higher amounts of IL-2Rβx compared to an IL-2 column, presumably owing to its higher affinity for the receptor. Because endoglycosidase F treatment of IL-2Rβx significantly reduced the apparent mass of 31 kDa to about 26 kDa (data not shown), and because automated N-terminal sequencing detected only a single sequence, the broad band observed on SDS/PAGE is likely a reflection of heterogeneity in glycosylation. Reverse-phase HPLC analysis of the protein also indicated a single protein species under nonreducing conditions (not shown). Analytical gel filtration and equilibrium sedimentation analyses both confirmed that even at micromolar concentrations IL-2Rβx remained monomeric, simplifying the interpretations of subsequent structural and ligand binding studies. This is in contrast to the behavior of the ectodomain of the p55 IL-2Rα subunit, which forms a disulfide-linked homodimer at high concentrations (Kato & Smith, 1987).

Structural predictions suggested that the hematopoietic receptor family primarily consisted of β-sheet secondary structure and captured ligand between a sandwich of immunoglobulin-like β-domains (Bazan, 1990). The cocrystal structure of the HGH/HGHRx complex (De Vos et al., 1992) confirmed the secondary structural content, but revealed a mode of ligand binding quite different from the prediction: the complex possesses a stoichiometry of one ligand to two receptor subunits. Mechanistically, the binding of ligand to the receptor homodimer was achieved in a sequential manner via two independent binding sites on HGH. The results of CD analysis of the solution conformation of IL-2Rβx (Figure 5) also suggest predominantly β-sheet with little or no α-helix, consistent with inclusion in this family of receptors.

Soluble versions of the receptors for a number of cytokine and polypeptide hormones, including IL-2Rα and IL-2Rβ, have been identified (Rubin et al., 1985; Honda et al., 1987, 1990). In one study (Tsudo et al., 1990), the IL-2 binding capacity of a 37-kDa soluble form of IL-2Rβ produced by NIH-3T3 cells was examined by the displacement of an IL-2Rβ monoclonal antibody (MIK-β1) via competitive ELISA. On the basis of this assay, it was observed that the affinity of IL-2 for the immobilized soluble receptor was approximately 2 orders of magnitude lower than that for the intermediate-affinity receptor ($K_d \approx 1 \times 10^{-9}$ M), but no value for the dissociation constant could be calculated. Later, it was reported that when full-length IL-2Rβ was expressed on the surface of fibroblasts, the relatively low affinity of IL-2 for the full-length receptor was due to its rapid dissociation rate (Ringheim et al., 1991). Although a dissociation constant can be determined for immobilized forms of soluble receptors, in solution, ligand binding may occur with kinetics different from those observed for receptors on a solid support or anchored at the plasma membrane (Goldstein et al., 1989).

In order to determine a solution dissociation constant (K_{ds}) for the receptor, we employed a competitive receptor binding assay (Figure 5). Since the soluble receptor was competing for IL-2 binding at a single cell-surface site of known affinity (the intermediate-affinity site on YT 2C2 cells; Teshigawara et al., 1987), we were able to calculate a dissociation constant from the IC_{50} value determined in the competition binding curve (Johnson et al., 1994). This value ($K_{ds} = 5.3 \times 10^{-7}$ M) is approximately 10-fold lower (stronger) than the K_{ds} value obtained in a related study employing the same methods (Johnson et al., 1994) and may reflect the tag epitope-modified nature of the IL-2Rβx protein used in that report. More importantly, our K_{ds} value is 10-fold higher (weaker) than the cell-surface value for the full-length protein expressed on fibroblasts (Ringheim et al., 1991). This may result from the surface contribution on binding to the transfected cells (Goldstein et al., 1989) and/or the presence of a transmembrane and cytoplasmic domain that may enhance the β-subunit affinity. It should be noted, however, that this study also reported a K_d value for cells transfected with both α- and β-subunits that was significantly lower than the values reported for this "pseudo-high-affinity" site by other groups (Matsuoka et al., 1993; Takashita et al., 1992; Minamoto et al., 1990).

Gel filtration anlysis of the IL-2Rβx/IL-2 complex (Figure 3) indicated that the apparent stoichiometry of ligand binding is 1:1. As anticipated from the ligand binding analysis, a large excess (>1000-fold) of IL-2Rβx was required to form the complex. Although rapid dissociation was evident, the use of a radioactive tracer allowed the detection of a peak eluting at time (33.5–34 min) corresponding to a 1:1 stoichiometry of association (expected MW, 47 100). This may seem unexpected in light of the fact that the p55 α-subunit can exist as a dimer in solution, and we have previously reported that the α/β IL-2R heteromeric complex exists preformed on the cell surface (Landgraf et al., 1992). Although the α-subunit may exist as a disulfide-linked dimer (Kato & Smith,

1987), this could result from the high concentrations achieved during isolation, since both alkylation and mutagenesis experiments reveal that α-subunit dimer formation has no effect on ligand binding (Rusk et al., 1988). Futhermore, although the disulfide-linked α-subunit dimer is also evident in the preliminary IL-2/p55 cocrystal structure, the stoichiometry of ligand binding remains 1:1 (Lambert et al., 1989); thus, it is not unusual that this is also the case for the β-subunit in solution.

The results of our solution IL-2Rβx competitive binding assays confirm the relatively low solution affinity of this ectodomain for IL-2 compared to any of the physiological cell-surface sites. The difference in affinities between the binding of IL-2 to IL-2Rβ transfected fibroblasts and to the intermediate-affinity site on NK cells is due to the participation of IL-2Rγ in the formation of the intermediate-affinity site (Takeshita et al., 1992; Voss et al., 1992). Likewise, a series of kinetic IL-2 binding studies performed with full-length IL-2 receptors reconstituted on both fibroblastoid and lymphoid cell lines indicated the involvement of IL-2Rγ in controlling both the association rate and dissociation rate constants in the high-affinity complex as well (Matsuoka et al., 1993). Preliminary results in a related study suggest that β-γ subunit cooperativity in ligand binding can also be observed in solution using surface plasmon resonance and that the γ-subunit retards the dissociation rate of IL-2 from IL-2Rβx in a fashion similar to that observed on the cell surface (Johnson et al., 1994). This suggests that different sites on IL-2 interact with each of the three receptor subunits. Supporting this model, different residues of the IL-2 protein have been implicated in selective binding to α, β, and γ IL-2R subunits (Collins et al., 1988; Zurawski et al., 1990; Suave et al., 1991; Buchli et al., 1993). In total, however, only a few residues have been implicated in receptor interaction, despite extensive mutagenesis. Since three subunits are involved in IL-2 binding and each contact the ligand, it is likely that much of the solvent-exposed surface of the protein participates in receptor binding. It now appears that the ectodomains of the receptor subunits are also capable of ligand binding in solution. Therefore, it may be possible to cocrystallize the ectodomains with IL-2, either individually (this has already been reported for the α-subunit; Lambert et al., 1989) or in multimeric fashion, and obtain high-resolution information for these complexes.

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