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Identification and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor

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Interleukin-15 (IL-15) is a novel cytokine of the four-helix bundle family which shares many biological activities with IL-2, probably due to its interaction with the IL-2 receptor β and γ (IL-2Rβ and γ_c) chains. We report here the characterization and molecular cloning of a distinct murine IL-15Rα chain. IL-15Rα alone displays an affinity of binding for IL-15 equivalent to that of the heterotrimeric IL-2R for IL-2. A biologically functional heteromeric IL-15 receptor complex capable of mediating IL-15 responses was generated through reconstruction experiments in a murine myeloid cell line. IL-15Rα is structurally similar to IL-2Rα; together they define a new cytokine receptor family. The distribution of IL-15 and IL-15Rα mRNA suggests that IL-15 may have biological activities distinct from IL-2.

Keywords: cytokine/IL-15 receptor/IL-2 receptor/sushi domain

Introduction

IL-15 is a recently identified cytokine with biological activities similar to IL-2 (Grabstein *et al.*, 1994). IL-15 has been shown to support proliferation of natural killer (NK) cells and activated peripheral blood T lymphocytes, induce lymphokine-activated killer (LAK) activity in NK cells, effect generation of cytolytic effector cells and serve as co-mitogen for proliferation and differentiation of activated B lymphocytes (Carson *et al.*, 1994; Grabstein *et al.*, 1994; Armitage *et al.*, 1995). Molecular modeling studies and the genomic structure of IL-15 (Anderson *et al.*, 1995) predict that IL-15, like IL-2, belongs to the four-helix bundle cytokine family. The overlapping activities of IL-15 and IL-2 suggest that IL-15 might utilize components of the IL-2 receptor (IL-2R) for binding and signal transduction. The high affinity IL-2R complex comprises three subunits, designated the IL-2Rα, β and γ chains (for a recent review, see Minami *et al.*, 1993). The β and γ chains are required for high affinity IL-2 binding and IL-2 signaling and are members of the hematopoietin receptor superfamily. The α chain (or p55) is a low affinity, non-signaling binding subunit, and the only cytokine receptor member of a large family of binding proteins whose members include complement receptor proteins (Davie *et al.*, 1986; Perkins *et al.*, 1988). We have demonstrated that IL-15 requires the β chain of the IL-2R for all the biological activities tested, but that the α chain of the IL-2R is not required (Giri *et al.*, 1994; Grabstein *et al.*, 1994). Moreover, the γ chain of the IL-2R, which has been shown recently to be shared by receptors for several other cytokines [IL-4, IL-7, IL-9 (Kondo *et al.*, 1993, 1994; Noguchi *et al.*, 1993a; Russell *et al.*, 1993, 1994)] and designated the common γ chain or γ_c, is also required for IL-15 signal transduction (Giri *et al.*, 1994) and efficient internalization (Kumaki *et al.*, 1995).

Although similar in function to IL-2, IL-15 lacks primary sequence homology with IL-2 and displays a broader tissue distribution of mRNA expression (Grabstein *et al.*, 1994). Several observations suggest the existence of a specific IL-15R protein in addition to IL-2Rβ and γ_c. Many non-lymphoid cell types bind IL-15 but not IL-2, while some murine cells with functional IL-2 receptors are not capable of responding to IL-15 (Giri *et al.*, 1994). Epstein–Barr virus-immortalized B cell lines from patients with X-linked severe combined immunodeficiency (XSCID), which is caused by a defect in γ_c (Noguchi *et al.*, 1993b), bind IL-15 with high affinity in the absence of γ_c (Kumaki *et al.*, 1995). This binding is unlikely to be due to IL-2Rβ on these cells, since IL-2Rβ expressed alone in transfected COS-7 cells does not bind detectable levels of IL-15 (Giri *et al.*, 1994). These observations led us to search for a unique IL-15R. Herein we describe the molecular cloning and characterization of a murine IL-15 binding protein that constitutes the predicted α chain of the IL-15R complex. This molecule is structurally related to the α chain of the IL-2R. Together, the α chains of the IL-2R and IL-15R define a new cytokine receptor family. In contrast to the low affinity IL-2Rα, the murine IL-15Rα is sufficient for the high affinity binding of IL-15. This observation, in conjunction with differences in distribution of mRNA for both IL-15 and IL-15Rα relative to IL-2 and IL-2Rα, suggests that IL-15 has additional biological activities and mechanisms of action beyond those that it shares with IL-2.

Results

Elevated expression of an IL-15-specific binding protein on a murine helper T cell line

IL-15 was originally identified in culture supernatants of a monkey kidney epithelial cell line, CV1/EBNA, based on its ability to stimulate the proliferation of the murine T cell line, CTLL-2. Comparison of the activity of recombinant simian IL-15 cloned from this source with human IL-2 showed that the two molecules had similar abilities to induce the proliferation of several antigen-

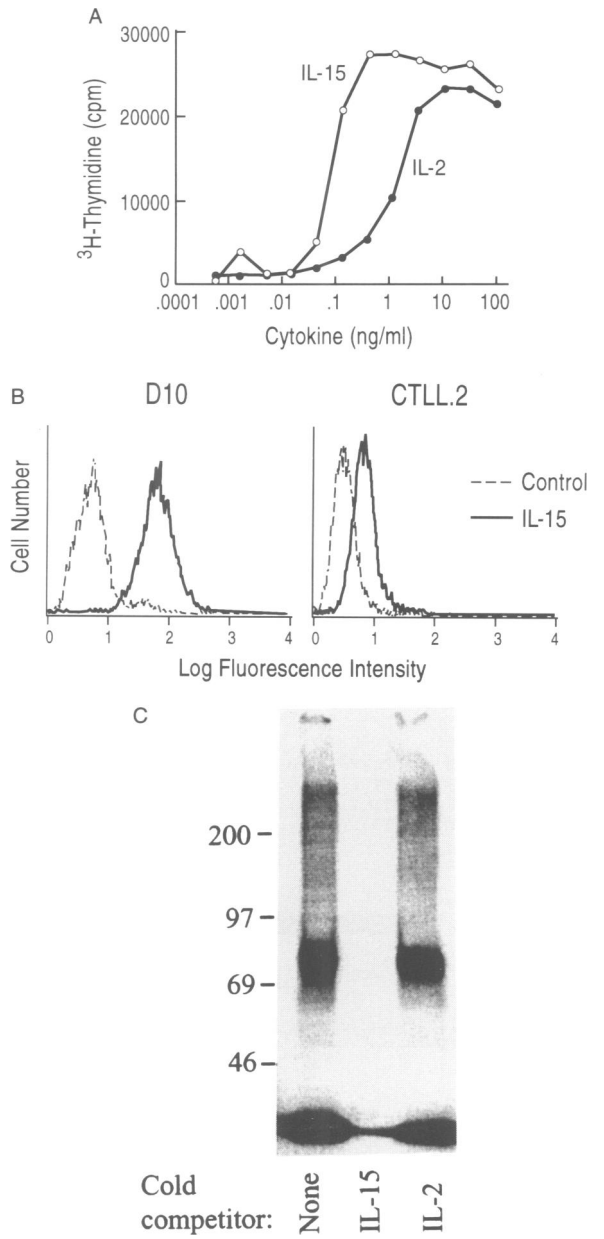

Fig. 1. IL-15 binding and responsiveness in murine Th2 clone D10. (**A**) Proliferation of D10 cells in response to IL-15 and IL-2. [^{3}H]TdR incorporation in D10 cells in response to the indicated amounts of recombinant Flag IL-15 and human IL-2. (**B**) Comparison of IL-15 binding on D10 and CTLL-2 cells. Cytofluorometric profiles of D10 and CTLL-2 cells showing binding of Flag-IL-15 (solid line) relative to control (dotted line). Binding of Flag-IL-15 was carried out at 4°C for 1 h, followed by staining with anti-Flag antibody M2, second step staining with goat anti-rat IgG FITC and cytofluorometric analysis as described in Materials and methods. (**C**) Cross-linking of radiolabeled IL-15 to D10 cells. Binding and cross-linking of [^{125}I]IL-15 to D10 cells in the presence or absence of excess unlabeled IL-2 or IL-15 was performed as described in Materials and methods.

dependent T cell lines. The D10 helper T cell clone (Kaye *et al.*, 1984), however, manifested close to a 10-fold difference in the dose response to IL-15 compared with IL-2 (an EC_{50} of ~0.1 ng/ml for IL-15 compared with ~1 ng/ml for IL-2, Figure 1A). The enhanced proliferative response of D10 cells to IL-15 correlated with higher levels of IL-15 binding compared with CTLL-2 cells, as estimated by cytofluorometric analysis of IL-15 binding (Figure 1B). The specificity for IL-15 of the major IL-15 binding protein on D10 cells was demonstrated by binding and cross-linking experiments as shown in Figure 1C. The major cross-linked complex of radiolabeled simian IL-15 to D10 cells detected by SDS–PAGE was between 76 and 78 kDa, suggesting a binding protein of ~58–60 kDa, smaller than the known size of the murine IL-2Rβ or γ_c chains, which have apparent molecular weights of ~110 and 75 kDa, respectively (Kono *et al.*, 1990; Kumaki *et al.*, 1993). This protein bound specifically to IL-15; an excess of IL-2 could not prevent IL-15 binding (Figure 1C). Low levels of high molecular weight cross-linked products were also present, which may represent heteromeric complexes with the murine IL-2Rβ and γ_c chains. Direct binding experiments with ^{125}I-labeled IL-15 revealed 5000–15 000 high affinity sites on D10 cells (see below). Pre-incubation of these cells with a neutralizing antibody against the IL-2Rα chain (mAb 7D4) had no effect on the number of IL-15 binding sites (not shown), confirming previous results indicating that IL-2Rα is not involved in IL-15 binding (Giri *et al.*, 1994; Grabstein *et al.*, 1994). The number of high affinity IL-2 receptors on D10 cells, which provides an estimate of the level of IL-2R β and γ_c chains present, was measured by Scatchard analysis at <500 receptors/cell (not shown). Hence, the large number of IL-15 binding sites on these cells could not be explained by binding of IL-15 to complexes containing IL-2Rβ and γ_c chains alone. Together, these results suggest that a 58–60 kDa IL-15-specific binding protein is over-expressed on the D10 cell line.

Cloning of an IL-15 binding protein and comparison with IL-2Rα

Binding of [^{125}I]simian IL-15 to COS-7 cells transfected with a D10 direct expression cDNA library was used to identify pools of clones encoding an IL-15 binding protein. Positive pools were partitioned and rescreened until a single clone (designated D5) was identified which directed synthesis of a cell surface protein capable of binding radiolabeled IL-15. The sequence of the D5 open reading frame and deduced amino acid sequence is shown in Figure 2A. This cDNA encodes a type I membrane protein with a predicted signal peptide of 32 amino acids, a 173 amino acid extracellular domain, a single membrane-spanning region of 21 amino acids and a 37 amino acid cytoplasmic domain. The sequence contains a single site for *N*-linked glycosylation, yet the size from cross-linking predicts extensive post-translational modification, which may be due to *O*-linked glycosylation. An independent cDNA clone isolated by hybridization from a CTLL cDNA library was found to have the identical sequence.

Initial searches using the full-length protein sequence against available protein and DNA databases did not yield any meaningful matches. Subsequent searches performed with regions of the extracellular domain produced fragmentary matches with the IL-2Rα chain and several complement receptor and blood coagulation proteins (CR2, complement factor H, β-2-glycoprotein I). In these searches, the highest scoring match identified was with the bovine IL-2Rα chain: 45% similarity (28% identity) over 41 residues. Detailed comparisons of the IL-15 binding protein sequence with the IL-2Rα and the comple-

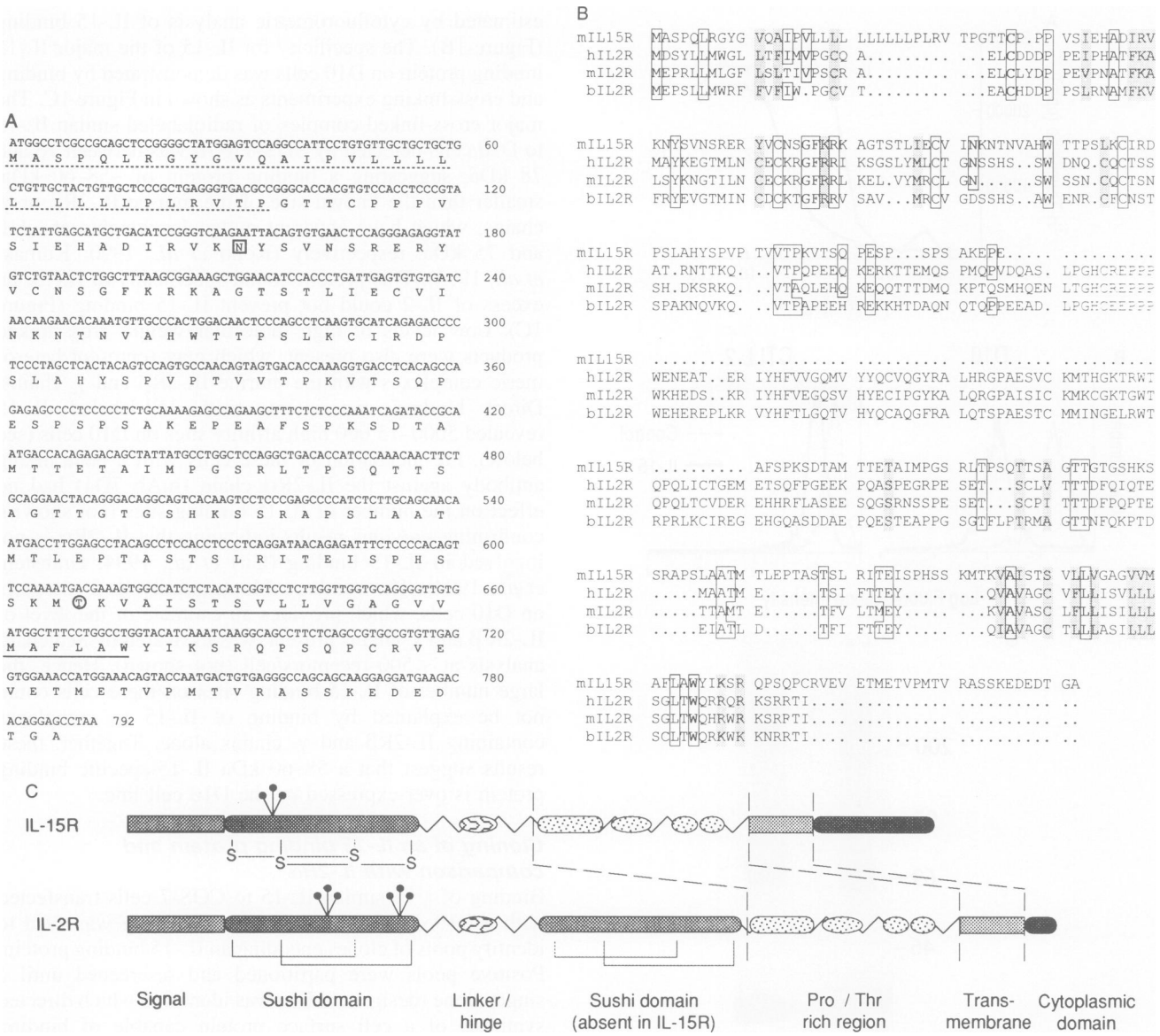

Fig. 2. Sequence analysis of murine IL-15R cDNA. (**A**) Sequence of the coding region and predicted amino acid sequence of clone D5. The predicted signal peptide is indicated by a dotted underline; the predicted membrane-spanning region by a solid underline; the single predicted site of *N*-linked glycosylation by a box; and the truncation site for expression of soluble receptor by a circle (see text, Figure 6 for details). Signal peptide and transmembrane domains were predicted with the SIGNALPEP and TRANSMEMBRANE programs (Genetics Computer Group, 1991). DNA sequencing was performed using the ABI DyeDeoxy Terminator Cycle Sequencing kit on an automated Applied Biosystems DNA Sequencer Model 373A (The Perkin-Elmer Corp., Foster City, CA). The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence databases under the accession number U22339. (**B**) Multiple sequence alignment. The murine IL-15R, human, mouse and bovine IL-2R were aligned as described in the Results. In the alignment, open boxes indicate residues that are identical between the IL-15R sequence and at least two of the IL-2R sequences; shaded boxes denote conserved residues. The sequence similarity for the IL-15R and IL-2R proteins is highest in the sushi domain; the Pro/Thr-rich region was identified using the MACAW alignment program (Schuler *et al.*, 1991). (**C**) Structural schematic. A structural comparison based upon the sequence alignment between the IL-15R and the IL-2R as shown above. Disulfide bridges characteristic of the sushi domain are shown below the structure, and potential *N*-linked glycosylation sites are indicated by the ball-and-stick symbols above the structure. The IL-15R contains only one sushi domain, whereas the IL-2R contains two.

ment receptor proteins revealed the presence of a conserved protein binding motif, the 'sushi domain,' also known as a GP-I motif or 'SCR' for short consensus repeat (Davie *et al.*, 1986; Perkins *et al.*, 1988). This homology with IL-2Rα, therefore, suggested that we had cloned the murine IL-15Rα chain. A multiple sequence alignment of the murine IL-15Rα and IL-2Rα chains from human, mouse and cow is shown in Figure 2B. The primary structural distinctions between IL-15Rα and IL-2Rα are the absence of a second sushi domain in IL-15Rα, which is present in the IL-2Rα sequences, and a longer cytoplasmic tail for IL-15Rα (shown schematically in Figure 2C).

Comparison of mRNA distribution for IL-15Rα and IL-2Rα chains

The cDNA insert of clone D5 was labeled with ^{32}P and used to probe Northern blots containing polyadenylated RNA from various cell lines and tissues. We detected an ~1.7 kb IL-15Rα transcript in several cell types such as

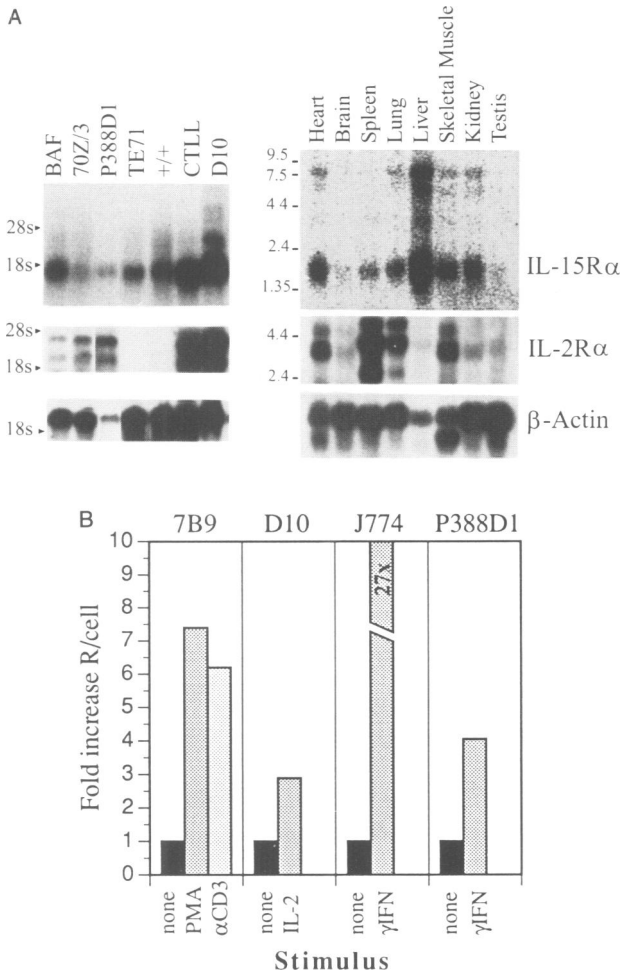

Fig. 3. Analysis of IL-15R expression. (**A**) Comparison of IL-15Rα and IL-2Rα mRNA levels in murine cell lines and tissues. Left panel: pro-B cell lines BAF-B03 (Hatakeyama *et al.*, 1989) and 70Z/3 (ATCC TIB 158), macrophage line P388D1 (ATCC CCL 46), thymic epithelial line TE71 (Giri *et al.*, 1994), bone marrow stromal line +/+ (Anderson *et al.*, 1990), and T cell lines CTLL-2 and D10. The sizes of 28 and 18S rRNAs are indicated on the left. Right panel: murine tissue blot (Clontech, Palo Alto, CA) containing mRNAs from the indicated tissue sources. Sizes of mRNA standards are indicated at the left of the panel. β-Actin probing results are shown for comparison of RNA loading. (**B**) Induction of surface IL-15R expression. Receptor numbers were estimated by Scatchard analysis of radiolabeled IL-15 binding to cells cultured in the absence or presence of known stimulatory agents for each cell type. Hamster anti-CD3 (500A2, gift of Dr J.P.Allison, UC Berkeley, Berkeley, CA) was purified at Immunex. The estimated number of receptors/cell for untreated 7B9, D10, J774 and P388D1 cells were 890, 7900, 360 and 2170 receptors/cell, respectively.

T cell lines (D10 and CTLL), a macrophage line (P388D1), B cell lines (BAF-B03 and 70Z/3), non-lymphoid lines such as thymic (TE71) and bone marrow stromal lines (+/+), and a wide tissue distribution including heart, spleen, lung, skeletal muscle and an especially abundant mRNA level in liver (Figure 3A). The pattern of IL-15Rα mRNA expression was consistent with the receptor distribution estimated by IL-15 binding (Giri *et al.*, 1994). The presence of additional high molecular weight bands (>6 kb) on the commercial tissue blot, with relative intensities proportional to the ~1.7 kb IL-15R mRNA species, may be due to RNA aggregation. Bands of this size were not detected on other blots. The blots were also probed for IL-2Rα expression. No IL-2Rα mRNA was detected in the stromal lines which express IL-15Rα. Liver, the most abundant source for IL-15Rα mRNA, had very low or undetectable levels of IL-2Rα mRNA, while spleen, the most abundant source for IL-2Rα mRNA, had only moderate levels of IL-15Rα mRNA. More detailed examination of the cell types in the tissue preparations contributing to the observed mRNA levels is needed; the presence of contaminating cell types such as connective tissue, fibroblasts or blood cells may bias the results.

IL-2Rα expression has been shown to increase dramatically upon T cell activation (Leonard *et al.*, 1985). In our initial attempts to detect IL-15 binding on various cell lines and clones, in particular T cell lines, we observed a striking increase in IL-15 binding after activation. As summarized in Figure 3B, several methods of activating T cell lines resulted in increased IL-15Rα expression, such as pre-treatment with anti-CD3 antibodies or with phorbol myristyl acetate (PMA) as shown for the 7B9 helper T cell clone, or with more specific activators like IL-2, as shown for the D10 clone. For macrophage lines like P388D1 and J774, treatment with interferon-γ (IFN-γ), an important activator of macrophages and monocytes, resulted in significantly elevated receptor numbers, from 4- to 27-fold. Similar results were obtained for human peripheral blood T cells and human antigen-dependent clones stimulated with phytohemagglutinin (PHA) and anti-CD3, as well as human lipopolysaccharide (LPS)-treated monocytes and vascular endothelial cells treated with IFN-γ (Giri *et al.*, 1994). These results suggest that the expression of IL-15Rα, like that of IL-2Rα, may be similarly regulated and is greatly enhanced following exposure to activation signals.

A functional role for the α subunit of the IL-15R on murine cells

In initial binding experiments with COS-7 cells transfected with the murine IL-15Rα cDNA clone D5, in excess of 5×10^5 receptors/cell were detected, a level too high to obtain accurate measurements of IL-15 binding to these cells. To demonstrate the role of IL-15Rα in the IL-15R complex more directly, and to obtain more accurate measurements of its affinity for IL-15, we used as a model system the murine IL-3-dependent 32D cell line, which constitutively expresses the IL-2R α and γ_c chains, but failed to respond to IL-15 (Grabstein *et al.*, 1994). We derived 32D cells stably expressing various components of the IL-2 and IL-15 receptors and tested their ability to proliferate in response to IL-15 (Figure 4). Our original 32D cell line responded to IL-2, but for these experiments we used a subline, 32D-01, which had lost the ability to respond to IL-2 (Figure 4A, top panel), presumably because it no longer expressed sufficient levels of IL-2Rβ (Figure 4A, lower panel). We introduced the murine IL-2Rβ chain into 32D-01 and derived a line designated 32Dmβ-5 (Figure 4B), which had the ability to proliferate in response to IL-2 but not IL-15. No detectable IL-15 binding to 32D-01 or 32Dmβ-5 was seen by cytofluorometric analysis, suggesting that the level of IL-15Rα was very low on these cells. Direct binding with [^{125}I]IL-15 confirmed this result (see below). To test the role of IL-15Rα, we transfected 32D-01 with the IL-15Rα cDNA,

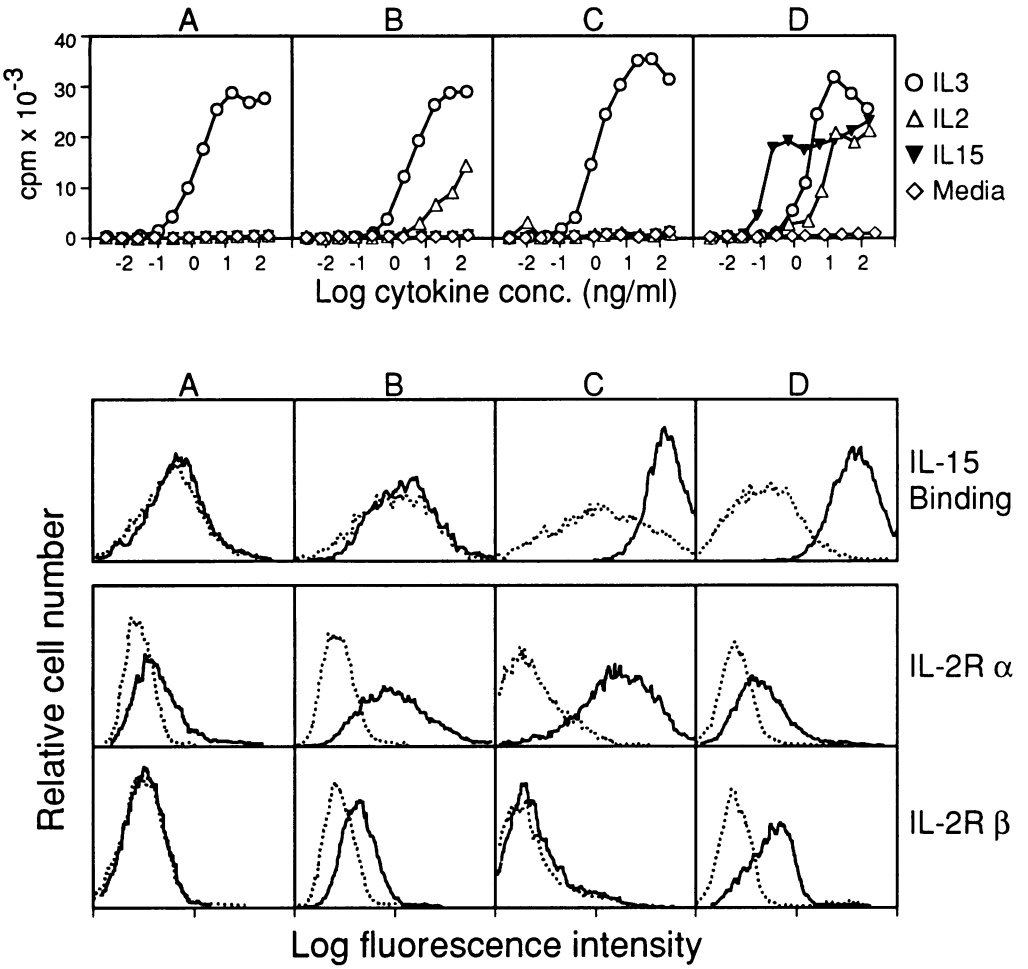

Fig. 4. Functional analysis of recombinant IL-15R. Top panel: proliferation of 32D sublines expressing various components of IL-15 and IL-2 receptors, in the presence of the indicated growth factors. (**A**) 32D subline 01; (**B**) 32Dmβ-5, transfected with murine IL-2Rβ; (**C**) 32Dm15Rα-102, transfected with IL-15Rα; and (**D**) 32Dmβm15Rα-3, co-transfected with both IL-15Rα and IL-2Rβ. Bottom panel: analysis of surface expression of IL-2R and IL-15R components. Cytofluorometric analysis of binding of Flag-IL-15, anti-IL2Rα mAb 7D4 or anti-IL2Rβ mAb TM-β1 was performed as described in Materials and methods. Control staining is indicated by dotted lines.

which resulted in a line expressing the α chain, 32Dm15Rα-102 (shown in Figure 4C). Although these cells bound high levels of IL-15, as evidenced by both cytofluorometric analysis (Figure 4C) and radiolabeled IL-15 binding (Figure 5A), they were unable to proliferate in response to IL-15. As seen in Figure 4C, the 32Dm15Rα-102 cells, like the parental 32D-01, did not express detectable levels of IL-2Rβ. We then derived 32Dmβm15Rα-3, co-expressing both IL-15Rα and IL-2Rβ (γ_c is constitutively expressed), and we were able to reconstitute proliferation in response to IL-15 and IL-2 (Figure 4D), with a pattern similar to proliferation of the D10 cell line shown in Figure 1. This result demonstrates that the ability of murine cells to respond to simian IL-15 is dependent on the level of IL-15Rα expression and confirms the requirement for IL-2Rβ.

IL-15Rα binds IL-15 with high affinity

Preliminary equilibrium binding experiments with [^{125}I]simian IL-15 indicated that the IL-15Rα chain alone was binding IL-15 with very high affinity, which prompted us to reassess the optimal binding conditions necessary to measure this affinity accurately under equilibrium conditions, as well as to measure whether a receptor complex containing the β and γ_c chains along with the IL-15Rα chain exhibited an enhanced affinity for IL-15. Using conditions detailed in Materials and methods, we first examined the parental 32D-01 cell line. These cells expressed an average of 100 ± 33 IL-15 binding sites per cell, with an affinity (K_a) of 1.4 ± 0.4×10^{11}/M (data not shown), which is similar to the affinity of IL-2 binding to the IL-2Rα/β/γ_c complex. The 32Dm15Rα-102 cells, transfected with the IL-15Rα chain (Figure 5A), exhibited a much higher level of IL-15 binding with the same very high affinity (average of 15 300 ± 3700 sites per cell with a K_a of 1.5 ± 0.9×10^{11}). Given the low expression of IL-2Rβ on these cells, the majority of these sites must reflect binding to the IL-15Rα chain alone. This suggests that the low amount of IL-15 binding on the 32D-01 cells is due to endogenous IL-15Rα. The affinity of the receptors on both of these 32D lines is very similar to the affinity of the native IL-15R on the D10 cells (Figure 5B) from which the IL-15Rα subunit was cloned (average K_a of 1.3 ± 0.5×10^{11}). Although D10 cells express several hundred copies of IL-2Rβ, inferred from the number of high affinity IL-2 binding sites (see above), we were

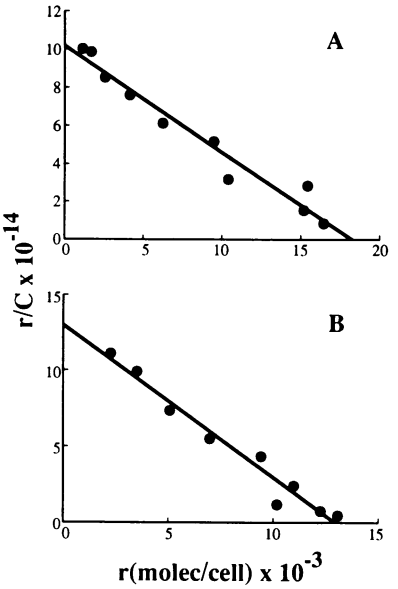

Fig. 5. Analysis of IL-15 binding to native and recombinant IL-15 receptors on murine cells. Scatchard analysis of equilibrium binding of [^{125}I]IL-15 to (**A**) 32Dm15Rα-102 cells expressing recombinant murine IL-15Rα, and (**B**) D10 cells. 32Dm15Rα-102 cells were used at 6.67×10^4 cells/ml diluted 1:200 with Daudi cells, while D10 cells were used at 1.33×10^5 cells/ml diluted 1:100 in Daudi cells. Binding was performed and assayed as described in Materials and methods. r, receptors bound per cell; C, molar concentration of radiolabeled IL-15.

unable to detect a second component of binding in these cells which might correspond to a higher affinity α/β or α/β/γ_c complex. This result was substantiated by analysis of the 32Dmβm15Rα-3 cells, co-expressing both recombinant IL-15Rα and IL-2Rβ subunits. These cells showed binding characteristics very similar to those exhibited by the 32Dm15Rα-102 cells (Figure 5A), with an average K_a of 2.2 ± 0.3×10^{11}/M, and 12 800 ± 2700 receptors/cell (data not shown). In both D10 and 32Dmβm15Rα-3 cells, overexpression of the IL-15Rα relative to the β subunit might serve to obscure a small higher affinity component. This possibility was addressed by analyzing binding to the 32Dmβ-5 cell line, which had been transfected with the β subunit alone. These cells showed a single high affinity binding site that was essentially identical to the parental 32D-01 line, with an average K_a of 1.9 ± 0.5×10^{11} and 40 ± 15 sites/cell, presumably due to low level expression of endogenous IL-15Rα. Although these data could be interpreted to suggest that the β subunit plays a minimal role as an affinity converter in the IL-15R complex, the very high affinity binding of the IL-15Rα on its own stretches the ability of the binding techniques used here to measure effectively and accurately interactions that may be of an even higher affinity, thus obscuring a definitive answer as to the contribution of the β subunit. The observation that the 32Dmβ-5 cell line did not display any additional IL-15 binding sites relative to the 32D-01 parent line did indicate, however, that simian IL-15 is unable to bind with any detectable affinity to complexes of murine β and γ_c, in the absence of the IL-15Rα chain.

Characterization of a soluble form of recombinant IL-15Rα

To test further the role of the IL-15R α chain, we prepared a soluble version consisting of the entire extracellular domain, as indicated in Figure 2A, and tested the ability of this soluble receptor to antagonize IL-15 binding to responsive cells. As shown in Figure 6A, the truncated soluble receptor is expressed in the supernatants of transfected CV1/EBNA cells as a protein of ~55–60 kDa, comparable in size with the membrane receptor on D10 cells. Similar to the membrane-anchored receptor, this size is larger than the size (20–25 kDa) predicted from the sequence shown in Figure 2A; more detailed biochemical characterization is required to explain this discrepancy. Supernatants from the transfected CV1/EBNA cells were very effective at inhibiting the binding of IL-15 to CTLL cells (Figure 6B), and were capable of specifically inhibiting IL-15- but not IL-2 induced proliferation of CTLL-2 cells (Figure 6C).

Discussion

The discovery of an IL-15-specific binding protein further extends the analogy between IL-2 and IL-15 and their receptor systems. Both receptor complexes appear to consist of three subunits, shared β and γ_c chains, and distinct α chains that bind only to their specific ligands. The receptor for IL-15 does not have the common features of the hematopoietin receptor family (Cosman, 1993), but rather shares structural similarities with the IL-2Rα subunit. Both proteins contain a short consensus repeat called a 'sushi domain'. Proteins containing this motif, such as C1s, C1r, C4BP, factor XIII, factor B and factor H, are proteins that bind to other proteins. In these proteins, the sushi domains are often found repeated multiple times: complement factor H, for example, consists entirely of 20 sushi domains (Perkins *et al.*, 1988). In addition, the motif itself is similar to other protein binding motifs, notably the protease kringle and fibronectin type II structures (Patthy *et al.*, 1984). Interestingly, the IL-15Rα contains a single sushi domain while the IL-2Rα contains two. Mutagenesis and antibody mapping experiments with the IL-2Rα chain have identified several residues in the first sushi domain as being directly involved in binding IL-2 (Moreau *et al.*, 1987; Robb *et al.*, 1988). Although no direct experimental evidence exists for IL-15Rα, the juxtaposition of several of these residues is conserved between the common sushi domain in the IL-15Rα and IL-2Rα sequences. The α chains of the IL-2R and IL-15R therefore define a new family of binding proteins for helical cytokines. It remains to be discovered if this family of molecules plays a role in other cytokine receptors, particularly those that are capable of utilizing γ_c. In this regard, it is interesting to note that there have been reports of as yet uncharacterized binding proteins for both IL-4 and IL-7 (Armitage *et al.*, 1992; Fanslow *et al.*, 1993).

In the reconstituted 32D murine cell model described in these studies, we have shown that expression of sufficient levels of the murine IL-15Rα subunit, along with the murine IL-2Rβ chain, is obligatory for conferring biological responsiveness to IL-15. This demonstrates that the IL-15 binding protein that we cloned is capable of performing the role of a specific IL-15Rα subunit to

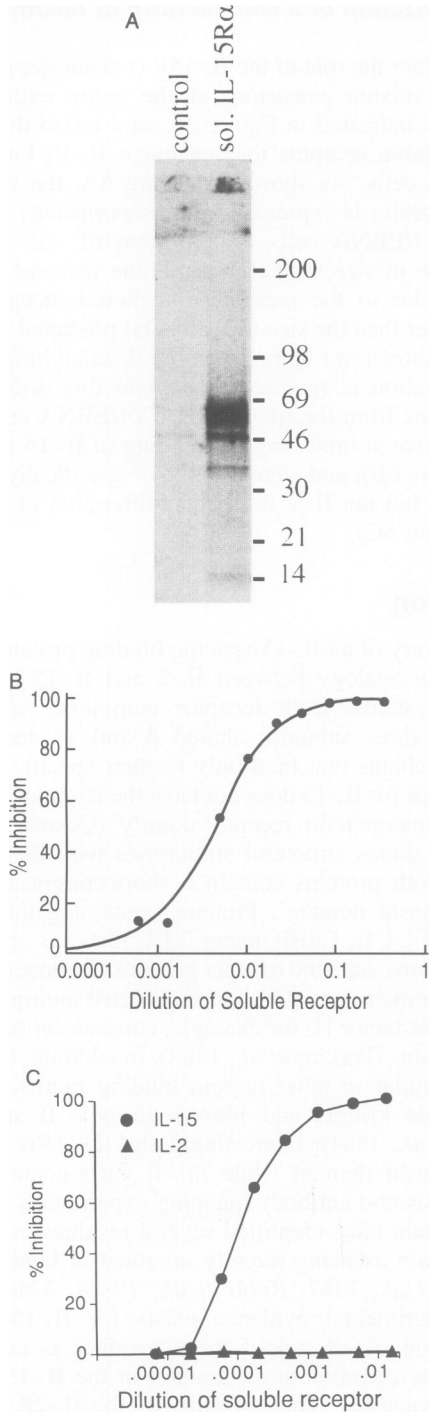

Fig. 6. Inhibition of IL-15 binding and function by a soluble form of IL-15R. (**A**) Expression of recombinant soluble IL-15R. The extracellular domain of IL-15R (Figure 2A) with five C-terminal histidine residues added for purification by metal chelate chromatography was expressed in transfected COS-7 cells, precipitated from metabolically labeled 3-day supernatants with Ni^{2+} NTA agarose, and analyzed by SDS–PAGE and autoradiography. Control is from COS-7 cells transfected with empty vector. The sizes of protein molecular weight standards are indicated on the right. (**B**) Inhibition of IL-15 binding to CTLL-2. Soluble IL-15R (10-fold concentrate of transfected COS-7 supernatant) inhibition of binding of 0.5 nM [^{125}I]IL15 for 1 h at 4°C. (**C**) Inhibition of CTLL-2 proliferation. Soluble IL-15R (10-fold concentrate of transfected COS-7 supernatant) tested for its ability to inhibit proliferation in response to 10 ng/ml IL-15 or IL-2.

mediate formation of a functional heteromeric complex with β (and probably γ_c). This system has certain limitations, however, in that we are utilizing simian IL-15 for binding to the murine components of the IL-15R. As has been demonstrated previously for human IL-2 (Kumaki *et al.*, 1993), we have shown that simian IL-15 is not capable of binding to a complex of the mouse β and γ_c chains, whereas it is clearly capable of binding to a complex of the human β and γ_c chains in the absence of the IL-15Rα subunit (Giri *et al.*, 1994). Therefore, in the 32D cells containing murine β and γ_c, the IL-15Rα chain may be obligatory for conferring responsiveness to simian IL-15 primarily because it is absolutely required to allow simian IL-15 to bind. We do not yet know whether murine IL-15 might be able to bind to, and signal through, a complex of murine β and γ_c in the absence of the IL-15Rα, nor do we know the extent of the role the α subunit may play in conferring responsiveness and/or specificity in such a system.

We observed a potential species-specific difference in sensitivity of IL-15 responsiveness between the 32D cells transfected with the murine β that we describe in this report, and murine BAF-B03 cells in a previous study (Giri *et al.*, 1994) in which we reconstituted IL-15 responsiveness by transfecting in the human β subunit. Both cell lines express a similar low level of endogenous IL-15Rα (<200 sites per cell); however, the BAF-B03 cells transfected with the human β chain were able to respond to simian IL-15, whereas the 32D cells were unresponsive to simian IL-15 until they were induced to express higher levels of the α chain by transfection with the IL-15Rα cDNA clone. This result may reflect the ability of simian IL-15 to interact in a more productive fashion with a complex containing a human rather than murine β subunit. This interpretation could explain a discrepancy in the results we obtained with 32D cells and those obtained by Bamford *et al.* (1994). These investigators have identified a cytokine, termed IL-T, which is secreted by the human cell line HuT-102, and has a number of properties which suggest that it could be equivalent to IL-15. They showed that 32D cells which are unresponsive to either IL-2 or IL-T can be induced to become responsive by transfecting the cells with the human β subunit alone. It is possible that the 32D cell line they used expresses a higher endogenous level of IL-15Rα subunits than our 32D line (and therefore might have a greater sensitivity to IL-15 upon introduction of the β chain). It is also possible that the difference is due to their use of the human β chain versus our use of the murine β chain.

IL-2 and IL-15 are at present the only T cell growth factors that have clearly identified heterotrimeric receptors, and their biological activities appear to be similar in promoting proliferation and functional activation of T and B lymphocytes (Grabstein *et al.*, 1994; Armitage *et al.*, 1995). One explanation for this apparent redundancy may be in the different distribution of both IL-15 and IL-15Rα compared with the more restricted expression of IL-2 and IL-2Rα. IL-2 and IL-15 may exert their effects at different stages of differentiation and on different populations of T and B cells. The ubiquitous expression of the IL-15Rα gene in various cell lines and tissues implies that *in vivo* the expression must be strictly controlled to regulate the immune response. As shown in studies of the IL-2Rα

chain (reviewed in Greene and Leonard, 1986), we found that the expression of IL-15Rα is also greatly enhanced after activation of T cells. It will be of interest in understanding T cell activation to investigate whether IL-2 and IL-15 may compete for a limited number of the common IL-2Rβ and γ_c subunits and, if so, what the biological consequences may be. Although IL-2 and IL-15 may share overlapping signaling mechanisms given their common usage of the β and γ_c chains, it will also be important to determine whether the IL-15Rα chain plays any role in signal transduction. No role for the extremely short (13 amino acid) cytoplasmic portion of the IL-2Rα chain has been demonstrated to date; however, the cytoplasmic domain of IL-15Rα is somewhat longer (37 amino acids), and it is possible that it plays some cooperative and/or regulatory role in signaling mechanisms which are mediated primarily through the β and γ_c subunits.

Despite the obvious resemblance and overlap in the heteromeric IL-2 and IL-15 receptor complexes, the striking difference in the affinities of the two α subunits for their respective ligands, as well as the wide distribution of mRNA for both IL-15 and its receptor, suggests additional, unidentified roles for IL-15. The IL-2Rα and IL-15Rα chains, although similar in general structural features, differ in their affinity for their respective ligands by 1000-fold or more. The average binding affinity of murine IL-15Rα alone, at a K_a of $\sim 1\times10^{11}$, is equivalent to the affinity generated by the complete IL-2R heterotrimer, while IL-2Rα alone has an affinity in the range of 1×10^8. The contribution of the β and γ_c chains to formation of high affinity IL-15R complexes could not be demonstrated due to the intrinsic high affinity of the IL-15Rα chain for IL-15. It remains to be determined if there is a difference in how IL-15 interacts with its α chain in the presence or absence of the shared IL-2R components. Further binding studies conducted in the context of our new knowledge regarding the very high affinity nature of the IL-15Rα subunit have revealed the affinity of binding of IL-15 across a range of murine cell lines to consistently average $\sim 1\times10^{11}$/M. The presence of high affinity IL-15 receptors on non-lymphoid cells such as thymic and bone marrow stromal lines raises the question of whether IL-15 can transmit a signal in cells which may not express the IL-2Rβ and γ_c chains. An equally intriguing question is the potential role that a high affinity IL-15Rα subunit expressed on the surface of cells lacking β or γ_c could play in the absence of the ability to signal or internalize. Such a high affinity binding protein might serve either to remove IL-15 from effective action in a local environment or, conversely, to serve as a source of presentation of IL-15 to neighboring populations of cells capable of responding to it. Soluble forms of IL-15Rα, which appear to be of high affinity with excellent IL-15 antagonist properties, could also be significant *in vivo*, whether endogenously generated or as specifically administered therapeutics.

We have described in this report the identification and cloning of a specific receptor for IL-15, which shares unique structural features with the α chain of the IL-2R. Together, the α chains of IL-2 and IL-15 define a new cytokine receptor family. IL-15 was initially cloned on the basis of functions it shared in common with IL-2, and the work presented here strengthens the basis for the commonality in function between these two molecules. Of equal interest, however, are the hints that the IL-15Rα gives us of potential differences that may exist in the functions of IL-15 and IL-2. Further study of the nature of the IL-15R complex and the location and regulation of both the receptor and its ligand should continue to generate important insights about the role of this new cytokine.

Materials and methods

Cell lines and culture conditions

Proliferation assays using the factor-dependent T cell lines CTLL-2 (Gillis and Smith, 1977) and 32D were carried out as described (Grabstein *et al.*, 1994). Briefly, cells were washed extensively, then grown for 24–48 h with the appropriate cytokines, and pulsed with [^{3}H]TdR 4–6 h before harvesting. The antigen-dependent cell line D10 (Kaye *et al.*, 1984) was cultured with irradiated allogeneic (C57/BL6) spleen cells and IL-2. For further stimulation in IL-2, D10 cells were removed from the spleen cells and cultured only in IL-2. For IL-15 binding experiments, the D10 cells were starved for 30 min after separation from the feeder layer or cultured overnight in the absence of IL-2 prior to separation from the feeder layer before initiation of binding incubations. COS-7 cells were cultured and transfected as described previously (Cosman *et al.*, 1984).

Growth factors

Yeast-derived Flag epitope-tagged simian IL-15 has been described (Grabstein *et al.*, 1994). Human IL-2 was purchased from Cetus (Emeryville, CA). Murine IL-3 and IFN-γ were obtained from Genzyme (Cambridge, MA).

Receptor binding analysis

^{125}I-Labeled simian IL-15 was prepared using the lactoperoxidase method previously described (Giri *et al.*, 1994) or using the Iodogen reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Labeled preparations were tested for biological activity in the CTLL proliferation assay as described (Giri *et al.*, 1994). Specific radioactivities of preparations were in the range $4–7\times10^{15}$ c.p.m./mmol. Binding assays were performed by a phthalate oil separation method and analyzed as described (Park *et al.*, 1986). Kinetic analysis of IL-15 binding at 4°C showed that at the low concentrations of IL-15 necessary to accurately measure high affinity binding, 4–5 h of incubation was required to reach equilibrium (data not shown). Unless otherwise noted, binding experiments were therefore performed standardly for long incubation times (4–5 h) at 4°C over an extended concentration range that went as low as the specific activity of the [^{125}I]IL-15 would allow (generally ~0.5 pM). When necessary, cells that expressed high levels of the IL-15Rα chain were diluted in incubation mixtures with a carrier cell that did not bind IL-15 [standardly the human B cell line Daudi (ATCC CCL 213)] in order to maintain proper conditions of ligand excess. Affinity cross-linking experiments were performed by binding of radiolabeled IL-15 to D10 cells at 4°C for 60 min followed by cross-linking with 1 mM disuccinimidyl suberate (Pierce) for an additional 30 min. Samples were then detergent solubilized in the presence of protease inhibitors according to established procedures (Sharon *et al.*, 1986) and analyzed by SDS–PAGE and autoradiography.

Flow cytometry

Cells analyzed by flow analysis for IL-15 binding were incubated with 1 μg/ml Flag epitope- (Hopp *et al.*, 1988) tagged simian IL-15 and 5 μg/ml anti-Flag antibody or with 10 μg/ml anti-IL-2R antibodies. Cells were washed, then stained with fluorescein-conjugated goat anti-rat IgG (Caltag, So. San Francisco, CA) and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Antibodies to murine IL-2Rα (7D4) and β (TM-β1) were purchased from Pharmingen (San Diego, CA). Control incubations were performed in parallel with isotype-matched antibodies from Jackson Immunoresearch (West Grove, PA).

RNA preparation and analysis

Total cellular RNAs were prepared by the guanidine thiocyanate–cesium chloride method, and polyadenylated RNAs were prepared by oligo(dT)–cellulose chromatography as described (Larsen *et al.*, 1990). RNA was electrophoresed through formaldehyde agarose, transferred by capillary blotting to a Hybond-N (Amersham, Arlington Heights, IL) and UV

cross-linked. ^{32}P-Labeled DNA and RNA probes were used in blot hybridizations as described (Anderson *et al.*, 1990). Blots were visualized by autoradiography on X-ray film or on phosphor screens (Molecular Dynamics, Sunnyvale, CA).

cDNA library preparation and screening

RNA was prepared as described above from D10 cells and used to prepare double-stranded, random-primed cDNA using a Pharmacia Timesaver cDNA kit (Pharmacia, Piscataway, NJ). The resulting cDNA was cloned into a mammalian expression vector using an adaptor method as previously described (Larsen *et al.*, 1990). The cDNA library was screened by a slide transfection method essentially as described (Gearing *et al.*, 1989). Briefly, plasmid DNAs prepared from pools of ~1000 colonies from the cDNA library were transfected into COS-7 cells grown in chamber slides. After 2 days of culture, the slides were incubated with 1 nM [^{125}I]IL-15 in binding medium for 1 h at 4°C. Slides were then washed with binding medium followed by phosphate-buffered saline (PBS), then fixed with 2.5% glutaraldehyde in PBS, dried, examined by autoradiography on phosphor screens, then dipped in autoradiographic emulsion. After 2–3 days, the slides were developed and examined microscopically for the accumulation of silver grains over individual COS-7 cells. Positive pools were divided and rescreened until a single positive clone, D5, was isolated.

Stable transfection of 32D cells

The murine IL-2R β chain cDNA (Kono *et al.*, 1990) was isolated from the murine lymphoma line EL-4 by RT-PCR and subcloned into expression vector pcDSRα (Takebe *et al.*, 1988) to yield pSRmβ-1. The IL-15Rα chain expression plasmid D5 and pSRmβ-1 were linearized with *Sal*I and the cleaved ends partially filled with dCTP and dTTP to generate 5′-TC overhangs. Compatible cohesive ends were generated by partial fill-in of *Bam*HI-digested pSV2neo with dATP and dGTP. Linearized D5 and/or pSRmβ-1 (20 μg) were ligated with 20 μg of linearized pSV2neo, and transfected into 32D cells by electroporation followed by selection in medium containing G418 (GIBCO BRL, Grand Island, NY). The resulting cell lines, 32Dm15Rα-102 and 32Dmβ-5, stably expressed the exogenous murine IL-15Rα and IL-2Rβ chains, respectively, while 32Dmβm15Rα-3 expressed both receptors.

Protein structural analysis

Database searches were performed against GenBank, GenPept (translated GenBank), SwissProt and the PIR protein databases using the FASTA (Genetics Computer Group, 1991) and BLAST (Altschul *et al.*, 1990) programs. Searches were performed using both the full-length conceptual translation of the IL-15R and the nested subsequences representing fragments of the extracellular domain only. A multiple alignment of murine IL-15Rα and IL-2Rα from various species was produced using the GCG program PILEUP and the MACAW multiple alignment tool (Schuler *et al.*, 1991). For these alignments, PILEUP was used to generate an initial estimate that identified the domain structure of the protein. MACAW was then used to refine alignments within delineated domains.

Soluble recombinant IL-15Rα

A plasmid encoding a soluble form of IL-15Rα was generated by PCR amplification of the D5 extracellular region (Figure 2A), with the incorporation of sequence at the 3′ end to encode five C-terminal histidine residues to allow purification by nickel chromatography (Hoffmann and Roeder, 1991). The amplified soluble IL-15Rα-encoding fragment was inserted into a mammalian expression vector and transfected into COS-7 cells as described above. Three-day supernatants containing secreted soluble IL-15Rα were used in cell binding and proliferation inhibition studies. ^{35}S-Labeled supernatants were generated from the transfected COS-7 cells as described (Giri *et al.*, 1994). Radiolabeled soluble receptor was purified from the supernatants by incubation with Ni^{2+} NTA agarose (Qiagen, Chatsworth, CA), following the manufacturer's instructions.

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