The EMBO Journal vol.13 no.12 pp.2822-2830, 1994

Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15

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Communicated by T.H.Rabbitts

We have recently cloned a novel cytokine, IL-15, with shared bioactivities but no sequence homology with IL-2. We found high affinity IL-15 binding to many cell types, including cells of non-lymphoid origin. Analysis of IL-15 interaction with subunits of the IL-2 receptor (IL-2R) revealed that the α subunit was not involved in IL-15 binding. We demonstrated directly in cells transfected with IL-2R subunits that both the β and γ chains are required for IL-15 binding and signaling. Hence, IL-15, like IL-2, IL-4 and IL-7, utilizes the common IL-2R γ subunit found to be defective in X-linked severe combined immunodeficiency in humans. IL-15 is the only cytokine other than IL-2 that has also been shown to share the β signaling subunit of IL-2R. The differential ability of some cells to bind and respond to IL-2 and IL-15 implies the existence of an additional IL-15-specific component.
Key words: IL-15 binding/receptor sharing/signaling

Introduction

We have recently identified a novel T cell stimulatory activity in culture supernatants of a monkey kidney epithelial cell line, CV-1/EBNA (Grabstein *et al.*, 1994). The protein was purified and, based on N-terminal sequence data, a cDNA encoding the cytokine was isolated and expressed in mammalian cells and yeast. The 14-15 kDa glycoprotein was designated IL-15. Human IL-15 has been cloned subsequently from a human stromal cell line, IMTLH, and found to have 97% amino acid identity with the simian protein (Grabstein *et al.*, 1994).

The biological activity of IL-15 appeared similar to that of IL-2, in particular its ability to stimulate proliferation of the established T cell line CTLL.2, as well as phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMC). In addition, IL-15 was also able to induce generation of cytolytic cells and lymphokine-activated killer cell (LAK) activity *in vitro*. This overlap in biological activities suggested the possibility that IL-15 might utilize components of the IL-2 receptor (IL-2R) for binding and signal transduction. It is important in this regard to note that IL-15 and IL-2 do not share any significant sequence homology, but modeling studies predict that IL-15 belongs to the family of four helix bundle type cytokines, which includes among others IL-2 and IL-4 and members of which bind to receptors of the hematopoietin superfamily (Bazan, 1992; Miyajima *et al.*, 1992; Cosman, 1993).

Because of the central role of IL-2 in both antigen-driven and nonspecific immune responses, both the cytokine and its receptor have been the subject of intensive investigation (Taniguchi and Minami, 1993). The IL-2R consists of at least three membrane proteins: the α chain (p55, Tac), the β chain (p75, IL-2Rβ) and the γ chain (p64, IL-2Rγ). The β and γ chains, but not the α chain, belong to the hematopoietin receptor superfamily (D'Andrea *et al.*, 1989; Hatakeyama *et al.*, 1989b; Takeshita *et al.*, 1992a) and are required for ligand internalization and signal transduction (Robb and Greene, 1987; Hatakeyama *et al.*, 1989a; Asao *et al.*, 1993). Different combinations of these receptor subunits on human cells give rise to three affinity classes: low affinity receptors (K_d of 10^{-8} M); intermediate affinity receptors (K_d of 10^{-9} M); and high affinity receptors (K_d of 10^{-11} M). The highest affinity class of receptors consists of all three chains of IL-2R. Intermediate affinity receptors expressed predominantly on NK cells which consist of the β and γ subunits have been shown to be functional (Voss *et al.*, 1992). Receptors which consist of the p55 and p75 subunits only (αβ), as in transfected fibroblasts, can bind IL-2 with relatively high affinity (K_d of 10^{-10} M), but do not appear to be capable of mediating IL-2 signals (Arima *et al.*, 1992; Takeshita *et al.*, 1992b; Asao *et al.*, 1993; Kumaki *et al.*, 1993). The essential role of the IL-2R γ chain is most dramatically demonstrated by the severity of X-linked combined immunodeficiency (XSCID), which has been localized to the IL-2Rγ gene (Noguchi *et al.*, 1993b).

In the experiments presented in this report, we have initiated studies aimed at the characterization of the receptor for IL-15 on various cells. In particular, due to the apparent similarities between the biological activities of IL-2 and IL-15, we have focused on investigating the possibility that IL-15 utilizes components of the IL-2R.

Results

Binding of IL-15 to cell surface receptors

Recombinant simian IL-15 was expressed in yeast and purified by passage over phenyl Sepharose and reversed phase HPLC, as described in Materials and methods. Fractions from HPLC were subjected to SDS-PAGE followed by silver staining as shown in Figure 1A, and the fractions with highest specific activity were radiolabeled with ^{125}I (Figure 1B) using immobilized lactoperoxidase (Park *et al.*, 1990). Radiolabeled IL-15 retained complete biological activity, as shown in Figure 1C, measured by the ability of IL-15 to stimulate proliferation of the CTLL.2 line. Similar results were obtained with human IL-15.

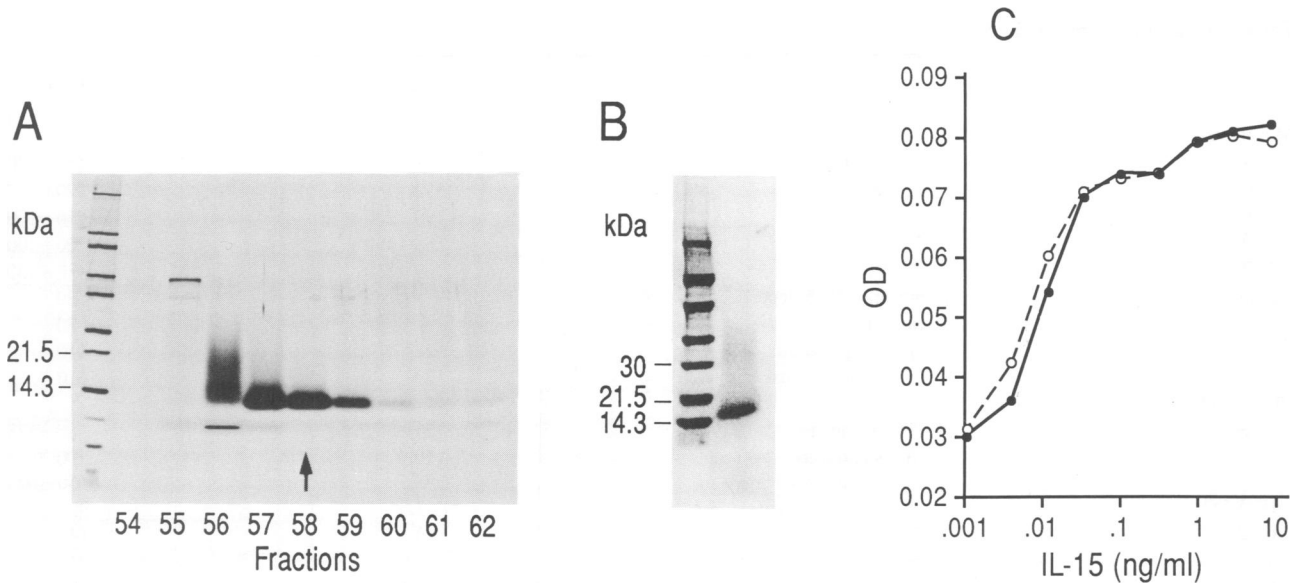

Fig. 1. Preparation of radiolabeled IL-15 for receptor binding. (**A**) 14% SDS–PAGE analysis of simian IL-15 fractions eluted from reversed phase HPLC C4 column and silver stained for protein detection. Arrow indicates fraction used for iodination. (**B**) SDS–PAGE (10%) of fraction 58 from panel (A) after iodination. (**C**) CTLL.2 proliferation assay with iodinated and 'mock' iodinated IL-15, using the MTT method (Roehm *et al.*, 1991). Filled circles, unlabeled fraction; open circles, ^{125}I-labeled IL-15. The two samples were treated in an identical manner, except that radioactive iodide was not added to the control.

Simian [^{125}I]IL-15 was used in receptor binding assays (Figure 2). Since IL-15 induces proliferation of the IL-2 dependent murine T cell line CTLL.2 with a similar dose response to IL-2 (Grabstein *et al.*, 1994), we compared binding of IL-2 and IL-15 to CTLL.2. Unlike IL-2, which has both high and low affinity binding sites, as shown in the representative experiment of Figure 2, only one type of high affinity binding site was observed with IL-15 with a K_d of 3.4×10^{-10} M and ~1420 sites/cell. Similar results were obtained with PHA-activated PBMC. A single class of high affinity IL-15 binding sites was detected on PHA blasts (Table I), while Scatchard analysis of IL-2 binding showed the presence of high and low affinity binding sites, as previously reported (Robb *et al.*, 1984).

In order to obtain an initial estimate of the distribution of IL-15 receptor expression, we sampled a large number of cell lines from various sources as well as antigen dependent human and murine T cell clones, as summarized in Table I. All cells listed that bound IL-15 did so with high affinity and with an apparent K_d between 1×10^{-11} and 5×10^{-10} M. In addition to established murine T cell lines such as S49 and CTLL, and antigen dependent clones, such as AB8, 7B9, RA8 and the human C.22 clone, binding to human B cells immortalized with Epstein–Barr virus (EBV), freshly isolated NK cells and monocytes was also detected. Specific binding of IL-15 was below detectable levels on several B cell lymphomas such as Daudi (Table I) and Raji and CB23 (not shown), as well as hepatoma lines like HepG2. Interestingly, high affinity binding to human endothelial cells, and to several murine thymic epithelial and fetal liver stromal lines, was also detected. The examples shown in Table I indicate that IL-15 receptors are found on many cell types of lymphoid and non-hematopoietic lineages. Also evident from the binding results presented in Figure 2 and Table I is the ability of simian (or the highly homologous human) IL-15 to bind to receptors on murine cells. We do not have any information at present regarding whether murine IL-15 can bind to receptors on human cells. Some murine cells appear to bind human or simian IL-15 with lower affinity than human cells, as shown in Table I.

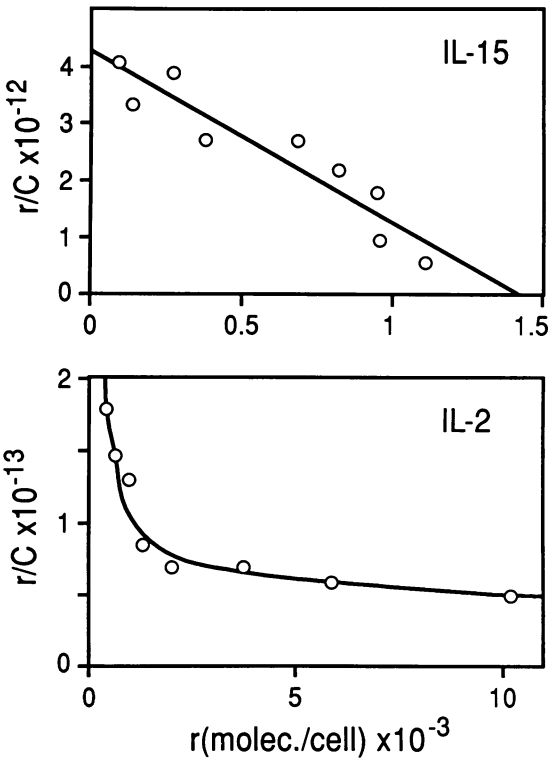

Fig. 2. Binding of IL-15 to receptors on CTLL.2 cells. Scatchard analysis of [^{125}I]IL-2 and [^{125}I]IL-15 binding to CTLL.2. Cell-associated cytokines on cells grown in the presence of IL-2 or IL-4 were removed by a rapid acid wash (0.05 M glycine, pH 3.5) followed by extensive washing in binding medium containing 3% BSA prior to IL-15 or IL-2 binding experiments.

Table I. Binding to murine and human cells

	Description	K_d (pM)	Sites/cell
Murine cells			
S49	T, lymphoma	41–83	140–300
RA8	T, clone	192	770
AB8	T, clone	190–230	670–2000
7B9	T, clone	163–490	700–1000
CTLL.2	T, clone	100–330	430–1300
32D	myeloid, IL-3 dependent	–	<30
TN	early T	–	<30
FL-10	thymic epithelium	22	110
TE71	fetal liver (stromal line)	29	1710
Human cells			
PBMC	PHA-stimulated	25–35.7	80–573
Monocytes	LPS-stimulated	10.0	190
NK	CD56+, enriched	55.5	50–80
HUVEC	vascular endothelial	33.3	990
C.22	T, clone	50	140
TF-1	myeloid	83.0	211
816	EBV-B	14.5	470
EBV-TC	EBV-B	12.5	1440
HepG2	hepatoma	–	<30
Daudi	B, lymphoma	–	<30
Jurkat	T, lymphoma	–	<30
THP.1	monocytic leukemia	10.3	60

Human monocytes and NK cells were derived from blood of normal donors. Monocytes were purified by elutriation and NK cells were enriched by selection with anti-CD56 antibodies (Becton-Dickinson, San Jose, CA) followed by removal of adherent cells. Monocytes were cultured for 24 h with 10 ng/ml LPS. The antigen-specific murine T cell clones were cultured and activated with anti-CD3 mAb as described by Grabstein *et al.* (1993). Clone 22 was generated from human PBMC stimulated with anti-CD3 mAb and maintained by stimulation with anti-CD3, heterologous PBMC and IL-2. Triple negative (TN) cells are described in Materials and methods. Fresh human endothelial cells were obtained from Cell Systems (Kirkland, WA) and cultured according to the supplier's directions. The 816 and EBV-TC lines were EBV-transformed B cell lines obtained from the University of Washington Medical Center Department of Pediatric Immunology. We considered <30 binding sites/cell as negative for IL-15 binding.

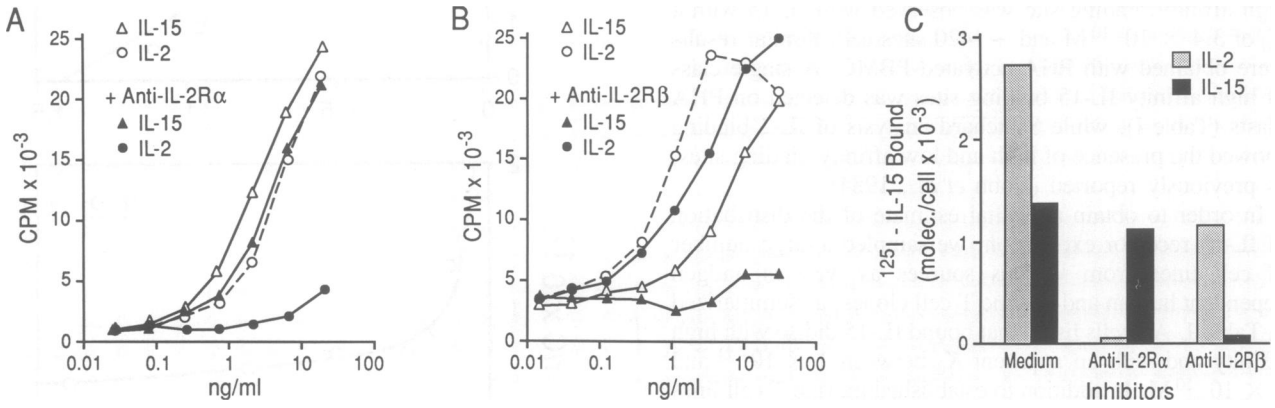

Fig. 3. Effect of neutralizing anti-IL-2R mAbs on IL-15 activity and binding to human PHA-treated PBMC. (**A**) Effect of anti-IL-2Rα mAb on IL-15-induced proliferation. Cells were incubated in the presence or absence of 20 μg/ml neutralizing anti IL-2Rα mAb 2A3 and increasing concentrations of cytokines for 48 h. For the last 4 h of incubation cells were pulsed with 0.5 μCi of [^{3}H]thymidine as previously described (Grabstein *et al.*, 1986). (**B**) Inhibition of IL-15-induced proliferation by anti-IL-2Rβ mAb. The neutralizing anti-IL-2Rβ mAb Mikβ1 was used at 50 μg/ml, as above. (**C**) Effect of anti-IL-2R antibodies on IL-15 binding. Cells were preincubated with antibodies for 30 min at 4°C prior to binding of iodinated cytokines. Binding results are corrected for non-specific binding in the presence of 100-fold excess of unlabeled cytokine; [^{125}I]IL-2 at 100 pM and [^{125}I]IL-15 at 500 pM were used in the experiment.

Interaction of IL-15 with the IL-2 receptor complex: the IL-2R p55 subunit is not involved in IL-15 binding

Because many of the same cells respond to and bind both IL-2 and IL-15, we tested the possibility that IL-15 binds to components of the IL-2 receptor complex. As seen from the experiments in Figure 3A and B, IL-2 and IL-15 appear very similar in their ability to promote the growth of human PBMC. Interaction of IL-15 with the low affinity IL-2R consisting of the p55 α subunit, or participation of this protein in IL-15 binding, was tested using a neutralizing mAb, 2A3, directed against the α subunit of IL-2R (Figure 3A). The 2A3 mAb (Dower *et al.*, 1985) completely abolished IL-2 dependent proliferation and binding of labeled IL-2 to activated PBMC, but the anti-IL-2Rα mAb had no effect on IL-15-induced proliferation (Figure 3A) or binding (Figure 3C). The β chain of the IL-2R, however, appears

to have a role in IL-15 binding, as a neutralizing antibody, Mikβ1 (Tsudo *et al.*, 1989), directed against the IL-2Rβ, inhibits IL-15 binding (Figure 3C) and IL-15-driven proliferation of PHA-treated PBMC (Figure 3B).

IL-15 also binds to the human NK-like cell line YT, as shown in Table II. The YT cell line (Yodoi *et al.*, 1985) has been well characterized and served as source for the cloning of the p75 (β chain) of the IL-2R (Hatakeyama *et al.*, 1989b). We have used in these experiments a subclone of the YT cell line, designated YTN17, that was selected for high expression of the IL-2R β chain. As summarized in Table II, by Scatchard analysis of IL-15 binding we estimated that there were ~300–400 high affinity (K_d = 30–60 pM) binding sites on YTN17 cells. Depending on culture conditions and, in particular, the level of cell surface β chain expression (estimated by staining with the anti-IL-2Rβ mAb Mikβ1), we also detected a variable level of lower affinity receptors (K_d of 270–2500 pM). The β chain of IL-2R is also a component of the low affinity IL-15 binding sites as they are not detected in the presence of the neutralizing anti-IL-2Rβ mAb Mikβ1 (data not shown). Cross-linking of iodinated IL-15 to receptors on the cell surface of YTN17 cells followed by SDS–PAGE resulted in a major complex of ~92 kDa (Figure 5C), which is similar in size to the complex obtained with iodinated IL-2 (Sharon *et al.*, 1986; Robb and Greene, 1987; Hatakeyama *et al.*, 1989b) and consistent with binding to a 74 kDa receptor protein, the approximate size of IL-2R β chain expressed on cells. (The size of the iodinated IL-15 was estimated at 18 kDa.)

Table II. Binding to receptors on YTN17 cells

	Affinity	pM	No. of binding sites
Experiment 1	k_1	60.6 ± 1.8	375 ± 93
	k_2	272 ± 22	1016 ± 334
Experiment 2	k_1	27.6 ± 10.8	307 ± 10
	k_2	2500 ± 500	2170 ± 635

Binding experiments were performed in duplicate. Cells were preincubated with the anti-IL-2Rα mAb 2A3 (40 μg/ml) for 60 min at 4°C prior to addition of radiolabeled cytokines to limit binding to intermediate affinity IL-2R (Robb and Greene, 1987).

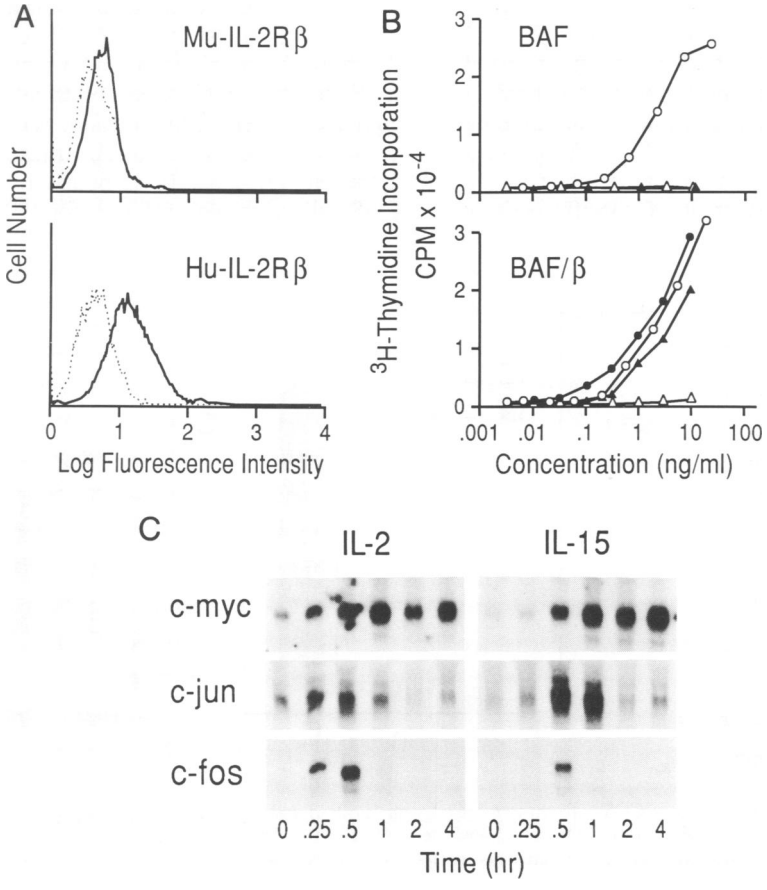

Fig. 4. Reconstitution of IL-15 binding and function by human IL-2R β subunit in transfected BAF cells. (**A**) Expression of human IL-2Rβ in murine BAF cells. Endogenous expression of murine IL-2Rβ, shown in the top panel in untransfected and human IL-2Rβ-transfected cells was assessed by cytofluorometry using biotinylated anti-mouse IL-2Rβ mAb from Pharmingen (San Diego, CA). To detect expression of human IL-2Rβ (bottom panel), cells were stained with the anti-human IL-2Rβ mAb Mikβ1 at 1 μg/ml, followed by FITC-coupled anti-mouse IgG (dotted line, untransfected control; solid line, transfected cells). (**B**) Proliferation of IL-2Rβ-transfected BAF/β cells in response to IL-15. Cells were cultured at 10^4 cells/ml with increasing concentrations of cytokines for 24 h, and pulsed with 1 μCi/ml [^{3}H]thymidine for the last 4 h of culture. Top panel, untransfected control BAF cells; bottom panel, IL-2Rβ-transfected cells. IL-2 is included as a control. ○, IL-3; ●, IL-2; ▲, IL-15; △, medium only. (**C**) Induction of proto-oncogene expression by IL-15 in BAF/β cells. Northern blot analysis of c-*fos*, c-*jun* and c-*myc* expression in total cellular RNA isolated from BAF/β cells at the indicated times following cytokine stimulation. Induction by IL-2 is included for comparison. RNA loading was normalized by UV visualization of 28S rRNAs (not shown).

The β chain of the IL-2R reconstitutes IL-15 responsiveness

To demonstrate directly that the β chain of the IL-2R is part of the receptor for IL-15, we have cloned the cDNA for the human β chain based on the published sequence (Hatakeyama *et al.*, 1989b) by RT-PCR, subcloned it into a mammalian expression vector as indicated in Materials and methods, and introduced it by electroporation into the murine pro-B cell line BAF/B03. The BAF/B03 cells are IL-3 dependent and express the IL-2R α and γ chains, but very little if any β chain (Hatakeyama *et al.*, 1989a). As shown in Figure 4B, these cells are not capable of proliferating in response to either IL-2 or IL-15. It has been demonstrated previously that expression of human IL-2R β chain in BAF cells results in the reconstitution of a functional IL-2R (Hatakeyama *et al.*, 1989a). In similar experiments (Figure 4) we have shown that BAF cells transfected with the human IL-2Rβ and expressing the β chain on their surface (Figure 4A) can proliferate not only in response to IL-2 but to IL-15 as well (Figure 4B). In addition, it was previously reported that IL-2 was able to induce proto-oncogene expression in BAF/β cells (Hatakeyama *et al.*, 1992). Figure 4C shows that both IL-2 and IL-15 were able to induce c-*myc*, c-*fos* and c-*jun* mRNA in BAF/β cells. We found that IL-15 binds to the IL-2Rβ transfected BAF/β cells with a K_d of 1.85×10^{-10} M, and ~140 binding sites are detected (binding to untransfected parental BAF cells is very low or undetectable, <30 binding sites/cell). As expected both high and low affinity binding sites for IL-2 are observed, with ~350 high affinity sites (with a K_d of 4.3×10^{-11} M) in the same range as the IL-15 binding sites and within error of specific activity estimates. The neutralizing anti-IL-2Rβ mAb Mikβ1 completely abolished IL-15 binding to BAF/β cells, confirming the dependence of IL-15 binding on the expression of the human β subunit of the IL-2R in these cells (data not shown).

The γ chain of IL-2R participates in IL-15 binding and signaling in transfected cells

We have shown that IL-15 binds YT cells which express the intermediate affinity class of IL-2R (heterodimers of β and γ subunits). In addition we were able to demonstrate the obligatory participation of the IL-2R β chain in IL-15 binding and signaling in the transfected BAF/β cells. To determine whether the γ chain is a component of the IL-15 binding complex, we subcloned the γ chain into an expression vector, in a similar manner to the β subunit, and examined IL-15 binding to COS cells transiently expressing the β chain alone, or co-expressing both β and γ chains. Under the conditions described in Figure 5, we detected very little or no cell associated binding of radiolabeled IL-15 to COS cells transfected with the β chain alone, but a dramatic increase in binding was evident on cells co-transfected with both β and γ IL-2R. The difference in binding of IL-15 was not due to lack of efficient cell surface expression of the β chain on cells transfected with cDNA for the β subunit alone. As shown by staining with mAb against the IL-2Rβ protein in Figure 5A, both types of transfected cells expressed equivalent levels of the β chain. We did not detect binding of either IL-2 or IL-15 to cells transfected with the γ chain alone. Both IL-2 and IL-15 bound to IL-2Rβγ expressed on COS cells with high affinity: for [^{125}I]IL-15 we estimated

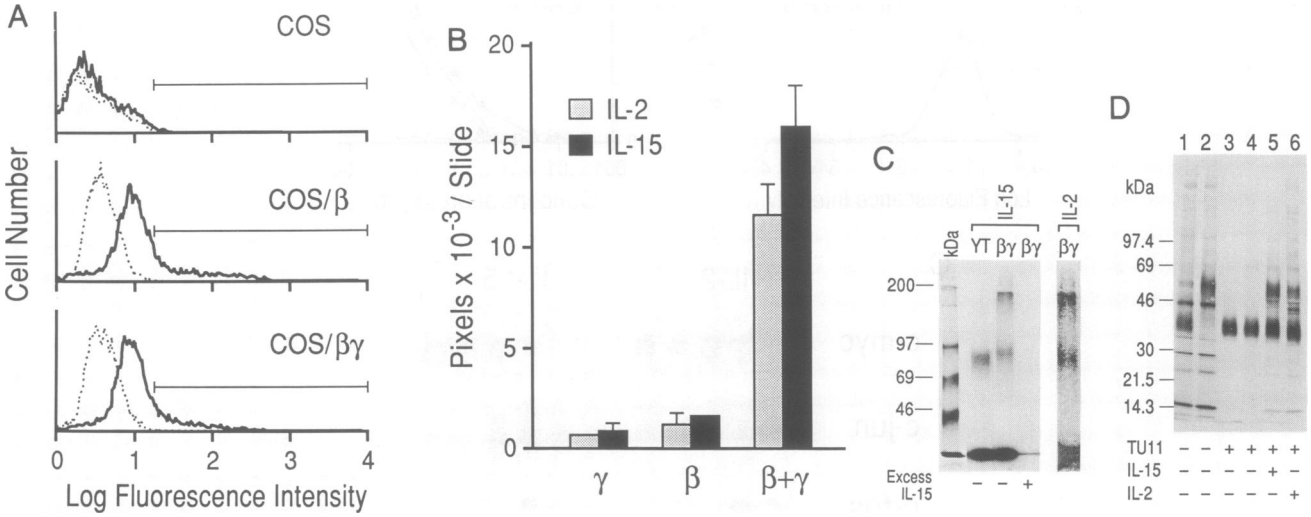

Fig. 5. Analysis of IL-15 binding and function in COS cells co-transfected with IL-2R β and γ subunits. (**A**) Comparison of cell surface expression of human IL-2R β chain in untransfected COS control, COS cells transfected with IL-2Rβ alone or co-transfected with IL-2Rβ and γ. The anti-human IL-2Rβ mAb Mikβ1 was used for cytofluorometry (dotted line represents an isotype antibody control; solid lines represent staining with Mikβ1). (**B**) IL-15 binding to transfected COS cells. The slide method for detecting receptor expression used in this experiment has been described previously (McMahan *et al.*, 1991). DNA was introduced into cells using DEAE–dextran. Iodinated IL-15 and IL-2 were used at 500 pM, and binding was carried out at 4°C for 1 h. The binding of iodinated ligands to the slides was measured using a Phosphorimager (Molecular Dynamics, Minnetonka, MN). (**C**) SDS–PAGE (8%) analysis of [^{125}I]IL-15 cross-linked to receptors on cells. Binding and cross-linking were carried out as described (Sharon *et al.*, 1986). Complexes of IL-15 cross-linked to receptors on COS cells co-transfected with IL-2Rβγ, or to YTN17 cells, were detergent solubilized and subjected to SDS–PAGE under reducing conditions. Results of cross-linking of [^{125}I]IL-2 to receptor proteins on IL-2Rβγ-transfected COS cells are shown for comparison. (**D**) IL-15 binding to soluble extracellular domains of IL-2R β and γ chains. CV-1/EBNA cells transfected with the extracellular domains of the IL-2R β and γ chains were metabolically labeled with [^{35}S]methionine and the supernatants were used as source of soluble receptors. Lane 1 shows supernatants from cells transfected with soluble β and lane 2 supernatants from cells transfected with soluble γ. Lanes 3–6 show proteins immunoprecipitated with the non-neutralizing anti-IL-2Rβ mAb TU11 prior to SDS–PAGE (8–16%). Control immunoprecipitation of soluble β with TU11 is shown in lane 3. Co-precipitation of soluble β and γ with TU11 is shown in the absence of IL-15 or IL-2 (lane 4) or with added 10 nM IL-15 (lane 5) or 10 nM IL-2 (lane 6).

320 binding sites with an affinity of 2.7×10^{-11} M and 1340 sites with a lower affinity of 2.8×10^{-10} M; with [^{125}I]IL-2 we detected 750 sites with a K_d of 6.0×10^{-11} M and 3050 binding sites with a K_d of 8.3×10^{-10} M. The presence of two types of binding sites for IL-15 and IL-2 is likely to be an artifact due to heterogeneity in co-expression levels of IL-2R β and γ chains in the transfected COS cell population, as both IL-2 and IL-15 showed the same anomaly in binding. In the transient expression system used, there are large variations in the number of receptors expressed on individual cells, and even single-chain receptors like IL-1R type I have been previously observed to bind with different apparent affinities (Sims *et al.*, 1989).

Analysis of [^{125}I]IL-15 cross-linking to receptors on IL-2R$\beta\gamma$-transfected COS cells is shown in Figure 5C. Under reducing conditions, a broad band between 92 and 96 kDa is observed, as well as a partially reduced larger complex of ~180 kDa. These sizes are comparable to the cross-linked receptor–IL-15 complexes observed on YT cells, or obtained by cross-linking IL-2 to receptors on $\beta\gamma$ transfected COS cells.

To analyze further the interaction of IL-15 with the $\beta\gamma$ subunits of IL-2R, we have expressed the soluble extracellular domains of the receptor proteins. As shown in Figure 5D, soluble β and γ chains can be co-precipitated with the non-neutralizing anti-IL-2Rβ mAb TU11 (Suzuki *et al.*, 1989), only in the presence of IL-15 or IL-2. The mixture of soluble β and γ chains was not immunoprecipitated with the TU11 mAb in the absence of IL-15 (Figure 5D, lane 4). This result suggests that IL-15, like IL-2, can induce dimerization of soluble β and γ IL-2R subunits, as previously demonstrated for the subunits expressed on the cell surface (Takeshita *et al.*, 1992b; Voss *et al.*, 1992).

We have further investigated whether the γ chain of IL-2R is also involved in IL-15 signaling, as suggested for the β chain in the experiments of Figure 4C. We compared IL-15- and IL-2-induced proto-oncogene expression in the murine L929 fibroblast line stably expressing the human IL-2R α and β chains in the presence or absence of γ subunit expression. As shown in Figure 6, an increase in the level of c-*myc* and c-*fos* mRNAs in response to IL-15 was detected only in the L929 cells co-expressing the γ chain, in agreement with the previously reported results for IL-2 in the same experimental system (Asao *et al.*, 1993). However, both $\alpha\beta$- and $\alpha\beta\gamma$-transfected L929 cells were able to bind IL-15 with similar affinity with K_d of 31 and 23 pM, respectively.

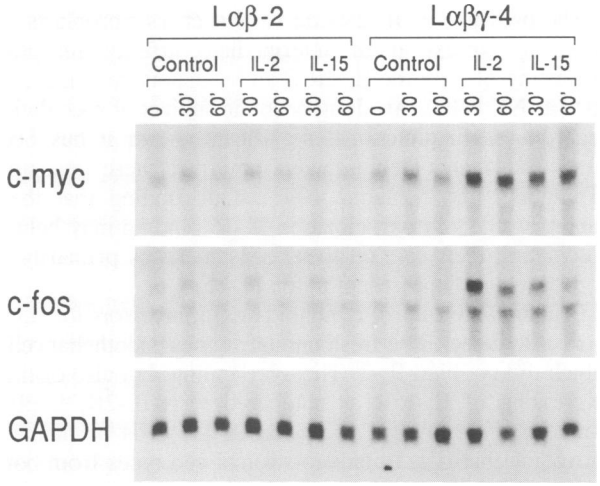

Fig. 6. Requirement for IL-2Rγ for IL-15 signaling. Expression of c-*myc* and c-*fos* proto-oncogenes in response to IL-2 and IL-15 was analyzed in transfected L929 fibroblasts. L$\alpha\beta$-2 and L$\alpha\beta\gamma$-4, stimulated with 1 nM IL-2 or IL-15 for 30 or 60 min. Northern blots were probed as in Figure 4C, using the relative amount of glyceraldehyde-3-phosphate dehydrogenase (GADH) mRNA as control for the amount of RNA applied to each lane.

Expression of functional high affinity IL-2R is not sufficient for IL-15 binding

We have shown that the IL-2R β and γ chains participate in IL-15 binding. The next question is whether these IL-2R subunits are sufficient to explain IL-15 binding to all cells examined. Several cell lines, such as the IL-3 dependent 32D

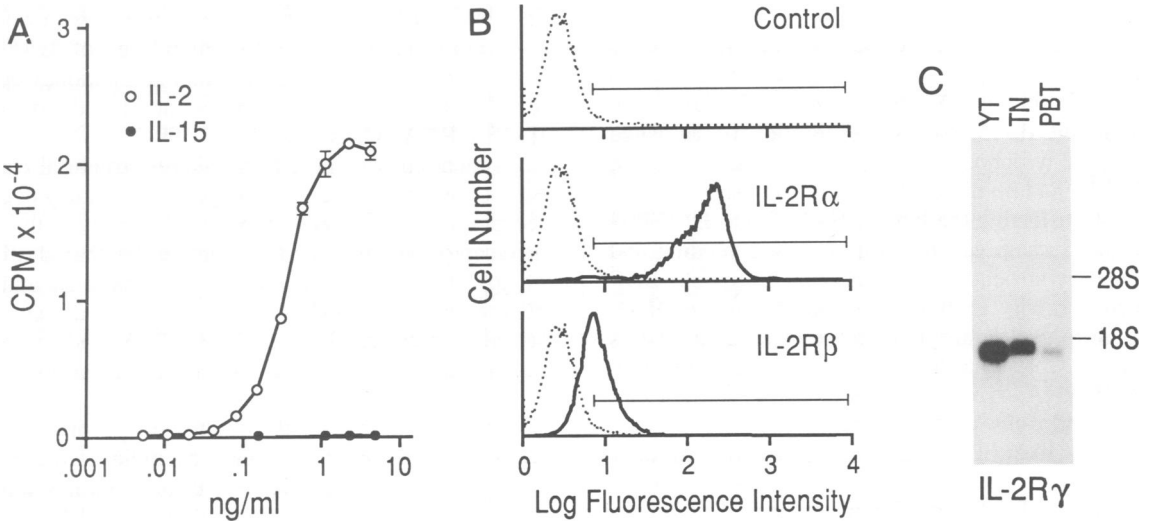

Fig. 7. Lack of IL-15 binding and responsiveness in TN cells. (**A**) Proliferation of triple negative (TN) cells (CD3$^-$, CD4$^-$, CD8$^-$) in response to IL-2 as compared with IL-15, measured by incorporation of [^{3}H]thymidine. (**B**) Expression of IL-2R α and β chains on TN cells estimated by cytofluorometry. Anti-mouse IL-2R mAbs were from Pharmingen (dotted line, isotype Ig control; solid line, anti-IL-2R mAb). (**C**) Northern blot analysis of IL-2R γ chain expression in TN cells.

cell line, which proliferates in response to IL-2, cannot proliferate in IL-15 (Grabstein *et al.*, 1994). We have examined more closely an early murine pre-T cell population derived from day 13 fetal liver which lacks CD3, CD4 and CD8 expression (TN cells). These cells, when cultured on a stromal cell layer, proliferate in response to IL-2 but, surprisingly, fail to respond to IL-15 (Figure 7A). Both the murine α and β IL-2R subunits are expressed on their cell surface (Figure 7B), and they express mRNA for the γ chain as well (Figure 7C). Scatchard analysis of IL-2 binding revealed the presence of 350–600 high affinity IL-2 binding sites and, because the cells proliferate in IL-2, they must express the necessary signaling components as well. In spite of the presence of functional IL-2Rs on these cells, the binding of IL-15 is undetectable (<30 binding sites/cell). Because other murine T cell lines and clones such as CTLL.2 and 7B9 bind human and simian IL-15 efficiently (Figure 2 and Table I), a difference in species of IL-15 is not likely to be a simple explanation for lack of binding to TN. Preliminary experiments using murine IL-15 confirm the results obtained with human and simian IL-15.

Discussion

We have presented in this report initial characterization of the binding properties of a novel cytokine, IL-15. This cytokine shares not only biological activities with IL-2, but as we have shown, it can also interact with two of the components of the IL-2R complex.

Cell surface receptors for IL-15 are expressed on a variety of T cells, such as murine antigen dependent T cell clones, and human and murine T cell lines, which express IL-2Rs as well. Low numbers of high affinity IL-15 receptors were also detected on peripheral blood monocytes, NK cells and PHA-activated PBMC. On many cells, such as the T cell lines and clones or fresh monocytes, higher numbers of receptors were detected after activation (with PHA, PMA, anti-CD3 for T cells, and LPS for monocytes). Direct comparison of IL-2 and IL-15 binding by Scatchard analysis on the same cell populations revealed that unlike IL-2, IL-15 exhibited only a single class of high affinity receptors on CTLL.2.

By several criteria we have established that IL-15 binds to the β chain of IL-2R, but that the IL-2R α chain has no effect on IL-15 binding. Antibodies to the β chain were shown to inhibit IL-15 driven proliferation of activated peripheral blood lymphocyte (PBL), but antibodies directed against IL-2Rα had no effect. We also demonstrated more directly, by transfecting the human IL-2Rβ into BAF/B03 cells, that the β chain can reconstitute IL-15 binding and responsiveness. Introduction of human IL-2Rβ into BAF cells, which normally grow only in the presence of IL-3, enabled them to proliferate in response to IL-2 as well as IL-15. Capacity for signal transduction was also shown by induction of mRNA expression not only for c-*myc*, but also for the c-*fos* and c-*jun* proto-oncogenes.

Since IL-15, like IL-2, was shown to bind to cells expressing predominantly the IL-2R consisting of β and γ subunits, such as YT cells and CD56$^+$ human NK cells, we also investigated the role of the γ chain in IL-15 binding and signaling. In COS cells transfected with IL-2R subunits, co-expression of both β and γ chains of IL-2R is needed for IL-15 binding. Moreover, the IL-2R γ subunit appears to be required for IL-15 signal transduction as demonstrated in L929 cells transfected with subunits of IL-2R. This result is important in view of the recent finding mentioned earlier that XSCID in humans has been mapped to the IL-2Rγ gene. Patients with XSCID have abnormal T cell populations and very few mature T cells (Cooper and Butler, 1989) as well as abnormal B cell responses. Because IL-2-deficient humans (Weinberg and Parkman, 1990) or IL-2-deficient mice generated by inactivation of the IL-2 gene (Schorle *et al.*, 1991; Kündig *et al.*, 1993) appear to have normal thymic development and T cell populations, it has been postulated that other cytokines in addition to IL-2 may utilize the IL-2R γ chain. Indeed several recent reports suggest that other cytokines involved in T lymphocyte growth and differentiation, such as IL-7, IL-4 and possibly IL-13, use the IL-2R γ chain as part of their receptors (Kondo *et al.*, 1993; Noguchi *et al.*, 1993a; Russell *et al.*, 1993).

The presence of IL-2Rs on cells such as fibroblasts, as well as expression of intermediate affinity receptors consisting of only the IL-2R β and γ chains on some cells such as NK cells, have long been the subject of speculation regarding their functional significance, and it has been suggested that other ligands may use these receptors (Taniguchi and Minami, 1993). Our finding that these subunits of IL-2R participate in IL-15 binding may help in elucidating their role on these cells, perhaps primarily as components of the receptor for IL-15.

In addition to cells of lymphoid origin, receptors for IL-15 were also detected on fresh human venous endothelial cells, which did not bind IL-2. The overlapping, but also distinct expression of IL-15R as compared with IL-2R, implies potential undiscovered roles for IL-15. It is particularly intriguing that IL-15 binds to stromal cell types from bone marrow, fetal liver or thymic epithelium and may be required for the functioning of these cells, suggesting a possible role for IL-15 in development of T and NK cells. One reason for the milder than expected *in vivo* effect of IL-2 deficiency on immune responses in mice (Schorle *et al.*, 1991; Kündig *et al.*, 1993) may be due to the ability of IL-15 to compensate for some IL-2 functions. It is also interesting to note in this regard that by Northern blot analysis a wider and distinct distribution of IL-15 gene expression was seen relative to IL-2. For example, unlike IL-2, high levels of IL-15 mRNA were found in muscle and placenta, and a kidney epithelial line, but T cells, the major source for IL-2, did not express IL-15 mRNA (Grabstein *et al.*, 1994).

As mentioned earlier, IL-15 shares structural characteristics of ligands for the hematopoietin superfamily of cytokine receptors. A common feature of this family is the sharing of receptor subunits, as for example the shared utilization of gp130 by IL-6, LIF, IL-11, Oncostatin M and ciliary neurotrophic factor (CNTF) receptors, and sharing of β subunits between human IL-3, IL-5 and granulocyte macrophage colony-stimulating factor receptors. Our discovery that IL-15 utilizes the β and γ subunits of IL-2R, which are the essential signal transducing components of the IL-2R system, provides a new example of receptor promiscuity and increases the number of cytokines able to use the common γ chain: IL-2, IL-4, IL-7 and IL-15.

Using the IL-2R paradigm, it is conceivable that IL-15 can also bind to receptors consisting of various combinations of subunits. As described earlier, IL-2 can bind to heteromeric receptors consisting of $\alpha\beta$ or $\beta\gamma$ subunits in

addition to the fully functional high affinity ($\alpha\beta\gamma$) class. Since in COS cells IL-2Rγ was needed for binding, we were surprised to find that IL-15 can bind with high affinity (K_d of 18−25 pM) to an EBV-B line established from a patient with XSCID, lacking functional IL-2R γ chains but expressing IL-2Rβ on their surface (S.Kumaki and J.Giri, unpublished). By analogy with 'pseudo' high affinity IL-2R comprised of $\alpha\beta$ chains, we suggest that an IL-15-specific subunit present on these cells can also associate with the IL-2R β subunit, forming high affinity receptors, which like IL-2R$\alpha\beta$ are not sufficient for signaling. This hypothesis is based on the observation that the β subunit of IL-2R alone, expressed in transfected cells, cannot bind IL-15 with high affinity or transduce IL-15 signals. Indirect evidence for an additional binding site for IL-15, not shared with IL-2, is also derived from the observation that IL-2 only partially inhibits IL-15 binding to YT cells, even at a concentration of 10^{-7} M (J.Giri and M.Ahdieh, unpublished observation).

We have found that some murine cells, such as the TN cells (described in Figure 7), cannot bind or respond to IL-15, although these cells apparently express fully functional IL-2Rs, as demonstrated by proliferation in response to IL-2 and by the presence of all three subunits forming the high affinity IL-2Rs. This suggests that in murine cells an additional, specific component may be required for IL-15 binding. The apparent contradiction between IL-15 binding to recombinant human β and γ chains (Figure 6) and the lack of IL-15 binding to TN cells could be explained if the murine $\beta\gamma$ chains, in the absence of other subunits, are not sufficient for detectable binding of simian or human IL-15 used in these experiments. A similar situation has been described for human IL-2 binding to mouse $\beta\gamma$ (Kumaki *et al.*, 1993).The murine $\beta\gamma$ subunits expressed in L cells were not able to bind human IL-2, while human $\beta\gamma$ chains were sufficient for binding. We have not as yet identified any human cells which bind IL-2 but not IL-15.

Several experiments suggest the existence of an additional, as yet unidentified, IL-15-specific receptor component. The precise molecular nature of the additional IL-15R component and its role in IL-15 binding and signaling will be the subject of future investigation. We have observed however, that L929 cells expressing human $\alpha\beta$, $\beta\gamma$ or $\alpha\beta\gamma$ chains of the IL-2R, bind simian IL-15 with very similar affinities, but only the complexes containing the γ chain can signal. This suggests that L cells express an additional IL-15R subunit which contributes to high affinity binding but not to signal transduction. The results we have obtained to date suggest that IL-15 depends for signal transduction on the β and γ subunits, the essential signaling elements of the IL-2R, but that on some cells, an additional protein may modulate binding.

Materials and methods

Cell lines and culture conditions

The continuous murine T cell line CTLL.2 was maintained as described (Gillis and Smith, 1977). The YTN17 line, a subclone of YT cells (Yodoi *et al.*, 1985), was obtained from Dr M.Caligiuri (Roswell Park Memorial Cancer Center, Buffalo, NY); the murine pro-B cell line BAF/B03 (Hatakeyama *et al.*, 1989a) was kindly provided by T.Taniguchi. The monkey kidney epithelial lines COS-7 and CV1/EBNA were maintained and transfected as described previously (Cosman *et al.*, 1984; McMahan *et al.*, 1991). Triple negative (TN) mouse cells were derived from 13 day fetal liver cultured in the presence of high levels of IL-7. The resultant cells are Thy1.2$^+$, CD25$^+$ (i.e. IL-2Rα^+) and CD44$^+$, and negative for CD3, CD4 and CD8 expression. This cell-surface phenotype is indicative of an immature T cell progenitor. PBMC obtained from heparinized blood of healthy donors were isolated by Ficoll-Hypaque density gradient centrifugation and cultured for 24−48 h with 1% PHA (Gibco).

Cytokines and receptor binding

Recombinant human IL-15 expressed in yeast (Grabstein *et al.*, 1994) was purified by passage over a Phenyl Sepharose CL-4B column (Pharmacia, Piscataway, NJ) followed by two passages over reversed phase HPLC C4 columns (Vydac), the first using a pyridine acetate/propanol buffer system, the second in a trifluoro-acetic acid/acetonitrile system. Fractions containing pure IL-15 were dried under nitrogen and radiolabeled using the Enzymobead iodination reagent (Bio-Rad, Richmond VA) as described by Park *et al.* (1990). The biological activity of radiolabeled IL-15 was assessed using the mitochondrial stain MTT [(3−4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or thiazol blue (Sigma, St Louis, MO) (Roehm *et al.*, 1991)] in an IL-15 dependent CTLL proliferation assay. Recombinant human IL-2 was purchased from R&D Systems (Minneapolis, MN), and radiolabeled human IL-2 was prepared as above or purchased (New England Nuclear). Binding experiments using the phthalate oil separation method and data analysis were performed as previously described (Dower *et al.*, 1984). Unless otherwise indicated, binding experiments were routinely performed at 4°C for 60 min. For affinity cross-linking of [^{125}I]IL-15 or IL-2 bound to cell surface receptor we used the irreversible bifunctional cross-linker BS^3. The cells were then detergent solubilized in the presence of protease inhibitors and analyzed by SDS−PAGE according to established procedures (Sharon *et al.*, 1986).

Antibodies

The anti-human IL-2Rα antibody 2A3 has been previously described (Dower *et al.*, 1985).The anti-human IL-2Rβ Mikβ1 (Tsudo *et al.*, 1989) was obtained from Nichirei Corp. (Japan), and anti-mouse IL-2R reagents for cytofluorometry were obtained from Pharmingen (San Diego, CA). The non-neutralizing anti-human IL-2Rβ mAb TU11 (Suzuki *et al.*, 1989) was kindly provided by Dr K.Sugamura (Tohoku University).

Establishment of IL-2R-expressing cell lines

The coding regions for the β (Hatakeyama *et al.*, 1989b) and γ (Takeshita *et al.*, 1992a) chains of human IL-2R were cloned by RT-PCR from human PBL mRNA, and subcloned into mammalian expression vectors pDC302 (Mosley *et al.*, 1989) and pDC409. Plasmid pDC409 is a derivative of pDC406 (McMahan *et al.*, 1991). BAF/β cells were obtained by electroporation of BAF/B03 with pDC302:IL-2Rβ plasmid DNA followed by selective growth in either IL-2 or IL-15. COS cells were transfected with pDC302:IL-2Rβ and pDC409:IL-2Rγ using DEAE-Dextran, as described previously (Cosman *et al.*, 1984). L929 murine fibroblasts stably transfected with IL-2R $\alpha\beta$ or IL-2R $\alpha\beta\gamma$ and designated L$\alpha\beta$-2 and L$\alpha\beta\gamma$-4, have been described previously (Asao *et al.*, 1993) and were obtained from the laboratory of Dr K.Sugamura.

Analysis of proto-oncogene induction

BAF/β cells were washed and placed in RPMI 1640/10% FCS with no added growth factors for 16 h prior to induction with 30 ng/ml of IL-2 or IL-15. A portion of each culture was removed and pelleted at established time intervals and lysed in guanidinium thiocyanate for the preparation of total cellular RNA. Northern blots were prepared as previously described (Larsen *et al.*, 1990), and probed with antisense RNA probes for c-*myc*, c-*jun* and c-*fos* proto-oncogene expression. Up-regulation of c-*myc* and c-*fos* expression in L$\alpha\beta$-2 and L$\alpha\beta\gamma$-4 cells was determined in the same manner.

Co-precipitation of IL-2Rβ and γ extracellular domains with IL-15

Mammalian expression plasmids were constructed to encode secreted forms of the extracellular binding domains of human IL-2Rβ and γ. The extracellular coding domain of huIL-2Rβ was truncated 9 bp upstream of the predicted transmembrane coding domain and subcloned into pDC409, resulting in a C-terminal fusion of nine additional amino acids (GRDLQICLN) provided by vector sequence at the cloning site. The entire extracellular coding domain of human IL-2Rγ was similarly subcloned into pDC409, generating the same C-terminal fusion in the encoded protein. Supernatants of CV-1/EBNA cells transfected with soluble receptor expressing plasmids were radiolabeled after 3 days with [^{35}S]cysteine and methionine at 100 μCi/ml for 5 h. Soluble receptors were incubated for 2 h with 10 nM IL-15, followed by immunoprecipitation with anti-IL-2Rβ mAb TU11 and protein G. Precipitated complexes were washed with PBS/0.05% Tween, and analyzed by SDS−PAGE.

Acknowledgements

The authors thank Bruce Hess for help with bioassays, Ray Paxton and Courtney Beers for help with purification of IL-15, Steve Dower for advice with receptor binding studies, and Steve Gillis and Carl March for critical review of the manuscript. We are also grateful to Dr K.Sugamura for cell lines and IL-2R antibodies.

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Received on March 1, 1994; revised on April 6, 1994