

Soluble Interleukin-15 Receptor α (IL-15R α)-sushi as a Selective and Potent Agonist of IL-15 Action through IL-15R β/γ

HYPERAGONIST IL-15·IL-15R α FUSION PROTEINS*

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Interleukin-15 (IL-15) is crucial for the generation of multiple lymphocyte subsets (natural killer (NK), NK-T cells, and memory CD8 T cells), and transpresentation of IL-15 by monocytes and dendritic cells has been suggested to be the dominant activating process of these lymphocytes. We have previously shown that a natural soluble form of IL-15R α chain corresponding to the entire extracellular domain of IL-15R α behaves as a high affinity IL-15 antagonist. In sharp contrast with this finding, we demonstrate in this report that a recombinant, soluble sushi domain of IL-15R α , which bears most of the binding affinity for IL-15, behaves as a potent IL-15 agonist by enhancing its binding and biological effects (proliferation and protection from apoptosis) through the IL-15R β/γ heterodimer, whereas it does not affect IL-15 binding and function of the tripartite IL-15R $\alpha/\beta/\gamma$ membrane receptor. Our results suggest that, if naturally produced, such soluble sushi domains might be involved in the IL-15 transpresentation mechanism. Fusion proteins (RLI and ILR), in which IL-15 and IL-15R α -sushi are attached by a flexible linker, are even more potent than the combination of IL-15 plus sIL-15R α -sushi. After binding to IL-15R β/γ , RLI is internalized and induces a biological response very similar to the IL-15 high affinity response. Such hyper-IL-15 fusion proteins appear to constitute potent adjuvants for the expansion of lymphocyte subsets.

IL-15³ is a cytokine that was originally described, like IL-2, as a T cell growth factor (1). Both cytokines belong to the four α -helix bundle family, and their membrane receptors share two subunits (the IL-2R/IL-15R β and γ chains) responsible for signal transduction (2). The IL-2R β/γ complex is an intermediate affinity receptor for both cytokines that is expressed by most NK cells and can be activated *in vitro* by

nanomolar concentrations of IL-2 or IL-15. The high affinity IL-2 and IL-15 receptors, such as those expressed on activated T cells, can be activated with picomolar concentrations of either cytokine, and additionally contain their own private α chain (IL-2R α and IL-15R α) that confers cytokine specificity and enhances the affinity of cytokine binding (3).

Both cytokines play pivotal roles in innate and adaptive immunity. Whereas initial *in vitro* experiments have shown a large functional overlap in the effects of the two cytokines (induction of the proliferation and cytotoxicity of activated lymphocytes and NK cells, co-stimulation of B cell proliferation and immunoglobulin synthesis, and chemoattraction of T cells) (1, 4–6), more recent experiments have indicated that they can exert complementary or even contrasting actions *in vivo*. Whereas IL-2 or IL-2R α knock-out in mice was associated with autoimmune phenotypes with increased populations of activated T and B cells, IL-15 and IL-15R α knock-out resulted in specific defects in NK, NK-T, intraepithelial lymphocytes, and memory CD8 T cells (7, 8). Furthermore, IL-2 promotes peripheral tolerance by inducing activation-induced cell death, whereas IL-15 inhibits IL-2-mediated activation-induced cell death (9), and, unlike IL-2, IL-15 is a survival factor for CD8 memory T cells (10). In line with these observations, it has been suggested that the major role of IL-2 is to limit continuous expansion of activated T cells, whereas IL-15 is critical for initiation of T cell division and survival of memory T cells (11). A novel mechanism of IL-15 action described recently is that of transpresentation in which IL-15 and IL-15R α are coordinately expressed by antigen-presenting cells (monocytes and dendritic cells), and IL-15 bound to IL-15R α is presented in *trans* to neighboring NK or CD8 T cells expressing only the IL-15R β/γ receptor (12). As a co-stimulatory event occurring at the immunological synapse, IL-15 transpresentation now appears to be a dominant mechanism for IL-15 action *in vivo* (13, 14) and appears to play a major role in tumor immunosurveillance (15).

The IL-15R α and IL-2R α subunits form a sub-family of cytokine receptors in that they comprise extracellular parts, so called “sushi” structural domains (one in IL-15R α and two in IL-2R α), at their N terminus, which are also found in complement or adhesion molecules (16). In both cases, these sushi domains have been shown to bear most of the structural elements responsible for cytokine binding. Whereas IL-2R α alone is a low affinity receptor for IL-2 ($K_d = 10$ nM), IL-15R α binds IL-15 with high affinity ($K_d = 100$ pM). Shedding of IL-2R α by proteolysis is a natural mechanism that participates in the down-regulation of lymphocyte activation. IL-2R α is cleaved by Der p1, a major mite allergen, thereby inhibiting Th1 cells and favoring an allergic envi-

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³ The abbreviations used are: IL-15, interleukin-15; IL-15R α , IL-15R α -chain; sIL-15R α -sushi, soluble IL-15R α sushi domain; ILR RLI, IL-15-sIL-15R α -sushi fusion protein; SPR, surface plasmon resonance; rIL, recombinant human IL-15; mIL, mouse interleukin; NK, natural killer cells.

ronment (17). IL-2R α cleavage by tumor-derived metalloproteinases also results in the suppression of the proliferation of cancer-encountered T cells (18). The soluble IL-2R α thus generated is a competitive inhibitor of IL-2 action *in vitro*. However, it remains a low affinity IL-2 binder and is not likely to efficiently participate in down-regulation of IL-2 activity *in vivo*.

We have recently shown that a soluble form of the human IL-15R α can also be naturally released from IL-15R α -positive cells by a shedding process involving matrix metalloproteinases (19). In contrast to soluble IL-2R α , this soluble IL-15R α receptor was able to bind IL-15 with high affinity and efficiently blocked proliferation driven through the high affinity IL-15R α / β / γ signaling complex. This result was consistent with the concept of sIL-15R α behaving, like its homolog sIL-2R α , as an antagonist, and with the inhibitory effects of mouse sIL-15R α *in vitro* or *in vivo* (20, 21). Here, we show that a soluble receptor consisting of the N-terminal structural domain of IL-15R α (sushi domain) has the opposite action; it is able to enhance the binding as well as the bioactivity of IL-15 through the IL-15R β / γ intermediate affinity receptor, without affecting binding and bioactivity through the high affinity receptor. In addition, we describe fusion proteins consisting of human IL-15 and human IL-15R α fused by flexible linkers that behave as potent superagonists of the IL-15R β / γ complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokines—Recombinant human IL-15 (rIL-15) was purchased from Peprotech Inc. (Rocky Hill, NJ). The Mo-7e myeloid leukemia (22) and TF-1 erythroleukemia human cell lines (ATCC CRL-2003) (kindly provided by Dr. Bruno Azzarone, Institut Gustave Roussy, Villejuif, France) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 1 ng/ml granulocyte macrophage-colony stimulating factor (R&D Systems, Abington, UK). TF1- β cells (23) were cultured in the same medium supplemented with 250 μ g/ml Geneticin. The Kit 225 human T lymphoma cell line (24) (obtained from Dr. Doreen Cantrell, University of Dundee, UK) was cultured in RPMI 1640 medium containing 6% fetal calf serum, 2 mM glutamine, and 10 ng/ml rIL-2 (Chiron, Emeryville, CA). 32D β cells (25) (kindly provided by Dr. Warren Leonard, Bethesda, MD) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 0.4 ng/ml mIL-3 (R&D Systems), 10 μ M 2-mercaptoethanol, 250 μ g/ml Geneticin.

sIL-15R α -IL-2, sIL-15R α -sushi-IL-2, and sIL-15R α -sushi—sIL-15R α -IL-2 was expressed in Chinese hamster ovary cells and prepared as described (26). A similar construction was made in which the sushi domain of IL-15R α (amino acids 1–66 of mature coding sequence) was linked to a molecule of human IL-2 (sIL-15R α -sushi-IL-2).

The sushi domain of IL-15R α was amplified by PCR. PCR products were purified, digested with BamHI and HindIII (Fermentas, Vilnius, Lithuania) and ligated into a pQE30 expression vector. Expression was performed in *Escherichia coli* SG13009 cells under isopropyl 1-thio- β -D-galactopyranoside induction. After cell lysis, inclusions bodies were washed, solubilized in 6 mM guanidine HCl, 20 mM sodium phosphate, pH 7.4, 20 mM imidazole, 150 mM sodium chloride, and 1 mM dithiothreitol. The IL-15R α -sushi was trapped in an nickel-nitrilotriacetic acid-agarose column (Qiagen) equilibrated with the solubilization buffer plus 1 mM reduced glutathione and 0.2 mM oxidized glutathione. It was then refolded via a gradient from 6 to 0 M guanidine HCl in the column buffer (27) and eluted with 250 mM imidazole.

RLI and ILR Fusion Proteins—The constructions of the fusion proteins are shown in Fig. 2E. The human IL-15R α -sushi domain (amino acids 1–77) and human IL-15 were separated by linker 20 (SGGS-

GGGSGGGSGGGSLQ) for RLI or by linker 26 (SGGSGGGSGGGSGGGSGGGSLQ) for ILR. A sequence coding for the FLAG epitope and Factor Xa binding site (DYKDDDDKIEGR) was added between the signal peptide and the coding sequences. The endogenous signal peptide of human IL-15R α was used for RLI and the signal peptide of bovine preprolactin for ILR. These constructions were inserted between the BamHI and the Hind III sites of a pFastBac 1 (Invitrogen) expression vector thus generating two expression vectors, which were recombined in baculovirus DNA using the Bac to Bac expression system (Invitrogen). The recombinant baculoviruses were used to infect SF9 cells, and fusion proteins were expressed in the SF 900 II medium and harvested 4 days post infection. The concentrations of the fusion proteins were measured by enzyme-linked immunosorbent assay with the anti-IL-15 monoclonal antibody 247 (R & D Systems) as the capture antibody and the anti-FLAG M2-peroxydase conjugate (Sigma) as the revealing antibody.

Surface Plasmon Resonance Studies—The SPR experiments were performed with a BIAcore 2000 biosensor (BIAcore, Uppsala, Sweden). rIL-15 was covalently linked to CM5 sensor chips, and the binding of increasing concentrations of sIL-15R α -IL-2, sIL-15R α -sushi-IL-2, or sIL-15R α -sushi was monitored. Analysis of sensorgrams was performed using BIAlogue kinetics evaluation software.

Proliferation Assays—The proliferative responses of Mo-7e, TF-1 β , and Kit 225 cells to rIL-15, rIL-2, RLI, or ILR were measured by [^{3} H]thymidine incorporation as described (19) after 4 h in cytokine-deprived medium, 48 h culture, and 16 h with [^{3} H]thymidine.

Apoptosis—The annexin V assay was performed using a FACScan flow-cytometer and the Annexin V-FITC Apoptosis detection kit (BD Biosciences). After cytokine starvation, cells were seeded in multiwell plates at 5×10^5 cells/well in 1 ml and cultured in medium supplemented with the various reactants (rIL-15, sIL-15R α -sushi, and RLI fusion protein). Data were acquired and analyzed using CellQuest software.

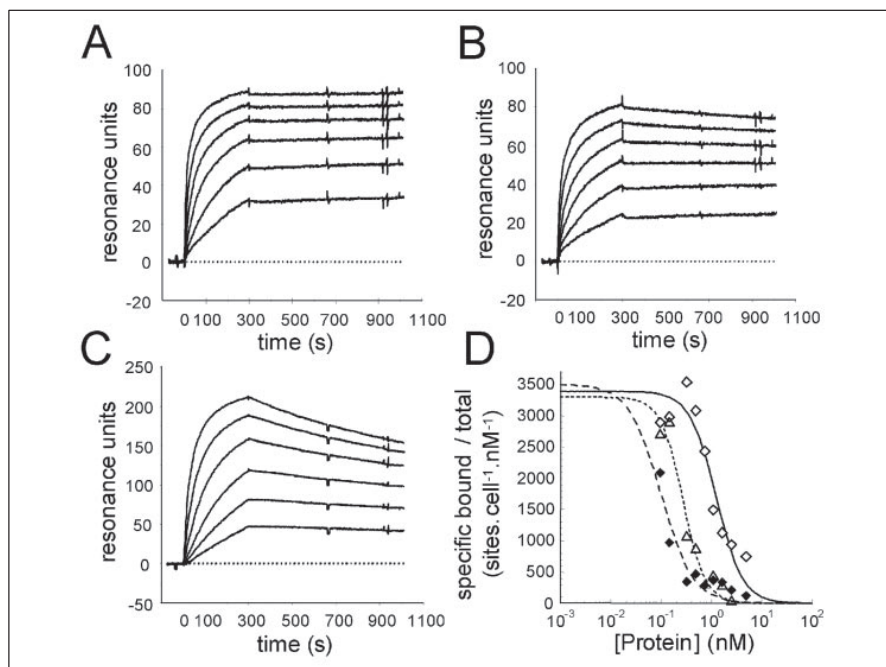
Binding Assays and Internalization—Labeling with [^{125} I]iodine of human rIL-15, sIL-15R α -sushi, and RLI fusion protein, as well as subsequent binding experiments, were performed as described previously (19). For internalization, cells were equilibrated at 4 °C with labeled sIL-15R α -sushi or RLI, and the temperature was switched to 37 °C. At different time intervals, two samples were washed and centrifuged. One of the cell pellets was treated with glycine-HCl buffer (0.2 M, pH 2.5), whereas the other was treated with phosphate-buffered saline (pH 7.4) at 4 °C for 5 min. After centrifugation, total ligand binding was determined from the pellet of the cells treated with PBS, whereas the membrane-bound and internalized fractions were determined, respectively, from the supernatant and pellet of cells treated with acid pH.

RESULTS

IL-15R α Binding to IL-15 Is Mainly Due to the Sushi Domain—In a previous study (28), we have shown that removal of the sushi domain encoded by exon 2 of IL-15R α resulted in a complete abrogation of IL-15 binding to membrane anchored IL-15R α , suggesting that the sushi domain was indispensable for binding. To directly measure the contribution of the sushi domain to IL-15 binding, soluble forms of IL-15R α containing the entire extracellular domain or only the N-terminal sushi domain were prepared and assayed for IL-15 binding in a competition assay and using SPR technology.

As shown in Fig. 1A, a sIL-15R α -IL-2 fusion protein produced in Chinese hamster ovary cells and consisting of the entire IL-15R α extracellular domain linked to a molecule of human IL-2 (used as a tag for purification) bound IL-15 with high affinity ($k_{on} = 3.7 \times 10^5$ M $^{-1}$ s $^{-1}$;

FIGURE 1. Binding affinities of the various soluble IL-15R α proteins for IL-15. SPR sensorgrams of the binding (association and dissociation phases) of increasing concentrations of rIL-15 (3.1, 6.2, 12.5, 25, 50, and 100 nM) to immobilized sIL-15R α -IL-2 (A), sIL-15R α -sushi-IL-2 (B), or IL-15R α -sushi (C). D, competition studies of sIL-15R α -sushi ($\diamond$), sIL-15R α -IL-2 ($\blacklozenge$), or sIL-15R α -sushi-IL-2 ($\triangle$) with radioiodinated rIL-15 (200 pM) binding to TF-1 cells.



$k_{\text{off}} = 1.4 \times 10^{-5} \text{ s}^{-1}$; $K_d = 38 \text{ pM}$). A similar construction linking the sushi domain of IL-15R α to human IL-2 also bound IL-15 (Fig. 1B) but with a 10-fold lower affinity, mainly due to a more rapid off rate ($k_{\text{on}} = 3.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{\text{off}} = 1.3 \times 10^{-4} \text{ s}^{-1}$; $K_d = 420 \text{ pM}$). A soluble sushi domain was also produced in *E. coli*. This sIL-15R α -sushi also bound IL-15 with a lower affinity ($k_{\text{on}} = 2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; $k_{\text{off}} = 3.8 \times 10^{-4} \text{ s}^{-1}$; $K_d = 1.5 \text{ nM}$) (Fig. 1C). These results indicate that the sushi domain is responsible for most of the binding affinity of IL-15 but that it does not fully reconstitute the high affinity binding displayed by the full-length extracellular domain.

Soluble IL-15R α Proteins Inhibit IL-15 Binding to Membrane-anchored IL-15R α —The three soluble forms of IL-15R α were tested for their ability to compete with radioiodinated IL-15 for binding to IL-15R α expressed by the human cell line TF-1 that also expresses the IL-15R γ chain but not the IL-15R β chain (Fig. 1D). The three proteins completely inhibited IL-15 binding to TF-1 cells with IC_{50} values that were similar to the K_d values measured by the SPR technology: 100 pM (sIL-15R α -IL-2), 270 pM (sIL-15R α -sushi-IL-2), and 1.3 nM (sIL-15R α -sushi).

sIL-15R α -sushi Increases IL-15-driven Cell Proliferation through the IL-15R β / γ Complex—Because the soluble sushi domain was easily produced in *E. coli* in high yields, it was selected for all further studies. It was first tested on cell lines that only express the IL-15R β / γ complex (the human Mo-7e cell line and the mouse 32D β cell line that expresses endogenous mouse IL-15R γ chain and transfected human IL-15R β chain). As expected, the Mo-7e cell line proliferated in response to nanomolar concentrations of rIL-15 or rIL-2 (Fig. 2, A and B). Unexpectedly, addition of a fixed concentration of sIL-15R α -sushi (10 nM) to the assay increased the proliferative response to lower concentrations of rIL-15 by ~ 4 -fold. By itself, sIL-15R α -sushi did not induce any proliferative response (data not shown). Similar results were obtained for 32D β with a shift of ~ 10 -fold (data not shown). The specificity was assessed by the fact that sIL-15R α -sushi did not affect the rIL-2-driven proliferation of Mo-7e cells (Fig. 2B). Fig. 2C shows that sIL-15R α -sushi dose dependently (with an IC_{50} of 3.5 nM, similar to its K_d for IL-15)

potentiated the effect of a fixed concentration of rIL-15 (1 nM) that alone induces only a low level of proliferation.

RLI and ILR Fusion Proteins Are Potent Inducers of Cell Proliferation through the IL-15R β / γ Complex—To evaluate whether the synergistic effect of sushi on IL-15 bioactivity could be transferred by a single molecule, molecular constructs encoding fusion proteins linking IL-15 and the sushi domain were elaborated. For the two constructions, a flexible linker was introduced between the C terminus of IL-15 and the N terminus of the sushi domain (ILR) or *vice versa* (RLI) (Fig. 2E). Molecular models illustrating the structures of these proteins are shown in Fig. 2F. These two fusion proteins were tested for their effect on the proliferation of Mo-7e cells. As shown in Fig. 2D, both proteins induced dose-dependent induction of Mo-7e cell proliferation, with EC_{50} s that were similar ($\sim 25 \text{ pM}$) and far lower than those of rIL-15 alone (3 nM), or of an equimolar mixture of rIL-15 plus sIL-15R α -sushi (0.9 nM). These results further confirm the synergistic effect of sIL-15R α -sushi on the actions of IL-15 and indicate that stabilizing the IL-15-sIL-15R α -sushi complex with a covalent linker markedly enhances this synergistic action.

sIL-15R α -sushi Increases IL-15-induced Prevention of Apoptosis, and RLI Efficiently Prevents Cellular Apoptosis—Following cytokine withdrawal, the fraction of apoptotic Mo-7e cells increased from 10 to 80% in 48 h (Fig. 3, A (panel a) and A (panel b)). When added at time zero, rIL-15 (5 nM) reduced this apoptosis to 70% (Fig. 3A, panel c). Alone, sIL-15R α -sushi (10 nM) had no effect (Fig. 3A, panel b). However, it markedly potentiated the anti-apoptotic effect of rIL-15 (35% apoptosis at 48 h, Fig. 3A, panel c). The synergistic effect of sIL-15R α -sushi on IL-15-mediated prevention of apoptosis was confirmed by a kinetic analysis (Fig. 3B) as well as by dose-response curves (Fig. 3C). rIL-15 acted with an IC_{50} of $\sim 1.5 \text{ nM}$, a value in agreement with the saturation of IL-15/ γ receptors. This IC_{50} was ~ 10 -fold lower (170 pM) in the presence of 10 nM sIL-15R α -sushi. The RLI fusion protein markedly prevented apoptosis (Fig. 3B). On a molar basis, it was even more active than the IL-15-sIL-15R α -sushi association, with an IC_{50} of $\sim 40 \text{ pM}$ (Fig. 3C).

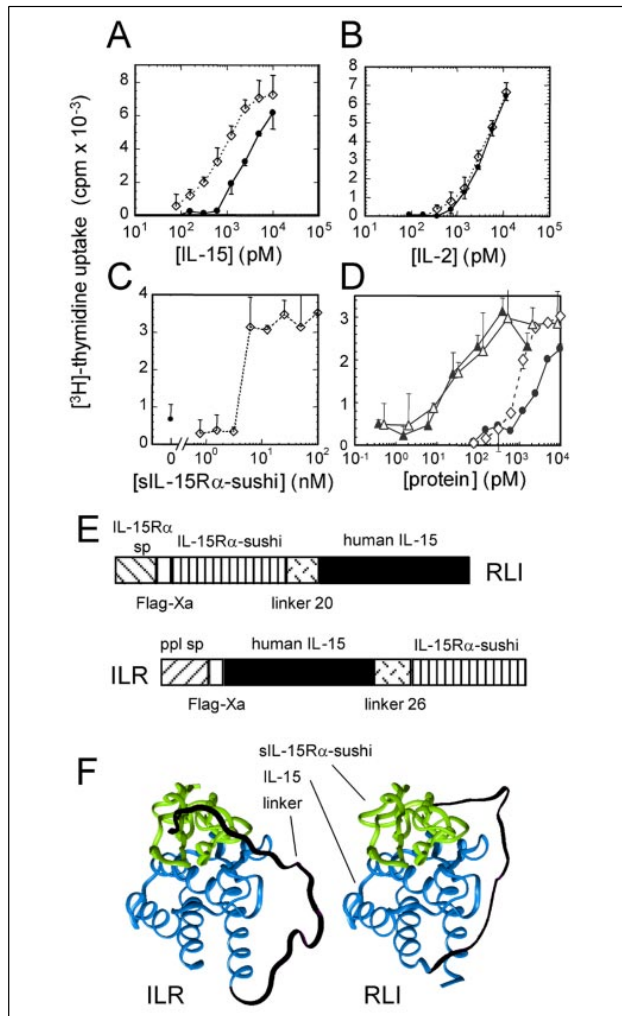


FIGURE 2. Effects on IL-15-induced proliferation through IL-15R β/γ . The proliferation of Mo-7e cells was evaluated by the incorporation of [^{3} H]thymidine. Cells were cultured with increasing concentrations of human rIL-15 (A) or human rIL-2 (B), in the absence (●) or presence (◇) of a fixed concentration (10 nM) of sIL-15R α -sushi. C, cells were cultured in the presence of 1 nM rIL-15, without (●) or with increasing concentrations of sIL-15R α -sushi (◇). D, cells were cultured with increasing concentrations of rIL-15 (●), an equimolar mixture of IL-15 and sIL-15R α -sushi (◇), RLI (▲), or ILR fusion protein (△). E, molecular constructs used to express RLI and ILR fusion proteins. IL-15R α sp, human IL-15R α signal peptide; ppl sp, bovine prolactin signal peptide. F, three-dimensional model structures of the fusion proteins.

sIL-15R α -sushi Increases IL-15 Binding to Mo-7e Cells, and the RLI Fusion Protein Binds to and Is Internalized by Mo-7e Cells—As expected, Mo-7e cells bound IL-15 with intermediate affinity ($K_d = 13.5$ nM), with a maximal binding capacity of 800 sites/cell (Fig. 4A). The addition of sIL-15R α -sushi (10 nM) increased the affinity of IL-15 binding ($K_d = 7$ nM) without significantly affecting the maximal binding capacity (1180 sites/cell). When using a radioiodinated RLI fusion protein (Fig. 4B), we detected binding to a similar number of receptor sites (730 sites/cell), and the affinity of binding ($K_d = 780$ pM) was markedly higher than that of IL-15. Fig. 4C shows that RLI can be efficiently and rapidly internalized. The fraction of cell-bound radioactivity decreased within ~20 min and was accompanied by a concomitant increase in intracellular radioactivity.

sIL-15R α -sushi Does Not Affect IL-15-driven Cell Proliferation nor Inhibition of Apoptosis through the High Affinity IL-15R $\alpha/\beta/\gamma$ Complex—The human lymphoma cell line Kit 225 expresses endogenous IL-15R α ,

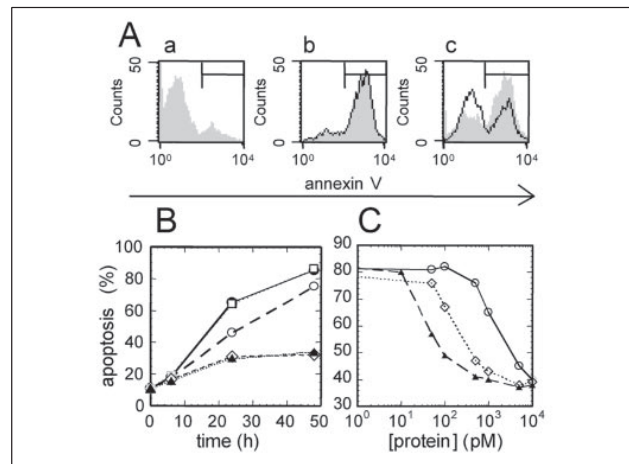


FIGURE 3. Effects on IL-15-induced prevention of apoptosis through IL-15R β/γ . Apoptosis was evaluated by Annexin V cell-surface expression using flow cytometry. In A: full histogram (a) represents annexin V staining on Mo-7e at the beginning of the experiment. Cells were cultured for 48 h, without (b) or with a fixed concentration of human rIL-15 (500 pM) (c), in the absence (full histogram) or presence of sIL-15R α -sushi (10 nM) (unbroken line). B, kinetics of Annexin V expression on Mo-7e cells in the absence of exogenous cytokine (●) or in the presence of rIL-15 (500 pM) (○), sIL-15R α -sushi (10 nM) (□), sIL-15R α -sushi (10 nM) plus rIL-15 (500 pM) (◇), or RLI (500 pM) (▲). C, Mo-7e cells were cultured with increasing concentrations of rIL-15, in the absence (○) or presence (◇) of a fixed concentration (10 nM) of sIL-15R α -sushi, or with increasing concentrations of RLI (▲).

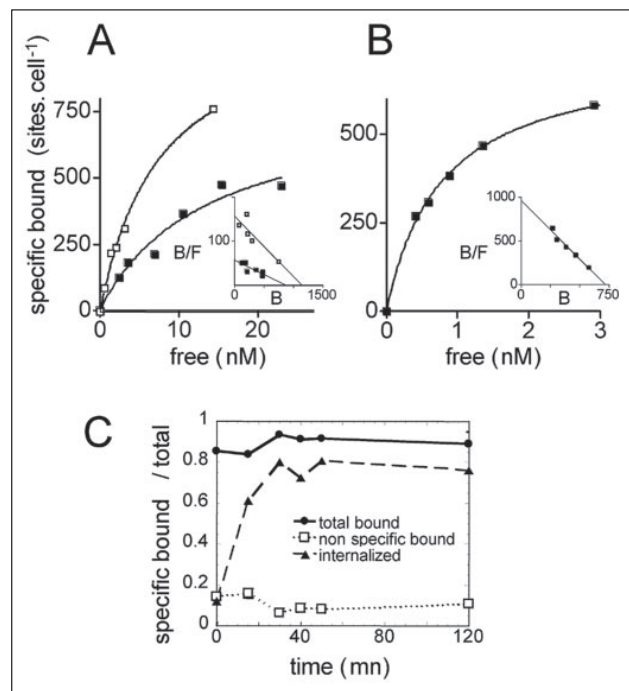


FIGURE 4. Agonistic effect of sIL-15R α -sushi on IL-15 binding to IL-15R β/γ : binding and internalization of RLI. A, binding of [^{125} I]-labeled rIL-15 to Mo7 cells in the absence (■) or presence of 10 nM sIL-15R α -sushi (□). B, binding of [^{125} I]-labeled RLI fusion protein. Scatchard plots are shown in insets. C, internalization of [^{125} I]-labeled RLI fusion protein (500 pM).

β , and γ chains, and the human TF-1 β cell line expresses endogenous IL-15R α and γ chains plus the transfected human IL-15R β chain. Consequently, these cell lines proliferate in response to low, picomolar concentrations of IL-15, as shown in Fig. 5 (A and B) ($EC_{50} = 19$ pM and 21 pM, respectively). In contrast to what was found using Mo-7e or 32D β

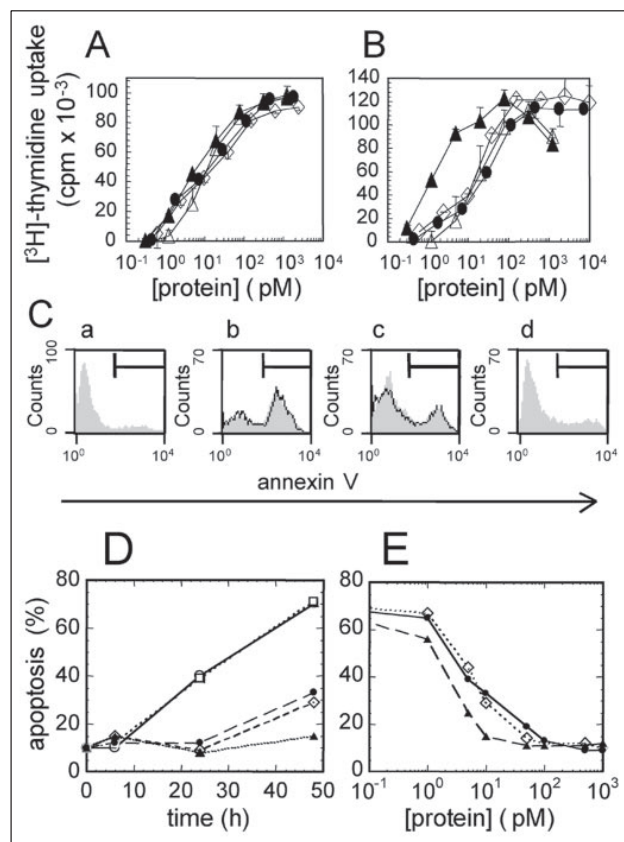


FIGURE 5. Effects on IL-15-induced cell proliferation and apoptosis through IL-15R α / β / γ receptors. [3H]Thymidine incorporation by Kit 225 (A) and TF-1 β (B) cells cultured with increasing concentrations of rIL-15 (●), an equimolar mixture of rIL-15 and sIL-15R α -sushi (◇), RLI (▲), or ILR fusion protein (△). In C: Annexin V staining of TF-1 β at the beginning of the experiment (a), at 48 h after culture without (b) or with a fixed concentration of human rIL-15 (500 pM) (c), in the absence (full histogram) or presence of sIL-15R α -sushi protein (10 nM) (unbroken line), or in the presence of 10 pM of RLI (d). D, kinetics of staining in the absence of exogenous cytokine (○) or in the presence of rIL-15 (10 pM) (●), sIL-15R α -sushi (10 nM) (□), sIL-15R α -sushi (10 nM) plus rIL-15 (10 pM) (◇), or RLI fusion protein (10 pM) (▲). E, staining at 48 h after culture with increasing concentrations of rIL-15, in the absence (●) or presence (◇) of a fixed concentration (10 nM) of sIL-15R α -sushi, or with increasing concentrations of RLI fusion protein (▲).

cells, addition of equimolar concentrations of sIL-15R α -sushi to IL-15 did not significantly affected the IL-15 dose-response curve on either cell type. The ILR fusion protein was as active as rIL-15 on the two cell lines. The RLI was also as active as rIL-15 on Kit 225 cells but was ~16-fold more efficient ($EC_{50} = 1.2$ pM) than rIL-15 on TF-1 β cells.

The effects of sIL-15R α -sushi and RLI were further analyzed on TF-1 β cell apoptosis induced by cytokine deprivation. Histograms showing these effects are given in Fig. 5C, whereas the kinetics and dose responses of these effects are shown in Fig. 5, D and E, respectively. rIL-15 dose and time dependently inhibited TF-1 β apoptosis. sIL-15R α -sushi alone had no effect and did not change the effect of IL-15 $IC_{50} = 6.5$ pM for rIL-15 or sIL-15R α -sushi plus rIL-15. The ILR fusion protein was as active as rIL-15, whereas RLI was about three times more active ($IC_{50} = 2.5$ pM).

IL-15, sIL-15R α -sushi, and RLI Binding to TF-1 β Cells—Insofar as IL-15R α -sushi did not affect IL-15 proliferation of TF-1 β , we examined its effect on IL-15 binding over a wide concentration range (Fig. 6A). Scatchard analysis of the saturation binding curve indicated the presence of two classes of IL-15 binding sites, compatible with the presence of a small number of high affinity binding sites (IL-15R α / β / γ com-

plexes, $K_d = 22$ pM, $B_{max} = 100$ sites/cell) plus higher amounts of intermediate affinity binding sites (IL-15R β / γ complexes, $K_d = 30$ nM, 2800 sites/cell). sIL-15R α -sushi induced an increase in IL-15 binding that, according to Scatchard analysis, was mainly due to an increase in the affinity of IL-15 binding for the intermediate-affinity component ($K_d = 3.5$ nM). To more specifically test the effect of sIL-15R α on the high affinity component, its effect was analyzed on the binding of a low concentration of radiolabeled IL-15 (200 pM) that mainly targets the high affinity receptor. As shown in Fig. 6B, sIL-15R α -sushi, at concentrations of up to 25 nM, did not affect this binding.

The binding of radiolabeled sIL-15R α -sushi to TF-1 β cells (Fig. 6C) revealed a specific binding component that was strictly dependent on the presence of rIL-15. In the presence of 1 nM rIL-15, the K_d reflecting sIL-15R α -sushi binding was 3.5 nM, a value compatible with its affinity for IL-15, with a maximal binding capacity (3300 sites/cell) compatible with the number of IL-15 intermediate binding sites. As further shown in Fig. 6D, the radiolabeled sIL-15R α -sushi was efficiently internalized. Radiolabeled RLI fusion protein also bound to TF-1 β cells (Fig. 6E). A single specific binding component was observed with a K_d of 250 pM and a maximal capacity (4000 sites/cell) again comparable to the number of IL-15 intermediate affinity binding sites. Once bound, the RLI was also efficiently internalized (Fig. 6F).

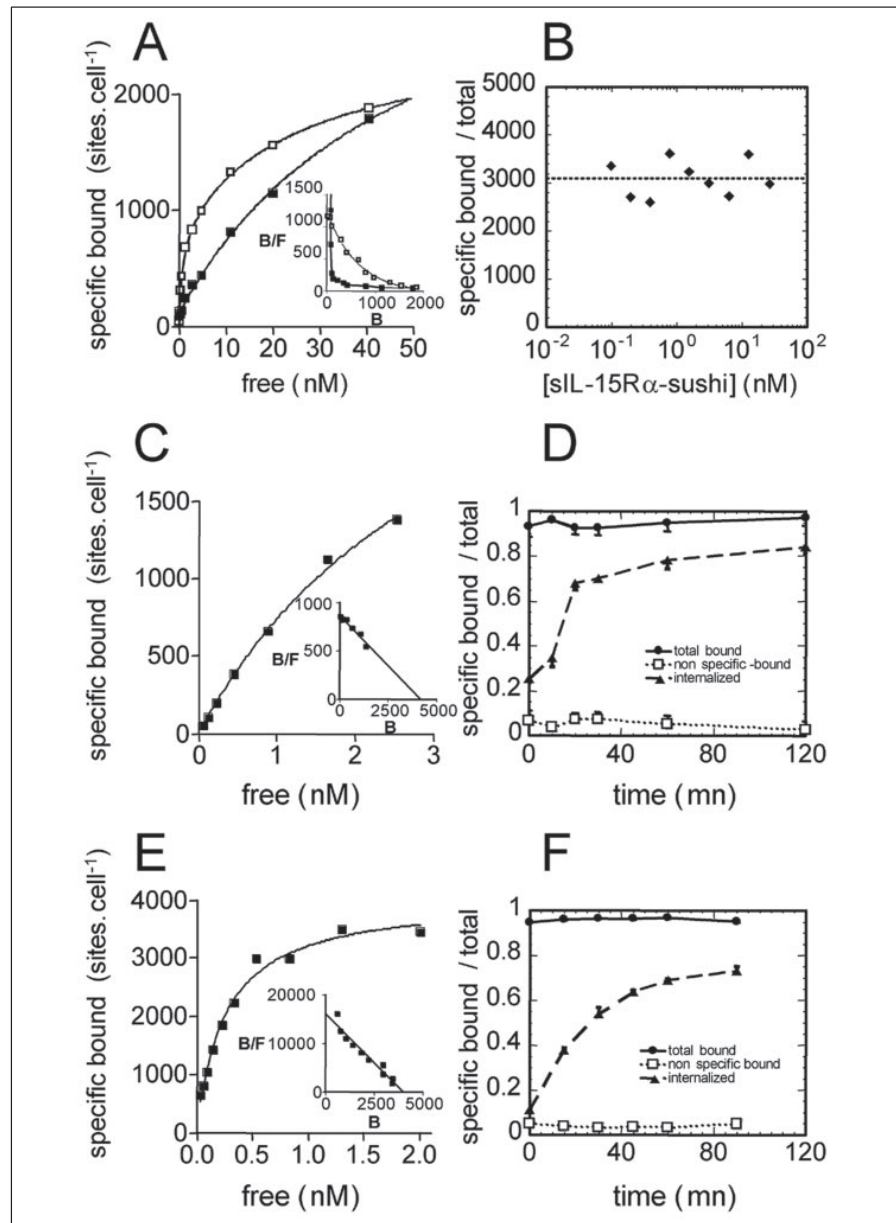
DISCUSSION

Deletion of the exon 2-encoded sushi domain of human IL-15R α was formerly shown to completely abrogate IL-15 binding, indicating the dispensable role of the sushi domain in cytokine recognition (28). Conversely, we show in this study that removal of the C-terminal tail (exons 3–5) of the extracellular part of IL-15R α (in the context of the sIL-15R α -IL-2 fusion protein) results in a 10-fold decrease in its binding affinity for IL-15, as seen by SPR, and a 3.5-fold decrease in its affinity as seen in a competition assay. In terms of thermodynamics, the 10-fold decrease in affinity was calculated to correspond to a 10% loss of the free energy of interaction of IL-15 with IL-15R α . Thus, the N-terminal structural domain encoded by exon 2 (sushi domain) bears most (90%) but not all of the IL-15 binding capacity. Recent data from our laboratory⁴ indicate that the domain encoded by exon 3 also contributes to IL-15 binding. The sIL-15R α -sushi produced in *E. coli* had an affinity that was 3- to 4-fold lower than that of sIL-15R α -sushi-IL-2 produced in Chinese hamster ovary cells. This difference cannot be explained by differences in the glycosylation status of the two proteins, inasmuch as the sushi domain does not contain any potential sites for *N*- or *O*-linked glycosylations (2). It is therefore likely to be due to differences in the structural folding of the two proteins.

While competing with IL-15 binding to membrane IL-15R α , sIL-15R α -sushi was found to exert agonistic effects by enhancing IL-15 action through the IL-15R β / γ complex. Studies on cells expressing either only intermediate affinity IL-15 receptors (Mo-7e and 32D β) or both high and intermediate affinity IL-15 receptors (TF-1 β and Kit 225) showed that the agonist action of sIL-15R α -sushi was specifically directed to the IL-15R β / γ complex: (i) it had no effect in the absence of IL-15; (ii) it bound to TF-1 β cells in the presence of IL-15 with a single affinity class of binding sites whose density was comparable to that of intermediate IL-15 binding sites; (iii) it increased the affinity of IL-15 for the IL-15R β / γ complex on Mo-7e cells and TF-1 β cells, whereas it did not affect the binding of IL-15 to the high affinity complex on TF-1 β cells; and (iv) it enhanced the efficiency of the biological actions of IL-15 (proliferation and prevention from apoptosis) through IL-15R β / γ on

⁴E. Mortier, unpublished results.

FIGURE 6. Binding and internalization of sIL-15R α -sushi and RLI in TF-1 β cells: effects of sIL-15R α -sushi on IL-15 binding. A, saturation binding curve of ^{125} I-labeled rIL-15 in the absence (■) or presence of 10 nM sIL-15R α -sushi (□). B, effect of increasing concentrations of sIL-15R α -sushi on the binding of a fixed concentration of radioiodinated rIL-15 (200 pM). C, saturation binding curve of ^{125} I-labeled sIL-15R α -sushi in the presence of 1 nM rIL-15 and D, subsequent internalization. E, saturation binding curve of ^{125} I-labeled RLI, and F, subsequent internalization.



Mo-7e cells but had no effect on the same biological effects mediated through the high affinity receptor on TF-1 β cells.

The functionality of this agonistic action was further supported by the fact that sIL-15R α -sushi, once bound in conjunction with IL-15 to IL-15R β / γ on Mo-7e cells, was efficiently internalized within the cell. Its potency was strengthened in the context of the ILR and RLI fusion proteins: (i) RLI bound to IL-15R β / γ with an affinity almost 20-fold higher than IL-15 itself; (ii) binding of RLI was followed by a rapid internalization of the fusion protein; and (iii) the RLI or ILR fusion proteins were much more potent than IL-15 in the functional assays. The dose-response curves of the two fusion proteins on Mo-7e cells were comparable to those of IL-15 through the high affinity receptor on Kit 225 or TF-1 β cells, indicating that these fusion proteins almost fully reconstituted the high affinity response on cells only expressing the intermediate affinity receptor.

These results therefore indicate that sIL-15R α -sushi and IL-15 form a complex that cooperatively increases their binding affinities to the IL-15R β / γ receptor. In contrast, sIL-15R α -sushi is not able to affect IL-15 binding and bioactivity once this latter is already associated with the high affinity membrane receptor complex. The possibility that sIL-15R α -sushi can still bind to IL-15 already engaged in this high affinity complex cannot, however, be excluded and could be tested with the availability of cells expressing mainly high affinity receptors, *i.e.* cells expressing similar levels of the three receptor subunits.

We have formerly shown that sIL-15R α expressed in COS cells or naturally produced by IL-15R α -positive cells, behaves as a powerful antagonist by binding IL-15 with high affinity ($K_d = 166$ pM) and inhibiting IL-15-induced proliferation of Kit 225 cells at low concentrations (IC_{50} between 3 and 10 pM) (19). These results are in contrast with the present study showing that sIL-15R α -sushi has no effect on the prolif-

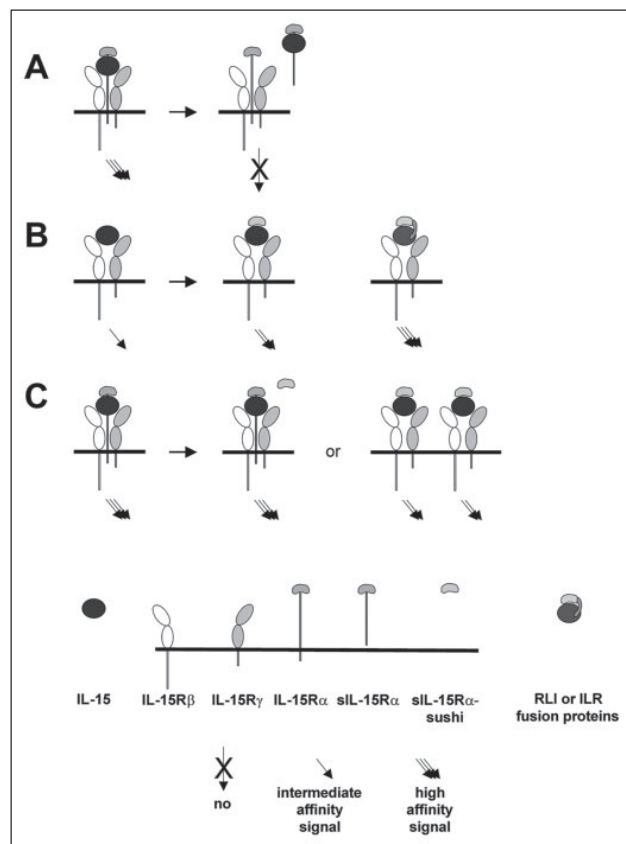


FIGURE 7. Proposed models for the differential effects of sIL-15R α and sIL-15R α -sushi. A, in the context of IL-15R α / β / γ receptors, sIL-15R α competes with membrane IL-15R α for binding IL-15. B, in the context of IL-15R β / γ receptors, sIL-15R α -sushi forms a complex with IL-15 that activates the IL-15R β / γ complex more efficiently than IL-15 alone. The RLI or ILR fusion proteins amplify this agonistic effect. C, in the context of IL-15R α / β / γ receptors, sIL-15R α -sushi is not efficient in competing with membrane IL-15R α , or it competes with membrane IL-15R α and the complex of sIL-15R α -sushi with IL-15 activates excess IL-15R β / γ complexes as in B.

eration of Kit 225 cells or of TF-1 β cells and acts as an agonist on Mo-7e cells. Another contrasting result was reported in a recent study showing that a mixture of rIL-15 and a recombinant human sIL-15R α (lacking exon 3)-Fc homodimeric chimera could have an anti-apoptotic effect on Mo-7e cells, whereas rIL-15 alone at the same dose had no effect (29). In an attempt to explain these differences of action, a model is proposed in Fig. 7. In this proposal, in the context of the high affinity IL-15 response, sIL-15R α acts as a competitor of membrane IL-15R α for the recruitment of IL-15 (Fig. 7A). The complex of sIL-15R α -sushi with IL-15, on the contrary, is able to associate with membrane IL-15R β / γ and enhance the biological effect of IL-15 (Fig. 7B). There are two ways of explaining the absence of the inhibitory effect of sIL-15R α -sushi in the context of the high affinity receptor (Fig. 7C). In the first, sIL-15R α -sushi has a lower affinity for IL-15 ($K_d = 1.5$ nM, this report) than has sIL-15R α ($K_d = 160$ pM) (19) and, therefore, is not able to efficiently compete with membrane IL-15R α on Kit 225 or TF-1 β cells. In the second, sIL-15R α -sushi can compete with membrane IL-15R α to bind IL-15 and form complexes with IL-15R β / γ similar to those formed on Mo-7e cells. Such complexes are less efficient, because they need higher concentrations of IL-15 to be activated ($IC_{50} = 750$ pM instead of 20 pM for high affinity receptors, Figs. 2 and 5). However, given the fact that IL-15R β / γ are in excess of IL-15R α in Kit 225 or TF-1 β cells, the lower efficiency of such complexes could be compensated for by their higher

density (~3000 intermediate affinity receptors instead of 100 high affinity receptors on TF-1 β cells, Fig. 6). This would result in no observable changes in terms of biological effects. Our observation that sIL-15R α -sushi does not affect IL-15 high affinity binding on TF-1 β cells (Fig. 6B) is, however, in favor of the first possibility. The functional differences between sIL-15R α and sIL-15R α -sushi indicate that the C-terminal tail of sIL-15R α plays a crucial role in competing with membrane IL-15R α and hence in the antagonistic action of sIL-15R α . This tail would either impede soluble IL-15R α association with IL-15R β / γ or allow such an association but result in a functionally inappropriate conformation of IL-15R β / γ . A similar mechanism has been proposed in the case of a soluble common γ chain (30). The inhibitory activity of this soluble γ chain (corresponding to the entire extracellular part of the γ chain) was abolished by removal of its C-terminal part or by mutations of the WSXWS motif, two regions not involved in cytokine binding.

The agonistic effects of sushi are reminiscent of the agonistic effects described for soluble receptors within the extended IL-6 family of cytokines (namely sIL-6R, sIL-11R, soluble ciliary neurotrophic factor receptor, and the IL-12p40 subunit) (31). However, such an agonistic action in the case of the IL-15R could not be anticipated given that all soluble receptors so far described within the γ c family (sIL-2R α , sIL-2R β , and sIL-4R), and sIL-15R α itself, behave as cytokine antagonists (19, 32). The present results therefore identify the soluble sushi domain of IL-15R α as an unexpected and efficient agonist within this family. It would be interesting to evaluate whether such an agonistic activity can be similarly obtained with a sIL-2R α -sushi protein.

The concept of cytokine trans-signaling was initially used in the case of IL-6, where soluble IL-6R was shown to enhance the sensitivity of IL-6-responsive cells to the action of IL-6 and to render cells that express gp130 but not membrane IL-6R responsive to IL-6 (33). This concept has been extended to other members of the gp130 cytokine family (IL-11R, ciliary neurotrophic factor receptor, and cardiotrophin-like cytokine) (34–37). In the case of IL-15, a mechanism of cytokine transpresentation has been shown (12) in which IL-15 produced by monocytes/dendritic cells is associated to membrane IL-15R α expressed by the same cells and can stimulate the proliferation of IL-15R β / γ ⁺IL-15R α [−] bystander cells. Recent reports have suggested that transpresentation is a dominant mechanism *in vivo* and necessitates expression of IL-15 and IL-15R α by the same cells (13, 14, 38, 39). It bears some similarity with the trans-signaling concept, in that trans-presented IL-15/IL-15R α complexes can sensitize IL-15R β / γ ⁺IL-15R α [−] cells to physiological concentrations of IL-15. In this respect, membrane IL-15R α acts as an agonist of IL-15 action by increasing its avidity for the IL-15R β / γ complex and the efficiency of signaling (12). Our data show that sIL-15R α -sushi behaves similarly, sensitizing IL-15R β / γ ⁺IL-15R α [−] cells to the actions of IL-15. This suggests that the sushi domain of membrane IL-15R α is crucial for transpresentation. We have shown that sIL-15R α , produced by IL-15R α -expressing cells and consisting of the entire extracellular part of IL-15R α , is inhibitory to the actions of IL-15 (19) and likely constitutes a negative feedback mechanism that limits the biological effects of IL-15. In contrast, the sIL-15R α -sushi described in this study displays an agonistic effect. If such soluble sushi is generated by IL-15R α -expressing cells, it could participate in the IL-15 transpresentation mechanism. The existence of such naturally produced soluble sushi domains has not yet been described but is supported by the facts that (i) different isoforms of the membrane IL-15R α have been described, including some that lack the tail (encoded by exons 3–5) linking the sushi domain to the transmembrane domain (3, 28, 40) and (ii) generation of soluble counterparts for some of them by shedding has been demonstrated (19). Thus, sIL-15R α and sIL-15R α -sushi could

have opposing regulatory effects and, as such, participate in both controlling the magnitude and duration of IL-15-mediated biological actions.

A fusion protein (hyper-IL-6) consisting of the sIL-6R C terminus fused by means of a flexible linker to the N terminus of IL-6 has been described as a novel approach to render the IL-6/sIL-6R complex more stable and more active than the combination of unlinked IL-6 and sIL-6R (41). A similar approach has also been used in the case of IL-11 (34). We show that this approach is also valid in the case of IL-15. A molecular model of IL-15 with the sushi domain was generated (Fig. 2)⁵ that helped to design a flexible linker enabling to link the C terminus of IL-15 to the N terminus of sushi (ILR fusion protein) or *vice versa* (RLI fusion protein). The model also predicted that the linker would not mask the areas of IL-15 that have been shown to be involved in binding to the IL-15R β and γ chains. As discussed above, the two fusion proteins turned out to be much more active than IL-15 and the combination of IL-15 plus sIL-15R α -sushi in activating the IL-15R β / γ complex on Mo-7e cells. When used on TF-1 β cells, and in the context of activation of the high affinity receptor, the ILR fusion protein was as active as IL-15, and the RLI fusion protein was even 10-fold more active, with an EC₅₀ as low as 1.2 pM in inducing cell proliferation. Due to their high activity, these hyper-IL-15 fusion proteins appear to constitute valuable tools for the expansion of lymphocyte subsets, and especially those (NK and CD8 memory T cells) for which transpresentation of IL-15 has been suggested to be the physiological activating process (13). Such fusion proteins might therefore be very efficient adjuvant molecules in therapeutic strategies aiming at curing patients with cancer, immunodeficiencies, or infectious diseases.

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⁵ A. Quemener, unpublished results.