Soluble Interleukin (IL)-15Rα Is Generated by Alternative Splicing or Proteolytic Cleavage and Forms Functional Complexes with IL-15*

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Interleukin 15 (IL-15) is a pleiotropic cytokine that is hardly detectable in biological fluids. Here, we show that IL-15 forms functional heterocomplexes with soluble high affinity IL-15 receptor α (IL-15Rα) chain in mouse serum and cell-conditioned medium, which prevents IL-15 detection by ELISA. We also demonstrate that two soluble IL-15Rα (sIL-15Rα) sushi domain isoforms are generated through a novel alternative splicing mechanism within the IL-15Rα gene. These isoforms potentiate IL-15 action by promoting the IL-15-mediated proliferation of the CTLL cell line and interferon γ production by murine NK cells, which suggests a role in IL-15 transpresentation. Conversely, a full-length sIL-15Rα ectodomain receptor α-converting enzyme (TACE)-dependent shedding of a natural soluble IL-15Rα gene is responsible for ligand binding (4). In the absence of the IL-2/15Rβ and γc subunits, IL-15Rα binds IL-15 with high affinity (Kₐ ~ 10⁻¹³ M⁻¹), which is in striking contrast to IL-2Rα that exhibits low affinity for IL-2 (Kₐ ~ 10⁻⁹ M⁻¹) (4). A number of distinct IL-15Rα isoforms were described in human and mice as a result of an alternative splicing within the IL-15Rα gene. These include deletions of exon 2, exon 3, an alternative usage of exon 7 or 7’ (5, 6), and deletion of a part of exons 3, 4, and 5 (7).

EXPERIMENTAL PROCEDURES

Reagents and Antibodies (Abs)—Recombinant IL-2, IL-15, DuoSet ELISA kits for murine sIL-15Rα, IFNγ, and human IL-15 detection were purchased from R & D Systems. Abs against IL-15Rα (N-19) and IL-15 (L-20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lipopolysaccharide (LPS), recombinant IL-15, recombinant IFNγ, IL-2, and recombinant IL-15Rα (sIL-15Rα) were kindly provided by W. Krönke, J. Krönke, and S. B. P. from the German Research Center for Biotechnology, Jena.

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‡ The Abbreviations used are: IL, interleukin; sIL, soluble interleukin; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon γ; LPS, lipopolysaccharide; GFP, green fluorescent protein; WT, wild type; DC, dendritic cell; WB, Western blot; CM, culture medium; MΦ, macrophages; TACE, tumor necrosis factor-α converting enzyme.

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charide (LPS) derived from Salmonella enterica serovar Friedenau was kindly provided by Dr. H. Brade (Research Center Borstel, Borstel, Germany). Phorbol 12-myristate 13-acetate and anti-β-actin Abs were from Sigma. The metalloproteinasin inhibitor GM6001, the respective negative control product, and anti-green fluorescent protein (GFP) Abs were purchased from Calbiochem. Recombinant sIL-15Ra was produced as described earlier (13). Rabbit antisera against 9 amino acids (RPLTESSTY) in the C terminus of the S2 isoform was generated by Sigma-Genosys. Newcastle disease virus was kindly provided by Dr. R. Zawatzky (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

Cell Culture, Stimulation, and Transfection Conditions—COS-7, RENCA, and CTLL-16 cell lines were obtained from ATCC and L929 cells were from European Collection of Cell Cultures. WT and IL-15Ra−/− mouse embryonic fibroblasts were described elsewhere (14). NK cells were purified from mouse spleens using CD49b (DX5) microbeads (Miltenyi Biotech), yielding cell purities of >93%. Concanavalin A blasts were prepared by incubation of splenocytes with 2 μg/ml concanavalin A for 48 h. Bone marrow-derived dendritic cells (DCs) and macrophages (Mφ) were generated as described previously (15, 16). Cells were cultured in RPMI 1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM 1-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen). Transfection efficiency was evaluated by fluorescent microscopy and WB was about 60%. After 48 h, Supernatants and cells were harvested for ELISA or ELISA. Cells were incubated for another 4 h. Supernatants and cells were harvested for ELISA or ELISA.

Plasmid Construction—Plasmid constructs were amplified from RENCA and L929 cDNA libraries using primers located in exon 2 (5′-AACACTCCACCTGATTGAGTGT-3′) and exon 7 (5′-GTTCATTCTGATTGAGTGT-3′). Cloning of different isoforms (named IL-15RaΔ4, IL-15RaΔ3,4, and IL-15RaΔ3,4,5) from murine mast cells was published earlier (7). The same isoforms and two additional S1 and S2 isoforms were isolated from L929 and RENCA cell lines using a rapid amplification of cDNA ends (3′-rapid amplification of cDNA ends kit; Invitrogen). Briefly, mRNAs were converted into cDNA using reverse transcriptase and an oligo(dT) adapter primer provided with the kit. Then an “anchored” PCR was performed, using upper gene-specific primer (5′-CATCCTCTGATTGAGTGT-3′) and lower abridged universal amplification primer (5′-TCCTCACTGATTGAGTGT-3′) provided with the kit. The resulting products were ligated into pcRII-TOPO vector (Invitrogen), transfected into Escherichia coli, purified, and sequenced. XhoI/BamHI fragments from each construct were then inserted into the pcDNA3.1 (Invitrogen) or pEGFP-N1 (BD Clontech) expression vectors. Cloning of murine IL-15Ra WT and human IL-15 was performed as described previously (17, 18).

For the generation of the exon 2 deletion mutant, a 65-amino acid sequence corresponding to the cytokine-binding domain (5′-GTTCPPVSIEHADRNVSNRSRYVCMNGKRKAGTSTLIECVNKNTVNAWTTPSGLC-3′) was deleted using inverse PCR strategy. The pair of primers (sense, 5′-AGAGACCTCTCCCTGACTGAC-3′; antisense, 5′-CGGGCTCACCCTCAGCCGGAG-3′) in the IL-15Ra coding sequence was designed in such a way that, after PCR amplification, the complete plasmid pEGFP-N1 was obtained again, lacking only the bases located between the two primers. The obtained PCR products were subsequently phosphorylated and ligated. The identity of the deletion-containing construct was verified by standard DNA sequencing.

Reverse Transcription-PCR—RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA using oligo(dT) as a template and the SuperScriptII™ kit (Invitrogen). cDNA was amplified by a standard PCR procedure as described previously (7). The following primers were used: murine IL-15Ra WT (524 bp) (sense, 5′-AACACTCCACCTGATTGAGTGT-3′; antisense, 5′-GTTCATTCTGATTGAGTGT-3′), S2 (283 bp) (sense, 5′-GGCCATTCTCTGTGGTTGC-3′; antisense, 5′-AGATCGTCAGTTCCATGAGGGAG-3′), and S1 (240 bp) (sense, 5′-GGTGGGGCCGCCGCCCCAGGAGGAC-3′; antisense, 5′-CTCCTTAATGTCAGCGGGCTGCTC-3′). The primers were purchased from Invitrogen. Reverse transcription semiquantitation PCR was performed from 35 cycles, 15 μl of cDNA, and the 30 cycles were continued. A mock PCR (without cDNA) was included to exclude contamination in all experiments. The expected sizes of PCR products were 524, 283, and 240 bp, respectively. The PCR product from the mock reaction was visualized on a 2% agarose gel. The PCR products were cloned 10-fold using 10 kDa cut-off filtration units (Vivaspin; Vivascience). Nonidet P-40 (0.5% final concentration) and a protease inhibitor cocktail (Roche Applied Science) were included in all experiments. Mice were bled from the tail vein, and sera were collected. Immunoprecipitation was performed for 2 h at 4 °C. Immunoprecipitation and WB analysis was performed as described elsewhere (7, 8).

CTLL Assay—Serial 2-fold dilutions of the supernatant were concentrated 10-fold using 10 kDa cut-off filtration units (Vivaspin; Vivascience). Nonidet P-40 (0.5% final concentration) and a mixture of protease inhibitors were added to supernatants, and immunoprecipitation was performed for 2 h at 4 °C. Immunocomplexes were captured on protein A/G-agarose (Pierce). To analyze glycosylation, the samples were treated with 250 milliunits of 0.25 mg/ml N-glycosidase F (Roche Applied Science) for 3 h at 37 °C according to the manufacturer's instructions. WB analysis of precipitates and protein lysates was performed as described elsewhere (7, 8).

Flow Cytometric Analysis—Adherent cells were harvested from culture plates using acutase (PAA Laboratories). IL-15Ra expression was evaluated by incubation of cells with biotinylated goat anti-mouse IL-15Ra Abs followed by incubation with streptavidin-fluorescein isothiocyanate and analyzed by flow cytometry.
IL-15Rα Forms Functional Complexes with IL-15

The ability of recombinant sIL-15Rα to bind exogenous and endogenous IL-15, preventing the IL-15-specific interaction with IL-15R complex and the IL-15-mediated downstream signaling, is well documented (13, 19, 20). Given that endogenous sIL-15Rα is

flow cytometry using FACScalibur (BD Biosciences) and CELLQuest software. Negative controls consisted of biotinylated isotype-matched Abs (BD Pharamingen). The fluorescence signal of the labeled cells was calculated as median fluorescence intensity of the cell population.

ELISA—Concentration of mouse sIL-15Rα, IFNγ, and human IL-15 in cell supernatants, lysates, and mouse sera was evaluated by DuoSet ELISA kits according to the manufacturer’s recommendations. IL-15Rα-IL-15 heterocomplexes were detected by a two-site ELISA. Plates were coated with monoclonal anti-IL-15Rα Abs, and IL-15Rα-IL-15 heterocomplexes in samples were detected using polyclonal biotinylated goat anti-human IL-15 Abs followed by incubation with streptavidin-peroxidase. Chromogenic substrate (R&D Systems) was used for visualization, and the reaction was stopped after 20 min of incubation by the addition of 1 N H2SO4. Optical density was determined at 450 nm using an ELISA reader (Dynatech). Anti-IL-15Rα or anti-IL-15 Abs did not show cross-reactivity for IL-15 or IL-15Rα, respectively.

The reference standard used for the two-site ELISA was a dilution of recombinant sIL-15Rα and recombinant soluble IL-15Rα, consisting of an extracellular domain of this receptor subunit. These two molecules were mixed at a fixed molar ratio of 1:1 and incubated for 2 h at room temperature with the ELISA plate. In addition, a control curve was also established for IL-15Rα, respectively, and a standard curve using IL-15-IL-15Rα complex and the IL-15Rα component alone (IL-15 or IL-15Rα, respectively).

Data Analysis—All experiments were performed in at least three independent assays, which yielded highly comparable results. Semiquantitative PCR data were quantified using ImageQuant TL software (Amersham Biosciences). Data are summarized as mean ± S.D. Statistical analysis of the results was performed by Student’s t test for unpaired samples. A p value of <0.05 was considered statistically significant.

RESULTS

sIL-15Rα Forms Heterocomplexes with IL-15—The ability of recombinant sIL-15Rα to bind exogenous and endogenous IL-15, preventing the IL-15-specific interaction with IL-15R complex and the IL-15-mediated downstream signaling, is well documented (13, 19, 20). Given that endogenous sIL-15Rα is
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A

B

1  
S1 ATGGCTCCGAGCAGCTCCGGTATCTGATGCCAGCCATTCCTGGTGTGCTGTCCATTCTTGGCTGTTGACGAGCTGGAGG  
S2 ATGGCTCCGAGCAGCTCCGGTATCTGATGCCAGCCATTCCTGGTGTGCTGTCCATTCTTGGCTGTTGACGAGCTGGAGG  

87  
S1 GGTGACGCGACGGAGGACAGCTGCTACCCTCCAGTGATATCCTATGCTGTAATGCTGACATCCGGTGAAGATGCAAGG  
S2 GGTGACGCGACGGAGGACAGCTGCTACCCTCCAGTGATATCCTATGCTGTAATGCTGACATCCGGTGAAGATGCAAGG  

173  
S1 AGAAGATTGTCCCTAGAATGCTGCTTTAAGCGGAAAGCTGGAACATCCACCCGAGAAGAAGAAG  
S2 AGAAGATTGTCCCTAGAATGCTGCTTTAAGCGGAAAGCTGGAACATCCACCCGAGAAGAAGAAG  

259  
S1 GCCACCTGGAACAATCCGTTGCTTAAGCGGAAAGCTGGAACATCCACCCGAGAAGAAGAAG  
S2 GCCACCTGGAACAATCCGTTGCTTAAGCGGAAAGCTGGAACATCCACCCGAGAAGAAGAAG

E

F

G

H

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present in mouse serum (8) and the high affinity of IL-15Rα toward IL-15, we questioned whether this soluble receptor molecule associates with IL-15 in biological fluids and cell supernatants, affecting ligand half-life and functional properties and causing difficulties in IL-15 detection. To test this hypothesis, mouse serum samples were assessed by a specific ELISA for IL-15 and sIL-15Rα concentration and by a two-site ELISA for IL-15/sIL-15Rα interactions, respectively. In a direct two-site ELISA, the 96-well plates were covered with monclonal coating Abs that recognize the extracellular domain of IL-15Rα. After the incubation with serum samples, polyclonal detection Abs targeting IL-15 were added. In inverted setup, monoclonal anti-IL-15 Abs served as coating Abs, and polyclonal anti-IL-15Rα served as detection Abs. Direct two-site ELISA showed that varying amounts of endogenous IL-15 are constitutively associated with sIL-15Rα in mouse serum, ranging from 100 to 900 pg/ml (Fig. 1A), whereas specific ELISA for IL-15 or inverted ELISA did not generate any signal (data not shown), presumably due to the inability of anti-IL-15 monoclonal coating Abs to bind sIL-15Rα-associated IL-15. This suggests that binding sites on IL-15 are inaccessible to these Abs and indicates that most if not all IL-15 molecules in mouse serum are presumably bound to sIL-15Rα. Thus, sIL-15Rα in mouse serum forms heterocomplexes with IL-15, and sIL-15Rα prevents IL-15 recognition by monoclonal Abs.

We have earlier demonstrated that mouse sera contain varying amounts of sIL-15Rα using an ELISA not commercially available at that time, and a commercial ELISA for sIL-15Rα displayed different serum sIL-15Rα levels (0.3–2 ng/ml), which were detected by two-site ELISA. The reason for such heterogeneity remains unclear. Not only was sIL-15Rα undetectable by specific ELISA in serum from IL-15Rα−/− animals, which were used here for control and comparison, but also IL-15 was absent, suggesting that IL-15Rα is required for IL-15 liberation and might play an important role in cellular mechanism(s) controlling this process.

Next, we tested whether IL-15-IL-15Rα heterocomplexes exist in primary cells from normal and IL-15Rα−/− animals or cells and cell lines of various origin. These experiments revealed that murine MΦ, mouse embryonic fibroblasts, DCs, and L929 cells express IL-15Rα and, to a lesser extent, IL-15, whereas IL-15Rα was undetectable in the cell lysates of mouse embryonic fibroblasts and DCs from IL-15Rα−/− animals (Fig. 1B).

Interestingly, the IL-15 level was also rather low in these cells, as detected by specific ELISA. The concentration of IL-15 in the cell lysates was always higher according to two-site versus specific ELISA, indicating that at least some IL-15 molecules associate with IL-15Rα already within the cell. Furthermore, sIL-15Rα-associated IL-15 (~100 pg/ml) was also detected by two-site ELISA in cell-conditioned medium from MΦ and DCs but not L929 fibroblasts, whereas specific ELISA for IL-15 did not generate any signal (Fig. 1C). Remarkably, all of these cells abundantly release sIL-15Rα, as evaluated by specific ELISA for this receptor chain (~320–550 pg/ml). The ability of sIL-15Rα to form heterocomplexes with IL-15 in intracellularly was also confirmed by WB analysis after immunoprecipitation from the cell lysates using specific anti-IL-15Rα Abs and indicates that most if not all IL-15Rα-competent DCs (Fig. 1D) may associate with IL-15Rα−/− cells did not precipitate IL-15 in these results show that sIL-15Rα is in the cytoplasm and forms complexes with IL-15 in mouse serum and cell lysates of different primary murine cells.

**FIGURE 2. Cloning of novel soluble IL-15Rα isoforms.** A, reverse transcription-PCR amplification of different IL-15Rα isoforms from RENCA and L929 cells. A mock PCR (without cDNA) was included to exclude contamination. B, comparative sequence analysis of S1 and S2 IL-15Rα isoforms. The sequence corresponding to exon 1 is in the gray box, and the sequence corresponding to exon 2 (sushi domain) is in boldface type. The start codon is underlined. The glycosylation site is indicated by an asterisk. C, cDNA libraries were analyzed by WB using anti-IL-15Rα Abs and anti-S2 antisera. Detection of β-actin on the same blot was used as a loading control. D, detection of S2 isoform in 50-fold concentrated conditioned medium from L929 cells byWB using S2 antisense. The supernatants from COS-7 cells transfected with a construct coding for S2 isoform or mock-transfected (empty vector) served as a positive or negative control, respectively. E, expression of IL-15Rα WT and S2 isoforms in different mouse tissues was analyzed by semiquantitative PCR. Expression levels are plotted as a ratio between specific cDNA amplification and amplification of control gene (β-actin). F and G, regulation of expression of IL-15Rα WT and S2 isoforms in different cells. DCs and MΦ were stimulated with LPS (100 ng/ml) for 4 or 8 h (E). L929 fibroblasts were stimulated for 3 or 6 h with LPS (100 ng/ml) or Newcastle disease virus (NDV) (2.4 hemagglutinating units) (F). Total RNA was extracted from cells, reverse-transcribed, and subjected to semiquantitative PCR amplification using specific primers for IL-15Rα WT and S2 as described under "Experimental Procedures." The image shows the amplified bands after 35 cycles. The amount cDNA was equalized by PCR amplification of β-actin. A mock PCR (no cDNA) was included as a negative control. H, the S2 isoform is present in murine sera in vivo, as detected by specific ELISA for sIL-15Rα in which monoclonal anti-IL-15Rα Abs targeting the extracellular domain of multiple IL-15Rα isoforms served as capture Abs, whereas anti-S2 Abs served as specific detection Abs for S2 protein. Detection of IL-15Rα WT is shown as a positive control. Detection of both isoforms in IL-15Rα−/− mice represents negative controls.
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tide (96 bp) and the sushi domain (Fig. 2B). These isoforms are slightly different in length (the S2 isoform is longer by 7 amino acids) and exhibit minor variations at the 3’ end of mRNA, whereby serine and lysine in the S1 isoform are replaced by arginine and proline in the S2 isoform, respectively. The open reading frame in the S1 and S2 isoform shares with the extracellular region of IL-15Rα (518 bp) the first 293 bp until isoleucine at position 97, flanking in S1 what is presumably an unspliced adjacent intron 2 that terminates the open reading frame following the lysine residue. The S2 isoform also seems to an end in unspliced intron 2, although the corresponding coding sequence is located more distantly (data not shown). The predicted molecular mass of S1 and S2 is about 12–15 kDa. L929 and RENCA cells were also found to express IL-15Rα WT as well as IL-15RαΔ4, IL-15RαΔ3,4, and IL-15RαΔ3,4,5 isoforms (Fig. 2A) recently identified and characterized in murine mast cells (7).

To confirm the existence of novel sIL-15Rα isoforms at the protein level, polyclonal Abs of rabbit origin directed against the unique last 9 amino acids of the S2 isoform were generated. As shown in Fig. 2C, varying amounts of S2 protein were detected in L929 fibroblasts, mouse embryonic fibroblasts, DCs, and MΦ but not in concanavalin A-activated T cell blasts. IL-15Rα WT protein was also detected in these cells. Remarkably, the expression of S2 isoform was considerably higher in DCs, whereas no S2 isoform was detected in DCs from WT and IL-15Rα−/− animals (Fig. 2C). Moreover, S2 protein was also detected in the concentrated culture medium of COS-7 cells (Fig. 2D). Given that the S1 isoform differs from IL-15Rα WT and S2 only by 2 amino acids, IL-15Rα isoform could not be generated using monoclonal Abs. Immunofluorescent confirmation of S1 protein expression in these experiments demonstrated that the antibody binding mechanism is responsible for the generation of novel sIL-15Rα sushi domain isoforms.

To gain information about the mechanism triggering sIL-15Rα and to analyze the pattern of expression of the novel S2 isoform, we surveyed a wide variety of cell types by semiquantitative reverse transcription-PCR for the expression for S2 isoform mRNA, using lower primer targeting the unique sequence at 3’ end of S2. Concomitantly, the expression of IL-15Rα WT mRNA was assessed for control and comparison (Fig. 2E). Both IL-15Rα WT and S2 isoform were highly expressed in lymph nodes and testis, whereas the highest level of IL-15Rα WT expression was found in the heart. Relatively low levels of expression of both proteins were detected in muscle and brain. Interestingly, the level of the S2 isoform was relatively high in thymus as compared with IL-15Rα WT. Furthermore, stimuli, such as LPS and Newcastle disease virus, differentially regulated expression of the alternatively spliced IL-15Rα S2 isoform and IL-15Rα WT at the mRNA level. Both stimuli up-regulated IL-15Rα WT expression in DCs, MΦ, and L929 cells, whereas the S2 isoform was down-regulated upon LPS stimulation (Fig. 2, F and G). This fact suggests that cells can reciprocally regulate the production of distinct IL-15Rα isoforms according to particular environmental challenges.

We also confirmed that the S2 isoform is present in murine sera in vivo using specific ELISA for sIL-15Rα, in which monoclonal anti-IL-15Rα Abs targeting the extracellular domain of multiple IL-15Rα isoforms served as capture Abs, whereas anti-S2 Abs served as specific detection Abs for S2 protein. This approach has an advantage of enhanced sensitivity as compared with immunoprecipitation and Western blotting analysis. It should be mentioned that the concentration of S2 protein in serum was rather low (below 100 pg/ml) (Fig. 2H). However, the fact that anti-S2 Abs target the last 9 unique amino acids of the S2 isoform essentially limits the sensitivity of the assay, making more precise detection of the S2 isoform a technically daunting task.

Next, we set forth to investigate whether IL-15Rα WT, IL-15RαΔ4, IL-15RαΔ3,4, and IL-15RαΔ3,4,5 as well as novel S1 and S2 isoforms are released as soluble proteins to the CM and form complexes with IL-15. To this end, COS-7 and HeLa cells were transfected with constructs encoding these IL-15Rα chains. Indeed, these proteins were effectively translated in COS-7 and HeLa cells and released to the cell supernatants, as detected by WB of the cell lysates and CM, with the exception of the IL-15RαΔ3,4,5 isoform, which was absent in the CM (Fig. 3, A and B; data not shown). These supernatants from these cells were tested by specific ELISA for IL-15Rα. Interestingly, all isoforms except IL-15RαΔ3,4 isoforms were present in rather high concentration in both CM and cell lysates (Fig. 3C). As expected, expression vectors, whereas IL-15RαΔ3,4 isoform remained intact (Fig. 3D). The Novel IL-15Rα Isoforms Show Usage of Their N-Glycosylation Site—IL-15Rα has single N-glycosylation and multiple O-glycosylation sites in the extracellular domain (5). Reportedly, a number of IL-15Rα isoforms are N-glycosylated, whereas no O-glycosylation was observed (7). To study the role of N-glycosylation in the post-translational processing of S1 and S2 isoforms, these were transiently transfected into COS-7 cells. The whole cell lysates or concentrated CM from transfected cells were treated with N- and O-glycosidases, and the expression products were analyzed by WB. Fig. 3E demonstrates that indeed both novel IL-15Rα isoforms are N-glycosylated in the cell lysates and CM, since treatment with N-glycosidase shifts corresponding protein bands to a lower molecular mass position.

IL-15Rα Is Essential for IL-15 Release—Reportedly, both IL-15 and IL-15Rα must be co-expressed by the same cells to transpresent IL-15, indicating a requirement of IL-15Rα at a cellular level for IL-15 elaboration (22). Thus, we sought to investigate whether IL-15Rα could affect IL-15 secretion using co-transfection experiments. For this purpose, COS-7 cells were co-transfected with vectors coding for IL-15 and IL-15Rα WT as well as novel S1 and S2 isoforms. Interestingly, these experiments showed that IL-15Rα expression was indeed essential for IL-15 release into the CM (Fig. 4A). Specific ELISA for IL-15Rα demonstrated that various con-
concentrations of sIL-15Rα WT, S1, and S2 isoforms are present in the CM. Notwithstanding, the release of IL-15 to the CM was observed only upon concomitant transfection with a respective IL-15Rα construct. Given that this effect was also seen in cells transfected with S1 or S2 isoforms, this indicates that distinct cellular mechanism(s) orchestrate and control the coordinated release of both IL-15Rα and IL-15. Notably, the amount of IL-15 was much lower compared with its high affinity chain (about 100–170 pg/ml versus 2.7–8.6 ng/ml, respectively).

**FIGURE 3.** Analysis of expression and shedding/secretion of different IL-15Rα isoforms in transfected COS-7 and HeLa cells. Cells were transfected with different constructs as indicated. Empty vector (mock)-transfected cells were used as a negative control, transfected with IL-15Rα WT construct as a positive control. Expression of membrane-bound IL-15Rα isoforms (A) and sIL-15Rα (B) isoforms was analyzed in cell lysates (L) and culture medium (CM), respectively, from COS-7 by WB using anti-GFP Abs. C, expression and shedding/secretion of IL-15Rα isoforms in L and CM from transfected COS-7 and HeLa cells were analyzed by specific ELISA. *, p < 0.05 versus IL-15Rα WT. D, effect of phorbol 12-myristate 13-acetate (PMA) on the surface expression of IL-15Rα isoforms in transfected COS-7 cells. Cells were treated with 200 ng/ml phorbol 12-myristate 13-acetate for 2 h, and expression of IL-15Rα was analyzed by fluorescence-activated cell sorting analysis. Untreated cells were used as a control. E, glycosylation pattern of S1 and S2 IL-15Rα isoforms. Samples were treated with N-glycosidase or left untreated as described under “Experimental Procedures.” After treatment, protein lysates were analyzed by WB using anti-GFP Abs.
IL-15Rα Forms Functional Complexes with IL-15

**A**

**IL-15Rα**

[Graph showing concentration of IL-15Rα in a mock and treated condition.]

**IL-15**

[Graph showing concentration of IL-15 in a mock and treated condition.]

**B**

**sIL-15Rα**

[Graph showing concentration of sIL-15Rα in control and GM6001 treated conditions.]

**IL-15**

[Graph showing concentration of IL-15 in control and GM6001 treated conditions.]

**C**

[Graph showing concentration of specific IL-15Rα, specific IL-15, and two-site IL-15 in mock, WT, S1, and S2 conditions.]

**D**

[Graph showing concentration of IL-15 in mock, WT, and Δ2 conditions.]

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Next, we tested whether blocking of IL-15Ra shedding by a broad spectrum metalloproteinase inhibitor, GM6001, affects IL-15 secretion. To this end, COS-7 cells transfected as above were treated with this chemical compound, and the supernatants were tested by ELISA for IL-15 or sIL-15Ra. In fact, GM6001 considerably reduced IL-15Ra WT shedding (an about 4-fold decrease), which was accompanied by a comparable decline in IL-15 release (Fig. 4B). Conversely, GM6001 did not affect the release of S1 and S2 isoforms. Importantly, the release of IL-15 to the supernatants from IL-15Ra S1- or S2-transfected cells also was not affected by GM6001 (Fig. 4B). This is in accord with the fact that these isoforms lack membrane-proximal and transmembrane regions and thus cannot undergo proteolytic cleavage.

To validate further the ability of IL-15Ra to form complexes with IL-15 and affect IL-15 secretion, cell-conditioned medium from transfected COS-7 cells was tested by specific or two-site ELISAs for the presence of sIL-15Ra, IL-15, or sIL-15Ra:IL-15 complexes, respectively. Specific ELISA for IL-15Ra showed that this protein is present (~5.5–6.8 ng/ml) in the CM of all transfected cells (Fig. 4C). Direct two-site ELISA for IL-15 confirmed the release of IL-15 in rather high concentrations (~3.5–4.2 ng/ml) to the CM. IL-15Ra S1 and S2 isoforms were almost equipotent in sustaining IL-15 release as compared with IL-15Ra WT (Fig. 4C). Conversely, specific ELISA for IL-15 detected considerably lower cytokine concentrations (~1–200 pg/ml). Thus, overexpression studies demonstrated that IL-15 associates with sIL-15Ra in the CM, and IL-15Ra is essential for IL-15 release.

Exon 2-encoding Sushi Domain Is Required for IL-15 Secretion—Next, we isolated murine NK cells and tested using the same supernatant from COS-7 cells transfected as above, the production of IL-15 in these cells (Fig. 5A). Remarkably, recombinant sIL-15Ra in a lower concentration (0.1 ng/ml) did not show any stimulating capacity. Again, the addition of recombinant IL-15Ra (0.1 ng/ml) to the CM was without effect. The production of IL-15 by NK cells stimulated with IL-15 or sIL-15Ra S1 isoforms was comparable with IL-15 alone (100 ng/ml), lying in the range of 80–100 versus 50–60 pg/ml, respectively (Fig. 5B). Remarkably, recombinant IL-15 in a lower concentration (0.1 ng/ml) also did not show any stimulating capacity. Again, the addition of recombinant sIL-15Ra had no effect, although it efficiently prevented stimulating action of IL-15 (data not shown). These results demonstrate that naturally produced and elaborated IL-15Ra:IL-15Ra S1 or S2 heterocomplexes stimulate CTLL cell proliferation and IFNγ production by NK cells, thereby implicating them in IL-15 transpresentation.

**DISCUSSION**

In this study, we show for the first time that an alternative splicing mechanism within the **IL-15Ra** gene is responsible for the generation of natural sIL-15Ra sushi domain isoforms. Thus, sIL-15Ra in mice is produced through a dual mechanism that includes both proteolytic processing of the membrane-tethered receptor and alternative splicing. We
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**Figure 5. Effect of soluble IL-15Rα ectodomains on proliferation of CTLL cells.** COS-7 cells were transfected with plasmids encoding different IL-15Rα isoforms. A, conditioned media from COS-7 cells transiently expressing WT, Δ4, Δ3,4, Δ3,4,5, S1, S2, or N/A recombinant IL-15Rα (re sIL-15Rα) were incubated with an excess of recombinant sIL-15Rα (re sIL-15Rα) at 1 μg/ml for 48 h, equilibrated for IL-15 content, and tested in a CTLL proliferation assay. Samples containing IL-15-S1 and IL-15-S2 complexes were incubated with an excess of recombinant sIL-15Rα (re sIL-15Rα) at 1 μg/ml for 1 h prior to the assay as indicated. * p < 0.05 versus vector control samples. B, NK cells were incubated with conditioned media from COS-7 cells for 24 h. Supernatants were collected, and concentration of IFNγ was evaluated by ELISA. Incubation of cells in the presence of IL-15 (0.1 and 100 ng/ml) was used as a control. * p < 0.05.

Also demonstrate that heterocomplexes consisting of IL-15 and sIL-15Rα are present in mouse serum and cell culture supernatants, which suggests that maintenance of a dynamic equilibrium between serum levels of sIL-15Rα and IL-15 is important for IL-15 biology. Given that natural sIL-15Rα sushi domain promotes IL-15 activity, inducing the IL-15-mediated proliferation of CTLL cells and IFNγ production by NK cells, transpresentation of IL-15 in such soluble agonistic complexes may constitute a heretofore unappreciated novel mode of action for this pleiotropic cytokine. Finally, IL-15Rα is required for a coordinated secretion of IL-15, suggesting that distinct control mechanisms orchestrate this process.

Thus, although murine sIL-15Rα is predominantly generated through TACE-dependent proteolysis of the membrane IL-15Rα, both proteolytic cleavage and alternative splicing coexist in the mouse system, adding to our understanding of this process (Fig. 6). It remains to be elucidated whether such a dual mechanism of sIL-15Rα generation also exists in humans. Whereas proteolysis might primarily be responsible for the generation of sIL-15Rα capable of inhibiting IL-15 action (8, 24), the secreted sIL-15Rα sushi domain exhibits agonistic qualities. This fact is in agreement with the reported ability of recombinant sIL-15Rα sushi domain to act as a potent and selective agonist of IL-15 function through the intermediate affinity IL-2/IL-15Rβγ heterodimer (23). It has been suggested that, if naturally produced, such sIL-15Rα sushi domains might be involved in the IL-15 transpresentation mechanism.

IL-15/sIL-15Rα complexes might play a particular role in distinct physiologic and/or pathologic conditions and alter in the first place IL-15 action on the cells expressing membrane-bound IL-15R complex. Depending on the particular sIL-15Rα subtype (i.e. sushi domain or full-length ectodomain), these soluble molecules may compete for IL-15 with the cellular receptors and inhibit its activity or, contrariwise, promote IL-15 action, especially in cells expressing intermediate affinity IL-15Rβγ complex. Thus, in addition to the ability of membrane-anchored IL-15Rα to transpresent IL-15 in trans to neighboring cells during direct cell–cell contact (15), IL-15/sIL-15Rα complexes might also perform a similar function when they are secreted in soluble form. This might also extend the complex biology of IL-15 to IL-15Rα generation and maintenance of critical immune effector cells, such as memory CD8+ T cells and NK cells. In fact, injection of recombinant IL-15/sIL-15Rα heterodimer complexes in vivo induces strong proliferation of memory CD8+ T cells and NK cells (26) and that IL-15Rα forms complexes with IL-15Rα in such agonistic complexes in vivo may represent an important mechanism of IL-15 action.

Although shed sIL-15Rα ectodomain acts as an inhibitory molecule, it might also provide increased molecular stability to IL-15, leading to the reduced activity decay, and expand IL-15 action from autocrine or juxtacrine to paracrine or endocrine modes, resulting in local or systemic effects of the cytokine and influencing the nature and/or duration of the signaling event. Given that sIL-15Rα ectodomain could extend the bioavailability of IL-15 by prolonging cytokine half-life, whereas dissociation of IL-15 from the complex under conditions that favor this process may serve to provide physiological concentrations of the cytokine in tissues, the precise role of proteolytically generated sIL-15Rα awaits further studies.

Despite widespread expression of IL-15 mRNA, detection of significant amounts of IL-15 in cell culture supernatants has proven to be extremely difficult (2). Because sIL-15Rα prevents recognition of IL-15 by monoclonal coating Abs in inverted ELISA, it appears likely that sIL-15Rα is present in excess over IL-15, and the majority of IL-15 molecules in mouse serum are presumably associated with sIL-15Rα, suggesting that these two molecules exist in a dynamic equilibrium. Considering that human IL-15 has at least two binding sites for recombinant sIL-15Rα (26) and that IL-15Rα can, at least partially, oligomerize (27), it is theoretically conceivable that at a low molar ratio, sIL-15Rα forms complexes with IL-15 where IL-15 could still be detected by distinct anti-IL-15 Abs, whereas at higher molar
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FIGURE 6. Schematic diagram of the IL-15Rα gene and synopsis of various IL-15Rα isoforms cloned by Anderson et al. (5); **, isoforms described by Dubois et al. (6) in the human system; *#, soluble IL-15Rα generated by proteolytic cleavage of the membrane-bound form (18), inhibitory sIL-15Rα, which shows nuclear co-localization with IL-15Rα.

ratios, most commercially available anti-IL-15 Abs would detect IL-15 in a specific or two-site ELISA setup. Our results provide the first conclusive evidence that IL-15 liberation is governed at multiple levels by abnormalities in IL-15 expression (31). Given that production of IL-15 is burdened by multiple complex regulatory mechanisms and is tightly governed at multiple levels (18), inhibitory sIL-15Rα may serve as an additional important protective control point to limit excessive and/or undesired IL-15 activity.

Experiments using IL-15- and IL-15Rα-deficient mixed chimera showed that IL-15 and IL-15Rα must be expressed by the same cells to present IL-15 in trans to neighboring cells, which indicates that IL-15Rα is required on a cellular level for the elaboration of IL-15 bioactivity (6). Our data conclusively demonstrate a direct requirement of IL-15Rα, in particular its sushi domain, for IL-15 secretion. Such a secretory process, in addition to multiple control mechanisms, may exist yet unknown control mechanism. This ensures IL-15 liberation is essentially in the presence of, and possibly under direct or indirect regulation by, IL-15Rα. However, it remains unclear why recombinant sIL-15Rα is not able to inhibit the ability of IL-15-sIL-15Rα sushi domain complexes to induce CTLL cell proliferation and IFNγ production by NK cells, NKT cells, NK cells, and certain subsets of intestinal intraepithelial lymphocytes, playing important roles in both innate and adaptive immunity (1, 29, 30). Given that IL-15 is associated with a wide range of immunopathological reactions, it has been suggested that this cytokine may be at the apex of a cytokine cascade that includes downstream production of IL-1, IL-6, granulocyte-macrophage colony-stimulating factor, and other biologically active substances (2). The fine tuned balance between antagonistic and agonistic IL-15-sIL-15Rα may be altered in distinct pathological conditions and can probably serve as a prognostic marker in certain types of diseases, including allergy and inflammation. Diverse neoplastic and inflammatory diseases, including adult T cell leukemia and certain autoimmune disorders like rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and pulmonary sarcoidosis, are characterized by abnormalities in IL-15 expression (31). Given that production of IL-15 is burdened by multiple complex regulatory mechanisms and is tightly governed at multiple levels (18), inhibitory sIL-15Rα may serve as an additional important protective control point to limit excessive and/or undesired IL-15 activity.

Upon the cell surface, IL-15 coupled to IL-15Rα may undergo proteolytic processing and enter the circulation either alone or together with IL-15. Conversely, alternatively spliced soluble IL-15Rα sushi domain may similarly associate with IL-15 in the cell and follow a secretion route whereby resulting complexes act as highly specific and potent agonists of IL-15 function. Such dual properties of sIL-15Rα isoforms might play an important role in the complex biology of IL-15, negatively or positively affecting the IL-15-mediated biological responses (Fig. 7).

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In summary, our data demonstrate that sIL-15Rα in mouse is generated by both alternative splicing and proteolytic cleavage. Qualitatively distinct sIL-15Rα molecules form functional soluble heterocomplexes with IL-15 capable of inhibiting or potentiating IL-15 action. Thus, IL-15/sIL-15Rα sushi domain heterocomplexes might play an important role in transpresentation of IL-15 and the generation and maintenance of multiple lymphocyte subsets, including memory CD8+ T cells and NK cells. This highlights new dimensions in our understanding of how this pivotal cytokine exerts its pleiotropic biological functions and identifies novel targets for the therapeutic manipulation of the pathologic conditions linked with abnormal IL-15 activity.

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WITHDRAWN

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