Natural Splicing of Exon 2 of Human Interleukin-15 Receptor α-Chain mRNA Results in a Shortened Form with a Distinct Pattern of Expression*  

We report the existence of eight different interleukin-15 receptor α-chain (IL-15Ra) transcripts resulting from exon-splicing mechanisms within the IL-15Ra gene. Two major classes of transcripts can be distinguished that do or do not (Δ2 isoforms) contain the exon 2-coding sequence. Both classes were expressed in numerous cell lines and tissues (including peripheral blood lymphocytes) at comparable levels and could be transcribed in COS-7 cells, and the proteins were expressed at the cell surface. Both receptor forms displayed numerous glycosylation states, reflecting differential usage of a single N-glycosylation site as well as extensive O-glycosylations. Whereas IL-15Ra bound IL-15 with high affinity, Δ2IL-15Ra was unable to bind IL-15, thus revealing the indispensable role of the exon 2-encoded domain in cytokine binding. A large proportion of IL-15Ra was expressed at the nuclear membrane with some intranuclear localization, supporting a potential direct action of the IL-15-IL-15Ra complex at the nuclear level. In sharp contrast, Δ2IL-15Ra was found only in the non-nuclear membrane compartments, indicating that the exon 2-encoded domain (which is shown to contain a potential nuclear localization signal) plays an important role in receptor post-translational routing.

Together, our data indicate that exon 2 splicing of human IL-15Ra is a natural process that might play regulatory roles at different levels.

Interleukin (IL)-15 is a cytokine that was discovered through its capacity to replace IL-2 in supporting the growth of the murine IL-2-dependent CTLL cell line (1, 2). Both cytokines belong to the short cytokine-binding hormone family. IL-2Rα, the α-chain, is a natural process that might play regulatory roles at different levels.

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The abbreviations used are: IL, interleukin; IL-2Ra, IL-2β, and IL-2γ; interleukin-2 receptor α-, β-, and γ-chains, respectively; IL-15Ra, interleukin-15 receptor α-chain; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); NLS, nuclear localization signal.
EXPERIMENTAL PROCEDURES

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from various human cell lines and tissues using guanidinium thiocyanate/phenol as described (21). Total RNAs from human liver, brain, and small intestine were purchased from CLONTECH (Basingstoke, United Kingdom). Reverse transcription and PCR amplifications were performed as described previously (22). For the other conditions were as follows: denaturation for 1 min at 94 °C; annealing for 1 min at 66 °C (E1/E7), 68 °C (E7/E2), and elongation for 1 min at 72 °C (30 cycles). The resulting primers were used: E1-5'-AG-TCCAGCCTGTTGCTGGTG; E7, 5'-TCATAGGGTGGAGACCT; E7, 5'-TCACACAGCCCTCCCACCTG; E4, 5'-GAATCCACGATGCCG; p2BGH, 5'-TAGAAGCACAATCGGACG; ββ (sense), 5'-GGTGCTGCAGCCAGGG; ββ (antisense), 5'-TGTCTGGTACCCAGAC-AC; 5E4.1, 5'-GCCAGCTTCACTCCCAC; and E7.2, 5'-TAGGGTGGA-GAGC.

Molecular Constructs

The four products resulting from RT-PCR using primers E1 and E7 were ligated into the pN5TA7 plasmid, leading to pN51R, pN51R3, pN51R2, and pN51R235. PCR amplification of pN51R with primers 5E4.1 (located in exon 4) and E7.2 enabled the elimination of the receptor stop codon. The resulting PCR product was purified and inserted into pN5TA7, leading to pN51R*. SmoI fragments from pN51R, pN51315R, pN51215R, and pN52315R were inserted into SmoI-digested and dephosphorylated pN515R*. The XbaI fragments from each construct were then inserted into the pcDNA3/TOPO vector (pDNA3m; Invitrogen, Groningen, Netherlands), yielding pcDNA5Rh, pDNA3315Rh, pcDNA3215Rh, and pcDNA-323315Rh, respectively.

Cell Culture and Transfections

The U937 human histiocytic lymphoma cell line, the SAOS-2 human osteogenic sarcoma cell line, and COS-7 monkey kidney epithelial cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium containing 10% fetal calf serum and 2 mM glutamine. Cells were then plated (5 × 10^6 cells/well) and increasing concentrations of labeled IL-15 in a final volume of 50 µl. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled cytokine. Regression analysis of the binding data was accomplished using a one-site equilibrium binding analysis. The IC50 values for each were calculated. IL-15 binding was determined by enhanced chemiluminescence (ECL kit, Roche Molecular Biochemicals) and exposure to X-Omat films (Eastman Kodak Co.).

IL-15 Binding Assays

Human recombinant IL-15 (Peprotech, Rocky Hill, NJ) was iodinated as described (25) with a specific radioactivity of ~2000 cpm/fmol. Binding experiments were carried out for 75 min at 4 °C in phosphate-buffered saline/bovine serum albumin (PBBSA) as described previously (26) using 10^5 cells/well and increasing concentrations of labeled IL-15 in a final volume of 50 µl. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled cytokine. Regression analysis of the binding data was accomplished using a one-site equilibrium binding equation (Graft, Erithacus Software, Staines, UK).

IL-15 Receptors and Solubilization from Transfected COS-7 Cells as above were purified by nickel-nitriiotriacetic acid (Qiagen, Chatsworth, CA) affinity chromatography (27) according to the manufacturer’s instructions. The IL-15Rα and 321L15Rα were solubilized from transfected COS-7 cells as above by using 1% sodium dodecyl sulfate (SDS) and 0.5 M KCl. IL-15 receptor complexes were then electrophoretically separated on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and probed with anti-IL-15Rα antibody (1 µg/ml well) and 1:2000 dilution of horseradish peroxidase-conjugated antihuman IgG. After washes, the membranes were developed by enhanced chemiluminescence (ECL kit, Amersham Pharmacia, Buckinghamshire, UK).

Proliferation Assays

Transfected Kii 225 cells were washed twice to remove IL-2 or IL-15 and starved for 1 h in RPMI 1640 medium with 6% fetal calf serum and 2 mM glutamine. Cells were then plated (5 × 10^5 cells/well) and cultured for 48 h in culture medium containing 750 µg/ml Genetcin and supplemented with increasing concentrations of IL-2 or IL-15. They were pulsed for 6 h with 4 µCi of [3H]thymidine and harvested onto Whatman filters. Cell-associated [3H]thymidine was measured using a Microbeta counter (Wallac, Turku, Finland).
to two new mRNA species, one lacking exon 2 (641 bp; D15R) and one lacking both exons 2 and 3 (542 bp; D2D3IL-15R), respectively. The two lower bands corresponded to full-length IL-15Rα and the IL-2Rβ/IL-2Rγ transducing subunits. IL-2Rβ and IL-2Rγ each contain one hematopoietic receptor domain with two conserved disulfide bonds and a consensus WSXWS motif. In IL-15Rα is shown the exon 2-encoded sushi domain characterized by two interwoven disulfide bonds (C-1-C-3 and C-2-C-4). This domain is absent in Δ2IL-15Rα.

**RESULTS**

**Characterization of New IL-15Rα Transcripts Lacking Exon 2**—Different IL-15Rα isoforms using alternate C-terminal exons (exons 7 and 7') and containing or not exon 3 have already been described (Fig. 1) (12). Using oligonucleotide primers corresponding to the N-terminal end of exon 1 (primer E1) and the C-terminal end of exon 7 (primer E7) or 7' (primer E7'), RT-PCR amplifications were carried out on mRNAs prepared from different cell lines and tissues (Fig. 2). For each couple of primers (E1/E7 or E1/E7'), four amplification products were detected. The products from the E1/E7 amplification carried out on human peripheral blood mononuclear cells were cloned and sequenced. The two upper bands (834 and 735 bp) corresponded to full-length IL-15Rα and IL-15Rα lacking exon 3 (Δ3IL-15Rα), respectively. The two lower bands corresponded to two new mRNA species, one lacking exon 2 (641 bp; Δ2IL-15Rα) and one lacking both exons 2 and 3 (542 bp; Δ2Δ3IL-15Rα). Sequence analysis showed that exon 2 and/or exon 3 deletions did not change the reading frame (data not shown).

The four products obtained from E1/E7' amplification had sizes that were ~100 bp lower than those obtained from the E1/E7 amplification, suggesting that they corresponded to similar IL-15Rα isoforms (full-length, Δ3, Δ2, and Δ2Δ3), but using the alternate exon 7', which is 100 bp shorter than exon 7 (12). The structures of the coding sequences of the eight mRNA isoforms are shown in Fig. 1.

These eight isoforms were detected in most of the cell lines and tissues examined (Fig. 2), except fetal bone marrow and the choriocarcinoma JAR cell line. The respective expression levels of the different isoforms varied from one cell/tissue type to another. Whereas, in most cases, the full-length and Δ3 isoforms appeared to predominate, the mRNAs corresponding to the Δ2 and Δ2Δ3 forms were as abundant as the others in a number of cell types (Kit 225 lymphoma cells and SAOS-2 osteocarcinoma cells) and even more in normal human peripheral blood mononuclear cells. The eight isoforms were also expressed by normal human T cell clones, either CD4+ or CD8+ (data not shown).

**Biochemical Analysis of IL-15Rα and Δ2IL-15Rα Expressed in COS-7 Cells**—The different IL-15Rα Myc/polyhistidine-tagged cDNA isoforms were transfected in COS-7 cells, and the expression products were analyzed by Western blotting on whole cell extracts (Fig. 3A). With the IL-15Rα cDNA (E7 isoform), two doublets of bands were revealed (best seen in panel c) at 59–62 and 39–41 kDa. Treatment with N-glycosidase induced a disappearance of the 62- and 41-kDa bands, leaving the 59- and 39-kDa bands unaffected, whereas treatment with O-glycosidase induced a disappearance of the 59- and 62-kDa bands, but not of the 39- and 41-kDa bands. Together, these results suggest that the 62- and 41-kDa bands are N-glycosylated forms of the 59- and 39-kDa bands, respectively, and that the 59–62-kDa bands are highly O-glycosylated forms of the 39–41-kDa bands.

In the case of Δ2IL-15Rα, similar results were observed. The lower band at 32.5 kDa corresponds to an unglycosylated form of the receptor. Its size is ~7 kDa lower than the corresponding 39-kDa band of IL-15Rα, in agreement with the deletion of the exon 2-encoded domain (12). The band at 35 kDa represents Δ2IL-15Rα with a carbohydrate linked to the single N-glycosy-
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Subcellular Localization of the IL-15Ra Isoforms in COS-7 Cells—The localization of the receptor was first analyzed by confocal microscopy. As shown in Fig. 4, IL-15Ra (in green) was mainly found associated with the nuclear membrane, giving a ring-like pattern. By comparison, localization of the nuclear protein p300 (in red) was purely intranuclear. There was also some co-localization of IL-15Ra and p300 (in yellow), suggesting that part of the receptor was localized at the inner side of the nuclear membrane and/or in the intranuclear space. Δ2IL-15Ra behaved very differently, with an expression pattern suggesting localization within the endoplasmic reticulum, Golgi, and cytoplasmic vesicles, but not with the nuclear membrane or intranuclear space. Accordingly, it did not co-localize with p300. Other experiments (data not shown) indicated that exon 3 deletion had no influence on the localization of the receptor: Δ3IL-15Ra and Δ2Δ3IL-15Ra showed patterns similar to those of IL-15Ra and Δ2IL-15Ra, respectively.

To support these observations, Western blot analyses were carried out on subcellular fractions prepared by biochemical means (Fig. 3B). In the membrane fraction prepared from isolated nuclei, IL-15Ra was expressed at a much higher level than the Δ2 isoform. Conversely, Δ2IL-15Ra was predominant in the membrane fraction prepared after depletion of nuclei (non-nuclear membrane fraction), and in comparison, IL-15Ra was expressed at a much lower level in that fraction. Both unglycosylated and glycosylated forms of the receptors were observed. Examination of the cytosolic extracts did not show detectable expression of either receptor form (data not shown).

Confocal microscopy did not reveal detectable expression of IL-15Ra or Δ2IL-15Ra at the plasma membrane. However, this could be due to low cell-surface receptor density as often observed with cytokine receptors. To analyze this point further, cells were externally labeled with [125I]iodine before lysis and immunoprecipitation with anti-histidine antibody (Fig. 3C). Specific bands at 62 and 53 kDa were precipitated from IL-15Ra-transfected cells and a specific band at 48 kDa from Δ2IL-15Ra-transfected cells, indicating that both receptor isoforms are routed to the plasma membrane and exposed at the cell surface as glycosylated proteins.

Deletion of Exon 2 Results in a Loss of IL-15 Binding—To
evaluate the function of the sushi domain encoded by exon 2, we first analyzed the binding of radioiodinated IL-15 to transfected COS-7 cells (Fig. 5A). Mock-transfected COS-7 cells did not bind IL-15, whereas cells transfected with IL-15Rα expressed ∼1200 high affinity receptors (Kd = 60 pm). COS-7 cells transfected with the Δ2 isoform did not show any detectable specific IL-15 binding. As far as both receptors were expressed at the cell surface (Fig. 3C), these results suggested a loss of IL-15-binding capacity as a result of exon 2 deletion. To rule out the possibility that a low expression level of Δ2IL-15Rα as compared with that of IL-15Rα at the cell surface could account for the absence of detectable cytokine binding, IL-15Rα and Δ2IL-15Rα were solubilized from transfected COS-7 cells and purified by nickel affinity chromatography, and their IL-15-binding capacities were assessed. An enzyme-linked immunosorbent assay using anti-Myc antibody as coating antibody and anti-histidine antibody as tracer antibody showed similar binding capacities were assessed. An enzyme-linked immunosorbent assay using anti-Myc antibody as coating antibody and anti-histidine antibody as tracer antibody. C, the same fractions were analyzed for their ability to bind 125I-labeled IL-15 (2 nM) using an anti-histidine antibody/protein A precipitation assay. Nonspecific immunoprecipitation was evaluated by including a 100-fold excess of unlabeled cytokine. An irrelevant rabbit antibody used instead of anti-histidine antibody served as a negative control. D, shown is the inhibition of the binding of 125I-labeled IL-15 (2 nM) to IL-15Rα by increasing concentrations of unlabeled IL-15.

**DISCUSSION**

Recently, three different IL-15Rα isoforms have been described that either lack exon 3 and/or use an alternate exon 7 (exon 7′) (12). Here, by RT-PCR amplification using appropriate sets of oligonucleotide primers, we confirm the existence of these isoforms and show, in addition, the existence of novel IL-15Rα transcripts corresponding to the deletion of exon 2. This new deletion can combine with the ones already described, leading to eight different transcripts corresponding to all the possible combinations of exon 2 deletion, exon 3 deletion, and alternate usage of exon 7 or 7′. In agreement with previous reports (12, 13), IL-15Rα transcripts were found to be expressed by various cell lines and tissues. Each positive tissue or cell line expressed the eight different IL-15Rα transcripts, although at relative levels that varied from one cell/tissue type to another.

Expression of human IL-15Rα in COS-7 cells gave rise to several protein bands, in agreement with a previous report suggesting several bands for a soluble form of mouse IL-15Rα (13). We show in this study that these different products are due to alternate N- and O-glycosylations of a 39-kDa precursor. Similarly, Δ2IL-15Rα gave rise to different glycosylated bands from a 32.5-kDa precursor. The size difference between IL-
IL-15 binding. They fully complement earlier work showing that exon 3-encoded linker sequence as well as exon 7-encoded cytoplasmic domains were dispensable for binding and signaling (12).

IL-15Ra was expressed at the cell surface, although at low density (−1000 sites/cell). Unexpectedly, confocal immunofluorescence studies and analysis of subcellular fractions showed that most of IL-15Ra was associated with the nuclear membrane. A large proportion of this nuclear receptor was heavily O-glycosylated, suggesting that it was routed to the nuclear membrane through the Golgi. Some co-localization with the nuclear protein p300 was also observed, indicating that part of the receptor was inside the nucleus, possibly at the inner side of the nuclear membrane. Due to the relatively large size (−60 kDa) of the glycosylated receptor, this observation suggests that an active mechanism is involved in its nuclear translocation, rather than passive diffusion through the nuclear pores. In support of this, we found, within the human IL-15Ra sequence, the presence of a putative nuclear localization signal (NLS). This sequence consists of two clusters of polycationic residues separated by a spacer (RERYCNSGFK23, amino acids 24–36) (12). Such putative NLSs have been demonstrated in the sequence of a number of ligands and receptors, including those that activate Stat transcription factors (29, 30). In this study, the possible involvement of this putative NLS in the nuclear routing of IL-15Ra is supported by the fact that the exon 2-truncated receptor (which does not contain this putative NLS motif located in the sushi domain) does not show nuclear localization. More specific deletions/mutations of this sequence are, however, required to demonstrate its role as an NLS.

Of major interest with respect to these findings is the recent demonstration that a newly identified isoform of IL-15 that uses a short (21 amino acids) signal peptide (SSP-IL-15) is not directed to the secretory pathway, but rather is stored intracellularly, appearing in the cytoplasm and nucleus (31). The other IL-15 isoform containing a long (48 amino acids) signal peptide (LSP-IL-15), in contrast to SSP-IL-15, has no nuclear localization and is directed to the secretory pathway. The two isoforms have also distinct tissue distribution and are likely to be generated by the usage of alternate promoters rather than by alternative splicing, suggesting that they might serve different roles. Our observations raise the interesting possibility that IL-15Ra, which has a high affinity on its own for IL-15, might bind SSP-IL-15 inside the cell and, through its putative NLS, translocate the cytokine-receptor complex in the nuclear compartment. Following that hypothesis, IL-15Ra might be involved in the different roles postulated for the two forms of IL-15, at the cell surface for LSP-IL-15 and at the nuclear level for SSP-IL-15.

Exon 2-truncated IL-15Ra has completely lost its capacity to bind IL-15, therefore raising the question of its biological role. Two findings suggest that it might serve some biological function: (i) a number of cell lines and tissues expressed Δ2IL-15Ra mRNAs at levels comparable to full-length IL-15Ra mRNAs; and (ii) upon transfection in COS-7 cells, Δ2IL-15Ra was expressed at the plasma membrane with similar efficiency as IL-15Ra. It is therefore possible that Δ2IL-15Ra might compete with IL-15Ra for recruitment of the transducing subunits IL-2Rg and IL-2Ry. Similar observations have been made in the case of IL-2Ra. A naturally occurring truncated form of IL-2Ra has been described that lacks the exon 4-encoded domain (second sushi domain) as a result of alternative mRNA splicing (32). This truncated IL-2Ra, whose function is presently unknown, was transported to the cell surface and was unable to bind IL-2. It was, however, expressed at low levels in human T cells. In this study, preliminary experiments on Kit 225 cells showed that Δ2IL-15Ra transfection did not affect IL-15- or IL-2-induced proliferation. However, a potential regulatory effect of transfected Δ2IL-15Ra in this system might have been masked by the fact that Kit 225 cells already express endogenous IL-15Ra and Δ2IL-15Ra. Additional studies addressing
signal transduction, proliferation, and apoptosis are required to determine the functional role of the Δ2IL-15Rα isoforms.

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