The Long Signal Peptide Isoform and Its Alternative Processing Direct the Intracellular Trafficking of Interleukin-15*

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Two isoforms of interleukin (IL)-15 exist: one with a short and another with a long signal peptide (LSP). Experiments using combinations of the LSP and mature proteins IL-2, IL-15, and green fluorescent protein revealed complex pathways of intracellular trafficking. In one pathway, the LSP was unprocessed, and IL-15 was not glycosylated, remained in the cytoplasm, and was degraded. The second trafficking pathway involved endoplasmic reticulum entry, N-linked glycosylation, and alternative partial LSP processing. The third pathway involved endoplasmic reticulum entry, followed by glycosylation, complete processing, and ultimate secretion. The complex intracellular trafficking patterns of LSP-IL-15 with its impediments to secretion as well as impediments to translation may be required due to the potency of IL-15 as an inflammatory cytokine. In terms of a more positive role, we propose that intracellular infection may relieve the burdens on translation and intracellular trafficking to yield effective IL-15 expression.

Interleukin (IL)1-15 is a 15–17-kDa member of the four-α-helix bundle family of cytokines that stimulate T and NK cell proliferation and activity (1–3). In parallel with the other interleukins (IL-2, IL-4, IL-7, and IL-9) that stimulate T cells, IL-15 utilizes the common γ-receptor subunit for its action (4). In addition, IL-15 and IL-2 share the IL-2/15 β-chain receptor subunit in these cells and consequently share many biological activities (1, 4). Nevertheless, there are major differences between these two cytokines in terms of their cellular origins and the mechanisms controlling their synthesis and secretion. IL-2 is produced by activated T cells and is predominantly controlled at the levels of mRNA transcription and stabilization, whereas the widely distributed IL-15 has a much more complex, multifaceted control of expression with regulation at the levels of transcription, translation, and intracellular trafficking (5–12). In contrast to the predominantly T cell-restricted expression of IL-2 transcripts, there is widespread constitutive expression of IL-15 mRNA in a variety of tissues, including placenta, skeletal muscle, kidney, lung, and heart, and fibroblasts, epithelial cells, and activated monocytes. Despite this widespread expression of IL-15 mRNA, detection of significant quantities of IL-15 protein in cell culture supernatants has proven to be extremely difficult. For example, IL-15 mRNA is induced in lipopolysaccharide/interferon-γ-activated monocytes, yet the supernatants as well as the lysates of such cells contain only trace amounts of IL-15, suggesting that IL-15 synthesis and secretion are negatively regulated at multiple levels (7). It has been proposed that the dominant control of IL-15 expression is post-transcriptional, i.e. at the levels of message translation and intracellular protein trafficking (5–12). We (5–7) and others (8–10) have reported that IL-15 mRNA is heavily burdened by a number of potential regulatory elements, including a 5'-untranslated region with multiple upstream AUG codons and complex secondary structure and a negative regulatory sequence within the protein coding sequence. Furthermore, we and others have demonstrated that the negative regulation of IL-15 protein expression is associated with the use of an unusually long (48 aa) signal peptide (LSP). In particular, the LSP with its associated start codon with a poor Kozak configuration impedes IL-15 translation (5–11).

Recently, a second IL-15 isoform with a typically short (21 aa) signal peptide (SSP) was detected in small cell lung carcinoma cell lines, T cell lines, and a human testicular cDNA library (8–11). This isoform was also detected in mouse cells (12). In contrast to most signal peptides that are encoded by a single exon, IL-15 leader sequence isoforms are encoded by at least two exons. The LSP is encoded by exons 3–5 of the human IL-15 gene, whereas the SSP is encoded by exon 5 as well as by an additional 119-nucleotide sequence termed exon 4a located in intron 4 (9–12). Thus, the two signal peptides share 11 amino acids encoded by exon 5. The presence of exon 4a disrupts the LSP sequence by inserting a premature termination codon and then provides an alternate initiation codon with a positive role, we propose that intracellular infection may relieve the burdens on translation and intracellular trafficking to yield effective IL-15 expression.

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Recently, a second IL-15 isoform with a typically short (21 aa) signal peptide (SSP) was detected in small cell lung carcinoma cell lines, T cell lines, and a human testicular cDNA library (8–11). This isoform was also detected in mouse cells (12). In contrast to most signal peptides that are encoded by a single exon, IL-15 leader sequence isoforms are encoded by at least two exons. The LSP is encoded by exons 3–5 of the human IL-15 gene, whereas the SSP is encoded by exon 5 as well as by an additional 119-nucleotide sequence termed exon 4a located in intron 4 (9–12). Thus, the two signal peptides share 11 amino acids encoded by exon 5. The presence of exon 4a disrupts the LSP sequence by inserting a premature termination codon and then provides an alternate initiation codon with a poor Kozak context. Previous studies reported that protein associated with the SSP was not secreted, but rather was stored intracellularly and appeared in nuclear and cytoplasmic components as detected by confocal microscopy (8–11).

Signal peptides classically function to target the endoplasmic reticulum (ER) (13). In the present study, expression constructs encoding combinations of the long signal peptide and mature proteins including those of IL-2, IL-15, and GFP were used to address the intracellular trafficking pathways and subcellular organelle fate of IL-15. LSP-IL-15 was relatively inefficiently translocated into the ER compared with IL-2; and therefore, some of LSP-IL-15 remained unprocessed in the cytoplasm, where the protein was degraded in proteasomes. Some of LSP-IL-15 entered the ER and was partially processed
to yield a proposed 19-aa 3’-element of the LSP linked to the mature glycosylated protein. This alternatively cleaved IL-15 was not found to be secreted into the medium in this form. An additional trafficking pattern involved translocation of LSP-IL-15 into the ER, where the LSP was fully processed, and the protein was N-glycosylated and ultimately secreted as a 17-kDa cytokine by the classical Golgi route. The observed complex patterns of IL-15 trafficking suggest that control of intracellular trafficking must be added to the control of translation as a level of negative control of the expression of this inflammatory cytokine, whose indiscriminate expression might result in a serious inflammatory disorder. Furthermore, the demonstration that there are both secreted and intracellular fates for IL-15 is consistent with the view that this lymphokine may act both as an intercellular signaling agent and in intracellular signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA).

**Antibodies and Cytokines**—Polyclonal rabbit anti-human IL-15 and monoclonal mouse anti-GFP antibodies used for immunoprecipitation were purchased from Serotec (Raleigh, NC) and CLONTECH (Palo Alto, CA), respectively. For indirect immunofluorescence, the polyclonal rabbit anti-IL-15 antibody was from Harmane Sharma (Genzyme, Cambridge, MA); the polyclonal rabbit anti-IL-2 antibody was from Genzyme; fluorescein-conjugated affinity-purified donkey anti-rabbit IgG, Texas Red-conjugated affinity-purified donkey anti-mouse IgG, and normal donkey serum were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); and recombinant human IL-15 was from Peprotech, Inc. (Rocky Hill, NJ).

**IL-15 Biological Assay Using CytL-2 Cells**—The murine cytokine-dependent CytL-2 cell line was used to quantitate IL-15 activity as described previously (11). COS cell transfection was performed as described (1).

**Brefeldin A Treatment**—A total of 8 × 10⁶ COS cells were electroporated (240 V, 950 microfarads) with the indicated construct with 10 μg of plasmid DNA and distributed evenly into two tissue culture dishes with 5 ml of complete RPMI 1640 medium in each. 72 h post-transfection, the supernatant was removed, and 1 ml of fresh complete medium was added to each dish with or without the addition of brefeldin A (Epitects Corp., Madison, WI) at 0.5 μg/ml for 8 h. After 8 h, supernatants were harvested and checked for IL-15 concentrations by an enzyme-linked immunosorbent assay (Genzyme).

** Constructs**—The pEF-Neo/ΔAUG-IL-15 expression construct used to express IL-15LSP-IL-15mp (where mp is mature protein) and pEF-Neo/IL-2SP-IL-15mp were described previously (5). The pEF-Neo/GFP construct was also described previously (11). For generation of the pEF-Neo constructs for IL-15LSP-GFP, IL-2SP-GFP, IL-15LSP-IL-15mp-GFP, and IL-2SP-IL-2mp-GFP, coding sequences for both the signal peptide and mature protein were polymerase chain reaction-amplified from existing constructs. Primer designs were as follows: IL-15LSP, primer 1 (sense), 5’-GCCATCGAT-ACCAGTGAATTTTGAAACCACAT-3’ (Cla I site underlined), and primer 2 (antisense), 5’-GCCACGCCGTGTCGCTGGTCTTTTATGAAAGC-3’ (Mlu I); IL-2SP, primer 3 (sense), 5’-GCCATCGATACCAGT-TACAGGAATTCACT-3’ (Cla I), and primer 4 (sense), 5’-GCCACGCC-GTTGCACTGTGTTGACAATGAAGC-3’ (Mlu I); IL-15LSP-IL-15mp, primer 1 above (sense) and primer 5 (antisense), 5’-GCCACCGGTTAGAAGTTGTTGAAAGCCAT-3’ (Mlu I); IL-2SP-IL-2mp primer 3 above (sense) and primer 6 (antisense), 5’-GCCACCGGTTAGAAGTTGTTGAAAGCCAT-3’ (Mlu I). The polymerase chain reaction-amplified fragments above were TA-cloned into pcR2.1 (Invitrogen, San Diego, CA) and subsequently subcloned into pEF-Neo/GFP.

**Pulse-Chase Studies**—Unless otherwise indicated, 1 × 10⁶ COS cells in 0.4 ml of RPMI 1640 medium were electroporated with 15 μg of the indicated construct (250 V, 950 microfarads). Electroporated cells were equally aliquoted into five 60-mm dishes in 3 ml of complete RPMI 1640 medium used for transfection. After 48 h, cells were Cys/Met-starved for 30 min in Cys/Met-free RPMI 1640 medium with 10% dialyzed fetal calf serum in the presence or absence of tunicamycin at a 5 μg/ml final concentration. For experiments involving lactacystin, starvation was for 1 h in presence or absence of lactacystin (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) at a 20 μM final concentration. Each dish was then pulsed with 150 μCi of [35S]Cys/Met (Express labeling mixture, NEN Life Science Products). After 10–15 min of labeling at 37 °C, cells were washed three times in ice-cold phosphate-buffered saline, and then 3 ml of complete RPMI 1640 medium was added as a cold chase, with tunicamycin or lactacystin maintained in the corresponding dishes. Lysates were made at the indicated time points in 1 ml of radioimmune precipitation assay buffer (50 mM Tris·HCl (pH 7.4), 1.0% Nonidet P-40, 0.25% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, 0.1% SDS, 1 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml apronin, and 1 μg/ml leupeptin). Lysates were immunoprecipitated with the indicated antibodies using Ultra-link-immobilized protein A/G beads (Pierce). Procedures, including incubation times, antibody concentrations, and washings, closely followed the manufacturers’ recommendations. For all studies except those requiring EndoH digestion, proteins were eluted from the protein A/G beads in SDS sample buffer. For EndoH experiments, proteins were eluted from the beads in EndoH buffer (0.1 M sodium phosphate (pH 6.1) containing 20 mM EDTA, 0.1% Triton-X, and 0.2% SDS) by heating to 95 °C for 10 min. Digestion with EndoH (Oxford GlycoSystems, Abingdon, United Kingdom) at a 0.1 milliunit/μl final concentration at 37 °C was carried out overnight, followed by the addition of SDS sample buffer. Unless otherwise stated, IL-2SP-IL-15mp and IL-15LSP-IL-15mp proteins were electrophoresed on 16% Tris/glycine-SDS gels, and all GFP chimeric proteins were run on 12% Tris/glycine-SDS gels. Gels were fixed, incubated in Enlightening autoradiography enhancer (Du Pont), dried, and exposed to film.

**RESULTS**

There were multifaceted controls of IL-15 expression at the levels of transcription, translation, and intracellular trafficking. The patterns of trafficking and secretory control alone are exceedingly complex. To obtain insights into these diverse patterns, experiments were performed employing different combinations of the LSPs and mature proteins, including IL-2, IL-15, and GFP. In preliminary experiments using SDS-PAGE, we characterized the intracellular forms of LSP-IL-15 biosynthesized by COS cells transfected with a pEF-Neo/LSP-IL-15mp construct containing the native IL-15 coding sequence, including the LSP. Following a 15-min labeling with [35S]Cys/Met, IL-15 was immunoprecipitated with the polyclonal anti-IL-15 antibody and resolved by SDS-PAGE. At least six species of IL-15 were observed that, as shown below, reflect N-linked glycosylation and alternative processing of the LSP of IL-15. Experiments were designed to facilitate the identification of the particular contributions of these two processes to the complex protein patterns observed with IL-15. First, to examine the contribution of N-glycosylation, COS cells were transiently transfected with a pEF-Neo/IL-2SP-IL-15mp construct consisting of the human IL-15 mature protein coding sequence linked to the sequence encoding the IL-2 signal peptide that does not manifest alternative processing. Parallel studies focusing on the processing of the IL-15 LSP were performed in the presence of tunicamycin to prevent N-linked glycosylation.

**IL-15 Has at Least Two Functional N-Linked Glycosylation Sites**—IL-15 has three potential glycosylation sites (Asn-Ser/Thr) in the mature protein coding sequence based on its sequence analysis (Fig. 1a). The number of functional sites was determined by transient transfection into COS cells with the fusion construct pEF-Neo/IL-2SP-IL-15mp. In this experiment, substitution of the 48-aa LSP of IL-15 with that of IL-2 enabled us to separate glycosylation events from signal peptide processing events and simplified our analysis of the number of bands on SDS-PAGE. Proteins were expressed in COS cells in the absence (Fig. 1b) or presence (Fig. 1c) of tunicamycin, an inhibitor of N-linked glycosylation, and were recovered by immunoprecipitation using the polyclonal anti-human IL-15 antibody. Based on the difference in the number of bands on SDS-PAGE between untreated cells (three bands; Fig. 1b) and tunicamycin-treated cells (one band; Fig. 1c), the number of functional N-linked glycosylation sites on IL-15 is two or more.
Furthermore, the amino acid sequence in this area (Thr27-comparable to that of IL-15SSP-GFP, which has a 21-aa SP. philicity profile (Fig. 4) and on the size of the band that is double-underlined 

lation sites are a sites.

Gly30 of the signal sequence based on the Kyle-Doolittle hydro-

protein. This alternative cleavage may occur between Ala29 and

located upstream of the cleavage site that yields the mature

29.2-kDa band results from an endoprotease cleavage point

(Fig. 3). In the IL-15LSP-GFP lanes, it would appear that the one band was obtained for GFP without the LSP (27.9 kDa).

The unglycosylated fully processed protein ran at 12.7 kDa in Fig. 1 (b and c).

The Long Signal Peptide of IL-15 Is Cleaved at Two Distinct Sites—We next examined the species obtained when COS cells were treated with tunicamycin following transfection with pEF-Neo/IL-2SP-IL-15mp, which consists of the native IL-15 coding sequence with the LSP. Instead of a single species on SDS-PAGE, as had been obtained with IL-2SP-IL-15mp when tunicamycin was present, we now obtained three unglycosylated species with IL-15LSP-IL-15mp in the presence of tunicamycin at 18.8, 14.5, and 13.0 kDa (Fig. 2). The constructs differed only in the coding sequence for the SP. We therefore considered the possibility that these forms arose from differences in SP cleavage.

To investigate whether these forms arose from differences in SP cleavage, a pEF-Neo fusion construct was synthesized in which the coding sequence for the LSP of IL-15 was fused with the GFP coding sequence (expected molecular mass for the fusion protein of 33 kDa). The GFP translation start site was removed, enabling translation to occur at the start site of the IL-15 LSP. Furthermore, since GFP has no potential glycosylation sites, it provided us a simpler means to interpret bands observed by gel electrophoresis. In addition, the use of the IL-15LSP-GFP fusion protein allowed us to use the monoclonal anti-GFP antibody for immunoprecipitation, thus eliminating the possibility that the three bands seen in Fig. 2 were non-specific products immunoprecipitated by the monoclonal anti-human IL-15 antibody. We compared IL-15LSP-GFP with a GFP control in the same vector (Fig. 5). Two bands could be seen for IL-15LSP-GFP from cell lysates (32.4, 29.2, and 27.9 kDa and correspond to uncleaved, alternatively cleaved, and fully cleaved LSP species. GFP control protein electrophoresed at 27.9 kDa.

The hydrophilicity profile of LSP-IL-15. The proposed alternative cleavage site of the LSP is shown C-terminal to Ala45 in the 48-aa SP and is indicated with an arrow in the hydrophilicity profile. The mature protein cleavage site (between Ala 46 and Asn47) is also shown with an arrow in the same profile.

More support for alternative cleavage within the LSP of IL-15 comes from experiments with IL-15LSPΔEx5-GFP, an artificial engineered deletion construct in which the amino acids encoded by exon 5 have been eliminated, thus removing the mature protein cleavage site. We compared this construct with a GFP control in the same vector (Fig. 5). Two bands could be seen for IL-15LSPΔEx5-GFP (at 30.2 and 27.9 kDa). The lower of these bands (27.9 kDa) migrated just slightly higher than the single band in the GFP control lane (at 27.3 kDa). The presence of the 27.9-kDa band in the IL-15LSPΔEx5-GFP lane therefore shows that although LSP(ΔEx5) lacks the mature protein cleavage site, an alternative cleavage can still occur within the signal peptide. The slightly higher migration of this band relative to the GFP control band would be in accordance with the few amino acids C-terminal to the proposed alternative cleavage site encoded by exon 4, namely
GHHVFILG, which would remain attached to GFP following cleavage.

Previous in vitro translation experiments in cell-free rabbit reticulocyte and wheat germ systems (in the absence of microsomes) performed with an IL-15LSP-IL-15mp construct yielded a single band on SDS-polyacrylamide gel with a molecular mass that indicated that translation initiates at the first AUG start site (5). Therefore, the bands suspected to result from alternative cleavage of the LSP (at 14.5 kDa in Fig. 2, 29.2 kDa in Fig. 3, and 27.9 kDa in Fig. 5) do not appear to be the result of alternative initiation.

Absence of Stepwise Processing of the Long Signal Peptide of IL-15—We wished to determine whether the species with the fully cleaved (48-aa) LSP (13.0 kDa in Fig. 2) arose from a stepwise processing of the alternatively cleaved form (at 14.5 kDa in Fig. 2). To address this question, COS cells were transfected with pEF-Neo/IL-15LSP-IL-15mp and subjected to a short pulse of [35S]Cys/Met (and, at selected times, half of the immunoprecipitated material was subjected to EndoH digestion to remove high mannose N-linked sugars). Multiple bands were visible (data not shown). The ratio of the 15.3- and 13.6-kDa bands was relatively constant throughout the experiment. This result suggests that the alternative cleavage occurs as a separate event and that the majority of the fully cleaved form is not the result of further processing of the alternatively cleaved form.

Multiple ER-retained Species of IL-15 Exist in COS Cells—In the absence of tunicamycin, six distinct IL-15 forms were generated from pEF-Neo/IL-15mp in the COS cell translation system precipitated with the polyclonal anti-human IL-15 antibody (at 29.1, 21.9, 20.1, 19.1, 17.6, and 16.1 kDa in Fig. 6). In an effort to determine which of the forms represent ER species, the aliquots of immunoprecipitated IL-15LSP-IL-15mp obtained from tunicamycin-un-treated cell lysates was digested with EndoH. EndoH cleaves the high mannose N-linked glycosylation as well as LSP N-linked site. As mentioned above (Fig. 1), a maximum of two N-linked sites are used by IL-15 at any time.

Following EndoH treatment (Fig. 6), three bands were obtained, which is compatible with the situation observed with tunicamycin in Fig. 2; the molecular masses of these forms were 19.1, 14.3, and 12.0 kDa. The band migrating at 19.1 kDa was EndoH-resistant and is thought to represent material that was uncleaved and uncleaved, alternatively cleaved, and fully cleaved LSP species. The 19.1-kDa band appeared to be EndoH-resistant.

would be 14.8 and 16.8 kDa, respectively, where 12.8 kDa is the calculated molecular mass of the mature form of IL-15 and ~2.5 kDa are contributed per N-linked site. As mentioned above (Fig. 1), a maximum of two N-linked sites are used by IL-15 at any time.

To determine which, if any, of these species exited the cell via the Golgi, we metabolically labeled COS cells transfected with the IL-15LSP-IL-15mp construct for a 4-h period, instead of 15 min, after which time the supernatants were harvested. Immunoprecipitated protein obtained from the supernatants using the polyclonal anti-IL-15 antibody were electrophoresed on SDS-polyacrylamide gel. The modifications of glycan that occur in the medial Golgi result in EndoH resistance. All but one of the six forms of IL-15LSP-IL-15mp displayed full EndoH sensitivity. Only the 19.1-kDa band appeared EndoH-resistant. Although only the 0 time point is shown in Fig. 6, the Endo H sensitivity pattern was maintained throughout the chase. Based on the molecular masses of the three highest bands at the 0 time point in the absence of EndoH in Fig. 6 (at 24.9, 21.9, and 20.1 kDa), it would appear that at least some of IL-15 is capable of translocating across the ER membrane and becoming glycosylated with all or part of its signal peptide intact. The sizes of these three species are larger than the predicted molecular masses of an ER species with its SP fully cleaved and glycosylated at 1 or 2 N-linked sites, which

FIG. 5. IL-15LSP(ΔEx5)-GFP is cleaved at the alternative site. Shown are the results from pulse-chase studies with IL-15LSP(ΔEx5)-GFP versus GFP control. COS cells transfected with the indicated pEF-Neo constructs were studied as described in the legend to Fig. 3. Bands in the IL-15LSP(ΔEx5)-GFP lanes migrated at 30.2 and 27.9 kDa and correspond to species with an uncleaved LSP (ΔEx5) and an alternative cleavage within the LSP, respectively. The GFP control protein migrated at 27.3 kDa.

FIG. 6. Retention of some IL-15 species in the ER. COS cells were transfected with pEF-Neo/IL-15LSP-IL-15mp. At 48 h post-transfection, the cells were pulsed with [35S]Cys/Met, and the resulting lysates were immunoprecipitated with the polyclonal anti-human IL-15 antibody; half of the immunoprecipitated material was subjected to EndoH digestion to remove high mannose N-linked sugars. Multiple bands without EndoH at 24.9, 21.9, 20.1, 19.1, 17.6, and 16.1 kDa represent IL-15 with differences in both N-linked glycosylation as well as LSP cleavage. Bands treated with EndoH that lacked N-linked glycosylation at 19.1, 14.3, and 12.0 kDa correspond to uncleaved, alternatively cleaved, and fully cleaved LSP species. The 19.1-kDa band appeared to be EndoH-resistant.
48 h post-transfection, the cells were subjected to [35S]Cys/Met pulse-chase labeling in absence or presence of lactacystin as described under “Experimental Procedures.” 1.0% Nonidet P-40 lysates were immunoprecipitated with the polyclonal anti-human IL-15 antibody; half of the immunoprecipitated material was subjected to EndoH digestion. The band at 19.1 kDa (with EndoH) in the absence of N-linked glycosylation appears to correspond to IL-15 with its LSP uncleaved and is, at least in part, a cytosolic species undergoing degradation by the lactacystin-sensitive proteasome. The bands at 14.3 and 12.0 kDa represent ER species with alternatively cleaved and fully cleaved LSP, respectively, in the absence of N-linked sugars.

**FIG. 7.** Effect of lactacystin on degradation of the 19.1-kDa band. COS cells were transfected with pEF-Neo/IL-15LSP-IL-15mp. At 48 h post-transfection, the cells were subjected to [35S]Cys/Met pulse-chase labeling in absence or presence of lactacystin as described under “Experimental Procedures.” 1.0% Nonidet P-40 lysates were immunoprecipitated with the polyclonal anti-human IL-15 antibody; half of the immunoprecipitated material was subjected to EndoH digestion. The band at 19.1 kDa (with EndoH) in the absence of N-linked glycosylation appears to correspond to IL-15 with its LSP uncleaved and is, at least in part, a cytosolic species undergoing degradation by the lactacystin-sensitive proteasome. The bands at 14.3 and 12.0 kDa represent ER species with alternatively cleaved and fully cleaved LSP, respectively, in the absence of N-linked sugars.

**FIG. 8.** EndoH resistance of the IL-15 molecule in supernatants from COS cells transfected with pEF-Neo/IL-15LSP-IL-15mp. COS cells were transfected with the indicated construct. At 48 h post-transfection, the cells were Cys/Met-starved, followed by [35S]Cys/Met labeling for 4 h. After 4 h, supernatants were harvested and immunoprecipitated with the polyclonal anti-human IL-15 antibody, and the immunoprecipitated material was subjected to EndoH digestion. The band at 17 kDa (with EndoH) in the absence of N-linked glycosylation appears to correspond to IL-15 with its LSP uncleaved and is, at least in part, a cytosolic species undergoing degradation by the lactacystin-sensitive proteasome. The bands at 14.3 and 12.0 kDa represent ER species with alternatively cleaved and fully cleaved LSP, respectively, in the absence of N-linked sugars.

**DISCUSSION**

IL-15 manifests very complex patterns of intracellular trafficking into diverse cellular compartments, including the ER, cytoplasm, and nucleus, based on alternative usage of distinct signal peptides as reported previously (11). The SSP isoform of IL-15 does not enter the ER and is not secreted. Rather, SSP-IL-15 appears in the cytoplasm and enters the nucleus. The SSP isoform of LSP-IL-15 enters multiple intracellular trafficking pathways leading to alternative destinations. The complex patterns of intracellular trafficking manifested by IL-15 contrast with those of most cytokines including IL-2, where an effective mRNA translation is followed by efficient translocation into the ER and expeditious secretion of the cytokine (6, 7). Nevertheless, there is considerable precedence for the sorting of the same protein into different cellular compartments. Examples include membrane and secreted forms of immunoglobulin, stem cell factor, colony-stimulating factor-1, epidermal growth factor, and tumor necrosis factor-α (14–16). In addition, Belin et al. (17) described both a cytosolic and a secreted N-glycosylated form of plasminogen activator inhibitor-2. They defined multiple independent interactions with the translocation machinery, with the initial binding of the mouse plasminogen activator inhibitor-2 signal sequence followed by a second relatively inefficient SRP-mediated ER-targeting event. Due to the inefficiency of this second event, there are both secreted and cytosolic forms of plasminogen activator inhibitor-2. In another system not involving a signal peptide, IL-1α precursor yields both nuclear and exogenous cytokines (15). In addition, fibroblast growth factor-2 manifests differential localization between the cytosol and nucleus that is attributable to alternative fibroblast growth factor-2 translation products (18). Furthermore, in the case of Int-2, a fibroblast growth factor-related oncoprotein, two different signal peptides are generated by the usage of different start codons associated with the alternative transport of the protein into either the secretory pathway or the nucleus (19). The case of IL-15 seems very similar to that of Int-2.

The intracellular trafficking patterns of LSP-IL-15 are quite complex. In one pathway, LSP-IL-15 in its unprocessed form is demonstrable in the cytoplasm and to a lesser extent in the nucleus, which it appears to enter by diffusion rather than by an active process. Theoretically, the form of cytoplasmic LSP-IL-15mp could represent protein that initially entered the ER, followed by passage from the ER into the cytoplasm. However, the alternative possibility is that a proportion of LSP-IL-15 is translated, but fails to be translocated into the ER; but defaults into the cytoplasm appear more likely. Furthermore, the cytoplasmic unglycosylated form of IL-15 appears to be rapidly degraded by cytoplasmic proteasomes in light of the inhibition of this process by lactacystin, a specific active-site inhibitor of proteasomal activity. The suggestion that some LSP-IL-15 is not translocated into the ER, but is translated and released directly into the cytoplasm, implies a relatively inefficient translocation process compared with an efficiently translocated molecule such as preprolactin. This inefficient translocation process may be viewed in the context of the unusual characteristics of the LSP, a signal peptide that is exceptionally long (48 aa) compared with the SP of most secreted molecules. The LSP of IL-15 also differs from prototypic signal sequences in its provisional secondary structure in that it appears to contain two hydrophobic elements. Plasminogen activator inhibitor-2 has a similar long SP that also includes two hydrophobic domains (17). Plasminogen activator inhibitor-2, in turn, manifests inefficient translocation, yielding both cytosolic and secreted forms of the protein. We have preliminary evidence supporting less than optimal IL-15 translocation using a cell-free system. In these studies directed toward examining IL-15 translation and translocation, we examined the impact of the addition of SRPs into a wheat germ in vitro translation assay system. The translation of LSP-15 was only slightly inhibited by the highest SRP concentration, in contrast to the positive control preprolactin, which was significantly inhibited by SRP even at low concentrations. These data support the COS cell studies with LSP-IL-15 and explain in part the existence of cytosolic IL-15, which defaults after insignificant SRP-mediated translational arrest. These observations add relatively inefficient translocation into the ER to the multiple impediments to IL-15 synthesis and secretion that had been identified previously.

A second pathway involving LSP-IL-15 involves translocation into the ER, followed by N-glycosylation and alternative processing with the putative elimination of 29 aa of the LSP. On the basis of studies with tunicamycin and EndoH, these alternative processed LSP-IL-15 species are N-glycosylated, in accord with their presence in the ER. A number of experiments support the proposed alternative, partial cleavage of the LSP. In contrast to the single species on SDS-PAGE obtained with IL-2SP-IL-15mp when tunicamycin was present, three species were observed with IL-15LSP-IL-15mp in the presence of tunicamycin at 18.8, 14.5, and 13.0 kDa. Cleavage of the IL-15...
LSP was also shown to occur at two sites when a fusion construct was used in which cDNA coding for the LSP was linked to GFP (32.4 uncleaved, 29.2 alternatively cleaved, and 27.9 kDa fully cleaved forms were observed). Further evidence for alternative cleavage within the LSP of IL-15 came from experiments in which an IL-15(LSPΔEx5)-GFP construct was utilized in which exon 5 was eliminated, thereby removing the mature protein cleavage site. As noted above, the intermediate sized bands appear to occur as a result of alternative cleavage of LSP rather than as a result of an alternative initiation site. Based on the size of the proteins on SDS-PAGE and an analysis of the LSP sequence (Fig. 4), we suggest that the alternative cleavage may occur between Ala29 and Gly30 of the signal peptide, i.e. 19 aa upstream of the start site for the mature protein. Indeed, the amino acid sequence at this site (Thr27-Glu28-Ala29) is identical to the tripeptide at the mature protein complete cleavage site (Thr46-Glu47-Ala48).

Partial and complete processing of leader sequences of comparable length to the IL-15 LSP are observed with proteins that are translocated from the cytoplasm to mitochondria (20, 21). In the case of mitochondria, however, there is stepwise processing of the N-terminal targeting leader peptide, with partial processing observed during the initial transfer into the mitochondrial organelle and with full processing subsequently occurring in association with a second transmembrane translocation into the correct organellar compartment (20, 21). In the present case, there does not appear to be a comparable stepwise processing of the LSP of IL-15. The alternatively cleaved form became evident subsequent to the first appearance of the fully cleaved form. Thus, in contrast to the pattern for mitochondrial signal peptides, the alternative cleaved LSP does not appear to represent the precursor of the majority of the fully processed protein. Although we have no data supporting this view, we consider the possibility that the alternatively processed and fully processed forms of LSP-IL-15 have distinct trafficking pathways and fates. The fully processed glycosylated form is secreted directly. However, IL-15 retaining the alternatively processed LSP was not demonstrable as such in cell supernatants. It is possible that it is retained in vesicles for subsequent intracellular release or, alternatively, secretion with a final processing step occurring at this terminal stage. Another possibility is that the alternatively processed form, like poorly folded proteins, is transferred out of the ER into the cytoplasm, where it may be degraded (22, 23).

The third pathway of LSP-IL-15 trafficking is the classical one for a secreted interleukin molecule. In this pathway, LSP-IL-15 enters the ER and is N-glycosylated at two sites; the signal peptide is fully processed; and glycosylated mature IL-15 is secreted. The following evidence supports this pathway of secretion mediated by a conventional ER-to-Golgi route. Fully processed glycosylated IL-15 is the single form of IL-15 demonstrable in culture supernatants of LSP-IL-15-transfected COS cells or cells such as CV1-EBNA or Huh7-102 that secrete IL-15 (2, 3). In addition, brefeldin A treatment that collapses the Golgi stacks virtually abrogated IL-15 secretion from COS cells transfected with the LSP-IL-15 construct. Furthermore, no difference in the migration of the secreted form of IL-15 was seen after EndoH treatment, indicating that this species does pass through the medial Golgi prior to secretion. Although IL-15 is demonstrable in the culture supernatants, we have emphasized previously that the time required for passage through the ER with transfer to the Golgi is much greater for IL-15 than for IL-2 with the IL-2 SP, which is rapidly secreted (5). Thus, the data support the view that at many stages, the intracellular trafficking and secretory pathways of IL-15 are very inefficient ones. Onu et al. (8) came to a similar conclusion using various IL-15 constructs. This group did not note any secretion of IL-15 when the IL-15 LSP was employed. However, after replacing the IL-15 LSP with that of the CD-33 molecule, translation and secretion increased, supporting the view that IL-15 expression is mainly controlled post-transcriptionally at the levels of translation and secretion (8).

The present studies, taken in concert with those we have reported previously (5–7), suggest that as in the case of insulin and IL-15 (24–26), there is multifaceted regulation of IL-15 translation, intracellular trafficking, and secretion. There are multiple impediments to IL-15 translation that have been identified, including elements of the 5′-untranslated region, the LSP with its start codon in a poor Kozak configuration, and the 3′-coding sequence of IL-15 (5). Furthermore, in cells so far examined, we have observed inefficient IL-15 trafficking in the ER, with subsequent secretion. Thus, there are a broad array of negative regulatory controls affecting IL-15 expression. These negative regulatory features may be required due to the potency of IL-15 in inducing the expression of tumor necrosis factor-α, IL-1β, interferon-γ, and other cytokines and chemokines involved in the inflammatory response (26–29). If these molecules were indiscriminately or constitutively expressed, they could be associated with serious inflammatory disorders including autoimmune diseases. However, removal of these negative regulatory controls in an integrated fashion might give rise to a major increase in IL-15 synthesis and secretion. Thus, in terms of a more positive role for IL-15, as a hypothesis we propose that by maintaining translationally and translocationally inactive IL-15 mRNA and protein pools, diverse cells may respond rapidly to an intracellular infection or other stimulus by transforming IL-15 mRNA and protein into ones that can be effectively translated, translocated, and secreted. The IL-15 protein secreted could, in turn, convert CD8 T and NK cells into effective killer cells, which may provide an effective host response to the infectious agents.

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