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The 5′ Untranslated Region, Signal Peptide, and the Coding Sequence of the Carboxyl Terminus of IL-15 Participate in Its Multifaceted Translational Control

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We previously reported that the AUG-burdened 5′ untranslated region (UTR) of IL-15 mRNA impedes its translation. Here we demonstrate that the nucleotide or protein sequences of the IL-15 signal peptide and carboxyl terminus also contribute to the poor translation of IL-15 transcripts. In particular, the exchange of the IL-15 signal peptide coding sequence with that of IL-2 increased cellular and secreted levels of IL-15 protein 15- to 20-fold in COS cells, while IL-2 transcripts with the IL-15 signal peptide generated 30- to 50-fold less IL-2 protein than wild-type IL-2. Furthermore, the addition of an artificial epitope tag to the 3′ coding sequence of IL-15 increased its protein production 5- to 10-fold. Combining these two IL-15 message modifications, in addition to removing the 5′ UTR, increased IL-15 synthesis 250-fold compared with a wild-type construct with an intact 5′ UTR. These data suggest that IL-15 mRNA, unlike IL-2 mRNA, may exist in translationally inactive pools. By storing translationally quiescent IL-15 mRNA, cells might respond to intracellular infections or other stimuli by rapidly transforming IL-15 message into one that can be efficiently translated.


Immune responses are regulated by a series of proteins, termed cytokines, that exhibit a high degree of redundancy and pleiotropy. These cytokines play critical roles in the regulation of a wide range of functions in various cell types. Recently, a novel cytokine, IL-15, was codiscovered in two laboratories (1–3). The action of IL-15 on T and NK cells requires the expression of both IL-2Rα and γc shared with IL-2 (2–4). Due in part to this sharing of receptor subunits, there is considerable redundancy in the actions of IL-2 and IL-15. Both cytokines activate the proliferation and differentiation of T, NK, and B cells (3, 5, 6). Initially, it was not clear why two cytokines with such similar actions should have evolved and been retained. However, further analysis revealed dramatic differences between these two cytokines in terms of their cellular sites of synthesis and the levels of control of their synthesis and secretion (7, 8). IL-2 is produced by activated T cells, and its expression is controlled predominantly at the level of mRNA transcription and message stabilization. In contrast with the T cell pattern of IL-2 mRNA expression, IL-15 mRNA expression is widespread. In particular, Northern blot analysis revealed a broad constitutive expression of IL-15 mRNA in diverse tissues, including placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells, and activated monocytes (3, 7). Despite the almost ubiquitous expression of IL-15 mRNA, it has been difficult to demonstrate IL-15 protein in the supernatants of many cells that express message for this cytokine, one of the hallmarks of translational regulation. In earlier studies, we observed that even though LPS/IFN-γ-activated monocytes express high levels of IL-15 message, the culture supernatants from these cells contain little or no IL-15 protein as assessed by either an IL-15 specific ELISA or a CTLL-2 proliferation assay (7). This suggested that normal IL-15 protein production is predominantly regulated posttranscriptionally. The discordance between IL-15 message expression and IL-15 protein production led us to examine IL-15 mRNA for translational impediments. We previously reported that the IL-15 message contains a complex 5′ UTR with 10 upstream AUGs (7). COS cells transfected with an expression construct lacking the 5′ UTR produced four- to fivefold more IL-15 protein than cells transfected with a construct that retained the 5′ UTR. However, the levels of IL-15 protein synthesized and secreted were still very low (~3 logs less than those obtained with a comparable IL-2 construct), suggesting the possibility that additional regulatory elements exist (7). Here we demonstrate that IL-15 expression is posttranscriptionally impeded not only by the 5′ UTR, but also by the coding sequence and/or protein sequence of its signal peptide (SP) and mature protein (mp) carboxyl terminus (C terminus).

Materials and Methods

Cell lines and cell culture

The simian kidney epithelial cell line, COS, and the cytokine-dependent murine cytotoxic T cell line, CTLL-2, were both purchased from the American Type Culture Collection (Rockville, MD). Conditions for CTLL-2 bioassays were previously described (1), and COS transfection conditions are described below.

Abs and cytokines

The rabbit anti-human IL-15 polyclonal Ab used for Western blot analysis was a gift from Harmesh Sharma (Genzyme, Cambridge, MA), and the

1 This work partially satisfied R.N.B.’s Ph.D. requirements in genetics at George Washington University (Washington, DC).

2 Address correspondence and reprint requests to Dr. Richard N. Bamford, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-1374.

3 Abbreviations used in this paper: UTR, untranslated region; mp, mature protein; SP, signal peptide; CDS, coding sequence; TET, transcription and translation; ER, endoplasmic reticulum; SRP, signal recognition particle.
rabbit anti-human IL-15 polyclonal used for immunoprecipitation was purchased from Serotec. Recombinant human IL-15 was purchased from Peprotech, Inc., and recombinant IL-2 was a gift from Hoffmann-La Roche, Nutley, NJ.

IL-15 and IL-2 expression constructs

The generation of the pEF-Neo/ΔUG-IL-15 expression construct was previously described (7). pEF-Neo/IL-2 CDS was constructed by amplifying the IL-2 CDS (nucleotides 45–509) using the sense primer (primer 1) 5′-ATCGAATTCACAATGTCAGGATGCAACTCC-3′ and the antisense primer (primer 2) 5′-GATCCGGATCCAGAATTTCAGGATGTCAGGATGCAACTCC-3′. The antisense primer was directly replaced with a NarI restriction site. The IL-2 construct with the NarI site was a gift from Dr. J. Reiner (1). The NarI site was then digested out of the vector with NarI and blunt ended. The NarI site was then ligated into the NarI sites of pEF-Neo and pEF-Neo/ΔUG-IL-15 to generate pEF-Neo/ΔUG-IL-2 and pEF-Neo/ΔUG-IL-15. The antisense primer (primer 1) 5′-GATCCAATTCAATGTCAGGATGCAACTCC-3′ (ΔUG-IL-2) and the sense primer (primer 2) 5′-GATCCGGATCCAGAATTTCAGGATGTCAGGATGCAACTCC-3′ (ΔUG-IL-15) were PCR amplified from existing constructs. For IL-15, the antisense primer (primer 3) 5′-GATCAGAGCTTGGTTCTGTTTAGAAGC-3′ (ΔUG-IL-2) and the sense primer (primer 4) 5′-GATCCGGATCCAGAATTTCAGGATGTCAGGATGCAACTCC-3′ (ΔUG-IL-15) were also PCR amplified from existing constructs.

IL-15 and IL-2 transfectants generated roughly 350,000 pg of secreted IL-2 and IL-15 proteins in 60-mm plates (five per construct) in 2.5 ml of complete DMEM. At 48 h, each plate was pulsed with 150 μCi of [35S]Cys/[35S]Met (New England Nuclear, Boston, MA, express labeling mix) in 2.5 ml of Cys/Met-free DMEM with 10% dialyzed FCS (cells were Cys/Met starved before the addition of [35S]Cys/Met). After 1 h in [35S]Cys/Met at 37°C, cells were washed twice in PBS and then 3 ml of complete DMEM was added at 37°C. Lysates were made at the indicated time points in 1 ml of RIPA buffer.

Western blotting, ELISAs, and immunoprecipitation

For IL-15 and IL-2 transfectants, supernatants were collected for assay. For Western analyses and mRNA stability studies, the DEAE-dextran protocol was scaled up to 1.6 × 10⁶ cells in 75-cm² flasks. Cellular lysates generated for Western blots were made by adding 1 ml of RIPA buffer/flask (50 mM Tris-HCl (pH 7.4), 1.0% Nonidet P-40, 0.25% NaDOC, 0.15 M NaCl, 1 mM EGTA, 1 mM PMSE, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1% SDS). For mRNA analyses, see below. For these studies the culture media for IL-2 and IL-15 ELISAs, COS cells were transfected using Lipofectamine (Life Technologies, Grand Island, NY) following the manufacturer’s recommendations scaled up for 1.6 × 10⁶ cells in 75-cm² flasks. Twenty-four hours after transfection with IL-15 and IL-2 constructs, supernatants were harvested, and the COS cells were trypsinized and washed in PBS. One-fifth of the cells were lysed in RIPA buffer, and the remainder were aliquoted equally into four 60-mm plates with 2.5 ml of fresh complete DMEM. One plate was then harvested (supernatants and lysates) every 24 h for 120 h. To lessen the likelihood of cytokine degradation, supernatants were collected from the remaining plates throughout the time course and replaced with fresh medium.

For IL-15 protein degradation studies, 1 × 10⁵ COS cells (in 400 μl of 1× PBS) were electroporated with 15 μg of the indicated construct (950 μf, 240 V). Electroporated cells were evenly aliquoted into 60-mm plates (five per construct) in 2.5 ml of complete DMEM. At 48 h, each plate was pulsed with 150 μCi of [35S]Cys/[35S]Met (New England Nuclear, Boston, MA, express labeling mix) in 2.5 ml of Cys/Met-free DMEM with 10% dialyzed FCS (cells were Cys/Met starved before the addition of [35S]Cys/Met). After 1 h in [35S]Cys/Met at 37°C, cells were washed twice in PBS and then 3 ml of complete DMEM was added at 37°C. Lysates were made at the indicated time points in 1 ml of RIPA buffer.

Results

The immunoprecipitation of IL-15 from [35S]labeled pulse/chase lysates used Ultrasilk immobilized protein A/G (Pierce). Procedures, including incubation times, anti-IL-15 Ab concentrations, and washings, closely followed the manufacturer’s recommendations.

Detection of mRNA and mRNA stability

Total RNA was isolated from transfected COS cells using TRizol (Life Technologies). RNA samples (5 μg) were assayed by Northern blot analysis for IL-15, IL-2, and β-actin mRNA following a published protocol (10). For mRNA stability studies, COS cells were replated on 60-mm plates (five per construct) 24 h posttransfection. At 72 h, 5 μg/ml of actinomycin D (Life Technologies) in 3 ml of complete DMEM was added to the cells, and total mRNA was isolated at the indicated time points. Relative levels of mRNA expression were determined using a Storm-840 (Molecular Dynamics, Sunnyvale, CA).

Results

Elements in addition to the 5′ UTR impede the expression of the IL-15 protein

To demonstrate that elements in addition to the 5′ UTR negatively influence IL-15 protein production, we compared the translational efficiencies of IL-2 and IL-15 constructs in a COS cell expression system. Specifically, IL-15 and IL-2 coding sequences lacking their 5′ and 3′ UTRs were subcloned into the expression vector, pEF-Neo, that uses the strong elongation factor promoter. The IL-15 construct, pEF-Neo/ΔUG-IL-15, and the IL-2 construct, pEF-Neo/IL-2 CDS, were transiently transfected into 2 × 10⁶ COS cells using DEAE-dextran in 35-mm plates, and supernatants were harvested 72 h later. The quantities of IL-2 and IL-15 protein in the supernatants were determined by their ability to stimulate CTL-2 cell proliferation ([3H]Tdr incorporation) compared with that of IL-15 and IL-2 standards. We determined in this study that the IL-2 transfectants generated roughly 350,000 pg of secreted IL-2
protein, while the IL-15 transfectants generated only about 360 pg, a 1,000-fold difference (Fig. 1). Subsequent studies assessing IL-2 and IL-15 mRNA expression in transfected COS cells over time showed virtually equal levels of transcript for the two cytokines throughout the time course despite the disparity in protein production observed (Fig. 2). Additionally, IL-15 transcripts generated from pSP64pA/ΔAUG-IL-15 were readily translated in a wheat-germ lysate, in vitro transcription and translation (TnT) system, but were poorly translated in a rabbit reticulocyte TnT system. In contrast, IL-2 transcripts generated using the same vector system expressed virtually equal quantities of IL-2 protein in the wheat-germ and rabbit reticulocyte TnT systems (data not shown). Collectively, these data suggest that in mammalian systems (i.e., COS cells and rabbit reticulocyte lysates) there are inhibitory/regulatory factors in addition to those in the 5' UTR that interfere with the efficient synthesis of IL-15.

Role of IL-15 SP and C terminus in the posttranscriptional regulation of IL-15 expression

We next examined the IL-15 mRNA for specific elements that might impede IL-15 expression and focused on the putative IL-15 SP. IL-15 has been shown to have an unusually long 48-amino acid SP compared with those of other secreted proteins, whose SPs average 15 to 30 amino acids in length. Therefore, we considered the possibility that the IL-15 SP and/or its coding sequence is a negative regulator of IL-15 generation (8). To test this hypothesis, we prepared expression constructs that exchanged the SP coding sequences of IL-2 and IL-15 so that they were linked to the alternate mp coding sequence. The resulting chimeric cDNAs were subcloned into pEF-Neo (i.e., the IL-2 SP with the IL-15 mp coding region (pEF-Neo/IL-2sp/IL-15 mp) and reciprocally pEF-Neo/IL-15sp/IL-2 mp) in which the coding sequences for their SPs had been exchanged. The chimeric constructs were compared with wild-type IL-15 and IL-2 constructs (pEF-Neo/ΔAUG-IL-15 and pEF-Neo/IL-2CDS). COS supernatants were harvested after 72 h, and the influence of the SPs was determined for IL-15 (A) and IL-2 (B) by assessing the total quantity of cytokine in the supernatants using a CTLL-2 bioassay.
protein over a 120-h time course. Supernatants and lysates were harvested every 24 h for 5 days, and ELISAs were used to measure the total IL-15 and IL-2 contents in the supernatant and lysate ([supernatant volume] × (ng/ml of cytokine in supernatant) + (lysat volume) × (ng/ml of lysate)). It was determined from these studies that regardless of the SP (its own or IL-2’s), IL-15 was secreted more slowly than wild-type IL-2 or chimeric IL-2 with the IL-15 SP (Fig. 4). However, at the latter time points (90 and 120 h), the majority of IL-15 protein produced from either construct had been secreted. Furthermore, even though the IL-15 SP contributed to a minor, but meaningful, increase in the percentage of wild-type IL-15 or chimeric IL-2 (IL-15sp/IL-2 mp) retained in the cell over time (compare Fig. 4A with 4B and Fig. 4C with 4D), the primary effect of the IL-15 SP was on the total amount of these cytokines generated. In particular, at the 72 h time point, roughly 45% of wild-type IL-15 was retained in the cell, but only a total of 370 pg (cellular plus secreted) was detected. However, at the same time point, 30% of the IL-15 chimeric with the IL-2 SP was retained within the cell, but a total of 6430 pg of IL-15 protein was present in the cell lysates and secretions, representing a 17-fold increase in total protein synthesized compared with that observed with wild-type IL-15. Similar observations were made with wild-type IL-2 vs chimeric IL-2. Specifically, the IL-15 SP slightly increased the proportion of chimeric IL-2 protein retained in the cell, but dramatically decreased, by roughly 30-fold, the total amount of IL-2 produced compared with the effect of wild-type IL-2. In these studies the ELISA results closely paralleled those with CTLL-2 bioassays that used the same constructs. Therefore, it can be concluded that the IL-15 SP and/or its coding sequence act to impede IL-15 protein expression, but the SP per se does not lead to an extensive accumulation of IL-15 within intracellular pools or on the cell surface.

It was noted from the above data that the inclusion of the IL-15 SP coding sequence on IL-2 did not reduce the expression of IL-2 to that of IL-15. This observation led us postulate that other elements in addition to the SP and 5′ UTR were negatively affecting IL-15 protein generation. In support of this, it was discovered serendipitously that a negative element may also exist in the C terminus of the IL-15 mp coding sequence and/or protein. Specifically, for the purpose of Ab detection, the coding sequence for the artificial epitope, FLAG, was engineered into the 3′ end of IL-15’s coding sequence. Surprisingly, we noted that the presence of FLAG (ΔAUG-IL-15-FLAG) increased total IL-15 production (cellular plus secreted) 5- to 10-fold over that produced by ΔAUG-IL-15 when they were transiently transfected into COS cells in the pEF-Neo expression system (Fig. 5A). Both these constructs lack any natural 3′ UTR nucleotides, so only the IL-15 coding sequence is affected by FLAG’s coding sequence. Additionally, to the best of our knowledge, there is no example of the FLAG epitope coding sequence enhancing the translation of mRNAs tagged with it. These data, therefore, suggest the existence of additional negative regulatory elements within the 3′ coding sequence or C terminus of IL-15. Apparently the presence of FLAG disrupts this putative, as yet undefined, negative regulatory mechanism.
To determine whether the SP and C terminus modifications to IL-15 transcripts could act synergistically, we engineered an IL-15 pEF-Neo construct with the IL-2 SP and FLAG epitope coding sequence. As with the other constructs, the IL-2sp/IL-15 mp/FLAG construct was transfected into COS cells, and the total intracellular and secreted quantities of protein were assessed by ELISA. The combination of the SP and carboxyl modifications acted cooperatively to boost IL-15 protein generation. Specifically, the IL-2sp/IL-15 mp/FLAG construct generated 40 to 50 times more protein than ΔAUG-IL-15 (data not shown), whereas the FLAG only modification, as demonstrated earlier, enhanced expression by only 5- to 10-fold, and the SP modification enhanced expression by only 17- to 20-fold. IL-2sp/IL-15 mp/FLAG was also compared with an IL-15 construct with its full complement of upstream AUGs in a COS cell transfection. The chimeric transcripts generated >250-fold more protein than the IL-15 transcripts with the upstream AUGs, emphasizing the full inhibitory potential of these negative elements (Fig. 5B). Additionally, lysates from the same transfected COS cells were assessed by Western blot analysis for IL-15 protein generation. The differences in intracellular IL-15 protein levels previously noted using the ELISA technique were also easily visualized by Western blot analysis (Fig. 6A). Importantly, to control for the possibility of a transcriptional difference between the various IL-15 constructs, Northern blot analysis for IL-15 was conducted on the same transfectants (Fig. 6B). There was virtually no difference between levels of IL-15 message generated with the alternative constructs, indicating that the protein expression differences seen with the constructs were not transcriptional, but were due to posttranscriptional events.

Based on the above studies alone, it appeared that the IL-15 SP and C terminus interfered with efficient IL-15 expression, but the exact molecular level of this regulation was not precisely defined and was open to many alternative interpretations. Possibilities included 1) instability of the mRNA within this region of coding sequence, 2) SP mediated rapid catabolism of the nascent proteins, or 3) inhibition of initiation, elongation, or termination of mRNA translation mediated by the SP. These alternatives are addressed below.

**IL-15 and IL-2 protein expression differences are not explained by differences in mRNA stability**

Since we demonstrated relatively equal levels of IL-15 transcript expression for the various IL-15 and IL-2 constructs, it seemed unlikely that differences in mRNA stability could play a major role in the differences seen in the quantities of IL-15 protein generated. However, to address this issue more critically, COS cells were transfected with the above constructs, and after 72 h, the antibiotic, actinomycin D (5 μg/ml), was added to the cells. Actinomycin D arrests transcription in eukaryotic cells, allowing for assessment of sequence. As with the other constructs, the IL-2sp/IL-15 mp/FLAG construct was transfected into COS cells, and the total intracellular and secreted quantities of protein were assessed by ELISA. The combination of the SP and carboxyl modifications acted cooperatively to boost IL-15 protein generation. Specifically, the IL-2sp/IL-15 mp/FLAG construct generated 40 to 50 times more protein than ΔAUG-IL-15 (data not shown), whereas the FLAG only modification, as demonstrated earlier, enhanced expression by only 5- to 10-fold, and the SP modification enhanced expression by only 17- to 20-fold. IL-2sp/IL-15 mp/FLAG was also compared with an IL-15 construct with its full complement of upstream AUGs in a COS cell transfection. The chimeric transcripts generated >250-fold more protein than the IL-15 transcripts with the upstream AUGs, emphasizing the full inhibitory potential of these negative elements (Fig. 5B). Additionally, lysates from the same transfected COS cells were assessed by Western blot analysis for IL-15 protein generation. The differences in intracellular IL-15 protein levels previously noted using the ELISA technique were also easily visualized by Western blot analysis (Fig. 6A). Importantly, to control for the possibility of a transcriptional difference between the various IL-15 constructs, Northern blot analysis for IL-15 was conducted on the same transfectants (Fig. 6B). There was virtually no difference between levels of IL-15 message generated with the alternative constructs, indicating that the protein expression differences seen with the constructs were not transcriptional, but were due to posttranscriptional events.

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After the addition of actinomycin D, total RNA was isolated at 0, 2.5, 5.0, 7.5 and 10 h and was quantitated by Northern blot analysis for IL-15 and \( \beta \)-actin transcripts. The levels of IL-15 message were comparable using the different constructs (Figs. 7 and 8). In particular, the stability of the IL-15 transcripts was not significantly altered by SP or FLAG epitope coding sequence modifications, with the half-life of each of these transcripts exceeding at least 8 h. As would be predicted, the half-life of \( \beta \)-actin transcripts easily exceeded 16 h in these same samples (Figs. 7 and 8). Additionally, there was no significant difference between the survival of IL-2 transcripts linked with the IL-2 or, alternatively, with the IL-15 signal coding sequence (data not shown). These data eliminate differences in mRNA stability as the dominant contributor to the disparity in IL-15 and IL-2 protein expression observed in this system.

Instability of processed protein does not explain the differences in IL-15 protein expression

Another possible explanation for the differences in the quantities of IL-15 and IL-2 protein expressed is that SP and FLAG had an effect on the proteins at the levels of their intracellular stability and their rates of breakdown. To address this possibility, \( \Delta \text{AUG-IL-15} \), IL-2sp/IL-15 mp pEF-Neo constructs, and IL-15/FLAG were transfected into COS cells followed at 48 h by a 1-h \([35 \text{S}]\text{Cys/Met} \) pulse labeling with a subsequent cold chase. To enhance IL-15 expression in these studies, COS cells were transfected by electroporation, not DEAE-dextran. Additionally, the \( \Delta \text{AUG-IL-15} \) construct was given an initiator codon with a higher Kozak context; however, this modification did not alter the coding sequence. Cell lysates using RIPA buffer were prepared 0, 30, 60, 90, and 120 min after the cold chase. The lysates were subsequently immunoprecipitated with a rabbit anti-human IL-15 polyclonal and resolved on SDS-PAGE. These studies indicate that the modifications to IL-15 do not change the stability of the processed protein compared with that of wild-type IL-15 protein (Fig. 9). In fact, even 120 min after the cold chase, the level of wild-type IL-15 protein was not substantially reduced relative to levels of the

**FIGURE 7.** Stability of IL-15 mRNA with SP and C terminus modifications. COS cells were transfected with the indicated pEF-Neo construct (A–C). After 72 h, cells were treated with 5 \( \mu \text{g/ml} \) of actinomycin D. RNA was isolated at the indicated time points and assessed for IL-15 transcripts (column 1) by Northern blot analysis. The same filter was then rehybridized with \( \beta \)-actin (column 2).

**FIGURE 8.** Quantitation of IL-15 and \( \beta \)-actin mRNA levels over a 10-h time course in actinomycin D. Relative amounts of mRNA (from Fig. 7) were determined using a Storm-840 (Molecular Dynamics) and are represented as a percentage of the time zero mRNA level. Lines represent a least squares fit with a 95% confidence interval. \( \beta \)-Actin levels are an average of results of the three experiments.

**FIGURE 9.** Protein stability assessment of IL-15 with SP and C terminus modifications. COS cells were transfected with the indicated pEF-Neo constructs. After 48 h, cells were subjected to a \([35 \text{S}]\text{Cys/Met} \) pulse/chase labeling (as detailed in Materials and Methods). A–C indicate the time course of decline in protein concentrations in: A, wild-type IL-15 protein (showing three glycosylated species); B, IL-15-FLAG (the presence of the FLAG epitope slightly increases the m.w. of three species); and C, IL-2sp/IL-15 mp (showing two predominant glycosylated species).
FLAG- and SP-modified IL-15 proteins. However, these data clearly demonstrate the presence of more than one glycosylated species of IL-15; interestingly, wild-type IL-15 protein and FLAG-modified IL-15 show three species, whereas IL-15 protein modified with IL-2 SP shows only two. These data suggest that IL-15 SP may be processed unusually, and in fact, this is supported by recent observations (R. N. Bamford and G. Kurys, unpublished observations). Nevertheless, the rate of intracellular IL-15 catabolism does not appear to be decreased by the presence of the IL-2 SP or the FLAG epitope.

Collectively, the above studies suggest that the negative regulatory influence of the IL-15 SP and the C terminus on IL-15 protein generation are not at the levels of either mRNA or protein instability, but rather reside in the control of IL-15 translation initiation, elongation, and/or termination.

Discussion
IL-15 is a recently discovered cytokine that shares many features with IL-2; they both are members of the four α-helix bundle cytokine family, they require receptor subunits IL-2Rβ and γc for their action, and they activate the proliferation and differentiation of T, NK, and B cells (1–3). However, further analysis revealed dramatic differences between these two cytokines in terms of their cellular sites of synthesis and the molecular levels of regulation of their expression (7, 8). IL-2 is predominantly controlled at the level of message transcription and stabilization. The regulation of IL-15 appears to be much more complex, with controls at the levels of message transcription, message translation, and protein secretion. Although IL-15 expression is regulated in part at the level of transcription, it appears that the predominant regulation of IL-15 expression is posttranscriptional, since there is a marked discordance between the high levels of widely expressed IL-15 message and the little or no IL-15 protein demonstrable in the cytoplasm or supernatants of cytokine message-expressing cells.

We considered four possible posttranscriptional mechanisms that might impede the synthesis and secretion of IL-15, including IL-15 mRNA instability, protein instability, retention of IL-15 in intracellular pools, and impeded IL-15 mRNA translation. We conclude that the first three mechanisms are not the predominant factors affecting IL-15 synthesis and secretion. Therefore, we focused on the alternative possibility that the major impediment to IL-15 synthesis is at the level of mRNA translation.

Control of translation has been observed with a variety of proteins, and this regulation can occur at all levels of translation (e.g., initiation, elongation, and termination). Examples of both the control of global mRNA translation rates and the regulation of translation of individual or small groups of mRNAs have been reported (11). Most mRNA-specific translational regulation has involved cis-acting RNA sequence elements that mediate regulation. Such regulatory sequences in the 5′ or 3′ UTR of the mRNAs as well as the mature coding sequence (12) have been observed in transcripts for ferritin (13), erythroid 5-aminolevulinate synthase (13), thymidylate synthase (14), and murine p53 (15).

In addition to specific regulatory protein/RNA interactions, there is considerable precedence for an inhibitory effect on translation manifested by upstream AUGs in the 5′ UTR upstream of the authentic initiation codon (16, 17). The 5′ UTR of effectively translated messages are short, simple, and unencumbered by AUGs upstream of the initiation AUG. In contrast, AUG-burdened 5′ UTRs, including those encoding many proto-oncogene, transcription factors, a variety of receptor proteins, and signal transduction components, are poorly translated (16, 17). It has been suggested that some of these transcripts may not use cap-depen-
transfected with the wild-type IL-15 coding sequence (ΔAUG-IL-15) at 120 h, at least 90% of the IL-15 protein (though at low levels) could be identified in the culture supernatants, and <10% was retained within the cell, as evaluated by sensitive ELISA assays. These data reemphasize that IL-15 protein is a secreted molecule, but its overall production is very low. Thus, noting the large discrepancy between the total quantities of IL-15 protein generated from ΔAUG IL-15 and chimeric IL-15 (IL-2sp/IL-15 mp) transcripts, it appears that the predominant level of regulation of IL-15 synthesis mediated by the SP is at the level of translation, with a lesser component at the level of intracellularly trafficking and secretion per se.

The SP has been shown to be a participant in a normal phenomenon that involves a transient impediment to translation elongation that affects secreted and cell surface membrane proteins. However, it has not been a major focus of studies on the differential regulatory mechanisms that lead to the induction of specific protein expression. The binding of the signal recognition particle (SRP) to the SP causes signal sequence-dependent and site-specific arrest of chain elongation (22). The SRP binds to the SP as soon as the peptide emerges from the ribosome. This causes a pause in protein synthesis, which presumably gives the ribosome enough time to bind to the ER membrane before synthesis of the polypeptide chain is completed, thereby ensuring that the protein is not released into the cytoplasm. This translational arrest is released when SRP binds its receptor, or docking protein, that is exposed on the cytosolic surface of the rough ER membrane. The binding results in chain completion and transfer into the ER.

The mechanisms underlying the SP coding sequence- and/or protein sequence-mediated regulation of IL-15 translation have not been defined. However, with preliminary in vitro translation studies, we observed that the addition of canine microsomal membranes did not result in IL-15 chain completion and translocation into microsomes in contrast to the situation with the prototypical secretory protein, pre/prolactin, which was fully translocated and processed, (data not shown). Therefore, a number of events or factors may be required for efficient IL-15 mRNA translation/translocation. It is possible that a translational activator(s) for chain elongation and translocation may be needed. Alternatively, a translational repressor or a stable secondary structure in the mRNA may prevent efficient IL-15 mRNA elongation and translocation. Furthermore, inefficient initiation of translation may contribute partially to the low levels of IL-15 protein generated in transfected COS cells. This stems from the observation that the start codon for the IL-15 coding sequence has a weak Kozak context (GTA ATGA) (16, 17). In fact, modifying the start codon to a higher context (ACCATGG or GCCGCCCATGA) increased IL-15 protein production four- to fivefold in transfected COS cells (data not shown).

In terms of regulation at the level of translation for specific proteins, 70-kDa heat shock protein mRNA translation in chicken reticulocytes has been shown to be controlled at the level of elongation (23). IL-1β expression is also regulated in part at the chain elongation termination phases of translation (24). Translational control has been demonstrated with other cytokines, including TNF-α (25), TGF-β3 (26), TGF-β1 (27), and granulocyte-macrophage CSF (28). Additionally, it has been reported that one of the multiple levels of insulin biosynthesis regulation includes a glucose-dependent, signal recognition, particle-mediated translational arrest (29).

Our studies also reveal that the FLAG epitope modifications made to the C terminus of the IL-15 constructs enhanced the generation of IL-15 protein. As with the SP, the FLAG coding sequence does not appear to substantially affect mRNA stability, nor does the FLAG peptide sequence affect protein secretion or stability, suggesting that the effect is on translation. Noting the position of the FLAG epitope coding sequence, it can be suggested that its presence may enhance the rate or the efficiency of translation termination compared with the situation with wild-type IL-15. Interestingly, it has been reported for the CMV gene, gp48, that an upstream open reading frame in the transcript does not efficiently terminate translation, and ribosomes stack up behind the paused ribosome (30). This inhibitory effect is mediated by the amino acid coding information in the vicinity of the stop codon, and it can be eliminated by adding a single codon to the C terminus of the peptide (31). Although this would be one possible explanation for FLAG’s effect on IL-15 translation, others may include the disruption of a critical secondary structure or protein binding site in IL-15 mRNA by the FLAG coding sequence.

The present studies taken in concert with those we have previously reported (7, 8) suggest that a major factor in the control of IL-15 expression is multifaceted regulation at the level of IL-15 mRNA translation. In particular, elements of the 5’ UTR, including 10 upstream AUGs, the SP, and the 3’ coding sequence of IL-15, participate in its posttranscriptional regulation. When the above three IL-15 message modifications were combined into a single construct, at least 250-fold more protein was generated than with the wild-type IL-15 construct with an intact 5’ UTR. Furthermore, although the total IL-15 production was increased with the modified construct, the ratio of secreted to retained cytokine was not significantly influenced, suggesting that the major focus of this regulation is at the level of translation rather than a dominant effect on protein secretion. These studies indicate that the translational control of IL-15, like that of insulin, occurs at multiple distinct levels. The removal of these negative control mechanisms in an integrated fashion may give rise to a major increase in IL-15 synthesis. The broad array of negative regulatory features controlling IL-15 expression may be required due to the potency of IL-15 in inducing the expression of TNF-α, IL-1, IFN-γ, and other cytokines involved in the inflammatory response that if indiscriminately expressed would be associated with serious disorders, including autoimmune diseases (32). In terms of a more positive role for IL-15, as a hypothesis, we propose that by maintaining a pool of translationally inactive IL-15 mRNA, diverse cells might respond rapidly to an intracellular infection or other stimuli by transforming IL-15 message into one that can be effectively translated. The IL-15 protein produced could, in turn, convert T and NK cells into effective killer cells, which may provide an effective host response to infectious agents.

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References


