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Regulated Release of L13a from the 60S Ribosomal Subunit as A Mechanism of Transcript-Specific Translational Control

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Summary

Transcript-specific translational control is generally directed by binding of *trans*-acting proteins to structural elements in the untranslated region (UTR) of the target mRNA. Here, we elucidate a translational silencing mechanism involving regulated release of an integral ribosomal protein and subsequent binding to its target mRNA. Human ribosomal protein L13a was identified as a candidate interferon-*G*amma-*A*ctivated *I*nhibitor of *T*ranslation (GAIT) of ceruloplasmin (Cp) mRNA by a genetic screen for Cp 3′-UTR binding proteins. In vitro activity of L13a was shown by inhibition of target mRNA translation by recombinant protein. In response to interferon-γ in vivo, the entire cellular pool of L13a was phosphorylated and released from the 60S ribosomal subunit. Released L13a specifically bound the 3′-UTR GAIT element of Cp mRNA and silenced translation. We propose a model in which the ribosome functions not only as a protein synthesis machine, but also as a depot for regulatory proteins that modulate translation.

Introduction

Most studies of acute or chronic inflammatory disease processes focus on the causes of inflammation, e.g., microbial infection, imbalance of proinflammatory cytokines, and disturbance of proinflammatory signaling pathways. Because of this emphasis, much more is known about processes that initiate and sustain inflammation than about processes that limit or terminate it. Understanding the latter processes are important because defects may contribute to inflammatory disease progression, and because these processes present alternative targets for therapy. Monocyte-derived macrophages, by virtue of their antimicrobial activity and their clearance of toxic debris, have a central role in inflammation. Activation of these cells depends on a complex suite of interactions with cytokines, and the consequent induction of specific genes that produce a toxic microenvironment for the invading organism. However, if the inflammatory process is not well-controlled or resolved in a timely fashion, it can lead to host tissue damage (Nathan, 2002).

Ceruloplasmin (Cp) is an acute phase protein synthesized and secreted systemically into plasma by hepatocytes, and locally in inflammatory sites by cytokine-stimulated macrophages (Fleming et al., 1991; Mazumder et al., 1997). Cp contains 6–7 copper atoms per molecule and utilizes them for diverse oxidative functions (Bielli and Calabrese, 2002). By virtue of its ferroxidase activity that facilitates iron loading into transferrin, liver-derived plasma Cp has an important role in plasma iron homeostasis (Osaki et al., 1966). In the absence of Cp, for example in patients with hereditary Cp deficiency, pathological iron accumulation leads to neural defects, blindness, and diabetes (Gitlin, 1998). The function of macrophage-derived Cp is less clear. A likely possibility is bactericidal activity, possibly due to oxidative damage by Cp copper or to iron restriction (Klebanoff, 1992). We and others have shown that Cp copper causes oxidative modification of macromolecules, including low-density lipoprotein (Mukhopadhyay et al., 1997). Cp-mediated oxidation may underlie the observation that Cp is an independent risk factor for cardiovascular disease including atherosclerosis, carotid restenosis after endarterectomy, and myocardial infarction (Salonen et al., 1991; Mezzetti et al., 1999).

Consistent with a role of Cp in macrophage-mediated host defense, interferon (IFN)-γ induces Cp mRNA and protein expression in U937 monocytic cells and peripheral blood monocytes (Mazumder et al., 1997). We have shown that Cp induction is subject to a unique translational silencing mechanism in which synthesis is selectively terminated about 16 hr after IFN-γ treatment, even in the presence of abundant Cp mRNA (Mazumder and Fox, 1999). A 29 nt, IFN-*g*amma-*a*ctivated *i*nhibitor of *t*ranslation (GAIT) element in the Cp 3′-untranslated region (UTR) is necessary for IFN-γ-mediated translational silencing of Cp, and is sufficient to convey the silencing response to a heterologous transcript (Sampath et al., 2003). We have also shown that translational silencing of Cp requires the essential elements of mRNA circularization; removal or inactivation of any of the components of end-to-end transcript closure, i.e., the poly(A) tail, eukaryotic initiation factor (eIF) 4G, or poly(A) binding protein (PABP), prevents translational silencing (Mazumder et al., 2001). Finally, we have shown delayed appearance of a cytosolic protein (or complex) in IFN-γ-treated U937 cells that specifically binds the GAIT element. On the basis of these results, we have proposed a mechanism of translational control in which Cp mRNA circularization brings a cytosolic, 3′-UTR binding inhibitor protein (GAIT) into the proximity of the 5′-UTR where it blocks the initiation of translation (Mazumder et al., 2001, 2003).

Here, we identify ribosomal protein L13a as GAIT, a molecular switch of the translational silencing event that blocks Cp expression. We further show that IFN-γ induces delayed phosphorylation of L13a and causes uncoupling of L13a from the large ribosomal subunit. The

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released L13a binds to the GAIT element in the Cp 3′-UTR and silences Cp mRNA translation.

Results

Identification of Ribosomal Protein L13a as a Candidate GAIT

We have identified candidate GAIT cDNAs using the yeast three-hybrid screen. In this genetic method, the binding of a bifunctional RNA to each of two-hybrid proteins activates transcription of a reporter gene in vivo (SenGupta et al., 1996). This method has been used to detect and identify several specific RNA binding proteins (Martin et al., 1997). For bait we used an RNA hybrid of the Cp 3′-UTR spanning nt 50 to 150 (Cp 3′-$UTR_{(50–150)}$) coupled with MS2 RNA. The prey was contained in a cDNA library from IFN-γ-activated U937 cells in tandem with a B42 activation domain. Binding of activation domain-tagged protein to the Cp 3′-$UTR_{(50–150)}$ bait results in activation of *HIS3* gene. 10^6 clones were screened to yield 10^3 primary positive clones. These clones were further screened for Cp 3′-UTR-dependent *HIS3* activation by their sensitivity to 5-fluoroorotic acid (FOA). 5-FOA causes the rejection of the *URA3*-containing bait plasmid, hence clones that depend on the presence of bait RNA for the activation of *HIS3* gene will not be able to grow in 5-FOA-containing, histidine-deficient medium. Sequencing of these clones identified fifteen 5-FOA-sensitive clones containing significant open reading frames (ORFs) in-frame with the B42 activation domain. The clones were replicated in uracil-containing medium to reject the *URA3*-containing bait RNA plasmid and to express RNA binding prey protein in unbound form. Translational silencing activity of candidate clones was assessed as the ability of yeast lysates to inhibit in vitro translation of the chimeric reporter transcript, Luc-Cp 3′-$UTR_{(50–150)}$-poly(A) in a rabbit reticulocyte lysate. A single clone, designated ORF2, reproducibly inhibited translation of the reporter but did not alter translation of a control transcript that lacked the GAIT element, T7 gene 10 (Figure 1A). The interaction of ORF2 with the Cp 3′-UTR was shown by RNA electrophoretic mobility shift assay (EMSA) using radiolabeled Cp 3′-$UTR_{(50–150)}$ as probe (Figure 1B). Specific binding was shown by competition by a 25-fold molar excess of in vitro-synthesized, 29 nt GAIT element (Cp 3′-$UTR_{(78–106)}$), but not by excess GAIT element containing the inactivating U87C mutation that blocks *cis*-acting translational silencing activity (Sampath et al., 2003) (Figure 1B). These functional and binding activities of ORF2 suggest that it is a likely GAIT candidate.

To verify that the observed activity was ORF2 plasmid-driven and not due to a secondary alteration or mutation in the yeast clone, ORF2 plasmid DNA was recovered from the clone and retransformed in yeast with the bait. Yeast cotransformed with vectors containing ORF2 and Cp 3′-$UTR_{(50–150)}$ grew in histidine-depleted, 3-AT-containing media, but vector-transformed yeast did not grow (Figure 1C, middle). All transformants grew in histidine-supplemented medium (Figure 1C, right). Translational silencing activity was verified in lysates prepared from yeast retransformed with ORF2 plasmid. The lysate contained functional protein as shown by inhibition of Luc-Cp 3′-$UTR_{(50–150)}$-poly(A) translation and by specific binding to radiolabeled Cp 3′-UTR probe (data not shown). These results indicate that the ORF2 protein product is responsible for the observed silencing activity.

Sequence analysis of the ORF2 plasmid DNA showed that it encoded full-length human ribosomal protein L13a (Figure 1D). L13a, also known as mouse tum^- transplantation antigen P198, is an integral protein of the large ribosomal subunit. It is a highly basic, 23 kDa protein containing 202 amino acids (Price et al., 1992). Two consensus motifs have been described in L13a (Figure 1E), a leucine zipper at the N terminus possibly involved in homo- or heterodimerization and a basic domain leucine zipper that may be involved in RNA or DNA interactions (Chan et al., 1994). The role of L13a in ribosome function is not known, but it is not near the peptidyl transferase site or the peptide exit tunnel, and thus may not have a direct role in the formation of nascent proteins (Ban et al., 2000; Moore and Steitz, 2003).

L13a Binds the Cp 3′-UTR GAIT Element in Cells and Is Required for Translational Silencing Activity

The binding of endogenous U937 cell L13a to the Cp 3′-UTR GAIT element was examined in vitro by an RNA "supershift" assay using a rabbit polyclonal antihuman L13a antibody prepared against a 16 amino acid peptide near the C terminus (Figure 1D). Cytosolic extracts were prepared from U937 cells treated with IFN-γ for 8 or 24 hr and incubated with radiolabeled, 29 nt GAIT element as probe. As observed previously, an RNA-protein complex formed in the presence of lysates from cells treated with the cytokine for 24 hr, but untreated cells or cells treated for 8 hr did not express binding activity (Mazumder et al., 2001; Sampath et al., 2003; Mazumder and Fox, 1999). The complex formed by the 24 hr lysate was supershifted by anti-L13a antibody indicating that L13a is in the GAIT element binding complex (Figure 2A). A minor complex was seen in some experiments and was not shifted by the antibody. The translational silencing activity of endogenous L13a in the cell lysate was tested by immunodepletion using anti-L13a antibody. Western analysis of the depleted extract showed that essentially all L13a was removed (data not shown). The extract was tested for its ability to inhibit translation of the chimeric Luc-Cp 3′-$UTR_{(50–150)}$-poly(A) reporter transcript in a reticulocyte lysate in vitro. As seen previously, lysates from cells treated with IFN-γ for 24 hr, but not for 8 hr, were inhibitory (Mazumder et al., 2001; Sampath et al., 2003; Mazumder and Fox, 1999). Also, the silencing activity was specific since it did not block translation of the phage T7 gene 10 control transcript that does not contain the GAIT element. Immunodepletion completely removed translational silencing activity indicating that L13a is GAIT or a component of a GAIT complex that silences Cp translation (Figure 2B).

The in vivo interaction between L13a and Cp mRNA in stimulated U937 cells was investigated. The cells were treated with IFN-γ for 8 or 24 hr, extracts were prepared, and L13a was immunoprecipitated with anti-L13a antibody. RNA was extracted from the immunoprecipitate, and reverse-transcribed with oligo-dT and amplified by PCR using primers specific for human Cp 3′-UTR. A PCR

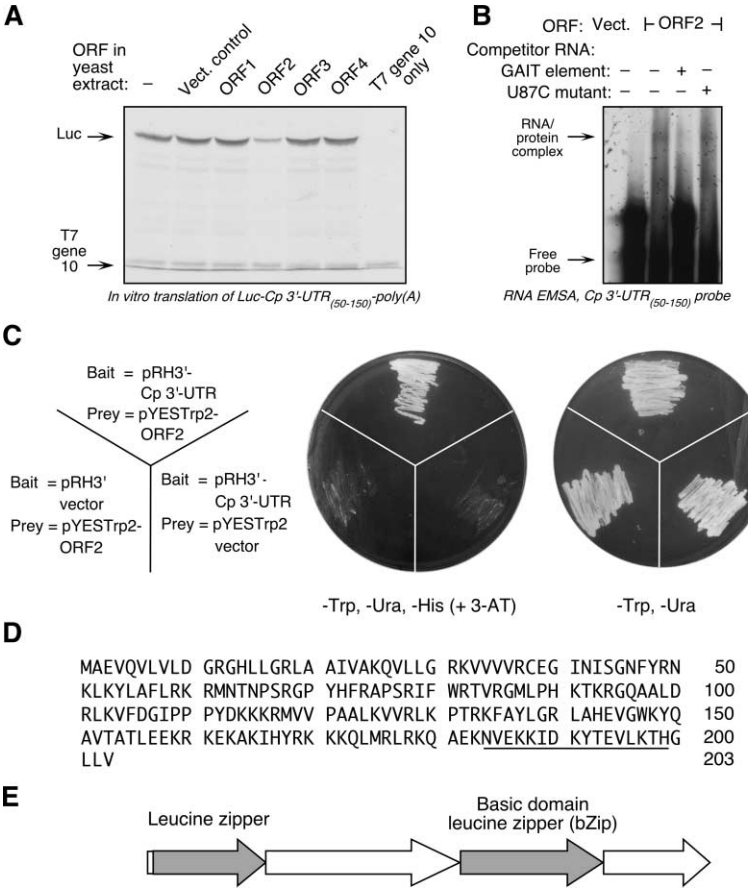

Figure 1. Identification of a Candidate GAIT by the Yeast Three-Hybrid Screen

(A) Inhibition of GAIT element-mediated translation by ORF2. Capped, Luc-Cp 3′-UTR$_{(50–150)}$-poly(A) cRNA was subjected to in vitro translation in a reticulocyte lysate containing [^{35}S]methionine in the presence of cytosolic extracts (4 μg) from selected yeast clones (ORFs 1–4), and from yeast transformed with the vector (vect.) control. Capped, T7 gene 10 cRNA, which lacks the GAIT element, was added to each sample as a loading and specificity control. Aliquots of the translation reaction were subjected to 10% SDS-PAGE and autoradiography.

(B) Binding of yeast ORF2 protein to the GAIT element. Lysate from the yeast clone containing ORF2 was subjected to RNA EMSA using radiolabeled Cp 3′-UTR$_{(50–150)}$ as probe. RNA-protein complexes were resolved by electrophoresis on a nondenaturing 5% polyacrylamide gel and detected by autoradiography. In competition experiment, the lysates were preincubated with a 25-fold molar excess of unlabeled, 29 nt GAIT element, or the U87C mutant element, before addition of radiolabeled probe.

(C) Retransformation of yeast with ORF2 and Cp 3′-UTR restores survival in histidine-deficient medium. Competent L40uraMS2 was cotransformed with vectors expressing ORF-2 as prey and Cp 3′-UTR$_{(50–150)}$ as bait, or with either one of these vectors plus control vector (left). Transformed yeast were grown either in histidine-deficient, 3-AT-containing medium (center) or in histidine-containing medium without 3-AT (right).

(D) Deduced amino acid sequence of human L13a obtained from the ORF-2 cDNA. Peptide used to raise a rabbit polyclonal antihuman L13a antibody is underlined.

(E) Putative domain structure of human L13a.

fragment corresponding to the Cp 3′-UTR was observed only in the immunoprecipitate from cells treated with IFN-γ for 24 hr (Figure 2C, left image). The identity of the amplified product was confirmed by sequencing. RT-PCR of extracts not subjected to immunoprecipitation showed that cells treated with IFN-γ for either 8 or 24 hr expressed Cp mRNA, as expected, but that the transcript did not bind L13a in 8 hr extracts (Figure 2C). As a control, no amplification product was seen in any RNA sample not subjected to reverse-transcription (Figure 2C, second image). Specificity of the interaction was shown by the absence of the RT-PCR product of Cp mRNA in extracts immunoprecipitated with antibody against another ribosomal protein, L28 (Figure 2C, third image). To show equal recovery of RNA from immunoprecipitated samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, a nonspecific, high-abundance transcript, was amplified using additional PCR cycles (Figure 2C, right image). Taken together, these results show that L13a binds specifically, and in a delayed fashion, to the Cp 3′-UTR in IFN-γ-treated U937 cells.

Recombinant L13a Has Translational Silencing Activity

The translational silencing activity of L13a was tested using recombinant protein expressed in *E. coli* and in baculovirus-infected insect cells, and partially purified by gel filtration chromatography. Both systems expressed substantial amounts of soluble recombinant protein, but insect cell-derived protein had slightly retarded mobility compared to *E. coli*-derived protein, suggesting a possible posttranslational modification by insect cells (Figure 3A). *E. coli*-derived protein did not affect in vitro translation of the chimeric reporter transcript Luc-Cp 3′-UTR$_{(50–150)}$poly(A) (Figure 3B). However, insect cell-generated L13a inhibited translation of the reporter transcript to about the same extent as an active cytosolic extract from U937 cells treated with IFN-γ for 24 hr (Figure 3B). The silencing activity of recombinant L13a was specific and did not block translation of the phage T7 gene 10 control transcript (Figure 3B).

IFN-γ Causes Delayed Phosphorylation of L13a in U937 Cells

Our finding that L13a does not bind the target transcript, or block its translation, until 8 to 24 hr of IFN-γ treatment suggests a delayed induction or activation of the protein. To examine the effect of IFN-γ on L13a expression, U937 cells were incubated with IFN-γ for 8 or 24 hr and RNA was isolated and subjected to Northern analysis using radiolabeled, full-length L13a cDNA as probe. IFN-γ treatment did not alter the steady-state level of L13a mRNA during this treatment period (Figure 4A). To exam-

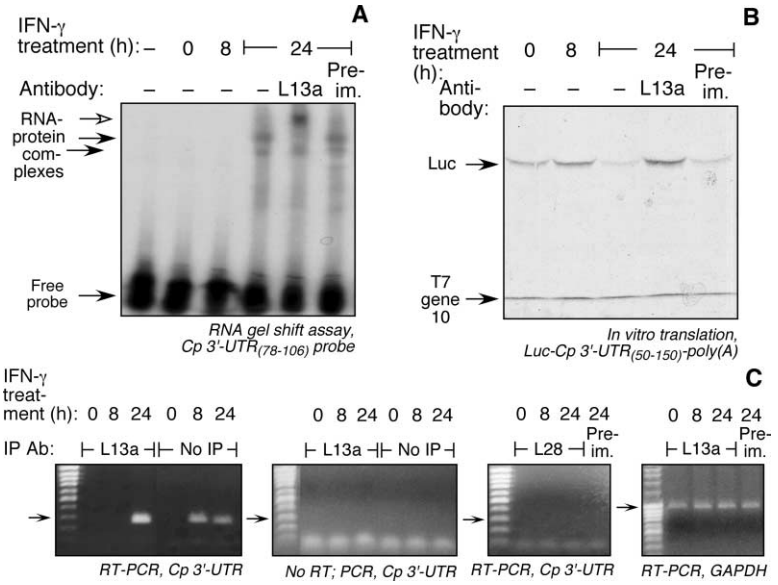

Figure 2. Endogenous U937 Cell L13a Binds to GAIT Element and Is Required for Translational Silencing

(A) RNA EMSA supershift analysis shows L13a in GAIT element binding complex in U937 cells. U937 cells were treated with IFN-γ for 8 or 24 hr and cytosolic extracts were incubated with radiolabeled, 29 nt GAIT element. To show the presence of L13a, the 24 hr extract was preincubated with polyclonal rabbit antihuman L13a antibody or with preimmune serum (Pre-im.) before addition of radiolabeled probe. Shifted complex is indicated (open arrow).

(B) Immunodepletion of L13a removes translational silencing activity from U937 cell lysates. Cytosolic extracts were made from U937 cells treated with IFN-γ for 8 or 24 hr. The 24 hr extract was immunodepleted with anti-L13a antibody or with preimmune serum as control. The effect of extracts on in vitro translation of capped, Luc-Cp 3′-UTR$_{(50–150)}$-poly(A) cRNA was determined by addition to a reticulocyte lysate containing [^{35}S]methionine. Capped, T7 gene 10 cRNA was added to each reaction as a specificity control.

(C) Demonstration of intracellular binding of L13a to Cp mRNA in U937 cells by immunoprecipitation (IP) and RT-PCR. U937 cells were treated with IFN-γ for 8 or 24 hr. Aliquots of the cytosolic extracts were subjected to immunoprecipitation with rabbit antihuman L13a antibody (Ab). RNA was extracted from the immunoprecipitate or from extracts not subjected to immunoprecipitation (No IP). RNA was subjected to reverse transcription with Superscript (Invitrogen) and oligo-dT, and then to PCR amplification with primers specific for the Cp 3′-UTR (left image) or GAPDH (right image). In other controls, the Cp 3′-UTR was amplified by PCR in the absence of the RT reaction (second image), and Cp 3′-UTR was amplified in cytosolic extracts that were immunoprecipitated with anti-L28 antiserum or with preimmune serum (third image).

ine posttranscriptional regulation, extracts from IFN-γ-treated U937 cells were subjected to immunoblot analysis with anti-L13a antibody. IFN-γ treatment did not significantly alter L13a expression level; however, the electrophoretic mobility of L13a was markedly decreased after treatment with the cytokine for 24 hr, suggesting a possible delayed posttranslational modification (Figure 4B).

In view of the regulation of RNA binding activity of several proteins by phosphorylation (Dominski et al., 2002; Law et al., 2003; Zhang et al., 2003; Ostrowski et al., 2000; Ostareck-Lederer et al., 2002; Siomi et al., 2002), we considered the possibility that L13a phosphorylation was responsible for the observed shift in electrophoretic mobility. L13a phosphorylation was measured by metabolic labeling with a 6 hr pulse of ^{32}P-orthophosphate followed by immunoprecipitation with anti-L13a antibody, SDS-PAGE separation, and autoradiography. L13a was phosphorylated with a time course consistent with its mobility shift (Figure 4C). To test the role of phosphorylation, we determined the effect of phosphatase treatment on L13a activity. U937 cells were treated with IFN-γ and active, 24 hr lysates were incubated with alkaline phosphatase. Immunoblot analysis showed that the phosphatase restored the original electrophoretic mobility of L13a, indicating that the shift was due to phosphorylation and also showing effectiveness of the treatment (Figure 4D). The phosphatase-treated lysate did not inhibit in vitro translation of the Luc-Cp 3′-UTR$_{(50–150)}$-poly(A) reporter, indicating a critical role of L13a phosphorylation in silencing activity (Figure 4E). Addition of phosphatase inhibitor (cocktail set II at a 1:100 dilution plus 5 mM levamisole) with the phosphatase prevented the mobility shift of L13a and restored its translational silencing activity (data not shown). To confirm the role of L13a phosphorylation in translational silencing activity, we considered the possibility that the difference in activity (and electrophoretic mobility) of recombinant L13a protein from two sources was due to differential phosphorylation. Metabolic labeling with ^{32}P-orthophosphate and immunoprecipitation with anti-L13a antibody revealed that active, insect cell-derived L13a was phosphorylated, whereas inactive, *E. coli*-derived protein was unmodified (Figure 4F). Treatment of insect cell-derived L13a with alkaline phosphatase increased the electrophoretic mobility of the protein (data not shown) and blocked its silencing activity (Figure 4G). Our results show that the entire U937 cellular pool of L13a is phosphorylated after prolonged IFN-γ treatment, and that the modification is required for translational silencing activity.

IFN-γ Induces Release of L13a from the 60S Ribosomal Subunit

Our findings raise a question about the form of L13a that binds the target transcript, namely, whether L13a remains associated with the large ribosomal subunit or is present in a cytosolic, nonribosomal form after IFN-γ treatment. Lysates from IFN-γ-treated U937 cells were fractionated on a sucrose cushion to separate the ribosomal fraction from ribosome-free cytosol (Mazumder and Fox, 1999; Wormington, 1991). Both fractions were subjected to SDS-PAGE and immunoblot analysis with anti-L13a antibody. L13a was primarily in the ribosome fraction of untreated cells or cells treated with IFN-γ for 8 hr (Figure 5A). In contrast, after 24 hr, L13a was present only in the ribosome-free, low-molecular weight fraction (and primarily in the phosphorylated form). As a control

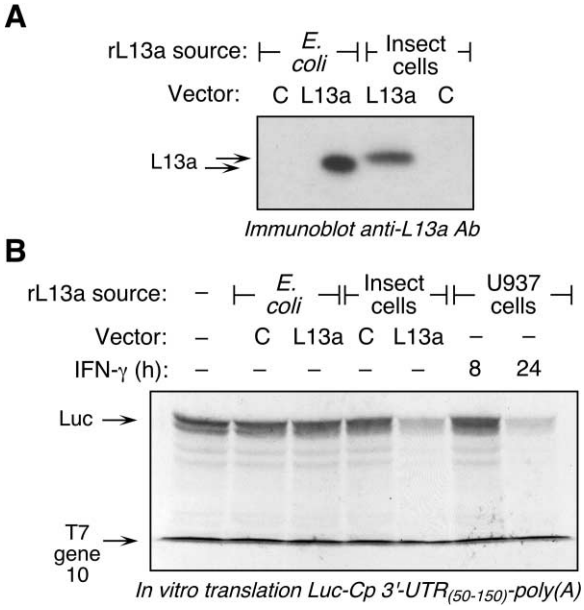

Figure 3. Translational Silencing Activity of Recombinant Human L13a

(A) Expression of recombinant L13a by *E. coli* and insect cells. Full-length human L13a ORF cloned into pET-17b vector (or vector control, C) was used to transform *E. coli* BL21(DE3)pLysS that were induced with IPTG. L13a cDNA was cloned into pFASTBAC1 and expressed in High Five insect cells. Soluble recombinant L13a (rL13a) in cell lysates was partially purified by size-exclusion chromatography. rL13a expression was shown by immunoblot analysis with rabbit antihuman L13a antibody.

(B) Translational silencing activity of insect cell-derived L13a. Capped, Luc-Cp 3′-UTR$_{(50–150)}$-poly(A) cRNA was subjected to in vitro translation in a reticulocyte lysate. The translation mixtures also contained cytosolic lysates (4 μg) of partially purified, rL13a made by transformed *E. coli* or by baculovirus-infected insect cells. Translation of capped, T7 gene 10 cRNA served as a specificity control. A cytosolic lysate from U937 cells treated with IFN-γ for 24 hr served as a positive control for translational silencing activity.

for specificity, another 60S ribosomal protein, L28, was found to be primarily associated with ribosomes at all times (Figure 5B). The absence of 28S and 18S ribosomal RNA in the ultracentrifugal supernatant verified that the procedure efficiently removed all intact ribosome subunits (Figure 5C). Together these results show that upon prolonged treatment of U937 cells with IFN-γ, L13a selectively dissociates from the 60S ribosomal subunit as a free, phosphorylated protein capable of binding the Cp 3′-UTR GAIT element and silencing translation.

Discussion

We have identified large ribosomal protein L13a as an essential component in IFN-γ-mediated, transcript-selective, translational silencing of Cp. Our results allow us to propose a mechanism for L13a-mediated silencing of translation. About 2–4 hr after treatment of U937 cells with IFN-γ, Cp mRNA is induced and translation begins (Mazumder et al., 1997; Mazumder and Fox, 1999); during this period L13a is present in the nonphosphorylated form bound to 60S ribosomal subunits (Figure 6A). About 16–24 hr after IFN-γ treatment, L13a is phosphorylated and released from the ribosome. At least two sequences of induced release are possible: (1) ribosome bound L13a is phosphorylated which causes release, or (2) L13a dissociates from the ribosome and then is phosphorylated, causing a shift in the equilibrium away from the ribosome bound form (Figure 6B). Free, phosphorylated L13a then binds the Cp 3′-UTR GAIT element, and possibly similar structural elements in other transcripts (Sampath et al., 2003). Interaction of L13a, or an L13a-containing GAIT complex, with the GAIT element silences target mRNA translation (Figure 6C). Previously, we have shown that silencing requires mRNA circularization, or more precisely, the elements required for transcript closure, i.e., poly(A) tail, PABP, and eIF4G (Mazumder et al., 2001). We have proposed that a function of transcript circularization is to juxtapose the 3′-UTR binding protein with the 5′-initiation site where it can exert translational control (Mazumder et al., 2001, 2003). The specific mechanism by which GAIT blocks translation-initiation has not been identified, but at least four possibilities can be considered (Figure 6C). GAIT may (1) inhibit function of the cap binding complex, eIF4F; (2) block recruitment of the 43S preinitiation complex, the mechanism responsible for inhibition of ferritin translation by iron-responsive protein-1 (Muckenthaler et al., 1998); (3) prevent scanning of the 43S complex to the initiation codon, the mechanism of inhibition of *Drosophila* msl-2 mRNA translation by sex-lethal (Gebauer et al., 2003); or (4) block 60S ribosomal subunit joining, the mechanism of inhibition of 15-lipoxygenase translation by hnRNP K and E1 (Ostareck et al., 2001).

IFN-γ-Inducible Phosphorylation of L13a

L13a joins the list of other eukaryotic ribosomal proteins that undergo phosphorylation (Lee et al., 2002; Remacha et al., 1995; Saenz-Robles et al., 1988; Diaz et al., 2002; Jefferies et al., 1997; Campos et al., 1990) or other post-translational modifications including ubiquitination (Finley et al., 1989; Spence et al., 2000), acetylation (Arnold et al., 1999), and methylation (Lhoest et al., 1984). In several cases, the modification regulates translation. The best-studied example is phosphorylation of the 40S ribosomal subunit protein S6. Ribosomes containing phosphorylated S6 exhibit a translational preference for transcripts containing the 5′-terminal oligopyrimidine sequence (Jefferies et al., 1997). Herpes simplex virus induces S6 phosphorylation, which increases the efficiency of viral transcript translation (Masse et al., 1990). A second case is phosphorylation of eukaryotic proteins P0, P1, and P2, which constitute the ribosomal stalk that interacts with elongation factors during protein translation (Ballesta et al., 1999). All are present in the phosphorylated form in ribosomes, but the function of the modification is unclear. Mutagenesis studies in yeast indicate that P0 phosphorylation is not necessary for overall ribosome translational activity, but may influence the translation of specific mRNAs (Ballesta et al., 1999). Likewise, the effects of phosphorylation of P1 and P2 are transcript-specific, and complete deletion of these genes in *Saccharomyces cerevisiae* results in only subtle changes in protein expression (Remacha et al., 1995). A recent ribosome-wide survey of herpes simplex-infected HeLa cells showed phosphorylation of the 40S

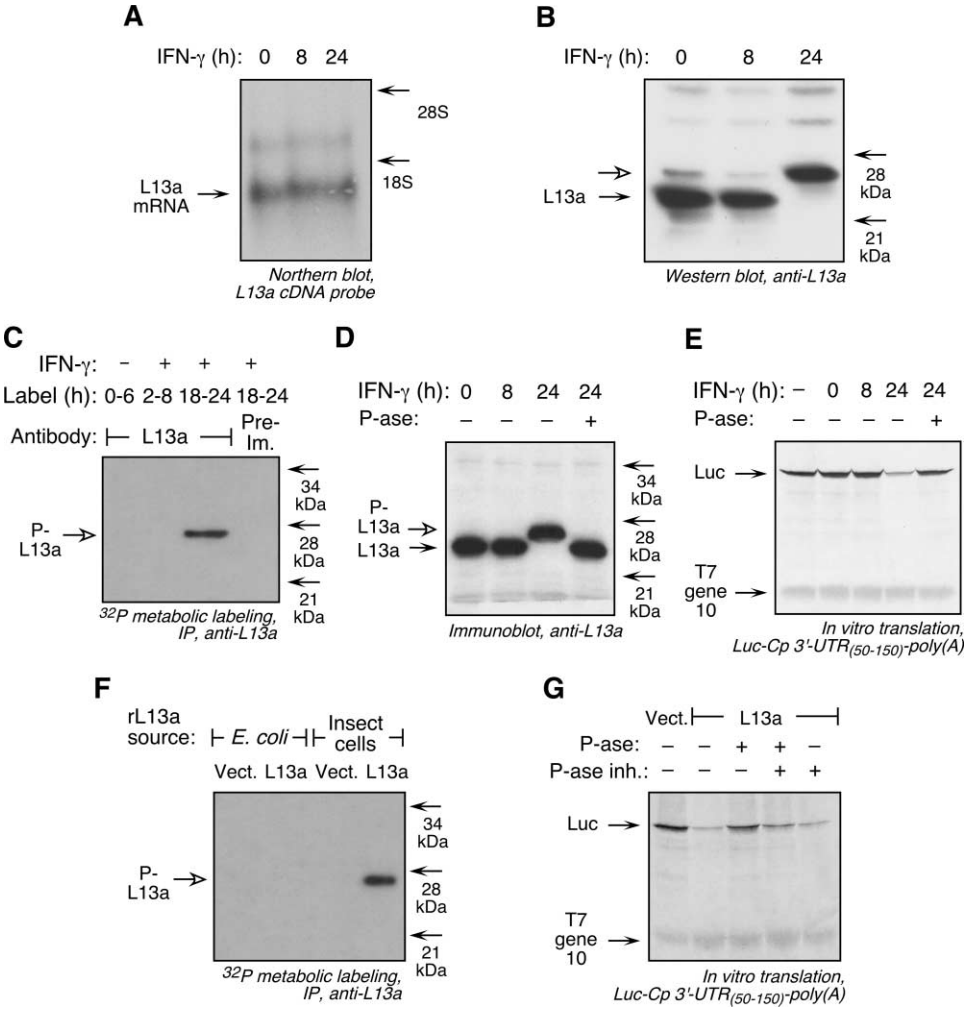

Figure 4. Regulation of L13a Expression and Phosphorylation by IFN-γ in U937 Cells

(A) IFN-γ treatment of U937 cells does not alter steady-state L13a mRNA expression. U937 cells were treated with IFN-γ for 8 or 24 hr. RNA was isolated and subjected to Northern blot hybridization with full-length, radiolabeled human L13a cDNA as probe.

(B) IFN-γ treatment causes posttranslational modification of L13a. U937 cells were treated with IFN-γ for 8 or 24 hr and extracts were subjected to SDS-PAGE using 12% polyacrylamide gel. Immunoblot analysis was done using polyclonal rabbit antihuman L13a antibody. The position of modified L13a is indicated (open arrow).

(C) IFN-γ induces delayed phosphorylation of L13a in U937 cells. U937 cells were treated with IFN-γ and metabolically labeled with a 6 hr pulse of ^{32}P-orthophosphate. Lysates from labeled cells were immunoprecipitated with anti-L13a antibody or preimmune (Pre-im.) sera, and immunoprecipitated protein was resolved by SDS-PAGE using 12% polyacrylamide gels and autoradiography. The position of phosphorylated L13a (P-L13a) is indicated (open arrow).

(D) Alkaline phosphatase restores electrophoretic mobility of L13a. U937 cells were incubated with IFN-γ for 8 or 24 hr. Cytosolic extracts from 24 hr-treated cells were treated with alkaline phosphatase (P-ase, 3 U) for 90 min and subjected to immunoblot analysis with anti-L13a antibody. The position of phosphorylated L13a is indicated (open arrow).

(E) Alkaline phosphatase inhibits translational silencing activity of U937 cell extracts. Cytosolic extracts from IFN-γ-treated cells were incubated with alkaline phosphatase as in (A). Capped Luc-Cp$_{(50–150)}$-poly(A) and T7 gene 10 cRNAs were subjected to in vitro translation in a rabbit reticulocyte lysate as in Figure 2B.

(F) Phosphorylation of recombinant human L13a by baculovirus-infected insect cells. L13a expressing *E. coli* BL21(DE3)pLysS and baculovirus-infected High Five cells, and their vector controls (vect.), were pulse-labeled for 4 hr with ^{32}P-orthophosphate. Cell lysates were immunoprecipitated with anti-L13a antibody followed by SDS-PAGE and autoradiography.

(G) Translational silencing activity of insect cell-derived L13a is phosphatase-sensitive. Capped Luc-Cp$_{(50–150)}$-poly(A) and T7 gene 10 cRNAs were subjected to in vitro. Partially purified, recombinant L13a (4 μg) made by baculovirus-infected insect cells was treated with shrimp alkaline phosphatase (P-ase, 3 U) in presence or absence of phosphatase inhibitor (P-ase inh.) cocktail for 90 min. After phosphatase treatment, recombinant insect cell-derived L13a was added to the translation reaction. Translation of capped T7 gene10 cRNA was carried out in the same reaction and served as a specificity control.

proteins S6, S2, Sa, and S3a, and a single 60S protein, L30 (Diaz et al., 2002). Together these data suggest that ribosomal protein phosphorylation (excluding P0, P1, and P2) is uncommon under basal conditions, but that specific ribosomal proteins are modified upon cell activation. Furthermore, phosphorylation of ribosomal proteins causes transcript-specific, rather than global, translational regulation consistent with altered mRNA selectivity not overall translational activity. The results are also consistent with the recently proposed "ribo-

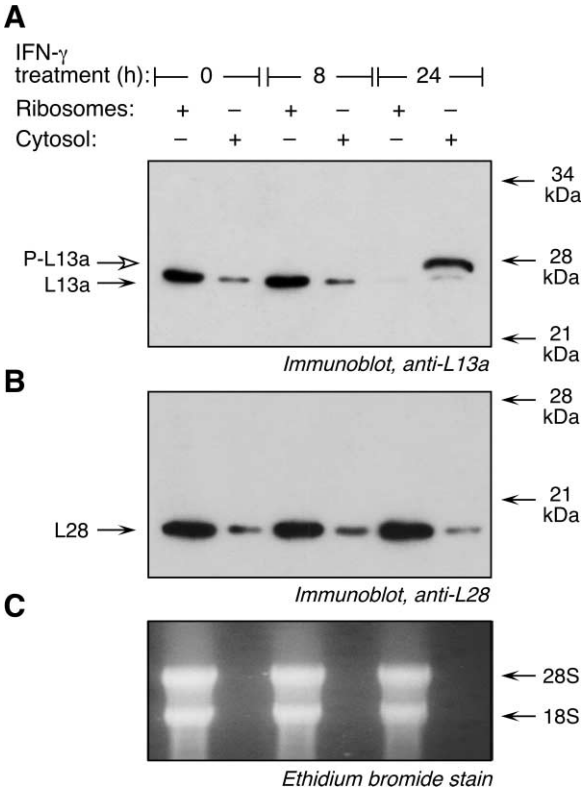

Figure 5. IFN-γ Induces Release of L13a from the 60S Ribosomal Subunit

(A and B) L13a is specifically released from ribosomes. U937 cells were treated with IFN-γ for 8 or 24 hr. The cells were lysed in the presence of cycloheximide and centrifuged at low speed. Ribosomes were precipitated by ultracentrifugation at 150,000 × g for 2 hr through a sucrose cushion. To remove contaminating ribosomes, the supernatant was recentrifuged under the same conditions. Ribosome and nonribosome (cytosol) fractions were subjected to immunoblot analysis with anti-L13a (A) or with anti-L28 (B) antibodies. The positions of phosphorylated (open arrow) and unmodified (closed arrow) L13a are indicated.

(C) RNA was extracted from ribosomal and cytosolic fractions by Trizol and resolved on an agarose-formaldehyde gel under denaturing conditions. Ribosomal RNA was visualized by ethidium bromide staining.

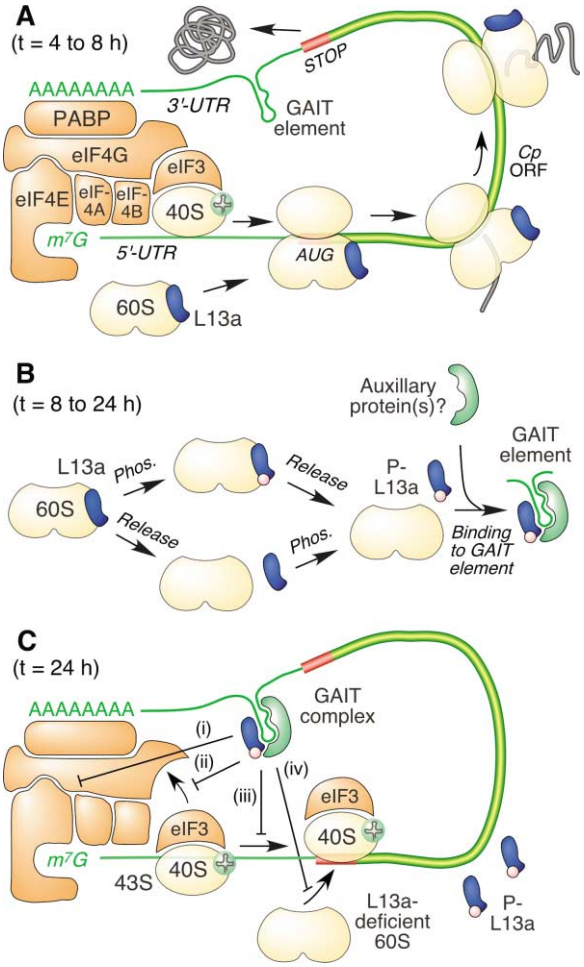

Figure 6. Model of L13a Release from Ribosomes and Its Role in Translational Silencing

(A) Time = 4 to 8 hr after IFN-γ addition: Cp mRNA is induced and efficiently translated. Circularization of transcript is mediated by 5′-3′-interactions between PABP and poly(A) tail, and between PABP and eIF4G.

(B) Time = 8 to 24 hr after IFN-γ addition: L13a is phosphorylated and released from the 60S ribosomal subunit. Phosphorylated L13a binds to the GAIT element, possibly in a complex with other cytosolic proteins.

(C) Time = 24 hr after IFN-γ addition: 5′-3′-interactions juxtapose GAIT, or the GAIT complex, with the translation-initiation region where it silences Cp mRNA translation by one of the following mechanisms: (1) inhibiting function of one or more components of the cap binding complex, eIF4F; (2) blocking recruitment of the 43S preinitiation complex; (3) preventing scanning of the 43S complex to the initiation codon; or (4) blocking 60S ribosomal subunit joining.

some filter hypothesis" in which structural differences among ribosomes in different cells, or under various conditions, leads to altered affinity for specific mRNAs (Mauro and Edelman, 2002).

Phosphorylation of RNA binding proteins influences their binding to mRNA, which in turn influences transcript translation, stability, and processing (Siomi et al., 2002; Ostareck-Lederer et al., 2002; Zhang et al., 2003; Li et al., 2002; Dominski et al., 2002). For example, phosphorylation of heterogeneous nuclear RNA binding protein K decreases binding to the 15-lipoxygenase 3′-UTR DICE element and derepresses translation (Ostareck-Lederer et al., 2002). In other examples, phosphorylation of Fragile X mental retardation protein-1 increases binding to specific transcripts and their translation (Zalfa et al., 2003; Siomi et al., 2002), phosphorylation of QKI inhibits interaction with myelin basic protein mRNA and increases transcript turnover (Zhang et al., 2003), and phosphorylation of plant abscisic-acid-activated protein kinase-interacting protein-1 increases dehydrin mRNA binding, mRNA stability, and transport (Li et al., 2002). The specific function of L13a phosphorylation is not known but, analogous to the examples above, it may facilitate binding to the GAIT element, either directly or via binding to other proteins in a GAIT element binding complex.

Release of L13a from the 60S Subunit: the Ribosome as a Depot for Translational Control Proteins

Our results show that phosphorylated L13a is present as a ribosome-free cytosolic protein. Previous reports

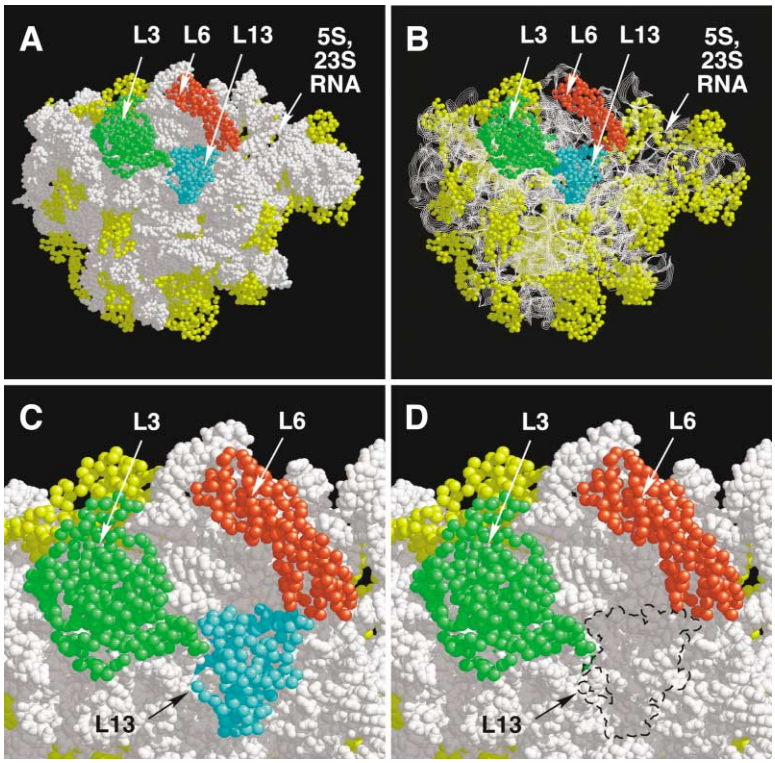

Figure 7. Structure of L13, the Bacterial Homolog of L13a, and Its Environment in the 50S Subunit of *Haloarcula Marismortui*

(A) Relationship of L13 to other 50S ribosomal proteins and RNA. Space-filled, shadowed model is generated using X-ray crystallographic coordinates (Ban et al., 2000). L13 (red) is adjacent to L3 (green) and L6 (blue) on the RNA (white) surface. Other 50S proteins are also shown (yellow).

(B) Same as (A), but RNA is depicted as strands to reveal proteins in the interior of the 50S ribosome.

(C) Detail of 50S ribosome surface showing relationship between L13a, L3, and L6.

(D) Same as (C) but L13 is removed from the model to reveal underlying 23S RNA surface (dashed outline).

of L13a are consistent with extra-ribosomal localization and function, for example, L13a is a tumor cell surface antigen in mice (Sibille et al., 1990) and in human melanoma cells (Li et al., 2001), and it is present in the cytosol of fibroblasts (Li et al., 2001). In carcinoma cells, L13a interacts with the heregulin receptor ErbB-3, a transmembrane kinase of the epidermal growth factor receptor family (Yoo and Hamburger, 1999). Interestingly, IFN-γ activates ErbB-3 in a developmentally regulated fashion, and thus the receptor is a candidate for the kinase that phosphorylates L13a (Kritzik et al., 2000).

There is accumulating evidence for extra-ribosomal functions of ribosomal proteins, particularly as participants in RNA translation and processing, and in DNA replication, transcription, and repair (Wool, 1996; Wool et al., 1996). Synthesis of *E. coli* ribosomal proteins is regulated by an "autogenous control" mechanism in which ribosomal proteins bind their own mRNA and inhibit translation of operons encoding groups of ribosomal proteins (Zengel and Lindahl, 1994). Similarly, yeast ribosomal protein L30 and *Thermus thermophilus* S15 autoregulate translation of their own transcripts by binding to *cis*-acting elements that may mimic their docking site in ribosomal RNA (Serganov et al., 2003; Vilardell et al., 2000). Phosphoproteins P1 and P2 also exchange between the cytosol and the 60S subunit during translation (Remacha et al., 1995). However, unlike L13a phosphorylation, phosphorylation of P1 and P2 does not influence their interaction with ribosomes (Ballesta et al., 1999). In these cases, the protein released from ribosomes influences translation of specific proteins (or a group of proteins in the case of P1 and P2) without altering global protein synthesis. We have found a similar result for L13a; treatment of U937 cells with IFN-γ for 24 hr does not inhibit global protein synthesis, as measured by metabolic labeling of protein with [^{35}S]methionine despite the complete absence of ribosome bound L13a (data not shown).

The finding that liberation of ribosomal proteins does not alter overall protein synthesis rate is consistent with recent studies of ribosome structure and function. Recent evidence indicates that the ribosome is a ribozyme in which catalysis, i.e., peptidyltransferase activity, is conducted by the RNA (Noller et al., 1992; Maden et al., 1968). In support of this conclusion, X-ray crystallographic studies of the 50S subunit of *Haloarcula marismortui* show that most of the protein mass is present as discrete globular domains on the surface of the RNA core, far from the site of catalysis (Ban et al., 2000; Moore and Steitz, 2003). Several proteins have extensions that penetrate between the ribosomal RNA helices of multiple domains and may contribute to structure stabilization or to ribosome function. However, other 50S proteins lack RNA-penetrating tails and appear to "float" on the RNA surface. None of these surface proteins have been assigned a specific function in translation, and potential extra-ribosomal functions remain largely elusive. A comparable picture is emerging from lower resolution structural studies of eukaryotic ribosomes (Spahn et al., 2001). One interpretation of these data is that some ribosomal proteins may represent an evolutionary embellishment of the RNA core, adopted by the ribosome from preexisting proteins with defined functions (Wool et al., 1996).

Insight into possible mechanisms of ribosomal interaction and release of L13a can be gained from inspection of L13, the bacterial homolog of eukaryotic L13a (Ban et al., 2000). *H. marismortui* L13 is a globular protein

at the surface of the 50S subunit (Figure 7A). Visual replacement of the RNA in the space-filled model with strands does not expose any submerged L13 domains, showing that it rests entirely on the surface and lacks an RNA-penetrating extension (Figure 7B). L13 has only minimal contact with domains of two nearby surface proteins, L3 and L6 (Figure 7C). Removal of L13 from the model exposes a shallow depression in the RNA surface and shows that L13 does not interact with any buried proteins (Figure 7D). The binding of ribosomal proteins to RNA is thought to depend on RNA surface topology and not specific RNA sequences (Moore and Steitz, 2003). Also, L13 is far from the sites of chain elongation and polypeptide exit, consistent with its lack of effect on global protein synthesis. Thus, the structure and position of L13 are consistent with unhindered release from the ribosome. Little is known about the structure and function of L13a in the eukaryotic 60S subunit. Low-resolution analysis of the *Saccharomyces cerevisiae* 80S ribosome suggests that ribosomal protein L16, the yeast homolog of vertebrate L13a, is located at the ribosome surface (Spahn et al., 2001). Furthermore, a protease sensitivity study of rat ribosomes suggests that surface exposure of L13a increases during chain elongation (Marzouki et al., 1990).

In summary, our results indicate that the ribosome, in addition to functioning as a protein synthesis machine, also acts as a depot for releasable regulators of protein translation. The function of delayed translational silencing of Cp is not known. An attractive possibility is suggested by the finding that Cp bactericidal activity is effective only in a narrow concentration range (Klebanoff, 1992). Alternatively, uncontrolled accumulation of Cp in inflammatory sites may have injurious consequences consistent with the ability of Cp copper to oxidize lipoproteins (Ehrenwald and Fox, 1996; Mukhopadhyay et al., 1997). The translational silencing mechanism may have evolved to terminate or limit macrophage expression of Cp and other inflammatory proteins. Interestingly, several known mechanisms of inflammation-termination involve translational control (Kirkpatrick, 2002). For example, long-term activation by IFN-γ may have adverse consequences on the cellular environment and thus IFN-γ mRNA autoregulates its translation by formation of an RNA pseudoknot (Ben-Asouli et al., 2002). Likewise, we speculate that macrophage products of IFN-γ activation, e.g., Cp, may have injurious consequences and require rapid downregulation.

Experimental Procedures

Reagents

Rabbit reticulocyte lysate, methionine-minus amino acid mixture, and RNasin were purchased from Promega (Madison, WI). Human IFN-γ was from R & D Systems (Minneapolis, MN). Trizol and Superscript were from Invitrogen (Gaithersburg, MD). Capped transcripts were synthesized using Message Machine from Ambion (Austin, TX). Translation-grade [^{35}S]methionine was purchased from NEN-DuPont (Boston, MA) and [^{32}P]orthophosphate was from ICN (Costa Mesa, CA). Rabbit polyclonal antibody against human ribosomal protein L28 was purchased from Santa Cruz (Santa Cruz, CA). Shrimp alkaline phosphatase was from Fermentas. Phosphatase inhibitor cocktail set II (containing 1 M imidazole, 0.1 M sodium fluoride, 0.115 M sodium molybdate, 0.2 M sodium orthovanadate, and 0.4 M sodium tartrate) was from Calbiochem (San Diego, CA). Levamisole and other reagents were from Sigma (St. Louis, MO).

Plasmid Construction

The bait RNA plasmid pRH3′-Cp 3′-UTR$_{(50–150)}$ used in the yeast three-hybrid screen was made by PCR amplification of pcDNA3-Cp 3′-UTR (Mazumder and Fox, 1999) using appropriate Cp 3′-UTR primers also containing AvrII and XmaI sites. The amplified product was cloned into the AvrII and XmaI sites of pRH3′ (Invitrogen). The construction of pcDNA3-Cp 3′-UTR$_{(50–150)}$ was described previously (Sampath et al., 2003). PSP64-Luc-Cp 3′-UTR$_{(50–150)}$-poly(A) was used to synthesize cRNA for in vitro translation, and was prepared from PSP64-Luc-Cp 3′-UTR-poly(A) (Mazumder et al., 2001). The parental construct was digested by StuI and XhoI, and the 247 bp 3′-UTR insert and the digested vector were gel-purified. The insert was amplified by PCR using primers to give a fragment spanning Cp 3′-UTR nucleotides 50–150, and containing StuI and XhoI sites. The fragment was ligated into the digested parental construct.

pET-17b-L13a and pFASTBAC1-L13a were used to express recombinant human L13a in *E. coli* and insect cells, respectively. pET-17b-L13a was made by cloning the full-length ORF of human L13a into the Nde1 and BamH1 sites of pET-17b (Novagen, Madison, WI). pFASTBAC1-L13a was made by cloning L13a into the BamH1 and Xho1 sites of pFASTBAC1 (Invitrogen).

Construction of U937 Cell cDNA Library for Yeast Three-Hybrid Screen

mRNA from U937 cells treated with IFN-γ for 8 and 24 hr was isolated and combined. The cDNA was synthesized by reverse transcription with oligodT-XhoI and random-XhoI primers, ligated with EcoR1 adapters (DNA Technologies, Gaithersburg, MD), and treated with XhoI and T4 polynucleotide kinase. Digested samples were subjected to exclusion chromatography using Sephacryl S-400 (Pharmacia). Fractions containing DNA 400 bp or larger were pooled and ligated to pYESTrp2 (Invitrogen), digested with EcoR1 and XhoI, and electroporated into *E. coli* DH5α. The entire library was plated on ampicillin-containing plates after recovery in SOC media for 1 hr. Colonies were scraped and resuspended in 15 ml of LB ampicillin containing 15% glycerol, and frozen. Aliquots of the frozen stock were regrown and plasmids representing the cDNA library were isolated (Qiagen Miniprep Kit).

Yeast Three-Hybrid Screen for Cp 3′-UTR Interacting Proteins

Saccharomyces cerevisiae strain L40uraMS2, that has the *HIS3* gene under the control of LexA operon, and expresses the hybrid MS2 coat protein fused to LexA DNA binding domain, was cotransformed with the plasmid pRH3′-Cp 3′-UTR$_{(50–150)}$ and pYESTrp2-cDNA library. The first plasmid expresses the Cp 3′-UTR$_{(50–150)}$ bait RNA fused to MS2 RNA, and contains *URA3* as auxotrophic marker. The second plasmid expresses prey proteins from IFN-γ-treated U937 cells encoded by the cDNA library as a fusion protein with the B42 activation domain and contains *TRP1* gene as auxotrophic marker. Double transformants were selected in medium deficient in histidine, uracil, and tryptophan, and to minimize false-positives screening was done in the presence of 3-AT. To select for Cp 3′-UTR$_{(50–150)}$ RNA-dependent activation, positive clones were counterscreened in media lacking tryptophan and histidine but containing uracil and 5-FOA, which causes rejection of the URA3-containing bait plasmid. The cDNA inserts were amplified from 5-FOA-sensitive clones with pYESTrp2 vector primer pair and sequenced. Clones containing the insert in-frame with the B42 activation domain were selected and grown in tryptophan-deficient medium. The clones were harvested and lysed with glass beads in 50 mM Tris, [pH 7.6], 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol. Lysates were centrifuged at 10,000 $\times$ g and supernatants collected for binding and activity measurements.

Culture of U937 Cells and Preparation of Cytosolic Extracts

Human U937 monocytic cells (American Type Culture Collection, Rockville, MD; CRL 1593.2) were cultured in RPMI 1640 medium containing 10% fetal bovine serum. For preparation of cytosolic extracts, 1×10^7 cells were preincubated for 1 hr in 10 ml of medium containing 2% fetal bovine serum and then IFN-γ (500 units/ml) was added for up to 24 hr. The cells were collected by low-speed centrifugation and suspended in 0.5 ml buffer containing 50 mM

NaCl, 1 mM PMSF, 1 mM dithiothreitol, and 50 mM Tris, [pH 7.6]. The suspension was subjected to three freeze-thaw cycles, passed several times through a 26 gauge needle, and centrifuged at 100,000 × g for 30 min. Supernatant protein concentration was adjusted to 1 mg/ml and 4 μg was used in the RNA EMSA and in the in vitro translation reaction.

In Vitro Transcription

Radiolabeled Cp 3′-UTR$_{(50–150)}$ cRNA was prepared by in vitro transcription of XhoI-linearized pcDNA3-Cp 3′-UTR$_{(50–150)}$ in the presence of [α-^{32}P]UTP (MaxiScript kit, Ambion, TX) using T7 RNA polymerase. The transcript was purified on a 5% acrylamide gel containing urea. For preparation of the 29 nt GAIT element (and the U87C mutant), transcription templates were prepared by hybridizing complementary DNA oligomers corresponding to nt 78–106 of the Cp 3′-UTR in buffer containing 1 mM EDTA, 0.1 M NaCl, and 10 mM Tris-HCl, [pH 8.0]. The templates were engineered to contain the T7 promoter at the 5′-end. Annealed, partially double-stranded templates were used for in vitro transcription using T7 RNA polymerase (MegaShortScript, Ambion, TX). Unincorporated nucleotides were removed using Micro Bio-spin P-30 columns (BioRad), and transcripts were purified by phenol/chloroform extraction and ethanol precipitation.

Luc Cp 3′-UTR$_{(50–150)}$-poly(A) cRNA was generated by linearizing PSP64-Luc Cp 3′-UTR$_{(50–150)}$-poly(A) using PvuII, and subjecting it to in vitro transcription using Sp6 polymerase (MegaScript, Ambion, TX). The cRNA transcripts were capped by adding cap analog m^{7}G(5′)ppp(5′)G and GTP at a 4:1 ratio (Message Machine, Ambion, TX). Capped, T7 gene 10 cRNA was made by in vitro transcription of pGEMEX-2 (Promega) by T7 RNA polymerase (Message Machine). Full-length cRNA transcripts were purified by electrophoresis on a 5% acrylamide gel containing 8 M urea.

RNA Electrophoretic Mobility Shift Assay

[α-^{32}P]UTP-labeled Cp 3′-UTR$_{(50–150)}$ or Cp 3′-UTR$_{(78–106)}$ (20 fmol) was incubated for 30 min at 4°C with U937 cell extract (4 μg of protein) in 20 μl of buffer containing 15 mM KCl, 0.25 mM DTT, 5 mM $MgCl_2$, 0.1 mM PMSF, 200 mg/ml of yeast tRNA, 40 U of RNasin, 10% glycerol, and 12 mM HEPES, [pH 8.0]. In competition experiments, unlabeled RNA segments were added to the extract at a 25-fold molar excess 10 min before addition of radiolabeled probe. RNA-protein complexes were resolved by native gel electrophoresis (5% polyacrylamide in 0.5 × Tris-buffered EDTA) at 4°C. The gel was dried and the probe position determined by autoradiography.

In Vitro Translation of cRNA by Reticulocyte Lysate

For in vitro translation, gel-purified Luc Cp 3′-UTR$_{(50–150)}$-poly(A) cRNA (200 ng) was added to 35 μl of rabbit reticulocyte lysate, 20 μM methionine-free amino acid mixture, 40 U RNasin, 20 μCi translation-grade [^{35}S]methionine, and U937 cell extract (4 μg of protein) in a total volume of 50 μl for 60 min at 30°C. To each reaction was added cRNA encoding T7 gene 10 (100 ng) as a loading and specificity control. Oligonucleotide decoys were added at 50-fold molar excess. An aliquot (5 μl) was resolved by SDS-PAGE using 10% polyacrylamide. The gel was fixed, treated with Amplify (Amersham), dried, and radiolabeled bands detected by autoradiography.

Preparation of Recombinant L13a

E. coli BL21(DE3)pLysS (Novagen) were transformed with pET-17b-L13a plasmid and induced with isopropyl β-d-thiogalactoside (IPTG). High Five insect cells (Invitrogen) were infected with pFAST-BAC1-L13a. Lysates were made from both L13a-expressing cells and from cells expressing empty vectors as controls. The lysates were partially purified by Sephadex G-200 size-exclusion chromatography. Eluted fractions were monitored by Coomassie blue stain and by immunoblot analysis using anti-L13a antibody.

Preparation of Rabbit Polyclonal Antihuman L13a Antibody and Immunoblot Analysis

A peptide near the C terminus of human L13a, NVEKKIDKYT EVLKTHG, was synthesized and antigenic complexes made (Biosynthesis, Lewisville, TX) by the solid-phase multiple antigen method (Tam, 1988). Rabbits were immunized, antiserum was prepared from the 8-week bleed, and antibody was purified by peptide affinity chromatography. For immunoblot analysis, cell lysates were resolved by SDS-PAGE using 12% polyacrylamide gels and transferred to Immobilon-P (Millipore) by a semidry transfer protocol. The blot was probed with anti-L13a (1:5000) or anti-L28 antibody.

Determination of In Vivo Interaction of L13a and Cp mRNA

The interaction of L13a with endogenous Cp mRNA was determined by immunoprecipitation and RT-PCR. Lysates from IFN-γ-treated U937 cells (500 μg protein in 150 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.05% Triton X-100, 50 mM HEPES, [pH 7.5]) were subjected to immunoprecipitation using 10 μl of affinity-purified anti-L13a antibody. L13a-bound mRNA was isolated by Trizol extraction and subjected to reverse transcription using oligo-dT primer (Superscript kit, Invitrogen) and PCR amplification using a primer-pair specific for amplification of the full-length, 247 nt Cp 3′-UTR.

Metabolic Labeling of L13a by [^{32}P]Orthophosphate

U937 cells (8 × 10^6 cells in 4 ml of RPMI 1640 medium) were treated with IFN-γ (500 units/ml) for up to 24 hr. The cells were collected by centrifugation at 7000 × g, resuspended in phosphate-free medium (Invitrogen), and metabolically labeled with a 6 hr pulse of [^{32}P]orthophosphate. The cells were pelleted by centrifugation at 7000 × g. The cells were lysed by suspension in 0.5% NP40, 50 mM NaCl, 1 mM PMSF, 1 mM dithiothreitol, and 50 mM Tris, [pH 7.6], subjected to three freeze-thaw cycles, and passed several times through a 26 gauge syringe, and centrifuged at low speed to remove debris. L13a was immunoprecipitated from the supernatant using rabbit antihuman L13a (or preimmune serum) and protein A-Sepharose in buffer containing 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 50 mM Tris, [pH 7.6]. Proteins were resolved by 12% SDS-PAGE, and the gel dried and allowed to expose MS film (Kodak, Rochester, NY). *E. coli* BL21(DE3) pLysS transformed with the plasmid pET-17b-L13a and baculovirus-infected High Five insect cells transfected with the plasmid pFAST-BAC1-L13a were metabolically labeled with [^{32}P]orthophosphate using the same procedure but without IFN-γ treatment.

RNA Blot Analysis

U937 cells (1 × 10^8 cells) were treated with IFN-γ for 8 or 24 hr, and total RNA was extracted with Trizol reagent (Invitrogen) and subjected to polyA selection using an OligoTex mRNA kit (Qiagen, Stanford, CA). The mRNA isolated from 100 μg of total RNA was fractionated on a 1% agarose-formaldehyde gel and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). The blot was hybridized with a random primer-labeled, full-length human L13a probe.

Ribosome Fractionation

Ribosome and nonribosome fractions were collected essentially as described (Wormington, 1991). In brief, U937 cells (5 × 10^8 cells) were homogenized in 5 ml of buffer containing 20 mM Tris, [pH 7.4], 10 mM $MgCl_2$, 300 mM KCl, 10 mM dithiothreitol, 100 units/ml RNasin, and 100 μg/ml cycloheximide. After centrifugation at 10,000 × g for 15 min to remove mitochondria and debris, the supernatant was layered over a sucrose (20% w/v) cushion containing cycloheximide and centrifuged at 149,000 × g for 2 hr. The ribosome-containing pellet and nonribosomal supernatant were collected. To remove any ribosome contaminants, the supernatant was subjected to a second centrifugation at 149,000 × g. RNA was extracted from both fractions by Trizol and resolved in denaturing 1% agarose-formaldehyde gel. The gel was stained with ethidium bromide to visualize 18S and 28S RNA representing RNA in the 40S and 60S subunits, respectively. For immunoblot analysis, ribosome pellets were resuspended in Laemmli's buffer and cytosolic fractions were concentrated by vacuum centrifugation and mixed with Laemmli's buffer.

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