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Noncanonical Function of Glutamyl-Prolyl-tRNA Synthetase: Gene-Specific Silencing of Translation

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Summary

Aminoacyl tRNA synthetases (ARS) catalyze the ligation of amino acids to cognate tRNAs. Chordate ARSs have evolved distinctive features absent from ancestral forms, including compartmentalization in a multisynthetase complex (MSC), noncatalytic peptide appendages, and ancillary functions unrelated to aminoacylation. Here, we show that glutamyl-prolyl-tRNA synthetase (GluProRS), a bifunctional ARS of the MSC, has a regulated, noncanonical activity that blocks synthesis of a specific protein. GluProRS was identified as a component of the interferon (IFN)-*g*amma-*a*ctivated *i*nhibitor of *t*ranslation (GAIT) complex by RNA affinity chromatography using the ceruloplasmin (Cp) GAIT element as ligand. In response to IFN-γ, GluProRS is phosphorylated and released from the MSC, binds the Cp 3′-untranslated region in an mRNP containing three additional proteins, and silences Cp mRNA translation. Thus, GluProRS has divergent functions in protein synthesis: in the MSC, its aminoacylation activity supports global translation, but translocation of GluProRS to an inflammation-responsive mRNP causes gene-specific translational silencing.

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Introduction

Translational control of gene expression can be global and regulate synthesis of many proteins, or it can be transcript selective and influence expression of a small subset of proteins (Standart and Jackson, 1994). Transcript-selective translational control permits rapid, fine, and reversible adjustment of gene expression. In most cases, a cytosolic RNA binding protein binds a *cis*-element in the 5′- or 3′-untranslated region (UTR) of the target transcript and inhibits translation, generally by blocking initiation (Mazumder et al., 2003b; Preiss and Hentze, 2003). Translational control elements usually comprise stem-loop structures with limited regions of required sequence. The presence of an element in multiple transcripts permits coregulation of protein families, a system functionally analogous to a bacterial operon (Keene and Tenenbaum, 2002).

Ceruloplasmin (Cp) is a copper-containing plasma protein synthesized and secreted by hepatocytes and activated macrophages. It is a multifunctional oxidase with important roles in inflammation and iron homeostasis (Bielli and Calabrese, 2002). Synthesis of Cp by macrophages or U937 monocytic cells is induced by IFN-γ but is subsequently suppressed by a mechanism involving translational silencing (Mazumder and Fox, 1999; Mazumder et al., 1997). Suppression of Cp expression may help to terminate inflammation and prevent injury caused by uncontrolled accumulation of the oxidase (Mukhopadhyay et al., 1997). Translational silencing requires binding of the interferon-*g*amma-*a*ctivated *i*nhibitor of *t*ranslation (GAIT) protein (or complex) to a 29 nt element in the Cp mRNA 3′UTR (Sampath et al., 2003). The GAIT element has a stem-loop structure containing two invariant residues essential for activity (Sampath et al., 2003). We have shown by a yeast three-hybrid screen that ribosomal protein L13a interacts with the Cp 3′UTR GAIT element and that L13a is required for translational silencing activity in IFN-γ-treated cells (Mazumder et al., 2003a). Furthermore, L13a phosphorylation causes its release from the large ribosomal subunit.

Here, we have used the GAIT element as an RNA affinity ligand to purify additional GAIT element binding proteins and have identified glutamyl-prolyl-tRNA synthetase (GluProRS) as a key component of the GAIT complex. Aminoacyl tRNA synthetases (ARS) catalyze the ligation of amino acids to specific tRNAs and are biochemically responsible for implementation of the genetic code (Ribas de Pouplana and Schimmel, 2001). The bifunctional GluProRS is present in higher eukaryotic cells, and its catalysis of two amino acids to their cognate tRNAs is unique (Cerini et al., 1991). It is a 163 kDa monomer consisting of the catalytic domains separated by a peptide linker. GluProRS is a component of the aminoacyl-tRNA multisynthetase complex (MSC) containing seven other ARSs and three noncatalytic components, p18, p38, and p43 (Han et al., 2003; Robinson et al., 2000). The functions of these smaller proteins are uncertain, but p38 may provide a scaffold for assembly and maintenance of the MSC (Kim et al., 2002).

Several vertebrate ARSs have activities unrelated to aminoacylation (Francklyn et al., 2002). The majority of ARSs with noncanonical functions have appended domains not present in the ancestral proteins (Shiba, 2002). However, a noncanonical function for GluProRS has not been reported. In this report, we elucidate a pathway of GluProRS activation involving phosphorylation and release from the MSC, and subsequent formation of a complex with three other proteins. This complex binds the 3′UTR GAIT element of Cp mRNA and specifically suppresses its translation.

Results

Identification of the Protein Components of the GAIT Complex

We tested whether ribosomal protein L13a by itself binds the GAIT element by RNA electrophoretic mobility shift assay (EMSA). As shown before (Sampath et al., 2003), lysates from U937 cells treated with IFN-γ for 24 hr but not for 8 hr contain a GAIT element binding protein or complex (Figure 1A). Recombinant, insect cell-derived human L13a, which is phosphorylated and silences in vitro translation of a GAIT element-containing reporter transcript (Mazumder et al., 2003a), did not by itself bind the GAIT element (Figure 1A). However, binding was reconstituted upon coaddition of phospho-L13a and cytosolic extract from cells treated with IFN-γ for 8 hr; extracts from untreated cells were inactive. Binding specificity was shown by competition with wild-type but not mutant GAIT element oligonucleotides. *E. coli*-derived L13a, which is not phosphorylated and does not inhibit reporter transcript translation, did not bind the GAIT element under any conditions. Thus, a component in the 8 hr lysate is required for interaction of phospho-L13a with the GAIT element.

To assess whether the GAIT mRNA ribonucleoprotein complex (mRNP) contains additional proteins, the molecular mass was determined. Cytosol from cells treated with IFN-γ for 24 hr was subjected to gel filtration (Figure 1B, top). The peak of GAIT element binding activity, measured by RNA EMSA, corresponded to a size of about 450 kDa (Figure 1B, middle). Transcript-specific translation inhibition was measured in an in vitro translation assay using luciferase (Luc)-Cp GAIT element-poly-A RNA as a heterologous reporter (Figure 1B, bottom). Specificity was shown by simultaneous translation of T7 gene 10, a transcript lacking the GAIT element. The results were consistent with the binding data and indicated that the GAIT mRNP is approximately 450 kDa.

GAIT mRNP proteins were isolated by affinity chromatography using 5′-biotinylated GAIT element RNA as ligand (Figure 2A, left). We took advantage of a mutated (U87C) GAIT element that is inactive with respect to mRNP formation and translational silencing activity (Sampath et al., 2003). Cytosol from IFN-γ-treated cells was applied to columns containing 5′-biotinylated wild-type or mutant GAIT element and eluted with salt. The eluates were resolved by SDS-PAGE and were silver stained (Figure 2A, center). Protein bands specifically eluted from the wild-type column were subjected to tryptic digestion and sequenced by liquid chromatography-tandem mass spectrometry. Three GAIT element binding proteins were identified: GluProRS, NS1-associated protein-1 (NSAP1, also called heterogenous nuclear ribonucleoprotein Q1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). We were unable to obtain mass spectrometric data from one stained band that specifically eluted from the wild-type column (open arrow); its electrophoretic mobility was similar to that of phospho-L13a, and immunoblot analysis showed L13a in the wild-type eluate (Figure 2A, right).

The presence of the four proteins in the GAIT mRNP was tested by immunodepletion of the cytosolic extract, followed by RNA EMSA; formation of the RNA-protein complex was markedly inhibited by each antibody (Figure 2B). Their presence was confirmed by supershift analysis using the same antibodies (data not shown). The function of the depleted lysates was also markedly suppressed, as shown by in vitro translation of a heterologous reporter (Figure 2C). Together, these results show that GluProRS, GAPDH, and NSAP1, in addition to L13a, are components of the functional GAIT mRNP.

IFN-γ Mobilizes GluProRS from the MSC and Induces Two-Stage Assembly of the GAIT mRNP

To explore the mechanism of induction of translational silencing activity, we examined whether IFN-γ induced expression of the GAIT mRNP components. We have previously shown that L13a is not induced (but is phosphorylated) upon IFN-γ treatment (Mazumder et al., 2003a). Immunoblot analysis showed that neither the expression nor the apparent molecular size of GluProRS, NSAP1, and GAPDH was altered by IFN-γ (Figure 3A). We considered the possibility of regulated translocation of GAIT mRNP components from other cytosolic complexes. A strategy coupling size fractionation to immunoblot analysis permitted unbiased detection of movement of all mRNP components. About 90% of the cellular GluProRS in untreated cells is in high molecular weight fractions consistent with the 1.5-mDa MSC, a finding confirmed by colocalization of p38, an integral MSC protein (Figure 3B, top panels). The remainder was in the low molecular weight fractions consistent with free enzyme. L13a was primarily in high molecular weight fractions consistent with its ribosomal localization. NSAP1 and GAPDH were almost exclusively in low molecular weight fractions consistent with an unbound form. None of the four GAIT proteins was detected in the intermediate fractions corresponding to the active fractions. Treatment of cells with IFN-γ for 8 hr showed shifts in two proteins. About 55% of the GluProRS originally in the high molecular weight fractions translocated to the region corresponding to active fractions (Figure 3B, middle panels). Likewise, the majority of the NSAP1 shifted from the low molecular weight fractions to colocalize with GluProRS in the active fractions. The elution positions of GAPDH and L13a were unchanged. These results suggest formation of a binary, pre-GAIT complex consisting of GluProRS and NSAP1 by 8 hr of IFN-γ treatment. After 24 hr, all four proteins were detected in the fractions containing the active GAIT complex (Figure 3B, lower panels). Consistent with our previous report, essentially all L13a in the high molecular weight, ribosomal fraction was mobilized (Mazumder et al., 2003a).

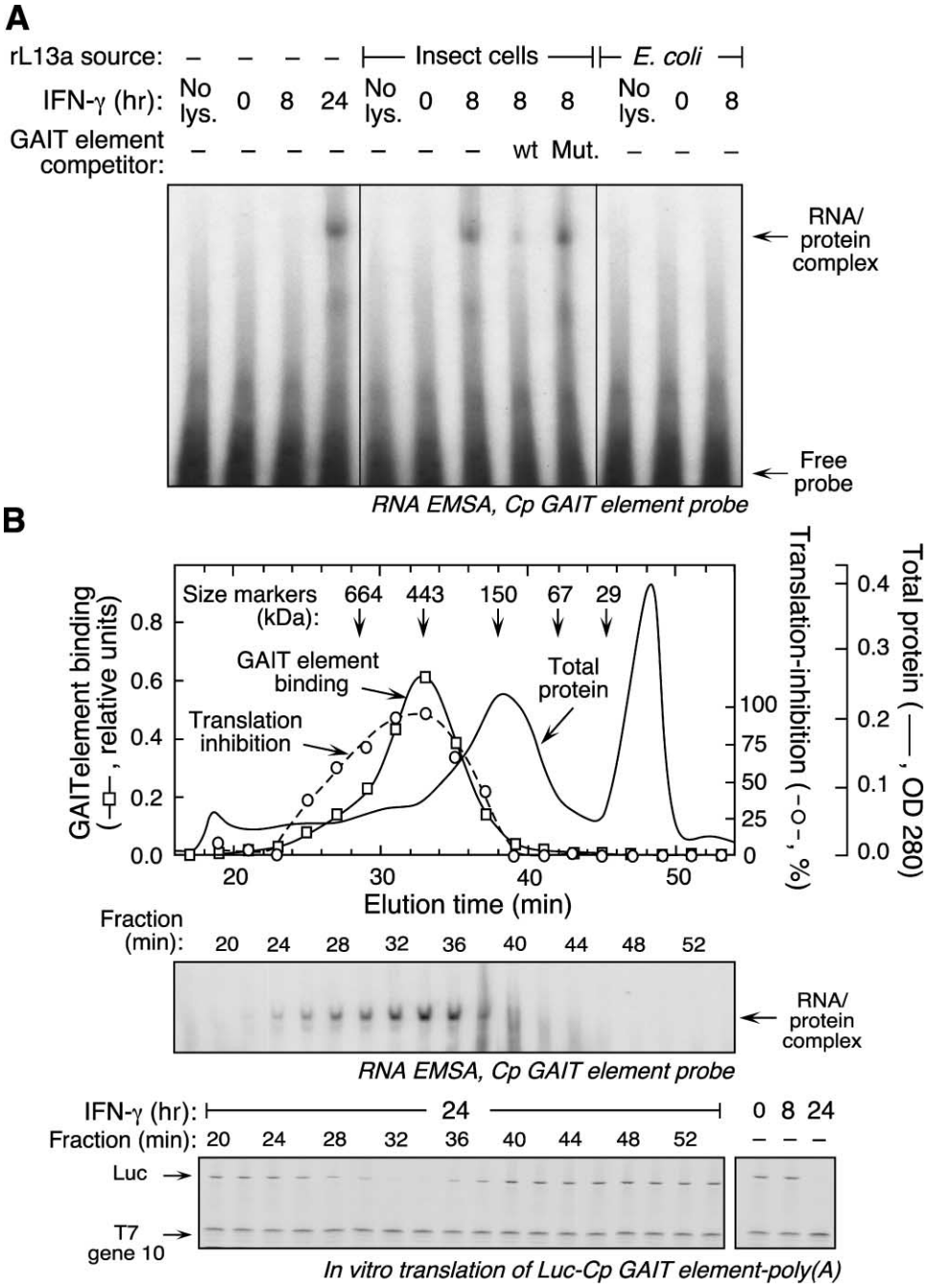

Figure 1. The GAIT mRNP Is a Large, Multicomponent Complex

(A) L13a by itself does not bind the GAIT element. U937 cells were incubated with IFN-γ for 8 or 24 hr and cytosolic lysates (lys.) prepared. Recombinant, full-length human L13a (rL13a) was expressed and partially purified from baculovirus-infected insect cells or *E. coli*. Binding of cell lysates and recombinant L13a to ^{32}P-labeled, 29 nt GAIT element RNA was analyzed by EMSA. Specificity was tested by competition with a 10-fold molar excess of unlabeled, wild-type (wt), or mutant (Mut.) GAIT element.

(B) Size exclusion chromatography indicates that GAIT is a large, multicomponent complex. Cells were treated with IFN-γ for 24 hr and the S100 fraction of the cell lysate (3 mg protein) subjected to Superose 6 size exclusion chromatography. Eluted protein was detected by absorbance at 280 nm and compared to protein size markers (top, line alone). Binding of fractions to radiolabeled, 29 nt human Cp 3′UTR GAIT element was determined by RNA EMSA (middle) and quantitated by densitometry (top, □). Translational silencing activity of the fractions (bottom left) (and unfractionated cell lysates, bottom right) was measured by in vitro translation of capped, Luc-Cp GAIT element-poly(A) cRNA in rabbit reticulocyte lysate containing [^{35}S]methionine (top, ○). Capped, T7 gene 10 cRNA was added as a control to show specificity of the inhibition.

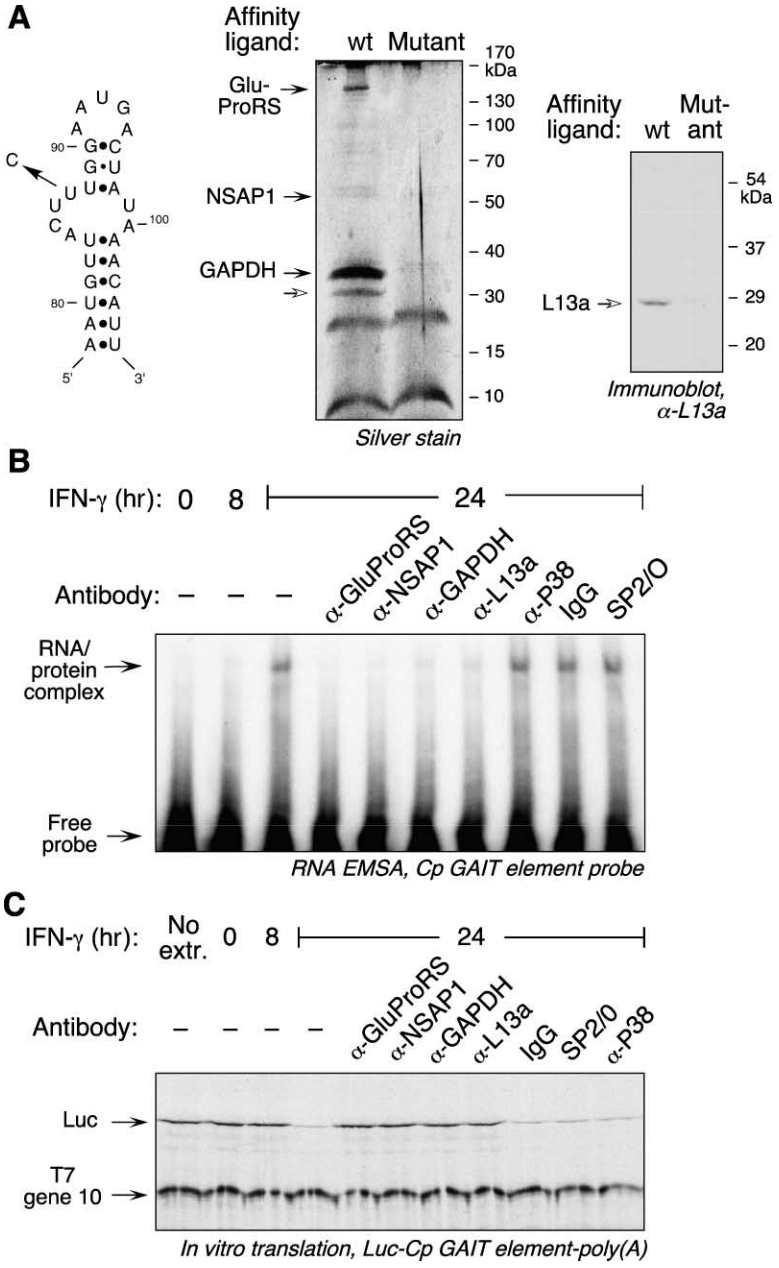

Figure 2. RNA Affinity Chromatography Identifies GluProRS, NSAP1, and GAPDH as Components of the GAIT mRNP

(A) Determination of specifically bound proteins. Cells were treated with IFN-γ for 24 hr, and the lysate (4 mg of protein) was precleared with scrambled GAIT and mutant GAIT element oligomers. Cleared lysate was incubated with biotinylated wild-type or mutant GAIT element (left) and then with streptavidin microbeads. Specifically bound protein was eluted with buffer containing 300 mM NaCl and samples subjected to SDS-PAGE and silver staining (center). Trypsinized bands were analyzed by liquid chromatography-tandem mass spectrometry to give peptide sequences. A major silver-stained band had an electrophoretic mobility consistent with phosphorylated L13a (center, open arrow) but was not identifiable by mass spectrometry. Immunoblot analysis of samples with anti-L13a antibody shows L13a in the GAIT mRNP (right).

(B) Verification of proteins in the GAIT mRNP by RNA EMSA. U937 cells were treated with IFN-γ for 8 or 24 hr. The 24 hr cell lysate was subjected to gel filtration, and active fractions were pooled and subjected to immunodepletion with antibodies against candidate proteins GluProRS, NSAP1, GAPDH, and L13a or with anti-p38, IgG, and monoclonal antibody SP2/O as control antibodies. Binding to radiolabeled GAIT element was determined by RNA EMSA.

(C) In vitro translation confirms that identified proteins are components of a functional GAIT mRNP. The loss of translational silencing activity of the cell lysates after specific immunodepletion was examined by their effect on in vitro translation of the chimeric Luc reporter as described above.

These results suggest a two-stage assembly of the GAIT complex.

The appearance of GluProRS in the intermediate fractions coincides with its disappearance from the high molecular weight fractions, suggesting that the MSC is the source of GluProRS in the pre-GAIT complex. Two other sources of GluProRS in the pre-GAIT complex should be considered: the free cytoplasmic pool and protein from de novo synthesis. The size fractionation experiment excludes the free pool as a major source of GluProRS, since the amount is insufficient to account for that subsequently found in the active fractions. To test de novo synthesis as a source of GluProRS, pre-GAIT complex formation was determined by coimmunoprecipitation of NSAP1 and GluProRS, under conditions in which protein synthesis was blocked. The coimmunoprecipitation procedure verified that GluProRS and NSAP1 are interacting partners in the pre-GAIT complex (Figure 4A, top left). Cycloheximide, at a concentration that inhibited total protein synthesis by about 90%, did not inhibit formation of this complex (Figure 4A, top right). Immunoblot analysis with LysRS (Figure 4A, middle panel) and NSAP1 (Figure 4A, bottom panel) as markers of the multisynthetase and pre-GAIT complexes, respectively, verified the effectiveness of the immunoprecipitation procedure and the specificity of the GluProRS shift. Thus, GluProRS in the pre-GAIT complex is not derived from de novo synthesis. Together these experiments show that IFN-γ not only mobilizes L13a from the large ribosomal subunit but also induces release of GluProRS from its parent MSC.

Our finding that a substantial amount of GluProRS in

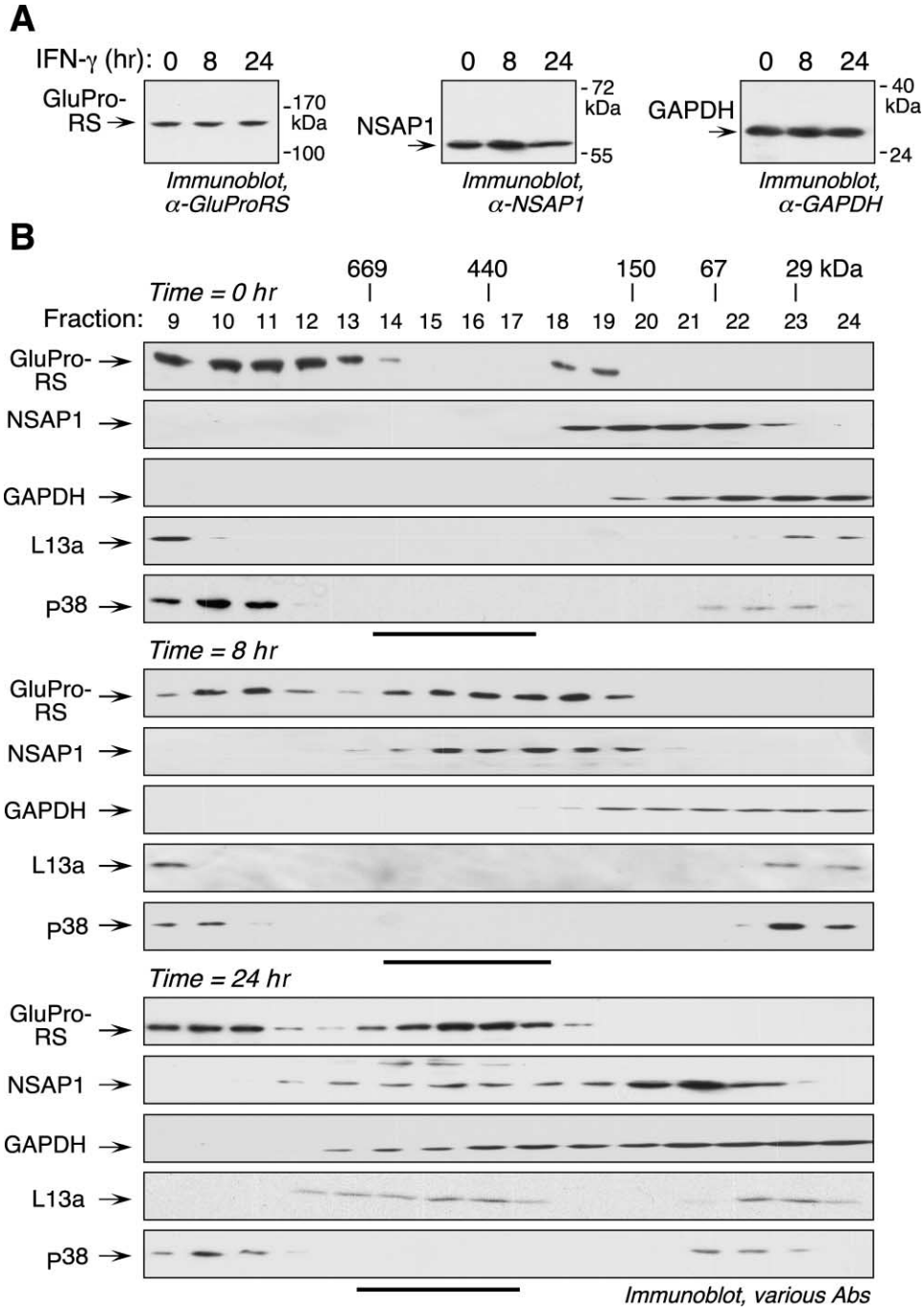

Figure 3. IFN-γ Induces Release of GluProRS from the MSC

(A) IFN-γ does not alter expression of GAIT mRNP components. Cell lysates from IFN-γ-treated U937 cells were subjected to immunoblot analysis using anti-GluProRS, -NSAP1, and -GAPDH antibodies.

(B) IFN-γ induces stepwise mobilization of proteins into pre-GAIT and GAIT complexes. Lysates from U937 cells treated with IFN-γ for 0 (top), 8 (middle), or 24 (bottom) hr were subjected to Superose-6 gel filtration chromatography and 2 ml fractions were collected. Fractions corresponding to active fractions (numbers 14–17) in the 24 hr lysate are denoted by bars. Fractions were analyzed by immunoblot analysis using antibodies (Abs) against GAIT mRNP components and anti-p38 as an indicator of the MSC.

IFN-γ-treated cells leaves the MSC raises the possibility that global protein synthesis is perturbed. However, metabolic labeling with [^{35}S]Met shows that the overall rate of protein synthesis is unchanged (Figure 4B). Thus, IFN-γ induces release of GluProRS from the MSC (and L13a from the ribosome) without compromising the overall cellular capacity for translation.

Cofractionation does not prove interaction of the GAIT complex proteins. These interactions were examined by immunoprecipitation studies. Fractions in the active

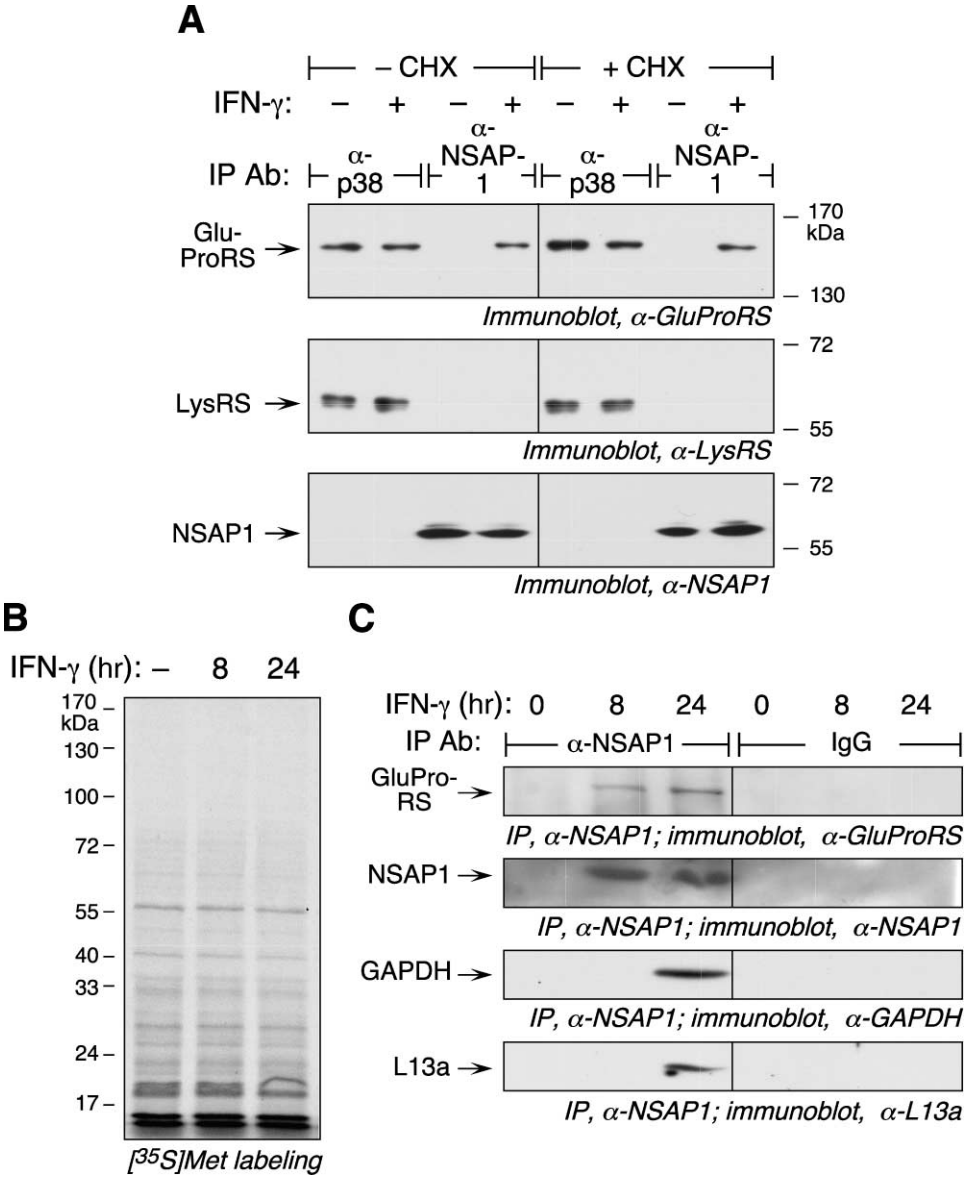

Figure 4. Protein-Protein Interactions in the Pre-GAIT and GAIT Complexes

(A) GluProRS in the pre-GAIT complex is derived from the MSC, not from de novo synthesis. U937 cells were preincubated with cycloheximide (50 μg/ml) for 30 min and then with IFN-γ for 8 hr. Cell lysates were immunoprecipitated with antibodies against p38 and NSAP1 to isolate the multisynthetase and pre-GAIT complex, respectively. Isolated complexes were probed by immunoblot analysis using antibodies against GluProRS (top), LysRS (middle), and NSAP1 (bottom).

(B) Release of GluProRS from the MSC does not alter global protein synthesis. U937 cells were incubated with IFN-γ for 8 or 24 hr and then metabolically labeled by incubation for 2 hr with [^{35}S]methionine in methionine-free medium. Control cells were labeled without IFN-γ treatment. An aliquot of the lysates was resolved by SDS-PAGE and detected by fluorography.

(C) Coimmunoprecipitation shows interaction of GluProRS with NSAP1 in the pre-GAIT complex and interaction of all four proteins in the active GAIT mRNP. Cells were treated with IFN-γ and subjected to gel filtration as in Figure 3B. Column fractions corresponding to the active fractions (numbers 14–17) were pooled and immunoprecipitated (IP) using anti-NSAP1 antibody (or IgG as control) and protein-A Sepharose beads. Precipitates were analyzed by immunoblot using antibodies against GluProRS (top panel), NSAP1 (second panel), GAPDH (third panel), or L13a (bottom panel).

region of the elution profile were pooled, immunoprecipitated with antibody directed against NSAP1, and subjected to immunoblot analysis. The experiment confirmed that NSAP1 interacted only with GluProRS during the assembly of the pre-GAIT complex and that all four proteins were subsequently joined to form the active GAIT complex (Figure 4C).

GluProRS Release from the MSC Requires Phosphorylation

Several ARSs, including GluProRS (Venema and Traugh, 1991), are subject to phosphorylation, but in no case is the function of phosphorylation known (Pendergast and Traugh, 1985). Metabolic labeling of cells with ^{32}P-orthophosphate showed that IFN-γ induced phosphorylation

of GluProRS by 8 hr and that phosphorylation continued for at least 24 hr (Figure 5A). A radiolabeled band consistent with phospho-L13a was also observed, in agreement with our previous findings (Mazumder et al., 2003a). Immunoprecipitation of GluProRS and immunoblot analysis with phosphospecific antibodies showed phosphorylation of GluProRS on serine but not on threonine or tyrosine (Figure 5B).

The temporal relationship between phosphorylation and release of GluProRS was examined. GluProRS phosphorylation, detected with anti-phosphoserine antibody, was seen after 1 hr of IFN-γ treatment (Figure 5C, first and second panels). GluProRS mobilization from the MSC was measured by its appearance in the pre-GAIT complex in association with NSAP1. Lysates were immunoprecipitated with anti-NSAP1 antibody and bound GluProRS detected by immunoblot analysis (NSAP1 was probed as recovery control). GluProRS was detected in the pre-GAIT complex after 2 hr of IFN-γ treatment, showing that phosphorylation precedes mobilization by about 1–2 hr (Figure 5C, third and fourth panels). Since appearance of GluProRS in the pre-GAIT complex may not coincide with its release from the MSC, the latter was examined directly. The MSC was immunoprecipitated with anti-p38 antibody and GluProRS detected by immunoblot analysis (LysRS was probed as recovery control). The disappearance of GluProRS from the MSC nearly coincided with its appearance in the pre-GAIT complex (Figure 5C, fifth and sixth panels). Densitometric analysis indicates that 60%–80% of the GluProRS is released, a conclusion consistent with the 55% release calculated by size fractionation (Figure 5C, bottom panel).

To determine whether GluProRS phosphorylation is required for IFN-γ-mediated translocation, cells were pretreated with inhibitors of serine/threonine kinases: staurosporine, a relatively nonspecific inhibitor; and H-7, an inhibitor with partial selectivity for protein kinases A and C (Hidaka et al., 1984). In a control experiment, staurosporine and H-7 completely blocked IFN-γ-induced GluProRS phosphorylation (Figure 5D, top panel). HA-1004, a structural analog of H-7 with much lower activity, was ineffective. The release of GluProRS from the multisynthetase and its appearance in the pre-GAIT complex were determined by immunoprecipitation with anti-p38 and anti-NSAP1 antibodies, respectively. The precipitates were probed with anti-GluProRS antibody and with anti-LysRS and -NSAP1 antibodies as controls. Staurosporine and H-7 (but not HA-1004) completely inhibited GluProRS release from the MSC and its appearance in the pre-GAIT complex, indicating that GluProRS phosphorylation is required for release (Figure 5D, lower three panels). Lysates were treated with alkaline phosphatase to determine whether dephosphorylation of GluProRS could dissociate the pre-GAIT complex. In a control experiment, phosphatase treatment completely dephosphorylated GluProRS (Figure 5E, top panel). The multisynthetase and pre-GAIT complexes were isolated and probed as above. The phosphatase completely dissociated GluProRS from NSAP1, indicating that GluProRS phosphorylation is required for the interaction and that phosphorylation is an initiating event in the formation of the GAIT mRNP (Figure 5E, lower three panels).

The GAIT Complex Binds the Cp 3′UTR via an Interaction of GluProRS with the GAIT Element

We have examined the specific role of GluProRS in inhibiting Cp translation. Direct binding of GluProRS to the Cp GAIT element is an attractive possibility in view of the three RNA binding domains in GluProRS, i.e., the two tRNA binding sites and the central linker, which has nonspecific RNA binding activity (Cahuzac et al., 2000; Jeong et al., 2000). Protein size was determined by ultraviolet crosslinking to radiolabeled GAIT element RNA. A labeled complex of about 170 kDa, a size consistent with GluProRS, was formed in the presence of wild-type but not mutant GAIT element probe (Figure 6A). As expected, crosslinking was seen after IFN-γ treatment for 24 hr but not for 8 hr. To verify the presence of GluProRS, the crosslinked complex was immunoprecipitated, in the presence of detergent, with antibodies against the GAIT mRNP proteins. Only anti-GluProRS precipitated the labeled RNA/protein complex (Figure 6B). Since crosslinking can result from proximity rather than actual binding, we tested purified GAIT proteins by RNA EMSA. GluProRS formed an RNA-protein complex with the wild-type GAIT element, but NSAP1, GAPDH, and phospho-L13a all failed to bind (Figure 6C). Thus, GluProRS is the GAIT mRNP protein that directly binds the GAIT element.

In vivo binding of the GAIT complex to Cp mRNA was examined. Lysates were immunoprecipitated with anti-GluProRS, and extracted RNA was subjected to RT-PCR using Cp 3′UTR-specific primers (Mazumder et al., 2003a). Amplification of lysates not subjected to immunoprecipitation showed that Cp mRNA was induced in cells treated with IFN-γ for 8 or 24 hr, as expected (Figure 6D, top). Cp mRNA was complexed to GluProRS only in cells treated with IFN-γ for 24 hr. In control experiments, immunoprecipitation with IgG or elimination of the reverse transcriptase step (Figure 6D, bottom) gave no amplification of Cp mRNA. Thus, GluProRS in the holo-GAIT complex (present at 24 hr) but not in the pre-GAIT complex (present at 8 hr) binds the Cp 3′UTR in IFN-γ-treated cells.

Discussion

Our experiments suggest a two-stage pathway for formation of the GAIT mRNP in IFN-γ-treated monocytic cells (Figure 7). During the early stage, which takes about 4 hr, GluProRS becomes phosphorylated and escapes from the MSC. GluProRS phosphorylation induces binding to NSAP1 to form the binary, pre-GAIT complex that is not competent to bind the Cp GAIT element. About 12 hr later, L13a is phosphorylated and released from the 60S ribosomal subunit (Mazumder et al., 2003a) to join GAPDH and the pre-GAIT complex, forming the mature GAIT mRNP. This complex, via interaction of GluProRS with the GAIT element, binds Cp mRNA and blocks its translation. The four GAIT components are housekeeping proteins abundant in most if not all cells. This observation may explain the paradoxical finding that phospho-L13a by itself inhibits in vitro translation of a GAIT element-containing reporter RNA (Mazumder et al., 2003a) but does not bind the GAIT element in an RNA EMSA. In the translation assay, phospho-L13a is

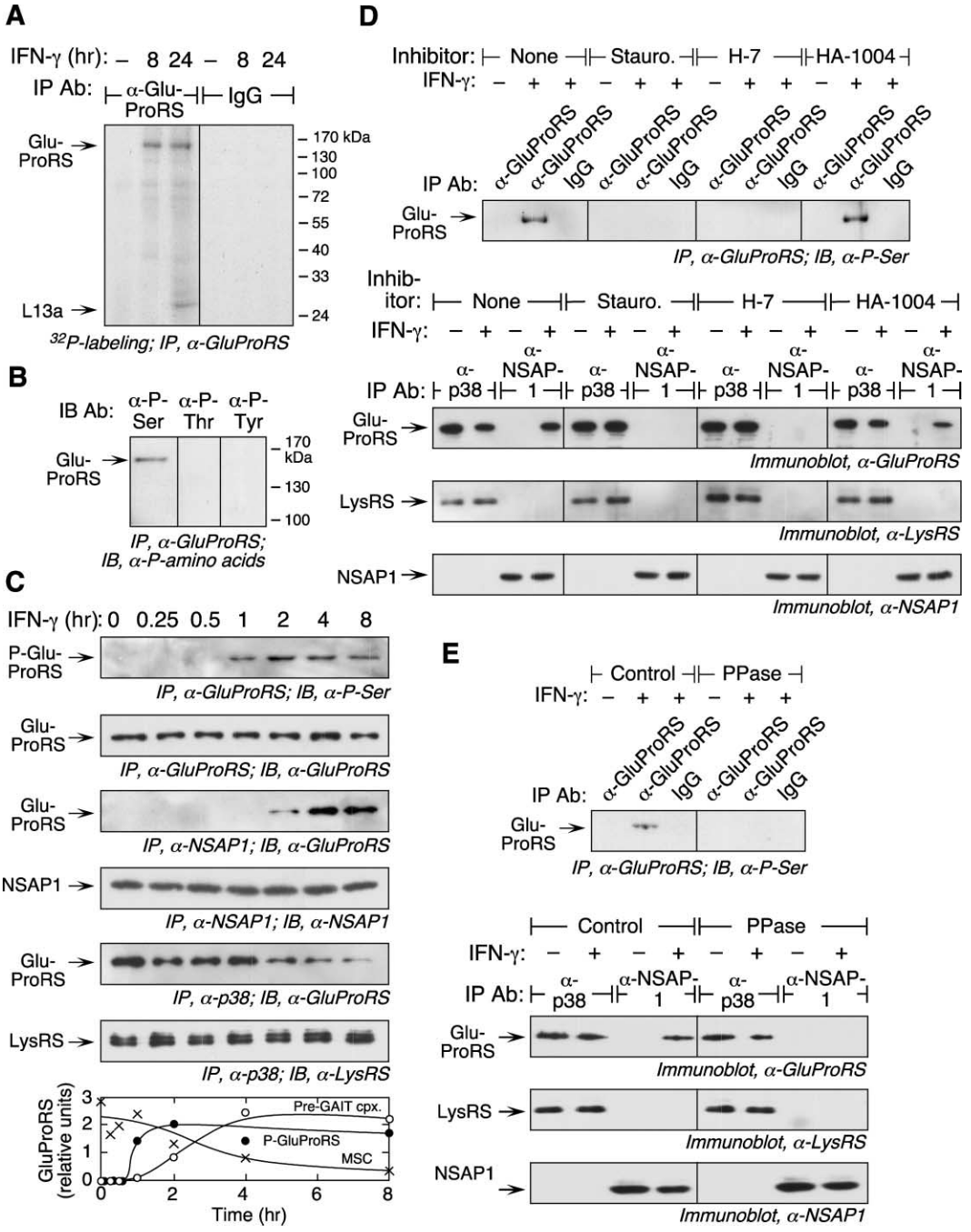

Figure 5. Phosphorylation Is Required for GluProRS Release from the MSC

(A) Metabolic labeling shows that IFN-γ induces GluProRS phosphorylation. U937 cells were incubated with IFN-γ and then with a 6 hr pulse of ^{32}P-orthophosphate between 5 and 11 hr (denoted 8 hr) or between 21 and 27 hr (denoted 24 hr). Lysates were immunoprecipitated with anti-GluProRS antibody (or IgG) and labeled proteins detected by autoradiography.

(B) IFN-γ induces serine phosphorylation of GluProRS. Lysates from cells treated with IFN-γ for 8 hr were immunoprecipitated with anti-GluProRS antibody and immunoblotted (IB) with antibodies targeted against phosphoserine (P-Ser), phosphothreonine (P-Thr), or phosphotyrosine (P-Tyr).

(C) Phosphorylation of GluProRS precedes its release from MSC. U937 cells were treated with IFN-γ for up to 8 hr, and lysates were prepared. To determine GluProRS phosphorylation, the lysate was immunoprecipitated with anti-GluProRS antibody and the pellet immunoblotted with antibodies against P-Ser (top panel) and GluProRS (second panel). To determine GluProRS binding to NSAP1, the lysate was immunoprecipitated with anti-NSAP1 antibody and the pellet immunoblotted with anti-GluProRS (third panel) and anti-NSAP1 (fourth panel). To examine the release of GluProRS from the MSC, the lysate was immunoprecipitated with anti-p38 antibody and the pellet immunoblotted with anti-GluProRS (fifth panel) and anti-LysRS (sixth panel) antibodies. The relative amount of GluProRS in each pool was quantitated by densitometry (bottom panel). Phospho-GluProRS (P-GluProRS) was normalized by total GluProRS (●), GluProRS in the pre-GAIT complex (cpx.) was normalized by NSAP1 (○), and GluProRS in the MSC was normalized by LysRS (×).

(D) GluProRS phosphorylation is required for release from the MSC. Cells were incubated with IFN-γ for 0.5 hr and then staurosporine (1 μg/ml, Stauro.), H-7 (25 μM), or HA-1004 (25 μM) was added for an additional 3.5 hr and lysates prepared. To show efficiency of the kinase

likely to be complemented by the GAIT proteins in the reticulocyte lysate. More importantly, our findings support the principle that common, constitutive proteins, even integral components of complexes, e.g., the MSC and ribosome, are sources of context-specific regulatory proteins mobilized to form new complexes with extraordinary functions.

Phosphorylation of GluProRS and Release from the MSC

The MSC of higher eukaryotes contains nine ARS activities and three noncatalytic proteins (Quevillon et al., 1999; Rho et al., 1999). The function of the complex, and the basis for selective inclusion of fewer than half of the ARSs in the complex, remains poorly understood. A clue may be found in studies in yeast that have a smaller tRNA synthetase complex consisting of MetRS, GluRS, and Arc1p, a homolog of p43 (Galani et al., 2001). When assembly of the ARSs into the ternary complex is prevented by removal of a noncatalytic appendage, all three components enter the nucleus. Thus, a major function of the MSC may be sequestration of constituent ARSs in the cytoplasm. Alternatively, the complex may restrict the access of specific synthetases to cytoplasmic targets other than tRNA. This idea is consistent with recent observations that several multisynthetase components in their unbound forms have activities unrelated to protein synthesis; compartmentalization in an inaccessible complex may provide a mechanism for regulation. There are several examples of vertebrate multisynthetase components that exhibit alternate activities in an unbound form: free MetRS in the nucleolus facilitates ribosomal RNA synthesis (Ko et al., 2000), transforming growth factor-β induces translocation of p38 to the nucleus where it inactivates c-*myc* (Kim et al., 2003), and LysRS binds the mast cell transcription factor MITF and maintains it in an inactive state (Lee et al., 2004).

The mechanism of release from the MSC is not known for any ARS. Removal of the peptide appendage from certain yeast and mammalian ARSs induces dissociation from the MSC (Galani et al., 2001; Robinson et al., 2000), but proteolysis as a release mechanism has not been reported in vivo. Several ARSs, including GluProRS and other members of the MSC, are phosphorylated in vitro by protein kinases (Venema and Traugh, 1991). In vivo phosphorylation has been described in reticulocytes (Pendergast et al., 1987) and in liver (Berg, 1990), but phosphorylation may be cell type- or condition-specific, since attempts to show phosphorylated ARSs in several cell lines have not been fruitful (Mirande et al., 1985). The primary function of ARS phosphorylation is not likely to be regulation of synthetase activity, since the effects are generally small (Clemens, 1990); however, other functions have not been seen. We have established that IFN-γ treatment induces GluProRS phosphorylation and that the modification is essential for release from the MSC. The mechanism underlying GluProRS release is unknown. Since GluProRS phosphorylation precedes its release, the kinase likely recognizes GluProRS while bound to the MSC. In view of our finding that GluProRS phosphorylation is required not only for release from the MSC but also for binding to NSAP1, the interaction with NSAP1 may facilitate GluProRS release.

Two-Stage Mechanism of Assembly of GAIT Complex

The pre-GAIT complex of GluProRS and NSAP1 is formed after 2–4 hr of IFN-γ treatment. NSAP1 has been observed in several RNA binding protein complexes, and it may have a broad role in RNA-related regulatory processes (Blanc et al., 2001; Grosset et al., 2000; Mourelatos et al., 2001). Our experiments show that the pre-GAIT complex does not bind the Cp mRNA GAIT element and regulate its translation. This result appears to conflict with the crosslinking studies that show a direct interaction of GluProRS with the GAIT element. We surmise that NSAP1 obstructs the binding site or alters the conformation of GluProRS such that it fails to bind the GAIT element. A similar inhibitory mechanism has been shown for GRY-RBP (a slightly larger NSAP1 isoform) in the apolipoprotein B RNA editing system. In this case, GRY-RBP binds apobec-1 complementation factor and inhibits its binding to apolipoprotein B RNA, thereby preventing RNA editing (Blanc et al., 2001).

The interacting domains of GluProRS and NSAP1 have not been identified. Human GluProRS consists of two catalytic structures separated by a central linker of three 50 amino acid WHEP domains (Cerini et al., 1991; Fett and Knippers, 1991). NMR studies of individual WHEP domains show a helix-turn-helix structure with basic residues lining one face (Cahuzac et al., 2000; Jeong et al., 2000). The linker domain may provide the binding site for NSAP1, since it is structurally related to NS1, an NSAP1 target (Harris et al., 1999). The linker also may provide the site for binding the GAIT element, since RNA binding activity has been reported (Cahuzac et al., 2000; Jeong et al., 2000). The physiological significance of the RNA binding linker domain has not been elucidated, but one report suggests it binds the 3′UTR of GluProRS mRNA, possibly to autoregulate at the level of translation (Schray and Knippers, 1991). It is tempting to speculate that the GluProRS linker binds both NSAP1 and the GAIT element, and successful competition by the former prevents the latter. The specific functions of GAPDH and L13a in the translational silencing mechanism are unknown. GAPDH is an integral component of the OCA-S

inhibitor, an aliquot of lysate was immunoprecipitated with anti-GluProRS antibody and subjected to immunoblot analysis with anti-phosphoserine antibody (top panel). Lysates were immunoprecipitated with antibodies directed against p38 and NSAP1 to isolate the multisynthetase and pre-GAIT complexes, respectively. The precipitates were subjected to immunoblot analysis with anti-GluProRS (second panel); the precipitates were also probed with anti-LysRS (third panel) and anti-NSAP1 (bottom panel) antibodies to show recovery of the multisynthetase and pre-GAIT complexes, respectively.

(E) GluProRS phosphorylation is required for its interaction with NSAP1 to form the pre-GAIT complex. Lysates from cells treated with IFN-γ for 4 hr were incubated with shrimp alkaline phosphatase (PPase) at 37°C for 1 hr and then subjected to immunoprecipitation and immunoblot analysis as in (D).

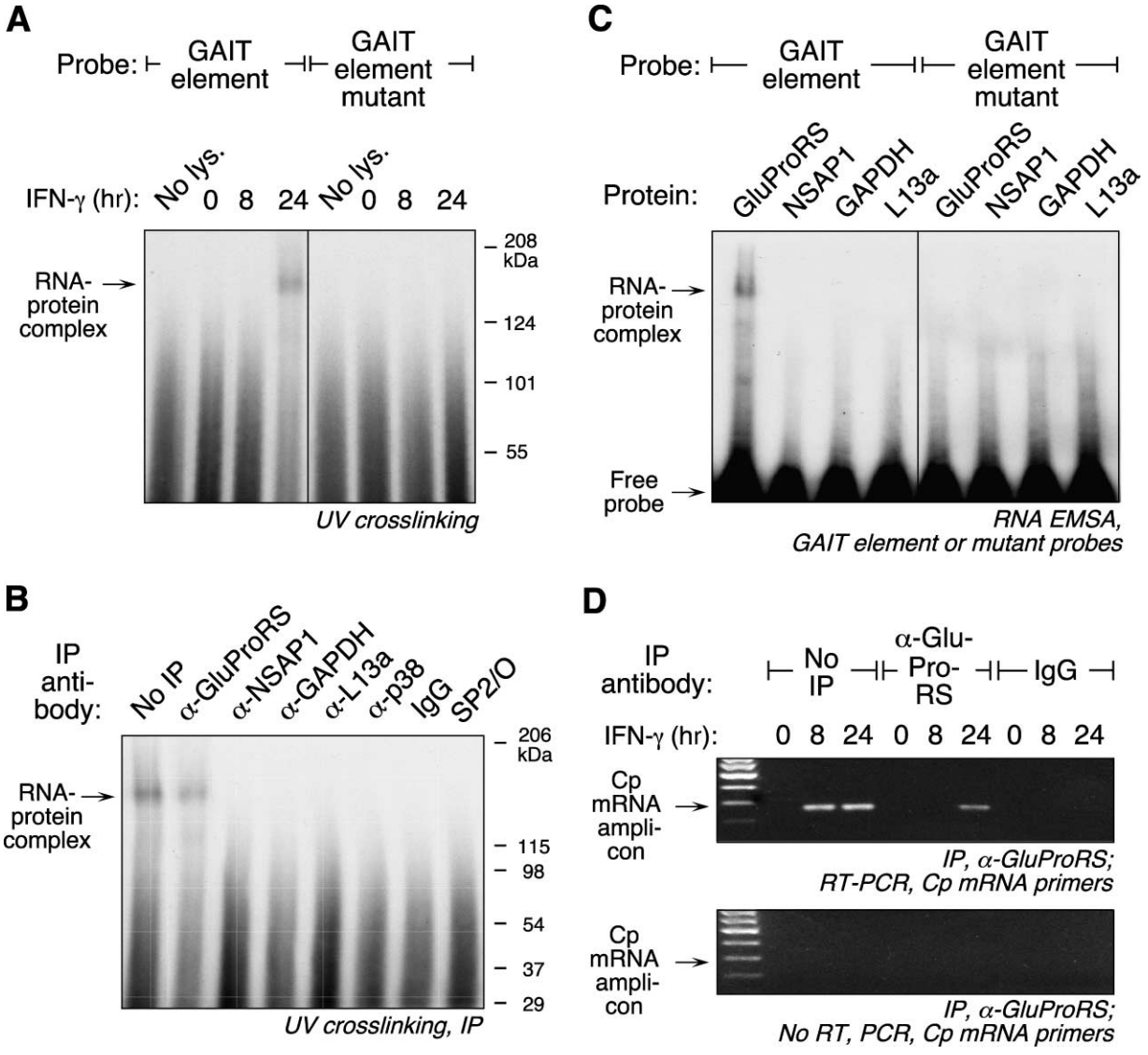

Figure 6. GluProRS Interacts with the GAIT Element

(A) Crosslinking identifies an approximately 170 kDa protein that binds the GAIT element. Cells were treated with IFN-γ for 0, 8, or 24 hr. Cytosolic lysates (lys.) were incubated with wild-type or mutant ^{32}P-labeled GAIT element and subjected to crosslinking by exposure to ultraviolet light. Proteins were resolved on SDS-PAGE and RNA-protein complexes visualized by autoradiography.

(B) Immunoprecipitation analysis identifies GluProRS as the GAIT element-interacting protein. Cytosolic extract from cells treated with IFN-γ for 24 hr was subjected to ultraviolet crosslinking. The protein mixture was immunoprecipitated under detergent-containing, denaturing conditions, with antibodies directed against GluProRS, NSAP1, GAPDH, L13a, or p38 (or IgG). Radiolabeled proteins were resolved and detected by autoradiography.

(C) Purified GluProRS binds the GAIT element in vitro. Purified rat GluProRS (81% identical to human amino acid sequence), rat NSAP1 (99% identical to human), purified rabbit GAPDH (95% identical to human), and recombinant insect cell-derived human phospho-L13a (100 ng of each protein) were subjected to RNA EMSA using radiolabeled wild-type (left) and mutant (right) Cp GAIT element probes.

(D) GluProRS binds the GAIT element in vivo. U937 cells were treated with IFN-γ for 8 or 24 hr. Cytosolic extracts were subjected to immunoprecipitation with anti-GluProRS antibody or IgG control. RNA was extracted from the immunoprecipitate or from extracts not subjected to immunoprecipitation (No IP). RNA was subjected to RT-PCR amplification with primers specific for the Cp 3′UTR (top). As a control, the Cp 3′UTR was amplified by PCR in the absence of the RT reaction (bottom).

coactivator complex and is a redox-sensitive regulator of transcription (Zheng et al., 2003). GAPDH may have a parallel function in translational control, a mechanism consistent with the known oxidation functions of Cp. L13a could, by binding ribosomal RNA, act as a "ribosome trap," thereby preventing either release or initiation of the large ribosomal subunit. Several mechanisms by which protein complexes exert transcript-specific translational control have been identified (Preiss and Hentze, 2003); however, the translational silencing mechanism used by the GAIT complex is not yet known.

Noncanonical Function of GluProRS in Translation Inhibition

Several chordate ARSs have noncanonical activities, i.e., cellular functions unrelated to tRNA aminoacylation

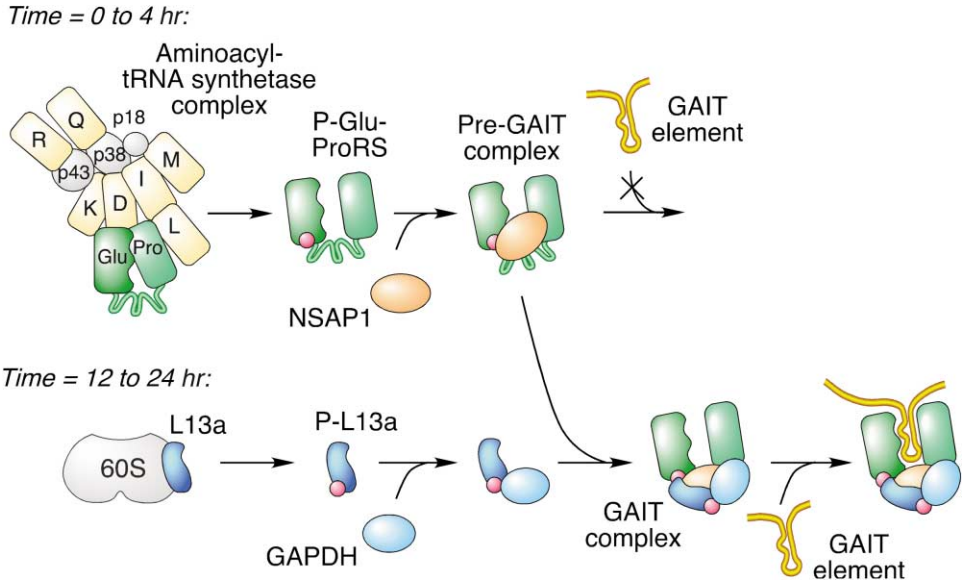

Figure 7. Two-Stage Model of GAIT Complex Formation

(Francklyn et al., 2002; Ko et al., 2002). Recent attention has focused on ARSs containing appendages similar to the WHEP domains in the linker of GluProRS, i.e., TrpRS, HisRS, MetRS, and GlyRS (Shiba, 2002). In chordates, these ARSs all contain a single copy at either the N or C terminus, except for GluProRS, which has three tandem copies in the central linker. In most cases, the appendage does not contribute to aminoacylation activity and thus may be involved in other functions. Indeed, all but GluProRS have been implicated in noncanonical activities. Two have extracellular functions related to cell movement: HisRS is chemotactic for leukocytes (Howard et al., 2002), and a TrpRS fragment inhibits angiogenesis (Otani et al., 2002). The N-terminal appendage of GlyRS binds to RNA not associated with its aminoacylation function (Wu et al., 1995), and mutations cause inherited neuropathies (Antonellis et al., 2003). Finally, MetRS enhances ribosomal RNA synthesis (Ko et al., 2000) and may facilitate transfer of aminoacylated tRNA to elongation factor EF-1α (Kaminska et al., 2001). Thus, GluProRS joins the other WHEP domain-containing ARSs in expressing a noncanonical function. Noncanonical functions are not restricted to ARS with this motif; human TyrRS, which contains a distinct peptide appendage, induces angiogenesis (Wakasugi et al., 2002), and GlnRS has antiapoptotic activity (Ko et al., 2001). GluProRS and L13a join an expanding family of translation machinery components, e.g., eIF2α and eIF4E/4E-BP, that are switched by phosphorylation from facilitators of global translation to gene-selective modulators of translation (Dever, 2002; Mazumder et al., 2003a).

Translational silencing of Cp may be a mechanism by which pathological accumulation of the protein is prevented. Excess Cp in inflammatory sites, in the presence of superoxide, may cause oxidative damage due to its surface copper (Mukhopadhyay et al., 1997). Alternatively, limiting Cp accumulation may maximize its bactericidal activity, which is suppressed at high concentrations (Klebanoff, 1992). Thus, the delayed translational silencing activity may have evolved to limit or terminate synthesis of Cp and possibly other inflammatory proteins. Identification of coregulated transcripts may provide clues to the physiological function of GAIT-mediated translational silencing. A pattern search for the GAIT element based on structural and sequence features reveals candidate elements in the 3′UTR of about 30 human transcripts (Sampath et al., 2003). Several are interesting due to their relevance to inflammation and injury, e.g., death-associated protein kinase, a serine/threonine kinase induced by IFN-γ in U937 cells (Deiss et al., 1995); and Mox1, an NADPH oxidase that produces superoxide (Suh et al., 1999). Multiple inflammatory proteins are controlled at the level of translation. For example, epithelial cell production of interleukin-18, a specific inducer of IFN-γ transcription, is translationally regulated (Garcia et al., 2003). IFN-γ mRNA negatively regulates its own translation by formation of an RNA pseudoknot, possibly to prevent adverse consequences of long-term activation by IFN-γ (Ben-Asouli et al., 2002). Finally, macrophage products themselves may require rapid downregulation, and IFN-γ-stimulated expression of inducible nitric oxide synthase, like Cp, is under strict translational control (El-Gayar et al., 2003). We speculate that the GAIT mRNP and as-yet undiscovered translational control complexes may be critical inhibitors of gene expression necessary for the timely resolution of inflammation.

Experimental Procedures

Reagents

Rabbit reticulocyte lysate, methionine-free amino acids, and RNasin were from Promega (Madison, WI). Human IFN-γ was obtained from R&D Systems (Minneapolis, MN), RNase H and Superscript was from Invitrogen (Gaithersburg, MD), and shrimp alkaline phosphatase from Fermentas. Translation grade [^{35}S]methionine was from NEN-DuPont (Boston, MA). H-7 and HA-1004 were purchased from Biomol. Staurosporine, rabbit muscle GAPDH, and other reagents were from Sigma (St. Louis, MO). GluProRS was purified from rat liver (Ting et al., 1992). Recombinant human L13a was expressed in *E. coli* or insect cells (Mazumder et al., 2003a). Antibodies against human GluProRS (Kim et al., 2002); GAPDH (Novus); LysRS (Kim

et al., 2002); L13a (Mazumder et al., 2003a); NSAP1; and against phospho-Ser, phospho-Thr, and phospho-Tyr (Biodesign) were used in immunoassays.

To prepare recombinant NSAP1, a plasmid DNA encoding rat GRY-RBP, a larger isoform that contains the entire NSAP1 sequence, was obtained from Donna Driscoll. Primers with flanking restriction sites were used to amplify NSAP1 by PCR. The product was gel purified, cloned into the HTa baculovirus vector (HTa-NSAP1), and used to infect High Five insect cells (Invitrogen). Lysates were made from cells expressing NSAP1 or empty vector and purified by Y-PER 6xHis Fusion Protein Purification Kit (Pierce). Eluted fractions were tested by immunoblot analysis using anti-NSAP1 antibody.

Binding and In Vitro Translation Assays

Cytosolic lysates were prepared from human U937 monocytic cells cultured in RPMI 1640 medium containing 10% fetal bovine serum (Sampath et al., 2003). For RNA EMSA, cell lysate (or 100 ng of purified protein) was incubated with radiolabeled, synthetic transcripts of wild-type and mutant Cp 3′UTR GAIT elements prepared by oligonucleotide-directed transcription (Sampath et al., 2003). For in vitro translation assays, gel-purified Luc-Cp GAIT element-poly(A) (200 ng) and T7 gene 10 (100 ng) cRNAs were incubated with cell extracts (4 μg protein) in 35 μl of rabbit reticulocyte lysate (Sampath et al., 2003).

Gel Filtration Chromatography of Cell Lysates

Cytosolic extract (3 mg protein) was applied to a Superose-6 FPLC column and eluted at a flow rate of 0.5 ml/min in buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM PMSF, and 1 mM DTT. Thyroglobulin (664 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were molecular weight standards.

Identification of GAIT Element Binding Proteins by RNA Affinity Chromatography and Tandem Mass Spectrometry

U937 cell lysate (4 mg of protein) was precleared by incubation for 30 min at 4°C with a 5′-biotinylated, scrambled GAIT element RNA oligomer and then with a 5′-biotinylated, U87C mutant GAIT element oligomer (Dharmacon, 2 μg of each). The cleared lysate was incubated with biotinylated, wild-type or mutant GAIT element, and then with μMACs magnetic streptavidin microbeads (200 μl, Miltenyi) for 10 min. The mixture was applied to a magnetic column and the column washed with buffer. Specifically bound proteins were eluted using buffer containing 300 mM NaCl. The eluate was desalted and concentrated using Ultra-4 columns (Amicon).

Eluates were subjected to SDS-PAGE and silver staining. Bands present only in the wild-type sample were trypsinized and peptide sequences determined by capillary liquid chromatography-electrospray mass spectrometry and collisionally-induced dissociation. Spectra were analyzed using TurboSequest software to search the NCBI nonredundant protein database. Matching spectra were verified by manual interpretation using Mascot and FASTA software.

Immunoanalysis of GAIT mRNP Components

For immunoprecipitation, samples were incubated with antibodies (1:500) directed against human GluProRS (Kim et al., 2002), NSAP1, or p38 (Kim et al., 2002) (or IgG) using Seize X IP Kit (Pierce) in buffer containing NP-40 (0.05%), NaCl (150 mM), and Tris-HCl (50 mM [pH 7.6]) for 1 hr at 4°C. Protein A-Sepharose beads (40 μl) were added and incubated with rotation for 4 hr. The beads were washed three times with buffer containing NP-40 followed by a brief, low-speed centrifugation. In some experiments, samples were immunoprecipitated under denaturing conditions in buffer that also contained 0.5% sodium deoxycholate and 0.1% SDS. In experiments requiring immunodepletion, samples were incubated with antibody (at 1:500) for 1 hr at 4°C. Protein-A Sepharose beads (40 μl) were preequilibrated in phosphate-buffered saline and added for 4 hr with rocking. After a brief, low-speed centrifugation, the supernatant was collected, and the process was repeated twice to assure complete depletion.

For immunoblot analysis, samples were denatured in Laemmli buffer containing SDS and resolved on a 10% SDS-PAGE. After transfer, blots were probed with rabbit polyclonal antibodies and developed with the ECL system (Amersham Pharmacia).

Analysis of GAIT Element Binding Protein by Ultraviolet Crosslinking

^{32}P-labeled wild-type or mutant GAIT element RNA oligomers (250,000 cpm) were incubated for 30 min at 4°C with cytosolic extract (20 μg of protein) in 20 μl of buffer containing KCl (15 mM), DTT (0.25 mM), $MgCl_2$ (5 mM), PMSF (0.1 mM), RNasin (40 U), glycerol (10%), and 12 mM HEPES (pH 8.0). The mixture was crosslinked by 10 min exposure to ultraviolet light (1,800 J/cm^2) in a Stratalinker (Stratagene) at room temperature. Samples were denatured in SDS-PAGE buffer under reducing condition, and RNA-protein complexes were analyzed by 10% SDS-PAGE and autoradiography.

Determination of In Vivo Interaction of GluProRS and Cp mRNA by Immunoprecipitation and RT-PCR

Cytosol from U937 cells was subjected to immunoprecipitation using 10 μl of anti-GluProRS antibody. GluProRS bound mRNA was amplified by RT-PCR using primers specific for the 247 nt human Cp 3′UTR (Mazumder et al., 2003a).

Metabolic Labeling by [^{35}S]Methionine and [^{32}P]Orthophosphate

U937 cells (8×10^6 cells in 4 ml of RPMI 1640 medium) were collected by centrifugation at 7000 × g, resuspended in methionine-free medium (Invitrogen), and metabolically labeled with a 2 hr pulse of [^{35}S]methionine. A 5-μl aliquot was resolved on 10% SDS-PAGE and subjected to fluorography. For [^{32}P]phosphate labeling, cells were incubated with a 6 hr pulse of [^{32}P]orthophosphate in phosphate-free medium (Invitrogen).

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