2008

An Insight into GAIT Complex Mediated Translational Silencing

Purvi Kapasi
Cleveland State University

Follow this and additional works at: https://engagedscholarship.csuohio.edu/etdarchive

Part of the Biology Commons

How does access to this work benefit you? Let us know!

Recommended Citation
https://engagedscholarship.csuohio.edu/etdarchive/557

This Thesis is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETD Archive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.
AN INSIGHT INTO GAIT COMPLEX MEDIATED TRANSLATIONAL SILENCING

PURVI KAPASI

Bachelor of Science in Microbiology
Sardar Patel University
May, 2000

Master of Science in Environmental Sciences
Sardar Patel University
May, 2002

Submitted in partial fulfillment of requirements for the degree
MASTER OF SCIENCE IN BIOLOGY
at the
CLEVELAND STATE UNIVERSITY
December, 2008
This thesis has been approved for the Department of Biological, Geological, and Environmental Sciences and for the College of Graduate Studies of Cleveland State University by

Dr. Barsanjit Mazumder, BGES/CSU
Thesis Chairperson

Date: ____________________

Dr. Anton Komar, BGES/CSU
Advisory Committee Member

Date: ____________________

Dr. Crystal Weyman, BGES/CSU
Advisory Committee Member

Date: ____________________
ACKNOWLEDGEMENTS

This thesis would not have been possible without the excellent guidance of my advisor Dr. Barsanjit Mazumder. He has encouraged me in every step of my research and has boosted my confidence even in the worst times. His expertise and thorough knowledge has helped me to develop an intrinsic understanding of science. His passion and dedication for the research in science have helped me mould I am sure that by having such a wonderful person as my advisor in the early stages of my career will help me immensely ahead.

I would also like to thank my committee members Dr. Crystal Weyman and special thanks to Dr. Anton Komar who helped me with their expert suggestions. I would also like to express my gratitude to former graduate advisor Dr. Jeffrey Dean for helping me with the department documentation during my earlier days.

I am grateful to my laboratory members, Dr. Sujan Chaudhuri, Keyur Vyas and Priyanka Das for helping me with the experiments and for being there as friends and supporters at times of failure. I would also like to thank lab members of Dr. Weyman, Dr. Komar and former lab members of Dr. Fontes for the experimental and moral support.

Last but not the least my husband Bhavin Gandhi who has supported my decision to do graduate studies, for his understanding and patience and my parents to whom I am indebted forever for their love and support.
AN INSIGHT INTO GAIT COMPLEX MEDIATED TRANSLATIONAL SILENCING

PURVI KAPASI

ABSTRACT

Transcript-specific translational control restricts macrophage inflammatory gene expression. The pro-inflammatory cytokine IFN-γ induces the phosphorylation of human ribosomal protein L13a and its subsequent release from 60S ribosome. L13a is a component of the interferon-gamma-activated inhibitor of translation (GAIT). The GAIT complex binds a defined element in the 3’-untranslated region (UTR) of ceruloplasmin (Cp) mRNA and causes delayed silencing of translation. In this research, we elucidate the molecular mechanism underlying L13a translational silencing activity. L13a mediates translational silencing particularly, when driven by internal ribosome entry sites (IRESs) that requires the initiation factor eIF4G, but is resistant to silencing when driven by eIF4F- independent IRESs. This demonstrates a critical role of the scaffold protein – eIF4G. Global inhibition of protein synthesis by targeting eIF4G is well appreciated in virus infection and apoptosis; however interaction of L13a with eIF4G blocks the 43S complex recruitment showing a unique role of eIF4G in gene specific translational silencing.
TABLE OF CONTENTS

ABSTRACT........................................................................................................ iv
LIST OF FIGURES.............................................................................................. vi
LIST OF ABBREVIATIONS............................................................................... vii

CHAPTERS

I BACKGROUND AND INFORMATION......................................................... 1
   Translational control of gene expression.................................................. 1
   Ceruloplasmin............................................................................................. 2
   Significance of the findings......................................................................... 5

II MATERIALS AND METHODS................................................................. 7

III RESULTS.................................................................................................. 11
   P-L13a blocks the 80S recruitment.......................................................... 11
   P-L13a blocks the recruitment of 43S ribosome complex to
     GAIT element bearing mRNA............................................................... 15
   Assembly of eIF4F in the presence of P-L13a bound RNA...................... 17
   P-L13a targets eIF4G for its translational silencing activity...................... 21
   eIF4G domain for translational silencing by P-L13a................................. 24
   L13a interacts with eIF4G with its elf3 binding site................................... 27

IV DISCUSSION.............................................................................................. 31

V GENERAL REFERENCES.......................................................................... 36
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eukaryotic protein translation………………………………………… 2</td>
</tr>
<tr>
<td>2</td>
<td>Schematic representation of L13a being phosphorylated, released and binding to GAIT…………………………………… 4</td>
</tr>
<tr>
<td>3</td>
<td>Schematic representation of different possibilities of GAIT complex mediated silencing…………………………………… 5</td>
</tr>
<tr>
<td>4</td>
<td>Translational Silencing by P-L13a Blocks 80S Recruitment to Reporter mRNA Containing a GAIT Element…………………… 13</td>
</tr>
<tr>
<td>5</td>
<td>P-L13a Blocks the Assembly of 48S Complex…………………………………… 16</td>
</tr>
<tr>
<td>6</td>
<td>Formation of Cap-Binding Complex eIF4F Is Not Disrupted by P-L13a………………………………………………………… 19</td>
</tr>
<tr>
<td>7</td>
<td>Virus IRES-Driven RNAs Show an eIF4F Component Is Required for Translational Silencing by P-13a………………………… 22</td>
</tr>
<tr>
<td>8</td>
<td>eIF4G C-Terminal Domain Is Necessary and Sufficient for Translational Silencing by P-L13a…………………………………… 25</td>
</tr>
<tr>
<td>9</td>
<td>L13a and eIF3 Shares a Common eIF4G-Binding Site……………………… 29</td>
</tr>
<tr>
<td>10</td>
<td>Global and Transcript-Specific Translational Silencing targeting eIF4G…………………………………………………... 35</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-L13a</td>
<td>Phosphorylated large ribosomal protein 13a</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon- gamma</td>
</tr>
<tr>
<td>GAIT</td>
<td>Gamma activated inhibitor of translation</td>
</tr>
<tr>
<td>3'UTR</td>
<td>3' Untranslated region</td>
</tr>
<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
</tr>
<tr>
<td>eIF4G</td>
<td>eukaryotic initiation factor 4G</td>
</tr>
<tr>
<td>eIF3</td>
<td>eukaryotic initiation factor 3</td>
</tr>
<tr>
<td>eIF4F</td>
<td>eukaryotic initiation factor 4F</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly A binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>hnRNP</td>
<td>heterogenous nuclear ribonucleoproteins</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3- phosphate dehydrogenase</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major histocompatibility class II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RRL</td>
<td>Rabbit Reticulocyte lysate</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>miRNP</td>
<td>micro Ribonucleoprotein</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription–Polymerase chain reaction</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>HRV-2A</td>
<td>Human rhinovirus 2A</td>
</tr>
<tr>
<td>CrPV</td>
<td>Cricket paralysis virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>HEK 293T</td>
<td>Human embryonic kidney T cells</td>
</tr>
<tr>
<td>U937 cells</td>
<td>Human leukemic monocyte lymphoma cell line</td>
</tr>
<tr>
<td>HA</td>
<td>Haemaglutinin</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>Mnk1</td>
<td>MAP kinase-interacting kinase 1</td>
</tr>
<tr>
<td>Hsp27</td>
<td>Heat shock protein 27</td>
</tr>
<tr>
<td>NSP3</td>
<td>Non structural rotavirus protein 3</td>
</tr>
<tr>
<td>NS1</td>
<td>Non structural protein 1</td>
</tr>
</tbody>
</table>
CHAPTER I

BACKGROUND AND INFORMATION

TRANSLATIONAL CONTROL OF GENE EXPRESSION

Translational Control is an important mechanism of post-transcriptional regulation. It offers a range of response levels even in the presence of a constant amount of mRNA. Translational regulation can be broadly divided into global and transcript specific translational control. In global translational control most cellular transcripts are affected whereas in transcript specific translational control cellular RNA – binding protein/s bind to the transcript and inhibit the protein synthesis. In eukaryotes, initiation is the rate – limiting step of translation and is often the target of control. Translation initiation is the process of ribosome assembly on mRNA (1) (2) (3). Initiation begins with the assembly of the heterotrimeric eIF4F complex on the 5’UTR of mRNA. The eIF4F complex contains a cap binding protein eIF4E, RNA – dependent helicase eIF4A and its cofactor eIF4B, and a scaffold protein eIF4G. eIF4G is the backbone of the complex as it binds to both cap binding protein eIF4E and the RNA helicase eIF4A. Moreover, it also
binds to poly (A) tail binding protein – PABP which facilitates the interaction of 3’ poly (A) tail and 5’ cap making the transcript circular via end – to – end interaction (4). The 43S pre – initiation complex contains multisubunit eIF3 complex, small ribosomal subunit 40S, eIF1/eIF1A and a pre-charged ternary complex of eIF2, GTP and initiator met-tRNA. This 43S complex is recruited to the mRNA by interaction of eIF3 with eIF4G of eIF4F complex (5). The complex then scans the 5’UTR to identify the AUG initiation codon. Codon recognition triggers hydrolysis of eIF2 and eIF5B – bound GTP and permits joining of the 60S ribosomal subunit to form an elongation competent 80S complex that can extend the peptide chain (fig 1).

Fig1. Protein Translation: Eukaryotic protein translation showing all the initiation factors, recruitment of 43S complex on mRNA and joining of 60S and 80S ribosomal subunit

**CERULOPLASMIN**

Ceruloplasmin (Cp) is an inflammatory, acute-phase plasma protein made by hepatocytes in the liver and by activated monocytes/macrophages (6). It is a 132kDa monomer with 7 copper atoms per molecule, and accounts for nearly about 95% of the circulating copper in healthy adult humans. Though its physiological function is
uncertain, experimental evidences from several laboratories suggests its role in bactericidal activity, coagulation, iron homeostasis, vascular relaxation and defense against oxidant stress and lipoprotein oxidation (7). Cp induces oxidation of low density lipoprotein (LDL) (8) (9) (10), which is considered to be a critical event during atherogenesis. Cp is an acute phase protein and its high concentration in the serum has been shown to pose an independent risk factor for atherosclerosis (11) (12). The synthesis of Cp, a copper-containing protein in monocytes is regulated by the inflammatory cytokine IFN-γ (13). Enhanced production of Cp by the activated monocytes–macrophages may be due to an inflammatory response. The precise regulation of the synthesis of Cp by activated monocytes–macrophages is critical because any defects in regulation may lead to accumulation of Cp protein. Series of studies from our laboratory have shown that monocytes have an endogenous mechanism to terminate the IFN-γ induced Cp synthesis at the level of translation (14) and a ribosomal protein L13a was identified as a molecular switch for this silencing (15).

Recently new evidences have started to emerge showing translational control may play a key role in the resolution of cellular inflammation. For example 15- Lipoxygenase, a pro-atherogenic molecule (16) is translationally silenced by binding of heterogeneous ribonuclear proteins (hnRNP) K and E1 to the DICE element of 3’UTR, which prevents the joining of 60S ribosomal subunit (17) (18). Similarly, using genetic and biochemical approaches our group discovered a novel pathway showing the translational silencing of Cp. Previous experimental studies showed that Cp protein synthesis is induced in response to pro-inflammatory cytokine IFN-γ, but the synthesis stops after about 16 hours, even in the presence of abundant Cp mRNA. These studies led to the identification of four protein constituents of the Interferon Gamma Activated Inhibitor of Translation (GAIT) complex as ribosomal protein – L13a (15), Glutamyl-prolyl-tRNA synthetase (EPRS), NS-1 associated protein-1 (NSAP-1) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (19). The complex binds to a specific element called as GAIT element in the 3’UTR of Cp mRNA. A 29 nt, GAIT element in the Cp 3’-untranslated region is necessary for the IFN-γ mediated translational silencing of ceruloplasmin and is sufficient to convey the silencing response to a heterologus transcript. We have also
shown that translational silencing of Cp requires the essential elements of mRNA circularization, i.e., the poly(A) tail, eukaryotic initiation factor (eIF) 4G or poly(A) binding protein (PABP). In IFN-γ induced monocytic cells, L13a is phosphorylated and released from 60S ribosomal subunit. Phosphorylation of L13a is required for translational silencing activity because the silencing activity is sensitive to phosphatase. The free P-L13a incorporates into the GAIT complex that binds to the GAIT element thereby inhibiting the protein synthesis of Cp (fig 2). In addition, recombinant L13a expressed in E.coli in unphosphorylated form is inactive; however when the same protein expressed in baculovirus in phosphorylated form has been found to be fully active for translational silencing in vitro translation assay. My research focuses on elucidating the molecular mechanism underlying the translational silencing activity of this large ribosomal protein - L13a.

Fig 2: Schematic representation of L13a being phosphorylated, released and binding to GAIT element (Mazumder et al., 2006, ref # 6).

To elucidate the silencing mechanism of P-L13a, we hypothesized the following potential mechanisms (fig 3):

(i) P-L13a blocks the recruitment of 43S pre-initiation complex on eIF4F
(ii) P-L13a prevents the scanning of 43S complex to the initiation codon
(iii) P-L13a blocks the joining of 60S ribosomal subunit
(iv) P-L13a inhibits the function of eIF4F assembly
Our basic findings were that P-L13a inhibits the formation of 48S pre-initiation complex on the GAIT element containing RNA in a Poly(A) dependent manner. Also, P-L13a binds the C-terminal domain of translation initiation factor eIF4G, thereby blocking the recruitment of eIF3 containing 43S complex. This exhibits a unique role of eIF4G in gene specific translation silencing. Moreover, the results suggest that eIF4G fragments containing the eIF3 binding site can also bind L13a indicating that eIF3 and L13a share the same or neighboring binding sites on eIF4G (20).

**SIGNIFICANCE OF THE FINDINGS**

Increasing evidences suggests the potential role of IFN-γ in the pathogenesis of atherosclerosis. For example: IFN-γ can stimulate the expression of VCAM-1, MHC-II, lipoprotein receptors and tissue factor, all potential pro-atherogenic molecules (21) (22) (23). Monocyte chemoattractant protein (MCP)-1 a pro-atherogenic factor, critical in development of atherosclerosis is chemotactic for monocytes and T-cells, and its expression is induced dramatically by IFN-γ (24). Genetic studies in gene knock out mice are also consistent with IFN-γ as a pro-atherogenic cytokine (25). In a model of allogenic transplantation, genetic absence of IFN-γ markedly reduces the extent of intimal expansion (26). In apoE knockout mouse, a genetic model of cholesterol-induced
atherosclerosis, targeted disruption of IFN-γ receptor gene that results in reduced atherosclerosis (27). The molecular mechanisms underlying IFN-γ induced atherosclerosis are unknown. We have shown that the synthesis of Ceruloplasmin (Cp), a copper-containing protein in monocytes, is regulated by IFN-γ (28), while others have shown that Cp is required for LDL oxidation by these cells (29) (30). In addition, Cp is an inflammatory protein and its inflammatory response may enhance the production of Cp by the activated monocytes-macrophages.
CHAPTER II
MATERIALS AND METHODS

Reagents
RRL, methionine-free amino acids, and RNasin were from Promega (Madison, WI). Shrimp alkaline phosphatase, reverse transcriptase, Taq polymerase, and restriction enzymes were from Fermentas (Hanover, MD). SP6 Message Machine kit for in vitro transcription was from Ambion (Austin, TX). Translation grade [35S]methionine and transcription grade [α-32P]UTP were from Amersham (Piscataway, NJ). Cycloheximide, GMP-PNP, and all other reagents were from Sigma (St.Louis, MO). Rabbit anti-peptide polyclonal antibody against human L13a was purified on a peptide column (44). Anti-eIF4G antibodies were from Simon Morley and Matthias Hentze. Anti-eIF4A antibody was provided by Hans Trachsel and Michael Altmann. Anti-eIF3 antibody was a gift from John Hershey. Anti-eIF4E was purchased from Abcam (Cambridge, MA). HRV-2A protease was from Tim Skern. Anti-Flag and anti-HA antibodies were from Sigma and Roche, respectively.

Plasmid Construction
Construction of the plasmids PSP64-Luc-GAIT-poly(A) and PSP64-Luc-GAIT (mut.)-poly(A) were described previously (19). These plasmids were modified and used to generate capped, poly(A)-tailed (A50) RNAs m7G-Luc-GAITpoly(A) and m7G-Luc-
GAIT (mut.)-poly(A). IRES-containing plasmids were made by PCR amplification of nt 265-828 of EMCV genome using pGEM-Cat-EMCV-Luv (from Graham Belsham), nt 40-373 of HCV genome using pHCV(40-373).NS from Tatyana Pestova (35), and nt 6029-6219 of CrPV genome using pCup1 LEU2 IGR URA3 from Peter Sarnow. PCR products containing IRES sequences were cloned into Pst1 and BamH1 sites of PSP64-Luc-GAIT-poly(A) and PSP64-Luc-GAIT (mut.)-poly(A) (19) to give plasmids EMCV(or HCV or CrPV)-Luc-GAIT-poly(A) and EMCV(or HCV or CrPV)-Luc-GAIT (mut.)-poly(A). These plasmids were used to generate RNAs containing ApppG at the 5’ cap. Plasmids PSP64-α-globin-GAIT-poly(A)50 and PSP64-α-globin-GAIT (mut.)-poly(A)50 were made by PCR amplification of the 429-nt α-globin fragment from PHST101, excision of Luc, and cloning into the BamH1 and Stu1 sites of PSP64-Luc-GAIT-poly(A) and PSP64-Luc-GAIT (mut.)-poly(A) (19). These plasmids were used to generate m7G-α-globin-GAIT-poly(A)50 and m7G-α-globin-GAIT (mut.)-poly(A)50 RNAs. GST-fusion proteins containing specific eIF4G1 domains were produced from plasmids pGEX-4G-GST-eIF4G1 (688-1133), pGEX-4G-GST-eIF4G1 (688-1560), and pGEX-4G-GST-eIF4G1 (1075-1560), made by cloning the appropriate eIF4G1 sequences into the Sal1 and Not1 sites of pGEX-5x-1. HA-tagged plasmids containing the three eIF4G1 domains were made by cloning the appropriate DNA sequences into BamH1 and Not1 sites of pcDNA3.1-HA. pGEX4G1404 was used as a template for PCR amplification of individual eIF4G1 domains. A plasmid expressing HA-tagged eIF3e was generated by inserting the eIF3e open reading frame from the vector pNOp48Nde (from John Hershey) into the BamH1 and Not1 sites of pcDNA3.1-HA. A plasmid expressing HA-tagged eIF3j in pcDNA5/FRT/HA-p35 was a gift from John Hershey. pFlag-L13a was made by cloning full-length L13a ORF into the EcoRI and BamH1 sites of pFlag-CMV-5 (Sigma, MO).

**Preparation of Recombinant L13a:** pET-17b-L13a and pET-15b-L13a were used to express recombinant, unphosphorylated L13a and His-tagged L13a in E. coli (44). pFASTBAC1-L13a and pFASTBACHTb-L13a were used to express phosphorylated, recombinant L13a and His-tagged L13a in baculovirus-infected insect cells. The
construction of these plasmids and protein purification were described previously (44). His-tagged L13a was purified by Ni affinity chromatography.

**In Vitro Transcription and Translation**

m7G-Luc-GAIT-poly(A) RNA was produced by *in vitro* transcription using Sp6 RNA polymerase. PSP64-Luc-GAIT-poly(A) as a template was linearized by PvuII. m7G-α-globin-GAIT-poly(A) RNA was made by *in vitro* transcription of PSP64-α-globin-GAIT-poly(A) after linearization by EcoRI. The same RNA, but without the poly(A) tail, was made by linearization of the same plasmid by SacI prior to addition of Sp6 RNA polymerase. Sp6 Message Machine kit was used to make m7G-capped transcripts. The IRES-containing RNA ApppG-EMCV (or HCV or CrPV)-Luc-GAIT-poly(A) was made by *in vitro* transcription using Sp6 RNA polymerase from PvuII-linearized EMCV(or HCV or CrPV)-Luc-GAIT-poly(A) plasmid. Sp6 Maxiscript kit (Ambion) was used with ApppG (unmethylated cap, Ambion) with limiting GTP concentration to perform the *in vitro* transcription reaction. The RNAs were purified by LiCl precipitation and washed with 75% ethanol. m7G-T7 gene 10 RNA was made by *in vitro* transcription of pGEMEX-2 (Promega) by T7 RNA polymerase using T7 Message Machine kit. PSP64-α-globin-GAIT-poly(A) and PSP64-α-globin-GAIT (mut.)-poly(A) plasmids were linearized by either EcoRI or SacI. The linearized plasmids were transcribed by SP6 RNA polymerase using SP6 Message Machine kit in the presence of [α-32P]UTP to generate radiolabeled of m7G-α-globin-GAIT and m7G-α-globin-GAIT (mut.) RNA with and without the poly(A) tail.

Purified RNA (200 ng) produced by *in vitro* transcription was added to RRL (35 μl) for translation. 20μM methionine-free amino acid mixture, 40 U of RNAsin, 20 μCi of translation grade [35S]methionine, and 5 μg of recombinant L13a were also incubated in a total volume of 50 μl for 60 min at 30°C. An aliquot (5 μl) was resolved by 7% SDS-PAGE. The gel was fixed, treated with Amplify (Amersham), dried, and radiolabeled bands detected by autoradiography.
**Sucrose Gradient Fractionation**

In vitro-synthesized RNA (500 ng) was incubated in RRL (100 ml) in the presence of 0.5 mM cycloheximide for 5 min at 30°C. The initiation reaction was stopped by placing on ice and layered with a 10%–25% linear sucrose gradient in buffer containing 100 mM KCl, 5 mM MgCl₂, 20 mM HEPES (pH 7.4), 2 mM dithiothreitol, and 0.5 mM cycloheximide. After ultra-centrifugation (Beckman) for 16 hrs at 20,000 rpm, the gradients were unloaded by upward displacement using a programmable density gradient system with a UA-6 detector (ISCO). Fractions were collected, precipitated by TCA, and followed for western blot analysis.

To detect the 48S ribosomal complex, 32P-labeled a-globin reporter RNAs (300,000 cpm) were incubated with RRL (100 ml) in the presence of GMP-PNP (2 mM) or cycloheximide (0.5 mM). Reaction products were resolved on a 5%–25% linear sucrose gradient by ultra-centrifugation for 18 hrs at 20,000 rpm. Fractions (0.75 ml) were collected, and radioactivity was determined by scintillation counting. For eIF3 detection, fractions were subjected to TCA precipitation, followed by SDS-PAGE and immunoblot analysis with anti-eIF3 antibody.

**Determination of eIF4F Assembly on GAIT Element-Containing RNA**

m7G-Luc-GAIT-poly(A) or m7G-Luc-GAIT (mut.)-poly(A) RNA (500 ng) was incubated with RRL (25 ml) and His-tagged L13a (10 mg) in the presence of 2 mM GMP-PNP at 30°C. After 5 mins, the reaction was terminated with ice-cold buffer containing 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂, 0.05% Triton X-100, and 50 mM HEPES (pH 7.5). Immunoprecipitation was done using 10 ml of monoclonal anti-His antibody (Novagen). From one aliquot, the GAIT element containing Luc RNA was extracted by Trizol (Invitrogen) and subjected to reverse transcription using Luc-specific primers. Another aliquot was subjected to SDS-PAGE, followed by immunoblot analysis with anti-eIF4G, anti-eIF4E.
CHAPTER III

RESULTS

P-L13a BLOCKS THE 80S RECRUITMENT

Translation initiation reactions of chimeric reporter mRNA containing m7G cap, a 29nt Cp mRNA GAIT element, and a 50 nt poly(A) tail (Luc-GAIT-poly[A]) were reconstituted in RRL (rabbit reticulocyte lysate), using luciferase GAIT reporters (Figure 4A). A construct containing inactive, mutated GAIT element (U87C replacement in the 3′UTR) was used as an additional control (Luc-GAIT [mut.]-poly[A]) All the reactions were performed in the presence of phosphorylated or unmodified L13a. The chimeric reporter mRNA was also translated in RRL with T7 gene 10 mRNA as a specificity control. Phosphorylated L13a (P-L13a) markedly inhibited translation of Luc-GAIT-poly(A), but not the reporter containing the mutant element or T7 gene 10 (Figure 1B). Unphosphorylated L13a, from E. coli or alkaline phosphatase-treated, insect cell-derived L13a was inactive. These results also showed the importance of phosphorylation for L13a containing GAIT element mediated silencing. It has been shown that GAIT element-mediated translational silencing by a U937 cell lysate requires the circularization of transcript (48). To examine the requirement of poly(A) tail for P-L13a silencing we performed experiments in absence of poly(A) tail. Its absence did not influence the basal
translation of the Luc-GAIT reporter, however translation of the Luc-GAIT reporter containing a poly(A) tail was selectively inhibited by P-L13a (Figure 4C). P-L13a bound the Luc reporter irrespective of the presence of a poly(A) tail; however, unphosphorylated L13a did not bind RNA (Figure 4D). Efficient binding of P-L13a to a poly(A)-minus reporter indicates that binding is not sufficient for translational silencing, but the poly(A) tail may contribute to effective presentation of P-L13a.

To determine at what stage P-L13a blocks the translation, we began to explore at the initiation step, starting with the termination initiation event i.e. the formation of 80S ribosome complex. The initiation complexes of chimeric reporter mRNA and P-L13a or UnP-L13a were resolved by sucrose density gradient in the presence of cyclohexamide. The identity of the 80S peak (Figure 4E, top) was authenticated by RT-PCR amplification of 28S and 18S rRNA (Figure 1E, bottom). Reporter RNA was determined by RT-PCR amplification using Luc-specific primers. Rapid formation of 80S on the Luc reporter containing wild-type or mutant GAIT element was observed (Figure 4F, top). P-L13a prevented 80S formation only on a reporter bearing wild-type GAIT element whereas unphosphorylated L13a was ineffective (Figure 4F, panels 3–6). Migration of P-L13a in the light, pre-80S fractions was consistent with binding to GAIT element-containing RNA and shifting with the RNA to pre-80S fractions (Figure 4F, second panel from bottom). Unmodified L13a does not bind the GAIT element reporter, and it is not part of an RNP complex; it also migrates in the light fractions (Figure 4F, bottom). Therefore, 80S formation on GAIT element-containing transcripts is impaired in the presence of P-L13a, and translation must be blocked at this or an earlier initiation step.
Figure 4 Translational Silencing by P-L13a Blocks 80S Recruitment to Reporter mRNA Containing a GAIT Element
(A) Schematic representation of Luc reporter RNAs used in translation initiation reactions.

(B) Translational silencing of GAIT element-containing reporter RNA by P-L13a. Luc RNAs (200 ng) were subjected to in vitro translation using [35S]methionine and RRL. T7 gene 10-poly(A) RNA (100 ng) was cotranslated in the same reaction as control. Recombinant P-L13a (5 mg) made by baculovirus-infected insect cells was treated with shrimp alkaline phosphatase (3 U) for 90 min.

(C) Poly(A) tail is required for translational silencing by P-L13a. Luc RNA with T7 gene 10 RNAs were subjected to in vitro translation by an RRL. Phosphorylated or unmodified L13a (5 mg) was added to the in vitro translation reaction.

(D) Poly(A) tail is not required for L13a recruitment to the GAIT element. Translation initiation reactions were reconstituted in RRL, using Luc reporters (500 ng) with or without a poly(A) tail, in the presence of His-tagged, phosphorylated or unmodified L13a (10 mg). L13a was immunoprecipitated (IP) with monoclonal anti-His antibody, and bound RNA was detected by RT-PCR with the Luc-specific primers.

(E) RNA detection in sucrose density gradient fractions. Initiation reactions were done as in (D) in the presence of 0.5mM cycloheximide. The reaction was subjected to sucrose density gradient centrifugation using 10%–25% gradient. The 80S peak is marked by a horizontal bar. The authenticity of the 80S fractions was confirmed by RT-PCR amplification using 28S- (middle) and 18S- (bottom) specific rRNA primers.

(F) Assembly of 80S ribosome complex. Initiation reactions were done as in (D) and subjected to sucrose density gradient centrifugation. Fractions(1 ml) were collected from the top. Total RNA was isolated and subjected to RT-PCR using Luc-specific primers (top six panels). Migration of P-L13a in the gradient was determined by precipitation of the fractions with trichloroacetic acid (TCA), followed by immunoblot (IB) analysis with anti-His antibody (bottom two panels).
P-L13a BLOCKS THE RECRUITMENT OF 43S RIBOSOME COMPLEX TO GAIT ELEMENT BEARING mRNA

Prior to 80S complex formation, 43S complex is recruited to the 5’cap-bound eIF4F complex to form 48S pre-initiation complex. Hence, now we had 2 possibilities: Either P-L13a blocks 43S recruitment or/And L13a interferes with eIF4F assembly on the target mRNA. To determine whether L13a blocks 43S recruitment, we analyzed initiation complex assembly in RRL in the presence of a nonhydrolyzable GTP analog, GMP-PNP, which prevents 80S formation. In a preliminary characterization, 48S and 80S peaks were detected in the presence of GMP-PNP and cyclohexamide, respectively (Figure 5B). In the presence of GMP-PNP, P-L13a markedly reduced 48S formation on a GAIT element-containing reporter (Figure 5 C). The inhibition was specific because 48S formation was not reduced by unmodified L13a or mutated GAIT element. We made constructs with α-globin GAIT (WT or MT) reporters with or without the poly(A) tail. P-L13a failed to inhibit 48S complex formation on an α-globin reporter RNA carrying the GAIT element but lacking a poly(A) tail (Figure 5 C). This result confirms that inhibition of 48S formation by P-L13a is poly(A) dependent and suggests an important role for transcript closure in the inhibitory process. To further verify the inhibition of 48S formation by P-L13a, 43S recruitment to target RNAs was determined as an association of eIF3 (43S component that binds cap-bound eIF4G) with GAIT element-containing α-globin reporter RNA. Addition of P-L13a to the RRL, when a GAIT element reporter RNA was used, shifted eIF3 from the 48S fractions to the lower-density RNP fractions whereas in control experiments, eIF3 shifting was not observed (Figure 5 D). As eIF4G is required for 48S formation, comigration of eIF4G and P-L13a in the sucrose gradients was examined by immunoblot analysis. In the presence of GAIT element-containing RNA, but in the absence of P-L13a, eIF4G comigrated with 48S complex. However, P-L13a shifted eIF4G from the 48S fractions to the low-density, RNP-containing fractions (fig 5 E). These results show that P-L13a inhibits 43S recruitment to a transcript containing a 3’UTR-located GAIT element. Also the inhibition was specific because 48S formation was not reduced by unmodified L13a or mutant GAIT element.
Figure 5 P-L13a Blocks the Assembly of 48S Complex
(A) Schematic of α-globin RNA constructs used in translation initiation reactions.

(B) Ribosomal complex formation on GAIT element-containing RNA. Translation initiation reactions containing $^{32}$P-labeled, α-globin-GAIT-poly(A) reporters (300,000 cpm) were reconstituted in RRL in the presence of GMP-PNP (black, 2 mM) or cycloheximide (red, CHX, 0.5 mM) and resolved by sucrose density gradient centrifugation.

(C) P-L13a blocks 48S complex formation on a GAIT element-containing RNA in a poly(A)-dependent way. Translation initiation reactions were reconstituted in an RRL with GMP-PNP (2 mM) as in (B), but in the presence of His-tagged P-L13a (5 mg). Conditions tested were as follows: α-globin-GAIT-poly(A) (red), α-globin-GAIT-poly(A) + L13a (blue), α-globin-GAIT-poly(A) + P-L13a (black), α-globin-GAIT (mut.)-poly(A) + P-L13a (green) and α-globin-GAIT (no poly[A] tail) + P-L13a (orange). The reaction was subjected to sucrose density gradient centrifugation, fractions were collected (0.75 ml), and radioactivity was determined.

(D) P-L13a prevents formation of eIF3-containing 48S complex. Gradient fractions from experiments with a-globin-GAIT-poly(A) reporter and buffer control (top), P-L13a (second panel), unmodified L13a (third panel), and other controls (fourth and fifth panels) were subjected to TCA precipitation, followed by immunoblot analysis with anti-eIF3 antibody. Two eIF3 subunits (44 and 110 kDa) are indicated.

(E) Migration of eIF4G and P-L13a in the sucrose gradient. Gradient fractions from (C) containing a-globin-GAIT-poly(A) reporter and buffer control (top) or P-L13a (second panel) were subjected to TCA precipitation, followed by immunoblot analysis with anti-eIF4G antibody. Migration of P-L13a in the presence of a-globin-GAIT-poly(A) (third panel), a-globin-GAIT (mut.)-poly(A) (fourth panel), or a-globin-GAIT (bottom) was determined by immunoblot analysis with anti-His antibody.
ASSEMBLY OF eIF4F IN THE PRESENCE OF P-L13a BOUND RNA

Considering the other possibility of L13a interference with eIF4F assembly, we started looking at the assembly of eIF4F complex in the presence or/and absence of P-L13a. Initiation of GAIT-element containing reporter RNA was reconstituted in RRL in the presence of P-L13a and GMP-PNP (Figure 6 A). Binding was measured by immunoprecipitation with anti-His, followed by RT-PCR with Luc-specific primers. P-L13a bound the RNA target containing Wild type GAIT, but not mutant GAIT element (Figure 6 B). Also, as expected for a protein that binds a 3’UTR element, P-L13a binding to RNA was not disrupted by interfering with complex assembly at the 5’cap using exogenous m7G or human rhinovirus (HRV)-2A protease. To investigate whether eIF4F remains bound to L13a-silenced reporter RNA, we identified eIF4F components by immunoprecipitation with anti-His antibody and by immunoblot analysis using anti-eIF4G, -eIF4E, and -eIF4A. All three eIF4F components remained bound to a GAIT element reporter in the presence of PL13a (Figure 6 C). The authenticity of the eIF4F complex was examined by assessing cap-dependent interaction with the P-L13a silenced reporter. Cap dependence was shown by the loss of interaction in the presence of exogenous m7G, which blocks eIF4E binding to the cap (Figure 6 C). Furthermore, cleavage of eIF4G with HRV-2A protease, which prevents cap-dependent assembly of eIF4F, blocked the interaction of P-L13a with eIF4F components eIF4A and eIF4E, while permitting a weak interaction with eIF4G (Figure 6 D, top). Thus, P-L13a blocks 43S recruitment to GAIT element-bearing transcripts without disrupting eIF4F complex assembly and an intact eIF4G is necessary for interaction of P-L13a with eIF4F.
Figure 6 Formation of Cap-Binding Complex eIF4F is Not Disrupted by P-L13a
(A) Schematic representation of initiation reactions reconstituted on GAIT element-bearing RNA and subsequent procedures.

(B) Binding of P-L13a to GAIT element-bearing RNA in RRL. Translation initiation reactions were reconstituted in RRL as above. L13a was immunoprecipitated with anti-His antibody, followed by RT-PCR with Luc-specific primers.

(C) Analysis of eIF4F components interacting with RNA-bound P-L13a. L13a was immunoprecipitated with anti-His antibody, and precipitates were subjected to immunoblot analysis with anti-eIF4G (top), anti-eIF4E (second panel), anti-eIF4A (third panel) and anti-L13a (bottom) antibodies.

(D) HRV-2A protease sensitivity of eIF4F complex assembly. Translation initiation reaction was reconstituted in RRL with or without HRV-2A protease (2 mg). Immunoprecipitation and immunoblot procedures were as in (C).
P-L13a TARGETS eIF4G FOR ITS TRANSLATIONAL SILENCING ACTIVITY

Our results indicate that L13a blocks initiation at a step after eIF4F assembly on the mRNA 50 cap, but before recruitment of 43S complex. Moreover, the presence of eIF4F on the silenced transcript suggests that L13a targets an eIF4F constituent. To more precisely define the inhibition locus, we took advantage of 5'-situated internal ribosome entry sites (IRESs) that initiate translation in the absence of specific eIF4F components. Initiation driven by the cricket paralysis virus (CrPV) IRES (31) (32) (33) (34) and hepatitis C virus (HCV) IRES (35) supports 43S and 60S recruitment independent of any eIF4F component. In contrast, initiation by encephalomyocarditis virus (EMCV) IRES requires eIF4A and the central domain of eIF4G, but not cap-binding protein eIF4E (36) (37). As expected, translation of capped Luc RNA and T7 gene 10 RNA constructs (Figure 7 A) were sensitive to exogenous cap, but translation of RNAs driven by CrPV, HCV, and EMCV IRESs were not sensitive (Figure 7 B). Thus, IRES-driven translation of GAIT element-bearing reporter RNAs is efficient in an RRL. P-L13a did not inhibit CrPV and HCV IRES mediated translation of reporter RNAs, indicating that one or more eIF4F components were required (Figure 7 C). In contrast, EMCV IRES-driven translation was specifically blocked by P-L13a. The effect of P-L13a on 80S assembly on IRES-driven reporters was examined by density gradient fractionation (Figure 3.4 D, representative gradient shows EMCV IRES-containing RNA). P-L13a blocked association of 80S ribosomes with transcripts driven by EMCV IRES, but not by CrPV or HCV IRES (Figure 7 E). These results suggests that an eIF4F component required for EMCV IRES-mediated translation, i.e., eIF4G, is required for silencing by P-L13a and may be its target.
Figure 7 Virus IRES-Driven RNAs Show an eIF4F Component Is Required for Translational Silencing by P-L13a
(A) Schematic of ApppG-capped, Luc-GAIT element-poly(A) RNAs with CrP, HCV, or EMCV IRES sequences.

(B) In vitro translation of IRES-driven reporters. IRES-driven and m7G-capped reporter RNAs (200 ng) were subjected to in vitro translation in RRL in the presence or absence of exogenous cap analog, m7G (200 mM).

(C) Sensitivity of IRES-driven reporters to repression by P-L13a. ApppG-IRES-Luc-GAIT-poly(A) RNAs (200ng) were subjected to in vitro translation in RRL with capped, T7 gene 10-poly(A) RNA (100ng) in the presence of recombinant phosphorylated or unmodified L13a (5 mg).

(D) Ribosomal RNA detection in sucrose density gradient fractions. Initiation reaction of IRES-driven Luc RNA reporters (500 ng) was done in the presence of CHX (0.5 mM) and subjected to sucrose density gradient centrifugation. A representative 80S peak is shown.

(E) Effect of P-L13a on 80S assembly on IRES-driven RNAs. Initiation reactions of Luc RNA-GAIT element reporters (500 ng) driven by m7Gcap (top), CrPV IRES (second panel), HCV IRES (third panel), or EMCV IRES (bottom) were reconstituted in RRL in the presence of P-L13a (10 mg) and CHX (0.5 mM) and were resolved by sucrose density gradient centrifugation. Total RNA isolated from 1 ml fractions was subjected to RT-PCR analysis using Luc-specific primers.
The eIF4G domain required for translational silencing by P-L13a was investigated. We took advantage of HRV-2A protease that cleaves eIF4G in the C terminus near the eIF3-binding site of the 43S complex (38). The central eIF4G domain permits the EMCV IRES-driven translation, and is resistant to HRV-2A protease. (39) (40). EMCV IRES-containing Luc reporter constructs are shown in Figure 5A. Immunoblot analysis showed eIF4G in RRL was cleaved by HRV-2A protease into 110 kDa C-terminal and 72 kDa N-terminal fragments (Figure 8 B). The identity of the cleavage products were verified by antibodies raised against eIF4G N- and C-terminal peptides. EMCV IRES-driven translation of the Luc-GAIT-poly(A) reporter was unaffected by eIF4G cleavage, but translation of cap-driven T7 gene 10 was blocked (Figure 8 C, lane 3). P-L13a blocked translation of EMCV IRES-driven reporter even after protease cleavage of eIF4G (Figure 8 C, lane 6). These results implicate the C-terminal domain of eIF4G as the L13a target. The inhibition of EMCV IRES-driven translation by P-L13a was poly(A) tail dependent (Figure 8 D, lanes 3 and 9). Interestingly, the inhibition of translation was poly(A) tail independent when eIF4G was cleaved with HRV-2A protease (Figure 8 D, lanes 6 and 8). Taken together, these results suggest the importance of intact eIF4G for
Figure 8. eIF4G C-Terminal Domain Is Necessary and Sufficient for Translational Silencing by P-L13a
(A) Schematic of ApppG-capped, Luc-GAIT element RNAs.

(B) Cleavage of RRL eIF4G by HRV-2A protease. Recombinant HRV-2A protease (2 mg) was incubated with RRL (100 mg) for 30 mins at 37°C and subjected to SDS-PAGE and immunoblot analysis with anti-eIF4G antibody raised against peptide sequences from N (aminoacids 175–200) and C (amino acids 1179–1206) termini of eIF4G1.

(C) Inhibition of EMCV IRES-driven translation by P-L13a in the presence of HRV-2A protease. ApppG-capped Luc RNA (200 ng) containing GAIT element with EMCV IRES and m7G-capped T7 gene 10 RNA (above) were translated in RRL with phosphorylated or unmodified L13a, and in the presence or absence of exogenous m7G cap and HRV-2A protease. (D) Inhibition of EMCV IRES-driven translation by P-L13a is poly(A) independent when eIF4G is cleaved. Translation was done as in (C) in the presence or absence of phosphorylated L13a and HRV-2A protease.
L13a INTERACTS WITH eIF4G AT ITS eIF3 BINDING SITE

Our co-immunoprecipitation studies *in vitro* suggest that L13a interacts with eIF4G in RRL (Figure 6C). The interaction was also investigated *in vivo*, human embryonic 293T kidney (HEK 293T) cells transfected with a vector driving expression of Flag tagged L13a. Immunoprecipitation with anti-Flag antibody, followed by an immunoblot with anti-eIF4G antibody, showed specific interaction of L13a with eIF4G (Figure 9 A). The eIF4G domain that binds L13a was investigated using HRV-2A protease to cleave eIF4G. His-tagged P-L13a was added to HRV-2A pretreated RRL, L13a was immunoprecipitated with anti-His antibody and probed with anti-eIF4G. P-L13a interaction with the C terminus of eIF4G was shown by co-immunoprecipitation of a 110 kDa, C-terminal fragment (Figure 9 B). The L13a interaction site was investigated by cotransfection of 293T cells with Flag- tagged L13a and vectors expressing HA-tagged eIF4G domains overlapping the initiation factor-binding sites (Figure 9 C). L13a co-immunoprecipitated with eIF4G(688–1560) and eIF4G(970–1560), but not with eIF4G(1075–1560) (Figure 9 D, top). Controls showed uniform expression of Flag-L13a (Figure 9 D, middle) and all eIF4G deletion fragments (Figure 9 D, bottom).

The eIF3-binding site in eIF4G, between amino acids 975 and 1065, is present in eIF4G(970–1560) but not in eIF4G(1075–1560), indicating that this domain is critical for L13a binding. To verify the importance of this interaction in translational silencing, dominant-negative activity of eIF4G deletion fragments was determined. C-terminal eIF4G fragments were expressed as GST fusion proteins in E. coli (Figure 9 C), preincubated with recombinant P-L13a, and added to an in vitro translation reaction driven by m7G-Luc-GAIT-poly(A) RNA. eIF4G(688–1131) and eIF4G(688–1560) blocked translation repression by P-L13a, but eIF4G(1075–1560) was ineffective (Figure 9 E, top). Fusion proteins added in the absence of P-L13a were inactive (Figure 9 E, bottom). Thus, only eIF4G fragments containing the eIF3-binding site inhibited P-L13a activity, indicating that eIF3 and L13a share the same or neighboring binding sites on eIF4G. To test whether P-L13a competes with eIF3 for binding to eIF4G, we took advantage of the finding that the eIF3e subunit of eIF3 binds with high affinity to eIF4G.
and is required for 43S recruitment (41). U937 cells were transfected with plasmids expressing HA tagged eIF3e and eIF3j (as negative control) and were treated with IFN-γ for 20 hr. Overexpression of eIF3e, but not eIF3j, substantially blocks the inducible binding between L13a and eIF4G as determined by co-immunoprecipitation (Figure 9F, top). Control studies show nearly equivalent expression of transfected eIF3 subunits (Figure 9F, middle) and consistent immunoprecipitation of L13a under all conditions (Figure 9 F, bottom). These results suggest that competition of P-L13a with eIF3e for binding to eIF4G is the mechanism by which L13a blocks 43S recruitment and silences translation.
Figure 9  
L13a and eIF3 Shares a Common eIF4G-Binding Site
(A) L13a binds eIF4G in 293T cells. 293T cells were transfected with pcDNA3-Flag-L13a or pcDNA3-Flag constructs. After 20 hrs, cell extracts were prepared and subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitations were resolved by SDS-PAGE, followed by immunoblotting with anti-eIF4G antibody.

(B) L13a binds the C-terminal fragment of eIF4G. eIF4G in RRL was cleaved by HRV-2A protease. His-tagged P-L13a was incubated with RRL in the presence or absence of HRV-2A protease, followed by immunoprecipitation with anti-His antibody and immunoblotting with anti-eIF4G antibody, or SP2/O as control antibody (top). Immunoprecipitation efficiency was shown by reprobing the blot with anti-L13a antibody (bottom).

(C) Schematic showing eIF4G deletion fragments made as GST fusion proteins or HA-tagged protein is indicated below.

(D) Mapping the interacting domain/s of eIF4G with L13a. 293T cells were cotransfected with Flag-L13a and HA-tagged eIF4G(688–1560), eIF4G(970–1560) or eIF4G(1075–1560). Cell lysates were immunoprecipitated with anti-Flag antibody, then were resolved by SDS-PAGE and subjected to immunoblot analysis with anti-HA (top) or with anti-Flag (middle) antibodies. Aliquots of lysates without immunoprecipitation were probed by immunoblot with anti-HA antibody (bottom).

(E) Exogenous eIF4G C-terminal fragment overcomes repression by P-L13a. P-L13a (5 mg) was preincubated with partially purified GST fusion proteins (50 mg) eIF4G(688–1131), eIF4G(688–1560), and eIF4G(1075–1560) and with GST control protein. Preincubated solutions were added to RRL, and cap-Luc-GAIT element-poly(A) (200 ng) and T7 gene 10-poly(A) were subjected to in vitro translation (top). As a control, partially purified GST fusion proteins containing eIF4G domains were added to in vitro translation reaction in the absence of P-L13a (bottom).

(F) Overexpression of eIF3e blocks binding of L13a to eIF4G. U937 cells were transfected with HA-tagged eIF3e or eIF3j by electroporation using Amaxa method. U937 cells (53106) were treated with IFN-γ for 4 or 20 hrs. Cell lysates were subjected to immunoprecipitation with anti-L13a antibody and immunoblotting with anti-eIF4G (top), anti-HA (middle), or anti-L13a (bottom) antibodies.
CHAPTER IV

DISCUSSION

We have determined a molecular mechanism by which the inflammatory protein Cp is translationally silenced in cells in response to prolonged treatment with IFN-γ. This repression is important because uncontrolled Cp expression may have injurious consequences due to the oxidative properties of the copper protein (42) (43). Here, we show that L13a is the GAIT complex protein directly responsible for translational silencing of Cp mRNA and possibly other target transcripts.

Transcript Circularization and Other Requirements for Silencing Activity of L13a

We took advantage of our previous findings, that recombinant L13a produced by baculovirus-infected insect cells is phosphorylated and active in an in vitro translation assay, whereas recombinant L13a from E.coli is unmodified and inactive (44). L13a phosphorylation is required for the GAIT complex mediated inhibition, and the RRL itself likely contributes the other three GAIT proteins that are all constitutive products of “housekeeping” genes. The requirement for a poly(A) tail on the target transcript is of substantial interest. In addition to its role in cap dependent translation, the poly(A) tail enhances the translation of EMCV IRES, indicating a possible communication between 5’
and 3’ termini during IRES-mediated translation (45), (46), (47). Therefore, it is not unexpected that P-L13a silences EMCV IRES-driven translation of GAIT element-containing RNA. However, our finding that P-L13a silences EMCV IRES-mediated translation even after the cleavage of eIF4G was surprising because the remaining C-terminal fragment of eIF4G does not bind to PABP (Poly(A)) binding protein) or facilitate end-to-end interaction. A possible explanation is that P-L13a has a very high affinity for the C terminus of the cleaved eIF4G fragment that remains bound to the EMCV IRES, and this high-affinity binding may compensate for the loss of mRNA circularization in the absence of a poly(A) tail. This possibility is consistent showing equal efficiency of translation of EMCV IRES driven mRNAs with and without poly(A) tails under the conditions of eIF4G cleavage by HRV-2A protease (47). In both cases, eIF4G cleavage causes translation (or repression) of EMCV IRES-driven mRNAs to be poly(A) independent. The efficient translation they observed may be due to increased affinity of eIF3e for the cleaved eIF4G fragment, which overcomes the need for the poly(A) tail. We speculate that, in our studies, P-L13a (which shares with eIF3e the same eIF4G-binding site) may also have an increased affinity for cleaved eIF4G, which similarly overcomes the requirement for a poly(A) tail.

Our results show that the poly(A) tail is not necessary for binding P-L13a to the GAIT element (Figure 4D), but rather it is necessary for end-to-end transcript closure by a succession of interactions with PABP and eIF4G (48). Similarly, a recent report on translational repression by miRNA shows that activity of the inhibitory, 3’UTR-binding miRNP complex requires target mRNA polyadenylation (49). Transcript closure may be necessary to carry P-L13a from the distal 3’UTR into the vicinity of the 5’-localized translation initiation complex where it exerts its inhibitory activity. Our new result showing the eIF4F complex remains intact on a silenced transcript is consistent with this mechanism.

**P-L13a Targets eIF4G for Transcript-Specific Translation Inhibition**

eIF4G is an essential initiation factor for protein translation. It is a component of the cap-binding complex eIF4F, facilitates end-to-end transcript interactions, acts as a
scaffold for RNA-unwinding proteins and other initiation factors, and makes initial contact with the ribosome via its interaction with eIF3 of the 43S complex. The central position of eIF4G and its multiple interactions makes it a strategic target for translation regulation. In fact, eIF4G is a target, common to multiple pathways that inhibit global protein synthesis, e.g., cell responses to virus infection, apoptosis, and stress (Figure 10a). Most silencing mechanisms that target eIF4G ultimately leads to global inhibition of cap – dependent protein synthesis. For example, eIF4G is proteolytically cleaved upon virus infection by viral proteases like 2A protease encoded by poliovirus, rhinovirus and cox – sackie virus, and L protease encoded by foot-and-mouth disease virus (50). Similarly, caspase – 3 cleaves eIF4G in apoptotic cells (51) (52). Other viral and cellular proteins bind eIF4G and inactivate it without cleavage, e.g., adenovirus 100-kDa protein binds eIF4G and displaces Mnk1, an eIF4E kinase that activates translation (53). NSP3 protein of rotavirus (54) and NS1 protein of influenza virus (55) bind the eIF4G N terminus and displaces PABP. Upon heat shock or other cell stress, the Hsp27chaperone protein binds eIF4G and traps it in insoluble complexes (56). In all the above examples, eIF4G is the targeted by RNA–independent mechanism to cause global inhibition of translation. Our results reveal that eIF4G is also a target for RNA–specific translation silencing by L13a (Figure 10b).

However, there are several aspects of the inhibition by L13a that differentiate it from global mechanisms. The requirement for a specific structural element makes this inhibitory mechanism transcript specific rather than global. Moreover, unlike most global inhibitors of translation, P-L13a does not alter initiation factors by proteolysis or by disruption of assembled complexes; translation inhibition by P-L13a occurs in the presence of an intact eIF4F complex. Our studies with HRV-2A protease fragments show that L13a binds the eIF4G C terminus. Detailed analysis of the binding site shows that L13a and eIF3 share the same binding site on eIF4G. We also observed that non P-L13a binds weakly to eIF4G in RRL even in the absence of RNA suggesting that L13a binds free eIF4G i.e. not associated with eIF4F complex with low affinity. But the binding to eIF4G is enhanced when it is associated with cap-bound eIF4F complex and when L13a is phosphorylated and bound to 3’UTR of mRNA as part of GAIT complex.
Overexpression of eIF3e (one of the subunit of eIF3), which exhibit high-affinity binding to eIF4G (41), blocks this interaction, supporting a model in which GAIT element-bound P-L13a and 43S-bound eIF3 compete for binding to cap-bound eIF4G. Unexpectedly, non-phosphorylated L13a binds weakly to eIF4G in an RRL even in the absence of RNA (Figure 6 C). Under this condition, L13a interacts with eIF4G, but not eIF4E or eIF4A, suggesting L13a binds free eIF4G not associated with the eIF4F cap-binding complex. In summary, L13a binds free eIF4G with low affinity, but binding is enhanced when eIF4G is associated with cap-bound eIF4F. Binding to eIF4G may be increased further when L13a is phosphorylated and bound to the transcript 3′UTR as a part of the GAIT complex (20).

Other Mechanisms that express Transcript-Specific Translational Control

Only a few systems of mRNA-specific translational control have been elucidated at the molecular level. However, it is already apparent that diverse mechanisms have evolved that inhibits initiation at multiple steps (57). The specific inhibitory mechanism driven by L13a has unique characteristics that differentiate it from previously described translation initiation regulators. P-L13a inhibiting the 43S recruitment distinguishes it from mechanisms in which initiation is blocked after 43S recruitment to the mRNA. For example, SXL binds to a poly(U)-rich region in the 5′UTR of msl-2 mRNA in D. melanogaster and inhibits 43S ribosome scanning (58). In a second example, binding of hnRNPs K and E1 to the 3′UTR DICE element of reticulocyte 15-lipoxygenase inhibits 60S ribosomal subunit joining to the 43S subunit (59). Inhibition of 43S recruitment has been reported for several translation regulators; however, their inhibitory mechanisms are distinct from that used by L13a. A complex containing cytoplasmic polyadenylation element binding protein (CPEB) and maskin binds the cytoplasmic polyadenylation element in the 3′UTR of target mRNAs during vertebrate oocyte development and silences their translation. The CPEB-maskin complex binds eIF4E and prevents its interaction with eIF4G, and thus blocks eIF4F assembly (60).
Our finding that a 3′UTR-bound inhibitory complex (GAIT) targets eIF4G and blocks 43S recruitment provides a new mechanism of transcript-specific translational repression. The presence of an intact eIF4F cap-binding complex, despite the interaction of L13a and eIF4G, is a notable feature that differentiates this mechanism from those in which attack on eIF4G prevents eIF4F assembly. Because of an integral eIF4F complex, the eIF4G component facilitates end-to-end transcript closure that is required for translation inhibition by L13a. In other cases in which eIF4E is targeted, 5′ to 3′ transcript interactions are mediated by the trans-acting factors themselves, not by the poly(A) tails. Thus, the requirement for an intact eIF4F complex for L13a-mediated repression may be related to the poly(A) dependency of the mechanism. It will be of interest to learn whether the requirement for an intact eIF4F is a common feature in translational control mechanisms that, like L13a, are poly(A) dependent and target general initiation factors.

**Figure 10**  Global and Transcript-Specific Translational Silencing targeting eIF4G

(A) Global inhibition of translation by targeting eIF4G. Virus infection, apoptosis, and cell stress target eIF4G by different mechanisms.  
(B) L13a targets eIF4G for transcript-specific inhibition of translation. L13a in the 3′UTRbound GAIT complex targets eIF4G without cleavage of eIF4G or disruption of cap-binding eIF4F complex. Binding of P-L13a to the eIF3-binding site of eIF4G blocks interaction of 43S ribosomal complex and represses translation.

(Kapasi et al., 2007, ref #20)
REFERENCES


