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MiniReview

Targeting internal ribosome entry site (IRES)-mediated translation to block hepatitis C and other RNA viruses ☆

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Abstract

A number of RNA-containing viruses such as hepatitis C (HCV) and poliovirus (PV) that infect human beings and cause serious diseases use a common mechanism for synthesis of viral proteins, termed internal ribosome entry site (IRES)-mediated translation. This mode of translation initiation involves entry of 40S ribosome internally to the 5′ untranslated region (UTR) of viral RNA. Cap-dependent translation of cellular mRNAs, on the other hand, requires recognition of mRNA 5′ cap by the translation machinery. In this review, we discuss two inhibitors that specifically inhibit viral IRES-mediated translation without interfering with cellular cap-dependent translation. We present evidence, which suggest that one of these inhibitors, a small RNA (called IRNA) originally isolated from the yeast *Saccharomyces cerevisiae*, inhibits viral IRES-mediated translation by sequestering both noncanonical transacting factors and canonical initiation factors required for IRES-mediated translation. The other inhibitor, a small peptide from the lupus autoantigen La (called LAP), appears to block binding of cellular transacting factors to viral IRES elements. These results suggest that it might be possible to target viral IRES-mediated translation for future development of therapeutic agents effective against a number of RNA viruses including HCV that exclusively use cap-independent translation for synthesis of viral proteins.
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Keywords: IRES-mediated translation; RNA viruses; Hepatitis C virus; Antivirals

1. Introduction

Picornaviruses and flaviviruses encompass a large variety of medically important human viruses, which include those inducing poliomyelitis (poliovirus, PV), infectious (hepatitis A) and chronic (hepatitis C, HCV) hepatitis, common cold (rhinoviruses), and myocarditis and encephalitis (coxsackieviruses). A common feature of these diverse groups of viruses is the strategy they employ for synthesis of viral proteins. While cellular capped mRNAs are translated by a cap-dependent "scanning" mechanism, the naturally uncapped viral RNAs use a cap-independent mechanism, which involves internal entry of ribosome within the 5′ untranslated region (5′UTR) of viral RNA, a process termed internal ribosome entry site (IRES)-mediated translation [1,2]. The observation that poliovirus RNA is not translated efficiently in the yeast *Saccharomyces cerevisiae* led to the identification and purification of a small (60 nt) RNA capable of specifically inhibiting viral IRES-mediated translation without interfering with cellular cap-dependent translation [3,4]. The ability of IRNA to discriminate between cap-dependent and cap-independent translation led to investigations towards

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understanding the mechanistic differences that exist between these two distinct modes of translation initiation and exploiting this fundamental difference to target hepatitis C virus (HCV) IRES-mediated translation as a novel approach in developing antiviral agents against HCV [5]. In this review, we will discuss the mechanism by which IRNA and a small peptide derived from the lulpus autoantigen La specifically inhibit viral IRES-mediated translation.

2. Viruses

Poliovirus is the prototype of a large variety of medically important human and animal RNA viruses (picornaviruses), which include those inducing common cold (rhinovirus), infectious hepatitis (Hep. A), coxsackie disease (Coxsackie virus), encephalomyocarditis (EMCV) and foot-and-mouth disease (FMDV) among others. These viruses are non-enveloped. Hepatitis C virus (HCV), on the other hand, is an enveloped RNA virus belonging to the flaviridae family. HCV infection frequently leads to chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [6–8]. There is currently no effective therapy or vaccine available to HCV-infected patients other than interferon (and ribavarin), which is only effective in a small percentage of infected patients. HCV has been a difficult virus to study due to the lack of an appropriate tissue culture system and an adequate, simple and low cost animal model. The RNA transcribed in vitro from a cDNA clone of HCV has been shown to be infectious when injected directly into the livers of chimpanzees [9,10].

Although PV and HCV belong to different groups of viruses, both contain single-stranded RNA genomes of plus polarity of approximately 7500 (PV) and 9500 (HCV) nucleotides. Additionally, the strategy of gene expression used by these viruses is remarkably similar at the molecular level. Both PV and HCV plus-strand genomic RNAs are translated as polyproteins that are proteolytically processed to mature structural and functional proteins. Both viruses encode RNA-dependent RNA polymerases that replicate the (+) strand RNA through a negative-strand RNA intermediate. Most importantly and pertinent to this review, both PV and HCV RNA genomes are translated by a common mechanism involving entry of ribosome to an internal sequence within the 5′ untranslated region (5′UTR) [2,11,12]. This mode of cap-independent translation is different from the cap-dependent translation, which involves 40S ribosome binding to the 5′ terminal cap structure on cellular mRNAs and scanning of the mRNA until the appropriate AUG is encountered. This fundamental difference could serve as a target for future development of antiviral drugs effective against such viruses as HCV [5,13,14].

3. Eukaryotic translation

3.1. Cap-dependent translation

The majority of the capped eukaryotic mRNAs are translated by the scanning mechanism in which the 43S complex, consisting of a 40S subunit bound to eIF-2: GTP: Met-tRNA$_i$, is recruited at the capped 5′ end of mRNA [15]. The binding of the 43S complex to the mRNA requires recognition of the mRNA 5′ cap by the cap binding protein complex, eIF-4F, consisting of three subunits: eIF-4E (the cap-binding protein), eIF-4A (an RNA helicase) and eIF-4G. The initiation factor eIF-4G is a modular scaffolding protein, which interacts simultaneously with eIF-4E, eIF-4A and several other proteins including the polyadenylate-binding protein (PABP). eIF-4G also interacts with ribosome through eIF-3, a multisubunit initiation factor. eIF-4B induced helicase activity of eIF-4A helps unwind the mRNA secondary structure leading to formation of single stranded region. eIF-4B then interacts with the 18S rRNA to guide the 40S ribosomal subunit to the single stranded region of the mRNA. Proteolysis of eIF-4G subunit of eIF-4F is the cause of inactivation of eIF-4F activity during poliovirus infection leading to host cell translation shut-off [16]. eIF-5 induces hydrolysis of eIF-2 bound GTP leading to formation of inactive eIF-2:GDP complex, which is recycled to eIF-2:GTP by the guanine exchange factor, eIF-2B. The joining of 60S ribosomal subunit is catalyzed by eIF-5B leading to the formation of the 80S initiation complex.

3.2. IRES-mediated translation

The development of bicistronic expression vectors containing the PV and EMCV 5′UTR located in the intercistronic region led to the demonstration that this region could confer internal initiation of translation on mRNA [1,2]. Since then, functional IRES elements have been identified in many other picornaviruses such as HRV, FMDV, HAV, TMEV, and Coxsackie virus [17]. The RNA genome of many other RNA viruses including HCV, classical swine fever virus, murine leukemia virus, simian immunodeficiency and cricket paralysis viruses use IRES-mediated translation [17]. Although capped, some cellular mRNAs including translation initiation factors, transcription factors, oncogenes, growth factors, homeotic genes, and survival proteins contain IRES elements in their 5′UTR sequences that may allow them to be translated under conditions when cap-dependent synthesis of proteins may be impaired. It is generally thought that phosphorylation of eIF-4F complex under conditions of stress, such as virus infection heat shock hypoxia, could shut down the cap-dependent mode translation

of some of these cellular mRNAs; the synthesis of the proteins encoded by these mRNAs can then be achieved by IRES-mediated translation that may not require the full participation of the subunits of the eIF-4F complex.

Comparison of IRES elements of picornaviruses indicates a lack of conservation of primary sequence with the exception of a short polypyrimidine tract. The structural requirements for translation initiation have led to two main groups of IRES structures: one for entero- and rhinoviruses, and the other for cardio- and apthoviruses. A number of studies have shown the importance of both RNA secondary and tertiary structures in viral cap-independent translation [18–26]. It is thus clear that the overall RNA structure of the IRES has an active role in internal initiation.

3.3. Mechanism of IRES-mediated translation: requirement of canonical initiation factors (eIFs) in IRES-mediated translation

The majority of viral IRES elements (with the exception of HAV IRES) do not require eIF-4E, the cap-binding protein for the 48S complex formation (association of the 43S complex with mRNA). In addition, various viruses require different combination of other canonical initiation factors. The canonical initiation factors eIF-2, eIF-3, eIF-4B and eIF-4F were found to be required for 48S complex formation with the EMCV IRES [27]. In the 48S complex formation assay, the cap-binding protein complex eIF-4F can be replaced by a complex consisting of eIF-4A and the central domain of eIF-4G (eIF-4G$_{697–949}$) [28]. Consistent with the interaction of eIF-4G$_{697–949}$ with picornavirus IRES elements, internal initiation promoted by these elements is efficient under conditions of eIF-4G cleavage that leads to host cell translation shut-off [16].

Similar analysis has shown that the 48S complex formation by the HCV IRES element requires only eIF-2: GTP: Met-tRNA$_i$, eIF-3 and 40S subunits [29]. The 40S ribosome appears to interact with HCV RNA at multiple sites including stems, loops, pseudoknots, as well as the initiator AUG. Ribosomal protein S5/S9 as well as eIF-2B and eIF-2γ have been identified as cofactors of HCV IRES-mediated translation [30,31]. Mutations of the IRES regions that constitute the binding site for proteins are detrimental to IRES activity [21].

The intergenic region of cricket paralysis virus (CrPV) and the 5′UTR element of PSIV (*Plautia stali* intestinal virus), a virus closely related to CrPV, have been shown to function as IRES elements [24,26]. Surprisingly, the in vitro binding of 40S ribosome to the CrPV IRES did not require any initiation factor. These results suggest that the IRES itself substitutes functionally for the role played by the initiator tRNA in the eIF-2:GTP:Met-tRNA$_i$ ternary complex.

3.4. Role of cellular transacting proteins in viral IRES-mediated translation

Although the studies described above have shed light on the minimum required canonical factors for viral IRES-mediated translation, it is apparent that transacting factors play an important role in modulating IRES activity [32]. Cellular proteins such as La (lupus autoantigen), PTB (polypyrimidine tract binding protein), PCBP2(poly rC binding protein), C23 (nucleolin) and unr have been shown to interact with viral IRES elements and stimulate IRES-mediated translation [33–42]. It has been hypothesized that the transacting proteins may act as "RNA chaperones" stabilizing IRES secondary and tertiary structures to allow efficient translation to take place [32]. In this review, we will focus on the La protein and consequently the role of other transacting proteins in cap-independent translation will not be discussed.

3.4.1. The La protein

La, a 52-kDa autoantigen in patients with systemic lupus erythematosus, was one of the first cellular proteins identified to interact with IRES-elements and stimulate PV and HCV IRES-mediated translation [34,35,42–44]. The majority of the La Protein is localized in the nucleus and appears to be involved in small RNA biogenesis including pre-tRNA maturation, stabilization of nascent RNAs, nuclear retention of nascent transcripts, and RNA pol III transcription termination [45]. However, cellular stress such as poliovirus infection causes redistribution of the La protein from nucleus to the cytoplasm possibly by removal of the C-terminal nuclear localization signal by a viral protease [42,46].

The 408aa long La protein contains three putative RNA recognition motifs (RRMs): the N-terminal 100 amino acids containing the highly conserved 60 amino acid long "La motif" (called RRM1), followed by a RNA recognition motif spanning amino acids 101–208 (RRM2), and RRM3 (amino acids 209–300). The C-terminus of La is highly charged and is required for La's ability to enhance poliovirus RNA translation [47]. However, both the N- and C-terminal halves bind the HCV 5′UTR and are required for efficient translation from the HCV IRES [35,48]. The N-terminal fragments of the human protein, consisting of the La-motif and RRM2 bind several RNA substrates with an affinity comparable to that of the full-length protein. Although the isolated "La motif" (amino acids 1–60) does not bind RNA, even small deletions within the La motif drastically decreases RNA binding affinity [49,50]. It is thought that the La motif may increase the affinity of RRM2 for RNA.

4. Mechanism of IRNA-mediated inhibition of viral IRES-mediated translation

4.1. IRNA sequesters a number of cellular transacting proteins involved in viral IRES-mediated translation

Studies directed at understanding the mechanism by which IRNA inhibits viral IRES-mediated translation indicated that IRNA could sequester a number of cellular RNA binding proteins thus making them unavailable to interact with the viral 5′UTR. These transacting proteins include the lupus autoantigen (La), polypyrimidine tract binding protein (PTB), nucleolin, and two other unidentified proteins having molecular masses of 70 and 38 kDa. In addition to its ability to inhibit viral IRES-mediated translation in vivo, replication of wt PV and a PV/HCV chimeric virus containing the HCV IRES was drastically inhibited in Huh-7 cells constitutively expressing IRNA [51,52]. IRNA has been shown to interact with the La protein very strongly and the IRNA-mediated inhibition of PV and HCV IRES-mediated translation can be reversed by the exogenous addition of the purified La protein in vitro. Additionally, the purified La protein forms a stable complex with the 5′UTR of both HCV and PV. Similarly, an RNA sequence (called SELEX RNA) that was selected for high-affinity binding to the La protein was found to sequester La and block HCV IRES-mediated translation [35]. Also, IFN treatment of cells leads to selective inhibition of HCV IRES-mediated translation, which correlates to a reduced level of La protein and transient expression of La in these cells completely restored the selective inhibition of HCV translation [53]. Taken together, these results provide strong evidence that La is needed for efficient translation of PV and HCV RNA in vivo and that IRNA blocks viral translation by sequestering the La protein.

4.2. Structure–function relationship of IRNA: structural alterations lead to loss translation inhibitory activity and altered protein binding

In order to gain insight into how IRNA blocks IRES-mediated translation, the secondary structure of IRNA was determined. The secondary structure of IRNA consists of two loops, a 7-base long stem, and a large bulge region (Fig. 1A) [54]. Mutational analysis of IRNA followed by secondary structure determination

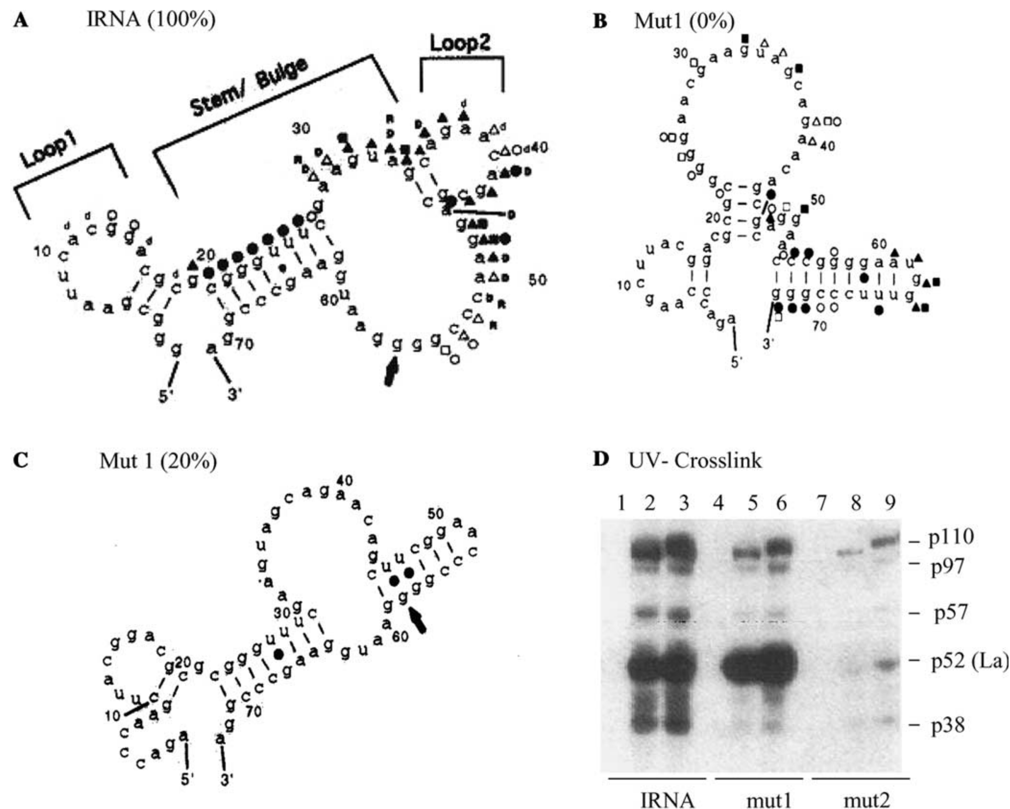

Fig. 1. Structural alterations of IRNA lead to loss of translation inhibitory activity and altered protein binding. (A–C) Proposed secondary structure models and enzymatic digestion maps of IRNA (A) and two mutants, mut1 (B) and mut2 (C). The numbers in parentheses indicate translation inhibition activity of IRNA and mutants. (D) UV cross-link analysis of RNA–protein interaction was performed with ^{32}P-labeled IRNA (lanes 1–3), mut1 IRNA (lanes 4–6), and mut2 IRNA (lanes 7–9) in the absence (lanes 1, 4 and 7) or presence of 30 μg (lanes 2, 5 and 8) and 60 and 9) HeLa cell-free extract. Reprinted with permission from Oxford University Press (Venkatesan et al. (1999) Nucleic Acids Res. 27, 562–572).

showed that the maintenance of the established secondary structure was required for both IRNA's ability to bind cellular transacting proteins and inhibit viral IRES-mediated translation. A mutant IRNA (mut1), which lacks loop 2 (nt 36–43) due to a 3 nt substitution (GCA to UUC), bound La extremely efficiently, but was defective in interacting with p110 (nucleolin), p57 (PTB), and p38 (Figs. 1B and D). This mutant is defective in inhibiting IRES-mediated translation by 80% compared to wt IRNA [54]. It is possible that loop 2 may not be involved in La binding but may play an essential role in binding other relevant proteins involved in IRES-mediated translation. Alternatively, the altered structure of mut1 IRNA enables it to bind so much La that the binding of other proteins is impaired. Indeed, such an exclusionary role for La has been posited in a slightly different context, where coating of mRNAs with La has been proposed to prevent binding of ribosomal initiation factors [54]. In the case of mut2 IRNA, the swapping of complementary sequences (5′-UUC-3′ and 5′-AAG-3′) within the stem of IRNA alters its structure due to formation of a new helix (Fig. 1C) [54]. Mut2 IRNA is 100% defective in inhibiting viral IRES-mediated translation and highly defective in binding almost all polypeptides, including La that normally interact with IRNA (Fig. 1D). One possibility for this global decrease of protein binding is that the structure is altered so extensively that these proteins are no longer capable of readily recognizing the RNA molecule. Alternatively, mut2 IRNA may be deficient in binding a protein that recruits the other proteins; thus, a deficiency in binding this protein leads to global decrease in protein binding by mut2 IRNA. Taken together, these results suggest that while interaction of La with IRES (or IRNA) is important, additional factors are almost certainly involved in IRES-mediated translation.

4.3. Identification of IRES-specific translation factors by IRNA affinity chromatography: IRNA interacts with both canonical and non-canonical factors

In order to identify additional cellular factors involved in IRES-mediated translation, HeLa translation lysates were fractionated through an immobilized IRNA affinity column. Bound proteins were eluted stepwise with 25 mM (fraction a, Fa), 100 mM (Fb), 300 nM (Fc), and 600 mM (Fd) KCl. The fractionated proteins were examined by Northwestern blot using ^{32}P IRNA as probe. Most of the proteins that interact with IRNA eluted in the Fb and Fc fractions (Fig. 2A). Passage of the cell-free lysates through a control column having the same matrix but not linked to IRNA, resulted in the elution of almost all IRNA binding proteins in the flow-through fraction (FT^m, Fig. 2A). Proteins eluted in Fa, Fb, Fc and Fd fractions from the mock column did not bind the labeled IRNA probe (data not shown). In contrast, the flow-through from the IRNA column had no detectable ^{32}P IRNA binding protein.

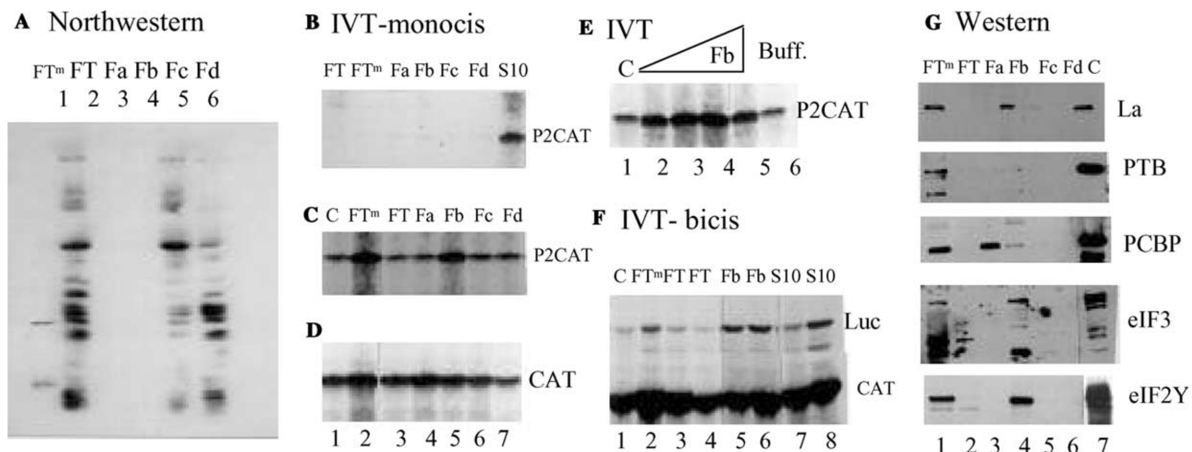

Fig. 2. Purification of HeLa cell proteins by IRNA affinity chromatography. (A) HeLa S10 proteins were purified through IRNA–Sepharose or mock-Sepharose columns. Flow-through from the mock column (FT^m, lane 1) and IRNA column (FT, lane 2) and fractions Fa, Fb, Fc and Fd from the IRNA column (lanes 3–6) were analyzed by Northwestern blot using ^{32}P IRNA probe. (B) Various fractions as indicated on top did not have any translation activity by themselves. The right most lane (#7) is a positive control showing translation of P2CAT (PV IRES linked to CAT RNA) in HeLa S10. (C) Translation of P2CAT in HeLa S10 (lane C) is stimulated by FT^m (lane 2) and Fb fraction (lane 5) but not by FT^m, Fa, Fc and Fd fractions. (D) Cap-dependent translation of CAT is not stimulated significantly by various fractions (lanes 2–7) compared to the control (lane 1). (E) Translation of P2CAT is shown in the absence (lane 1) and presence of 0.5, 1.0, 1.5 and 2.0 μg of Fb fraction, respectively (lanes 2–5). Lane 6 is buffer control. (F) Effect of Fb fraction on HCV IRES-mediated translation of luciferase (Luc) from a bicistronic RNA (cap-CAT-HCV IRES-Luc). Luc and CAT translation was monitored in limiting HeLa S10 translation extracts in the absence (lane 1) and presence of 1 μg FT^m (lane 1, C), 1 μg FT^m (lane 2), 0.5 and 1 μg FT (lanes 3 and 4), 0.5 and 1 μg Fb (lanes 5 and 6), and 0.5 and 1 μg S10 9lanes 7 and 8). (G) FT^m (lane 1), FT (lane 2), Fa (lane 3), Fb (lane 4), Fc (lane 5) and Fd (lane 6) fractions were analyzed by Western blot using anti-La, anti-PTB, anti-PCBP2, anti-eIF-3 and anti-eIF-2 as indicated. Western blot of each purified protein is shown in lane 7 for each panel as positive control.

4.3.1. Proteins that bind IRNA stimulate IRES-mediated translation

Different fractions from the IRNA affinity column were tested for their ability to stimulate IRES-mediated translation. Sub-optimal or limiting concentrations of HeLa S10 extracts were used for in vitro translation of P2CAT mRNA (containing PV 5′UTR) and the effect of addition of various fractions from the IRNA affinity column were determined. The fractions by themselves (in the absence of HeLa S10) had no P2CAT translation activity (Fig. 2B). Addition of the flow-through (FT) fraction from the IRNA column to limiting amount of HeLa S10 did not stimulate IRES-mediated translation significantly compared to the control (lanes 1 and 3, Fig. 2C). As expected, the flow-through fraction (FT^m) from the mock column did stimulate both IRES-mediated (8 fold) and cap-dependent (2 fold) translation (lane 2, Figs. 2C and D). Among all the fractions from the IRNA column, only the Fb fraction was found to stimulate PV IRES-mediated translation ~4-fold over the control (lane 5, Fig. 2C). None of these fractions stimulated cap-dependent translation significantly over the control (Fig. 2D). A titration of the Fb fraction showed up to 8-fold stimulation of IRES-mediated translation (Fig. 2E). However, at higher concentrations of the Fb fraction inhibition of translation was observed (lane 5, Fig. 2E). The buffer alone had no significant effect on translation (lane 7, Fig. 2E). Translation in reticulocyte lysates of a bicistronic RNA showed that the Fb fraction specifically stimulated HCV IRES-mediated synthesis of luciferase almost to the level observed with the HeLa S10 fractions (Fig. 2F). These results strongly suggest that factors retained by the IRNA affinity column are critical for IRES-mediated translation.

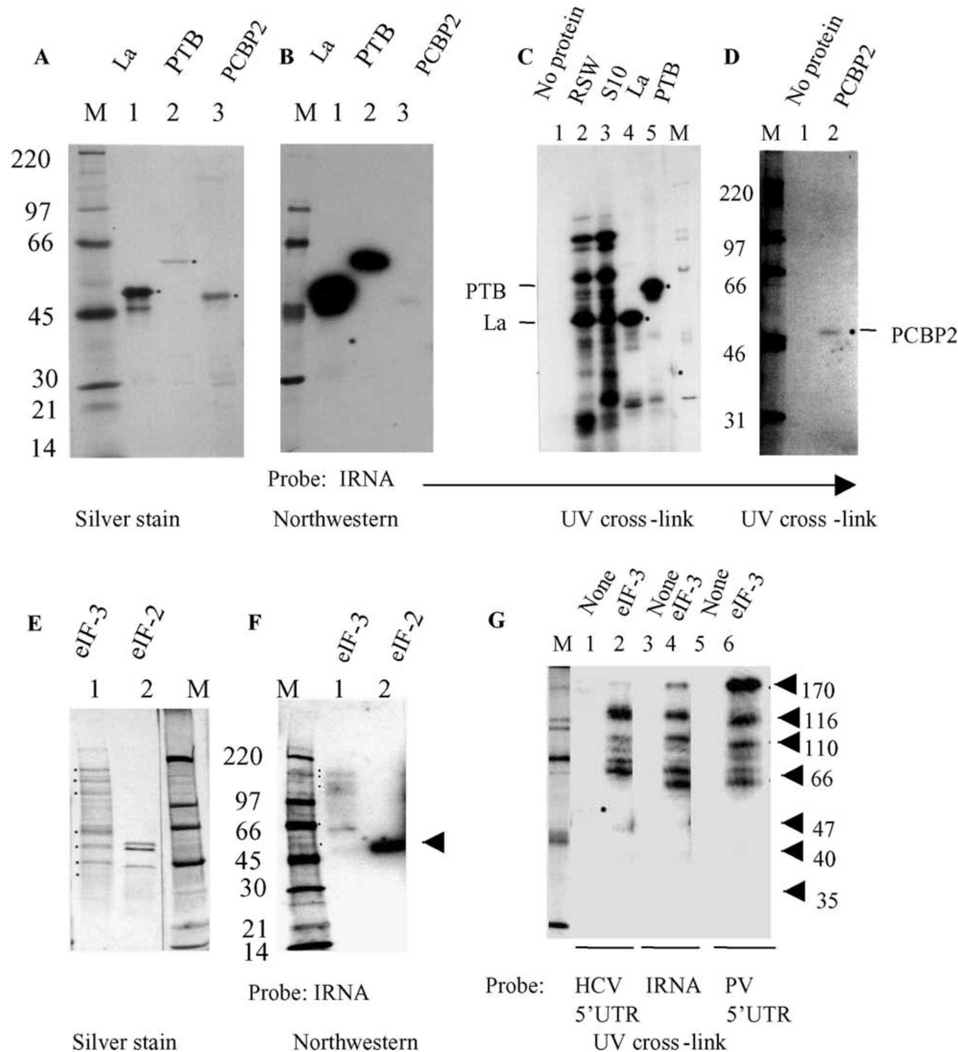

Fig. 3. Interaction of IRNA with noncanonical and canonical factors. (A) Purified La, PTB and PCBP-2 were analyzed by SDS–PAGE followed by silver stain. (B) A Northwestern blot of the same gel probed with ^{32}P-labeled IRNA is shown. (C, D) UV cross-link analysis of RNA–protein complex is shown using ^{32}P-labeled IRNA and purified La, PTB and ribosomal salt wash (RSW) and S10 (C), and purified PCBP-2 (D). (E) A silver stained gel of purified eIF-3 and eIF-3 are shown. (F) A Northwestern blot of the same gel probed with ^{32}P-labeled IRNA is shown. (G) UV cross-link analysis of RNA–protein complexes were performed using purified eIF-3 and ^{32}P-labeled HCV 5′UTR, IRNA, and PV 5′UTR as indicated.

4.3.2. Fraction Fb contains high levels of transacting proteins as well as eIF-3 and eIF-2

To determine the level of protein factors required for IRES-mediated translation, a series of Western blot analyses were performed with various fractions recovered from the IRNA- and mock-columns. As expected, the flow-through fraction (FT^{m}) from the mock-column contained significant amounts of La, PTB, PCBP-2, eIF-2 and eIF-3 (Fig. 2G, lane 1). However, the flow-through fraction from the IRNA column was depleted of most of these proteins except for some subunits of eIF-3 (Fig. 2G, lane 2). A number of eIF-3 subunits ranging from 66 to 170 kDa, the γ subunit of eIF-2, PTB and the La protein were detected in the Fb fraction (Fig. 2G, lane 4). PCBP-2 was mostly present in the Fa fraction (lane 3, Fig. 2G) although small amount of PCBP-2 was detected in the Fb fraction (lane 4). These results suggest that IRNA could block IRES-mediated translation through interaction with not only the transacting factors like La and PTB, but also canonical initiations factors (eIF-2 and eIF-3) required for cap-independent translation.

4.3.3. Purified La, PTB, eIF-3 and eIF-2 interact with IRNA

The results obtained from IRNA affinity chromatography were confirmed by direct binding of IRNA with various purified proteins. Both UV cross-link and Northwestern analyses demonstrated that purified La and PTB bound very efficiently to IRNA (Figs. 3A, B and C). The interaction of La and PTB with IRNA was specific as shown by competition binding studies (4, 51 and data not shown). However, in both RNA binding assays PCBP2 showed relatively weak interaction with IRNA (Figs. 3A and D). The 170, 116, 110, and a doublet migrating at approximately 66 kDa subunits of eIF-3 were cross-linked to PV and HCV 5′UTR and IRNA (Fig. 3G). Interestingly, PV 5′UTR bound p170 very strongly, but IRNA and HCV 5′UTR bound this subunit of eIF-3 relatively weakly. Northwestern analysis also confirmed the interaction of purified eIF-3 with IRNA (Fig. 3F). Direct binding of purified eIF-2 to IRNA, PV and HCV 5′UTR as measured by UV cross-link analysis was weak (data not shown), although a significant amount of eIF-2 was found to interact with ^{32}P-labeled IRNA by Northwestern analysis (Fig. 3F). These results suggest that IRNA interacts with at least two canonical translation factors involved in IRES-mediated translation.

5. Development of a novel peptide, which blocks viral IRES-mediated translation

To examine the region of the La protein critical for IRNA binding, we expressed and purified various deletion mutants of La and determined their ability to interact with IRNA, HCV IRES and PV IRES. These studies identified a highly conserved region of La (aa 11–28) within the N-terminus "La motif" (aa 1–60 within RRM1), which was absolutely necessary for La binding to all three RNAs [55]. We synthesized a synthetic peptide (called LAP for La peptide) corresponding to amino acids 11–28 of La, which efficiently and specifically blocked translation programmed by HCV and PV IRES elements both in vivo and in vitro without interfering with cap-dependent translation [55]. The purified LAP peptide did not bind IRNA, HCV IRES or PV IRES directly, but interfered with La binding to all three RNAs. LAP-induced inhibition of IRES-mediated translation in vitro could be completely reversed by addition of excess HeLa translation lysates but not by excess 5′UTR suggesting that LAP inhibits viral translation by interacting with proteins rather than the template RNA [55]. The amino acid sequence of LAP, ^{11}AALEAKICHQIEYYF GDF^{28}, is highly conserved in the La protein from a variety of species [55]. Single amino acid changes (the underlined amino acids Y23 and F25 mutated to Q) in the LAP totally abrogated its translation inhibitory activity [55]. When the Y23Q mutation was introduced into the full-length La protein, the purified mutant protein was found to be severely defective in binding HCV and PV IRES elements and was unable to stimulate IRES-mediated translation from the viral IRES elements. Mutation of the next tyrosine (Y24) to Q, however, did not alter La's ability to bind RNA and stimulate IRES-mediated translation.

5.1. LAP enters cells efficiently and blocks HCV IRES-mediated translation

We examined Lap's ability to transduce cells by incubating Huh-7 cells with FITC-labeled LAP. A non-specific peptide (called NSP) derived from a different region of the La protein was used as a negative control in these experiments. We were able to demonstrate that while LAP-FITC entered cells efficiently, neither the NSP-FITC nor free FITC could enter cells [55]. LAP appeared to be localized within the cytoplasm of Huh-7 cells as confirmed by staining the nuclei with Hoescht dye (Fig. 5). To determine the rate of LAP entry into cells, Huh-7 cells were incubated with LAP-FITC for various times and cells were then examined by flow cytometry. We were surprised to find that nearly 100% of the cells had internalized LAP by ~1.5 h [55]. We also found that the LAP-FITC entered cells at 4 °C, but at a slightly slower pace (30% slower), therefore making it unlikely that the peptide was being endocytosed. The entry of LAP into Huh-7 cells was confirmed by a functional assay demonstrating that preincubation of Huh-7 cells with LAP was sufficient to almost totally block HCV IRES-mediated translation of firefly

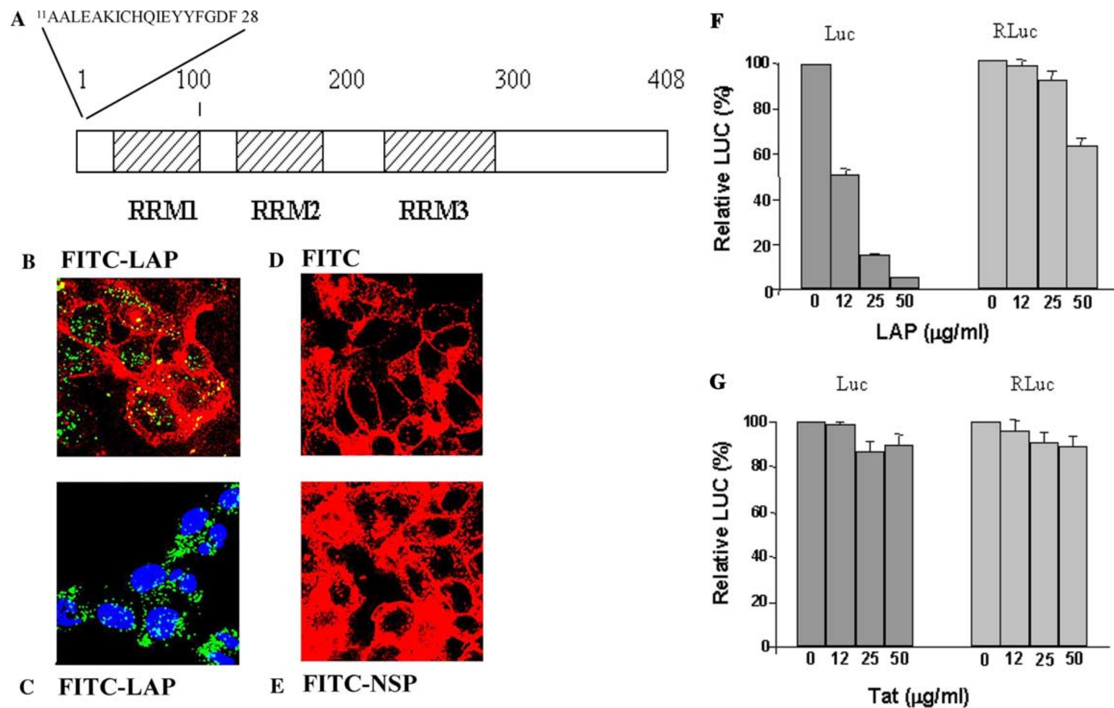

Fig. 4. Cell entry and inhibition of HCV IRES-mediated translation in vivo by LAP. Huh-7 cells were incubated overnight with 5 μM of FITC-labeled LAP (green) (A), or 5 μM unconjugated FITC (C), or 5 μM NSP-FITC (D). The cell membrane is stained (orange) with DiI. Cells were visualized by confocal microscopy as described in Materials and Methods. (B) Huh-7 cells were incubated overnight with LAP-FITC (green) as described above. The nuclei were stained with Hoeschst dye (blue). In this sample cell membrane was not stained with DiI. Huh-7 cells were pre-incubated with various concentrations of LAP (E) or the HIV-1 Tat peptide (F). After 2.5 h, the cells were washed free of peptides and transfected with the capped bicistronic RNA template (Cap-Rluc-HCV 5′UTR-Luc). At 6 h post-transfection, the cell lysates were harvested and measured for Renilla and firefly luciferase activity. Representative data from three separate transfections is shown. Reprinted with permission from the American Society for Microbiology (Izumi et al. (2004) J. Virol. 78, 3763–3776).

luciferase from a bicistronic mRNA in vivo (Fig. 4). Cap-dependent translation of *Renilla* luciferase was not significantly affected by LAP at lower concentrations. At the highest concentration tested, there was a 40% reduction in cap-dependent translation. An unrelated peptide (HIV Tat peptide, which enters cells freely) did not inhibit HCV IRES-mediated translation of Luc.

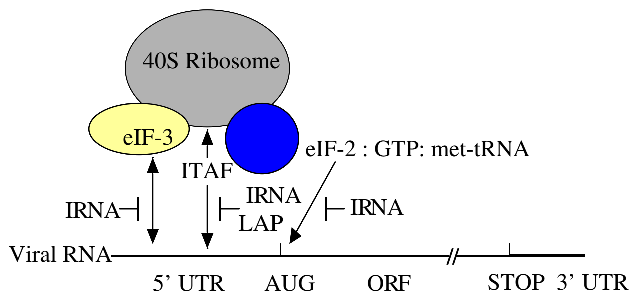

Fig. 5. Proposed mechanism for inhibition of viral IRES-mediated translation by IRNA and LAP. The illustration depicts possible steps where IRNA and LAP could interfere with binding of the 43S ribosomal complex to viral IRES elements. IRNA can sequester IRES transacting factors (ITAFs) as well as canonical initiation factors eIF-2 and eIF-3 thus blocking interaction of viral RNA with the 40S ribosome. The La peptide (LAP) can block binding of ITAFs to viral RNA. It is not known at present whether LAP also interferes with interaction of viral IRES elements with canonical factors.

We also made single, double and triple amino acid substitution mutants of LAP to determine which amino acids were critical for LAP's translation inhibitory activity. Although several double and triple substitution mutants completely lost translation-inhibitory activity, an interesting observation was that the single mutant Y23Q (#741) (but not Y24Q, # 633) was totally inactive in blocking viral translation in vitro (Table 1 and [55]). When the Y23Q mutation is introduced into full-length La protein, there is almost total loss of both RNA binding and translation stimulation by La [55]. Curiously, almost all mutations that interfered with Lap's viral translation inhibitory activity also interfered with its ability to enter cells (Table 1). Only one mutant (LAP 701) that had lost translation inhibitory activity could still transduce cells, however, with much lower efficiency compared to wt LAP.

Although translation from the HCV IRES has been reported to require only eIF-2, eIF-3, GTP and met-tRNA, a number of studies have shown a role of the La protein in the translation of HCV and PV RNA. Our in

Table 1
Translation inhibition and cell entry of various LAP mutants[e]

Peptide	Mutation[a]	Activity[c]	Cell entry[d]
LAP	AALEAKICHQIEYYFGDF	+	+++
702	**AALEAQICQQIEYYFGDF**	+	+++
701	AAL**Q**AKICHQI**Q**YYFG**Q**F	–	+
761	**QQQ**EAKICHQIEYYFGDF	+	+
762	**QQQ**E**Q**K**Q**CHQIEYYFGDF	–	–
703	AALEAKICHQIE**QQQ**GD**Q**	–	–
771	AALEAKICHQIEYY**Q**GD**Q**	–	–
772	AALEAKICHQIE**QQ**FGDF	–	–
741	AALEAKICHQIE**Q**YFGDF	–	–
633	AALEAKICHQIEY**Q**FGDF	+	+++
632	AALEAKICHQIEYY**Q**GDF	–	–
631	AALEAKICHQIEYYFGD**Q**	–	ND[b]

[a] Mutations are indicated by bold and underlined letters.
[b] ND, not determined.
[c] Activity was assayed by the ability of the peptides to block p2CAT translation.
[d] Cell entry was determined by confocal microscopy of FITC-labeled peptides.
[e] Reprinted with permission from American Society of Microbiology (Izumi et al. (2004) J. Virol. 78, 3763–3776).

vivo data that LAP could inhibit HCV IRES-mediated translation to almost the background level in vivo also suggest a role of La in HCV mRNA translation. However, the possibility still exists that like IRNA, LAP is able to interact with canonical factors eIF-2 and eIF-3. Recent results from our laboratory have provided evidence that suggest interaction of LAP with La and other transacting factors that play important roles in IRES-mediated translation [55]. This is suggested by the inability of the purified La protein to completely reverse the translation inhibitory effect of LAP; however, the proteins present in HeLa lysates could completely restore translation induced by LAP [55]. Additionally, the interaction of HCV and PV IRES with a number of proteins including La and PTB in the HeLa lysates were completely blocked by LAP(data not shown). Although the nature of all the polypeptides that interact with LAP are not known yet, it is quite possible that LAP could block interaction of a number of transacting proteins as well as eIF-2 and eIF-3 with the viral IRES element.

5.2. Acute toxicity and tissue distribution of LAP in animals

To examine Lap's potential as an IRES inhibitor in animals, LAP was tested in rats for acute toxicity, tissue distribution, and maximum concentration in various tissues. LAP had no serious side effects when administered to rats at 25 mg/kg body weight in terms of survival as well as abnormal pathology except for development of rash in the paw in 10% of the experimental animals. After a single intravenous injection of LAP at 10 mg/ml body weight, 50–60% of the dosed LAP was found to localize to the liver of rats and remained there for at least 6 h (data not shown). At 24 and 48 h post injection, approximately 13% and 1% remained in the liver. The maximum concentration of LAP in the liver was found to be ~160 μg/g. Currently we are testing suitable viruses that use IRES-mediated translation as well as infect liver cells in rats (or mice). Identifying such viruses will help determine the efficacy of LAP in mice.

6. Concluding remarks

The chance discovery that poliovirus RNA is not translated in the yeast *S. cerevisiae* has led to the discovery of a naturally occurring yeast RNA (IRNA) capable of specifically inhibiting viral IRES-mediated translation. The studies leading to understanding the mechanism of IRNA has consequently resulted in the development of another IRES-specific peptide inhibitor (LAP). Initial preclinical studies have shown that LAP has excellent potential to be developed as an inhibitor of HCV protein synthesis. LAP may also have applications in other human diseases that involve synthesis of cellular proteins by IRES-mediated translation. For example, the mRNA encoding the angiogenic factor, vascular endothelial growth factor (VEGF), is translated in an IRES-dependent fashion under hypoxic conditions induced by rapidly growing tumors [56]. Although the VEGF mRNA is capped, cap-dependent translation most probably does not contribute to VEFG synthesis in a fast-growing tumor due to phosphorylation (and inactivation) of the cap-binding protein under hypoxic condition. Thus, the IRES inhibitors LAP/IRNA should, in principle, inhibit formation of the VEGF protein ultimately leading to retarded tumor growth. Since LAP readily enters cells and appears to be relatively stable in vivo (animals), its efficacy in blocking formation of new blood vessels can be directly tested by injecting LAP into growing tumors. Future studies into the three-dimensional structure of LAP and IRNA may lead to design of novel therapeutic molecules more effective in inhibition of viral/cellular IRES-mediated translation.

Apart from the usefulness of IRNA and LAP in identifying and understanding the mechanisms by which transacting proteins participate in the IRES-mediated translation in mammalian cells, we have just begun to use these molecules to examine IRES-mediated translation in the yeast *S. cerevisiae*. Initial studies have identified four yeast proteins that interact strongly with IRNA. One of these proteins has been identified as Zuotin (Zuo1), a DnaJ molecular chaperone homologous to mammalian hsp40. Our recent studies using Zuo1 knock out yeast have shown that Zuo1 specifically stimulate IRES-mediated translation in yeast. The three other yeast proteins that interact with IRNA have also

been identified and all three appear to be involved in protein synthesis. Future studies will determine whether these proteins also participate in IRES-mediated translation in yeast.

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