RNA aptamers to initiation factor 4A helicase hinder cap-dependent translation by blocking ATP hydrolysis

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ABSTRACT

The mammalian translation initiation factor 4A (eIF4A) is a prototype member of the DEAD-box RNA helicase family that couples ATPase activity to RNA binding and unwinding. In the crystal form, eIF4A has a distended “dumbbell” structure consisting of two domains, which probably undergo a conformational change, on binding ATP, to form a compact, functional structure via the juxtaposition of the two domains. Moreover, additional conformational changes between two domains may be involved in the ATPase and helicase activity of eIF4A. The molecular basis of these conformational changes, however, is not understood. Here, we generated RNA aptamers with high affinity for eIF4A by *in vitro* RNA selection-amplification. On binding, the RNAs inhibit ATP hydrolysis. One class of RNAs contains members that exhibit dissociation constant of 27 nM for eIF4A and severely inhibit cap-dependent *in vitro* translation. The binding affinity was increased on Arg substitution in the conserved motif Ia of eIF4A, which probably improves a predicted arginine network to bind RNA substrates. Selected RNAs, however, failed to bind either domain of eIF4A that had been split at the linker site. These findings suggest that the selected RNAs interact cooperatively with both domains of eIF4A, either in the dumbbell or the compact form, and entrap it into a dead-end conformation, probably by blocking the conformational change of eIF4A. The selected RNAs, therefore, represent a new class of specific inhibitors that are suitable for the analysis of eukaryotic initiation, and which pose a potential therapeutic against malignancies that are caused by aberrant translational control.

Keywords: eIF4A; helicase; RNA aptamer; SELEX; translation initiation

INTRODUCTION

The initiation of protein synthesis in eukaryotes is a highly regulated process involving at least 12 protein factors (Hershey and Merrick 2000). The initial association of mRNA with the small (40S) ribosomal subunit requires the participation of at least three initiation factors (eIF4A, eIF4B, and eIF4F) and the hydrolysis of ATP (Gingras et al. 1999; Hershey and Merrick 2000). eIF4F is comprised of three subunits (eIF4E, eIF4A, and eIF4G) and binds to the cap structure (m⁷GpppN, where N is any nucleotide), which is present at the 5' end of all cellular mRNAs, via the cap-binding protein subunit, eIF4E. The eIF4A subunit is an RNA-dependent ATPase that cycles through the eIF4F complex

(Yoder-Hill et al. 1993; Pause et al. 1994). eIF4A, in cooperation with eIF4B, exhibits duplex RNA helicase activity (Rozen et al. 1990). Therefore, the apparent role of eIF4A is to facilitate the “melting” of secondary structure present in the 5' untranslated region of mRNAs that impedes translation initiation (Sonenberg 1996). There are three known eIF4A gene products—eIF4AI through eIF4AIII (Nielsen and Trachsel 1988; Li et al. 1999)—we focus on eIF4AI and refer to it as eIF4A throughout this text.

eIF4A is a member of the DEAD-box RNA helicase protein family. DEAD box (and related DEXH/D box; where X can be any amino acid) proteins contain eight highly conserved amino acid sequence motifs and have been implicated in a variety of biological processes involving RNA unwinding and/or rearrangement. These include transcription, ribosome biogenesis, pre-mRNA editing and splicing, RNA export to the cytoplasm, translation initiation and termination, RNA degradation, organelle gene expression, and virus propagation (Venema and Tollervey 1995; Py et al. 1996; Staley and Guthrie 1998). Motif I (AX₄GKT, where X can be any amino acid) is responsible for ATP binding;

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motif II (DEAD) is involved in ATP hydrolysis and couples ATP hydrolysis to helicase activity; motif III (SAT) is important for RNA helicase activity; and motif VI (HRI GRXXR) is required for RNA binding in a manner that is dependent on ATP hydrolysis (Pause and Sonenberg 1992, 1993; Pause et al. 1993; Lüking et al. 1998). It is assumed that the other motifs of eIF4A—Ia (PTRELA), Ib (TPGR), IV (FXXT), and V (RGXD)—are involved in RNA binding (Svitkin et al. 2001; Tanner and Linder 2001).

Crystal structures of superfamily I (SF1; Gorbalenya and Koonin 1993) RNA helicases—that is, the hepatitis C virus (HCV) NS3 protein (Yao et al. 1997; Cho et al. 1998; Kim et al. 1998), the DEAD box protein from the archaeobacterium *Methanococcus jannaschii* (Story et al. 2001), and fragments of eIF4A from yeast (Benz et al. 1999; Johnson and McKay 1999)—have been solved. The core structures of the SF1 and SF2 helicase proteins are largely superimposed (Korolev et al. 1997, 1998; Bird et al. 1998; Lin and Kim 1999). Caruthers et al. (2000) have completed the structure of full-length eIF4A; unlike other known helicases, it is a dumbbell structure consisting of two compact domains connected by an extended, 11-residue (~18 Å long) linker (see Fig. 7, below). Therefore, in solution, eIF4A is a distended molecule with a relative flexible linker between these two domains. They further modeled the compact structure for eIF4A by using the structures of other helicases as a template (Caruthers et al. 2000). This suggests juxtaposition of side chains of several amino acids residues of motifs V and VI required for coupling the interactions with ATP or ADP to conformational changes in the protein. A kinetic and thermodynamic analysis of the RNA-activated ATPase activity of eIF4A has shown that the binding of RNA and ATP to eIF4A is coupled (Rogers et al. 2001). This, along with changes in proteolytic digestion patterns (Lorsch and Herschlag 1998a,b), suggest that eIF4A undergoes a series of ligand-dependent conformational changes as it binds its substrates (RNA and ATP), hydrolyzes ATP, and releases products. The molecular basis of the conformational changes in eIF4A, however, is still lacking.

eIF4A ATPase activity is stimulated by single-stranded RNA (ssRNA); however, the binding of eIF4A to ssRNA is weak [$K_d \sim 100 \mu\text{M}$ (Abramson et al. 1987, 1988; Lorsch et al. 1998a)]. It is also clear that there is no defined RNA-binding module in eIF4A, in contrast to most other RNA-binding proteins (Mattaji 1993). Moreover, none of the substrate–protein interactions observed in the crystal structure of NS3 involve the recognition of specific nucleotide bases (Kim et al. 1998). Therefore, this might be a general feature of proteins involved in unwinding double-stranded nucleic acids. *A priori*, any sequence specificity would preclude the free movement of the helicase along the strand.

To characterize the RNA-binding property of eIF4A and its role in translation initiation, we selected for RNA aptamers with high affinity for eIF4A by in vitro RNA selection–amplification. Using these RNAs, we investigated the se-

quence and structural (or conformational) requirements in eIF4A and RNA aptamers for their high affinity interactions, and a possible constraint of the RNA on the activity of eIF4A and translation initiation. A causal relationship between aberrant expression of initiation factors and malignant transformation has been documented (Clemens and Bommer 1999; Dua et al. 2001). Consequently, if any of the RNA ligands were to be inhibitory to eIF4A-dependent initiation step, it could serve as a tumor-suppressing reagent.

RESULTS

High-affinity RNAs for eIF4A adapt a specific secondary structure

Four RNA selection experiments were performed using full-length eIF4A and RNA pools of either 30 or 40 random nucleotide positions (referred to as N30 or N40 RNA pool). In selections I(+) and I(–), RNA selection was carried out in the presence and absence of ATP, respectively, using a N40 pool of 5×10^{14} different RNA molecules. RNA molecules bound to eIF4A (His-tagged) were captured by affinity precipitation with Ni-NTA agarose beads. In selection II, the same procedures were used in the absence of ATP except that RNA–eIF4A complexes were trapped on nitrocellulose membranes in most selection steps. In selection III, RNA selection was carried out in the absence of ATP using a N30 pool; bound RNAs were trapped on nitrocellulose membranes. For each selection, RNAs with affinity to Ni-NTA agarose or nitrocellulose membrane were removed from an RNA pool by repeated washing in a preselection step.

From each selection, 48 RNA sequences were cloned. Sequences of several of the selected RNAs are shown in Figure 1A. It was surprising that the appearance of high-affinity RNA molecules was not accelerated in the presence of ATP in selection I(+) compared with selection I(–) (data not shown). Moreover, high-affinity RNA sequences obtained through selections I(+) and I(–) are very similar (Fig. 1A; therefore, the subsequent selections did not include ATP). These are classified into two groups, groups I and II (see Fig. 1A). Group I sequences have two conserved motifs, G/CU/AUUAG (element I) and C/GCUCCC (element II), whereas group II sequences have only element II. In selection II, clone no. 20 was most abundant (28/48), and nine other clones (nos. 21–29) are also identical to no. 20 except for one or two bases (Fig. 1A). Selection III enriched one class of sequences (16/48) that carry single base changes (Fig. 1A). Interestingly, selection-II and -III RNAs conserve the common sequence GAC/UCGCGC (element III).

The in vitro-selected RNAs were folded using the MFOLD program (Zuker 1989) to yield shared, as well as distinct, RNA secondary structures (Fig. 1B). RNA clones no. 1 and no. 11 represent the group I and group II RNAs,

FIGURE 1. Selected RNA sequences with affinity for eIF4A. (A) Representative RNA sequences selected from randomized RNA libraries using different selection procedures. In selection I(+) and I(-), RNAs were selected from N40 pool in the presence and absence of ATP, respectively, via affinity precipitation with Ni-NTA agarose. Selection II and III used N40 and N30 pools, respectively, and RNAs were selected in the absence of ATP via nitrocellulose membrane trapping. The frequency of each sequence in these selections is shown as numbers of each clone found in 48 independent isolates. Boxed sequences indicate the consensus elements I, II, and III. According to consensus elements, RNAs in selection I are classified into two groups, group I and II. (B) Predicted secondary structures of certain RNA ligands selected by eIF4A. Selected sequences are shown in capital letters. Lowercase letters indicate the constant sequence regions flanking the random sequence. Conserved elements I, II, and III are shaded.

respectively, and contain stem-loop structures. The conserved primary sequences, element I and element II, lie in defined stem regions to base-pair with a constant primer sequence. RNA clones no. 20 and no. 30 represent selection II and selection III RNAs, respectively, and contain stem-loop structures comprised of the conserved primary sequence GAC/UCGCGC (element III).

RNA aptamers bind eIF4A in the absence of ATP

To confirm that the in vitro-selected RNAs contain high-affinity binding sites for eIF4A, the apparent dissociation

constant of recombinant eIF4A for some selected RNAs was assessed by a nitrocellulose retention assay in the absence of ATP (Fig. 2A). The dissociation constants (K_d) were at ~ 3 μ M, ~ 8 μ M and ~ 1 μ M for RNA no. 1 (group I), no. 11 (group II), and no. 30 (selection III), respectively. For comparison, the K_d for an N40 RNA pool is ~ 80 μ M; this is consistent with the reported K_d value for ssRNA at ~ 100 μ M (Abramson et al. 1987, 1988; Lorsch and Herschlag 1998a). The dissociation constants for RNA no. 20 and no. 21 were estimated at 44 nM and 27 nM, respectively (Fig. 2A). Addition of ATP only slightly stimulated eIF4A binding to RNA no. 20, whereas addition of ADP or ATP γ S, the non-

FIGURE 2. RNA-binding specificity of wild-type and mutant eIF4A proteins. Shown mostly is the percentage of input RNA that bound to the nitrocellulose filter with various concentrations of the selected RNAs. Experiments were performed independently at least three times and the values are expressed with or without standard deviations. (A) Nitrocellulose filter-binding assays of selected RNAs for wild-type eIF4A. (X) N40 random pool; (■) no. 1; (●) no. 11; (▲) no. 20; (▼) no. 21; (◆) no. 30. (B) Binding affinity of RNA no. 20 in the absence (●) or presence of ATP (■), ADP (▲) and ATPγS (▼). (C) Sensorgrams of RNA no. 21 binding to eIF4A. Each RNA sample with the indicated concentration was injected to the flow cells immobilized with wild-type eIF4A as well as to the control cell. Experimental details are described in Materials and Methods. (D) Nitrocellulose filter-binding assays of RNA no. 20 for eIF4A fragments. eIF4A samples: (X) wild-type; (■) amino-terminal fragment N1–235; (●) carboxy-terminal fragment C216–406; (▲) N1–235 + C216–406. (E) Nitrocellulose filter-binding assays of RNA no. 20 for eIF4A mutants. eIF4A proteins: (X) wild-type; (■) R362Q; (●) DQAD; and (▲) PRRVAA. (F) Pull-down assay of eIF4A and eIF4G613–1560 binary complex. Ni-NTA agarose resin conjugated with eIF4A (0.4 μM) was mixed with Flag-tagged eIF4G613–1560 (0.4 μM) in the presence of N40 and selected RNAs, and formed complexes were spun down. Bound eIF4G613–1560 and eIF4A were detected by Western blotting using anti-Flag antibody (upper panel) and by Coomassie staining (lower panel), respectively. Experimental details are described in Materials and Methods. Proteins: (lane 1) eIF4A alone; (lane 2) eIF4G613–1560 alone; (lanes 3–9) eIF4A plus eIF4G613–1560. RNAs: (lanes 1–3) none; (lane 4) N40 (2 μM); (lane 5) N40 (10 μM); (lane 6) no. 1 (2 μM); (lane 7) no. 1 (10 μM); (lane 8) no. 20 (2 μM); (lane 9) no. 20 (10 μM).

hydrolyzable analog of ATP, did not stimulate the binding (Fig. 2B). Binding of eIF4A to other RNA sequences examined was not changed significantly by the addition of ATP (data not shown). These aptamers did not bind to other (control) proteins tested, such as bovine serum albumin and eIF4G613–1560 (data not shown).

The formation of RNA no. 21 complexes on the surface-coupled eIF4A was also monitored in the absence of ATP in real time with a BIACORE 2000 instrument based on the surface plasmon resonance (SPR) technique. eIF4A was covalently linked to the surface of the carboxy methyl (CM) dextran sensor chip via its primary amines (see Materials and Methods), and the formation of RNA complexes on this matrix was monitored as SPR signals. SPR signals caused by interaction of the selected RNA with eIF4A were observed in the absence of ATP (Fig. 2C). No positive signal was observed in a blank flow cell (data not shown). The K_d was estimated from this SPR profile is 58.8 nM, which is slightly higher than, but not inconsistent with that estimated by a nitrocellulose retention assay. These results indicate that the protein's affinity to selected RNAs is ATP-independent. Also confirming to this notion is the fact that selected RNA sequences did not depend on the presence of ATP.

Cooperative interactions between RNA aptamers and eIF4A domains

eIF4A may bind the RNA aptamers either through part of its potential RNA-binding region(s) that is split in the dumbbell structure, or its RNA-binding site formed in the compact structure. To distinguish between these two possibilities, the eIF4A sequence was cleaved at the linker site, and amino- and carboxy-terminal domains (N1–235 and C216–406, respectively) were expressed and purified. Their affinity to RNA no. 20 was assessed by the nitrocellulose retention assay. As shown in Figure 2D, neither of the domains showed high affinity for the RNA aptamer; mixing of both domains had only a slight effect, if any.

These findings indicate that the cooperative interactions between the selected RNA and both domains of eIF4A are important for the tight binding. One simple explanation might be that the selected RNA is bound to eIF4A through its compact form. Alternatively, the selected RNA interacts cooperatively with both domains of eIF4A in its dumbbell form, leading to an “induced fit” tight complex.

The compact structural model of yeast eIF4A suggests that a putative RNA-binding region is formed with an extended network of arginines including Arg-98 of motif Ia (according to the yeast numbering) (Caruthers et al. 2000). On this basis, three variant eIF4A proteins were investigated for their affinity: PRRVAA (motif Ia variant; see Fig. 7, below, colored in yellow), DQAD (motif II variant; see Fig. 7, below, colored in red), and R362Q (motif VI variant; see Fig. 7, below, colored in cyan). As shown in Figure 2E, the PRRVAA mutation stimulates the binding of eIF4A to RNA no. 20, whereas the other two mutations do not. Of these variant sites, the structural model predicts that motif Ia (PTRELA, in which R corresponds to the yeast Arg-98) is located in the RNA-contact region, whereas motif II (DEAD) and motif VI (HRIGRGGR) are not (Caruthers et al. 2000; Tanner and Linder 2001). This finding, along with the structural model, suggests that PRRVAA strengthens the extended network of arginines to increase its affinity for RNA. This, in turn, suggests that the selected RNA may be bound to eIF4A through the authentic substrate-binding site formed in the compact structure of eIF4A, although this possibility remains to be investigated with other mutations in the predicted RNA-binding site.

RNA aptamer does not interfere with eIF4A–eIF4G interaction

It is known that eIF4A binds eIF4G and forms a binary complex. We first examined whether the RNA aptamers are inhibitory to this interaction. eIF4A-conjugated resin was mixed with FLAG-tagged eIF4G613–1560 in the presence of increasing amounts of N40 (control) and selected RNAs. The resulting complexes were spun down, and eIF4G (bound to eIF4A) and eIF4A (bound to Ni-NTA agarose) were detected by Western blotting and Coomassie staining, respectively. Figure 2F shows part of these spin-down experiments. None of the selected RNAs examined, including those of moderate or high affinity for eIF4A, interfered with eIF4A's binding to eIF4G.

RNA aptamers inhibit ATPase activity of eIF4A

On the basis of the above results, it was important to examine the ATPase activity of eIF4A in the presence of RNA aptamers. First, we performed *in vitro* ATPase assays in the absence of poly(A) RNA, which is routinely used to activate the ATPase (Pause and Sonenberg 1992). Pi release was

measured with increasing amounts of selected RNAs. Under these conditions, no Pi release was detected (data not shown). This suggests that high-affinity RNA ligands are inhibitory to, or fail to activate, the ATPase, or both. Next, we performed the ATPase assay in the presence of saturating amounts of poly(A). As shown in Figure 3A, RNAs no. 20 and no. 21 severely inhibit the ATPase activity; the equimolar addition of either RNA relative to eIF4A inhibited 90% of hydrolysis. N40 RNA pool (control) does not affect the reaction. RNA no. 1 inhibits the ATPase, but 10 times less efficiently than RNAs no. 20 and no. 21. Nevertheless, this inhibition is still significant considering that the affinity for eIF4A is 100 times less compared with no. 21 ($K_d \sim 3 \mu\text{M}$ vs. 27 nM). Thus, most, if not all, of the RNA sequences selected in four different selection procedures inhibit, to some extent, the ATPase activity irrespective of their wide spectrum of K_d s among them for RNA–eIF4A interaction.

A series of point mutations and deletion variants of RNA no. 20 was constructed and characterized in this work (see below). Of these variants, M1 through M7 contain single- (or double in one case) base changes and only moderately weaken the affinity for eIF4A (shown below in Fig. 5A,C). Whereas M1, M4, M5, and M6 are still inhibitory to the ATPase, single-base changes in M2, M3, and M7 fail to inhibit (Fig. 3B). This suggests that the high affinity of selected RNAs is necessary but not sufficient to inhibit the ATPase activity. These high-affinity aptamers did not inhibit the ATPase activity of PriA, which is a DEXH-type DNA helicase from *Escherichia coli* (H. Masai, A. Oguro, and Y. Nakamura, unpubl.).

Nucleotide sequence and structure requirements for specific RNA binding

To identify the minimum sequence sufficient for high-affinity binding to eIF4A, several deletion variants were made from RNA no. 20. Deletion of the 3'-most six and 5'-most 23 bases (referred to as $\Delta 6\Delta 23$) did not diminish the affinity (Fig. 4B). The $\Delta 6\Delta 23$ is predicted to contain a 5' small stem-loop and a 3' extended stem-loop (Fig. 4A). When the $\Delta 6\Delta 23$ was cleaved at the three-base linker region (referred to as “bridge”) connecting the two stem-loop domains, each domain alone (5'R14 and 3'R41), nevertheless, does not bind eIF4A efficiently (Fig. 4B). Moreover, when the 3'-most nine bases are removed from RNA no. 20, the resulting variant (referred to as $\Delta 9$) is severely impaired in its binding potential (Fig. 4B). The affinity disappeared on deletion of the internal-loop 2 (ACAGGAG) or the bridge (ACA) sequence (see Fig. 4A for the positions; data not shown). The ongoing proton NMR spectroscopy analysis shows that each $\Delta 6\Delta 23$, 5'R14 and 3'R41 oligomers form highly stable stem-loop structures, which are consistent with the predicted secondary structure (T. Sakamoto, G. Kawai, A. Oguro, T. Ohtsu, and Y. Nakamura, unpubl.).

FIGURE 3. Inhibition of eIF4A ATPase by RNA aptamers. Pi release was measured with increasing amounts of selected RNAs in the presence of saturated amounts of poly(A). Shown is the percentage of control inorganic phosphate (Pi) release in the absence of RNA. (A) Inhibition by RNA aptamers. (X) 40N pool; (■) no. 1; (●) no. 20; (▲) no. 21. (B) Inhibition by no. 20 variant RNAs: M1 (□), M2 (○), M3 (△), M4 (●), M5 (■), M6 (▲), and M7 (◆). Base substitutions in these variants are shown in Figure 5.

These findings suggest that the nearly entire region of $\Delta 6\Delta 23$ is essential for the high affinity for eIF4A.

Apparent secondary structure of $\Delta 6\Delta 23$ was determined by probing the susceptibility of individual phosphodiester bonds with ribonucleases. RNase T1 and RNase A selectively hydrolyze single-stranded RNA at 3' of G and U/C residues, respectively. The 3'-end ³²P-labeled $\Delta 6\Delta 23$ was partially digested by RNase T1 and RNase A, and RNA digests were separated on a denaturing gel (Fig. 4C). Unstructured regions of the MFOLD prediction were hydrolyzed efficiently except for two regions (summarized in Fig. 4A); that is, the 5' loop composed of the conserved sequence ACCGCG, that is, element III (referred to as loop 1), and the 3' internal loop sequence GAGGC (referred to as internal-loop 1). These results with ongoing NMR analysis generally support the predicted secondary structure of RNA no. 20, and further suggest that loop 1 and internal-loop 1 regions may interact and participate in the tertiary structure (see Fig. 4A, broken lines).

To identify residues critical for eIF4A binding and to possibly improve the RNA affinity for eIF4A, several variants of RNA no. 20 were generated by mutagenic PCR. Over 100 variants were isolated and characterized. Of those, 15 variants are presented in Figure 5 as they are affected in their binding capacity. These are classified into two groups, either moderately affected (Fig. 5A,C), or severely impaired (Fig. 5B,D). Variants in the conserved sequence in loop 1 (M8, M9, and M11) fall into the latter severe group as expected. G→A changes at different positions in loop 1 (M3 and M4), however, fall into the former moderate group. These probably reflect the distinct functional and/or structural significance of each residue in this conserved loop 1. Most variants in the stem regions (M10, M12, M13, M14, and M15, except for M1, M2, and M7) also diminished the affinity. Moreover, the affinity disappeared

on deletion of the internal-loop 2 (ACAGGAG) or the bridge (ACA) sequence (see Fig. 5D for the positions; data not shown). These findings are interpreted as indicating that the whole sequence and structure of the selected RNA core region, $\Delta 6\Delta 23$, are crucial for the high affinity for eIF4A. (We assume that M1, M2, and M7 cause a moderate structural change as they affect the stem at residues proximal to single-strand regions.)

RNA aptamers inhibit cap-dependent translation in vitro

Next, we wished to determine the effect of RNA aptamers on in vitro translation. A bicistronic mRNA for which translation of the 5'-proximal chloramphenicol acetyltransferase (CAT) open reading frame (ORF) is cap-dependent, whereas translation of the second luciferase (LUC) ORF is cap-independent as it is directed by the HCV internal ribosome entry site (IRES), was used (Fig. 6A). HCV IRES-directed translation initiation is independent of eIF4A, eIF4B, eIF4F, and eIF4H (Pestova et al. 1998). When translated in a rabbit reticulocyte lysate (RRL) in the presence of increasing amounts of N40 (control), efficient translation of both CAT and LUC was observed (Fig. 6B). In contrast, the addition of RNA no. 20 and no. 21 inhibited preferentially cap-dependent CAT translation versus cap-independent LUC translation in a dose-dependent manner (Fig. 6D,E). This may be well correlated to the previous observation that a dominant-negative eIF4A variant PRRVAA inhibits cap-dependent CAT translation but not cap-independent LUC translation in vitro (Svitkin et al. 2001). It is, however, noteworthy that the LUC translation is slightly but significantly reduced by the RNA aptamer, but not by the dominant-negative eIF4A, by unknown reason. Under the same condition (at least up to 2 μM of RNA where 0.8 μM of RNA no. 20 or no. 21 is inhibitory), addition of RNA no. 1 was not inhibitory to CAT translation (Fig. 6C).

It is noteworthy that 0.8–1.2 μM amount of RNAs no. 20 and no. 21 is sufficient to inhibit CAT translation in the presence of 3.8 μM endogenous eIF4A in RRL (Pause et al. 1994). This inhibition is three to four times more efficient than that of the ATPase of eIF4A, which requires at least equimolar amounts of the RNA to achieve 90% inhibition (see Fig. 3A). Addition of wild-type eIF4A reversed the RNA-directed inhibition (Fig. 6F). However, 1.6 μM exogenous eIF4A—equimolar to RNA no. 21—only partially restored the inhibition; relief from the inhibition needs several molar-excess exogenous eIF4A. These findings suggest that the RNA aptamer inhibits the

FIGURE 4. Structural probing of selected RNA and requirement for affinity for eIF4A. (A) Secondary structure of $\Delta 6\Delta 23$ RNA examined by ribonuclease sensitivity assays. Closed and open arrows indicate major cleavage points with RNase T1 and RNase A, respectively. Arrowheads indicate minor cleavage points. Nucleotide position of $\Delta 9$ deletion is indicated. Broken lines between loop 1 and internal-loop 1 indicate putative base-pairings. (B) Nitrocellulose filter-binding assays of no. 20 deletions for eIF4A. Experimental procedures are the same as in Figure 2. RNA variants: (■) no. 20; (●) $\Delta 6\Delta 26$; (▲) $\Delta 9$; (□) 5'R14; and (○) 3'R41. (C) Autoradiogram of RNase probing pattern for $\Delta 6\Delta 23$ RNA. The 3'-end labeled RNA was partially digested with RNase T1 or RNase A, and subjected to 8% PAGE in the presence of 7 M urea. Alkaline digest ladders of $\Delta 6\Delta 23$ RNA are shown in left- and right-hand lanes (OH). Nucleotide positions are indicated.

CAT-dependent translation not only by blocking the ATPase of eIF4A in a stoichiometric fashion but also by preventing the function of eIF4F complex in a catalytic fashion.

DISCUSSION

In this study, high-affinity RNA aptamers for eIF4A were generated by in vitro RNA selection. These RNAs possess

FIGURE 5. Base changes in the selected RNA that affect affinity for eIF4A. Base substitutions of RNA no. 20 are generated by mutagenic PCR. Experimental procedures are the same as in Figure 2. (A) Filter-binding profiles of moderately affected variants. (B) Filter-binding profiles of severely affected variants. (C) Base changes of moderately affected variants. (D) Base changes of severely affected variants. M8 and M15 are double mutants, whereas M10 is triple mutant.

different binding affinities for eIF4A, with dissociation constants ranging from 10 nM to 10 μ M. Of these, selection II RNAs were the most prominent winners; the K_d for RNA no. 20 and no. 21 is at 44 and 27 nM, respectively, a value 10^4 -fold less than that for N40 (see Fig. 2A). Mutational studies with the selected RNAs, no. 20, indicate that the affinity sequence can be trimmed to 58 bases ($\Delta 6\Delta 23$), for which (nearly) overall structure and sequence seem to be required for the high-affinity binding. This notion is consistent with the structural probing data with RNase T1 and RNase A except for the lack of sensitivity in loop 1 and internal-loop 1 as well as the minor cleavages in stem 2 and stem 3 (see Fig. 4A). These unexpected sensitivity or tolerance could be attributed, at least in part, to the tendency of this aptamer to form dimers (T. Sakamoto, G. Kawai, A. Oguro, T. Ohtsu, and Y. Nakamura, unpubl.) as well as to potential interactions between loop 1 and internal-loop 1 in the tertiary structure. The ongoing NMR analysis shows that the predicted loop 1 and loop 2 regions form stable stem-loop structures (T. Sakamoto, G. Kawai, A. Oguro, T. Ohtsu, and Y. Nakamura, in prep.). Throughout this study, we noticed that the apparent maximum levels of binding between eIF4A and aptamers are <50% and somewhat variable from experiment to experiment. This is probably because when the RNA concentration is high, part of the aptamer tends to form dimers, which are inactive to bind to

eIF4A (T. Sakamoto, G. Kawai, A. Oguro, T. Ohtsu, and Y. Nakamura, unpubl.).

Although it is thought that when ATP binds eIF4A, the protein undergoes a conformational change that results in a compact structure, the molecular or thermodynamic basis of this transition is not known. It is noteworthy that RNA aptamers do not bind separately to each domain in eIF4A when split at the linker site, but do bind efficiently to the full-length eIF4A in the absence of ATP. Consistent with this, RNA sequences selected with or without ATP were essentially the same. These findings could be explained by assuming that the conformation of eIF4A is in the equilibrium state between dumbbell and compact structures in solution, and that the selected RNA may recognize the compact form and shifts the equilibrium toward the latter (Fig. 7). An intriguing idea is that ATP functions in a similar mechanism; its primary role may not be to induce a protein's conformational change but to stabilize the compact form and shift the equilibrium. The extended, flexible linker should be the structural basis for these rapid conformational changes. Alternatively, the RNA aptamer interacts cooperatively with amino- and carboxy-terminal domains of eIF4A in its dumbbell form, and triggers a conformational change to the compact form. This possibility cannot be excluded at present as the selected RNA $\Delta 6\Delta 23$ spans 45–70 Å depending on its conformation (A. Oguro, T.

FIGURE 6. Inhibition of cap-dependent translation by RNA aptamers. (A) Schematic diagram of capped CAT/HCV/LUC mRNA. (B–E) Translation products of capped CAT/HCV/LUC mRNA in RRL. Reaction mixtures were pre-incubated at 30°C for 3 min with increasing amounts (0, 0.4, 0.8, 1.2, 1.6, and 2.0 μ M in lanes 1–6, respectively) of N40 (B), no. 1 (C), no. 20 (D) and no. 21 (E) RNAs, followed by the addition of mRNA and [35 S]methionine and further incubation for 60 min at 30°C. Products were analyzed by SDS-PAGE (15%) and fluorography. [35 S]methionine incorporated into CAT and LUC are quantified using BAS-2000 Phosphorimager (Fuji Co.), and their relative values (CAT/LUC) are shown. The CAT/LUC ratio obtained in the absence of RNAs (lane 1, buffer control) was set as 100%. (F) Suppression of the inhibitory RNA action by exogenous eIF4A. Reaction mixtures were pre-incubated at 30°C for 3 min with increasing amounts of eIF4A (0, 0.4, 0.8, 1.6, 3.2, and 6.4 μ M in lanes 1–6 or 7–12, respectively) in the presence (lanes 7–12) and absence (lanes 1–6) of RNA no. 21 (1.6 μ M); followed by the addition of mRNA and [35 S]methionine and further incubation for 60 min at 30°C.

Ohtsu, and Y. Nakamura, unpubl.), which might be sufficient to interact with the distended dumbbell domains (<80 Å).

The most intriguing finding in this study is that almost all RNAs selected for their affinity for eIF4A—150 independent isolates—are inhibitory for ATPase activity. The degree of inhibition is generally high irrespective of the binding affinity (K_d), which varies by two or three orders of magnitude (see Fig. 2A). Of these, RNAs no. 20 and no. 21 were found to inhibit preferentially cap-dependent CAT translation relative to HCV IRES-directed LUC translation *in vitro*. (The slight but significant inhibition of HCV IRES-directed LUC translation by the RNA aptamer, unlike by the dominant-negative eIF4A variant, could be explained by assuming that another target protein of the RNA aptamer might be involved in the HCV IRES-directed LUC translation although other explanations might be possible.) This suggests that the unwinding of double-stranded RNA is hindered, as the helicase activity of eIF4A is ATP-dependent. We believe that the inhibition is not caused by a general inactivation of eIF4A but by the specific interaction between the protein and the RNA. This view is supported by the finding that selected RNAs do not interfere with the

interaction between eIF4A and eIF4G (see Fig. 2F). It is assumed that the inter-domain movement between amino- and carboxy-terminal domains of eIF4A is necessary for, or coupled with, ATP hydrolysis and the helicase action (Lorsch and Herschlag 1998b; Caruthers 2000). Therefore, it is likely that high-affinity RNA ligands possess the general potential to inhibit the ATPase activity presumably by hindering the conformational change. RNA aptamers recognize and bind to the full-length eIF4A but do not bind to each domain alone. Therefore, we suggest that RNA aptamers “staple” between two domains of eIF4A, leading to the inhibition of its inter-domain movement (Fig. 7). This is consistent with the prediction that any sequence specificity might hinder the movement of the helicase along the strand (Tanner and Linder 2001). It, nevertheless, cannot be excluded that some, if not all, of the selected RNAs sterically interfere with the access of ATP or block the catalytic amino acid residues as M2, M3, and M7 variants bind eIF4A but significantly reverse the inhibition (see Fig. 3B).

It is also likely that the inhibition of *in vitro* translation by RNA aptamers may not be solely accounted for by the ATPase inhibition as three to four times less amounts of

FIGURE 7. The schematic model for the mechanism of eIF4A inhibition by the RNA aptamer. We propose that eIF4A is in the equilibrium state between dumbbell and compact structures in solution, and that high-affinity RNA and ATP recognize the compact form and shift the equilibrium toward the latter. Whereas the ATP-bound state allows a flexible inter-domain movement between the amino- and carboxy-terminal domains necessary for the helicase action, the aptamer-bound state staples two domains and inhibits the conformational change necessary for ATP hydrolysis and the helicase action. Structures of full-length eIF4A are drawn in ribbon diagrams using the relevant coordinates of eIF4A (full-length, PDB ID code 1FUU; amino-terminal, ID code 1QDE; carboxy-terminal, ID code 1FUK) and RasMac Molecular Graphics. Motif Ia (PTRELA) is colored in yellow; motif II (DEAD), in red; and motif VI (HRIGRGGR), in cyan. In the *bottom* drawing, oligonucleotides and ATP are shown as space-filling model and ball-and-stick representation, respectively, according to the crystal structure of HCV NS3 protein complexed with oligo(dU) (PDB ID code 1A1V) and Caruthers et al. (2000).

RNAs are sufficient to inhibit translation in RRL compared with the ATPase inhibition. This means that the RNA not only hinders functionality of eIF4A alone in a stoichiometric fashion but also inhibits a subsequent step that involves eIF4A in a catalytic fashion. It is shown here that the selected RNAs do not interfere with eIF4A-eIF4G interaction (see Fig. 2F). Therefore, a possible explanation is to assume that the RNA is incorporated into eIF4F complexes comprising eIF4E, eIF4G, and eIF4A, and poisons eIF4F by preventing recycling of eIF4A as

shown for the dominant-negative eIF4A mutant (Pause et al. 1994).

eIF4B does not interact biochemically with eIF4A, but it stimulates the unwinding activity of eIF4A (Rozen et al. 1990; Pause and Sonenberg 1992), which is intrinsically very weak on its own (Rogers et al. 1999). The mechanism of this stimulation is not understood. eIF4A is the smallest among all DEAD helicase proteins (Tanner and Linder 2001). RNA helicases, however, often have very large amino- or carboxy-terminal extensions that can be longer than 500 amino acids. NS3 helicase has a carboxy-terminal extension, called domain 3. The crystallographic analysis reveals that in NS3, the RNA substrate appears to be bound in a cleft formed between domains 1–2 and domain 3 (Kim et al. 1998). Therefore, it is possible that eIF4B functions as an intermolecular domain 3 (Tanner and Linder 2001); this is consistent with the RNA-binding activity of eIF4B (Méthot et al. 1994). If this were the case, selected RNA with large secondary structures should interfere with the proper access of eIF4B to eIF4A or eIF4F complex. This remains to be tested but it is very likely that selected RNA can hinder multiple steps in translation initiation that involves eIF4A, that is, ATP hydrolysis, dsRNA unwinding, eIF4B entry, and eIF4A recycling. Taking these together, our findings demonstrate that the RNA aptamer could be prominent inhibitors of eIF4A, and thereby for cap- or eIF4F-dependent translation.

Recently, a causal relationship between aberrant expression of initiation factors and malignant transformation of mammalian cells has been reported. Human or rat cell lines can be transformed *in vitro* by overexpression of eIF4E (Lazaris-Karatzas et al. 1990) and eIF4G (Fukuchi-Shimogori et al. 1997) or by dephosphorylation of eIF2 α (Koromilas et al. 1992; Meurs et al. 1993; Barber et al. 1995; Donzé et al. 1995). The oncoprotein PI3K (phosphoinositide 3-kinase) and Akt (protein kinase B) induce oncogenic transformation of chicken embryo fibroblasts possibly via affecting translation; this involves the kinase mTOR and its target proteins; p70S6 kinase (S6K) and the eIF4E-binding proteins (4E-BPs) (Aoki et al. 2001). Also, increases in protein or RNA levels for initiation factors, such as eIF4E, eIF4G, and eIF4A, are observed in several carcinomas or tumor cell lines (Brass et al. 1997; Eberle et al. 1997; Benedetti and Harris 1999). Consequently, human initiation factors could be the targets for anticancer drugs or prophylactic agents. Selected RNAs with high affinity for these initiation factors might therefore serve in medical applications.

MATERIALS AND METHODS

Protein expression and purification

Amino-terminal (amino acid residues 1–235, referred to as N1–215) and carboxy-terminal (amino acid residues 216–406,

referred to as C216–406) fragments of mouse eIF4A were amplified by PCR using pairs of primers: N1–215, 5′-GGGGCATATGTCTGCGAGCCAGGATTC-3′ and 5′-GGCCGGATCCAGAATCCGAATGGGGT-3′; and C216–406, 5′-GGGGCATATGCCTTCTGATGTGCTTG-3′ and 5′-CGGGCGGATCCGCAACATTGAGGGG-3′. These DNAs were cloned into *NdeI*–*Bam*HI sites of expression plasmid pET15b (Novagen). Purification of wild-type, truncated or variant eIF4A proteins was performed as described previously (Pause & Sonenberg 1992) except that His-tagged proteins were purified using Ni-NTA agarose (Qiagen) and Resource Q (Amersham) column chromatography to >95% purity. GST–Flag–eIF4G (amino acid residues 613–1560, referred to as eIF4G613–1560; Morino et al. 2000) was purified on glutathione–Sepharose (Amersham). GST-tag was digested with PreScission protease (Amersham) overnight at 4°C. The remaining Flag–eIF4G613–1560 was purified using Resource Q column.

Selection-amplification

In vitro RNA selection was performed as described (Ellington and Szostak 1990; Tuerk and Gold 1990). Transcription templates were synthesized by PCR using synthetic oligonucleotides: 5′-GGGAGACAAGAATAAAACGCTCAA(40N)TTCGACAGGAGGCTCA CAACAGGC-3′ and 5′-GGGACACAACCGGACG(30N)TAACGGCCGACATGAGAG-3′, where 40N and 30N represent 40 and 30 random nucleotide positions, respectively. PCR primer sets used are: 40N template, 5′-TAATACGACTCACTATAGGGAGACAAGAATAAAACGCTCAA-3′ and 5′-GCCTGTTGTGAGCCTCC TGTCGAA-3′; 30N template, 5′-TAATACGACTCACTATAGG GACACAACGGACG-3′ and 5′-CTCTCATGTCGGCCGTTA-3′, where T7 promoter sequence is underlined. N30 and N40 RNA pools were prepared by in vitro transcription with T7 RNA polymerase (TaKaRa Co.). RNAs were refolded by heat denaturing (at 85°C) and slow cooling (to room temperature) in buffer A (20 mM Tris, pH 7.6, 80 mM potassium acetate, 2.5 mM magnesium acetate and 5% glycerol). Selections were performed in buffer A containing 100 Units RNase inhibitor (TaKaRa Co.) with [in selection I(+)] or without [in selections I(–), II, and III] 1 mM ATP. In selection I, eIF4A was mixed with 3 μL of Ni-NTA agarose (Qiagen; pre-equilibrated with buffer A) at room temperature for 30 min; after washing out unbound protein, N40 or each round RNA pool (50 μL) was added and incubated for 30 min at room temperature. RNA–protein complexes were isolated by centrifugation and washing, and eluted with buffer A containing 300 mM imidazole. RNAs were extracted with phenol/chloroform treatment and precipitated with ethanol. Then cDNAs were synthesized with RAV-2 reverse transcriptase (TaKaRa Co.), amplified by PCR using the N40 primer set, and followed by T7 transcription. Selections II and III employed essentially the same procedures except that N30 RNA pool was used in selection III and RNA–protein complexes were trapped on nitrocellulose membranes in both selections (50 μL). After washing out unbound RNA, RNA/protein-bound membranes were soaked and heated at 95°C for 5 min in buffer A containing 3.3 M urea/66.6% phenol, and the RNAs were extracted with 50% chloroform, followed by phenol/chloroform treatment and ethanol precipitation. Selection-amplification was repeated 12, 15, and 12 times for selections I, II, and III, respectively. As the selection cycle preceded, the stringency increased because of the decreased ratio of protein versus RNA. An initial molar ratio of 3 μM protein to 9 μM RNA (1:3) was raised to 0.15

μM protein to 3 μM RNA (1:20) in the final round. The last-round RNA in selection II was recovered by affinity with Ni-NTA agarose instead of membrane trapping. cDNAs for selected RNAs were cloned into plasmid pGEM-T Easy Vector (Promega) and sequenced.

Preparation of RNA aptamers for in vitro assays

Selected sequences were amplified by PCR using N40 and N30 primer sets. The PCR products were purified on a 12% polyacrylamide gel (PAGE) and used for in vitro transcription templates. Following transcription, DNA was removed with RNase-free DNase I (1 Unit/μg of template DNA). The RNA was extracted by phenol/chloroform treatment and recovered by ethanol precipitation in the presence of 0.3 M sodium acetate, pH 6.5. The RNA pellet was rinsed with 70% ethanol, dried, and dissolved in water and passed into spine column (Micro Bio-spine column P-30, BioRad). RNA transcripts used for filter-binding analysis were synthesized in the presence of [α -³²P]CTP (800 Ci/mmol) to a specific activity of 2×10^{17} cpm/mole RNA, and purified as mentioned above.

RNA structure analysis

RNA secondary structure was probed by RNase digestion as described (Kiga et al. 1998). The 3′ end of selected RNA (~0.3 μM) was labeled with T4 RNA ligase (TaKaRa Co.) in the presence of 0.3 μM of [α -³²P]pCp (3000 Ci/mmol) in 10 μL reaction buffer. Alkaline digestion was performed in 10 μL of 50 mM sodium carbonate (pH 9.0), 1 mM EDTA for 3 min at 90°C. The labeled RNA (~0.01 μM) was heated at 85°C, then slowly cooled to room temperature in buffer A, and incubated with increasing amounts of RNase T1 or RNase A for 3 min at room temperature. The resulting digests were subjected to an 8% PAGE in the presence of 7 M urea.

RNA aptamer variants

Point mutants of selected RNA were obtained by mutagenic PCR with 0.75 mM MnCl₂ against cDNA of RNA clone no. 20. PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. Deletion variants of clone no. 20 were manipulated by PCR using designed primers: Δ9, 5′-TAATACGACTCAC TATAGGGAGACAAGAATAAAACGCTCAA-3′ and 5′-GAGCC TCCTGTCTGAATCTA-3′; Δ6Δ23, 5′-TAATACGACTCACTATA GGAAGGGGACCGCGCCCC-3′ and 5′-TATGAGCCTCCT GTCG-3′. Truncated RNA segments of Δ6Δ23 were synthesized by the in vitro transcription using T7 primer (5′-GAATT TAATACGACTCACTATAG-3′) from oligonucleotide templates: that is, 5′-GGGGCGCGGTCCCCTATAGTGAGTCGTATTTAA TTC-3′ for the 5′-terminal 14-base RNA (referred to as 5′R14), and 5′-TGTGAGCCTCCTGTCTGAATGTATCTTTAGGAATCAC TCACCCTATAGTGAGTCGTATTTAAATTC-3′ for the 3′-terminal 41-base RNA (referred to as 3′R41).

Filter-binding analysis

Nitrocellulose retention assays were performed essentially as described (Méthot et al. 1994). Briefly, indicated amounts of proteins

were mixed with ~0.18 pmole of ^{32}P -labeled RNA substrate in 50 μL of buffer A containing 10 $\mu\text{g}/\text{mL}$ tRNA. The mixture was incubated for 30 min at 25°C and filtered through a pre-soaked nitrocellulose membrane (0.45 μm pore size; type HA; Millipore). The filter was washed with 1 mL of buffer A containing 10 $\mu\text{g}/\text{mL}$ tRNA, dried, and retained radioactivity was quantitated by scintillation counting. Each point is corrected for the amount of RNA retained in the absence of protein, which was typically <1% of the RNA input. The data sets were calculated using the software package Kaleida Graph (Synergy Software).

Assay for ATP hydrolysis

eIF4A protein (1 μM) was mixed with RNA aptamer (after refolding) in solution (10 μL) containing 20 mM Tris-HCl (pH 7.6), 80 mM potassium acetate, and 2.5 mM magnesium acetate. The mixture was pre-incubated at room temperature for 15 min, and after addition of 37.5 A_{260} units of poly(A) and 1.25 μCi of [γ - ^{32}P]ATP (6000 Ci/mmol), the reaction mixture was incubated at 37°C for 60 min. Released inorganic phosphate (^{32}Pi) was counted as described (Pause and Sonenberg 1992).

mRNA preparation and in vitro translation

The plasmid CAT/HCV/LUC (Svitkin et al. 2001) was linearized with *Apa*I, and transcribed by SP6 RNA polymerase. The RiboMAX system protocol for synthesis of capped RNA transcripts (Promega) was followed. In vitro translation in a rabbit reticulocyte lysate (RRL) (Promega) was performed as described (Svitkin et al. 2001) according to the manufacturer's instruction. Briefly, RRL was pre-incubated at 30°C for 3 min with or without selected RNA before being supplemented with mRNA (0.2 μg) and [^{35}S]methionine. Translation was performed at 30°C for 60 min. Samples were analyzed by SDS-PAGE followed by fluorography.

BIACORE experiments

The real-time measurement of the interaction between eIF4A and the selected RNA was performed using a BIACORE 2000 biosensor system (Biacore AB) at 25°C as described previously (Ishino et al. 2000) according to the manufacturer's instruction. Immobilization of eIF4A on a CM5 sensor chip (Biacore AB) was carried out according to the standard protocol of the amine coupling method (BIAApplication Handbook; Biacore AB). One uncoated flow cell was used to check for nonspecific binding of RNAs. For monitoring the interaction using the BIACORE instrument, all procedures were automated to create repetitive cycles of sample injection (30– μL injection samples, at a flow rate of 10 $\mu\text{L}/\text{min}$) and regeneration (10 μL regeneration buffer containing 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% tween20, and 2 M Urea, pH 7.4). Selected RNA no. 21 was diluted in various concentrations. Interaction was estimated by subtracting response units of the blank flow cell from response of the selected RNA for flow cell.

Pull-down assay

His-tagged eIF4A (0.4 μM) protein was incubated with Ni-NTA agarose (Qiagen; pre-equilibrated with buffer A) overnight at 4°C.

The mixture was centrifuged at 2,100g for 10 sec and the resin was washed three times with buffer A. The eIF4A-conjugated resin was dissolved in 25 μL of buffer A, and incubated with 2 or 10 μM of N40, no. 1 and no. 20 RNAs for 15 min at room temperature. Flag-tagged eIF4G613–1560 (0.4 μM) was then added and after 1 hr incubation at room temperature, the mixture was centrifuged at 2,100 g for 10 s. The resin was washed five times with 150 μL of buffer A containing 20 mM imidazole. Bound eIF4G613–1560 was eluted with 15 μL of buffer A containing 300 mM imidazole, subjected to SDS-PAGE (12%), and detected by standard Western blot techniques using anti-Flag antibody and secondary anti-mouse horseradish peroxidase-conjugated IgG (Amersham) according to the manufacturer's instructions. After washing with Tris-buffered saline-tween, the membranes were developed by means of enhanced chemiluminescence using ECL Western Blotting Detection System (Amersham) according to the manufacturer's instructions.

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