Structural analysis of 5'-mRNA-cap interactions with the human AGO2 MID domain

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In RNA silencing, microRNA (miRNA)-mediated translational repression occurs through mechanisms that do not invoke messenger-RNA (mRNA) target cleavage by Argonaute proteins. The nature of these mechanisms is unclear, but several recent studies have proposed that a direct interaction between the mRNA-cap and the middle (MID) domain of Argonautes is involved. Here, we present crystallographic and NMR data demonstrating that cap analogues do not bind significantly to the isolated MID domain of human Argonaute 2 (hAGO2) and are found in the miRNA 5'-nucleotide binding site in an implausible binding mode. Additionally, *in vitro* pull-down experiments with full-length hAGO2 indicate that the interaction with cap analogues is nonspecific.

Keywords: Argonaute; miRNA; MID domain; cap; translational repression

EMBO reports (2011) 12, 415–420. doi:10.1038/embor.2011.48

INTRODUCTION

MicroRNAs (miRNAs) are a class of genomically expressed small RNAs that post-transcriptionally regulate gene expression by recognizing partially complementary sequences, found mostly in the 3'-untranslated region of target messenger RNAs (mRNA; Bartel, 2009). miRNAs associate with Argonaute proteins (AGOs) to form the so-called miRNA-induced silencing complex (miRISC). The miRNA imparts sequence specificity to the miRISC and guides the complex to specific target mRNAs for post-transcriptional gene silencing. AGOs consist of amino-terminal, PAZ, MID (middle) and PIWI domains, in which the PAZ and MID domains interact

with the 3'- and 5'-ends of miRNAs, respectively (Hutvagner & Simard, 2008). In small RNAs that are complementary to the target, such as many short-interfering RNAs (siRNAs), the catalytic PIWI domain cleaves the target mRNA strand. However, most miRNAs show only partial complementarity towards the target, which disrupts cleavage activity, and in these situations mRNA repression occurs by other mechanisms.

Considerable efforts have been made to investigate the mechanisms by which miRISC represses protein synthesis of target mRNAs. It is well established that miRNAs cause mRNA degradation through deadenylation mediated by interactions with GW182 (Fabian *et al*, 2010). In fact, Guo *et al* (2010) recently used ribosome profiling and simultaneous measurements of mRNA levels to show that mRNA degradation accounts for most ($\geq 84\%$) of the miRNA-mediated decrease in protein levels. However, it seems that miRNAs can also hinder gene expression by inhibiting mRNA translation (Chekulaeva & Filipowicz, 2009; Fabian *et al*, 2010), although the mechanism of translational repression remains controversial, with different, often conflicting results found in the literature (Chekulaeva & Filipowicz, 2009; Fabian *et al*, 2010).

One model posits that the 5'-cap structure of the target mRNA interacts directly with AGO in miRISC, thereby competing with the cap-binding translation factor eIF4E and leading to translational repression at the initiation stage (Kiriakidou *et al*, 2007; Boland *et al*, 2010; Djuranovic *et al*, 2010). Kiriakidou *et al* (2007) suggested that two conserved residues (F470 and F505) in the human AGO2 (hAGO2) MID domain bind to the cap by stacking interactions analogous to those found in eIF4E and other cap-binding proteins. Mutating these residues prevented the cap interaction and inhibited translational repression. Subsequent studies have challenged these results by showing that F470 and F505 are buried in the hydrophobic core (Kinch & Grishin, 2009) and that mutating them also prevented AGO from interacting with both GW182 and miRNAs (Eulalio *et al*, 2008).

More recently, however, another study that supported the AGO-cap interaction hypothesis was published. Djuranovic *et al* (2010) found that binding of *Drosophila* AGO1 (dmAGO1) to m⁷ guanosine triphosphate (GTP)-Sephacrose resin was enhanced

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in the presence of miRNAs and that the interaction was localized to the MID domain. This led to a model of an allosterically regulated cap-binding site in AGOs, in which an initial interaction of miRNA with the 5'-phosphate binding site in the MID domain triggers a conformational change in AGO that makes available a second binding site specific for the cap (Djuranovic *et al*, 2010). In support of this model, the crystal structure of the MID domain from *Neurospora* Argonaute QDE-2 identified a sulphate ion occupying a binding site in close proximity to the 5'-nucleotide binding site (Boland *et al*, 2010).

Recently, we reported crystal structures of the hAGO2 MID domain in complex with nucleoside monophosphates (NMPs) that revealed the structural basis for the observed bias of uracil or adenine nucleotides at the 5'-end of eukaryotic miRNAs (Frank *et al*, 2010). Notably, the hAGO2 MID domain presented no evidence for a second binding site and no conformational changes were observed on nucleotide binding. However, it is possible that conformational changes were masked by the constraints of the crystal lattice or that the isolated MID domain was insufficient to engage the cap. This prompted us to analyse in more detail the interaction between the cap and the hAGO2 MID domain, by using biophysical methods and biochemical experiments with full-length hAGO2. We found that cap analogues do not significantly interact with either the isolated MID domain or full-length hAGO2, in the conditions used here.

RESULTS AND DISCUSSION

Biophysical analysis of hAGO2 MID domain–cap interaction

We had previously reported the affinities of NMPs to the MID domain and found that uridine monophosphate (UMP) and adenosine monophosphate (AMP) interact with an affinity of 125 and 250 μ M, respectively, whereas cytidine monophosphate (CMP) and guanosine monophosphate (GMP) bind more weakly, with approximately 3 mM affinity (Frank *et al*, 2010). Thus, we began by determining the dissociation constants of cap analogues with MID domain using ^1H – ^{15}N heteronuclear single-quantum coherence (HSQC) NMR titration experiments. Both dinucleotide cap analogues used in this study, m⁷GpppG and m⁷GpppA, bound to the MID domain with similar affinities of 1.57 and 1.37 mM, respectively (Fig 1A,B,D; supplementary Fig S1 online). Both affinities were lower than for the corresponding single nucleotides (m⁷GTP, GTP and ATP). This observation is in contrast to what is expected for a binding site specific for interaction with a capped mRNA. Furthermore, such weak affinities suggest that the interaction is not physiologically relevant, as comparable interactions of the cap with eIF4E and other *bona fide* cap-binding proteins are in the nM range (Cai *et al*, 1999; Worch *et al*, 2005). However, the affinities determined here for dinucleotide cap analogues are between those of weakly binding mononucleotides (GMP/CMP) and higher-affinity mononucleotides (UMP/AMP) at the miRNA 5'-end.

To determine the source of the stronger affinity for cap analogues over that of GMP, we solved the crystal structure of the complex between the hAGO2 MID domain and m⁷GpppG (see supplementary information online). The cap structure consists of a 7-methylguanosine linked to the first nucleotide of the mRNA by a 5'–5' triphosphate bridge (Fig 1C). The crystal structure showed that m⁷GpppG binds to the highly conserved, positively

charged 5'-nucleotide binding site that is responsible for interaction of AGOs with small RNAs (Fig 2A,B). Clear electron density was observed for the α -phosphate of the bound nucleotide, and only fragmented density was visible for one of the sugar moieties and base. The α -phosphate group interacts with the conserved phosphate-binding residues, as shown previously for NMPs (Frank *et al*, 2010). The β -phosphate was also partly visible and makes electrostatic interactions with Y529, K533 and K570. These additional phosphate contacts are probably responsible for the stronger affinities of cap analogues over those of GMP as the binding affinities of both ATP and GTP were also substantially increased relative to AMP and GMP, respectively (Fig 1D). The crystal structure of the MID domain in complex with ATP confirmed the additional interactions with the β -phosphate (supplementary Fig S2 online).

No electron density was observed for the second base of the cap. Due to the almost symmetrical nature of the m⁷GpppG molecule (Fig 1C) and lack of clear electron density for the base, its binding orientation could not be determined. Notably, no electron density that might indicate the presence of a second binding site was evident at other regions on the MID domain and no conformational changes relative to the nucleotide-free state were observed, consistent with our previously published structures of MID domain:NMP complexes (Frank *et al*, 2010).

The reduced affinity of m⁷GpppA and m⁷GpppG compared with the mononucleotides ATP, GTP and m⁷GTP (Fig 1D) is probably due to unfavourable interactions with the second base of the dinucleotide cap analogues (in miRNAs, the second nucleotide is linked by a 5'–3' linkage rather than 5'–5', see Fig 2C) on the surface surrounding the 5-nucleotide binding pocket. We expect these restrictions on cap binding to be accentuated in the context of full-length AGOs (Wang *et al*, 2008) due to the presence of additional domains adjacent to the 5'-nucleotide binding site. These domains are positioned in a manner that prevents an additional 5'–5'-linked nucleotide from occupying this site (supplementary Fig S3 online). This reasoning extends to the additional phosphates present in nucleotide triphosphates relative to NMPs, thus confining the miRNA 5'-binding site to interact only with its physiological ligands, namely monophosphorylated small RNAs. Indeed, chemical modification of the monophosphorylated 5'-end of miRNAs significantly reduces AGO cleavage activity (Nykänen *et al*, 2001; Martinez *et al*, 2002).

Next, we measured the affinity of m⁷GTP, to assess the effect of the methyl modification of the cap. Titration of m⁷GTP into the MID domain caused the same characteristic NMR peak shifts as observed with GTP (supplementary Fig S4 online), but slightly weaker affinity was observed for the interaction (Fig 1D). This confirms that m⁷GTP also binds to the 5'-nucleotide binding pocket in solution and suggests that a specific interaction between the cap and this site does not occur. In studies of *bona fide* cap-interacting proteins such as eIF4E or the cap-binding complex, the presence of the 7-methyl modification results in substantially increased affinity of cap analogues over guanosine (Cai *et al*, 1999; Worch *et al*, 2005).

To investigate the possible presence of a second miRNA-dependent allosteric binding site for the cap (supplementary Fig S5 online), we carried out ^1H – ^{15}N HSQC NMR experiments in which the MID domain was initially saturated with 5 mM UMP

Fig 1 | Binding affinity analysis of cap analogues with hAGO2 MID domain. (A) ^1H – ^{15}N HSQC NMR titration experiments with hAGO2 MID domain and m⁷GpppG. Significantly shifting peaks selected for analysis are marked with arrows. (B) Chemical shift differences were calculated and were plotted as a function of the molar ratio (nucleotide/protein). The data for multiple peaks were fit using the maximum shift and dissociation constant as adjustable parameters. (C) Chemical structure of the mRNA cap and miRNA 5'-nucleotide. (D) Dissociation constants (K_D) extracted from NMR titration experiments. Standard deviations from the fitting procedures of three different peaks are shown. GMP, guanosine monophosphate; GTP, guanosine triphosphate; hAGO2, human Argonaute 2; HSQC, heteronuclear single-quantum coherence; MID, middle; miRNA, micro RNA; mRNA, messenger RNA.

Fig 2 | Crystal structure of the hAGO2 MID domain in complex with cap analogue. (A) Charged surface representation. The nucleotide specificity loop is highlighted in yellow. (B) Difference electron density contoured at 2σ before inclusion of m⁷GpppG. Black sticks show the unmodelled portion of the cap. α -, β - and γ -phosphates are labelled in red. (C) Schematic view of potential cap binding compared with miRNAs. The surface of the hAGO2 MID domain is shown in grey. A small RNA 5'-nucleotide bound in the nucleotide-binding pocket is shown in cyan. The position of the second nucleotide of a bound small RNA is shown as white sticks, while the hypothetical position of a bound cap-like dinucleotide is shown as grey sticks. hAGO2, human Argonaute 2; MID, middle; miRNA, microRNA.

Fig 3 ^1H – ^{15}N HSQC NMR experiments assessing the presence of a second allosteric binding site. (A) MID domain alone (grey) and with 5 mM UMP (red). Significant peaks shifts due to UMP addition are marked with arrows. (B) MID with 5 mM UMP (red) overlaid with 3 mM $m^7\text{GpppG}$ (blue). HSQC, heteronuclear single-quantum coherence; MID, middle.

(miRNA mimic), followed by the addition of 3 mM $m^7\text{GpppG}$. The presence of a separate binding site specific for the cap would predict additional chemical shift changes after UMP saturation. This should be the case even in the presence of the high concentrations of UMP used here (which could in theory occupy the potential second binding site as well), as the addition of the cap should displace the UMP at the putative second site, which is ostensibly specific for the cap. We found that addition of $m^7\text{GpppG}$ to UMP-saturated MID domain did not cause any additional chemical shift changes in the NMR spectra (Fig 3). Furthermore, Hill coefficients determined from binding curves are close to 1, indicating no cooperativity of binding (supplementary Table S2 online).

In vitro pull-down experiments

The Djuranovic *et al* (2010) study showed that there is an increase in the binding of full-length dmAGO1 to $m^7\text{GTP}$ -Sephacrose in the presence of miRNA, leading to the postulation of a second allosteric binding site for the cap. Conversely, this effect was not seen for either GTP/GMP-agarose beads suggesting that this is a cap-specific interaction. It should be noted that $m^7\text{GTP}$ -Sephacrose beads (from GE Healthcare) differ in their chemical conjugation from GMP/GTP-agarose (from Sigma) in that $m^7\text{GTP}$ is linked to the matrix by the γ -phosphate, whereas both GMP and GTP are attached by either their 2'-OH or the 3'-OH of the ribose sugar. The effect of these different linkages on binding experiments is unclear, given the nature of the binding mode of ligands with the miRNA 5'-nucleotide binding sites, as described above. Additionally, differences between bead-linked nucleotides and free nucleotides used in other experiments could also contribute to the inconsistencies in the MID domain–cap interactions reported here.

Our biophysical analyses were restricted to the isolated hAGO2 MID domain because of the difficulties associated with studying full-length eukaryotic AGOs using structural techniques. In order to address this, and the inconsistencies with the Djuranovic *et al* (2010) study, we performed pull-down experiments of full-length hAGO2 as well as the isolated MID domain with $m^7\text{GTP}$ -conjugated beads, using concentrations and conditions similar to those in the Djuranovic *et al* (2010) study, where possible.

We performed pull-down experiments using 5 μg of hAGO2 MID domain as a hexahistidine/SUMO-fusion protein (approximately 0.75 μM in a 200 μl reaction) and 30 μl of $m^7\text{GTP}$ -Sephacrose beads (approximately 75 μM ligand concentration according to the manufacturer). Binding of MID domain to cap resin was nonspecific, as addition of free cap analogue did not abolish the interaction, which is observed in the case of the known cap-binding protein eIF4E (Fig 4A). Moreover, no increased binding was observed in the presence of GMP, and the presence of both GMP and $m^7\text{GpppG}$ did not alter the amount of recovered protein.

We proceeded to express and purify full-length hAGO2 (S34 to the carboxy-terminus, lacking the N-terminal proline-rich tract) from baculovirus-infected insect cells (supplementary Fig S6 online) for use in pull-down experiments. This protein was properly folded and active, as assessed by RNA cleavage experiments (supplementary Fig S6 online). We incubated approximately 1 μg (approximately 50 nM in a 200 μl reaction) of purified hAGO2 with 30 μl of $m^7\text{GTP}$ -Sephacrose beads in the presence or absence of $m^7\text{GpppG}$ and/or let-7 miRNA. We found that regardless of the combination of competitors included in the binding reactions, the same amount of hAGO2 was recovered (Fig 4B). Furthermore, recovery of hAGO2 from the resin was consistently less than 1% of the input, suggesting that this reflects background binding only. To confirm this result, we repeated the same set of experiments in the context of glutathione-conjugated Sephacrose beads (i.e., nonspecific binding control). We obtained a similar recovery of hAGO2 (Fig 4C), suggesting that the interaction of hAGO2 with $m^7\text{GTP}$ -Sephacrose beads is not specific. Notably, there was no increase of hAGO2 binding to $m^7\text{GTP}$ -Sephacrose beads in the presence of miRNA.

CONCLUSIONS

Here, we present crystallographic and NMR data using the hAGO2 MID domain together with pull-down experiments using full-length hAGO2. The observation that cap analogue binds to the known miRNA 5'-nucleotide binding site in the MID domain together with the low affinity of the interaction suggests that cap interaction with the hAGO2 MID domain is a consequence of its capacity to accommodate nucleotides. Additionally, comparison

Fig 4 | Full-length hAGO2 binds to m⁷GTP-Sepharose nonspecifically in *in vitro* pull-down experiments and at low levels, indicating background binding. (A) Pull-down experiments using recombinant GST-eIF4E and hexahistidine-SUMO-tagged hAGO2 MID domain with m⁷GTP-Sepharose. Western blots using eIF4E or His-tag antibodies are shown, respectively. (B) Recovery of hAGO2 during *in vitro* pull-down experiments with m⁷GTP-Sepharose is less than 1%. Binding is not affected by the presence of 10-fold excess of let-7 miRNA or by 500 μ M m⁷GpppG as a competitor. (C) Binding to glutathione-Sepharose as a negative control gives similar results to experiments with m⁷GTP-Sepharose, confirming that binding represents background levels. Full-length hAGO2 was detected on western blots using hAGO2 antibody. GMP, guanosine monophosphate; GST, glutathione-S-transferase; GTP, guanosine triphosphate; hAGO2, human Argonaute 2; MID, middle.

of the affinities of cap-like nucleotides (m⁷GpppG, m⁷GpppA and m⁷GTP) with non-cap-like nucleotides (ATP, GTP, AMP and GMP) shows that there is no specificity for a cap structure at this site. *In vitro* pull-down experiments suggest that the interaction of the mRNA cap structure with hAGO2 is not specific and reflects background binding to the Sepharose resin, rather than a specific interaction with m⁷GTP.

Our results are in disagreement with m⁷GTP-Sepharose pull-down experiments reported by Djuranovic *et al* (2010). Their study presented pull-down experiments with full-length and MID domain constructs from dmAGO1, which supported the notion of an allosterically regulated cap-binding site located in the MID domain, separate from the miRNA 5'-nucleotide binding site. One possibility for the observed discrepancies is that dmAGO1 and hAGO2 are inherently different in their mechanistic behaviours. However, analogous pull-down studies, previously performed by Eulalio *et al* (2008) and Iwasaki *et al* (2009) on dmAGO1, revealed that the interaction of dmAGO1 with cap-resin is non-specific, consistent with our findings here. Taken together, our results show that the isolated MID domain is insufficient to engage the cap and, moreover, that full-length AGO, under the conditions used here, cannot specifically bind to the cap. However, we do not discount the possibility that *in vivo*, there might be other cellular factors that could allow accommodation of capped mRNA by the miRISC.

METHODS

Chemical syntheses and protein preparation. Syntheses and purification of the cap analogues were performed as described previously (Darzynkiewicz *et al*, 1985).

The hAGO2 MID domain (residues 439–578 for crystallographic experiments and residues 432–578 for NMR studies) was cloned into the *Bam*HI and *Not*I sites of a pSMT3 vector (Höck *et al*, 2007), which contains an N-terminal Ulp1 cleavable His₆-Sumo tag. The protein was bacterially expressed using standard protocols and purified by Ni-affinity chromatography, cleavage of the tag, anion exchange chromatography and Superdex-75 size-exclusion chromatography in 25 mM Tris pH 8.0, 150 mM NaCl and 3 mM DTT (for crystallization), or in 25 mM MES pH 6.5, 200 mM NaCl and 3 mM DTT (for NMR). For pull-down experiments the hexahistidine/SUMO fusion protein was not cleaved with Ulp-1 and was purified by Ni-affinity and anion exchange chromatography.

Full-length hAGO2 (S34 to the C-terminus) was cloned into the *Bam*HI and *Not*I sites of the pFastBac HTb vector for expression in baculovirus-infected insect cells. Infected cells were harvested 72 h post-infection and protein was extracted by Ni-affinity chromatography, cleavage of the tag using tobacco etch virus protease and Superdex-200 size-exclusion chromatography in 25 mM Tris pH 8.0, 300 mM NaCl, 3 mM DTT and 10% glycerol. **Crystallization, data collection and structure determination.** Crystals (size: approximately 0.2 mm $\times$ 0.2 mm $\times$ 0.6 mm) of hAGO2 MID domain (439–578) were grown by hanging-drop vapour diffusion at 4 °C. Protein at 10–15 mg ml⁻¹ was mixed 1:1 with a well solution containing 0.1 M imidazole pH 8.0, 0.2 M NaCl, 0.46 M NaH₂PO₄ and 1.84 M K₂HPO₄. Protein-ligand crystals were obtained by soaking native crystals in a drop containing 0.2 M (NH₄)₂SO₄, 0.1 M Na-Cacodylate pH 6.5, 15% PEG8000, 20% glycerol and 20 mM ligand. Diffraction data for nucleotide-containing crystals were collected on a Rigaku rotating

copper-anode generator and processed using *HKL2000* (Otwinowski, 1997). The nucleotide complexes were solved by difference Fourier analysis using the native structure and refined using *CNS* (Brunger et al, 1998) and *Phenix* (Adams et al, 2010). For the m⁷GpppG complex, we arbitrarily chose to model the m⁷Gpp portion of the cap analogue, as the quality of the density for the base does not allow us to discern the orientation of the dinucleotide. The α-phosphate and G nucleotide portion were placed so as to minimize close contacts, and their occupancies were set to 0 during refinement.

NMR titration experiments. All NMR experiments were performed at 293 K using a Bruker 600 MHz spectrometer. NMR titrations were carried out by acquiring ¹H–¹⁵N HSQC spectra on samples of 0.10–0.25 mM ¹⁵N-labelled hAGO2 MID domain (432–578), with the addition of increasing amounts of unlabelled ligand. Chemical shift differences were calculated as $\Delta\delta = [(\Delta\delta_H)^2 + (0.2 \cdot \Delta\delta_N)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the observed chemical shift changes for ¹H and ¹⁵N, respectively. For determination of dissociation constants, $\Delta\delta$ was plotted as a function of the molar ratio (nucleotide/protein) and the data for multiple peaks were fit, using the maximum shift and dissociation constant as adjustable parameters.

For saturation with UMP and subsequent addition of m⁷GpppG, the MID domain was first saturated with 5 mM UMP by addition from a stock solution of 0.5 M UMP in water and a spectrum was recorded. After addition of 3 mM m⁷GpppG (from a 0.3-M stock solution of m⁷GpppG in water) to the UMP-saturated sample, another spectrum was recorded.

In vitro pull-down experiments. In all, 0.5 μg of GST-eIF4E, 5 μg of hexahistidine/SUMO-hAGO2 MID domain fusion protein and 1 μg of recombinant hAGO2 (S34 to the C-terminus) were diluted in PBS with 5 mM MgCl₂. Where indicated, we added a 10-fold molar excess of let-7 miRNA or nucleotides at a final concentration of 500 μM. Samples were then incubated for 15 min at 4 °C before addition of 30 μl of m⁷GTP-Sepharose beads (GE Healthcare) or glutathione-Sepharose, followed by rotation for 2 h at 4 °C. Samples were washed four times with 600 μl PBS containing 2 mM MgCl₂ and heparin (0.5 mg/ml) and bound proteins were eluted with SDS-loading buffer and analysed by western blot. Pull-down experiments were performed at least twice and representative results are shown.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 3QX9 and 3QX8.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank Katalin Illes for technical support in the production of full-length human AGO2, K. Gehring for comments on the manuscript and R. Szittner for technical support. NMR data were collected at the Québec/Eastern Canada High Field NMR facility. The hAGO2 antibody was a kind gift from Zissimos Mourelatos. B.N. is supported by a Canada Research Chair, a Career Development Award from the Human Frontiers Science Program (0018/2006-C/1) and an operating grant from the Canadian Institutes of Health Research (CIHR; grant MOP-82929). N.S. is funded by a CIHR grant. F.F. is supported by a Boehringer Ingelheim Fonds PhD Fellowship. E.D. is a Howard Hughes Medical Institute International Scholar (Grant No. 55005604). E.D., J.S. and J.J. are supported

by the Polish Ministry of Science and Higher Education (Grant No. N N301 096339).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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