Association of Argonaute proteins and microRNAs can occur after cell lysis

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ABSTRACT

MicroRNA (miRNA) target identification is a challenging but important endeavor. Global analyses of the direct mRNA targets of miRNAs have relied heavily upon immunopurification techniques, wherein a core protein component of the miRNA–protein complex, Argonaute (Ago), is immunoprecipitated to isolate associated RNAs. This approach involves the assumption that the selected RNAs were bound to the Ago protein *in vivo* and that the methodology did not significantly perturb endogenous interactions or produce novel interaction artifacts. To test whether RNAs that coimmunoprecipitate with human Ago were bound *in vivo* or could associate post-cell lysis, we used an experimental approach that distinguishes between these two origins of interaction. We show that a transfected miRNA mimic, but not a plasmid-expressed miRNA, can interact with human Ago proteins post-lysis. Our results have important implications for the design of miRNP immunoprecipitation experiments.

Keywords: microRNA; RIP; Argonaute

INTRODUCTION

The biological function of a microRNA (miRNA) is defined by the mRNAs that it targets. From the moment that miRNAs were discovered, the identities of the mRNAs they bind *in vivo* have been pursued (for a recent review, see Pasquinelli 2012). Unfortunately, miRNA target identification has proven to be challenging, due in part to the imperfect and variable nature of miRNA–mRNA base-pairing (Lewis et al. 2005; Didiano and Hobert 2006; Grimson et al. 2007; Shin et al. 2010; Chi et al. 2012). The limitations of bioinformatic miRNA target prediction necessitate the use of biochemical approaches. Immunopurification of ribonucleoprotein (RNP) complexes from cell lysates has been used extensively to study miRNAs and their mRNA targets (Rio et al. 2011).

MiRNA-mediated regulation is thought to require minimally the stable interaction of a mRNA with a miRNA and an Argonaute (Ago) protein, comprising a tightly associated miRNA-associated RNP (miRNP), which is the core of the RNA-induced silencing complex (RISC) (Pillai 2005;

Wang et al. 2008). Human cells possess four Ago proteins (Ago1–4), all ~100 kDa and ubiquitously expressed (for review, see Ender and Meister 2010). The four human Ago proteins have overlapping biological functions and are roughly equivalent in their affinity for bulged miRNA-type duplexes with mRNA (Su et al. 2009). Since an Ago protein must bind a single-stranded mature miRNA before this binary complex engages in mRNA target recognition, the miRNA–Ago interaction is a crucial step in determining the specificity of miRNA targeting (Czech and Hannon 2011).

The high affinity of association between Ago and RNAs ($K_d = 10$ –80 nM for tagged mammalian Ago2 binding to mature miRNAs) (Lima et al. 2009; Tan et al. 2009) is exploited in the biochemical identification of miRNA-targeted mRNAs, where the miRNP complex is precipitated with anti-Ago antibodies. The biggest assumption underlying this methodology is that the biochemical manipulations of the procedure generate selected complexes that are representative of the *in vivo* miRNP (Mili and Steitz 2004). In some cases, tagged Ago proteins and/or miRNAs (synthetic mature miRNA mimics, pre-miRNA mimics, or plasmid-expressed miRNAs) have been transiently or stably transfected or transduced into cells before anti-Ago immunoprecipitation (Easow et al. 2007; Karginov et al. 2007; Orom and Lund 2007; Hendrickson et al. 2008; Hong et al. 2009; Dolken et al. 2010; Hafner et al. 2010; Wang et al. 2010). Two major experimental protocols are then used:

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(1) a simple RNA coimmunoprecipitation (RIP), where mRNAs in the cell lysate are coprecipitated with Ago (Easow et al. 2007; Karginov et al. 2007; Hendrickson et al. 2008; Hong et al. 2009; Dolken et al. 2010; Wang et al. 2010), or (2) crosslinking and immunoprecipitation (CLIP), which incorporates an *in vivo* UV crosslinking step to covalently link Ago to miRNAs and mRNAs prior to immunoprecipitation (Chi et al. 2009; Hafner et al. 2010). The crosslinking step irreversibly locks the complexes in their *in vivo* state, permitting stringent purification of the RNP and identification of binding sites (Ule et al. 2005). However, it is argued by those who use RIP procedures that crosslinking is inefficient and can lead to sequence bias (Keene et al. 2006).

RIP experiments for the RNA-binding protein HuR demonstrated that, in the absence of crosslinking prior to preparation of the cell lysate, HuR artificially binds mRNAs *ex-vivo* (Mili and Steitz 2004). If the same holds true for Ago, then both miRNAs and their targets could be incorrectly identified in RIP-type experiments. Here, we asked whether Ago proteins can bind to miRNAs not present *in vivo* during a RIP procedure. We show that transfected, synthetic miRNA mimics used at manufacturer-recommended concentrations—but not plasmid-expressed miRNAs—can interact with Ago after cell lysis. Our findings have important implications for the design of miRNP selection experiments.

RESULTS AND DISCUSSION

A diagram of the experimental approach is depicted in Figure 1A. Separate HEK293T cell populations were transfected in parallel and then lysed together pairwise prior to immunoprecipitation. For our analyses, we chose the Epstein-Barr virus (EBV) miRNA BHRF1-2 (Pfeffer et al. 2004), which is expressed in EBV-infected cells such as Raji, but not in HEK293T cells (Fig. 1B). Mature BHRF1-2 originated from the transfection of either a double-stranded synthetic miRNA mimic or a plasmid containing the BHRF1-2 pri-miRNA genomic sequence (pcDNA3-BHRF1-2). We examined two concentrations of mimic: “high” (38 nM), the quantity recommended by the transfection reagent manufacturer, or “low” (2 nM). The pcDNA3-BHRF1-2 plasmid was transfected at the maximum recommended amount. A direct comparison of the amounts of mature BHRF1-2 produced in these experimental conditions is shown in Figure 1B. To distinguish transfected Ago from endogenous Ago proteins in the coimmunoprecipitations, we used HA-tagged Ago. We tested “HI” amounts (2 μ g of transfected plasmid) of either human Ago1 or Ago2, and a “LO” amount of Ago2 (100 ng of transfected plasmid). A direct comparison of the quantity of Ago proteins relative to endogenous levels is provided in Figure 1C.

To test whether Ago can bind miRNAs after cell lysis, we conducted four anti-HA coimmunoprecipitation experiments,

FIGURE 1. Design of coimmunoprecipitation experiments and controls. (A) Schematic diagram of the transfected HEK293T cell populations that were mixed before coimmunoprecipitation. Cells were transfected for 24 h with equal amounts of the noted plasmids and/or synthetic miRNAs. Each pair of separately transfected wells was harvested in PBS and mixed immediately prior to lysis and immunoprecipitation with anti-HA antibody. (B) Northern blot of 50 μ g of total RNA from Raji cells (first lane) or transfected HEK293T cells (remaining lanes) for miRNA BHRF1-2 relative to endogenous U6 snRNA (loading control). HEK293T cells were transfected with 2 μ g of empty vector (vector), 38 nM synthetic BHRF1-2 (high mimic), 2 μ g of pcDNA3-BHRF1-2 (plasmid), or 2 nM synthetic BHRF1-2 (low mimic). (C) Western blots using the indicated antibodies to determine the levels of endogenous (vector) or transfected HA-Ago proteins with a GAPDH loading control in HEK293T cells.

each exploring different HA-Ago proteins and/or miRNAs (Fig. 2). Using manufacturer-recommended quantities of HA-Ago2 plasmid and BHRF1-2 mimic in standard HEK293T transfections, immunoprecipitations of mixed cell lysates were carried out as depicted in Figure 1A. BHRF1-2 does not coprecipitate when HA-Ago2 is not present (Fig. 2A, lane 1), but does coprecipitate when the same cells were cotransfected with HA-Ago2 (Fig. 2A, lane 3). Strikingly, BHRF1-2 is also readily detected in the immunoprecipitates when the two wells were separately transfected with HA-Ago2 plasmid and BHRF1-2 mimic, but then mixed just before lysis and immunoprecipitation. This indicates that the association between Ago and BHRF1-2 is not representative of *in vivo* binding, having necessarily occurred after cell lysis (Fig. 2A, lanes 4). As expected, endogenous miR-16 is coimmunoprecipitated when HA-Ago2 plasmid is transfected in conditions 2–4, and endogenous U6 snRNA, which does not bind Ago, does not coprecipitate. Together, these data demonstrate that the

FIGURE 2. Coimmunoprecipitation experiments. Notations 1–4 refer to the transfected cell mixtures that are depicted in Figure 1A. (A) High amounts of the HA-Ago2 plasmid (2 μ g/well) and of the BHRF1-2 mimic (100 pmol/well) were transfected. (B) As in Figure 2A, but substituting FLAG/HA-Ago1 for HA-Ago2 plasmid. (C) High amounts of plasmids HA-Ago2 (2 μ g/well) and pcDNA3-BHRF1-2 (2 μ g/well) were transfected. (D) Low amounts of both HA-Ago2 plasmid (100 ng/well) and BHRF1-2 mimic (5 pmol/well) were transfected. In all cases, Northern blots were probed for BHRF1-2 miRNA (transfected), miR-16 miRNA (endogenous), and U6 snRNA (endogenous). Corresponding Western blots (WB panel) of cell lysate and immunoprecipitates were probed for HA-tagged Ago. In experiments A–C, IN stands for input cell lysate (10% for RNA and protein); IP stands for immunoprecipitated material (90% for RNA; 10% for protein). In experiment D, IN stands for input cell lysate (1.25% for RNA and protein); IP stands for immunoprecipitated material (95% for RNA; 5% for protein).

BHRF1-2 miRNA mimic can associate with Ago post-lysis when transfected in manufacturer-recommended quantities.

We asked whether in vitro association of miRNAs also occurs with other Ago proteins. Human Ago1–4 are generally viewed as being functionally redundant for miRNA-directed activities; all four proteins bind bulged miRNAs and mRNA partners in vivo (Su et al. 2009). The results of our coimmunoprecipitation experiments were the same when we substituted FLAG/HA-Ago1 for HA-Ago2 (Fig. 2B).

We next asked how a plasmid-expressed miRNA behaves in the mixed lysate immunoprecipitation assays. As previously observed for other miRNAs (Winter and Diederichs 2011), BHRF1-2 accumulated to higher levels when co-transfected with HA-Ago2 (Fig. 2C, lane 3 IN). However, we were unable to detect any BHRF1-2 coimmunoprecipitating after cell lysate mixing, even though endogenous miR-16 was clearly coprecipitated with HA-Ago2 (Fig. 2C, lane 4 IP). Thus, plasmid-expressed BHRF1-2 does not associate with Ago post-lysis. However, the relative fraction of endogenous miR-16 that was coprecipitated was much lower than that of transfected BHRF1-2 (Fig. 2C, lane 3, cf. IP and IN), whereas this difference is not observed when synthetic miRNAs are transfected (Fig. 2A–D, lanes 3). The reason for this difference is unknown.

Synthetic miRNA mimics are easily transfectable and bypass the canonical miRNA biogenesis pathway, allowing them to accumulate intracellularly to extremely high levels relative to endogenous miRNAs (Fig. 1B). Due to limitations of plasmid transfection and possible oversaturation of limiting biogenesis components such as Drosha, Dicer, and export proteins, plasmid-expressed miRNAs do not typically accumulate to comparably high levels (Fig. 1B). To ask whether reducing the quantity of transfected miRNA mimic affects its ability to bind Ago post-lysis, we repeated the coimmunoprecipitation experiment using 1/20th the amount of each miRNA mimic and HA-Ago2 plasmid (Fig. 2D). Despite the significantly reduced levels of both RNA and protein (Fig. 1B,C), the miRNA reproducibly associated with Ago post-lysis (Fig. 2D, lane 4 IP). Thus, the post-lysis association of BHRF1-2 mimic with Ago cannot be simply circumvented by lowering the abundance of both BHRF1-2 and HA-Ago.

CONCLUSIONS

We have demonstrated that antibodies directed against tagged human Ago1 and Ago2 can coprecipitate synthetic miRNA mimics that become available for binding only

after cell lysis (Fig. 2A,B,D). In contrast, even maximal levels of plasmid-expressed miRNAs did not exhibit this behavior in the same experimental context (Fig. 2C). We were surprised by these results because the binding of a miRNA to Ago2 is high affinity (Lima et al. 2009; Tan et al. 2009), and each makes a considerable contribution to the stability of the other (Fig. 2C; Winter and Diederichs 2011; Elkayam et al. 2012).

Artificial introduction of miRNAs or Ago protein inevitably alters the stoichiometry of RISC components in the cellular environment. Because transfected miRNAs compete for a limited pool of protein-binding partners to form miRNPs, miRNA transfection results in both the down-regulation of the mRNA targets of the transfected miRNA and global up-regulation of endogenous miRNA targets (Khan et al. 2009). Nonetheless, even transfection of low amounts of miRNA mimic and HA-Ago2 plasmid produced significant *ex vivo* association (Fig. 2D).

Because miRNA loading onto Ago precedes mRNA target loading (Czech and Hannon 2011), disruption of the Ago–miRNA association necessarily impacts the miRNA–mRNA interaction. Therefore, our findings have implications for both miRNA and mRNA target identification. We attempted to also explore the behavior of Ago-bound mRNAs upon mixing cell lysates. We found that, while known target mRNAs are enriched in immunoprecipitates from cells cotransfected with tagged Ago and full-length BHRF1 or firefly luciferase-LMP1 3' UTR mRNAs (Riley et al. 2012) (similar to Fig. 1A, scheme 3), the amount of coprecipitated mRNA in mixing experiments (similar to Fig. 1A, scheme 4) is highly variable from one experiment to the next (K Riley, T Yario, and J Steitz, unpubl.), thereby preventing firm conclusions. In contrast, high-throughput experiments that add a UV crosslinking step generate highly reproducible mRNA target data, including those using Ago and/or miRNA transfection (Chi et al. 2009; Hafner et al. 2010). In all cases, validation of miRNA targets by independent methods, such as luciferase assays with full-length 3' UTRs and Western blots of endogenous miRNA targets, is necessary. Thus, we have added another cautionary note to the growing list of concerns about miRNA target identification methods: artificial association with Ago post-lysis, an artifact occurring upon transfection of synthetic miRNAs and tagged Ago.

MATERIALS AND METHODS

Nucleic acids

We cloned 566 bp of EBV genomic sequence encoding BHRF1-2 and BHRF1-3 between pcDNA3 EcoRI and XbaI sites using Raji cell genomic DNA as a template with 5' PCR primer GACTGAATTCATCAGGGTCATTGGGCGCTG and 3' PCR primer GATCTCTAGAGGTTCTTCATCATTAACATAGCG to create plasmid pcDNA3-BHRF1-2. The empty control vector pcDNA3

(Invitrogen) was used as a control. N-terminally tagged HA-Ago2 in pCI-neo (Pillai et al. 2004) and FLAG/HA-Ago1 in pIRESneo (Meister et al. 2004) were kind gifts and were verified by sequencing. Synthetic BHRF1-2 mimic (IDT) was designed as previously described (Riley et al. 2012), using 20 μ M annealed guide (P-UAUCU UUUGCGGCAGAAUUGA) and passenger (AAUUCUGCCG CAAAAGACAGG) RNA strands.

Cell culture and transfections

HEK293T cells were grown in DMEM (Gibco) and Raji cells were grown in RPMI. Both media types were supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine. The day preceding transfection, HEK293T cells were seeded in 6-well plates for ~50% confluence at the time of transfection. Cells were transfected with 4 μ g of total plasmid (2 μ g of each type of plasmid; HI), 100 ng of total plasmid (LO), 100 pmol of BHRF1-2 mimic (HI), and/or 5 pmol of BHRF1-2 mimic (LO) per well using 10 μ L per well Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Coimmunoprecipitations

Pairs of wells of separately transfected cells were harvested together in the indicated combinations 24 h post-transfection and washed twice in phosphate-buffered saline. The cells were lysed in NET-2 buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% NP-40 with 1 $\times$ protease inhibitor cocktail [Roche]). The lysate was gently sonicated on ice (3 $\times$ 15 sec, low setting) and centrifuged at 16,000g and 4°C for 10 min. For each immunoprecipitation, 30 μ L of protein G-Sepharose bead slurry (GE) was pre-washed in NET-2 and blocked at 4°C for 30 min in a solution containing 0.2 mg/mL of glycogen, 0.1 mg/mL of yeast tRNA, and 2 mg/mL of BSA. The cleared lysate was mixed with pre-blocked beads and 5 μ L of anti-HA antibody (Covance). The mixtures were incubated 2–4 h at 4°C, the beads were centrifuged at 200g and 4°C for 2 min, and the supernatant was collected for analysis. The beads were washed three times in NET-2 buffer, and the samples (input, supernatant, and immunoprecipitate) were split as noted and either subjected to TRIzol extraction to isolate coimmunoprecipitated RNAs or mixed with protein gel loading buffer for standard Western blotting. Results were replicated in at least three independent experiments with indistinguishable results.

Northern blots

RNA was isolated from indicated fractions of the input cell lysates or antibody-coated beads with TRIzol reagent (Invitrogen). Northern blots were carried out as previously published (Riley et al. 2010). The ebv-miR-BHRF1-2 and hsa-miR-16 miRNAs were probed with 5' phosphate radiolabeled TCAATTTCTGCCG CAAAAGATA and CGCCAATATTTACGTGCTGCTA, respectively. U6 snRNA was probed as a loading control: GCAGGGGC CATGCTAATCTTCTCTGTATCG (Pfeffer et al. 2004).

Western blots

The indicated fractions of cell lysates were harvested in PBS, subjected to SDS-PAGE, and Western blots were performed as previously described (Riley et al. 2012). Primary antibodies were anti-HA (Covance), anti-GAPDH t4C10 (Cell Signaling Technology),

anti-Ago2 (Millipore), and anti-pan-Ago 2A8 (Nelson et al. 2007), a gift from Z. Mourelatos. HRP-conjugated secondary antibodies (mouse, rabbit) were from Pierce. Western blots were visualized using the G:BOX (Syngene) chemiluminescence imaging system.

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