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Expression of PACT and EIF2C2, Implicated in RNAi and MicroRNA Pathways, in Various Human Cell Lines

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MicroRNA and siRNA (small interfering RNA), representative members of small RNA, exert their effects on target gene expression through association with protein complexes called miRNP (microRNA associated ribonucleoproteins) and RISC (RNA induced silencing complex), respectively. Although the protein complexes are yet to be fully characterized, human EIF2C2 protein has been identified as a component of both miRNP and RISC. In this report, we raised antiserum against EIF2C2 in order to begin understanding the protein complexes. An immunoblot result indicates that EIF2C2 protein is ubiquitously expressed in a variety of cell lines from human and mouse. EIF2C2 protein exists in both cellular compartments, as indicated by an immunoblot assay with a nuclear extract and a cytosolic fraction (S100 fraction) from HeLa S3 lysate. Depletion of EIF2C1 or EIF2C2 protein resulted in a decrease of microRNA, suggesting a possible role of these proteins in microRNA stability or biogenesis. We also prepared antiserum against dsRNA binding protein PACT, whose homologs in *C. elegans* and *Drosophila* are known to have a role in the RNAi (RNA interference) pathway. The expression of PACT protein was also observed in a wide range of cell lines.

In recent years, small RNAs have provided a new paradigm for the regulation of gene expression, as well as an efficient way by which a gene of interest can be experimentally knocked out in mammalian cells (Elbashir et al., 2001) (reviewed in Dykxhoorn et al., 2003). The former are a new class of small RNAs, termed miRNAs (microRNAs), whereas the latter are siRNA (small interfering RNA), a key intermediate in the RNAi (RNA interference) pathway.

A few hundred miRNAs have been identified from various organisms, but their functions are not well understood (reviewed in Bartel, 2004). The current model for the mechanism of miRNA function was established by characterizing the first miRNAs identified, *lin*-4 (Lee et al., 1993) and *let*-7 (Reinhart et al., 2000) of *C. elegans*. Both miRNAs are derived from their precursors with characteristic stem-loop structures. They bind to their targets in the 3' untranslated region with imperfect complementarity and repress target expression at the translational stage without affecting the mRNA levels.

RNAi is a phenomenon in which expression of an endogenous gene is repressed by the homologous dsRNA (double stranded RNA) (Fire et al., 1998). dsRNA artificially introduced into a cell or derived from a virus or a transposon is processed into small RNA of approximately 21-nucleotides by an RNase III family enzyme Dicer. This small interfering RNA, termed siRNA, is then incorporated into a protein complex called RISC (RNA induced silencing complex). The siRNA-charged RISC is a sequence specific nuclease that targets mRNA for cleavage and thereby inhibits the expression of the target gene (reviewed in Hannon, 2002). In mammalian cells, introduction of long dsRNA induces the interferon response, which results in an overall block of cellular translation by inactivation of an elongation factor via the action of a protein kinase (Stark et al., 1998). This non-specific effect of long dsRNA, which overrides any sequence specific effect, can be avoided by using siRNA. Transfection of an siRNA into mammalian cells results in sequence-specific inhibition of the corresponding gene and provides a valuable tool to study a loss-of-function phenotype (Elbashir et al., 2001).

Even though miRNA and siRNA comprise two distinct classes of small RNAs, they share several common features. Besides their similar size, both are processed by Dicer and are implicated in the regulation of gene expression through base-pairing interactions with their

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targets. Although the conventional model distinguishes the translational repression by miRNA from the mRNA cleavage by siRNA, these two mechanisms are simply distinguished from each other by the degree of complementarity between the small RNA and its target, regardless of the source of small RNA (Doench et al., 2003; Hutvagner and Zamore, 2002; Saxena et al., 2003; Tang et al., 2003; Zeng et al., 2002). In other words, an siRNA bearing some mismatches to its target represses the translation. Conversely, a mRNA containing artificial target sequences of perfect complementarity to a miRNA can be cleaved by the miRNA.

siRNA and miRNA are known to exert their effects in association with protein complexes called RISC (Hammond et al., 2000) and miRNP (Mourelatos et al., 2002) (reviewed in Schwarz and Zamore, 2002), respectively. Although the exact molecular nature of these protein complexes is not yet fully understood, genetic and biochemical analysis of the complexes partially purified from *C. elegans, Drosophila*, and humans identified several protein components, which include the Argonaute family of proteins and dsRNA binding proteins.

Argonaute proteins make up a conserved family whose members have been implicated in RNAi and related phenomena in a number of organisms (reviewed in Carmell et al., 2002). Argonaute proteins are characterized by two common domains, PAZ and Piwi (Cerutti et al., 2000). The PAZ domain, also present in Dicer proteins, is now known to recognize 3' single-stranded overhangs of dsRNA, contributing to the efficiency of RNAi (Lingel et al., 2003; Song et al., 2003). In addition, several reports identified Argonaute family proteins as a core component of RISC or miRNP. *Drosophila* AGO2 protein is essential for RNAi and was the first protein component to be identified as part of the RISC in cultured *Drosophila* S2 cells (Hammond et al., 2001). Human EIF2C2 was detected as a protein component associated with siRNA (Martinez et al., 2002) or miRNA (Mourelatos et al., 2002).

Rde-4 protein was discovered during a screen for RNAi defective mutants in *C. elegans* (Tabara et al., 1999). Results from Rde-4 (Tabara et al., 2002) and its *Drosophila* homologue R2D2 (Liu et al., 2003) revealed that these proteins bind trigger dsRNAs and present them for subsequent steps of the RNAi pathway by interacting with Dicer or other protein factors. Sequence alignment assigned PACT (protein activator of the interferon-induced protein kinase) as a human homologue of Rde-4 and R2D2 (Tabara et al., 2002). However, a direct relationship between PACT and RNAi has not been reported.

In this paper, we prepared antisera against human EIF2C2 and PACT proteins. By immunoblot assay, the expression of these proteins was examined in various cell lines, and the sub-cellular localization of EIF2C2 was revealed. In the future, these antibodies will provide a valuable tool for the investigation of these proteins, the identification of any associated proteins, and the *in vitro* reconstitution of RNAi activity in human cell extract.

Materials and Methods

Overexpression and purification of antigenic polypeptide of EIF2C2 and PACT

To construct the plasmid DNAs for overproduction of antigenic polypeptides of EIF2C2 and PACT, cDNA containing the antigenic region (amino acid from 386 to 645 and from 104 to 313, respectively; Fig. 1) was amplified by PCR and cloned into pET-23a(+) (Novagen). Recombinant polypeptides with a hexahistidine tag were overexpressed in *E. coli* BL21(DE3) in the presence of 0.5 mM IPTG for 3 hours. Cells were harvested and resuspended in the cell lysis buffer (50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 10 mM imidazole, 10% glycerol and 2.86 mM 2-mercaptoethanol). Unless indicated, subsequent steps were performed at 4°C or less.

Cell lysis by sonication was followed by centrifugation at 15 krpm for 20 min in a Sorvall SS-34 rotor. The precipitate containing the majority of the recombinant polypeptide was resuspended in buffer A (25 mM HEPES-KOH, pH 7.8, 10% glycerol) containing 6 M guanidine hydrochloride, 10 mM imidazole and 50 mM NaCl. After resuspension, insoluble material was removed by centrifugation at 15 krpm for 20 min in a Sorvall SS-34 rotor. The resulting supernatant was mixed with nickel-charged chelating sepharose resin (Amersham Biosciences) which had been pre-equilibrated with the same buffer, and the mixture was incubated for 3 hours with constant rotating. Then, the mixture was packed into a column, and the polypeptide was eluted by increasing the concentration of imidazole in the same buffer. The eluted fractions were pooled and sent to Cocalico Biologicals Inc. for production of antisera in rabbit.

Preparation of cell extract and fractionation of nuclear extract and S100 fraction

Cells were harvested, washed once with PBS (phosphate-buffered saline), and lysed in the lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP40, 2 mM EDTA) with protease inhibitors. The protein concentration of lysate was measured with Bradford reagent (BioRad).

The nuclear extract and the S100 fraction were prepared from HeLa S3, as previously described (Dignam et al., 1983). The cell pellet, obtained from a 5 liter culture (2.5×10^9 cells), was purchased from the NCCC (National Cell Culture Center). The S100 fraction was further fractionated by stepwise precipitation by ammonium sulfate. All steps were performed at 4°C or less. 0.3 g of ammonium sulfate per ml were added to the S100 fraction, and the resulting precipitate was

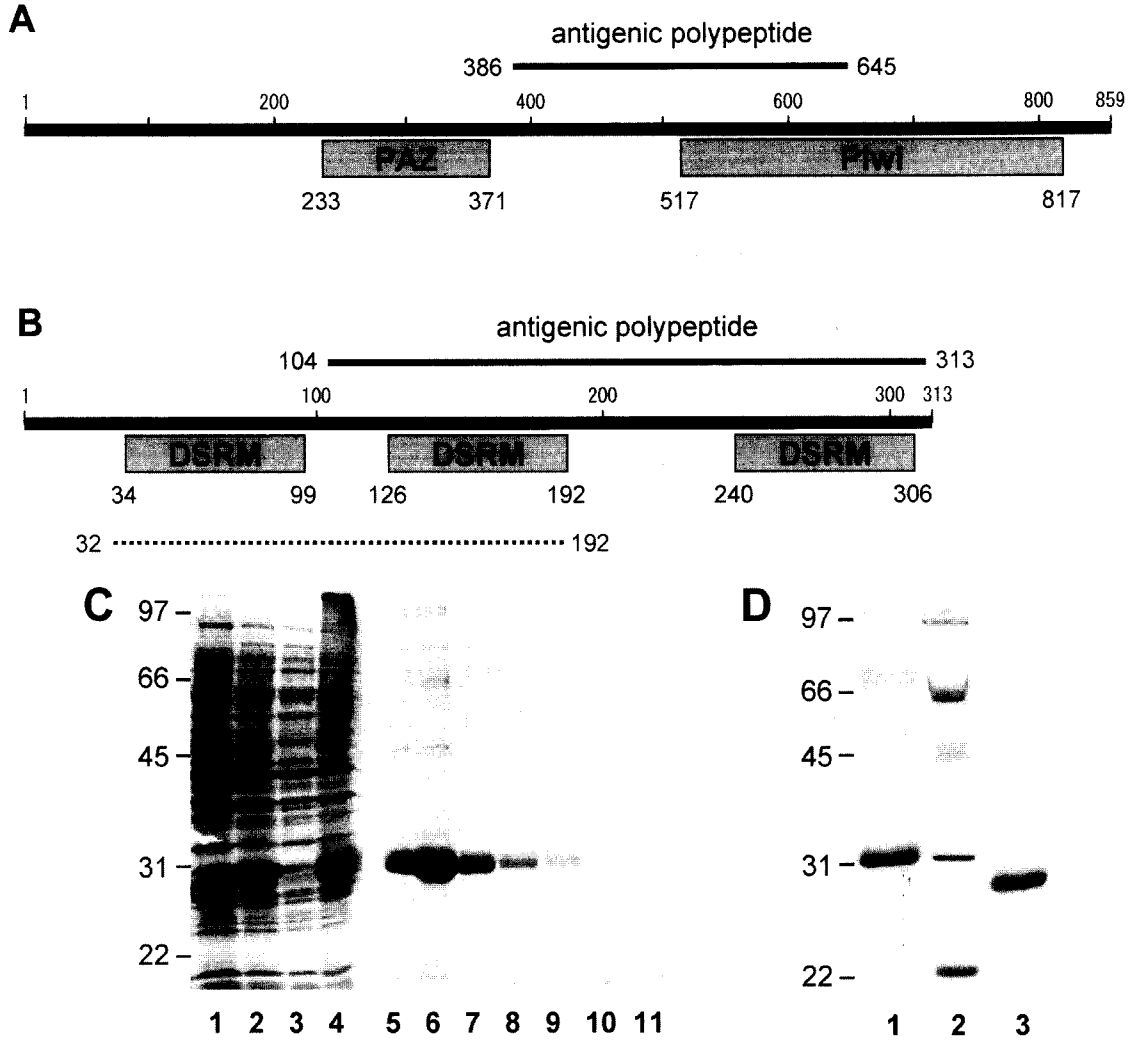

Fig. 1. Overexpression and purification of antigenic polypeptides of EIF2C2 and PACT. The primary structures of EIF2C2 (A) and PACT (B) are shown. The amino acid coordinates are denoted on the black bar. Conserved domains (PAZ, Piwi, DSRM) are indicated in grey boxes with the corresponding amino acid coordinates. The regions of antigenic polypeptides are indicated in solid lines. In panel B, the dotted line indicates a highly homologous region across several species. C, Samples from the purification steps of EIF2C2 antigen (see Materials and Methods) were subjected to 12% SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by Coomassie blue staining. Molecular size markers in kDa are indicated on the left. Lane 1, total cellular protein before IPTG induction; lane 2, after IPTG induction; lane 3, soluble fraction after cell lysis; lane 4, insoluble fraction which was extracted with 6 M guanidine hydrochloride; lanes 5-11, fractions eluted from Ni-charged chelating sepharose with increasing imidazole. D, Purified EIF2C2 antigen (lane 1) and PACT antigen (lane 3), as well as molecular size markers (lane 2), are visualized on 12% SDS-polyacrylamide gel as described above.

recovered by centrifugation at 35 krpm for 30 min in a Beckman Ti45 rotor. The precipitate was resuspended in buffer D (20 mM HEPES-KOH, pH7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM dithiothreitol) and labeled as "0.3 AS fraction". 0.2 g of ammonium sulfate per ml were added to the resulting supernatant and the above steps were repeated. This time, the resuspended pellet was labeled as "0.5 AS fraction".

siRNAs and transfections

siRNA duplexes targeting EIF2C1 and EIF2C2 were synthesized by Dharmacon. Transfection into HeLa cells was performed with 320 nM of siRNA duplex and oligofectamine reagent (Invitrogen Life Technologies) as manufacturer's instructions. The target sequences for siRNA duplexes are as following;

siEIF2C1-A: 5' aagccctggatgtggccatga 3'
siEIF2C1-B: 5' aagacttgtcctacatggtgc 3'
siEIF2C2-C: 5' aacggacaatcagacctcaac 3'
siEIF2C2-D: 5' aagcaggccttcgcactatca 3'

RNA isolation and primer extension assay

Total RNAs from cells were isolated with Trizol reagent

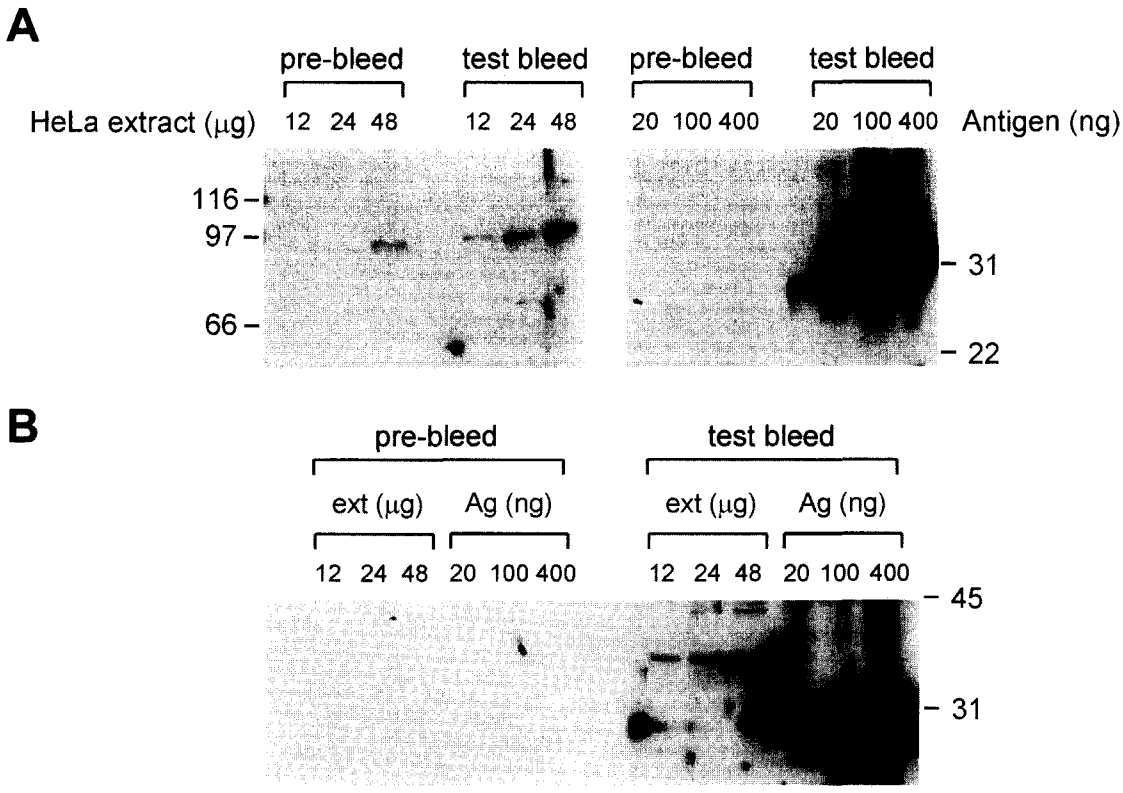

Fig. 2. Validation of antisera against EIF2C2 and PACT. Immunoblot assays with EIF2C2 (A) and PACT (B) test bleeds, in parallel with the corresponding prebleeds, were performed with the indicated amount of HeLa cell extract or purified antigen.

(Invitrogen Life Technologies) according to the manufacturer's instructions. Primer extension assay (Hutvagner et al., 2001) was performed with Superscript II reverse transcriptase (Invitrogen Life Technologies) as manufacturer's instructions with minor modifications. 20 μl of extension reaction contained 10 μg of total RNA and 0.1 pmol of the γ-^{32}P-labeled primer for *let*-7 miRNA (5'GCCCCAA-ACTATACAACCTACTAC3').

Extension reaction at 50°C for 50 minutes was quenched by addition of 20 μl of sequencing gel loading buffer (Sambrook, 1989). Extension products were resolved from the primer by electrophoresis in an 18% polyacrylamide gel with 7 M urea.

Results and Discussion

The primary structures of EIF2C2 and PACT proteins are shown in Fig. 1A and B, respectively. Like other Argonaute family proteins, EIF2C2 protein has PAZ and Piwi domains (Fig. 1A). The PACT protein has three copies of dsRNA binding motifs (DSRM in Fig. 1B; Patel and Sen, 1998), and the region encompassing the first two copies (dashed line) is conserved across several organisms (Tabara et al., 2002).

To begin investigating these proteins, antisera were prepared. Antigenic polypeptides (Fig. 1) were overproduced in *E. coli* and purified by nickel affinity chromatography (Materials and Methods). Fig. 1C represents the purification of EIF2C2 antigen (residues 386 to 645). Most of overproduced polypeptides (lane 2 in Fig. 1C) were detected in the insoluble fraction after cell lysis (lanes 3 and 4 in Fig. 1C). This insoluble material was extracted with 6 M guanidine hydrochloride, and the subsequent nickel affinity chromatographic step performed under this denaturing condition. Elution with imidazole yielded highly pure antigenic polypeptides (lanes 5 to 11 in Fig. 1C). Fig. 1D shows the purity of EIF2C2 and PACT antigens (residues 104 to 313), which were injected into rabbits to produce antisera.

The quality of antibody was validated by immunoblot assays (Fig. 2). Most importantly, both antisera recognize the protein bands of expected size (97.2 kDa for EIF2C2 and 34.4 kDa for PACT) from HeLa cell extract. In the case of EIF2C2, a band at a similar position is detected by the prebleed (left panel in Fig. 2A), but careful examination revealed that this band is of slightly faster mobility than the EIF2C2 detected by the test bleed. In addition, this nonspecific band is only visible at 48 μg of extract whereas EIF2C2 band is detectable at as low as 12 μg of extract. In the case of PACT, several other bands are also recognized by the test bleed, but the major band matches the expected size of PACT. In

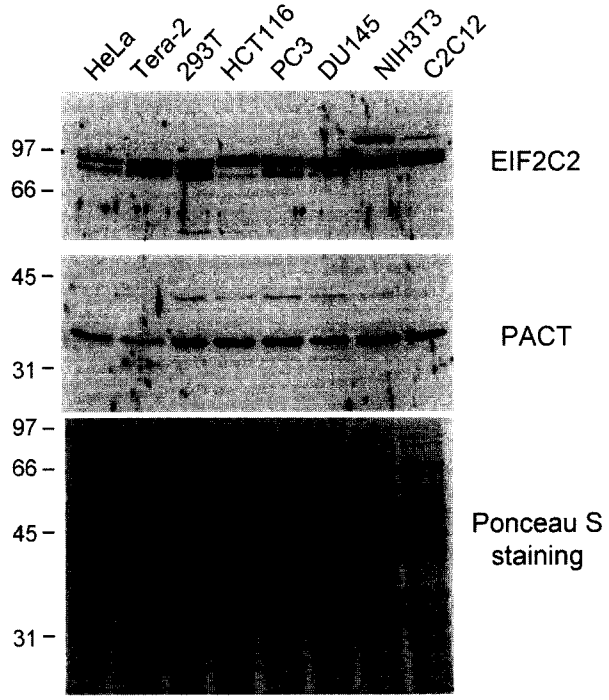

Fig. 3. Expression of EIF2C2 and PACT in various cell lines. Immunoblot assays were performed with 48 μg of each cell extract from various cell lines (denoted on top). EIF2C2 (top panel) and PACT (middle panel) are shown with the staining of all proteins by Ponceau S (bottom panel). Molecular size markers in kDa are indicated.

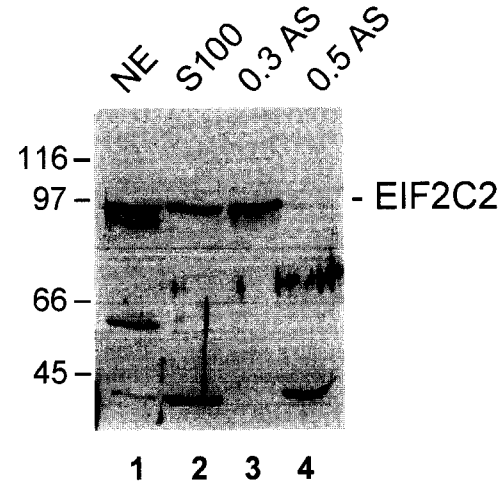

Fig. 4. EIF2C2 is present in both nuclear and cytoplasmic fraction. Immunoblot assay with EIF2C2 antibody was performed with 40 μg of each of the following; nuclear extract (NE, lane 1), S100 fraction (S100, lane 2), 0.3 g/ml ammonium sulfate fraction (0.3 AS, lane 3), and 0.5 g/ml ammonium sulfate fraction (0.5 AS, lane 4). Each fraction is described in Materials and Methods.

addition, no signal is observed by the prebleed (Fig. 2B). The specificity and titer were further confirmed by immunoblot assays with the antigens. Both of the test bleeds, but not the prebleeds, detect the corresponding antigens. Based on the strong signals from 20 ng of antigens, it seems likely that only a few nanograms or less would be detected in the immunoblot assay.

Both antibodies, qualified as specific in immunoblot assays, were used to compare intracellular levels of EIF2C2 and PACT proteins among various cell lines. Comparable levels of expression of both proteins are shown in all the cell lines tested in this experiment, including human cancer cell line derived from cervix (HeLa), kidney (293T), colon (HCT 116), prostate (PC-3 and DU 145), and embryo (Tera-2), as well as mouse fibroblast (NIH/3T3) and myoblast (C2C12) cell lines (Fig. 3). Both proteins are well conserved between human and mouse, with 98% identity in amino acid sequence. Furthermore, the antigenic portion of EIF2C2 protein is identical between the two organisms. As expected from the similarity, both antisera recognize mouse proteins as efficiently as human proteins.

EIF2C2 protein belongs to EIF2C/AGO subfamily in Argonaute family proteins (Sasaki et al., 2003). There are four members, from EIF2C1 to EIF2C4, in the human EIF2C/AGO subfamily. They are very closely related to each other with approximately 80% of amino acid sequence identities between the members. In addition, three of them are of almost identical molecular size. Therefore, we cannot rule out the possibility of cross-reactivity of our antibody across the EIF2C/AGO family members. In any case, EIF2C/AGO family member(s) is expressed ubiquitously in several cell lines at almost constant levels. This is in agreement with the observation that knock-down of a gene by siRNA is seen in all of the cell lines listed above (data not shown), and is consistent with the proposed role of EIF2C/AGO proteins and its homologues as core components of RNAi machinery (Doi et al., 2003; Hammond et al., 2001; Martinez et al., 2002).

According to a previous report (Patel and Sen, 1998), PACT mRNA was expressed at varying levels in several human cancer cell lines. In contrast, our result agrees with that of another report (Gupta and Patel, 2002), in showing that the protein levels of PACT are almost constant among various cell lines. While PACT levels among most cell lines were constant, a slightly lower level of PACT is apparent in Tera-2, an embryonic carcinoma cell line representative of undifferentiated cell lines. Noticeably, mouse embryonic stem cells (Yang et al., 2001) or embryonal teratocarcinoma cell lines (Billy et al., 2001) have been reported to lack the nonspecific interferon response and thus be capable of specific RNAi by long dsRNA. Taken together with the proposed role of PACT protein as an activator of PKR (Patel and Sen, 1998), the lower level of PACT in the Tera-2 cell line might be responsible for the inefficient activation of PKR and account for the lack of the nonspecific response in the undifferentiated cells.

Next, we fractionated HeLa S3 cells into nuclear

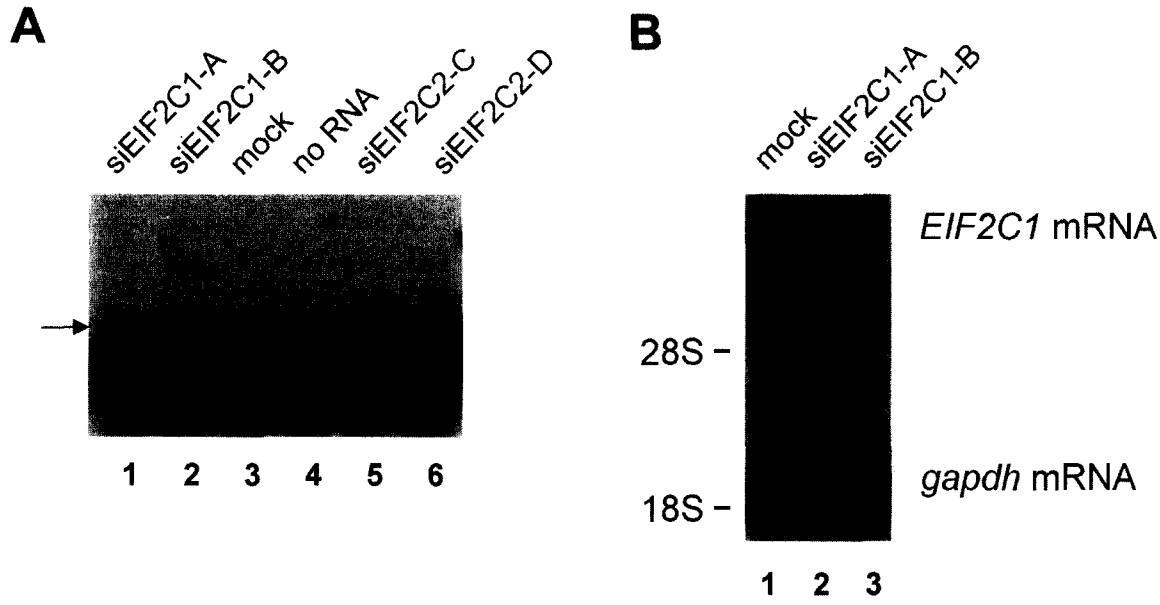

Fig. 5. The depletion of EIF2C/AGO results in decreased level of miRNA. A, Primer extension assays were performed with RNAs from HeLa cells transfected with the indicated siRNA oligonucleotides (lanes 1, 2, 5, 6) or without siRNA (lane 3), as described in Materials and Methods. The extension product, resolved from the free primer (lane 4; no RNA control), is indicated by arrow. B, mRNA levels of *EIF2C1*, as well as *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) as a control, were detected by Northern hybridizations with RNAs from HeLa cells transfected with the indicated siRNAs (lanes 2, 3) or without siRNA (lane 1). The locations of 18S and 28S ribosomal RNA are indicated in the left, and the identities of each band are indicated in the right.

extract and cytoplasmic fraction (S100) to examine the sub-cellular localization of EIF2C2 protein and possibly other EIF2C/AGO proteins. Although few other bands of smaller size appear, the EIF2C/AGO protein(s), which is approximately 97 kDa, is present in both nuclear and cytoplasmic fractions at comparable level (lanes 1 and 2 in Fig. 4). We cannot rule out that different members of the EIF2C/AGO family might be present exclusively in the nucleus or the cytoplasm. RNAi activity for degradation of target mRNA is known to be located in the cytoplasm (Hutvagner and Zamore, 2002; Zeng and Cullen, 2002); however, there are several reports on the effect of RNAi machinery on chromatin structure in yeast (reviewed in Ekwall, 2004) and in vertebrates (Fukagawa et al., 2004). It would therefore be interesting to test whether nuclear EIF2C/AGO protein(s) has any function in heterochromatin formation.

According to previous reports, an S100 fraction was used for *in vitro* reconstitution of RNAi in *Drosophila* and in human cells (Hammond et al., 2000; Hammond et al., 2001; Hutvagner and Zamore, 2002; Martinez et al., 2002). Therefore, we performed further fractionation of the S100 fraction. Most of the EIF2C/AGO protein(s) in the S100 fraction was precipitated with 0.3 g/ml ammonium sulfate (lane 3 in Fig. 4). This fractionation confers some degree of purification of EIF2C/AGO (compare lanes 2-4 in Fig. 4) and associated proteins, and provides highly concentrated material for *in vitro* RISC assay.

Previous results (Mourelatos et al., 2002) suggest that human EIF2C2 is a protein component in miRNP. To examine the effect of EIF2C/AGO proteins on the miRNA metabolism, we performed an RNAi experiment to deplete these proteins and observed the miRNA level by a primer extension assay (Fig. 5A). The presence of mature *let*-7 miRNA in total RNA produced an extension product (compare lane 3 and 4 in Fig. 5A), whose intensity reflects the amount of *let*-7. The depletion of EIF2C1 (Fig. 5B) or EIF2C2 (data not shown) resulted in a reduced level of *let*-7 (compare lane 3 with lanes 1, 2, 5, and 6). The equal amount of RNA in each lane was confirmed by agarose gel electrophoresis of total RNA (data not shown). Therefore EIF2C1 and EIF2C2 proteins may be important for miRNA biogenesis or accumulation.

One plausible scenario is that miRNP might simply be required for the stability of mature miRNA. Since EIF2C/AGO protein(s) has a PAZ domain that binds to 3' single-stranded overhangs of dsRNA (Lingel et al., 2003; Song et al., 2003), EIF2C/AGO protein(s), through a physical association with the miRNA end, might protect the miRNA from any nuclease attack. Another possibility is that EIF2C/AGO protein(s) contributes to the catalytic activity of the enzyme Dicer. The knock-down of Dicer also results in reduction of miRNA (data not shown; Hutvagner et al., 2001), and several groups (Doi et al., 2003; Ishizuka et al., 2002; Sasaki et al., 2003) reported the association of Dicer and Argonaute proteins. Recently,

a ternary complex including AGO1, Dicer-1, and pre-miRNA was observed in *Drosophila*, implicating a possible role of Argonaute protein in miRNA biogenesis (Okamura et al., 2004). It remains to be investigated how EIF2C/AGO protein(s) contributes to the stability or biogenesis of miRNAs.

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