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Short-Interfering-RNA-Mediated Gene Silencing in Mammalian Cells Requires Dicer and eIF2C Translation Initiation Factors

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Summary

RNA interference (RNAi) is the process of long, double-stranded (ds), RNA-dependent posttranscriptional gene silencing (PTGS) [1]. In lower eukaryotes, dsRNA introduced into the cytoplasm is cleaved by the RNaseIII-like enzyme, Dicer, to 21–23 nt RNA (short interfering [si] RNA), which may serve as guide for target mRNA degradation [2]. In mammals, long-dsRNA-dependent PTGS is applicable only to a limited number of cell types [3–7], whereas siRNA synthesized in vitro is capable of effectively inducing gene silencing in a wide variety of cells [8]. Although biochemical and genetic analyses in lower eukaryotes showed that Dicer and some PIWI family member proteins are essential for long-dsRNA-dependent PTGS [9–11], little is known about the molecular mechanisms underlying siRNA-based PTGS. Here, we show that Dicer and eIF2C translation initiation factors belonging to the PIWI family (eIF2C1–4) play an essential role in mammalian siRNA-mediated PTGS, most probably through synergistic interactions. Immunoprecipitation experiments suggest that, in human and mouse cells, complex formation occurs between Dicer and eIF2C1 or 2 and that the PIWI domain of eIF2C is essential for the formation of this complex.

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Results and Discussion

As the first step toward clarifying the molecular mechanisms of mammalian RNAi, we searched for possible human homologs of *Caenorhabditis elegans* RDE-1 [10] and *Drosophila melanogaster* ARGONAUTE2 (AGO2) [11]. Four components, highly similar in sequence to rabbit eIF2C [12], were identified (Figure 1A), two of which had been previously noted to correspond to eIF2C1 and eIF2C2 [13]. Genes encoding three of the four (*eIF2C1*, FLJ12765 [*eIF2C3*], KIAA1567 [*eIF2C4*]) were clustered near lp34-p35 on the first chromosome, with the other (*eIF2C2*) being situated on the eighth chromosome ([13] and National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Four mouse counterparts were also isolated by RT-PCR. Phylogenetic tree analysis (Figure 1A) shows that eIF2Cs are not the counterparts of either AGO2 or RDE-1, suggesting that mammalian genomes lack *AGO2* and *RDE-1* orthologs. Unlike AGO2 and RDE-1, eIF2Cs along with *Drosophila* AGO-1 and *C. elegans* ALGs share in common a new motif, PRP, in addition to the PAZ and PIWI domains (Figures 1A and 1B).

To knock down human and mouse *eIF2C*s and *dicer*, we designed a series of siRNA, siECx (x = 1–4) and siDCR, the former being for *eIF2Cx* (x = 1–4) knockdown and the latter for *dicer*. We used long sequences that had been conserved between human and mouse target genes so that each siRNA was capable of simultaneously knocking down human and mouse target mRNAs (see Experimental Procedures section). siRNAs cognate to the firefly luciferase gene (*luc*; siFL), *DsRed* (siRED), or *EGFP* (siGFP) were also prepared. Prior to systematically analyzing possible suppressor activity of siRNAs for *eIF2C* or *dicer*, we wanted to discover whether mammalian cells are constitutively expressing *eIF2C1–4* and *dicer* mRNA and, if so, whether these mRNAs and their protein counterparts would be specifically and effectively eliminated by transfection of cognate siRNA. HeLa cells were transfected with various siRNAs at the concentration of 100 nM and possible change in mRNA expression levels in cells were first examined via semi-quantitative RT-PCR. Cells were collected 36 hr after siRNA transfection. All four *eIF2C* genes, along with *dicer*, were found to be constitutively expressed, and expression was effectively and specifically knocked down by cognate siRNAs (Figure 2A). As with mRNA, Dicer protein (Figure 2B) and eIF2C protein (our unpublished data) were significantly reduced in HeLa cells 36 hr after cognate siRNA treatment. Tubulin was used as an internal control. F9 cells also exhibited similar siDCR-dependent concentration reduction of Dicer protein (Figure 2B). Thus, *dicer* and *eIF2C* activities are concluded to be virtually completely eliminated in human and mouse cells by cognate siRNA treatment and a subsequent 36 hr incubation.

We examined possible inhibitory effects arising from siRNA transfection by using the dual luciferase system [3], in which firefly *luc* provides target mRNA while *Renilla luc* serves as the control. *luc* constructs were trans-

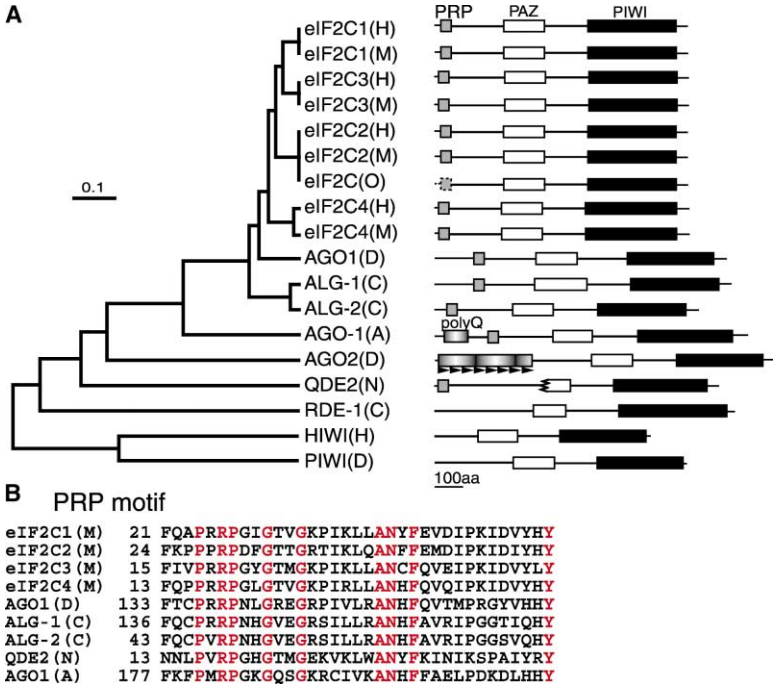

Figure 1. Phylogenetic Tree and Domain Structure of PIWI Family

(A) The phylogenetic tree of PIWI domains was constructed by the UPGMA method. Sequence data of PIWI family members other than those examined here were collected from a published database (National Center for Biotechnology Information). Abbreviations are as follows: eIF2C(O), rabbit (*Oryctolagus cuniculus*) eIF2C; D, *D. melanogaster*; A, *Arabidopsis thaliana*; N, *Neurospora crassa*; C, *Caenorhabditis.elegans*; H, *Homo sapiens*; and M, *Mus musculus*. Corresponding domain structures are shown on the right side. PAZ domains are shown as open boxes labeled with PAZ, and filled boxes represent PIWI domains. polyQ, Glutamine-rich sequences; the polyQ domain of *D. melanogaster* AGO2 includes eight repeats of 20–21 amino acids. PRP is a motif newly found in all 2C-type translation initiation factors so far examined. The amino acid sequence alignment of PRP is shown in (B), in which invariant amino acids are colored in red. Note that PRP in rabbit eIF2C is present in an N-terminal extension of the sequence published by Zou et al. (1998) [12]. Phylogenetic tree analysis (A) along with domain analysis (B) indicates that the *Drosophila* counterpart of eIF2Cs is not AGO2 but AGO1, whereas the *C. elegans* counterparts are ALGs.

fected simultaneously with siRNA, and the effect of siRNA treatment was examined 36 hr after transfection. As shown in Figure 2D, about 80% of firefly *luc* activity, normalized with *Renilla luc* activity, was abolished by siFL at the concentration of 20 nM, whereas 100 nM noncognate siRNAs (siGFP or siEC3) had only slight, if any, effects on firefly and *Renilla luc* expression in both NIH3T3 and HeLa cells (Figure 2C). One hundred nM siRNA for *EGFP*, each of the *eIF2C*s, or *dicer* was cotransfected with 20 nM siFL, and the effects on siFL-dependent knockdown of firefly *luc* activity in human (HeLa, P19) and mouse (NIH3T3, F9) cells were examined (Figure 2D). The RNAi effect due to siFL was almost completely abolished in all four cell lines subsequent to cotransfection of siDCR or siEC1, and the effect, if any, of siGFP (noncognate siRNA) was subtle. siRNAs corresponding to *eIF2C*s other than *eIF2C1* gave intermediate suppression effects.

siDCR and siEC1-dependent suppression of RNAi was also examined via systems other than the dual luciferase assay. Mouse F9 cells were doubly transfected with plasmids harboring *EGFP* and *DsRed* genes, and gene activity was knocked down by siGFP. Transfection efficiency was assessed as 80% and 40% after EGFP- and DsRed-positive cells, respectively, were counted; more than 99% of DsRed-positive cells were noted as being EGFP positive (Figure 2E$_{a–a''}$). Because of delayed DsRed fluorescence response, gene expression was examined 48 hr after transfection. Figure 2E$_{b–b''}$ indicates that effective and specific gene silencing is induced by siGFP. That cotransfection of siFL (noncognate siRNA) has little or no effect on siGFP-dependent gene silencing is evident from Figure 2E$_{c–c''}$. The effect of siGFP-dependent gene silencing was significantly abolished in the presence of siDCR (Figure 2E$_{d–d''}$) or siEC1 (Figure 2E$_{e–e''}$). siRED-dependent silencing of DsRed was also significantly abolished by siDCR or siEC1 (our unpublished data).

Based on these observations, we conclude that Dicer, eIF2C1, and probably other eIF2Cs are components essential for siRNA-mediated gene silencing in mammalian cells and hence that they are involved in the effecter step of mammalian RNAi. Recently, as with Dicer in

(*Figure 2 continued from page 43*)
siDCR, or siEC1–4 (100 nM each). As in (A), firefly *luc* activity relative to that of *Renilla luc* was normalized. As a normalization control, the firefly *luc* activity relative to that of *Renilla luc* in cells transfected with firefly and *Renilla luc* plasmids one of the following was used: siGFP, siDCR, or siEC1–4 (100 nM each). All four cell lines exhibited almost identical results so that they were averaged, and the average values are shown by thick horizontal bars.

(E) Suppression of siGFP-dependent posttranscriptional gene silencing by reduction of Dicer or eIF2C1 activity. F9 cells were transfected with plasmids directing the expression of target genes, EGFP and DsRed, in the presence (b–e) or absence (a) of various siRNA. (a–e) EGFP fluorescence signals are colored in green. (a′–e′) DsRed fluorescence signals are colored in red. (a″–e″) Merged pictures, in which yellow corresponds to cells strongly expressing both EGFP and DsRed. (a–a″) Control without siRNA transfection. About 80% and 40% of cells, respectively, were positive to EGFP (a) and DsRed (a′). Nearly all DsRed-positive cells appeared capable of expressing EGFP. (b–b″) Cells were transfected in the presence of siGFP. Virtually all EGFP signals disappered (b and b″), but DsRed signals were normal (b′). Panels in (c–c″) show that there is no substantial effect of cotransfection with siFL on siGFP-dependent posttranscriptional gene silencing. Compare (c) with (b) and (c″) with (b″). In contrast, siDCR (d–d″) and siEC1 (e–e″) exhibited strong suppression effects on siGFP-dependent posttranscriptional gene silencing.

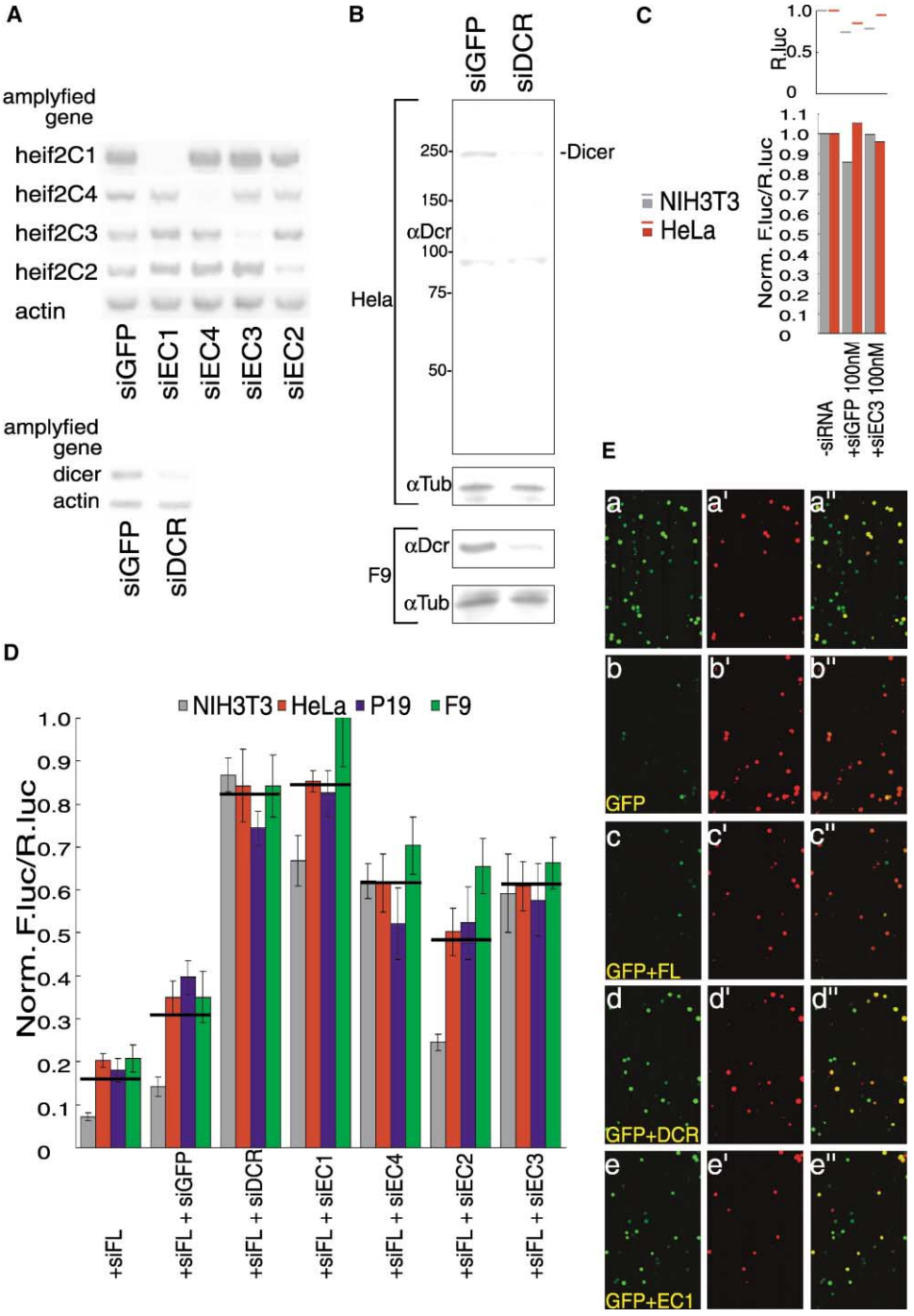

Figure 2. Effects of the Reduction of *dicer* or *eIF2C1-4* Activity on siRNA-Dependent Post-Transcriptional Gene Silencing

(A) *eIF2C1-4* and *dicer* are constitutively expressed in HeLa cells and that siRNA designed here are capable of cleaving target mRNA specifically. RT-PCR was carried out with RNA preparations without any substantial contamination of genomic DNA. The concentration of siRNA used for transfection was 100 nM.

(B) Dicer protein is specifically eliminated in HeLa and F9 cells by transfection of siDCR; irrelevant siRNA, siGFP, has no effect. Cells were lysed and analyzed by Western blotting with anti-Dicer and anti-Tubulin (control) antibodies.

(C) NIH3T3 and HeLa cells were cotransfected with plasmids directing the expression of firefly *luc* and *Renilla luc* in the presence or absence of siRNA. Note that there are no appreciable effects of 100 nM siRNA (siGFP or siEC3) unrelated in sequence to firefly *luc* on firefly *luc* expression. The value obtained in the absence of siRNA was used for normalizing *Renilla luc* activity. "Norm. F. luc/R. luc" is the normalized ratio of firefly and *Renilla luc* activity.

(D) Suppression of siFL-mediated posttranscriptional gene silencing upon cotransfection of siDCR or siEC1-4. Mouse (NIH3T3, F9) or human (HeLa, P19) cells were cotransfected with firefly and *Renilla luc* plasmids and a combination of siFL (20 nM) and one of the following: siGFP,

(*legend continues on page 42*)

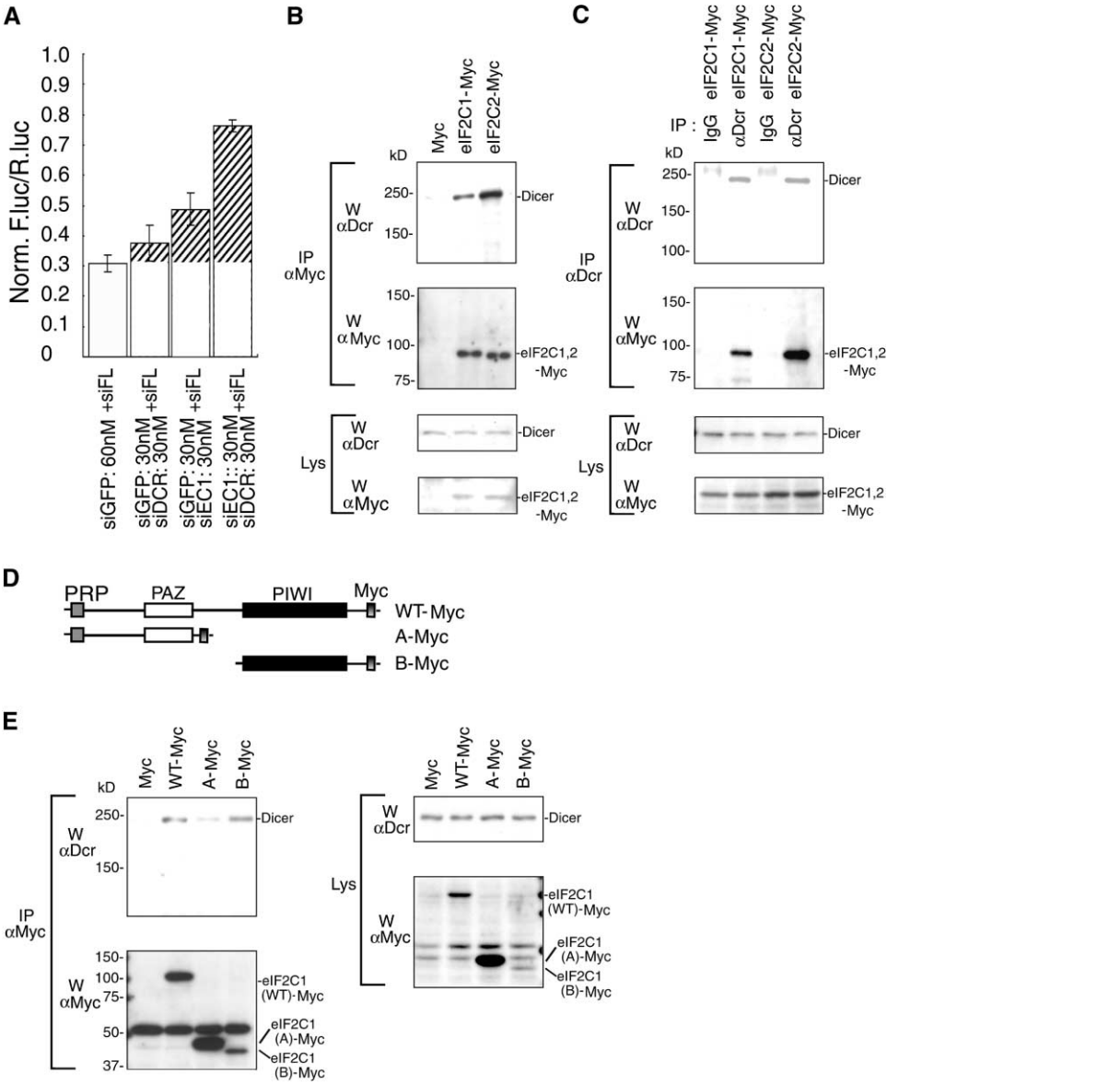

Figure 3. Genetic and Biochemical Analysis of Dicer/eIF2C1, 2 Interactions

(A) Possible synergistic interactions between Dicer and eIF2C1. F9 cells were cotransfected with *luc* expression plasmids and the indicated siRNAs. Relative *luc* activity was normalized as in Figure 2D. Note that siDCR or siEC1 at 30 nM gave only slight suppression effects (see hatched areas) on siFL-dependent gene silencing; siGFP serves as a negative control. In contrast, the suppression effect of double knockdown appears much greater than the sum of effects of single knockdown of *dicer* and *eIF2C1*.

(B and C) Coimmunoprecipitation experiments of Dicer and either eIF2C1 or eIF2C2 with anti-Dicer or anti-Myc antibodies. In (B), 293 cells transfected with the indicated constructs were lysed and analyzed by immunoprecipitation (IP) with anti-Myc followed by Western blotting (W) with anti-Dicer and anti-Myc. In (C), similar cell lysates were analyzed by IP with anti-Dicer or total IgG followed by Western blotting. These results strongly suggest that Dicer and eIF2C1 or 2 form complexes within 293 cells. In the lower panels labeled Lys, lysates were probed with anti-Dicer or anti-Myc.

(D) Diagrams of Myc-tagged deletion constructs of eIF2C1 are depicted. In A-Myc, the PIWI domain is deleted, whereas in B-Myc, PAZ and PRP are deleted. "WT-Myc" is Myc-tagged intact eIF2C1.

(E) 293 cells transfected with constructs depicted in (D) or an empty vector encoding only the Myc tag peptide were lysed and analyzed by anti-Myc IP followed by Western blot analysis (W) (left panels). The B-Myc/Dicer interaction appears much stronger than the interaction between WT-Myc and Dicer. In contrast, the A-Myc/Dicer interaction, if any, is very weak. Crude lysates (Lys) were also probed by Western blotting (right panels).

Drosophila [2], mouse Dicer was found to be required for long dsRNA cleavage in F9 cells [4]. Thus, the role of Dicer in mammalian RNAi may be 2-fold. It is required not only for the initial long dsRNA cleavage but also for subsequent effecter steps, which may include the formation of long dsRNA (potential Dicer substrate) by an RNA-dependent RNA polymerase [14, 15].

In *Drosophila* S2 cells, AGO2, a PIWI family member,

is a component of RNA-induced silencing complexes (RISC) and is capable of interacting with Dicer [11]. Thus, we investigated whether suppression of siFL-dependent gene silencing is enhanced in F9 cells doubly knocked down with *dicer* and *eIF2C1* (Figure 3A). Irrelevant siRNA, siGFP, was used to balance siRNA concentration. The concentration of siDCR and siEC1 for transfection was 30 nM, at which siFL-dependent gene silencing would only be slightly suppressed in single-knockdown cells. The occurrence of synergistic interactions between Dicer and eIF2C1 in siRNA-dependent mammalian gene silencing would be indicated by the result of double-knockdown cells (Figure 3A).

To further clarify this point, we carried out immunoprecipitation experiments by using the lysates of 293 cells transfected with Myc tag, Myc-tagged human eIF2C1, or mouse eIF2C2 constructs. Myc-tagged eIF2C1 and eIF2C2 were immunoprecipitated with anti-Myc antibody, and a clear association was found with Dicer in both cases (Figure 3B). Similarly, anti-Dicer antibody immunoprecipitated endogenous Dicer associated with Myc-tagged eIF2C1 or eIF2C2 (Figure 3C). Similar coimmunoprecipitation of Dicer and Myc-tagged eIF2C1 or eIF2C2 was also evident in HeLa and F9 cells (our unpublished data). It may thus follow that Dicer forms complexes with eIF2C1, eIF2C2, or possibly other eIF2Cs in mammalian cells.

Immunoprecipitation experiments were also carried out with the extracts of F9 and 293 cells cotransfected with siFL (an irrelevant siRNA) and constructs of Myc-tagged eIF2C1 or 2. That no apparent difference in the coimmunoprecipitation pattern of Dicer and Myc-tagged eIF2C proteins was found (our unpublished data) suggests that Dicer/eIF2C complex formation may occur independently of exogenous siRNA. A recent experiment indicated that miRNP, a ribonucleoprotein complex containing numerous micro RNAs, contains eIF2C2 as a protein constituent [16]. However, at present we do not know whether our putative Dicer/eIF2C complex is relevant to miRNP. Our experiment also could not reveal whether eIF2C proteins (eIF2C1–4) directly or indirectly interact with Dicer.

Finally, we assessed which domain of eIF2C proteins is essential for Dicer/eIF2C complex formation. The PAZ (with PRP) and PIWI domains of eIF2C1 were labeled with the Myc tag (Figure 3D), transiently expressed in 293 cells, and immunoprecipitated. Figure 3E shows that the PIWI domain (B-Myc) coimmunoprecipitated Dicer much more effectively than did intact eIF2C1 (WT-Myc), whereas only a marginal level of Dicer precipitation was induced by the PAZ domain (A-Myc). Note that production of B-Myc in cells was much less effective than that of A-Myc, whereas there was no apparent difference in Dicer production (see right panels). Thus, the PIWI domain is tentatively concluded to play a key role in Dicer/eIF2C interactions.

In conclusion, we found that Dicer and eIF2C1-4, capable of forming complexes in mammalian cells, are essential for siRNA-based gene silencing in mammals.

Experimental Procedures

Cell Culture

HeLa and NIH3T3 cells were cultured at 37°C in Earle's minimum essential medium (GibcoBRL) supplemented with 10% fetal bovine serum and Dulbecco's modified Eagle medium (DMEM, GibcoBRL) with 10% calf serum, respectively, whereas F9, 293, and P19 cells were cultured in DMEM supplemented with 10% fetal bovine serum. All mediums were supplemented with penicillin (30 μg/ml) and streptomycin (0.1 mg/ml). F9 and P19 were from IDAC at Tohoku University, and HeLa, 293, and NIH3T3 were from our laboratory stocks. Cells were regularly transferred to maintain exponential growth. A day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and transferred to 1.03 cm wells of the 48-well plate (Falcon).

Anti-Dicer Antibody Preparation

Anti-Dicer antibody was prepared with a C-terminal portion of human Dicer. A cDNA fragment of human *dicer* was PCR-amplified, digested with BclI and NotI, and a BclI/NotI fragment (position 4957–5772) was inserted into the BamHI/NotI site of pET-33b (Novagen). Bacterially expressed protein was isolated by Ni-NTA agarose (Qiagen) and used for immunizing rabbits. Antibody was used after affinity purification. Anti-Dicer antibody thus prepared was used for detection of human and mouse Dicer.

Cloning of Mouse and Human *dicer* and *eIF2C1-4* Genes

Total RNA was isolated with Trizol (Life Technologies) treatment from NIH3T3 or HeLa cells cultured in a 90 mm dish to a late log phase, treated with RQI DNase (Promega), and used for cDNA synthesis with Superscript II first strand synthesis systems. The nucleotide sequences of PCR primers used for amplification of mouse and human *dicer* and *eIF2C1-4* are available upon request. Amplified fragments were blunt-ended with T4 DNA polymerase and inserted into the EcoRV site of pBluescript II KS(−).

Plasmids and Recombinant Proteins

pGL3-Control and pRL-SV40 (Promega) encode firefly and *Renilla* luciferases, respectively. pEGFP-N1 encoding EGFP (enhanced green fluorescent protein) was from Clontech. pCAGGS-DsRed, an expression plasmid of DsRed, was a derivative of pDsRed1-1 (Clontech); the DsRed fragment of pDsRed1-1 was inserted into the EcoRI site of pCAGGS [17]. In pCAGGS-DsRed, gene expression is under the control of the CAG promoter. To produce Myc-epitope-tagged proteins in mammalian cells, pcDNAM (N.D., S.Z., and K.S., unpublished data), a derivative of pVP22/Myc-His (Invitrogen), was used. PCR-amplified *eIF2C1–4* genes or their derivatives were inserted into the BamHI/NotI site of pcDNAM.

siRNA Preparation

siRNAs were designed as 21 nt sense and antisense strands (dsRNA region: 19 bp) or 22 nt sense and antisense strands (dsRNA: 20 bp). siGFP corresponds in nucleotide sequence to position 290–312 of the EGFP coding sequence; siRED, 239–261 (DsRed coding sequence); siFL, 36–58 (firefly *luc*); siDCR, 60–82 (human *dicer*) or 30–52 (mouse *dicer*); siEC1, 262–285 (human *eIF2C1*) or 262–285 (mouse *eIF2C1*); siEC2, 746–769 (mouse *eIF2C2*) or 31–54 (human *eIF2C2* (XM_050334)); siEC3, 238–260 (human *eIF2C3*) or 238–260 (mouse *eIF2C3*); and siEC4, 201–224 (human *eIF2C4*) or 201–224 (mouse *eIF2C4*). Note that XM_050334 represents a truncated form of human eIF2C2. For siRNA annealing, sense and anti-sense single-stranded RNA (25 mM each) molecules were incubated in 10 mM Tris-HCl (pH 8.0) with 20 mM NaCl for 2 min at 90°C followed by 1 hr at 37°C. The concentration of resulting double-stranded siRNA was estimated by electrophoresis followed by ethidium bromide staining. The concentration of siRNA was presented based on that of single-stranded RNA.

Transfection

A day before transfection, cells (2×10^4 for HeLa and NIH3T3, 3×10^4 for F9 and P19, and 4×10^4 for 293) were trypsinized, diluted with fresh medium without antibiotics, and transferred to a 1.03 cm well. HeLa, 293, or NIH3T3 cells were transfected with plasmid DNA and/or siRNA by the use of LipofectAMINE PLUS (Invitrogen), and LipofectAMINE 2000 (Invitrogen) was used for F9 and P19.

luc Assay

The dual luciferase assay was performed as described previously [3]. In the case of HeLa, 293, or NIH3T3 transfection, 90 ng of pGL3-Control DNA and 10 ng of pRL-SV40 were used per well, whereas in F9 or P19 transfection, 160 ng of pGL3-Control and 40 ng of pRL-SV40 were used. Cell lysates were prepared 36 hr after transfection.

Immunoblotting of Endogenous Dicer

HeLa or F9 cells were collected 36 hr after cotransfection with 100 nM siDCR and irrelevant plasmid DNA (carrier DNA; 100 and 200 ng for HeLa and F9, respectively), washed with phosphate-buffered saline (PBS), and treated with 10% trichloroacetic acid. Cells were collected by centrifugation at 7,000 g for 20 min at 4°C. Pellets were suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, sonicated, clarified by centrifugation, neutralized with 1 M Tris, and subjected to SDS-PAGE and Western blotting. Monoclonal anti-Tubulin was purchased from ICN.

EGFP/DsRed Fluorescence Assay

F9 cells were transfected with 100 ng of pEGFP-N1 (Clontech) and 100 ng of pCAGGS-DsRed along with the indicated amounts of siRNA. Because of the delayed fluorescence response of DsRed, this assay was carried out 48 hr after transfection. Cells were trypsinized and loaded on a hemocytometer, and fluorescence was detected under a confocal microscope (Bio-Rad).

RT-PCR Assay of Endogenous *eIF2C1-4* and *dicer*

HeLa cells (2×10^4 cells/well) were transfected with 100 nM of a given siRNA and 100 ng of pEGFP-N1 encoding EGFP. The transfection efficiency of pEGFP-N1 was estimated at 60%–80%. After cells were collected from five wells and lysed with Trizol, a total RNA was prepared. The RNA solution was treated with RQI DNase and then with phenol-chroloform-isoamylalcohol. RNA was precipitated with ethanol and dissolved in RNase-free water. Half of the RNA sample thus obtained was used for first-strand cDNA synthesis, and the remaining sample was used for a negative control. The nucleotide sequences of RT-PCR primers are available on request.

Immunoprecipitation Assay

For coimmunoprecipitations, 293 cells (1.5×10^6 cells/60 mm dish) transfected with Myc constructs and collected 42 hr after transfection were lysed in 1% NP-40 lysis buffer (with 20 mM Hepes [pH 7.3], 1 mM $CaCl_2$, 1 mM $Mg(OAc)_2$, 125 mM KOAc, 5 mM EGTA, 1 mM dithiothreitol, and Complete Protease Inhibitor [Roche]). After being clarified by a centrifugation at 15,000 g for 20 min at 4°C, lysates were immunoprecipitated with anti-Dicer antibody, total IgG, or anti-c-Myc (Ab-1; oncogene) antibody followed by protein G sepharose (for anti-c-Myc, SIGMA) or proteinA agarose (for anti-Dicer and IgG, Gibco BRL). Pellets were washed four times with lysis buffer. After SDS-PAGE and Immobilon P (Millipore) transfer, proteins were visualized via immunoblotting with indicated antibodies and subsequent secondary-antibody treatment (AP-conjugated-goat-affinity-purified antibody to rabbit (or mouse) IgG [Cappel]) and chemiluminescence (CDP-Star detection reagent [Amersham Pharmacia Biotech]).

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Accession Numbers

GenBank accession numbers are as follows: human *dicer* (helicase-MOI), AB028449 [18]; mouse *dicer*, AB081470 (this work); human *eIF2C1* (*eIF2C1*), NM_012199; human *eIF2C3* (FLJ12765), XM_029051; human *eIF2C4* (KIAA1567), AB046787; mouse *eIF2C1*, AB081471 (this work); mouse *eIF2C2*, AB081472 (this work); mouse *eIF2C3*, AB081473 (this work); mouse *eIF2C4*, AB081474 (this work).