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Identification of Components of RNAi Pathways Using the Tandem Affinity Purification Method

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1. Introduction

RNA interference (RNAi) is rapidly becoming a standard laboratory technique for understanding and regulating the function of specific genes in evolutionarily diverse organisms, including plants, *Caenorhabditis elegans*, *Drosophila*, and mammalian cells ***(1–10)***. RNAi is initiated by the conversion of double-stranded RNA (dsRNA) into 21- to 23-nucleotide (nt) fragments of dsRNA by Dicer enzymes. These short, interfering RNAs, or siRNAs as they are known, are incorporated into an RNAi effector complex, the RNA-induced silencing complex (RISC), which uses them as guides to target and destroy complementary messenger RNA (mRNA). Recent findings point to a tight connection between microRNA (miRNA) and RNAi molecular machineries. Recent study also has led to the unmasking of a widespread biological regulatory mechanism involving miRNAs ***(11–17)***, and there is a wide agreement that the core RNAi machinery carries out numerous cellular functions by an endogenous pathway important for normal development in many organisms, including gene regulation, virus resistance, and chromatin remodeling ***(1–17)***. Although some components of the RNAi cellular machinery have been identified, the overall picture is far from clear. We describe the tandem affinity purification (TAP) method ***(18–20)*** to isolate protein components of RISC in cultured *Drosophila* Schneider-2 (S2) cells. This purification method has allowed us to identify several RISC components, including Argonaute 2 (AGO2), the *Drosophila* homolog of fragile X mental retardation protein (dFMR1), and a DEAD-box RNA helicase Dmp68 ***(21,22)***. Identification of components of RNAi/miRNA pathways by the TAP method eliminates the need for large-scale sample

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preparation and overcomes, for example, the potential hazards associated with the use of a radioisotope, thereby placing the method within the scope of the average laboratory.

The expression of TAP-tagged AGO2, which is an essential component for a siRNA-directed RNAi response ***(22,23)***, will be used to illustrate the method utilized for isolation of components of RNAi. This method will be useful for studying many aspects of RNAi/miRNA machineries.

2. Materials

1. *Drosophila* Schneider-2 (S2) cells.
2. pRmHa-C-FLAG-His expression vector.
3. pRmHa-TAP expression vector.
4. Schneider's *Drosophila* medium.
5. Spinner bottles.
6. CELLFECTIN.
7. pCoBlast.
8. Blastcidin S Hydrochloride.
9. TAP buffer: 10 m*M* Tris-HCl (pH 8.0); 150 m*M* NaCl; 0.5% Triton X-100 (can be replaced by 0.05–0.1% NP-40); protease inhibitors.
10. IgG Sepharose.
11. TEV protease.
12. TEV buffer: 10X Stock buffer and ditheithreitol (DTT) solution are supplied with TEV enzyme when purchased from Invitrogen. Prepare 1X TEV buffer from the stock solution according to the manufacturer's instruction.
13. Calmodulin affinity resin.
14. Calmodulin-binding buffer: 10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.5% Triton X-100; 10 m*M* β-Mercaptoethanol, 1 m*M* MgOAc, 1 m*M* Imidazole, 2 m*M* $CaCl_2$.
15. Calmodulin elution buffer: 10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.5% Triton X-100; 10 m*M* β-Mercaptoethanol; 1 m*M* MgOAc, 1 m*M* Imidazole, 2 m*M* EGTA (the concentration of EGTA can be higher (up to 10 m*M*) if the elution efficiency is lower than desired).
16. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
17. Coomassie brilliant blue staining solution: 0.22% Coomassie blue R; 50% Methanol; 10% Acetic acid.
18. SYPRO Ruby (Molecular Probes, Inc.). SYPRO Ruby staining is done according to the manufacture's manual (*see* **Subheading 3.4.**).
19. Silver staining kit (Wako, Inc.). Silver staining is done according to the manufacturer's manual (*see* **Subheading 3.4.**).

3. Methods

The methods described below outline (1) the construction of the TAP-tagged expression plasmid, (2) the transfection of the plasmid DNA and the induction of protein expression, (3) the purification of the TAP-tagged protein and its

associated proteins from S2 cells, and (4) the identification of associated proteins by mass spectrometric peptide sequencing.

3.1. Expression Plasmid

The pRmHa-C expression system was a kind gift of Dr. Frank Lafont ***(24)*** and is based on the pRmHa-3 ***(25)***. pRmHa-3 contains the *Drosophila* metallothionein promoter, which is activated by Cu^{2+} and a polyadenylation signal. pRmHa-3 was modified by introducing a multiple cloning site (*Eco*RI-*Sac*I-*Nhe*I-*Kpn*I-*Sma*I-*Bam*HI-Flag tag-*Eco*RV-10 × His-*Sal*I), FLAG-tag, and His × 10 tag downstream of the metallothionein promoter to generate pRmHa-C ***(25)***. The pBS1479 plasmid was a kind gift of Dr. Bertrand Seraphin ***(18,19)*** and is a yeast expression vector that contains a TAP tag consisting of two IgG-binding domains of *Staphylococcus aureus* protein A and a calmodulin-binding peptide (CBP) separated by a TEV protease cleavage site. A *Bam*HI-*Hin*dIII fragment containing the TAP tag of pBS1479 was subcloned into pBluScript SK (Stratagene, Inc.), which had been cut with *Bam*HI and *Hin*dIII. A recombinant plasmid was isolated and designated pBlue-TAP. A *Bam*HI-*Sal*I fragment of pBlue-TAP was subcloned into pRmHa-C that had been digested with *Bam*HI and *Sal*I, and the desired plasmid was designated pRmHa-TAP. A cDNA encoding a protein of interest (in this case, AGO2) was subcloned into a multiple cloning site (*Eco*RI-*Sac*I-*Nhe*I-*Kpn*I-*Sma*I-*Bam*HI) of the plasmid (*see* **Note 1**), which gave rise to a C-terminal TAP tag fusion gene (*see* **Fig. 1**).

3.2. Protein Induction

The next steps in this process involve the transfection of the Schneider line 2 (S2) of *Drosophila* with the expression plasmid for TAP-tagged AGO2 followed by induction with $CuSO_4$ to achieve the production of the fusion protein.

3.2.1. S2 Cells

The S2 line is a highly versatile expression system, useful for both the analysis of gene function and generating substantial quantities of expressed protein ***(25,26)***. It was originally derived from primary cultures of late-stage *Drosophila melanogaster* male embryos. S2 cells have also been demonstrated to be amenable to RNAi, thereby permitting selective silencing of gene activity.

The S2 cells were grown at room temperature (25°C) under normal atmosphere in Schneider's *Drosophila* medium (Gibco, Inc.) supplemented with 10% fetal calf serum (FCS) at densities between 5×10^5 and 5×10^6 cells/mL. Mass cultures were grown in 1-L spinner bottles in 400 mL of medium.

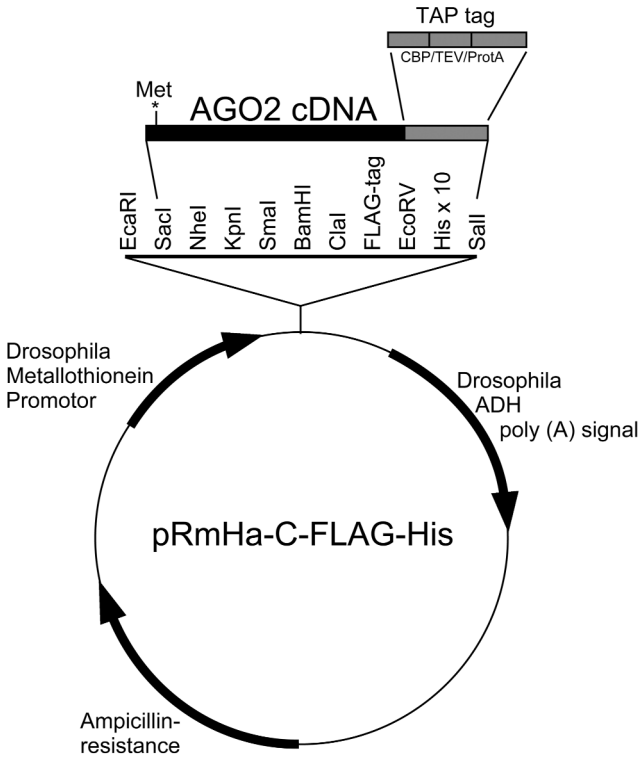

Fig. 1. Schematic drawing of pRmHa-AGO2-TAP. The TAP is tagged at the C-terminus of AGO2. CBP: calmodulin-binding peptide; TEV: TEV protease cleavage site; ProtA: two IgG binding domains of *Staphylococcus aureus* protein A.

3.2.2. Transfection

Transfection was performed using CELLFECTIN (Life Technologies, Inc.) as follows:

1. Put 250 µL of FCS-free medium in each of two 1.7-mL Eppendorf tubes.
   Tube 1: Add 2–6 µg of plasmid DNA into the tube and mix well by pipetting. If wishing to make a stable line, add 0.1–0.3 µg of a plasmid containing cDNA of a blasticidin-resistant gene (pCoBlast; Invitrogen, Inc.) additionally.
   Tube 2: Add 8 µL of CELLFECTIN into the tube and mix well by pipetting.
2. Add the well-mixed DNA solution into the CELLFECTIN solution tube and mix well by pipetting.
3. Incubate at room temperature (RT) for 20 min.
4. During the incubation time, wash S2 cells (about 1×10^7 cells) with FCS-free medium one or two times.
5. Resuspend the cells with 0.8 mL of FCS-free medium in a 15-mL conical tube.

6. Add the DNA/CELLFECTIN solution into the cell suspension and mix by gentle tapping.
7. Incubate the mixture for 3 h at 25°C.
8. After 3 h incubation, spin down the cells at 400–700*g* for 5 min. Discard the supernatant and resuspend the cells with 8 mL of S2 medium containing 10% FCS and incubate at 25°C.
9. For heterologous protein expression, on d 2, one-half of the transfected cells are incubated with $CuSO_4$ at 1 m*M*, and cells are harvested after 12 h.
10. To generate stably transfected S2 cell lines, on the same day, add blasticidin (Blastcidin S Hydrochloride; Waken, Inc.) at 25 µg/mL to the other half of the transfected cells. After 2–3 wk of selection, resistant clones have grown. Cells are replated in fresh medium every 4 d over the selection period. These clones are kept together and are used as polyclonal cell lines. Note that it is difficult to obtain monoclonal S2 cell lines mainly because S2 cells grow poorly at low densities. Frozen stocks are kept in 80% FCS, 10% dimethyl sulfoxide at –80°C.

3.3. TAP Purification

Protein purification by the TAP method is performed as follows.

1. Harvest S2 cells expressing the TAP-tagged protein of interest.
2. Wash the cells twice with cold PBS.
3. Resuspend the cells in cold TAP buffer. One milliliter of TAP buffer is needed per 10^7–10^8 cells.
4. Pass the cell suspension through a 25-gauge needle attached to a syringe five times. During this step, the sample should be kept on ice. If cells are not disrupted enough at this point, pass the sample several times through a 30-gauge needle after the 25-gauge needle passage to ensure cell lysis.
5. Transfer the sample to 1.7-mL microtubes and spin at 16,000*g* for 1–2 min at 4°C.
6. Take the supernatant and transfer it to new microtubes. This is the cytoplasmic lysate used for further purification steps. If you wish to obtain the whole-cell lysate at this point, the cell suspension at **step 4** should be sonicated on ice and **steps 5** and **6** then followed.
7. The supernatant is now mixed with IgG Sepharose™ 6 Fast Flow beads (Amersham Biosciences, Inc.) in microtubes and rocked for 1–2 h at 4°C. Prior to this step, an aliquot of the IgG beads (approx 30–40 µL) is placed into a 1.7-mL microtube, washed with TAP buffer several times, and kept on ice.
8. Wash the IgG beads with TAP buffer five times (*see* **Note 2**). After the last wash, spin the tubes briefly to collect residual buffers to the bottom, suck them up, and discard.
9. Wash with TEV buffer once. Drain the beads well, as in **step 8**. Add TEV buffer to the beads. The buffer volume used here is about 500 µL per tube.
10. Add TEV (Invitrogen) to the beads. The enzyme volume added is 1/100 vol of the buffer. Rock at 16°C for 2 h.
11. Spin at 16,000*g* for 1–2 min at 4°C. Transfer the supernatant into new microtubes.

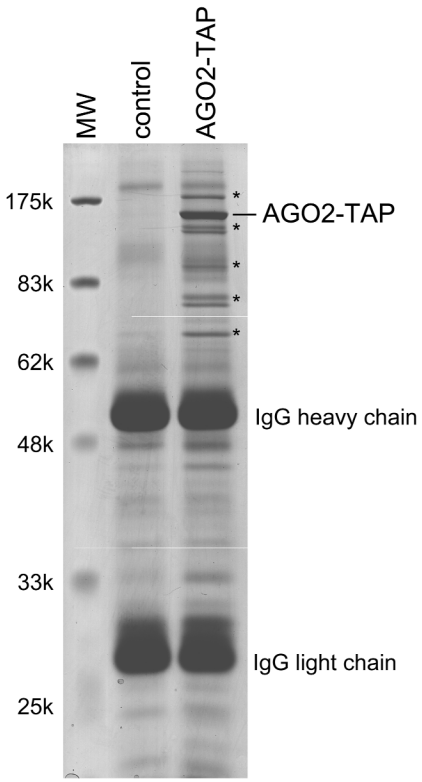

Fig. 2. Purification of proteins that associate with AGO2 by using a C-terminal TAP tag. The protein components in the "IgG bound" obtained from S2 cells expressing the C-terminal TAP-tagged AGO2 (AGO2-TAP) and the parental cells (control) were resolved on SDS-PAGE and visualized by Coomassie blue staining. Several distinct bands (indicated with asterisks) are observed only in the AGO2–TAP lane. The purification was done from 150 mL of S2 cell culture. The left lane represents molecular markers.

12. Add 100 µL of TEV buffer to the beads, and mix well by tapping. Take the supernatant after the spinning at 16,000*g* for 1–2 min at 4°C and combine it with the sample obtained at **step 11**.
13. Spin again at 16,000*g* for 1–2 min at 4°C to make sure all of the beads sediment at the bottom of the tubes. Take the supernatant and transfer it to new tubes.
14. Add 3 mL of calmodulin-binding buffer and 2 µL of 1 *M* $CaCl_2$ per 1 mL of the sample.
15. Mix with calmodulin affinity resin (Stratagene, Inc.) and rock for 1 h at 4°C. An aliquot of the calmodulin beads (approx 30–40 µL) is placed in each microtube and washed with calmodulin-binding buffer several times prior to use.
16. Wash the beads with calmodulin-binding buffer five times. After the last wash, spin briefly to collect the residual buffer to the bottom and drain well.

17. Add EGTA-containing elution buffer to the beads; rock at RT for 15 min or longer to elute the TAP complexes. If the volume is higher than desired at this point, perform a trichloroacetic acid (TCA) precipitation to concentrate the protein sample.
18. Run the sample on a protein gel and stain to visualize the protein bands.

3.4. Identification of TAP Affinity Purified Proteins

The final extracts were visualized on SDS-PAGE gels by Coomassie brilliant blue (ICN Biomedicals, Inc.) staining, SYPRO Ruby staining using a SYPRO Ruby Protein gel stain kit (Molecular Probes, Inc.), or silver staining using SilverQuest Silver Staining kit (Invitrogen) or Silver Stain II kit (Wako, Inc). Stained bands (*see* **Fig. 2**) were excised from the gels, digested with trypsin, and processed for mass spectrometric fingerprinting as described ***(21)***. Using mass spectrometry and the complete *Drosophila* genome (the Celera/Berkeley *Drosophila* protein database), it is relatively easy to identify each of the specific bands on the gels.

4. Notes

1. Usually, standard DNA cloning procedures can be used to introduce the C-terminal TAP tag in frame with the coding region of the protein of interest. The upstream sequence of the initiation ATG is often very important for getting sufficient expression levels of the TAP-tagged protein of interest. If the Kozak sequence of the gene of interest is not close to the consensus Kozak sequence of *Drosophila melanogaster*, you might need to modify the sequence upstream (and downstream) of the initiation ATG of the gene.
2. If the background is high when you visualize proteins recovered following TAP purification, you might use a higher salt concentration in the washing buffer. However, of course, a high salt level would disrupt the interaction of TAP-tagged proteins with proteins. Therefore, you might have to selectively adjust TAP purification conditions (salt and/or detergent concentrations, incubation time for binding, and so on).

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