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Post-transcriptional gene silencing activity of human GIGYF2

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ABSTRACT

In mammalian post-transcriptional gene silencing, the Argonaute protein AGO2 indirectly recruits translation inhibitors, deadenylase complexes, and decapping factors to microRNA-targeted mRNAs, thereby repressing mRNA translation and accelerating mRNA decay. However, the exact composition and assembly pathway of the microRNA-induced silencing complex are not completely elucidated. As the GYF domain of human GIGYF2 was shown to bind AGO2 in pulldown experiments, we wondered whether GIGYF2 could be a novel protein component of the microRNA-induced silencing complex. Here we show that full-length GIGYF2 coimmunoprecipitates with AGO2 in human cells, and demonstrate that, upon tethering to a reporter mRNA, GIGYF2 exhibits strong, dose-dependent silencing activity, involving both mRNA destabilization and translational repression.

1. Introduction

MicroRNA (miRNA)-mediated post-transcriptional gene silencing (PTGS) is a widespread, evolutionarily conserved, mode of gene expression regulation. A miRNA-loaded Argonaute protein (AGO1-4 in human) constitutes the core of the miRNA-induced silencing complex (miRISC), which directs miRNA annealing to fully or partially complementary sequences within hundreds of target mRNAs. In mammalian cells, PTGS can be achieved through AGO2-mediated cleavage of perfectly complementary targets. Otherwise, AGO recruits additional factors to induce PTGS through translational repression and mRNA destabilization. In this pathway, a protein of the GW182 family acts as a scaffold connecting AGO2 to downstream effector proteins involved in mRNA translation, deadenylation, decapping and 5′-to-3′ degradation (recently reviewed in Refs. [1,2]).

Substantial progress has been made recently in understanding how the miRISC may assemble and elicit PTGS in mammals. Human cells express three *GW182* paralogs, which encode the trinucleotide repeat-containing (TNRC) proteins TNRC6A, B and C. Each TNRC6 protein can be divided into an N-terminal AGO-binding domain (ABD) and a C-terminal silencing domain (SD). The PAN2-PAN3 and CCR4-NOT deadenylase complexes are recruited through interaction of TNRC6-SD with their PAN3 and CNOT1 subunits, respectively. The mRNA poly(A) tail is first shortened by the distributive action of PAN2-PAN3. Then, CCR4-NOT processively deadenylates mRNA. CNOT1 also binds the RNA helicase DDX6 (also known as p54/RCK), which associates with the catalytic core of the mRNA decapping complex DCP1-DCP2. DCP1 in turn interacts with the 5′-to-3′ exoribonuclease XRN1, leading to the complete degradation of deadenylated and decapped mRNA. However, besides triggering mRNA decay, the CCR4-NOT complex may be involved in the repression of mRNA translation, through activation of the ATPase activity of DDX6, as well as displacement of eIF4A from mRNA [1,2]. Nevertheless, it remains likely that not all miRISC components have been identified yet, and that there is no single way to build a PTGS-competent complex.

GIGYF2, also known as TNRC15, is a conserved, GYF domain-containing, mammalian protein discovered in a yeast two-hybrid screen using the proline-rich N-terminal region of mouse growth factor receptor-bound protein 10 (Grb10) as a bait [3]. GIGYF2, like TNRC6, is a W-rich protein harboring several Q-repeat regions. A characteristic peptide signature, GPF-X_4-[M/V/I]-X_2-W-X_3-GYF, hallmarks GYF domains, which recognize PPGΦ motifs in a structurally conserved manner. Indeed, the GYF domain of GIGYF2 is

Abbreviations: ABD, AGO-binding domain; AGO, Argonaute; Grb, growth factor receptor-bound; miRNA, microRNA; PABP, poly(A)-binding protein; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; SD, silencing domain; TNRC, trinucleotide repeat-containing.

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required for its interaction with Grb10. Beyond its implication in insulin-like growth factor 1 signal transduction, the biological activities of GIGYF2 have not been extensively investigated. Interestingly, the GYF domain of human GIGYF2 is related to that of Smy2, a yeast cytoplasmic protein suggested to control mRNA translation and decay. In addition, pulldown experiments identified AGO2 among proteins interacting with the GIGYF2 GYF domain in HeLa cell extracts [4]. Moreover, GIGYF2 has been implicated in a translational repressor complex in mammals [5]. These observations prompted us to ask whether GIGYF2 could be a novel component of the miRISC.

The involvement of various proteins in the miRNA-mediated silencing pathway has been studied through tethering to reporter mRNAs. In a founding experiment, an AGO2 protein fused to a 22-amino-acid peptide of bacteriophage λ N protein was tethered to the 3′-UTR of a luciferase reporter mRNA harboring five λN-binding *BoxB* sequences, which led to a tenfold reduction in luciferase expression in HeLa cells [6]. A similar approach later demonstrated that tethered TNRC6A, B or C is able to silence reporter mRNA expression in the absence of AGO [7,8]. In the same way, we decided to investigate whether GIGYF2 tethering to mRNA could induce PTGS.

2. Materials and methods

2.1. Cells

HEK293 Tet-On cells (Clontech 631182), stably expressing the tetracycline-regulated transactivator Tet-On, are cultured in Dulbecco's modified Eagle's medium supplemented with 10% [v/v] heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen).

2.2. Antibodies

We used as primary antibodies: mouse monoclonal anti-HA IgG (Sigma H3663) and rabbit polyclonal anti-AGO2 IgG (Upstate Biochemicals 07-590), and as secondary antibodies: horseradish peroxydase (HRP)-conjugated anti-mouse IgG (Sigma A4416) and fluorescein-conjugated anti-mouse IgG (Rockland 610-1202).

2.3. Plasmids

pBiFluo [9] was created by inserting the EGFP and DsRed cDNAs (Clontech) on both sides of the tetracycline-regulated bidirectional CMV promoter in pBi-Tet (Clontech). To construct pBiFluo-6BoxB [10], an *Xho*I linker, inserted into a *Bgl*II site in the *EGFP* 3′UTR of pBiFluo, was used to sequentially introduce three copies of a *Sal*I-*Xho*I synthetic DNA fragment including two *BoxB* motifs from the bacteriophage λ genome, following the stepwise cloning strategy designed by Gehring et al. [11]. pCIneo-λN-HA-GIGYF2 encodes HA-tagged GIGYF2 fused to a 22-amino-acid peptide of bacteriophage λ N protein. It was constructed by replacing the *Eco*RI-*Not*I fragment covering the AGO1 ORF in pCIneo-λN-HA-AGO1 (gift from W. Filipowicz, Basel, Switzerland) by an *Eco*RI-*Not*I fragment including the GIGYF2 ORF excised from the GIGYF2 variant 1 cDNA (Biovalley). Similarly, pCIneo-λN-HA-BFP was obtained by substituting the AGO1 ORF with the BFP ORF of pTagBFP-N (Evrogen). Excision of the λN-coding sequence of pCIneo-λN-HA-GIGYF2 by *Nhe*I + *Xho*I restriction, Klenow fill-in and religation produced pCIneo-HA-GIGYF2. pCIneo-λN-HA-TNRC6B (gift from E. Izaurralde, Tübingen, Germany) encodes TNRC6B variant 2 [7]. pCIneo-λN-HA-TNRC6B-ΔSD is a pCIneo-λN-HA-TNRC6B derivative deleted from the sequence encoding TNRC6B amino acids 995-1722 [10].

2.4. Transfection

HEK293 Tet-On cells were seeded at 10^4 cells/cm^2 and transfected 24 h later using Fugene reagent (Promega, 3 μl/μg DNA). In tethering experiments, 2 μg DNA were introduced per well (9 cm^2), including reporter plasmid + effector plasmid (or control DNA) at a 2:1 ratio. In dose-response experiments, the amount of reporter plasmid was unchanged, decreasing amounts of effector plasmid were introduced, and control DNA was added to reach 2 μg total DNA/well. Expression of the reporter plasmid was induced by doxycycline (Sigma D9891, 1 μg/ml) 24 h post-transfection. Cells were scraped in Hank's balanced salt solution (Invitrogen) diluted to 20% [v/v] in PBS (1.5 ml/well) 48 h post-transfection for analysis by spectrofluorimetry or immunoblotting.

2.5. Spectrofluorimetry

Cells were lysed by addition of NP40 to 1% [v/v], followed by shaking at 120 rpm for 30 min at 4 °C on a Labnet Shaker 20. Lysates were cleared by centrifugation at 20,000*g* for 10 min at 4 °C. Fluorescence intensities were measured with a Cary spectrofluorimeter (excitation/emission wavelengths: 489/508 nm for EGFP, 557/579 nm for DsRed).

2.6. Immunoblotting

Cells were centrifuged for 5 min at 400*g* and lysed in 2 volumes RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 0.5% [v/v] NP40, 1 mM EGTA) in the presence of Complete® protease inhibitor cocktail (Roche) at 4 °C. After shaking for 30 min at 120 rpm, lysates were cleared by centrifugation at 20,000*g* for 10 min at 4 °C. Proteins were quantified by the Bradford assay (BioRad) and stored in aliquots at −80 °C. Samples (30 μg proteins) were separated by standard SDS-polyacrylamide gel electrophoresis through 8% [w/v] polyacrylamide gels (or 12% for BFP detection) together with prestained protein molecular weight markers (Thermo Fisher Scientific 26619). Electrotransfer to nitrocellulose was performed in Tris-glycine buffer for 1 h at 200 mA. Incubation with primary antibody was set up overnight at 4 °C. HRP-conjugated secondary antibody was applied for 1 h at room temperature and revealed by incubation in Amersham ECL detection reagent, followed by exposure to Hyperfilms MP (GE Healthcare).

2.7. RT-qPCR

Total RNA was extracted 48 h post-transfection, using the SV Total RNA Isolation System (Promega Z3100) following manufacturer's instructions. Briefly, cells were scraped in 350 μl RLA buffer/well (9 cm^2), the cell lysate was treated with DNAase I, separated on a spin column, and RNA was eluted in 100 μl H_2O. 300 ng RNA were reverse-transcribed by ProtoScript II RT (Biolabs M0368) in the presence of 6 μM random hexamer primers (Promega C1181). cDNA was quantified by qPCR in a Light Cycler® 1.5 instrument (Roche) using the Light Cycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche 03515869001). Primer pairs used were: 5′-ATCCGCCACAACATCG-3′ and 5′-TGTGATCGCGCTTCTC-3′ for EGFP; 5′-TACTGCTCCACGATGGTGTAGTC-3′ and 5′-CAAGTCCATCTACATGGCCAAGAAG-3′ for DsRed; 5′-GACCTGACCTGCCGTCTAG-3′ and 5′-GCCCAGGATGCCCTTGAG-3′ for GAPDH (Eurogentec).

2.8. Immunoprecipitation

Cells were transfected in 150 cm^2-dishes with 10 μg effector plasmid (or control DNA). 48 h post-transfection, cells were scraped