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Human Dicer C-terminus functions as a 5-lipoxygenase binding domain

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ABSTRACT

Dicer is a multidomain ribonuclease III enzyme involved in the biogenesis of microRNAs (miRNAs) in the vast majority of eukaryotes. In human, Dicer has been shown to interact with cellular proteins via its N-terminal domain. Here, we demonstrate the ability of Dicer C-terminus to interact with 5-lipoxygenase (5LO), an enzyme involved in the biosynthesis of inflammatory mediators, *in vitro* and in cultured human cells. Yeast two-hybrid and GST binding assays delineated the smallest *5-l*ipoxygenase *b*inding *d*omain (5LObd) of Dicer to its C-terminal 140 amino acids comprising the double-stranded RNA (dsRNA) binding domain (dsRBD). The Dicer 5LObd–5LO association was disrupted upon Ala substitution of Trp residues 13, 75 and 102 in 5LO, suggesting that the Dicer 5LObd may recognize 5LO via its N-terminal C2-like domain. Whereas a catalytically active 5LObd-containing Dicer fragment was found to enhance 5LO enzymatic activity *in vitro*, human 5LO modified the miRNA precursor processing activity of Dicer. Providing a link between miRNA-mediated regulation of gene expression and inflammation, our results suggest that the formation of miRNAs may be regulated by 5LO in leukocytes and cancer cells expressing this lipoxygenase.

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1. Introduction

As a member of the ribonuclease (RNase) III family of enzymes, Dicer is a large multidomain protein that plays an essential role in the biogenesis of microRNAs (miRNAs) in the vast majority of eukaryotes, including humans [1–4]. Human Dicer is a protein of 1912 amino acid residues composed of several domains: an N-terminal putative ATPase/helicase domain containing a DECH box, a domain of unknown function (DUF283), a central PIWI/Ago/Zwille (PAZ) domain, and a C-terminal RNase III domain, composed of tandem RNase III motifs and a C-terminal double-stranded RNA (dsRNA) binding domain (dsRBD) (reviewed in [5]). Human Dicer has been proposed to recognize the terminal 3′ overhangs of miRNA precursors (pre-miRNAs) through its PAZ domain [6] and to process its substrates through intramolecular dimerization of its two RNase III motifs, assisted by the flanking RNA binding domains PAZ and dsRBD [4].

Although the RNase III processing activity and enzyme properties of human Dicer have been well characterized [2–4], the assessment and validation of its anticipated multiple functionalities remains incomplete. While the structure of its C-terminal RNase III domain (residues 1660–1852) has been reported [7], characterization of the several predicted domains of the 217-kDa human Dicer remains challenging and is currently being extrapolated mainly from studies performed on RNase III-related enzymes [8], ancestral, minimal or reengineered forms of Dicer from distantly related lower eukaryotes [6], such as *Giardia intestinalis* [9,10]. Highly conserved through evolution, Dicer exhibits striking differences at various levels. For example, the human genome harbors, like that of *S. pombe* and *C. elegans*, a single Dicer form, in contrast to two and four Dicer isoforms in *Drosophila melanogaster* and *Arabidopsis thaliana*, respectively [5].

Abbreviations: AD, activating domain; Ade, adenine; a.a., amino acids; ATP, adenosine triphosphate; BD, binding domain; C-term, C-terminal domain; ds, double-stranded; dsRBD, dsRNA binding domain; DTT, dithiothreitol; GSH, glutathione; GST, glutathione S-transferase; His, histidine; HPLC, high pressure liquid chromatography; IB, immunoblot; LO, lipoxygenase; miRNA, microRNA; nt, nucleotide; OAG, 1-oleoyl-2-acetylglycerol; PACT, Protein kinase R activator; PAZ, Piwi/Argonaute/Zwille; PC, phosphatidylcholine; PCR, polymerase chain reaction; RNAi, RNA interference; RNase, ribonuclease; 17-OH-22:4, 17(*S*)-hydroxy-7(Z),10(Z),13(Z),15(E)-docosatetraenoic acid; 5LObd, 5-lipoxygenase binding domain; 5-HPETE, 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid; 5-HETE, 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid

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These enzymes also differ in terms of sequences, domain composition and functionalities. For instance, the function of the dsRBD present in Dicer enzymes remains unclear in view of the observations that (i) the C-terminal dsRBD is dispensable for processing activity in *Escherichia coli* RNase III [11], (ii) *Giardia* Dicer lacks a dsRBD domains [9], and (iii) an RNase III naturally lacking the dsRBD functions in *B. subtilis* [12].

Concerning the molecular context in which Dicer operates in cells, three mammalian Dicer-interacting proteins have been identified so far: Argonaute 2 (Ago2), transactivating response RNA-binding protein (TRBP) and protein kinase R activator (PACT). Ago2 was found in immunoprecipitates prepared from *Drosophila* S2 cells expressing an epitope-tagged version of Dicer-1 (Dcr-1) [13]. A study by Tahbaz et al. [14] extended these findings to mammalian cells and determined that Dicer·Ago2 complex formation may involve a direct interaction between a subregion of the PIWI domain of Ago2 and the RNase III domain of Dicer. Using coimmunoprecipitation strategies in cultured human cells, two research groups reported the identification of TRBP as a Dicer-interacting protein [15,16]. TRBP was shown to facilitate Dicer-mediated cleavage of pre-miRNAs *in vitro* and to be required for optimal RNA silencing *in vivo* [15,16]. A similar role was proposed for PACT [17]. In fact, both TRBP and PACT were found to interact with the N-terminal region of Dicer that contains the putative ATPase/helicase domain.

Human Dicer partial cDNA clones were initially isolated from a yeast two-hybrid screen using 5-lipoxygenase (5LO) as bait [18]. In humans, 5LO is expressed mainly in differentiated inflammatory cells, such as granulocytes, monocytes/macrophages, mast cells, dendritic cells, and B lymphocytes, as reviewed in [19]. This lipoxygenase catalyzes the first two steps in the biosynthesis of leukotrienes, which are potent mediators of inflammation [20,21]. The 5LO enzyme activity depends on prosthetic iron in the C-terminal catalytic domain (residues 121–673), whereas its C2-like N-terminal β-sandwich (residues 1–114) binds Ca^{2+}, leading to Ca^{2+} stimulation of enzyme activity [22]. Previously shown to bind phosphatidylcholine (PC) [36], the 5LO β-sandwich was also found to mediate interaction with proteins, such as coactosin-like protein (CLP) [23].

In this study, we sought to validate and characterize the interaction between human Dicer and 5LO, and identified a 140-amino acid (a.a.), dsRBD-containing, C-terminal domain of Dicer (referred to as *5-l*ipoxygenase *b*inding *d*omain, or 5LObd) as a protein interacting module recognized by the N-terminal C2-like domain of 5LO. Whereas 5LO enzyme activity is slightly enhanced by a 5LObd-containing fragment of Dicer, 5LO modified the enzymatic properties of Dicer. Modulating each others enzymatic activity, the functional implications of the interaction between human Dicer and 5LO may provide a link between miRNA-mediated regulation of gene expression and inflammatory processes.

2. Materials and methods

2.1. Plasmid DNA constructs

Various deletion mutants of human Dicer were amplified by polymerase chain reaction (PCR) and cloned in frame into the BamHI/SalI or SalI sites of pACT2 (Clontech). The pGBT9-5LO 62-673 vector was prepared by cloning the PCR-amplified inserts in frame into the EcoRI/SalI sites of pGBT9 (Clontech). The presence and orientation of the insert was verified by restriction analysis and at least two bacterial clones were tested.

The open reading frames of human platelet-type 12LO (acc. no. M58704), rat brain 12LO (acc. no. L06040) and human 15LO type I (acc. no. M23892) were directionally cloned into pGBT9 and sequenced.

A cDNA fragment encoding human Dicer C-terminal domain (C-term; composed of a.a. 1238–1912) was amplified by PCR, digested and ligated into the BamHI/XhoI restriction sites of the pcDNA3.1-5′ Flag vector, as described previously [3]. A pcDNA3.1-5LO-HA expression construct was created by inserting the human 5LO open reading frame into a pcDNA3.1 vector containing a C-terminal HA epitope inserted into the XhoI/ApaI restriction sites. The constructs were verified by DNA sequencing.

2.2. Yeast two-hybrid system

Yeast two-hybrid strain (PJ69-4A), vectors (pGBT9-5LO, pGBT9-SNF1, pACT2-SNF4) and reporter gene assays were described previously [18,24,25]. The known two-hybrid interactors SNF1 and SNF4 were used as a positive control [18].

2.3. Protein determination and immunoblot analysis

The protein concentrations were determined by the method of Bradford [26] using the Bio-Rad dye reagent, with bovine serum albumin as standard.

Yeast protein extracts or protein suspensions were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) using the Mini Protean system (Bio-Rad), transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences), immunoblotted, and the immunoreactive proteins visualized as described previously [18,24,25,27].

2.4. Confocal immunofluorescence microscopy

Confocal immunofluorescence microscopy was performed essentially as described previously [3]. Briefly, HeLa cells were grown on sterile glass coverslips coated with poly-L-lysine in Dulbecco's minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator under 5% CO_2 at 37 °C. Cells were transfected with plasmid constructs encoding epitope-tagged 5LO and Dicer C-term proteins using Lipofectamine 2000 (Invitrogen) and harvested 20 h post-transfection. Cells were then washed in phosphate buffered saline (PBS), fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated in blocking buffer (PBS containing 10% FBS). Cells were subsequently incubated with mouse monoclonal anti-HA clone 12CA5 (dilution 1/200; Roche) and rabbit polyclonal anti-Flag (dilution 1/200; Santa Cruz Biotechnologies, Inc.) antibodies, respectively. After extensive washing in PBS, the cells were incubated with Alexa Fluor 488 (green)-conjugated goat anti-mouse or Alexa Fluor 546 (red)-conjugated goat anti-rabbit secondary antibodies (dilutions 1/500; Molecular Probes). After extensive washing in PBS, the coverslips were mounted on slides with Prolong Gold antifade reagent (Molecular Probes). Labeling of HeLa cells was visualized with an inverted Olympus IX70 microscope (90× magnification), and images were prepared with Image J 1.38× software.

2.5. Immunoprecipitation experiments

HEK 293 cells were transiently transfected with plasmid constructs encoding epitope-tagged 5LO, 5LO W13/75/102A and/or Dicer C-term proteins by the calcium phosphate method and harvested 48 h later. Cells were washed twice with ice-cold PBS and solubilised with 1 ml of lysis buffer composed of 50 mM Tris·HCl, 137 mM NaCl, 1% Triton X-100, 1 mM PMSF, pH 8.0, supplemented with complete protease inhibitor cocktail (Roche). The lysate was kept on ice for 15 min, centrifuged at 13,000 *g* for 1 min, and the supernatant preserved. An aliquot of the supernatant was kept for protein determination by Bradford and analysis of 5LO and Dicer C-term protein expression by immunoblotting. Cell lysates (2 mg proteins) were incubated with 1 µg of anti-Flag M2 antibody (Sigma) for 1 h at 4 °C under continuous rotation. Ten (10) µl of pre-washed Protein G agarose beads (Roche) was added and the incubations continued for an additional 3 h. For anti-HA immunoprecipitations, 15 µl of anti-HA affinity matrix (rat anti-HA 3F10 linked to agarose beads) (Roche) was used. The beads

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