Stimuli-dependent cleavage of Dicer during apoptosis

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miRNAs (microRNAs) play important roles in diverse physiological processes, including stress response, apoptosis and carcinogenesis. Even though the role of individual miRNAs has been demonstrated, expression of proteins involved in miRNA production in response to acute stress or harmful agents has not been extensively investigated. Here, we have studied the role of Dicer, one of the central proteins of the miRNA processing machinery during apoptosis, and show that down-regulation of Dicer results in accelerated apoptosis of HeLa cells, triggered by TNFα (tumour necrosis factor α). We have also investigated the integrity of Dicer, and provide evidence that Dicer is a target for caspases during apoptosis. The cleavage of Dicer is stimuli-dependent and more pronounced when apoptosis is induced by PKC (protein kinase C) inhibitors, and can also be observed in HIV-1-infected cells at late stages of infection. Thus the apoptotic machinery may regulate the miRNA pathway by affecting individual proteins, such as Dicer.

Key words: apoptosis, caspase, cleavage, Dicer, HIV, microRNA.

INTRODUCTION

Exposure of mammalian cells to acute stress or harmful agents induces compensatory activation of multiple intracellular signalling pathways. These effects can play critical roles in controlling cell survival in a stress-specific and cell-type-dependent manner and may lead to apoptosis [1,2]. Activation of TNFα (tumour necrosis factor α) is one of the prime endogenous stress signals that induce apoptosis [3–5]. Considerable progress has been made in understanding of the involvement of pathways, individual proteins and genes has been demonstrated. Much less, however, is known about the effect of stress-related apoptosis on the RNAi (RNA interference) machinery. This system is directly involved in control of expression of numerous genes and acts through miRNAs (microRNAs). miRNAs have been shown to play essential roles in diverse biological and pathological processes, including cell proliferation, differentiation, apoptosis and carcinogenesis [6–9]. In light of miRNA involvement in modulating cellular phenotypes, it is also believed that miRNAs might play a role in regulating the response to stress. Reaction of the cells to acute stress or other harmful stimuli requires the fast activation and repression of genes encoding appropriate proteins. Rapid activation of certain genes can be achieved through RNAi. Indeed, it has been shown that CAT-1 (cationic amino acid transporter 1) mRNA can be relieved from miRNA-122-mediated repression in human HuH7 hepatoma cells when they are subjected to different types of stress [10]. Furthermore, miRNA-23a, miRNA-27a and miRNA-24-2 were shown to be up-regulated during cardiac hypertrophy, and cardiac-specific miRNA-208 was required for response to stress and hormonal signalling [11]. Since several hundreds of miRNAs have been predicted and many are not characterized yet, especially in the context of apoptosis induction, it is difficult to estimate the contribution of each individual miRNA, and to make an appropriate conclusion.

An alternative approach for testing the importance of miRNAs is to knockdown enzymes responsible for processing miRNAs to their mature, active form. Mature miRNAs are generated by sequential processing by a series of RNase III-related enzymes. Drosophila produces a precursor miRNA hairpin transcript of 70 nt from a longer primary RNA [12]. This 70-nt hairpin is then cleaved by a second RNase III enzyme, Dicer, to yield the 22-nt mature miRNA [13,14]. Because there is only a single copy of Dicer in the human genome, knockdown of Dicer should theoretically produce cells that are deficient in miRNAs.

Here, we show that knockdown of Dicer significantly modulates the response to harmful agents and leads to accelerated apoptosis of HeLa cells treated with several pro-apoptotic agents. Moreover, Dicer can also be down-regulated through caspase-mediated cleavage during apoptosis. Our results point to an additional layer of regulation of apoptosis and suggest that Dicer, and RNAi in general, may be involved in this process.

MATERIALS AND METHODS

Cell culture

HeLa cells were grown in DMEM (Dulbecco’s minimal essential medium) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Antibodies and plasmids

Rabbit polyclonal antibodies against PARP (poly(ADP-ribose) polymerase) were purchased from Transduction Laboratories, mouse mAb (monoclonal antibody) against Dicer was from Clonogene and mouse mAbs against α-tubulin and β-actin were from Santa Cruz Biotechnology. Anti-c-Myc polyclonal rabbit antibody was purchased from Santa Cruz Biotechnology.

Abbreviations used: Ago, Argonaute; CHX, cycloheximide; CK18, cytokeratin 18; DCL1, Dicer-like 1 protein; dsRNA, double-stranded RNA; dsRBD, dsRNA-binding domain; GFP, green fluorescent protein; mAb, monoclonal antibody; miRNA, microRNA; MOI, multiplicity of infection; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; RDRC, RNA-directed RNA polymerase complex; RNAi, RNA interference; siDicer, siRNA target sequence of Dicer; siGFP, siRNA against GFP; siRNA, small interfering RNA; TNFα, tumour necrosis factor α; wt, wild-type; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone.

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Plasmid pcDNA3.1-5′-Myc-Dicer expressing human Dicer cDNA [15] was kindly provided by Professor P. Provost (Université Laval, Ste-Foy, QC, Canada).

### Apoptosis induction and inhibitors

Apoptosis in HeLa cells was induced with a combination of 10 ng/ml of TNFα (Sigma) and 20 μg/ml of CHX (cycloheximide; Sigma) or with 100 μM of etoposide (Biovision), 1 μM of staurosporine (Biovision) and 10 μM of chelerythrine (Biovision). The caspase inhibitor Z-DEVD-FMK (benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; Alexis Biochemicals) was used at a concentration of 40 μM.

#### siRNA (small interfering RNA)-mediated knockdown of Dicer and transfection

siRNA against GFP (green fluorescent protein) was purchased from Dharmacon. siDicer (siRNA target sequence of Dicer) has been published previously [16]. siRNAs or plasmid DNAs were transfected into cells with Lipofectamine™ 2000 (Invitrogen) or HiPerfect (Qiagen) according to the manufacturer’s instructions.

### Site-directed mutagenesis

Exchange of the aspartic acid residue at position 1644 of the Dicer amino acid sequence with an alanine residue was performed by using 5’-GATGTATGTTTGTCACTACGCTGCAGATAA-ACACTGAA-3’ oligonucleotide and processed by a Quick-Change® Multi Site-directed Mutagenesis kit (Stratagene) following the manufacturer’s instructions.

### ELISA

Supernatant from apoptotic cells was used for quantification of apoptosis by utilizing a catcher monoclonal ‘M5’ antibody directed against an epitope on the 284–396 fragment of CK18 (cytokeratin 18) and a horseradish peroxidase-conjugated ‘M30’ antibody directed against CK18Asp596 neoepitope in M30-Apoptosense ELISA (Peviva) following the manufacturer’s instructions. The relative levels of soluble caspase-cleaved fragments of CK18 are expressed as an increase in M30 antigen (fold) in treated cells compared with untreated control cells.

The activity of caspase 3 was determined in cell lysates by using a labelled caspase 3 substrate (Caspase 3 Colorimetric Assay kit; Alexis Biochemicals) and expressed as fold stimulation by dividing the caspase 3 activity in treated cells by the activity in untreated control cells.

### Western-blot analysis

Cells were lysed in RIPA buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 1 mM dithiothreitol) for 20 min on ice. Lysates were cleared by centrifugation for 10 min at 7000 g, and protein contents were estimated employing a Bradford reagent (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS/PAGE and blotted on to nitrocellulose membranes and probed with different antibodies.

#### In vitro cleavage assay

A 1 μl portion of recombinant Dicer (Stratagene) was incubated with 3 μl of recombinant caspase 3 (Biovision) or 10 μg of lysates extracted from non-treated or apoptotic HeLa cells in a single caspase 3 dilution buffer (Biovision) for 1 h at 37°C. The mixture was then separated by SDS/PAGE, and Western blotting was performed using anti-Dicer antibody.

### Viruses and viral infections

Poliovirus type 1 was provided by Dr J. Pavlovic (Institute of Medical Virology, Zurich, Switzerland) and used for infection of HeLa cells. HIV-1 IIIB was used for infection of the HTLV (human T-cell lymphotrophic virus)-1-transformed T-cell line C81/66. For infection, cells were washed with PBS and infected with HIV-1 at MOI (multiplicity of infection) = 0.01 for 60 min at 37°C or poliovirus at MOI = 0.1 for 60 min at 37°C. The inoculum was aspirated and cells were incubated with fresh medium. At the indicated time points, cells were trypsinized, proteins were extracted and Western blotting was performed using anti-Dicer antibody.

### Statistical analysis

Bars in the Figures represent the means ± S.D. for three independent experiments. Statistical analysis was performed using a Student’s t test.

### RESULTS

#### The rate of apoptosis in Dicer knockdown HeLa cells

In the present study, we examined the implication of Dicer in apoptosis induced by TNFα and other agents. Because TNFα activates not only death signals, but also survival signals that are mediated by the activation of NF-κB (nuclear factor κB) transcription factor [17], we used CHX to block survival signals mediated through protein synthesis. In order to study the function of Dicer, we have employed the technique of gene silencing using siRNA oligonucleotides. We transfected HeLa cells with siRNAs against Dicer, designated as siDicer, siGFP (siRNA against GFP) served as a control. We could achieve 70–80% down-regulation of Dicer as measured on the protein level (Figure 1A). At 48 h after transfection, the cells were treated with a combination of TNFα and CHX (TNFα/CHX). The treatment of transfected cells with TNFα/CHX resulted in increased mortality of siDicer-transfected cells compared with siGFP-transfected control cells (Figures 1B and 2A). To confirm that the increased rate of cell death was due to apoptosis, we tested the caspase-mediated cleavage of CK18 in Dicer knockdown cells by quantitative M30-Apoptosense ELISA assay and measured caspase 3 activity. In both cases, siDicer-transfected TNFα/CHX-treated cells were undergoing accelerated apoptosis compared with control siGFP-transfected cells (Figures 1C and 1D). Another hallmark of apoptosis is the caspase-mediated cleavage of the nuclear 116 kDa protein PARP to a smaller 85 kDa inactive form [18]. Immunoblot analysis revealed an increased cleavage of PARP in Dicer knockdown cells (Figure 1E). This cleavage was more pronounced at later points of TNFα/CHX-treatment (Figure 1F). These results show that down-regulation of Dicer leads to accelerated apoptosis of TNFα/CHX-treated cells.

#### Suppression of accelerated apoptosis in Dicer knockdown cells by caspase inhibitor

Apoptosis-related caspase activation can be efficiently suppressed by a cell-permeable, non-toxic inhibitor, Z-DEVD-FMK, which binds irreversibly to the activated caspase 3 and some other caspasas in apoptotic cells [19]. The pre-incubation of transfected cells with Z-DEVD-FMK prior to TNFα/CHX treatment reduced the enhanced level of cell death in siDicer-transfected cells.

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Apoptosis-dependent cleavage of Dicer

Figure 1 Knockdown of Dicer leads to accelerated apoptosis of TNFα/CHX-treated HeLa cells

(A) Efficiency of down-regulation of Dicer in HeLa cells. Cells were transfected with siRNAs against GFP and Dicer respectively. After 48 h, proteins were extracted from transfected cells, and Western-blot analysis was performed. (B) Knockdown of Dicer affects the survival of HeLa cells after treatment with TNFα/CHX. Cells were transfected with siRNAs against GFP and Dicer respectively and, 48 h later, incubated with TNFα/CHX for 0, 1, 2 and 4 h and analysed by light microscopy at 100× magnification. (C) Supernatants from treated cells were used in a quantitative M30-Apoptosense ELISA. The relative levels of soluble caspase-cleaved fragments of CK18 are expressed as an increase in M30 antigen (fold) in treated cells compared with untreated siGFP-transfected cells. (D) Cells were lysed 4 h post-treatment and lysates were subjected to a caspase 3 colorimetric assay. The activity of caspase 3 is expressed as fold stimulation by dividing the caspase 3 activity in treated cells by the activity in untreated siGFP-transfected cells. (E) Lysates were also analysed by Western blotting performed 3 h after TNFα/CHX treatment or (F) 4 h post-treatment by using an antibody recognizing full and cleaved forms of PARP (upper and lower bands respectively). Error bars represent the S.D. for three independent experiments, and statistically significant differences between siGFP- and siDicer-transfected cells at individual time points are indicated by asterisks (Student’s t test; ∗P < 0.05; ∗∗P < 0.01).

(C) Cleavage of Dicer during apoptosis

To study further the role of endogenous Dicer during TNFα/CHX-induced apoptosis, we analysed the pattern of expression of Dicer in HeLa cells using an antibody directed against the N-terminus of Dicer. We could detect an additional smaller product migrating as a 180–190 kDa band (whereas full-length Dicer migrates as a 240–250 kDa band), which appeared after treatment of cells with TNFα/CHX (Figure 3A). This product is specific for the N-terminus of Dicer, since a similar band was also seen in cells transfected with pcDNA3.1-5′Myc-Dicer (pcDNA-Dicer), and this fragment could be detected by anti-Myc antibody, recognizing Myc epitope on the N-terminus of overexpressed Dicer (Figure 3B). Interestingly, prolonged incubation of cells with TNFα/CHX showed that proteolytic cleavage of Dicer was increased in a time-dependent manner (Figure 3C).

The involvement of caspases in cleavage of Dicer

In order to study the involvement of caspases in degradation of Dicer, HeLa cells were pre-incubated with the caspase inhibitor Z-DEVD-FMK prior to the induction of apoptosis. As can be seen in Figure 4(B), Dicer cleavage product was not detected, suggesting that caspases are involved in the cleavage of Dicer. Since Dicer is cleaved into a fragment migrating as a 185 kDa band, we expected to identify at least one cleavage site for caspases on the C-terminus of Dicer. Using the GraBCas.jar program (http://www.uniklinikum-saarland.de/de/einrichtungen/fachrichtungen/humangenetik/Software), we could find a cleavage site for caspase 3/7 at the aspartic acid residue at position 1644 of the Dicer amino acid sequence (cleavage site DHPD↓A).

Overexpression of Dicer mutated at Asp1644 to an alanine residue (mutDicer), which destroys the caspase 3/7 cleavage site (DHPAA), generated a much weaker cleavage product (Figure 4C). This result suggests that Asp1644 is indeed a caspase 3/7 cleavage site. However, since the cleavage was not completely prevented, we cannot rule out the involvement of other apoptosis-activated proteolytic enzymes. To investigate further the possibility that caspases are responsible for Dicer cleavage, recombinant Dicer was incubated with recombinant caspase 3 or...
A. A. Matskevich and K. Moelling

Figure 2 Accelerated apoptosis of Dicer knockdown cells is suppressed by a caspase inhibitor

(A) HeLa cells were transfected with siRNAs against GFP or Dicer or mock-treated and, 48 h later, treated with 1% DMSO for 2 h. Cells were washed and subsequently treated with TNFα/CHX. At indicated time points, cells were trypsinized, Trypan Blue-stained, and live cells were counted. Error bars represent the S.D. for three independent experiments, and statistically significant differences between siGFP- and siDicer-transfected DMSO-treated cells at 3 and 4 h after treatment with TNFα/CHX are indicated by asterisks (Student's t-test; ***P < 0.01). (B) Similar to (A), but transfected cells were pretreated with the caspase inhibitor Z-DEVD-FMK (Z-DEVD), instead of DMSO. (C) HeLa cells were transfected with siRNAs against GFP or Dicer and treated 48 h later with TNFα/CHX for 1 h. Cells were washed and subsequently treated with 1% DMSO or Z-DEVD-FMK for 2 h. Trypan Blue-stained, and live cells were counted. Error bars represent the S.D. for three independent experiments, and statistically significant differences between siGFP- and siDicer-transfected cells are indicated by asterisks (Student's t-test; ***P < 0.01). (D) HeLa cells, treated as described in (C), were lysed and lysates were analysed by Western blotting.

Figure 3 Dicer is cleaved during apoptosis induced by TNFα/CHX

(A) HeLa cells were transfected with siRNAs against Dicer or mock-treated and, 48 h later, treated with TNFα/CHX for 1.5 h (+) or left untreated (−). Cells were then lysed and lysates were analysed by Western blotting. (B) HeLa cells were transfected with a plasmid expressing Dicer (pcDNA3.1-5′Myc-Dicer; pcDNA-Dicer) or empty vector (pcDNA), lysed 48 h later and lysates were analysed by Western blotting. (C) HeLa cells were transfected with TNFα/CHX and, at indicated time points (hours), cells were washed and fresh medium was applied. Cells were lysed 6 h later and lysates were analysed by Western blotting. Arrows point to full and cleaved forms of Dicer.

in the presence of a lysate extracted from apoptotic HeLa cells that contains active caspases [20]. Lysate obtained from non-treated cells served as a control. Although in this in vitro assay we could not detect the cleavage product (the 185 kDa fragment appears to be unstable under these conditions), the amount of intact Dicer was reduced not when control lysate was used but in the presence of caspase 3 and lysate extracted from apoptotic cells (Figure 4D). Taken together, these results suggest that caspases, and specifically caspase 3, are involved in degradation of Dicer. Another unexpected observation is that incubation of cells overexpressing either wtDicer (wild-type Dicer) or mutDicer with TNFα/CHX made cells slightly more susceptible to the treatment compared with control pcDNA-transfected cells (Figures 4E and 4F, please see the Discussion section).

Stimuli-dependent cleavage of Dicer after treatment with pro-apoptotic agents

Next, we investigated whether the cleavage of Dicer is stimulated dependent. We could observe only a small amount of cleavage product when HeLa cells were incubated with the apoptosis-inducing agent etoposide, an inhibitor of topoisomerase II [21] (Figure 5A). Since a link between TNFα and PKC (protein kinase C) has been demonstrated [22], we have also tested inhibitors of PKC. Incubation of HeLa cells with chelerythrine, an inhibitor of PKC [23], led to much stronger cleavage of Dicer.
Apoptosis-dependent cleavage of Dicer

**Figure 4** Caspases are involved in cleavage of Dicer

(A) Schematic representation of domains of human Dicer: helicase/ATPase (41–217 amino acids), DUF 283 (domain with unidentified functions; 423–592), PAZ (Piwi-Ago-Zwille; 881–1032), RNase IIIa (1266–1814), RNase IIIb (1656–1814), RB (dsRBD; 1839–1904). The arrow indicates the position Asp1644, a potential caspase 3 cleavage site. (B) HeLa cells were pre-incubated with Z-DEVD-FMK for 2 h and then treated with TNFα/CHX. At indicated time points (hours), cells were washed and fresh medium was added. Cells were lysed 6 h later, and lysates were analysed by Western blotting. (C) HeLa cells were transfected with a plasmid expressing wtDicer or mutDicer and, 48 h later, treated with TNFα/CHX for 1 h. Cells were then lysed and lysates were analysed by Western blotting. (D) Recombinant Dicer was incubated with recombinant caspase 3 or in the presence of lysates extracted from non-treated or apoptotic HeLa cells as described in the Materials and methods section. The mixture was then subjected to Western-blot analysis. (E) Cells were transfected with wtDicer, mutDicer or pcDNA and, 48 h later, incubated with TNFα/CHX for 0, 2 and 4 h. Supernatants from treated cells were used in a quantitative M30-Apoptosense ELISA. The relative levels of soluble caspase-cleaved fragments of CK18 are expressed as an increase in M30-antigen (fold) in treated cells compared with untreated pcDNA-transfected cells. (F) Cells were lysed 4 h post-treatment and lysates were subjected to caspase 3 colorimetric assay. The activity of caspase 3 is expressed as fold stimulation by dividing the caspase 3 activity in treated cells by the activity in untreated pcDNA-transfected cells. Error bars represent the S.D. for three independent experiments, and statistically significant differences between pcDNA- and wtDicer- or mutDicer-transfected cells at individual time points are indicated by asterisks (Student’s t test; n.s., not significant; *P < 0.05).

Cleavage of Dicer in virus-infected cells

Finally, we have attempted to investigate whether the cleavage of Dicer could be seen in other pathological situations, which lead to apoptosis. In doing so, we examined the pattern of expression of Dicer in HeLa cells infected with poliovirus type 1 and in the transformed C81/66 T-cell line infected with HIV-1. Since cleavage of Dicer in cells treated with pro-apoptotic agents was more pronounced at late stages of apoptosis, here we also focused on the late stages of infection. Cells infected with poliovirus were collected 36 h later, when more than 50% of the cells were dead. HIV-infected cells were analysed 15 days post-infection, when syncytia formation was observed, and HIV-1 p24 production reached the maximal level. As can be seen in Figures 5A and 6(B), cleavage of Dicer could be detected at late stages of infection with HIV-1, and not in poliovirus-infected cells. These results indicate that degradation of Dicer can be observed in virus-infected cells, and suggest that HIV-1 has developed a mechanism for inactivation of Dicer – the caspase-dependent cleavage at late stages of infection.

DISCUSSION

miRNAs are involved in various pathological processes, including cellular stress [10,25,26]; however, the expression of the miRNA machinery proteins in stress-related apoptosis has not been...
A. A. Matskevich and K. Moelling

Figure 5  Cleavage of Dicer during apoptosis is stimuli-dependent

(A–C) HeLa cells were incubated with (A) etoposide, (B) chelerythrine or (C) staurosporine. At indicated time points (hours) cells were lysed and lysates were analysed by Western blotting. Arrows point to full and cleaved forms of Dicer. (D–F) Cells were transfected with siRNAs against GFP and Dicer and 48 h later incubated with (D) etoposide, (E) chelerythrine or (F) staurosporine for indicated times. Supernatants from treated cells were used in a quantitative M30-Apoptosense ELISA. The relative levels of soluble caspase-cleaved fragments of CK18 are expressed as an increase in M30-antigen (fold) in treated cells compared with untreated siGFP-transfected cells. Error bars represent the S.D. for three independent experiments, and statistically significant differences between siGFP- and siDicer-transfected cells at individual time points are indicated by asterisks (Student’s t test; ∗P < 0.05; ∗∗P < 0.01). (G) Cells treated with etoposide and chelerythrine were collected 24 h post-treatment, and cells treated with staurosporine were collected 4 h post-treatment. Cells were then lysed, and lysates were subjected to a caspase 3 colorimetric assay. The activity of caspase 3 is expressed as fold stimulation by dividing the caspase 3 activity in treated cells by the activity in untreated siGFP-transfected cells. Error bars represent the S.D. for three independent experiments, and statistically significant differences between siGFP- and siDicer-transfected cells after individual treatments are indicated by asterisks (Student’s t test; ∗P < 0.05; ∗∗P < 0.01).

Extensively investigated. In the present study, we have examined the role of Dicer, one of the central proteins involved in the miRNA machinery, during apoptosis and show that down-regulation of Dicer results in accelerated apoptosis of HeLa cells. We also investigated the effect of apoptosis on the integrity of Dicer, and show that Dicer is a target for caspasases during apoptosis in HeLa cells.

The cleavage of Dicer occurs with similar kinetics to the cleavage of PARP. An interesting result we obtained is that a decreased level of Dicer (achieved either with artificial knock-down of Dicer by siRNA or by cleavage of Dicer at the late stages of apoptosis) correlated with a markedly increased amount of cleaved PARP compared with full-length PARP (Figures 1E, 1F and 5C). These results suggest that expression or stability of cleaved PARP during apoptosis can be controlled by Dicer. This direct or indirect regulation can be exerted through not yet identified miRNAs, production of which is strictly dependent on the function of Dicer.

We observed that overexpression of wtDicer led to the appearance of a 185 kDa fragment of Dicer in the absence of TNFα/CHX, and incubation of cells overexpressing either wtDicer or mutDicer with TNFα/CHX made cells slightly more susceptible to the treatment compared with control pcDNA-transfected cells (Figures 3B, 4E and 4F). These unexpected results can be
explained by the fact that the function of Dicer is dependent on assembly with two or more other factors [27,28], and over-expression of a single protein Dicer might titrate out these other factors, resulting in the increased formation of inactive complexes that contain only a subset of the required components [29]. The presence of such improperly assembled complexes may affect the RNAi machinery and other pathways, thus triggering the activation of caspases that cleave Dicer and making cells more susceptible to the treatment with pro-apoptotic agents.

In our study, both overexpression and down-regulation of Dicer led to accelerated apoptosis of TNFα/CHX-treated cells, suggesting that the balanced expression of Dicer may be required for optimal response to pro-apoptotic signals. A similar situation has been described for the scaffold-like protein ASK2 (apoptosis signal-regulating kinase 2) in serum-starved cells [30].

Cleavage of Dicer occurs between RNase IIIa and RNase IIIb domains, thus releasing a C-terminal fragment containing RNase IIIb and dsRBBDs [dsRNA (double-stranded RNA)-binding domains] (Figure 4A) [14]. It has been demonstrated that the fission yeast Dicer (Dcr1) is physically associated with the RDRc (RNA-directed RNA polymerase complex) via its C-terminal domain. Truncations of this domain abolish Dcr1–RDRC association, siRNA generation and gene silencing in vivo [31]. The requirement of a C-terminal dsRBD for RNA cleavage has been shown for human Dicer [14,15] and for in vivo function of Arabidopsis DCL1 (Dicer-like 1 protein) [32]. Implication of the C-terminus in protein–protein interactions between DCL4 and its dsRNA-binding partner in Arabidopsis was also demonstrated [33]. Thus cleavage of Dicer and release of a fragment containing RNase IIIb and dsRBD may disrupt not only miRNA production but also interaction of Dicer with other proteins and may lead to several effects [loss of signal transmission for miRNA production and destabilization of RISC (RNA-induced silencing complex)] and may affect other factors that interact with Dicer. It remains to be investigated whether the cleavage and thus inactivation of Dicer may lead to a local decrease in binding partners of human Dicer, such as Ago (Argonaute) proteins (proteins associated with miRNAs that bind miRNAs through partial base-pairings to primarily repress translation) and TRBP (HIV transactivating response RNA-binding protein) [27]. This situation might play a role during the late phases of apoptosis. Since destruction of Dicer may affect many processes, it is perhaps a more efficient way of blocking RNAi than inactivation of the several downstream effectors of Dicer.

Our results also suggest that induction of apoptosis may affect other proteins involved in miRNA production. It has been shown that most of the Ago proteins are distributed diffusely in the cytoplasm and, when cells are subjected to stress, Ago proteins accumulate with newly assembled structures known as stress granules in addition to processing bodies [34]. It was also recently shown that stress conditions could alter miRNA actions via relief of miRNA-mediated translational repression [10], an effect downstream of miRNA biosynthesis. On the other hand, it has been reported that hypoxia has no significant influence on expression of the key miRNA processing proteins Drosha, Dicer and Ago2 at the mRNA level; however, the authors could not rule out the possibility that hypoxia may regulate other miRNA biogenesis proteins, such as Pasha GW182 or Rck/p54 [35]. Stress might also modulate the kinetics of miRNA biogenesis enzymes or induce differential regulation of discrete miRNA species via selective miRNA biosynthetic cofactors or (yet unidentified) cell- or stress-specific regulators [11,35].

The cleavage of Dicer appears to be stimuli-dependent and more pronounced when apoptosis is induced by inhibitors of PKC, compared with other agents, suggesting that components of the RNAi machinery and protein kinases may directly or indirectly interact. More investigations are required in order to determine which PKC isoforms and other proteins are involved in this process. In the present study, we did not detect cleavage of Dicer in poliovirus-infected HeLa cells (Figure 6A); previously, we did not detect cleavage of Dicer in human alveolar epithelial A549 cells infected with influenza A virus [36]. However, we could observe the cleavage of Dicer in HIV-infected cells (Figure 6B), where involvement of PKC in HIV-1 gp120-induced apoptosis has been shown [37]. It has also been recently demonstrated that Dicer inhibited HIV-1 virus replication in latently infected cells. In turn, HIV-1 actively suppressed the expression of the polycistrionic miRNA cluster miRNA-17/92, which is required for efficient viral replication. However, no mechanism for such a phenomenon was provided [38]. A number of virus-encoded RNAi inhibitors have been identified [39–41], the virus-dependent saturation of RNAi has been demonstrated [42] and the down-regulation of Dicer mRNA by influenza A virus has been reported [36]. Our present results indicate that HIV may have developed yet another novel mechanism of RNAi inactivation, namely caspase-dependent cleavage of Dicer.

In summary, our results suggest that down-regulation of Dicer contributes to progression of apoptosis and indicate that the cell death machinery exerts its effect on the miRNA pathway by affecting individual proteins, such as Dicer.

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