Dicer is involved in protection against influenza A virus infection

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INTRODUCTION

RNA interference, RNAi, is a natural antiviral mechanism in plants and invertebrates. Based on the results obtained in plants, Drosophila and worms and because the RNAi machinery is present in all animals from nematodes to mammals, RNAi has often been proposed to be involved in the response to viral infection in vertebrates (Bennasser et al., 2005; Gatignol et al., 2005). Indeed, viral microRNAs, miRNAs, isolated from cells infected with Epstein–Barr virus and herpes simplex virus provided the first evidence of a role of RNAi machinery during infection with DNA viruses (reviewed by Cullen, 2006). Retroviruses provide another example, showing that cellular miRNA restricts the replication of the primate foamy virus in human cells (Lecellier, 2006). In addition, it has been shown that type III RNases Dicer and Drosha, responsible for miRNA processing, inhibited human immunodeficiency virus type 1 (HIV-1) replication in peripheral blood mononuclear cells (Triboulet et al., 2007). Another indirect support for a role of RNAi is the characterization of virus-encoded RNAi suppressors. Adenovirus-associated RNA I and RNA II act as RNAi suppressors (Andersson et al., 2005) and transfected HIV TAR RNA and Tat protein can attenuate the RNAi machinery in human cells (Bennasser et al., 2006). Despite all these findings, the hypothesis that RNAi could play a role in the defence against RNA viruses in mammals remains relatively untested and debatable. The validity of identified miRNA encoded by RNA viruses and the specificity of RNAi suppressors in mammals was also questioned (Cullen, 2006; Pfeffer et al., 2005). In contrast to viruses from plants and invertebrates, which encode RNAi-suppressing proteins to inhibit the degradation of viral RNA by the RNAi pathway (Wang & Metzlaff, 2005), it was thought that mammalian viruses might not need to interfere with the RNAi pathway because of the ubiquity and effectiveness of the IFN system, which exerts inhibitory effects on viral gene expression (Katze et al., 2002). Viruses, however, have developed strategies to circumvent the IFN response either by limiting IFN production or by blocking IFN actions (Goodbourn et al., 2000).

Interestingly, it has been suggested that RNA silencing and IFN response are partially overlapping, because: (i) some viral products efficiently inhibit both RNA silencing and IFN response by targeting their common elicitor, dsRNA, (ii) the TAR RNA-binding protein, which is a negative regulator of protein kinase R, is an essential component of the RNA-induced silencing complex, (iii) adenosine deaminase acting on RNA edits miRNA precursor and is also an effector of the IFN response (Saumet & Lecellier, 2006 and references therein), and (iv) expression of miRNA-155 can be induced by cytokines IFN-γ and IFN-α (O’Connell et al., 2007). These relationships between RNA silencing and IFN system indicate that RNAi may be involved in antiviral response.

In order to investigate the role of RNAi upon viral infection we addressed this problem from a different angle by focusing not on the identification of miRNAs or characterization of RNAi suppressors, but tested whether the presence of the essential RNAi component Dicer is important during infection with an RNA virus in the absence of IFN. The results demonstrate that in the absence of IFN, knockdown of Dicer leads to a modest increase of

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virus production and accelerated cell death upon infection with influenza A virus. They also show that in the IFN-producing cells influenza A virus targets Dicer at mRNA and protein levels. Thus, Dicer is important for the survival of influenza A virus-infected cells.

METHODS

Viruses, cells and viral infections. Avian influenza virus A/Bratislava(H7N7) was kindly provided by Dr Pavlovic (Institute of Medical Virology, Zurich, Switzerland) and used for infection of different cell lines. African green monkey epithelial cell line Vero and the human alveolar epithelial cell line A549 were grown in Dulbecco’s minimal essential medium (DMEM; containing 10 % fetal bovine serum, 100 U penicillin ml\(^{-1}\), 0.1 mg streptomycin ml\(^{-1}\)). For infection, cells were washed with PBS and infected with influenza A virus at an m.o.i. of 0.2 or 2 in DMEM for 60 min at 37 °C. The inoculum was aspirated and cells were incubated with fresh DMEM. At indicated time points cells were trypsinized, trypan blue stained and live cells were counted. The m.o.i. of 0.2 has been chosen to ensure prolonged survival of the influenza A virus-infected cells. The amount of infectious virus in cell supernatants was determined by measuring TCID\(_{50}\) and by real-time PCR or semi-quantitative RT-PCR. The TCID\(_{50}\) of influenza A virus was determined by using the classical method described previously (Leland & French, 1988).

UV irradiation of influenza A virus was performed as described previously (Li et al., 2005).

Antibodies, plasmids, apoptosis induction and inhibitors. Rabbit polyclonal antibodies against poly (ADP-ribose) polymerase (PARP) were purchased from Transduction Laboratories, mouse monoclonal antibody against Dicer was purchased from Clonetech and mouse monoclonal antibody against \(\alpha\)-tubulin and \(\beta\)-actin were purchased from Santa Cruz Biotechnology. Mouse anti-IFN type I receptor neutralizing antibodies against IFN type I receptor were purchased from Calbiochem and used at a 1:2 dilution and maintained throughout the experiments. Control anti-c-Myc polyclonal rabbit antibody was purchased Santa Cruz Biotechnology. All the antibodies were against human genes, and in Vero cells recognized bands at a similar size as those in A549 cells (approx. 250 kDa band for Dicer, 116 and 85 kDa for cleaved and uncleaved PARP, 50 kDa for \(\alpha\)-tubulin and 43 kDa band for \(\beta\)-actin). Sequence information about green monkey homologues of human PARP and Dicer is not available and therefore specific antibodies could not be generated.

Plasmid expressing firefly luciferase was from Ambion. pSuper-shLuc plasmid expressing short hairpin RNA against luciferase has been described previously (Lorger & Moelling, 2006). Recombinant IFN-\(\alpha\) was from Roche and used at a concentration of 1000 U ml\(^{-1}\) for 2 h after infection. The caspase inhibitor Z-DEVD-FMK (Alexis Biochemicals) was added to the medium at the concentration of 40 \(\mu\)M after aspiration of the inoculum and maintained throughout the experiments. Apoptosis in A549 cells was induced with 10 ng tumour necrosis factor alpha (TNF-\(\alpha\); Sigma) ml\(^{-1}\) and 20 \(\mu\)g cycloheximide (Sigma) ml\(^{-1}\).

Small interfering RNA (siRNA)-mediated knockdown of Dicer. All siRNAs were purchased from Dharmacon. siRNA target sequences of firefly luciferase and Dicer (siLuc and siDic, respectively) have been published previously (siDic in Hutvagner et al., 2001; siLuc in Chendrimada et al., 2005). The siRNAs were transfected into Vero or A549 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Efficiency of transfection was estimated by using fluorescein isothiocyanate (FITC)-conjugated siRNA (Dharmacon). Efficacy of Dicer knockdown was confirmed by semi-quantitative RT-PCR using primers specific for Dicer (primers sequences described in Chendrimada et al., 2005) and using \(\beta\)-actin as a control for relative expression levels (primers are described in Pang et al., 2003), or by Western blot analysis performed for Dicer. Sequence information about the green monkey homologue of human Dicer is not available; however, the efficiency of downregulation of this gene by siRNAs could be confirmed at mRNA or protein level. The RT-PCR amplification products obtained in Vero cells had similar size and sequence to that of A549 cells. Cells were used for RT-PCR or Western blot analysis 48 h post-transfection. Primers for Ago2 were described previously (Meister et al., 2004).

RNA isolation and RT-PCR. siRNA-transfected Vero or A549 cells were grown in 12-well-plates. For isolation of total cellular RNA QiAamp RNA Blood Mini kit (Qiagen), for isolation of viral RNA QiAamp Viral RNA Mini kit (Qiagen) and for semi-quantitative RT-PCR analysis Access RT-PCR kit (Promega) were used according to the manufacturer’s instructions. For real-time PCR, RNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems) and the amount of viral RNA was quantified by the real-time PCR assay using the ABI 7300 instrument (Applied Biosystems). The sequences of primers of influenza A virus for RT-PCR were published previously (van Elden et al., 2001).

ELISA. Supernatant from infected cells was used for quantification of apoptosis by using the M30-Apoptosense ELISA kit (Peviva) following manufacturer’s instructions.

Western blot analysis. Influenza A virus-infected cells were lysed 24 h post-infection (p.i.). The nuclei-containing fraction was obtained by using Nuclear Fractionation kit (BioVision) following the manufacturer’s instructions. Protein contents were estimated by employing the Bradford reagent (Bio-Rad). Equal amounts of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes, and cleavage of PARP was determined by using anti-PARP antibody. For analysis of Dicer expression, Cytosolic Fractionation kit (BioVision) was used following the manufacturer’s instructions and anti-Dicer antibodies applied.

Luciferase assay. Luciferase assay was performed using the Dual-Luciferase Reporter Assay kit (Promega) following the manufacturer’s instructions.

Statistical analysis. Bars represent the mean value of three independent experiments ± standard deviation (SD). Statistical analysis was performed using the Student’s t-test.

RESULTS

Generation of Dicer-knockdown cells

IFN is the major antiviral factor in mammalian cells. However, many viruses have been able to, at least partially, block the IFN pathway. Thus, to investigate the role of RNAi in the absence of IFN, we used Vero cells, which lack IFN-\(\alpha\) and IFN-\(\beta\) genes (Diaz et al., 1988). These cells were analysed upon influenza A virus infection. In order to study the putative role of the RNAi system, we knocked down Dicer, an essential component of the RNAi system. We transfected Vero cells with siRNAs against Dicer, designated siDic. siRNA against luciferase (siLuc) served as a control. We achieved 70–80 % downregulation of Dicer, as measured at the mRNA levels, and 60–70 %, as
measured at the protein level (Fig. 1a). Transfection efficiency in Vero cells was around 80% (Fig. 1b). Analysis of the cells showed that knockdown of Dicer did not significantly affect morphology or growth rate of the transfected cells (Fig. 1c and d). In order to functionally characterize activity of RNAi, we analysed the effect of Dicer knockdown on repression of exogenously added luciferase gene by short hairpin RNA against this gene (shLuc). Short hairpin RNAs delivered extracellularly require Dicer-dependent processing to trigger the silencing of target genes. Thus, cells were first transfected with siDic to knockdown Dicer, or si green fluorescent protein (GFP) as a control, and 36 h later co-transfected with plasmids expressing the luciferase gene (pLuc) and shLuc (pSuper-shLuc). Plasmid pSuper was used as a control and luciferase activity was measured 36 h after the second transfection. As shown in Fig. 1(e), the knockdown of Dicer resulted in a substantial decrease of the inhibition of luciferase activity by shLuc. Thus, knockdown of Dicer disrupts RNA silencing in Vero cells.

**Increased susceptibility of Dicer-knockdown Vero cells to influenza A virus infection**

As the next step, we transfected Vero cells with siLuc and siDic and 48 h later infected the cells with influenza A virus. Infection of the transfected cells with influenza A virus at a low m.o.i. of 0.2 resulted in significantly increased mortality of siDic-transfected cells compared with siLuc-transfected control cells (Fig. 2a and b). Indeed, 36 h after infection just 10% of siDic-transfected cells remained alive, compared with more than 60% live siLuc-transfected cells. RT-PCR analysis and testing the infectivity of the virions released by infected cells revealed that the virus was produced to higher levels in Dicer downregulated cells compared with siLuc cells at early (8 h) and intermediate stages (12–24 h) of infection (Fig. 2c and d). The effects were less pronounced at later stages (32 h), possibly due to excessive death of Dicer-knockdown cells compared with control siLuc-transfected cells. Similar effects were observed when cells were infected at the higher m.o.i. of 75x150.

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**Fig. 1.** (a) Efficiency of downregulation of Dicer in Vero cells. Cells were transfected with siRNAs against luciferase and Dicer. After 48 h, total RNA or proteins were extracted from transfected cells and RT-PCR (upper panels) or Western blot analysis (lower panels) were performed as described in Methods. (b) Efficiency of transfection in Vero cells. Cells were transfected with siRNAs against luciferase and control FITC-conjugated control siRNA, and analysed by fluorescent microscopy 18 h post-transfection. (c) Knockdown of Dicer has no visible effects on transfected cells as monitored by measuring growth rate 48 and 72 h post-transfection and (d) by observing the cells by light microscopy. (e) Knockdown of Dicer decreases inhibitory activity of RNAi. Cells were transfected with siRNAs against GFP or Dicer, and 36 h later co-transfected with plasmid expressing luciferase (pLuc) and plasmid expressing short hairpin RNA against luciferase (pSuper-shLuc). Plasmid pSuper was used as a control. Luciferase activity was measured 36 h after the second transfection. \(P = 0.017\) for siDic- versus siGFP-transfected cells (grey bars).
2 (data not shown). These results suggest the importance of Dicer for antiviral response in Vero cells.

**Effects of Dicer knockdown are decreased in the presence of IFN**

In view of the significant role of IFN in antiviral defence, we investigated the role of exogenously added recombinant IFN-α in influenza A virus-infected Vero cells. Treatment with IFN-α after infection significantly reduced the differences in survival rate and virus production between Dicer knockdown and control cells (Fig. 3a–c). These results were consistent with relatively weak differences between survival rates and virus production of siDic-transfected cells, compared with control siLuc cells observed in another IFN-producing cell line, the human alveolar epithelial A549 (Fig. 4a–d). However, the treatment of infected A549 cells with neutralizing antibody against IFN type I receptor, which blocks the biological effects of IFN-α and IFN-β (Colamonici & Domanski, 1993), led to accelerated death of Dicer-knockdown cells compared with the control cells (Fig. 4d, e) and to increased virus production (Fig. 4f). The effects were weak, possibly due to insufficient suppression of the IFN pathway by the neutralizing antibody, and due to the presence of other IFN-independent antiviral systems (Kim et al., 2002; Noyce et al., 2006). Taken together, these observations suggest that RNAi may also be involved in antiviral response, in addition to the IFN system.

**Knockdown of Dicer in Vero cells leads to accelerated apoptosis**

An important cellular signalling response commonly observed upon virus infections is the induction of programmed cell death. The significantly decreased viability of Dicer-knockdown Vero cells during influenza A virus...
propagation demonstrated here prompted us to investigate whether the observed phenomenon was related to apoptosis. It has previously been reported that influenza infection could trigger programmed cell death in Vero or A549 cells, and that induction of apoptosis is a crucial event for efficient influenza virus propagation (Wurzer et al., 2003).

To investigate the impaired survival of Dicer-knockdown cells, we tested the rate of apoptosis in siDic-transfected influenza A virus-infected Vero cells. A hallmark of apoptosis is the caspase-mediated cleavage of the nuclear 116 kDa protein PARP to a smaller 85 kDa inactive form (Bromme & Holtz, 1996). Immunoblot analysis of influenza A virus-infected Vero cells revealed an increase in cleavage of PARP in Dicer-knockdown cells. At 24 h p.i., cleavage of PARP in siDic-transfected cells was completed, while in control siLuc-transfected cells PARP was only partially cleaved (Fig. 5a, upper panels). Infection of all three transfected cell lines with UV-inactivated virus did not lead to cell death, indicating that apoptosis induction requires productive infection of the cells (data not shown).

To confirm that the increased rate of cell death was due to apoptosis, we tested the cleavage of cytokeratin-18, another hallmark of apoptosis, in Dicer-knockdown cells by using the quantitative M30-Apoptosense ELISA assay. Indeed, siDic-transfected influenza A virus-infected Vero cells were undergoing accelerated apoptosis compared with control siLuc and untransfected cells (Fig. 5b). Virus-induced caspase activity can be efficiently suppressed by a cell-permeable, non-toxic inhibitor, Z-DEVD-FMK, that binds irreversibly to activated caspase 3 and some other caspases in apoptotic cells (Wurzer et al., 2003). The treatment of influenza A virus-infected cells with Z-DEVD-FMK reduced the enhanced level of cell death and virus production in siDic-transfected cells (Fig. 5a, c and d), thus indicating that accelerated cell death of Dicer-knockdown cells was due to caspase activation. Taken together, these results show that downregulation of Dicer increases virus replication and subsequently leads to accelerated apoptosis of infected cells.

Dicer rapidly disappears in influenza A virus-infected cells

So far we have shown that artificial knockdown of a key RNAi component, Dicer, by siRNA led to accelerated apoptosis of influenza A virus-infected IFN-deficient cells and to an increase of virus production. However, downregulation of Dicer could also be observed naturally in virus-infected Vero cells. Indeed, Dicer mRNA disappeared more rapidly compared with actin and Ago2 mRNAs in infected cells, suggesting that the expression of Dicer could be targeted by influenza A virus (Fig. 6a). A similar situation was seen at the protein level (Fig. 6b). Furthermore, in IFN-producing A549 cells Dicer also rapidly disappears both at mRNA and protein levels at late stages of infection with influenza A virus (Fig. 6c). Indeed, 40 h p.i. the remaining 50 % of live-infected cells had very little Dicer mRNA left, and the amount of Dicer protein was significantly reduced. The induction of apoptosis with TNF-α and cycloheximide (Smith et al., 1999), which leads to the death of A549 cells, showed much weaker effects on Dicer expression (Fig. 6d), suggesting that expression of Dicer could be targeted by influenza A virus at mRNA and protein levels. Interestingly, significant reduction of Dicer at late stages of influenza A virus infection coincided with increased rate of apoptosis (Fig. 6e) and maximal level of virus production (Fig. 6f). Thus, natural virus-mediated downregulation of Dicer may play a role in increased virus production and accelerated apoptosis even in IFN-producing virus-infected cells.
DISCUSSION

The question as to whether RNAi plays any role in RNA virus-infected cells is still under debate. The major challenge for the current study was the controversial situation with the involvement of RNAi in antiviral response against RNA viruses in mammals, characterization of RNAi suppressors and identification of siRNAs encoded by RNA viruses. We left these problems out of the scope of the present study. However, most of the previous studies...
did not address the question of the activity of the RNAi pathway in virus-infected cells, and previous experiments have been performed in IFN-producing cells, where a powerful IFN system plays the major role and surpasses other potential defensive systems. Indeed, also in our experiments, IFN effectively decreases the differences between influenza A virus-infected Dicer knockdown and control cells (Figs 3 and 4). In the absence of IFN, however, as shown here in Vero cells or by using neutralizing antibodies against IFN type I receptor in A549 cells, knockdown of Dicer has a moderate effect on virus replication, and to a higher extent on apoptosis and the survival of infected cells (Figs 2 and 5). Thus, both RNAi and IFN systems may be involved in antiviral responses. Furthermore, Dicer rapidly disappears at late stages of infection and not after treatment with pro-apoptotic agents, suggesting that the key component of RNAi, Dicer, could be targeted by influenza A virus at its mRNA and protein levels both in IFN-deficient and IFN-producing cells (Fig. 6). Downregulation of Dicer by influenza A virus coincides with significantly accelerated death rate, indicating that Dicer, and RNAi in general, could be involved in influenza A virus-triggered IFN-independent (Vero cells) and IFN-dependent cell death (A549 cells). In the latter case, the effects of artificial knockdown of Dicer by siRNA appear to be overshadowed by the IFN system. It has been shown that IFN potentiates virus-induced apoptosis (Balachandran et al., 2000). Taken together, these results show the physiological significance of Dicer, as one of the factors involved in the control of virus replication and subsequently survival of the cells upon infection with lytic influenza A virus.

At the present time, the mechanism of downregulation of RNAi by influenza virus is not known. One possibility is that downregulation of Dicer is a consequence of virus-mediated cap-snatching mechanism, which may lead to Dicer mRNA degradation, similar to situations described for pre-IFN-mRNA (Marcus et al., 2005). It is also not clear how suppression of Dicer may affect the susceptibility of cells to virus infection. It has been shown recently that downregulation of Dicer in HEK293 cells leads to a modest upregulation of several hundred RNA transcripts, which could be directly or indirectly involved in the control of virus propagation and apoptosis of infected cells (Schmitter et al., 2006). On the other hand, downregulation of Dicer leads to impaired synthesis of miRNAs. Human miRNA, miRNA-136, expressed in lung has been predicted to have a potential binding site to the haemagglutinin (HA) gene of influenza A virus. HA is known to be critical for pathogenicity of influenza virus and immunosuppression (Scaria et al., 2006). It has also been demonstrated that expression of miRNA-155 can be induced by cytokines IFN-β and IFN-γ (O’Connell et al., 2007), and thus may be involved in the modulation of antiviral effects of infected cells. Several miRNAs (including miRNA-29b) were found to be predominantly localized to the nucleus, where influenza A virus replicates (Hwang et al., 2007). Finally, it has been recently shown that Dicer inhibited HIV-1 virus replication both in peripheral blood mononuclear cells and in latently infected cells. In turn, HIV-1 actively suppressed the expression of the polycistronic miRNA cluster miRNA-17/92, which is required for efficient viral replication (Triboulet et al., 2007). Since several hundreds of miRNAs have been predicted and many are not characterized yet,
especially in the context of viral infection, it is notoriously difficult to estimate the contribution of each individual miRNA in antiviral response, and to make an appropriate conclusion. The advantage of our approach is that it allows us to monitor summarily the effects of Dicer down-regulation, the situation, which could be seen in influenza A virus-infected cells. Furthermore, we cannot exclude the possibility that a key component of RNAi Dicer can participate in other processes in addition to miRNA biogenesis, such as regulation of the accumulation of pre-miRNAs, small nuclear RNAs or small nucleolar RNAs, as it has been suggested for dicer-like protein 1 in plants (Mlotshwa et al., 2005).

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REFERENCES


