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Sendai virus trailer RNA simultaneously blocks two apoptosis-inducing mechanisms in a cell type-dependent manner

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Induction of apoptosis during *Sendai virus* (SeV) infection has previously been documented to be triggered by initiator caspases (for strain F) or by a contribution of the cellular protein TIAR (T-cell-activated intracellular antigen-related) (for strain Z). Here, evidence was provided that both TIAR and caspases are simultaneously involved in apoptosis induction as a result of infection with SeV strain F. SeV F infection induced death in all tested cell lines, which could only be partially prevented through the pan-caspase inhibitor z-VAD-fmk. However, infection of seven different cell lines with the SeV mutant Fctr48z overexpressing a TIAR-sequestering RNA from the modified leader resulted in a cell type-dependent reduced cytopathic effect (CPE); in an earlier study a similar mutant derived from SeV Z was shown to prevent the induction of any CPE. Finally, blocking of caspases through z-VAD-fmk combined with Fctr48z infection led to complete abrogation of CPE, clearly demonstrating the existence of two separate mechanisms inducing cell death during SeV F infections. Interestingly, a cell type-specific interference between these two mechanisms could be detected during infection with the mutant virus Fctr48z: RNA transcribed from the mutated leader was able to *trans*-dominantly inhibit caspase-mediated apoptosis. Thus, virus-expressed factors enabling a well-balanced ratio of suppression and triggering of apoptosis seem to be essential for optimal virus replication.

INTRODUCTION

During the last decade, it has become evident that viral infection can function as a trigger of apoptosis, a common mechanism among higher eukaryotes to guarantee the survival of the whole organism (Ezoe *et al.*, 1981; O'Brien, 1998). On the other side, it has also been shown that certain viruses have evolved strategies to actively prevent the apoptotic cell response. Hence, viruses are involved in apoptosis in two opposing ways. They can either inhibit or delay cellular altruistic suicide early after infection in order to provide sufficient time for efficient virus replication or for persistent infection. Members belonging to this group of viruses are the well-studied adenoviruses (McNees & Gooding, 2002) and baculoviruses (Clem, 2001), as well as herpes simplex virus type 1 (HSV-1) (Galvan *et al.*, 1999) and others (O'Brien, 1998). Alternatively, they can stimulate lysis of infected cells, promoting release and spread of virus progeny throughout the infected organism. During this process, caspases (proteases that support and execute cellular breakdown) are frequently activated. This mechanism has been reported for bovine herpesvirus type 1 (Devireddy & Jones, 1999) and for coxsackievirus (Carthy *et al.*, 1998), *Sindbis virus* (Nava *et al.*, 1998), human immunodeficiency virus type 1 (HIV-1) (Bartz & Emerman, 1999) and other viruses (O'Brien, 1998). However, viruses known to induce apoptosis might also be able to delay the onset of cellular destruction during the first steps of an infection.

Sendai virus (SeV), consisting of a single-stranded, negative-sense RNA genome, belongs to the genus *Respirovirus* within the family *Paramyxoviridae* and can cause severe respiratory illness in rodents. As shown for many other viruses within this family, such as respiratory syncytial virus (O'Donnell *et al.*, 1999), canine distemper virus (Moro *et al.*, 2003), measles virus (Servet-Delprat *et al.*, 2000) and Newcastle disease virus (Lam, 1996), SeV acts intracellularly by inducing apoptosis (Bitzer *et al.*, 1999, 2002; Tropea *et al.*, 1995).

Little is known to date about how apoptosis is induced during SeV infection. Experiments with a recombinant SeV strain Z (SeV Z) mutant, rSeV^{GP48}, that contains an exchange of the first 48 nt of the genomic leader with the equivalent antigenomic complementary trailer sequence (ctr), result in complete abrogation of programmed cell death during infection of various cell lines (Iseni *et al.*, 2002). Interaction between a short viral RNA expressed from the mutated leader and the cellular T-cell-activated intracellular antigen-related (TIAR) protein was identified as causing this effect. Cells infected with wild-type SeV Z or overexpressing TIAR still died through apoptosis, as

demonstrated by phosphatidylserine exposure on the outer leaflet of the plasma membrane (Garcin *et al.*, 1998; Iseni *et al.*, 2002).

As well as this involvement of TIAR, data from SeV strain F (SeV F) infection, another laboratory strain, have described the activation of certain caspases involved in pathways that lead to apoptosis (Bitzer *et al.*, 1999, 2002). Surprisingly, during immunohistochemical experiments SeV F seemed to induce cell death much faster and to a greater extent than SeV Z (R. Sedlmeier, personal communication).

The apoptotic cascade involving various caspases at defined steps has been relatively well described. However, whether and where TIAR is involved in these pathways, and to what extent, still has to be determined. While induction of apoptosis during SeV Z infection has been found to involve TIAR, caspases have been detected during infection with SeV F. Whether these observations are SeV strain-specific characteristics or whether cellular reactions during virus infection can vary depending on cell type has yet to be elucidated. Thus, apoptosis induction by SeV Z or F could be restricted to only one mechanism in a cell type-dependent manner, or could occur simultaneously via two mechanisms (TIAR involvement and caspases), which might somehow be connected with each other.

In this report, we investigated the cytotoxic effect of a specifically designed SeV mutant (Fctr48z) that combined the TIAR-inactivating leader region with the caspase-activating genomic backbone from SeV F. Based on these data, we determined that induction of cell death during infection with wild-type SeV F occurred via two processes that were simultaneously activated. We demonstrated a cell type-dependent interference between both mechanisms by infection of cells with mutant Fctr48z. This interference seemed to be based on the suppressive action of ctr RNA transcribed from the mutated leader region of Fctr48z and led, in the case of LLC-MK_2 cells, to a complete abrogation of cell destruction.

METHODS

Cells and viruses. All cell lines originated from the ATCC. Vero, HeLa, BHK-21, LLC-MK_2 and HepG2 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10 % fetal calf serum (FCS; Gibco-BRL). A549 and 293 cells were grown in Medium 199 (Gibco-BRL) with 10 % FCS.

SeV Z was obtained from M. F. G. Schmidt (Berlin, Germany). SeV F was recovered from a cDNA clone derived from SeV D52 (ATCC).

For construction of the mutant virus Fctr48z cDNA containing the mutated leader sequence, two single PCR reactions were first performed whose products also included the sequence complementary to nt 1–48 from the SeV Z trailer (antigenomic). Template for the reactions was a pUC plasmid containing the entire SeV genome (strain F). The following primer pairs were used: (i) standard pUC/M13 R 5′-AGCGGATAACAATTTCACACAGG-3′ (forward) and 5′-AGACAAGAAAATTTAAAAGGATACATATCTCTTAAACTCTTGT-CTGGTCCCTATAGTGAGTCGTATTACG-3′ (reverse), and (ii) 5′-GTATCCTTTTAAATTTTCTTGTCTGGATTTTAGGGTCAAAG-TATCCAC-3′ (forward) and 5′-CCATGAGAGATACAAGGC-3′ (reverse) from inside the N gene. A fusion PCR was then performed to join both fragments using the forward primer of the first reaction and the reverse primer of the second. The resulting PCR product contained a unique restriction enzyme recognition site at each end for introduction into the viral cDNA.

Generation of recombinant SeV from cDNA was carried out and all viruses were propagated in Vero cells as documented previously (Leyrer *et al.*, 1998).

Virus replication studies. The number of virus particles released from infected cells was quantified using a haemagglutination (HA) test and the infectious fraction by $TCID_{50}$ assays, as described previously (Bitzer *et al.*, 1997; Neubert & Hofschneider, 1983). Under our conditions, 40 HA U ml^{-1} was equivalent to 10^7 virus particles ml^{-1} and 1 $TCID_{50}$ ml^{-1} was equivalent to 5×10^3 cell infectious units (CIU) ml^{-1}.

Cell viability assay. A modified version of the MTT assay (Mosmann, 1983) was used for determining the viability of infected cells. After incubation for 72 h, medium from infected cells grown in 96-well plates (20 000 cells per well) was replaced with medium containing MTT [tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; Sigma-Aldrich] at a final concentration of 5 mg ml^{-1}. After incubation for 3·5 h at 37 °C, the formazan crystals formed were dissolved by the addition of 2-propanol with 0·04 M HCl and thoroughly resuspended to homogeneity. The absorbance of formazan at 590 nm was measured using an ELISA reader (Dynatech MR7000). Each value was determined from three parallel infection experiments and absorption by the medium (blank) was subtracted each time.

When the caspase inhibitor z-VAD-fmk [*N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone; Merck Biosciences] was added to the medium of infected cells, a final concentration of 50 μM was used and renewed every 24 h. The remaining MTT assay was performed as described.

Sequence determination of the mutated leader. The original 3′-terminal sequence of the virus genome from nt 1 to 55 was determined using a 5′-RACE kit (Invitrogen).

Total RNA was extracted from infected cells using TRIzol reagent (Invitrogen). Only the antigenomic SeV RNA strand was reverse transcribed to cDNA with Superscript II (Invitrogen). The primer used for this reaction started from inside the N gene towards the antigenomic 5′ end. Next, the 3′ end of the generated cDNA was elongated by adding guanosine residues with the enzyme terminal transferase (Roche). Subsequent amplification of the fragments containing the viral sequence from nt 1 to 55 was performed according to the manufacturer's recommendations. The 5′-RACE products were analysed directly and also after subcloning into pUC29. DNA sequencing was performed by Medigenomix.

RESULTS

SeV F induces a stronger CPE than SeV Z

Inhibition of an apoptotic response during SeV Z and F infections was achieved in two different ways (Bitzer *et al.*, 1999, 2002; Garcin *et al.*, 1998; Iseni *et al.*, 2002). As apoptosis could not be completely prevented by pan-caspase inhibitors during SeV F infections of CV-1 and MCF-7 cells (Bitzer *et al.*, 1999, 2002), SeV appeared to induce cell

death via more than one mechanism. In addition to this incomplete apoptotic block, the observation that SeV F provoked a significantly stronger CPE than SeV Z, which could be related to an additional CPE-inducing potential, qualified this strain for further investigation of apoptosis induction during SeV infection. Therefore, we first had to verify the enhanced cytopathic potential of SeV F. Fig. 1(a) showed that there was a clear strain-dependent, variable, detrimental impact on various cell lines (BHK, LLC-MK_2 and Vero). The extent of the variation in CPE, however, was surprising, since such differences have so far only been reported between pathogenic field isolates (Hamamatsu and Ohita) and SeV laboratory strains (Harris, Fushimi and Z) (Fujii *et al.*, 2001; Itoh *et al.*, 1997; Sakaguchi *et al.*, 1994), and not among laboratory strains, which share 99 % nucleotide sequence identity. While cells infected with SeV Z developed a distinct but slow CPE after 72 h, the effect of SeV F infection appeared to be much more detrimental towards host cells, and a strong CPE could be observed by 24 h for BHK, Vero and LLC-MK_2 cells (data not shown). Quantification of viability of infected Vero cells, using MTT assays, confirmed these observations (Fig. 1b). While MTT values for mock-infected cells remained constant for 72 h, the viability of virus-infected cells decreased, as expected. However, the intensity of the decline induced by the two viruses clearly differed; whereas the viability of SeV Z-infected cells was only reduced by 55 % after 3 days, almost all cells infected with SeV F had died after the same period.

To exclude the possibility that this effect was simply a result

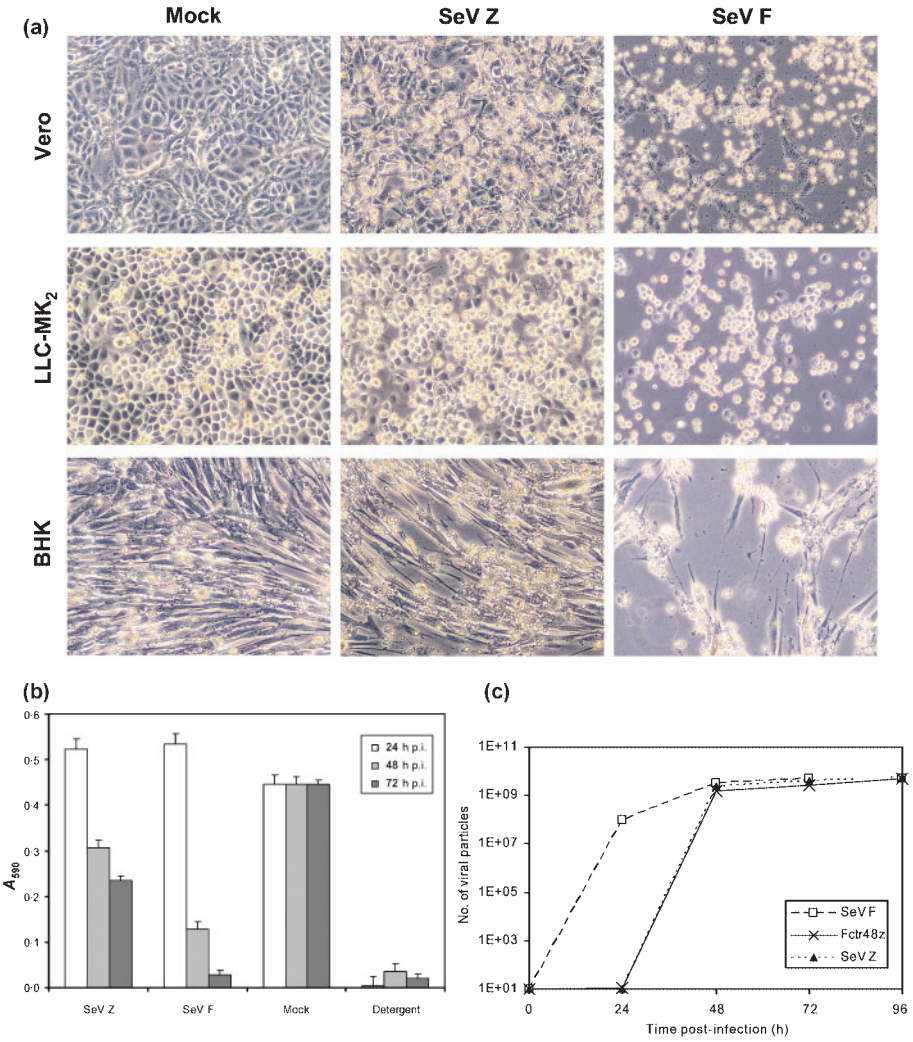

Fig. 1. Comparison of virulence and replication of SeV Z and F. Vero, LLC-MK_2 and BHK cells were mock infected or were infected with 3 CIU per cell SeV Z or F. (a) Morphology of infected cells at 72 h p.i. (b) Viability of Vero cells measured using MTT assays at the indicated times after infection with 3 CIU per cell SeV Z or F. The detergent 'Desinpure' (Interchem AG) was used as a positive control, and mock-infected cells as a negative control. (c) Release of progeny virus from Vero cells infected with 3 CIU per cell SeV Z or F, or with mutant Fctr48z, determined every 24 h and summed up to 96 h p.i. Comparable results were obtained from three independent experiments.

of unequal production of virus progeny, we investigated the replication efficiency of both strains in Vero cells. During incubation of infected cells for 96 h, supernatant was removed at the indicated time points and analysed for the release of virus particles (Fig. 1c). Although the time course of virus release differed slightly between SeV Z and F, the overall number of particles produced was very similar. This was also the case when ratios of infectious to non-infectious particles were analysed (data not shown). We therefore concluded that factors other than unequal production of virus particles were responsible for the observed variation in CPE (Fig. 1b). Although there is a 99 % overall nucleotide sequence identity between SeV Z and F, 44 nt exchanges lead to changes in the amino acid sequence (Fig. 2), which could thus account for the observed differences in viral pathogenicity. One such candidate is the lysine at position 461 within the haemagglutinin–neuraminidase glycoprotein of strain Z, which is replaced by glutamic acid in strain F. Glutamine at this position has previously been characterized as a stabilizing factor of SeV F neuraminidase function (Fujii *et al.*, 2002; Takahashi *et al.*, 1992; Thompson & Portner, 1987) and could thus contribute to the more destructive phenotype of SeV F compared with SeV Z.

In conclusion, SeV F induced a much stronger CPE than SeV Z, possibly reflecting an apoptotic response triggered by more than one mechanism. Thus, we used this strain for further studies of apoptosis induction during SeV infection.

Generation of mutant Fctr48z and analysis of virus replication

After selection of strain F, we constructed the SeV mutant Fctr48z consisting of two portions: the first part from nt 1 to 48, schematically shown in Fig. 2, corresponded to the complementary sequence of the last 48 nt from the genomic 5′ end (trailer) of SeV Z and was designated ctr. This sequence is reported to cause abrogation of apoptosis during infection with an analogous mutant derived from strain Z (Garcin *et al.*, 1998). The second part of the mutant, from nt 49 to 15384, represented the wild-type sequence of SeV F. This part of the genome is thought to harbour elicitors of caspase activation, as demonstrated previously (Bitzer *et al.*, 1999, 2002). Generation of recombinant Fctr48z was performed as described in Methods. Despite attenuated replication early after infection, growth of Fctr48z in Vero cells was comparable to SeV Z and F at 48 h post-infection (p.i.) (Fig. 1c).

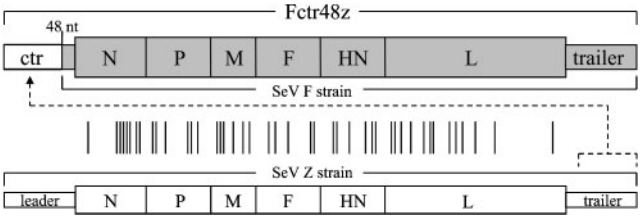

Fig. 2. Schematic presentation of the genome of mutant Fctr48z compared with SeV Z. The first 48 nt of mutant Fctr48z consist of the equivalent sequence from SeV Z trailer (antigenomic nt 1–48). Nt 49–15384 represent the genomic sequence of SeV F. The 44 aa differences between the genomes of Fctr48z and SeV Z (indicated by vertical bars between the genomes) are shown at the approximate nucleotide position within each gene.

In order to ensure the genetic stability of the mutant under selective conditions, we analysed viral genomes after 10 passages on Vero cells. The 5′-end sequence of the viral antigenomic RNA was reverse transcribed and amplified using a 5′-RACE reaction including the outermost nucleotides of the leader. In all viral RNA samples, the correct sequence of the mutated leader could be confirmed except for a nucleotide reversion from C to U (original F strain leader) at position 42 that occurred in approximately 50 % of the genome population.

Cell type-dependent reduced CPE during infection with mutant Fctr48z

Next, we investigated whether the exchange of the leader sequence combined with the SeV F genome also caused abrogation of CPE, as reported for the Z strain (Garcin *et al.*, 1998).

Therefore, CPE was monitored during infection of seven different cell lines (Vero, LLC-MK_2, BHK, 293, A549, HepG2 and HeLa) with Fctr48z. SeV F, which causes a severe CPE (as shown above), was used as a positive control. The CPE in cells infected with either virus was analysed for 72 h (Fig. 3a). This analysis of Fctr48z- or SeV F-infected cells revealed a widely varying CPE. Almost all cells infected with SeV F were dead by 72 h. Fctr48z-infected cells, however, exhibited a cell type-dependent, variable CPE that could be classified into two categories: a strong and quickly developing CPE, similar to SeV F infection, was observed in BHK, A549 and HeLa cells, while a slightly attenuated and more slowly developing CPE was seen in 293, HepG2, Vero and LLC-MK_2 cells (data not shown). Incubation of infected cells with serum-free or FCS-containing medium did not result in significant alterations to the CPE.

Lack of correlation between virus replication and CPE

Next, we wanted to exclude the possibility that the variation in CPE was a result of cell type-dependent virus replication. Although release of SeV F and Fctr48z particles was comparable in Vero cells (Fig. 1c), the observed CPE differed clearly as shown in Fig. 3(a). Hence, in Vero cells, which are known to allow effective replication of paramyxoviruses, a direct correlation between the number of virus particles produced and CPE was not detected.

In order to test whether this was also the case in the other cell lines, virus replication was monitored in these lines over a period of 4 days. The number of virus particles

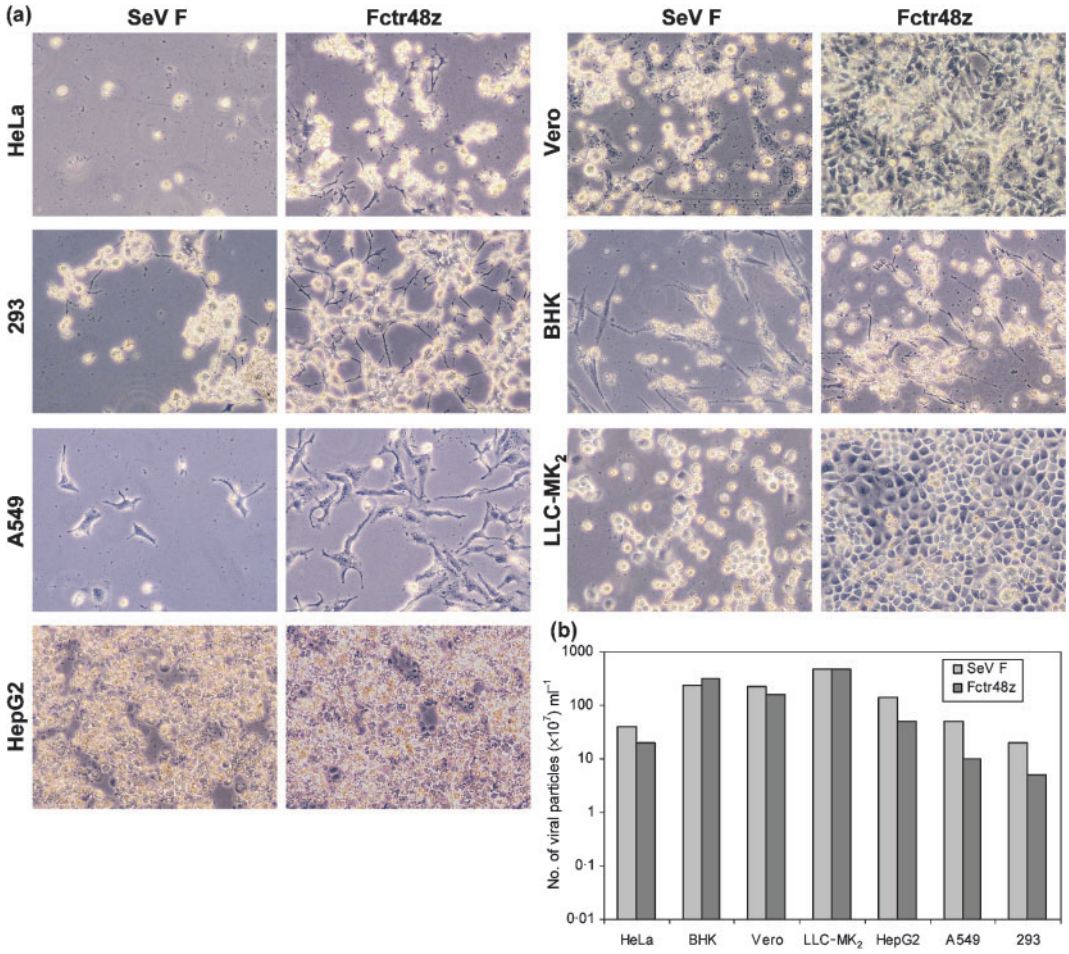

Fig. 3. Comparison of CPE among seven different cell lines and number of virus particles released following infection with SeV F or Fctr48z. (a) Morphology of cells from seven different cell lines infected with 3 CIU per cell SeV F or Fctr48z at 3 days p.i. (b) Total number of virus particles released from seven different cell lines infected with 3 CIU per cell SeV F or Fctr48z, determined by HA test every 24 h and summed up until 96 h p.i. Similar amounts were obtained from three independent experiments.

produced during SeV F or Fctr48z infection was found to differ depending on the cell type, as shown in Fig. 3(b). However, comparison of the CPE in these replication studies (Fig. 3) clearly showed that there was no general correlation between the efficiency of virus replication and the observed CPE. This was most obvious in infection of LLC-MK_2 cells with Fctr48z: by far the highest production of viral progeny was seen for this cell line, but a CPE was barely detectable (Fig. 3). BHK cells, in contrast, showed a strong CPE after Fctr48z infection and still produced a large number of virus particles. During Fctr48z infection of HeLa, 293 and A549 cells, however, a low level of virus replication coincided with a massive CPE (Fig. 3a and b). Western blot analysis of Vero and LLC-MK_2 cells, which both produced large numbers of virus particles, also showed a high level of viral protein synthesis during wt and Fctr48z infections, verifying a high cellular burden (data not shown).

The above results indicating that the CPE of mutant virus-infected cells varied with cell type over a broad range (Fig. 3a) and did not correspond to the burden resulting from viral propagation (Fig. 3b) was confirmed by evaluating cell viability. The viability of Vero and LLC-MK_2 cells was examined during infections with the mutant Fctr48z or with SeV F as representative cell lines able to survive infection with the mutant virus for a long time, while still producing high numbers of virus particles (Fig. 4). While almost all Vero cells infected with wild-type SeV F had died by 72 h p.i., approximately one-third of those infected with the mutant were still alive. Viability of LLC-MK_2 cells infected with SeV F clearly decreased up to 72 h p.i. When infected with Fctr48z, however, only a slight decline in viability could be observed during the first 2 days of infection, and there was a full recovery by 72 h p.i.

SeV F induces CPE by two different mechanisms

The above data clearly showed that mutant virus Fctr48z still induces a CPE, albeit sometimes attenuated. Thus,

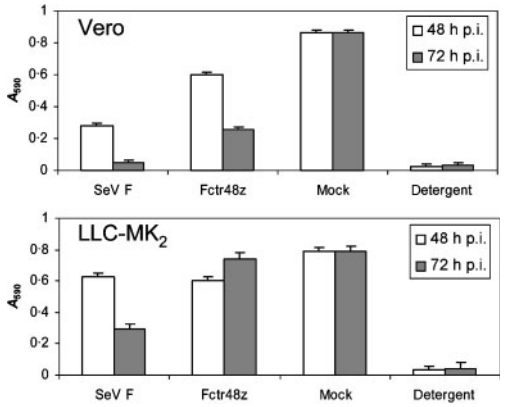

Fig. 4. Evaluation of viability of Vero and LLC-MK$_2$ cells during infection with SeV F or Fctr48z. The viability of infected Vero and LLC-MK$_2$ cells was determined at 48 and 72 h p.i. by MTT assay. Mock-infected cells served as a negative control and cells incubated with a detergent as a positive control.

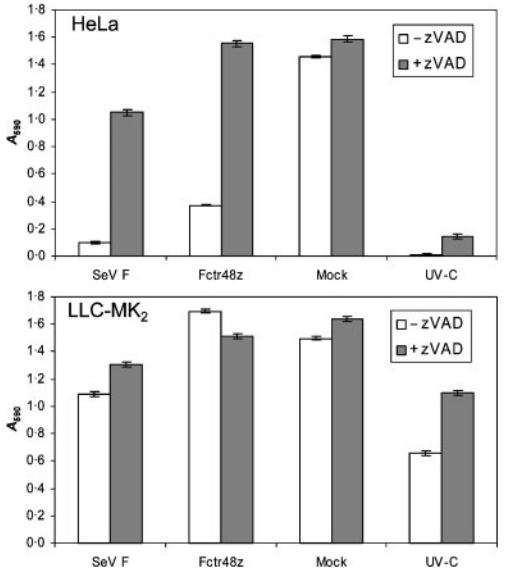

Fig. 5. Effect of pan-caspase inhibitor z-VAD-fmk on viability of cells infected with SeV F or Fctr48z. HeLa and LLC-MK$_2$ cells infected with 3 CIU per cell SeV or Fctr48z were incubated for 72 h with or without the caspase inhibitor z-VAD-fmk (50 μM) present in the medium. After 24 and 48 h, the medium including z-VAD-fmk was renewed. At 72 h p.i., cell viability was determined by MTT assay. The mock-infected negative control and the positive control (cells irradiated with UV-C light at 40 J m^{-2}; Stratalinker, Stratagene) were treated in the same way. Comparable results were obtained from three independent experiments.

abrogation of pathogenicity through interaction between the transcribed viral leader and the cellular protein TIAR, as described for an analogous mutant based on the SeV Z strain (Garcin *et al.*, 1998), did not seem to have the same impact during infection with our Fctr48z mutant. This led us to the conclusion that other factors may contribute to the induction of CPE. Caspases have been shown to be involved in apoptosis induced by SeV F (Bitzer *et al.*, 1999, 2002) and could represent an additional mechanism to the above-mentioned TIAR-related pathway. Thus, we tried to shed some light on a possible interaction between TIAR and caspases and to analyse their influence on prevention of apoptosis during SeV infection.

Viability of HeLa and LLC-MK$_2$ cells, representative of cells showing a strong or no CPE (Figs 3 and 4), respectively, was determined by MTT assays during infection with Fctr48z in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. SeV F (wild-type) was used for comparison (Fig. 5). SeV F infection without inhibitor reduced the viability of LLC-MK$_2$ cells by about one-third and the viability of HeLa cells almost completely. When z-VAD-fmk was present, viability clearly increased, but was still diminished for LLC-MK$_2$ (by one-fifth) and HeLa cells (by one-third). Remarkably, infection with Fctr48z led to different results: viability of HeLa cells was reduced by two-thirds in the absence of z-VAD-fmk and almost completely retained in the presence of z-VAD-fmk, resembling an accumulative effect of blocking TIAR and caspases. Most strikingly, the viability of Fctr48z-infected LLC-MK$_2$ cells was not impaired, regardless of the presence or absence of z-VAD-fmk. Here, synthesis of ctr RNA was obviously sufficient to prevent CPE or apoptosis induction, including activation of caspases. Thus, the expressed ctr RNA appeared also to act as an apoptosis blocker in the context of SeV F infection, and not only with strain Z, as indicated by Iseni *et al.* (2002).

In conclusion, our results provide strong evidence that an apoptotic response in SeV F-infected cells can be simultaneously triggered via different mechanisms – one of which is caspase independent – that can interfere with each other in a cell type-specific way.

DISCUSSION

While infection with our SeV mutant Fctr48z provoked a cell type-dependent variable CPE, which was almost completely abrogated within LLC-MK$_2$ cells, all cells infected with wild-type SeV F exhibited a massive and lethal CPE. These variable degrees of CPE could originate from an unequal cellular burden due to different amounts of viral progeny produced during infection. Evaluation of virus replication, however, showed no correlation between a large number of viral progeny and a strong CPE. Thus, virus replication was most efficient in Vero and LLC-MK$_2$ cells, which were not or were only slightly affected by infection. In contrast, HeLa, 293 and A549 cells, in which there was poor replication of virus, exhibited a fast-developing and strong CPE (Figs 3 and 4). In conclusion, efficient virus replication within host cells is not inevitably linked to the development of a strong CPE.

Another way in which CPE could develop during Fctr48z infection is linked to defective interfering (DI) particles. During SeV infection, high levels of non-infectious virus particles are produced that lead to interference with wild-type virus replication (Leppert *et al.*, 1977; Tuffereau & Roux, 1988) and reduction of CPE has consequently been linked to the diminished virus replication. Thorough consideration of the present results, however, allows exclusion of a comparable situation for Fctr48z infection. Firstly, the CPE caused by Fctr48z infection was reduced in a cell type-dependent manner and only for LLC-MK_2 cells was the CPE drastically diminished. Secondly, the production of virus particles was slightly reduced only in some cell lines (from two- to fivefold). However, DI particles are typically reported to diminish viral particle production by up to 100-fold (Tuffereau & Roux, 1988). Furthermore, while infections with DI particles contribute to the establishment of persistent infections without restrictions on the cell types infected (Perrault, 1981; Roux & Waldvogel, 1981), in our experiments only one cell line (LLC-MK_2) survived and constantly produced virus particles over a period of at least 13 passages. Fctr48z infection thus did not result in a situation comparable to typical DI particle infections. The enhanced fraction of non-infectious particles (four- to 10-fold) could represent particles containing full-length antigenomic rather than fragmentary genomic RNA (like DI particles). The introduction of a stronger promoter in Fctr48z might initiate synthesis of antigenomic viral RNA more efficiently (see below).

After excluding variable degrees of virus replication as a regulator of the cell type-specific CPE during infections with Fctr48z and SeV F, our investigation focused on cell type-specific apoptotic reactions. Two pathways or factors involved in SeV-induced apoptosis have been described so far.

First, during infection studies with SeV F, induction of apoptosis in MCF-7 and CV-1 cells was shown to coincide with the activation of caspases (Bitzer *et al.*, 1999, 2002). In our experiments, however, using HeLa and LLC-MK_2 cells infected with SeV F, the presence of the pan-caspase inhibitor z-VAD-fmk only partially reduced the CPE (Fig. 5). Hence, SeV F appeared to trigger apoptosis via at least one additional, caspase-independent mechanism.

Evidence for the induction of apoptosis in a caspase-independent manner has recently been provided. The mitochondrial apoptosis-inducing factor was identified as a possible inducer (Joza *et al.*, 2001; Susin *et al.*, 1999) and linked to a serine protease able to damage the mitochondrial membrane (Egger *et al.*, 2003) or to the calcium-dependent proteinase calpain, which cleaves cytoskeletal proteins or the pre-apoptotic protein Bax (Vanags *et al.*, 1996; Wolf *et al.*, 1999). However, the exact positions at which these factors could be integrated into the apoptotic pathway have not yet been determined. Similarly, for infections with several viruses such as HIV-1, coxsackie B3 and HSV-1 d120 mutant (Carthy *et al.*, 1998; Galvan *et al.*, 1999; Roumier *et al.*, 2002), the induction of apoptosis and typical signs of a CPE, even in the presence of caspase inhibitors, have been reported. Our results showing that apoptosis could not be completely inhibited by z-VAD-fmk during SeV F infection, combined with the findings that caspase 9 is cleaved (Bitzer *et al.*, 2002) but subsequently not activated (Wolf *et al.*, 1999), support the idea of the existence of additional caspase-independent apoptosis induction during SeV F infection.

A second factor has been identified during infection studies with SeV Z mutants. Viral RNA, transcribed from a mutated leader, was found to bind to the cellular protein TIAR, which had been proposed to be involved in apoptosis induction during SeV Z infection (Garcin *et al.*, 1998; Iseni *et al.*, 2002). Binding of RNA to TIAR seems to influence its activity negatively, thereby diminishing the virus-induced CPE. As a prerequisite for this mechanism to function efficiently, enough molecules of ctr RNA have to be generated during viral transcription (Lamb & Kolakofsky, 1996), otherwise unbound TIAR proteins could still be functional, contributing to the induction of apoptosis. In the present studies, LLC-MK_2 was the only cell line, among seven lines tested, showing no sign of CPE during infection with the mutant Fctr48z (Fig. 3). Therefore, we infected BHK cells – as a representative cell line exhibiting a strong CPE – with an enhanced m.o.i. of 10 in order to provide initially more available templates for transcription of ctr RNA. However, no significant reduction in CPE could be observed (data not shown). A loss of functionality of the genetically modified leader sequence within Fctr48z due to mutations could be excluded after monitoring genetic stability for 10 virus passages. We concluded that triggering of an apoptotic response by the virus mutant Fctr48z did not solely depend on the TIAR-induced pathway.

The assumption that SeV F induces apoptosis via more than one mechanism, one of which is caspase independent, as recently shown for adenovirus (Zou *et al.*, 2004), was confirmed by results obtained from infection of HeLa cells with our Fctr48z mutant. Compared with SeV F, the apoptotic response in Fctr48z infection was only slightly reduced, but was completely abolished when z-VAD-fmk was added (Fig. 5). Thus, prevention of apoptosis in HeLa cells was only prevented when both mutated leader RNA transcribed during Fctr48z infection and z-VAD-fmk were present. This lends further support to apoptosis induction via two mechanisms in an additive manner (Fig. 6a).

Interestingly, results obtained by infection of LLC-MK_2 cells with Fctr48z revealed interference with both mechanisms (Fig. 6b). While induction of apoptosis in LLC-MK_2 cells by SeV F was partially reduced in the presence of the pan-caspase inhibitor, infection of the same cells with Fctr48z completely abrogated apoptosis, even in the absence of z-VAD-fmk (Fig. 5). In this case, the mutated leader transcript alone was obviously sufficient to protect these cells from apoptosis. Therefore, both apoptosis-inducing

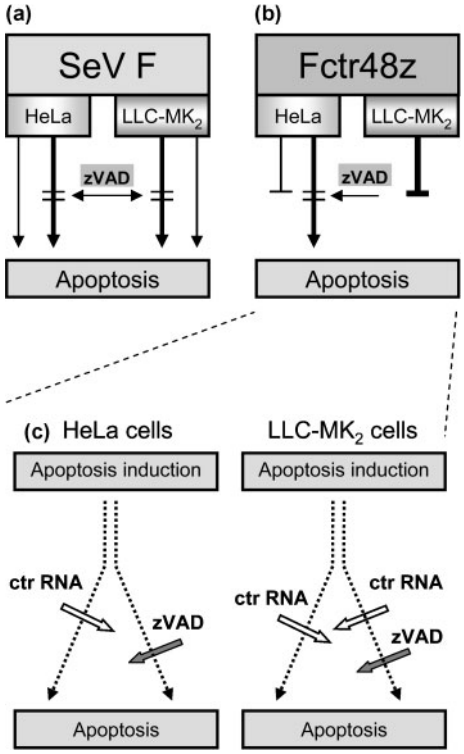

Fig. 6. Schematic model of apoptosis induction in HeLa and LLC-MK_2 cells infected with SeV F or Fctr48z under the influence of apoptosis inhibitors. In infected cells, different mechanisms (depicted by arrows) can lead to an apoptotic reaction. (a) SeV F triggers apoptosis in both cell types via two pathways, one of which can be reversibly blocked by z-VAD-fmk. (b) During Fctr48z infection, apoptosis induction is blocked in a cell type-specific way. In HeLa cells, there is complete inhibition by zVAD. In LLC-MK_2 cells, there is complete abrogation of apoptosis, even in the absence of zVAD. Bold and thin lines/arrows represent the relative weighting of each mechanism inside cells. (c) Possible explanation of cell type-specific apoptosis inhibition (in HeLa and LLC-MK_2 cells) through a different spectrum of activity of the inhibitors ctr RNA and zVAD.

mechanisms were somehow inhibited simultaneously during infection. Two explanations to interpret the observed results are conceivable. In case of HeLa cells, apoptosis during SeV infection can be induced via (at least) two mechanisms, one of which can be inhibited by ctr RNA and the other by z-VAD-fmk (Fig. 6c). In contrast to HeLa cells, ctr RNA in LLC-MK_2 cells seems to have the potential to block both apoptosis-triggering mechanisms, whereas z-VAD-fmk can only partially abrogate apoptosis (Fig. 6c). However, the TIAR-induced mechanism seems to play a more dominant role in LLC-MK_2 cells compared with HeLa cells.

Support for complete abrogation of the apoptotic response via a caspase-independent mechanism comes from studies performed with mutants of SeV Z. This virus has been demonstrated to induce apoptosis with involvement of the cellular protein TIAR (Iseni *et al.*, 2002). The only known characteristics of TIAR so far are its ability to bind RNA, to regulate mRNA translation and to be involved in alternative splicing of several pre-mRNAs (Forch & Valcarcel, 2001; Gueydan *et al.*, 1999; Piecyk *et al.*, 2000). Direct intracellular interaction of TIAR proteins with transcribed viral RNA has been documented during infection with SeV (Iseni *et al.*, 2002) and the flavivirus West Nile virus (Li *et al.*, 2002).

Results from previous work with SeV Z and F (Bitzer *et al.*, 1999, 2002; Garcin *et al.*, 1998; Iseni *et al.*, 2002) combined with our data thus lead to the following conclusions: (i) so far unknown virus strain-specific characteristics determine which mechanism for induction of apoptosis is triggered and to what extent; and (ii) variable apoptosis-inducing mechanisms exist simultaneously during SeV infections. For the first time, we have demonstrated a combination of TIAR-induced and caspase-mediated apoptosis within virus-infected cells. During evolution of eukaryotic cells, the development of redundant pathways or mechanisms to trigger programmed cell death does not seem to be surprising: apoptosis marks an essential reaction for multicellular organisms to survive. On the other hand, viruses have to provide an efficient means of shutting off these cell death-inducing pathways.

While most apoptotic reactions involve the participation of caspases, as shown here for SeV infection (Fig. 5), the TIAR-related pathway can also influence virus-induced CPE, albeit to a lesser degree.

Interestingly, repression of the TIAR-related pathway of apoptosis induction alone can (*trans*)-dominantly block the caspase-related CPE, as shown for LLC-MK_2 infection with Fctr48z (Fig. 5). A complete block of apoptosis has previously also been shown for infection of various cell lines with SeV Z mutants (Garcin *et al.*, 1998; Iseni *et al.*, 2002). This phenomenon can only be detected if cells are infected with mutants that transcribe ctr RNA in larger amounts, such as Fctr48z, resulting in enhanced interaction with cellular TIAR proteins. During SeV wild-type infection, transcription of trailer RNA, corresponding to the first 48 nt of ctr RNA, starts before new genomes are synthesized. Genome amplification provides more templates for viral transcription enabling high-level protein synthesis, delineating a starting point for the development of a strong CPE. Thus, transcription of trailer RNA may ensure efficient viral amplification by delaying the early onset of an apoptotic reaction.

In conclusion, we have elucidated the co-existence and interrelationships of two different pathways of apoptosis induction using a SeV mutant genetically adapted to allow this investigation. Whether this and possibly other means of preventing or delaying apoptotic reactions play an important role during infections with other viruses remains to be explored.

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