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Viral protein synthesis is required for Enterovirus 71 to induce apoptosis in human glioblastoma cells

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Human glioblastoma cells (SF268) develop apoptosis, as characterized by DNA fragmentation and caspase activation, upon infection with Enterovirus 71 (EV71). To determine the step in virus replication that triggers apoptosis, the authors used ultraviolet (UV)-inactivated virus, inhibitors of protein and viral RNA synthesis, and chloroquine to block virus uncoating. Activation of caspase-3 was detected 24 h after infection with EV71 but not with UV-inactivated EV71. Apoptosis was inhibited when EV71-infected cells were treated with chloroquine, guanidine HCl, or cycloheximide. In summary, the authors studied the event(s) required to induce apoptosis in EV71-infected human glioblastoma cells, a subject much less studied than the possible role of viral proapoptotic genes, concluding that EV71 induces apoptosis in the infected SF268 cell in the presence of viral protein synthesis and virus replication, whereas virus adsorption, internalization, entry, uncoating, and viral RNA replication are all not required to trigger the apoptosis. *Journal of NeuroVirology* (2008) **14,** 53–61.

Keywords: apoptosis; Enterovirus 71; viral protein synthesis

Introduction

Enterovirus 71 (EV71) is a member of the genus enterovirus, family Picornaviridae, a large and diverse group of small RNA viruses. Poliovirus is the prototype virus of the genus enterovirus. Enteroviruses all contain a positive-strand RNA genome of approximately 7400 nucleotides. After infection of the host cell, the genome is translated in a cap-independent manner into a single polyprotein, which is subsequently processed by virus-encoded proteases into the structural capsid proteins and the nonstructural proteins, the latter are mainly involved in the replication of viral RNA. Possibly because of the limited coding capacity of picornavirus genomes, precursor polyproteins as well as mature cleavage products actively participate in viral replication (Wimmer and Nomoto, 1993; Bedard and Semler, 2004).

EV71 poses a global public health problem, for which there is no effective therapy and no vaccine. Outbreaks of infection with this virus have occurred around the world. EV71 infection is most frequently as a childhood exanthem known as hand, foot, and mouth disease (HFMD). However, acute EV71 infection can also be associated with severe neurological disease and significant mortality. Children under 5 years old are particularly susceptible to the most severe forms of EV71-associated neurological complications, including aseptic meningitis, brainstem and/or cerebellar encephalitis, acute flaccid paralysis (AFP), myocarditis, and rapid fatal pulmonary edema and hemorrhage. Such presentations as well as a poliomyelitis-like syndrome have been observed during outbreaks in Taiwan, Malaysia, Singapore, western Australia, Bulgaria, New York, and Europe (McMinn, 2002; Ho *et al*, 1999).

Apoptosis, or programmed cell death, is a tightly controlled cell suicide process, critical for the formation (embryonic development) and maintenance

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(tissue homeostasis) of multicellular organisms. Programmed cell death also plays a protective role in that it eliminates cells damaged by irradiation, chemical injuries, or virus infections (Li and Stollar, 2004). The elimination of damaged cells due to virus infection can be mediated by cytotoxic T-lymphocyte recognition, or by the expression of viral components, which can modify the intracellular dynamic balance between factors promoting and inhibiting apoptosis (White, 1996). On the other hand, viruses have evolved strategies to regulate apoptosis, either by blocking it to prevent premature death of the host cell and thus maximizing virus production or facilitating persistent infections, or by inducing apoptosis to spread virus progeny while limiting host inflammatory and other immune responses (Li and Stollar, 2004; Levine, 2002).

In the case of picornaviruses, the infections have been shown to exert both anti- and proapoptotic effects. Apoptotic cells are present in coxsackievirus (CV)-infected myocardium and central nervous system (CNS) (Gebhard *et al*, 1998; Feuer *et al*, 2003), and CV infection activates caspase-3 (Carthy *et al*, 1998). A proapoptotic effect has also been proposed for the CVB3 VP2 polypeptide, which interacts with the cellular protein Siva (Henke *et al*, 2000). Poliovirus infection of transgenic mice expressing the poliovirus receptor triggers neuronal apoptosis (Girard *et al*, 1999); both the 2A and 3C proteins of poliovirus are sufficient to induce cellular apoptosis in the absence of other viral gene products (Goldstaub *et al*, 2000; Li *et al*, 2002). However, picornaviral proteins may also exert an anti-apoptotic functions. The poliovirus 3A protein can inhibit tumor necrosis factor (TNF)–triggered apoptosis by eliminating the cytokine receptor from the surface of the infected cell (Neznanov *et al*, 2001), and the CVB 2B protein seems to exert intracellular antiapoptotic effects (Campanella *et al*, 2004). That individual poliovirus proteins can have both pro- and antiapoptotic effects indicates that apoptosis of poliovirus-infected cells is tightly regulated and delicately balanced (Blondel *et al*, 2005; Buenz and Howe, 2006). In addition, the prevention of apoptosis could be one of many requirements for the successful establishment of viral persistence (Griffin and Hardwick, 1997). Some strains of Theiler's murine encephalomyelitis virus (TMEV) express a unique protein called L protein, that has an antiapoptotic activity and is crucial for viral persistence (Ghadge *et al*, 1998).

EV71 has been demonstrated to induce apoptosis in several cell lines, such as Vero and HeLa cells, and it has been proposed that the cleavage of eIF4GI by the viral 2A protease causes apoptosis (Kuo *et al*, 2002). Work from our group showed that the 3C protease activity of EV71 triggers apoptosis in human neural SF268 cells via the mitochondrial pathway, which is mediated by activation and cleavage of caspase-9 (Li *et al*, 2002; Chang *et al*, 2004).

Given the importance of EV71 to human health, the development of strategies to prevent or possibly treat EV71 infection should be given high priority. Studies directed to a better understanding of EV71-induced apoptosis are likely to provide insights that will facilitate the development of such strategies. Although a number of reports have been concerned with proapoptotic genes of EV71 (Li *et al*, 2002; Kuo *et al*, 2002; Chan and Abubakar, 2003; Chang *et al*, 2004), little is known regarding exactly how the virus induces apoptosis in infected cells. To determine the step in EV71 replication that triggers apoptosis, we examined the requirements for induction of caspase-3 activation by EV71 in SF268 cells, using ultraviolet (UV)-inactivated virus, inhibitors of viral protein and RNA synthesis, and virus uncoating. Our results suggest that apoptosis is initiated by the expression of viral proteins and that virus adsorption, internalization, uncoating, and RNA synthesis are not required to induce the apoptosis in EV71-infected SF268 cells.

Results

EV71 induces apoptosis in SF268 cells

In addition to HFMD, EV71 infection causes severe neurological complications. Apoptosis has been implicated in virus induced neuronal diseases (Everett and McFadden, 2001; Li and Stollar, 2004). Work from our group and others has shown that EV71 infection induced apoptosis in infected cells (Kuo *et al*, 2002; Li *et al*, 2002; Chang *et al*, 2004). However, the correlation between virus replication and apoptosis remains unknown. To obtain further insight into the mechanism of EV71-induced apoptosis in human gliomablastoma cells, SF268 cells were infected with EV71 at an multiplicity of infection (m.o.i.) of 2 and the DNA analyzed at the indicated times after infection (Figure 1A). Samples from mock-infected or EV71-infected cells were electrophoresed in an 1.5% agarose gel. Intense internucleosomal fragmentation, indicative of cells undergoing apoptosis, was observed in EV71-infected SF268 cells at 24 and 48 h post infection and in 2 μg/ml actinomycin D–treated cells. No DNA laddering was observed in mock-infected cells or in cells infected with EV71 for 8 and 12 h. Because caspases play a central role in apoptosis, we further examined whether EV71 infection induces caspase-3 activation. The caspases exist as inactive procaspases that require proteolytic activation. To investigate the mechanism of caspase activation during EV71 infection and to determine whether there is any correlation between induction of caspase-3 activity and the processing of the procaspase to the proteolytic fragment, we examined in parallel samples from EV71-infected SF268 cells for both the cleavage of p32 precursor and the caspase-3 activity (Figure 1B and C). Cellular extracts obtained at the indicated times after infection were standardized for protein concentration (30 μg) and analyzed by

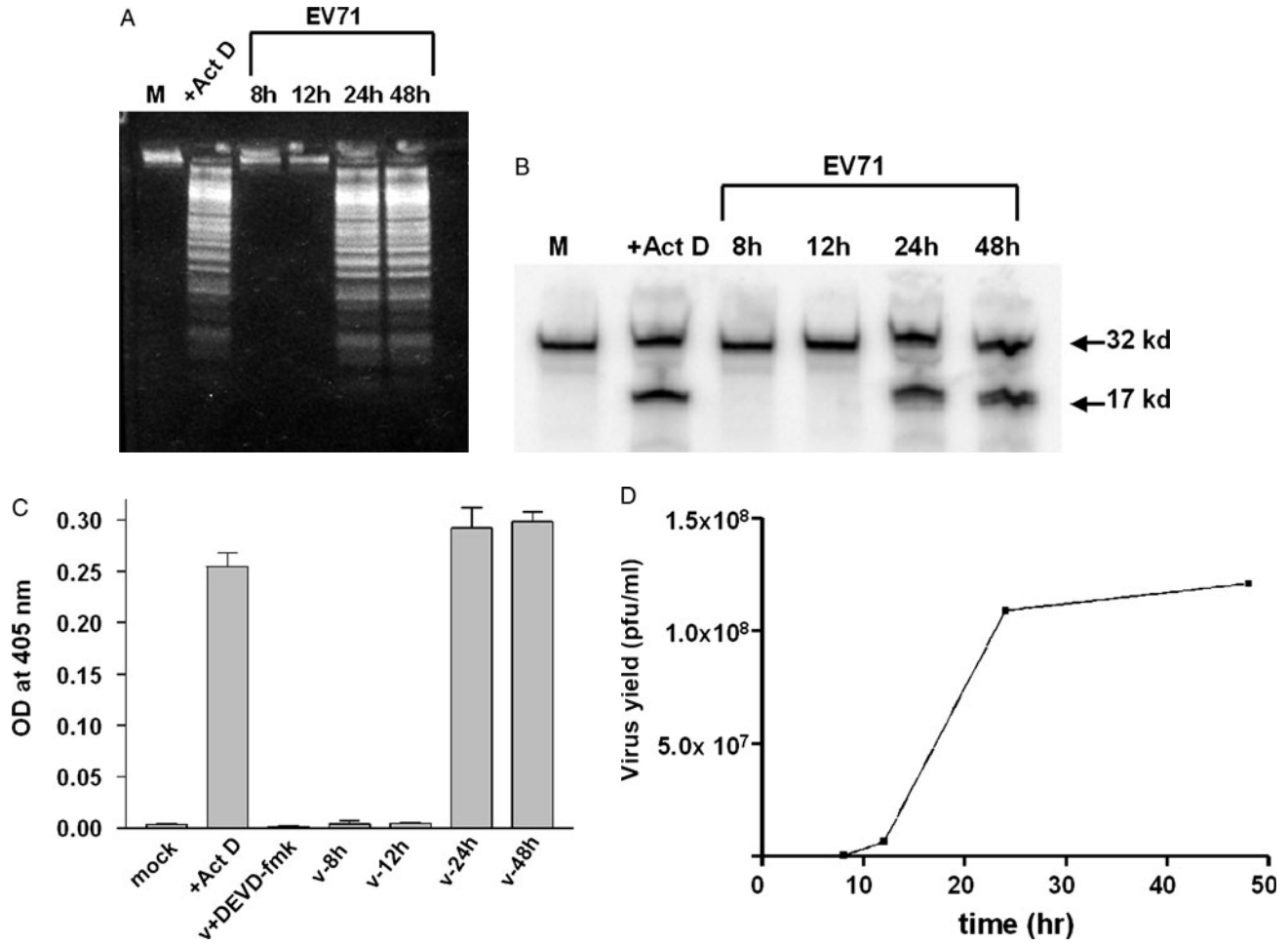

Figure 1 Induction of apoptosis in EV71-infected SF268 cells. (**A**) Agarose gel electrophoresis of the internucleosomal DNA laddering. At the indicated times after infection, DNA from mock- (M) or EV71-infected SF268 cells was obtained and subjected to electrophoresis on a 1.5% agarose gel. (**B**) EV71 infection induced the cleavage of caspase-3. Thirty micrograms of cell lysates from mock- or EV71-infected SF268 cells obtained at the indicated times after infection were subjected to 12% SDS-PAGE electrophoresis. Western blotting analysis with anti-caspase-3 antibody was carried out for the detection using an ECL-based method. (**C**) Caspase-3 activity in EV71-infected SF268 cells. Cell lysates from mock- or EV71-infected cells, or infected with UV-inactivated virus, were obtained at 24 h p.i. and analyzed for caspase-3 activity using the ApoAlert Caspase Colorimetric Assay Kits from Clonetech with caspase-3–specific substrate chromophore *p*-nitroaniline (pNA). The protease activity was measured using a spectrophotometer at 405 nm. Data from two independent assays were averaged and plotted. (**D**) Replication of EV71 in SF268 cells. Confluent cells were infected with EV71 at an m.o.i. of 2 as described in the text and incubated at 37°C. Medium was harvested at indicated time points and assayed for infectious virus by plaque formation on Vero cells.

Western blot using a specific antiserum anti-caspase-3, which can recognize both the 32-kDa precursor and 17-kDa active fragment (Calbiochem, La Jolla CA). Figure 1B shows that infection with EV71 induces the processing of caspase-3 from its 32-kDa precursor into an active fragment of 17 kDa at 24 and 48 h post infection. No processing of caspase-3 was found in mock-infected cells. To test the activation of caspase-3, the ApoAlert Caspase Clorimetric kit was used according to the manufacturer's instruction (Clontech, Mountain View, CA). Figure 1C shows that activation of caspase-3 was observed 24 and 48 h after EV71 infection in SF 268 cells. No caspase-3 activity could be detected at 8 or 12 h post infection or in the presence of caspase-3 inhibitor, the synthetic tetrapeptide DEVD-fmk. Figure 1B and C also show the temporal correlation between the cleavage of caspase-3 and its enzymatic activity.

Virus was harvested and titrated in Vero cells by standard plaque assay. As shown in Figure 1D, virus yield reached a maximum ($\sim 10^8$ plaque-forming units [PFU]/ml) by 24 h post infection (p.i.). Thus EV71-infected SF268 cells show evidence of apoptosis when the virus yield reaches its maximum.

Induction of apoptosis by EV71 infection does not require virus adsorption or internalization

To examine whether virus entry is required to induce apoptosis, stocks of [^{3}H]EV71 were prepared from SF268 cells, which were infected with EV71 at an m.o.i. of 1 and metabolically labeled with 20 μCi/100-mm plate of [^{3}H]uridine. Supernatant fluids

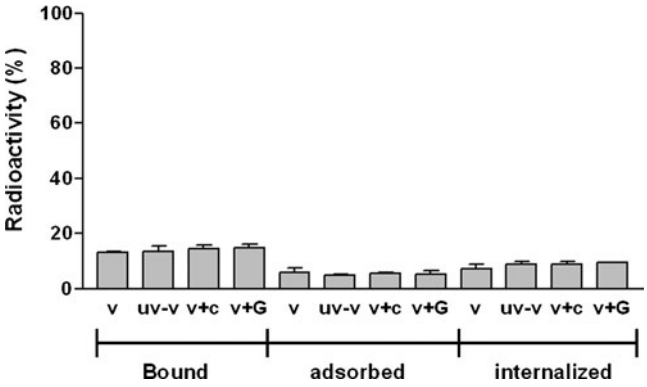

Figure 2 EV71 binding and internalization. SF268 cells were infected with [^{3}H]EV71 in the absence (v) or presence (v+c) of CHX (10 μg/ml) or guanidine HCl (v+G), or with UV-inactivated [^{3}H]EV71 (uv-v), and then assayed for EV71 binding and internalization as described in Materials and Methods. Mean values and standard errors for bound, adsorbed, and internalized radioactivity, from duplicate samples, are shown in the bar graph.

were collected at 4 days p.i. and the extracellular virus was purified by Percoll sedimentation, as described previously (Carrascosa *et al*, 2002).

SF 268 cells were grown in 6-well plates till confluent and then infected with 5×10^4 cpm of purified [^{3}H]EV71 for 4 h at 37°C. Cultures were extensively washed with phosphate-buffred saline (PBS) to remove the unbound material before incubation with Pronase E (1 mg/ml in PBS) for 1 h at 4°C and 15 min at 37°C to release the adsorbed virus. Radioactivity retained after the final washing of cell pellets was considered "internalized," and the sum of internalized plus the "adsorbed/eluted" radioactivity represented the "bound" virus.

As shown in Figure 2, the amount of virus that was internalized was not affected by UV inactivated of the virus, or by guanidine HCl or cycloheximide (CHX) treatment of the infected cells. In each case, about 15% of the virus was internalized.

Taken together, these studies indicate that induction of apoptosis by EV71 infection does not require virus adsorption or internalization.

Viral protein synthesis is necessary to trigger apoptosis in EV71-infected SF268 cells

The step after internalization is the uncoating and release of the viral genome into the cytoplasm. It is known that after entering into the cells, the virus is internalized in acidic endosomes. The genome then appears to transverse the endosomal membrane and enter the cytosol. Agents such as chloroquine, monensin, tributylin, or metabolic inhibitors that raise endosomal pH, dissipate proton gradients, inhibit acidification, or deplete ATP inhibit entry of the genome into the cytoplasm (Smyth and Martin, 2002).

To further determine the step after EV71 internalization needed to trigger the apoptosis, we studied the effect of chloroquine, an inhibitor of virus uncoating, on the induction of apoptosis by EV71 in SF268 cells. Confluent SF268 cells were pretreated with 1.2 mM chloroquine 1 h prior to infection and maintained throughout the infection course (Zeichhardt *et al*, 1985). Media were harvested at 24 h p.i. and virus yield was determined by plaque formation on Vero cells. Table 1 shows that chloroquine inhibited the virus yield by more than 10,000 fold, indicating the uncoating was blocked by this inhibitor. Viral RNA replication was examined by the incorporation of [^{3}H]uridine into newly synthesized viral RNA at the presence or absence of 1.2 mM chloroquine. As shown in Figure 3A, in the presence of chloroquine, the viral RNA synthesis was reduced by more than 90%. Cell lysates were analyzed by the ApoAlert Colorometric Kit for the activation of caspase-3 as described above. Our results indicated that chloroquine inhibited the activation of caspase-3 in EV71-infected cultures (Figure 3B).

Table 1 Replication of EV71 in the presence of chloroquine

Virus and treatment	Virus titer (pfu/ml)
EV71	2.6×10^8
UV-EV71	4.3×10^3
Chl+EV71	3.7×10^3

Note. Confluent SF268 cells were infected with EV71 at an m.o.i. of 2 in the presence or absence of 1.2 mM chloroquine. Media were harvested 24 h post infection and virus yield was determined by plaque formation on Vero cells.

To determine whether viral RNA replication is necessary to induce apoptosis in EV71-infected SF268 cells, we infected the cells with EV71 in the presence or absence of 2 mM guanidine HCl and evaluated the caspase-3 activity induced at 24 h after infection. To examine viral RNA replication, SF268 cells in 6-well dishes were infected with EV71 at an m.o.i. of 2, labeled with 10 μCi/plate of [^{3}H]uridine from 20 to 24 h post infection. Cells were then lysed and centrifuged. Supernatant was collected, nucleic acids were precipitated with trichloroacetic acid (TCA) and the radioactivity was counted by scintillation counter. As expected, labeled RNA was detected only in cultures infected with the active virus in the absence of guanidine HCl (Figure 4A). In another experiment (not shown), guanidine HCl blocked the production of infectious virus. As shown in Figure 4B and C, caspase-3 activity was elevated in EV71-infected cells but could not be detected when in the presence of guanidine HCl, indicating viral RNA synthesis is required for the induction of apoptosis in SF268 cells.

To determine whether viral protein synthesis could affect the EV71-induced apoptosis, we infected SF268 cells with EV71 in the presence or absence of CHX (10 μg/ml), and evaluated the caspase-3 activity induced at 24 h after infection. Expression of viral proteins was monitored by Western blotting using anti-3C antiserum. As shown in Figure 5A, there was no synthesis of 3C protein in cultures infected either with UV-inactivated EV71 or with the infective virus in the presence of CHX. As shown in Figure 5B

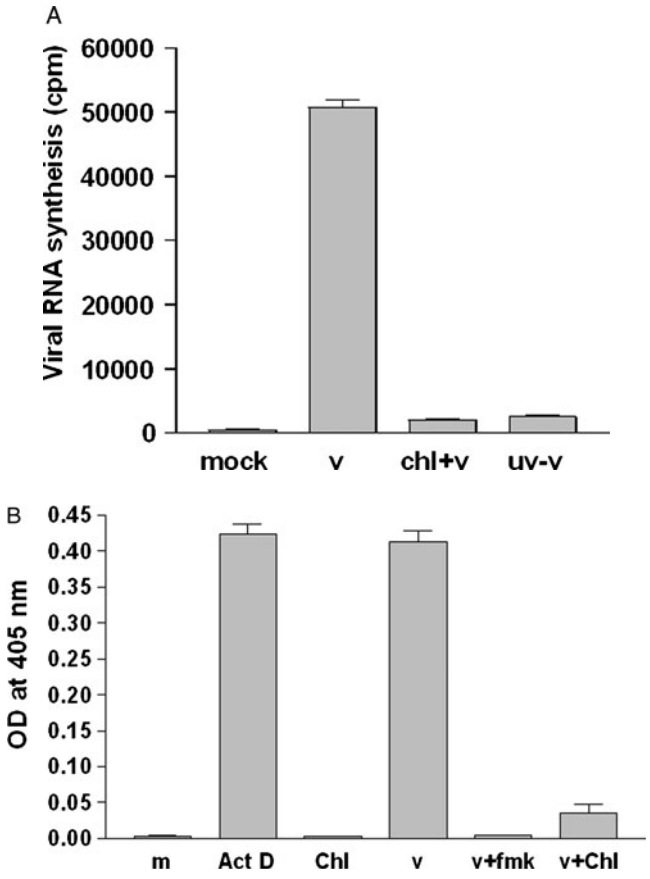

Figure 3 Effect of chloroquine on EV71-induced apoptosis. (**A**) Effect of chloroquine on viral RNA synthesis. SF268 cells, infected (v) or not (mock) with infective or UV-inactivated EV71 (uv-v) and labeled from 20 to 24 h p.i. with [^{3}H]uridine, in the absence (v) or presence of 1.2 mM chloroquine, were collected and resuspended in a solution of 0.6% NP-40 in PBS. After incubation at 4°C for 30 min, the nuclear fraction was removed by centrifugation and the acid-insoluble radioactivity was determined in the supernatant (cytoplasmic) fraction. Mean values and standard errors from duplicate samples are shown. (**B**) Caspase-3 activity in EV71-infected SF268 cells. Cell lysates from mock- or EV71-infected cells in the presence or absence of 1.2 mM chloroquine, or infected with UV-inactivated virus, were obtained at 24 h p.i. and analyzed for caspase-3 activity as described in Figure 1C.

and C, no caspase-3 activity could be detected in EV71-infected cells treated with CHX. CHX can inhibit cellular protein synthesis as well so that the inhibition of EV71-induced apoptosis could be due to the inhibition of host protein(s) whose synthesis is required for apoptosis. However, no caspase-3 activation was observed in mock-infected cells, suggesting host protein synthesis may not be required for EV71-induced apoptosis. We therefore conclude that viral protein synthesis is necessary to induce apoptosis in EV71-infected SF268 cells. Inhibition of apoptosis by chloroquine and guanidine HCl is due to the inhibition of uncoating and viral RNA replication that block the downstream viral protein synthesis.

On the other hand, the fact that the induction of caspase-3 was never observed with UV-inactivated EV71 (Figures 3B, 4B, 4C, 5B, and 5C) indicates that

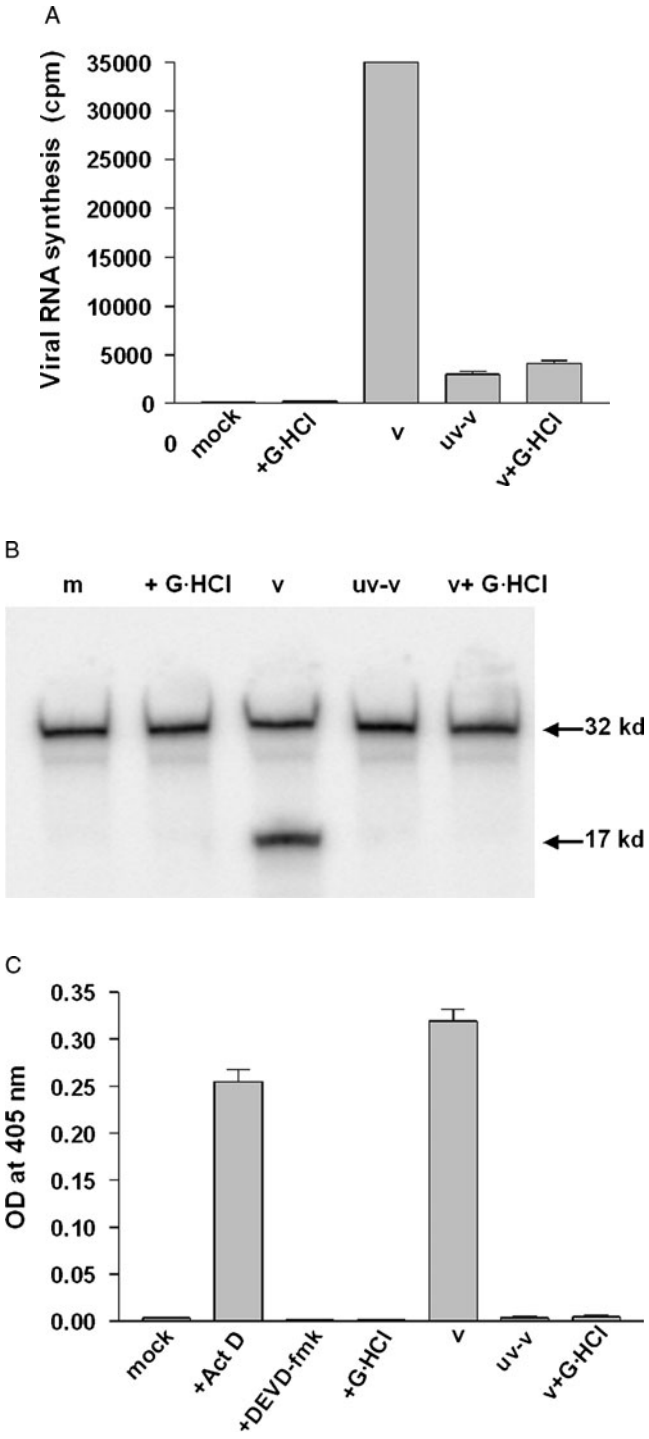

Figure 4 Effect of EV71 RNA replication on induction of apoptosis in EV71-infected SF268 cells. (**A**) EV71 RNA synthesis. Viral RNA from EV71-infected SF268 cells in the absence (v) or presence of 2 mM guanidine HCl (v+G·HCl), were determined as described in Figure 3A. (**B**) Cleavage of caspase-3. Thirty micrograms of cell lysates from mock- or EV71-infected SF268 cells in the presence or absence of guanidine HCl were subjected to 12% SDS-PAGE electrophoresis. Western blotting analysis was carried out to detect caspase-3 using an anti-caspase-3 antibody, which can recognize both the uncleaved (32-kDa) and cleaved (17-kDa) forms of caspase-3. (**C**) Activation of caspase-3. Lysates from mock- or EV71-infected cells in the presence or absence of guanidine HCl, or infected with UV-inactivated virus, were obtained and subjected to caspase-3 activity assay as described in Figure 1C.

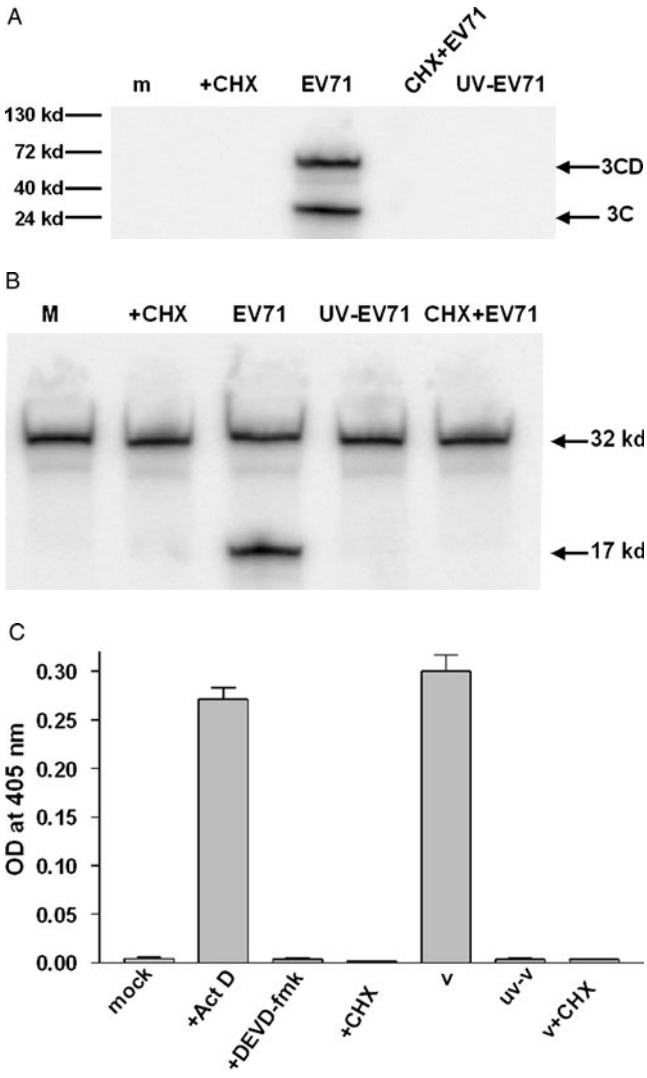

Figure 5 Viral protein synthesis is required for inducing apoptosis in EV71-infected SF 268 cells. (**A**) Expression of EV71 3C protein. Cell lysates from mock- or EV71-infected SF268 cells in the presence or absence of 10 μg/ml CHX, or UV-inactivated EV71, were subjected to 12% SDS-PAGE electrophoresis. Western blotting analysis was carried out using an anti-3C antibody. (**B**) Effect of CHX on the cleavage of caspase-3. Cell lysates from mock- or EV71-infected SF268 cells in the presence or absence of CHX (10 μg/ml) were separated on 12% SDS-PAGE and subjected to Western blotting using an antibody reacts with both the 32- and 17-kDa fragments of caspase-3. (**C**) Effect of CHX on aspase-3 activity. Lysates from mock- or EV71-infected SF268 cells in the presence or absence of 10 μg/ml CHX, or infected with UV-inactivated virus, were obtained and subjected to caspase-3 activity assay as described in Figure 1C.

apoptosis was not induced with these inactivated viral particles.

Discussion

Many reports have indicated that infection with EV71 induces apoptosis in both cultured cells and in the CNS of neonatal mice and cynomolgus monkey (Kuo *et al*, 2002; Nagata *et al*, 2002; Chen *et al*, 2004; Chang *et al*, 2004). In general, the ability of virus strains to induce apoptosis correlates with their neurovirulence, although both apoptosis and neurovirulence are age dependent, i.e., resistance increases with age (Yu *et al*, 2000). The cellular pathways and virus replication cycle involved in EV71-induced apoptosis are complex, and much remains poorly understood.

Apoptosis is the process whereby cells undergo systematic self-destruction in response to a wide variety of stimuli. In the case of virus-infected cells, the induction of early cell death would severely limit virus production and reduce or eliminate the spread of progeny virus in the host. Thus, many animal viruses have evolved strategies to evade or delay early apoptosis to allow production of high yields of progeny virus. However, apoptosis is also a very efficient mechanism by which the virus can induce cell death and spread progeny while limiting induction of inflammatory and immune responses (Everett and McFadden, 2001; Whitton *et al*, 2005; Buenz and Howe, 2006). Viruses struggle to maintain apoptotic homeostasis and simultaneously evade the host immune system. In the case of picornaviruses, reports of decreased tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) receptor expression (Neznanov *et al*, 2002) and inhibition of sorbitol-induced apoptosis by poliovirus infection (Koyama *et al*, 2001) suggest the presence of a potential viral mechanism to suppress the induction of apoptosis in infected cells. On the other hand, reports of DNA fragmentation and caspase activation indicate that picornaviruses are able to induce apoptosis (Blondel *et al*, 2005). Because less information is available on the process of EV71-induced apoptosis in terms of viral replication cycle and specific proteins involved, we have studied in the present work the mechanism by which the virus induces apoptosis in the infected cell. Interestingly, the fragmentation of the cellular DNA could only be detected 24 h after infection with EV71 in SF268 cells. Similar results were obtained in the analysis of caspase-3 activation, which was induced at 24 h after EV71 infection. The observation indicates that to ensure the production of progeny virus, the EV71-infected cells did not undergo the apoptosis at early times of infection. Thus, the apoptosis in EV71-infected cells must be tightly regulated and balanced by the expression of viral pro- and antiapoptotic genes through out the infectious cycle.

Activation of caspases is an event associated with apoptosis. Caspases are activated either via the receptor-mediated or the mitochondrial pathways. The receptor-mediated pathway leads to the activation of caspase-8. In the mitochondrial pathway, however, proapoptotic members of the Bcl-2 family associate with mitochondria and direct the release of cytochrome *c* and other proteins which activate procaspase-9, leading to the activation of

effector caspases such as caspase-3 (Kroemer *et al*, 1997; Zamzami *et al*, 1997). We report here that EV71 infection activates caspase-3 in SF268 cells. In a recent report (Chang *et al*, 2004), we have demonstrated that (1) during EV71 infection, cytochrome *c* was released into the cytosol of infected SF268 cells, and (2) caspase-9 was activated. These results suggest that apoptosis induced by EV71 involves the mitochondrial pathway.

Although cell death in the absence of viral replication has been reported for several virus infections (Li and Stollar, 2004; Carrascosa *et al*, 2002), viral replication is required for the induction of apoptosis by many types of viruses. In the previous case, apoptosis was triggered in an early step of the infective cycle, and usually structural proteins of virus were involved in the process, for example: sigma-1 attachment protein in type 3 reovirus (Tyler *et al*, 1995), virion L1R protein in vaccinia virus (Ramsey-Ewing and Moss, 1998), or envelop proteins E2 and E1 in Sindbis virus (Jan and Griffin, 1999). In the case of EV71, we found that viral replication is required to induce apoptosis in infected cells since activation of caspase-3 was not detected in cells infected with UV-inactivated virus. In contrast to the induction of apoptosis by UV-inactivated herpes simplex virus 1 in human HEp-2 cells (Aubert *et al*, 1999), UV-inactivated EV71 was not able to trigger apoptosis in SF268 cells.

As UV irradiation could have a deleterious effect also on viral proteins, resulting in a less efficient virus entry (Jan and Griffin, 1999), we therefore carried out a study of the process of EV71 entry. Interestingly, the statistics of virus binding and internalization were similar in all the virus infections, indicating that the presence of guanidine HCl or CHX or the UV-inactivation of EV71 did not affect the process of virus entry. This result rules out the possibility of induction of apoptosis by interactions between viral and cellular components involved in the process of EV71 entry, which should proceed normally in SF268 cells infected by UV-inactivated EV71 (in the absence of caspase-3 activation), and establishes a model for apoptosis induction different from those of human immunodeficiency virus (HIV)-1 or bovine herpesvirus-1, in which cell death was induced early in the infection, by the interaction of virus components with a cellular protein in the plasma membrane (Banda *et al*, 1992; Hanon *et al*, 1998).

Consequently, the next step to analyze in the EV71 cycle was the virus uncoating. It has been known that agents such as chloroquinine, monensin, tributylin, or metabolic inhibitors that inhibit acidification, or deplete ATP, interfere with the uncoating of virus particles (Smyth and Martin, 2002). In the present study, we have found that caspase-3 activation does not occur in EV71-infected SF268 cells incubated in the presence of chloroquine, indicating that uncoating of EV71 particles was needed to induce the apoptosis infected cells.

It is interesting to note that the activation of caspase-3 was not observed in EV71-infected SF268 cells in the presence of CHX, an inhibitor of protein synthesis. This observation indicates that apoptosis induced by EV71 infection is dependent upon the synthesis of the viral proteins. No caspase-3 activity was detected in mock-infected cells, indicating cellular protein synthesis may not be necessary for the EV71-induced apoptosis. The fact that caspase-3 activation could not be detected in EV71-infected SF268 cells in the presence of CHX rules out the role of viral uncoating and genome replication in induction of apoptosis. The inhibition of viral uncoating by chloroquine and interference with the viral genome replication by guanidine HCl, further inhibiting the viral protein synthesis, block the apoptotic process.

Our finding that viral protein synthesis is required for the induction of apoptosis in infected cells was further supported by other reports: Chan and Abubakar (2003) demonstrated that EV71 infection could induce apoptosis in Vero cells and the induction requires high level of EV71 infectivity and the presence of live virus particles, indicating the need for the expression of specific viral proteins for triggering apoptosis; report from Kuo *et al*, (2002) showed that transient expression of EV71 2A protease resulted in the induction of apoptosis in HeLa and Vero cells. In addition, our group reported that transient expression of EV71 3C protein triggered apoptosis in SF268 cells and that it is the proteolytic activity of 3C that induces the apoptosis in transfected cells (Li *et al*, 2002). Our finding in this report pinpoints to the requirement of viral protein synthesis for the induction of apoptosis in EV71-infected SF268 cells.

From these results we conclude that the apoptotic signal in EV71-infected SF268 cells is triggered in the presence of virus replication, after virus internalization, uncoating, viral RNA replication, and most probably during the virus protein synthesis.

Materials and methods

Cells and virus

SF268 (human glioblastoma) cells were cultured at 37°C in RPMI medium 1640 supplemented with 10% fetal calf serum (FCS) (Mediatech, VA). Vero cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Mediatech, VA). EV71 was propagated in SF268 cells. Cells were infected with EV71 at specified multiplicity of infection (m.o.i.) in a small volume of medium supplemented with 2.5% FCS for 2 h at 37°C, unbound inoculum was washed away, and cells were refed with fresh medium. Media from infected cultures were harvested at the indicated times, and virus titers were measured by plaque formation on Vero cells.

UV inactivation of EV71
EV71 was kept on ice and inactivated by exposing the virus to a portable UV lamp at 225 nm wavelength at a distance of 2 cm for 30 min. Inactivation was confirmed by titration of the virus before and after UV exposition, and by the absence of cytopathic effect after infection of Vero cells with UV-inactivated EV71.

Preparation of [^{3}H]EV71
Vero cells were infected with EV71 at an m.o.i. of 1 and labeled with 20 μCi/100 mm dish of [^{3}H]uridine. Medium was harvested at 4 days after infection. Virions were purified by 12.5%, 25%, and 60% sucrose step gradient and centrifuged at 25,000 rpm for 2 h.

Virus binding and internalization assays
Cells were grown on 6-well plates till confluent and then infected with 5×10^4 cpm of purified [^{3}H]EV71 for 4 h at 37°C. Cultures were extensively washed with PBS to remove the unbound virus before incubation with Pronase E (1 mg/ml in PBS) for 1 h at 4°C and 15 min at 37°C to release the adsorbed virus. Radioactivity retained after the final washing of cell pellets was considered "internalized." The amount of bound virus is the sum of internalized and the "adsorbed/eluted" radioactivity.

Drug treatments
All inhibitors were purchased from Sigma Chemical and used at the indicated concentrations unless specified. SF268 cell monolayers were pretreated with cycloheximide (CHX; 10 μg/ml), guanidine HCl (2 mM), or chloroquine (1.2 mM) for 1 h before EV71 infection. Inhibitors were also present at the indicated concentrations in the virus inoculum (during absorption of viral particles), in the radioactive medium for labeling, and in the culture medium along the infective cycle.

Metabolically radiolabeling of EV71 RNA
To examine the viral RNA replication, SF268 cells in 6-well dishes were infected with EV71 at an m.o.i. of 2, labeled with 10 μCi/plate of [^{3}H]uridine from 20 to 24 h post infection. Cells were then lysed with 0.6% NP-40 on ice for 30 min and centrifuged at 12,000 rpm for 10 min at 4°C. Supernantant was collected, nucleic acids were precipitated by TCA and the radioactivity was counted by scintillation counter.

Western blotting
To monitor the viral protein synthesis, confluent SF268 cells in 60-mm plates were infected with EV71 at an m.o.i. of 2 for 24 h. Expression of viral protein was examined by Western blotting using antibody against EV71 3C protein. Briefly, cells were collected in sample buffer and proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels and transferred to polyvinylidene fluoride membranes by wet transfer. Membranes were blocked with PBS containing 5% low-fat dry milk. Anti-3C antibody was then added, and the membranes were washed with PBS containing 0.2% Tween 20. Goat anti-mouse horseradish peroxidase–conjugated antibody (Pierce, Rockford, IL) and the enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ) were used to detect bound antibodies. Chemiluminescence was detected by exposure to Kodark x-ray film.

Characterization of apoptosis
For analysis of DNA fragmentation, DNA was isolated using the Blood and Cell Culture DNA mini kit (Qiagen, Valencia, CA) and analyzed on 1.5% agarose gel. Caspase activation and inhibition were analyzed using the ApoAlert Caspase Colorimetric Assay Kit (Clontech, Mountain View, CA). The protease inhibitor DEVD-fmk was purchased from Clontech.

The cleavage of procaspase-3 (32 kDa) to the active form of 17 kDa was detected in EV71-infected cell extracts by Western blotting, using a monoclonal antibody specific for human caspase-3 from Oncogene Research Products (San Diego, CA).

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