Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death

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Enterovirus 71 (EV71) is the causative agent of human diseases with distinct severity, from mild hand-foot-and-mouth disease to severe neurological syndromes, such as encephalitis and meningitis. Infection of several different cell lines with EV71 causes extensive cytopathic effect, leading to destruction of the entire monolayer and the death of infected cells. In this study, cell death processes during EV71 infection and the underlying mechanisms of them were investigated. The hallmarks of apoptosis, nuclear condensation and fragmentation, were observed 24 h after infection. Apoptosis in infected cells was also confirmed by detectable cleavage of cellular DNA and degradation of poly(ADP-ribose) polymerase. Transient expression of EV71 2A protease (2Apro) alone resulted in the induction of apoptotic change. Infection of EV71 or expression of EV71 2Apro leads to cleavage of the eukaryotic initiation factor 4GI, a key factor for host protein synthesis. This study added one more example to the growing list of human viruses that induce apoptosis by a virus-encoded protein.

Introduction

Enterovirus 71 (EV71) is a member of the genus Enterovirus, family Picornaviridae. It is associated with a mild childhood exanthema known as hand-foot-and-mouth disease (Hagiwara et al., 1978; Ishimaru et al., 1980), as well as a number of severe diseases, including encephalitis, aseptic meningitis, pulmonary oedema and poliomyelitis-like paralysis (Alexander et al., 1994; Lum et al., 1998; Chang et al., 1998; Hayward et al., 1989). Since the first report concerning EV71 infection in the United States in 1969 (Schmidt et al., 1974), several epidemic outbreaks have occurred throughout the world, including Bulgaria (Chumakov et al., 1979), Hungary (Nagy et al., 1982), Australia (Gilbert et al., 1988), Japan (Shimizu et al., 1999), Malaysia (Lum et al., 1998; Shimizu et al., 1999) and Taiwan (Ho et al., 1999; Shimizu et al., 1999; Wang et al., 1999; Liu et al., 2000).

EV71 is a small, nonenveloped, icosahedral RNA virus that possesses a single-stranded RNA genome of approximately 7400 nucleotides of positive polarity (Brown & Pallansch, 1995). The genome is predicted to comprise a 5’ untranslated region (UTR), a long open reading frame that encodes a protein of approximately 2100 amino acids, a short 3’ UTR and a polyadenylated tail (Brown & Pallansch, 1995). Although the replication mechanism of EV71 is largely unknown, studies from members in the Picornaviridae family revealed that the plus-strand RNA genome is translated into a single large polyprotein. Maturation cleavage of the polyprotein to generate functional viral proteins is mainly mediated by virus-encoded proteases, designated 2A (2Apro) and 3C (3Cpro). Most of the proteolytic reactions are accomplished by 3Cpro, whereas 2Apro catalyses only two cleavages on the polyprotein, one between the capsid protein precursor and itself and another on 3CD to generate 3C’ and 3D’.

Apoptosis, or programmed cell death, is a genetically determined cell death programme and provides a natural mechanism to remove damaged cells from tissue. The most typical signs involve a series of cellular events that include the...
nucleolytic internucleosome degradation of chromosomal DNA, compaction and fragmentation of chromatin, membrane blebbing and cellular shrinkage (Martin et al., 1994; Green, 2000). Recently, several reports focused on the ability of picornaviruses to affect the apoptosis-inducing activities within infected cells, most evidently exemplified by Theiler’s murine encephalomyelitis virus (TMEV) (Jelachich & Lipton, 1999), coxsackievirus B3 (CVB3) (Carthy et al., 1998) and poliovirus (PV) (Tolkskaya et al., 1995; Agol et al., 1998, 2000; López-Gurreo et al., 2000). Further investigation revealed that expression of 2Apro as the only PV component results in apoptotic cell death (Goldstaub et al., 2000). Moreover, infection of mice with TMEV or PV induces apoptosis in the central nervous system (CNS) and is associated with the neuroviral effect or fatal outcome in infected mice (Tsunoda et al., 1997; Girard et al., 1999).

Infection with picornaviruses can cause dramatic inhibition of host protein synthesis (Haller & Semler, 1995; Sachs et al., 1997), which is followed by a selective and efficient translation of the viral mRNA. Cellular mRNAs contain a 5′-terminal cap structure that is responsible for initiation of translation (Sonenberg & Gingras, 1998). In contrast, picornavirus RNAs have in their 5′ UTRs a complex structure known as an internal ribosome entry site (IRES) element, which directs a cap-independent mechanism of translation (reviewed by Jackson et al., 1994; Belsham & Sonenberg, 1996). An early event occurring during many picornavirus infections is cleavage of the eukaryotic translation initiation factor 4G (eIF4G), either directly or indirectly, by viral 2Apro (Etchison et al., 1982). There are two related eIF4G species, eIF4GI and eIF4GII, which appear to be functional homologues of each other but share only 46% identity (Gradi et al., 1998). IRES-directed initiation of virus protein synthesis is maintained following cleavage of the eIF4G, with concomitant inhibition of capped cellular mRNA translation (Ohlmann et al., 1996; Borman et al., 1997; Gradi et al., 1998; Roberts et al., 1998).

In this study, we showed that infection with EV71 induces apoptosis, as shown by the data from morphological and biochemical studies. Despite a moderate level of homology to poliovirus 2Apro, EV71 2Apro expression is sufficient to trigger cleavage of eIF4GI and apoptotic cell death.

Methods

- **Cell lines and virus.** The cell lines HeLa-229 (ATCC, CCL-2.1), Vero (ATCC, CCL-81) and RD (ATCC, CCL136) were cultured in modified Eagle’s medium (MEM) supplemented with 10% foetal bovine serum (FBS) in an atmosphere of 5% CO2 at 37 °C. EV71 (BrCr strain; ATCC, VR784) was propagated in Vero cells and virus titrated on Vero cell monolayers by virus plaque assay.

- **Infection with EV71.** Cells were seeded on culture Petri dishes a day before infection with EV71 at an m.o.i. of 0.1. After a 2 h adsorption, the cell layer was rinsed with PBS and MEM containing 2% FBS was added. Cells were then incubated at 37 °C throughout the infection.

- **Plasmid construction.** Total RNA was extracted from EV71 virus stock with 200 µl RNAzol B solution (TEL-TEST). The cDNA was first synthesized using 100 units of Moloney murine leukaemia virus reverse transcriptase (Promega) at 37 °C for 60 min. One-fifth volume of the product from the reverse transcription reaction was used as a template for PCR for the EV71 (BrCr strain) 2A-coding region (nt 3330–3779 relative to the predicted transcription initiation site; Brown & Pallansch, 1995), with the primers forward, 5′-CGGATCCATGGGAAATTCGGTCA-GCAGTC 3′, and reverse, 5′-GCTCGAATTCCTGCATCCTCGTTCC-TCAT 3′. The 5′ and 3′ primers were engineered to contain BglII and EcoRI restriction sites for cloning. The conditions for PCR were as follows: 94 °C for 3 min, then 35 cycles of a denaturation step at 94 °C for 1 min, an annealing step at 58 °C for 1 min 30 s and an elongation step at 72 °C for 3 min, and 1 cycle of an elongation step at 72 °C for 10 min. The RT–PCR product was subsequently digested and directionally cloned into the BglII and EcoRI sites of plasmid pEGFP-N2 (Clontech). This construct contains the 2A-coding sequences fused in frame to the N terminus of the green fluorescent protein (GFP) gene and is referred to as p2A-GFP. Plasmid p2A-ires-GFP was constructed by inserting the 2A cDNA fragment into pRES2-EGFP (Clontech), a plasmid harbouring the GFP reporter gene controlled by the IRES from encephalomyocarditis virus (EMCV). This permits both 2A and GFP genes to be translated from a single bicistronic mRNA. The authenticity of each construct was confirmed by automated DNA sequencing.

- **Immunofluorescence assay and Hoechst staining.** Infected cells were fixed with 3% paraformaldehyde for 30 min and 0.2% Triton X-100 for 5 min and then incubated with a mouse anti-EV71 monoclonal antibody (MAb) (Chemicon, 3324) at 37 °C for 30 min. Following two washes in PBS, cells were incubated with the secondary antibody, a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (ICN), at 37 °C for 30 min. Nuclei were counterstained with a DNA-binding dye, Hoechst 33258 (0.5 µg/ml, Sigma). Cell morphology was visualized under a fluorescence microscope equipped with both FITC and UV filters.

- **DNA analysis.** Cells were incubated in versene and then suspended in the buffer containing 20 mM EDTA and 10 mM Tris–HCl (pH 7.4). Next, cells were treated with 0.5% Triton X-100 at 0 °C for 20 min. After centrifugation (12,000 r.p.m. for 15 min at 4 °C), the supernatant was first treated with SDS (at a final concentration of 1%) and then with 10 µg RNase A at 37 °C for 30 min. DNA was then isolated by phenol extraction and ethanol precipitation. DNA was fractionated by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

- **DNA transfection.** Approximately 70% confluent monolayer of Vero or HeLa-229 cells grown on 100 mm diameter dishes were transfected with 30 µg of plasmid DNA using the calcium phosphate precipitation method (Sambrook et al., 1989). After incubation at 37 °C in 5% CO2 for the appropriate amount of time, the cells were analysed as indicated for each experiment.

- **Western blot analysis.** Vero, RD and HeLa-229 cells were harvested at various time-points following the infection of EV71 or transfection of 2A-expressing plasmids. Cell extracts were prepared by washing with cold PBS and scraped into the lysis buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.2% bromophenol blue), separated by SDS–PAGE and transferred onto nitrocellulose membranes. Blots were incubated with mouse anti-PARP [poly(ADP-ribose) polymerase] MAb (1:500 dilution; BD Transduction Laboratories) or mouse anti-eIF4GI antibody (1:250 dilution; BD Transduction Laboratories). Blots were then
Results

EV71 induces apoptosis in the infected cells

To investigate the effects of EV71 infection upon host cells, Vero and HeLa-229 cells were subjected to infection by EV71 at an m.o.i. of 0–1, followed by observation under a phase-contrast microscope. After infection for 24 h, the infected cells exhibited a cytopathic effect reaction with morphological changes, including rounding up, shrinkage, cytoplasmic blebbing, nuclear condensation and detachment from the culture dish, indicative of apoptotic cells (data not shown). To determine whether EV71 provokes an apoptotic response of host cells during infection, the nuclear shape in the infected Vero cells was observed by staining with a membrane-permeable nuclear fluorescent dye, Hoechst 33258. Concurrently, the infected cells were recognized by immunofluorescence microscopy using an EV71-specific MAb. The virus antigen was detectable in the cytoplasm of the infected cells as early as 24 h post-infection (p.i.) (Fig. 1a), with a significant increase in the number of EV71-positive cells detected at 48 h p.i. (Fig. 1c). Concomitant inspection of the dye-stained cells revealed appreciable nuclear morphological alterations, where condensed and fragmented nuclei coincided with the EV71-positive cells (Fig. 1b, d). In contrast, EV71-negative cells retained the normal rounded nuclei, as observed in the neighbouring, uninfected (Fig. 1b, d) and mock-infected (Fig. 1f) cells. A similar result was obtained in the infected (Fig. 1g–j) or mock-infected (Fig. 1k, l) HeLa-229 cells. These results indicate that apoptosis may be triggered in EV71-infected cells.

We then conducted two biochemical assays to ascertain whether the morphological changes observed in the EV71-infected cells were due to the induction of apoptosis. First, the characteristic features of cells undergoing the final stages of apoptosis include the fragmentation of chromosomal DNA into nucleosomal oligomers. We thus examined EV71-infected HeLa-229 cells for the presence of internucleosomal DNA cleavage. The low molecular mass genomic DNA extracted from the infected cells at various time-points p.i. was analysed...
Fig. 2. DNA fragmentation of cells infected with EV71. HeLa-229 cells (10^6) were mock-infected (lane 7) or infected with EV71 at an m.o.i. of 0–1 for 0, 12, 24, 36 and 48 h (lanes 2–6, respectively). Low molecular mass DNA was isolated from cells harvested at the indicated condition and subsequent DNA analysis was carried out on a 1.5% agarose gel. Lanes 1, 8 and 9 represent the DNA markers (USA MBI Fermentas) in 100, 100 and 500 bp ladders, respectively. M, mock-infected; I, EV71-infected.

by agarose gel electrophoresis. As shown in Fig. 2, a canonic oligonucleosome-sized DNA ladder was observed in cells harvested at 24 h p.i. and at time-points thereafter (Fig. 2, lanes 4–6). No significant DNA fragmentation was observed in either the cells infected for 12 h (Fig. 2, lane 3) or mock-infected cells (Fig. 2, lane 7). Another hallmark in apoptotic cells is the cleavage of PARP, a nuclear enzyme that is catalytically activated by DNA strand interruption (Kaufmann et al., 1993). Hence, as a second method, Western blot for PARP was performed on samples prepared from mock-infected or infected HeLa-229 cells. Only full-length PARP at the size of 116 kDa was detected in the extracts harvested at 12 and 48 h following mock infection (Fig. 3a, lanes 1 and 6, respectively). Conversely, the intact PARP protein was lost starting at 12 h p.i., with the concomitant appearance of the N-terminal cleavage product at an apparent size of 25 kDa (Fig. 3a, lanes 2–5). By analogy, the specific cleavage of PARP was observed in Vero and RD cells infected by EV71 (Fig. 3b, c, respectively). These data support the notion that infection of EV71 induced cell death by apoptosis.

2Apro plays a critical role in EV71-induced apoptosis

After confirming that EV71 infections result in apoptosis in several permissive cell lines, we then tried to identify the putative EV71-encoded protein(s) that may be responsible for the induction of apoptosis. Recent reports indicated that the expression of a single poliovirus protein, 2Apro, was sufficient to induce severe morphological changes, similar to those observed in poliovirus-infected cells (Barco et al., 2000; Goldstaub et al., 2000). Computer alignment of 2Apro sequences at the amino acid level from several picornaviruses revealed that the identities are 58–00, 74–66, 72–66, 73–33, 35–33 and 44–00% in those between EV71 (BrCr strain) and PV type 1 (Mahoney strain), CVB1, CVB3, CVB4, human rhinovirus type 2 (HRV-1) and HRV-14, respectively (Fig. 4; Kitamura et al., 1981; Iizuka et al., 1987; Lindberg et al., 1987; Jenkins et al., 1987; Skern et al., 1985; Stanway et al., 1984; Brown & Pallansch, 1995). Both the 2A proteases of EV and HRV are believed to be structurally similar to trypsin-like serine protease (Bazan & Fletterick, 1988; Ryan & Flint, 1997), and a catalytic triad has been identified in the PV 2Apro comprising His^{36}, Asp^{38} and Cys^{109} (Yu & Lloyd, 1991, 1992). Inspection of the sequences revealed that the catalytic triad is conserved in EV71 2Apro as well as in the other picornaviruses examined (Fig. 4). The results implied that 2Apro from EV71 may exhibit an analogous effect as those from other EV and HRV.
EV71 and its 2Apro induce apoptosis

Fig. 4. Amino acid alignments of 2Apro in representative members of picornaviruses. Identical residues are in white lettering on a black background. The residues marked with asterisks (*) represent the amino acids that form the catalytic triad of poliovirus 2Apro (His\(^{20}\), Asp\(^{38}\) and Cys\(^{109}\)). Residues are numbered according to the amino acid sequence of PV-1 Mahoney (M) strain; CVB; HRV; and EV71 (Kitamura et al., 1981; Iizuka et al., 1987; Lindberg et al., 1987; Jenkins et al., 1987; Skern et al., 1985; Stanway et al., 1984; Brown & Pallansch, 1995).

Fig. 5. Effect of EV71 2Apro on the nuclear morphology of transfected cells. HeLa-229 cells were transfected with p2A-GFP vector (a, b), empty vector pEGFP-N2 (c, d), p2A-IRES-GFP (e, f) or control plasmid pIRES2-EGFP (g, h), followed by nuclei staining with Hoechst 33258 dye 40 h post-transfection. Each arrow indicates a GFP-positive cell and its nucleus in the same field. Bar, 5 \(\mu\)M.
To explore the possibility that 2Apro from EV71 is involved in the induction of apoptosis, we cloned the EV71 2A-coding sequences under the transcriptional control of the cytomegalovirus IE promoter, which provides high-level expression in a variety of cell types. To monitor the ectopically expressed protein at the single-cell level, we employed a strategy where the loxivirus IE promoter, which provides high-level expression in a cell type-dependent manner, was placed under the control of the IRES of EMCV. 2Apro and GFP were therefore expressed as separate proteins in the same cells after transfection, with GFP being translated in a cap-independent manner. The cytotoxicity of 2Apro in transfected HeLa-229 cells was also analysed by Hoechst staining. Once again, nuclear condensation and fragmentation occurred in the GFP-expressing cells at 40 h post-transfection (Fig. 5e, f). In contrast, cells transfected with the empty plasmid, pIRES-EGFP, retained the normal nucleus morphology (Fig. 5g, h). Likewise, the findings were demonstrated in Vero cells (data not shown). One notable feature is that the signals from the IRES-controlled GFP (Fig. 5e) appeared to be generally stronger than those without the preceding IRES (Fig. 5a), suggesting that EV71 2Apro also exerts a suppressive effect on translation in a cap-dependent manner.

To gain biochemical evidence that overexpression of the 2A-induced apoptotic change, we also examined the degradation of PARP in transfected HeLa-229 cells. Cells were transfected with p2A-GFP or p2A-IRESGFP and then harvested at 18, 30, 42 and 54 h post-transfection. We detected the appearance of the PARP cleavage product at 30 h post-transfection and thereafter, with gradual loss of the intact form of PARP in both transfectants (Fig. 6a, b, lanes 3–5). This observation strongly suggests that EV71 2Apro plays an important role in apoptotic cell death.

**EV71 2Apro induces elf4G cleavage**

The trans-cleavage activity of EV and HRV 2Apro processes several cellular factors (Krausslich et al., 1987; Lloyd et al., 1988; Lamphear et al., 1993; Liebig et al., 1993); among them the cleavage of elf4G was well identified (Etchison et al., 1982; Pérez & Carrasco, 1992; Liebig et al., 1993; Aldabe et al., 1995). Thus, we sought to determine whether elf4G is also cleaved after EV71 infection. HeLa-229 cells were subjected to infection by EV71 at an m.o.i. of 0.1 and extracts prepared from the infected cells were harvested at 48 h p.i. By immunoblot analysis with mouse anti-elf4G MAb, it was found that full-length elf4G (220 kDa) was cleaved at this time-point (Fig. 7a, lane 1), while the protein remained intact in mock-infected cells (Fig. 7a, lane 2). We further investigated whether the cleavage of elf4G could be attributed to the activity of EV71 2Apro, as was demonstrated for those from other picornaviruses. To this end, HeLa-229 cells were transfected with 2Apro expression vectors, p2A-GFP or p2A-IRESGFP. Immunoblot analysis revealed that elf4G was degraded as early as 18 h post-transfection (Fig. 7b, c, lane 2), with gradual increases in the product of PARP. **Fig. 6.** Immunoblot analysis of PARP in cells transfected with p2Apro-expressing vectors. HeLa-229 cells were transfected with p2A-GFP (a) or p2A-IRESGFP (b). Total protein extracts were prepared from cells transfected with the 2Apro-bearing vectors for 18, 30, 42 and 54 h (a, b, lanes 2–5) or with the empty vectors pEGFP-N2 (a) or pIRESGFP (b) for 54 h (lanes 6). Proteins were separated by SDS–PAGE on a 13% gel and then probed with anti-PARP MAb. M, mock-transfected; Vector, empty vector-transfected; PARP, the full-length of the PARP; Cp, the cleavage product of PARP.
intensity of the cleavage product (ca. 100 kDa) at later time-points (Fig. 7b, c, lanes 3–5). Only the intact form of eIF4GI was detected in mock-transfected cells (Fig. 7b, c, lane 1) or cells transfected with the control plasmid (Fig. 7b, c, lane 6).

Discussion

EV71 is the most recently recognized member of the enteroviruses. Despite the clinical manifestations known to be associated with EV71 infection, the mechanisms of EV71 pathogenicity remain largely elusive. As a first step to unfold the cytopathology caused by EV71, we employed the cell culture systems to study the effect of EV71 infection on host cells. We showed that infection of EV71 triggered the development of apoptosis in several cell lines, where nuclear alterations, DNA fragmentation and PARP degradation took place. To localize the possible virus trigger(s) of apoptosis, we started with EV71 2Apro, whose counterpart in other picornaviruses has been investigated for its role in inducing apoptosis (Barco et al., 2000; Goldstaub et al., 2000). We adopted a system to study the effects of 2Apro on the cells by taking advantage of the autofluorescent peptide GFP, either fused to 2A or expressed as a bicistronic construct. The detectable number of GFP-positive cells ensured that the 2A–GFP fusion protein was expressed in the transfected cells in spite of their relatively weak signals, possibly impaired by 2A activity (Fig. 5). On the other hand, the IRES-controlled GFP permitted higher intensity of fluorescence, very likely owing to the situation that its translation is in a cap-independent fashion and is refractory to the inhibitory effect by 2Apro (Fig. 5). Nevertheless, the tracking of 2A expression was carried out on a single-cell level. More importantly, the cells displaying condensed or fragmented nuclei were found restricted to the GFP-positive cells, indicating that 2Apro alone plays a critical role in eliciting the apoptotic features. This argument was supported by the data where cells transfected with 2A-bearing plasmids induced PARP cleavage, a biochemical parameter for apoptosis (Fig. 6). This approach will be useful for gaining further insights into the process of apoptosis in EV71-infected cells, since it is provoked with a single gene product in the absence of additional virus products.

Cell death occurs by either necrotic or apoptotic pathways. Necrosis is accidental death characterized by rupture of the plasma membrane and release of cytoplasmic constituents. Apoptosis is an active and energy-dependent process of cell death in response to a wide variety of stimuli (Martin et al., 1994; Green, 2000). In vivo, apoptotic cell death is reported to be noninflammatory, while necrotic death is typically accompanied by inflammation (Melcher et al., 1999). The ability of numerous viruses to elicit apoptosis either directly or indirectly upon infection has been demonstrated (Teodor & Branton, 1997; O’Brien, 1998). This process may represent an important step in the spread of progeny to neighbouring cells, while limiting the inflammatory and immune responses (O’Brien, 1998), and protecting progeny virus from action of host antibodies. In some cases, specific viral proteins have been identified that are potent apoptosis inducers by themselves, such as E1A of human adenovirus and Tat and Nef of human immunodeficiency virus (Roulston et al., 1999; Teodor & Branton, 1997). In other cases, the presence of viral RNA, rather than viral proteins, is implicated as a trigger. Examples of these are the apoptotic action of the RNA-dependent 2-5A synthetase/RNase L system (Castelli et al., 1997) and the RNA-dependent protein kinase PKR (Lee & Esteban, 1994; Lee et al., 1997). Among human EV, PV was first described as being implicated in inducing apoptosis in several cultured cell lines (Ammendolia et al., 1999; López-Guerrero et al., 2000; Tolskaya et al., 1995), with its 2Apro being implicated in the apoptosis-inducing function (Goldstaub et al., 2000). In addition, CVB3 was shown to induce apoptosis of HeLa cells (Carthy et al., 1998). Its capsid protein VP2 was identified to

Fig. 7. Analysis of eIF4GI in cells infected with EV71 or transfected with EV71 2Apro-expressing vectors. HeLa-229 cells were infected with EV71 at an m.o.i. of 0.1 for 48 h (a) or transfected with p2A-GFP (b) or p2A-IRES-GFP (c). Total proteins were prepared from the infected or transfected cells harvested at the indicated time-points and subjected to analysis for eIF4GI by Western blot. M, mock-infected (a) or mock-transfected; Cp, the cleavage product of eIF4GI.
interact with the cellular pro-apoptotic protein Siva and may contribute to the induction of apoptotic events (Henke et al., 2000). We addressed herein that EV71 2A\textsuperscript{pro} is capable of triggering apoptosis, adding one more to the growing list of pro-apoptotic genes fromenteroviruses.

Elucidation of the exact molecular mechanism used by EV71 2A\textsuperscript{pro} to provoke apoptosis must take into account that this pro tease may cleave a variety of host proteins, including eIF4GI and PARP shown in this report. eIF4GI is a component of the cap-binding complex (eIF4F), which plays a pivotal role in the interaction between capped mRNA and the 40S ribosomal subunit, leading to the initiation of translation (Morley et al., 1997). Cleavage of eIF4GI results in the disruption of the eIF4F complex and, hence, inhibition of cap-dependent translation. In addition, it was demonstrated that recombinant 2A\textsuperscript{pro} of EV and HRV can cleave eIF4G directly in vitro, albeit to a much lesser extent (Bovee et al., 1998; Haghighat et al., 1996; Lamphere et al., 1993). This could be explained by the finding that eIF4G alone is a relatively poor substrate, as opposed to the eIF4F complex, which is cleaved efficiently by the HRV 2A\textsuperscript{pro} (Haghighat et al., 1996). It has been proposed that the shut-off of cap-dependent translation is a major mechanism of the execution phase of apoptosis, which leads to rapid cell death (Clemens et al., 1998; Marissen & Lloyd, 1998). Furthermore, it has been noted that eIF4GI also serves as a substrate for activated caspases when cells undergo apoptosis (Clemens et al., 1998; Marissen & Lloyd, 1998). However, the cleavage products (ca. 150 and 80 kDa) are distinct from those generated in EV71-infected or EV71 2A\textsuperscript{pro}-transfected cells (Fig. 7), consistent with the data documented previously for picornavirus-infected cells (Roberts et al., 2000). Also, it was shown that caspase inhibitors did not inhibit the cleavage of eIF4GI during PV infection (Roberts et al., 2000). These data suggested that apoptosis could be induced as a consequence of cleavage of eIF4GI in a caspase-independent manner.

With regard to PARP, another host protein susceptible to cleavage by 2A\textsuperscript{pro} reported herein, it is proteolytically cleaved by family members of the ICE-like cysteine protease family, in particular by caspase-3 during the process of apoptosis (LaZebnik et al., 1994). More interestingly, the caspase-3 cleavage products have a molecular mass of 85 and 25 kDa, reminiscent of those seen from EV71-infected (Fig. 3) or 2A\textsuperscript{pro}-transfected cells (Fig. 6). Moreover, recent reports showed that caspase-3 is activated after CVB3 infection of HeLa cells (Cardy et al., 1998) or PV infection of U937 cells (López-Guerrero et al., 2000). Thus, the mechanism by which EV71 2A\textsuperscript{pro}-induced apoptosis may be a caspase-3-dependent event. In fact, our preliminary study indicated that PARP remained intact in the EV71-infected or EV71 2A\textsuperscript{pro}-transfected MCF-7 cells deficient in caspase-3 (unpublished data). Taken together, it is tempting to assume that the cleavage of PARP and eIF4G isoforms occurs at distinct stages during the apoptotic process. In this case, eIF4G cleavage may be directed toward blocking de novo cap-dependent translation. It may initiate an apoptotic event without the action of caspases, while PARP cleavage is the consequence of caspase-3 activation at the later phase of apoptosis. Nonetheless, it is conceivable that EV71 2A\textsuperscript{pro} induces apoptosis indirectly by activation of other still unidentified cellular substrates pertinent to an endogenous cell suicide program. Further experiments are needed to better delineate the mechanism(s) by which EV71 2A\textsuperscript{pro} induces apoptosis.

Induction of apoptosis by virus infection is emerging as an important aspect of pathogenesis. Cell damage in the CNS that is caused by its response to a virus infection can involve apoptosis (Oberhaus et al., 1997; Tsunoda et al., 1997). Virus-induced apoptosis has been shown to be an important component of PV-induced cell death and tissue injury in the CNS of infected mice (Girard et al., 1999). EV71, like PV, invades the CNS to give rise to several syndromes, notably aseptic meningitis, encephalomyelitis and flaccid paralysis. Therefore, this work represents an initial attempt to unveil the relevance of EV71-induced apoptosis for human diseases. Moreover, discerning the trigger that initiates apoptosis in a virus-infected cell, in this case the EV71 2A\textsuperscript{pro}, should be of benefit to understanding virus pathology as well as the development of effective inhibitors targeted to this viral protein.

We thank Dr Shiau-Ting Hu for helpful discussion. This study was supported in part by grant NSC 89-2320-B-010-041 from the National Science Council and by grant VTY 90-P5-38 from the Veterans General Hospital-Taipei, Taiwan, Republic of China.

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Received 23 November 2001; Accepted 30 January 2002