Caspases are not involved in the cleavage of translation initiation factor eIF4GI during picornavirus infection

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Infection of cells by many picornaviruses results in the rapid inhibition of cellular protein synthesis due to cleavage of the translation initiation factor eIF4G. The poliovirus (PV) 2A and foot-and-mouth disease virus (FMDV) L proteases are each sufficient to mediate this cleavage, but the cleavage mechanism may be indirect, involving an unidentified cellular protease(s). eIF4G is also targeted for cleavage by caspase-3 during apoptosis. Here, it is shown that caspase inhibitors do not inhibit the cleavage of eIF4GI during PV or FMDV infection. Similarly, in transient-expression studies, the cleavage of eIF4GI induced by PV 2A or FMDV L was unaffected by these inhibitors. Furthermore, the cleavage of eIF4GI was observed in PV-infected MCF-7 cells lacking caspase-3. These data, and the fact that induction of apoptosis yields different eIF4GI cleavage fragments, indicate that caspases do not have a major role in the cleavage of eIF4GI during PV or FMDV infection.

Infection of cells by many picornaviruses (enteroviruses, rhinoviruses and aphthoviruses) leads to the rapid inhibition of cellular (cap-dependent) translation. This is accompanied by cleavage of the eukaryotic translation initiation factor 4G (eIF4G) (Etchison et al., 1982; Etchison & Fout, 1985; Devaney et al., 1988). There are two species of eIF4G, termed eIF4GI and eIF4GII, which appear to be functionally equivalent but are only about 50% identical (Gradi et al., 1998). eIF4G is a component of the cap-binding complex (eIF4F), together with the cap-binding protein eIF4E and the RNA helicase eIF4A. Cleavage of eIF4G results in disruption of the eIF4F complex and, hence, inhibition of cap-dependent translation. Picornavirus RNAs have in their 5′ untranslated regions a complex structure known as an internal ribosome entry site (IRES) element, which directs a cap-independent mechanism of translation (reviewed in Jackson et al., 1994; Belsham & Sonenberg, 1996). IRES-directed initiation of protein synthesis is maintained following the cleavage of eIF4G (Ohlmann et al., 1996; Borman et al., 1997; Gradi et al., 1998; Roberts et al., 1998).

The cleavage of eIF4G in entero-/rhinovirus-infected cells has been attributed to the action of the viral 2A protease (Kräusslich et al., 1987) and, in foot-and-mouth disease virus (FMDV)-infected cells, to the L protease (Devaney et al., 1988). These two proteases are unrelated and are members of different families of proteases; 2A belongs to the trypsin-like family of serine proteases whereas L belongs to the papain-like family of cysteine proteases (Ryan & Flint, 1997). The sites at which these proteases cleave eIF4GI in vitro lie just seven amino acids apart (Kirschweger et al., 1994; Lamphear et al., 1995). FMDV L cleaves eIF4GI at residues 635/636 and poliovirus (PV) 2A cleaves at residues 642/643 (numbering taken from Imataka et al., 1998).

It is currently unclear whether the 2A and L proteases cleave eIF4G directly in vivo, or whether the cleavage process is mediated by a cellular protease that requires prior activation by 2A or L. This uncertainty stems from the observation that eIF4G is cleaved in PV- and FMDV-infected cells without any virus replication (e.g. in guanidine-treated cells) and when the levels of 2A and L are undetectable (Bonneau & Sonenberg, 1987; Belsham et al., 2000). PV 2A alone is responsible for inducing the cleavage of eIF4G (Kräusslich et al., 1987; Lloyd et al., 1988). However, fractionation of PV-infected HeLa cell extract resulted in the separation of eIF4G-specific cleavage activity from PV 2A (Lloyd et al., 1986; Bovee et al., 1998a). In addition, an anti-2A antiserum that blocks PV 2A processing activity did not prevent eIF4G cleavage in an in vitro system (Kräusslich et al., 1987). The direct cleavage of eIF4G by recombinant PV 2A was reported recently to be very inefficient (Bovee et al., 1998b). However, it has been demonstrated that addition of eIF4E to eIF4GI enhances the susceptibility of eIF4GI to cleavage by PV 2A (Haghighat & Sonenberg, 1997). Taken together, these data leave open the possibility of the involvement of an unidentified cellular protease(s) in the cleavage of eIF4G induced by PV 2A.

It has been demonstrated recently that eIF4G is also targeted for cleavage in cells undergoing apoptosis (Clemens et al., 1998; Marissen & Lloyd, 1998; Morley et al., 1998). The
Fig. 1. Caspase inhibitors have no effect on PV- or FMDV-induced cleavage of eIF4GI. Picornavirus infections were performed in the absence or presence of caspase inhibitors. Cytoplasmic extracts were made at the times indicated post-infection (p.i.) and subjected to SDS–PAGE and Western blotting. Blots were probed with anti-eIF4G antisera as described in the text and detection onto X-ray film was achieved by using chemiluminescence. (A)–(B) PV infection of HeLa cells in the presence or absence of Z-VAD-FMK (200 µM) (A) or Z-DEVD-CMK (200 µM) (B). (C) FMDV infection of BHK cells in the absence or presence of Z-VAD-FMK (200 µM). The C-terminal cleavage fragment of eIF4GI is referred to as Ct.

protease responsible for the cleavage of eIF4G during apoptosis was identified as caspase-3 (a member of the caspase family of Asp-specific cysteine proteases) in cells and in vitro (Marissen & Lloyd, 1998; Bushell et al., 1999). Recombinant human caspase-3 was shown to cleave purified eIF4G within the eIF4F complex (Bushell et al., 1999). It has been shown recently that caspase inhibitors prevent PV-induced apoptosis in HeLa cells (Agol et al., 1998), implying a role for caspases in cell death during picornavirus infection. In the light of these findings, we set out to determine whether caspases were involved in the mechanism of eIF4GI cleavage in picornavirus-infected cells (mediated by either PV 2A or FMDV L protease). We now report that caspases play no major role in the mechanism of picornavirus-induced cleavage of eIF4GI.

HeLa cells and BHK cells were grown in modified Eagle’s medium (Gibco BRL) and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% foetal bovine serum (FBS) at 37 °C, in a humidified atmosphere of 5% CO₂ in air. MCF-7 cells were grown under similar conditions in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS. The cells were plated into 35 mm dishes 20 h prior to infection. PV type 1 (Mahoney, PV1) was added to HeLa or MCF-7 cells at approximately 10 p.f.u. per cell in their respective medium containing 2% FBS. After 1 h incubation with rocking, virus was removed and the cells were washed and incubated with fresh medium containing 10% FBS. Similarly, BHK cells were infected with FMDV (type O) at approximately 10 p.f.u. per cell using the same protocol. Mock-infected cells were treated similarly. Where indicated, the caspase inhibitors Z-Val–Ala–Asp–fluoromethylketone (Z-VAD-FMK; Bachem) or Z-Asp–Glu–Val–Asp–chloromethylketone (Z-DEVD-CMK; Bachem) were added to a final concentration of 50–200 µM to the cells in medium containing 2% FBS for 45 min prior to virus infection. To ensure the continued presence of the inhibitors throughout the experiment, the medium added to the cells after the virus had been removed was also supplemented, where appropriate, with the inhibitor at the concentrations indicated. After incubation at 37 °C, cells were harvested in Promega lysis buffer (400 µl) at the indicated times post-infection. Samples were analysed for eIF4GI...
cleavage by immunoblotting from 7% SDS–PAGE minigels (Laemmli, 1970). Following transfer, the membranes were incubated with rabbit anti-eIF4G antibodies raised against a C-terminal fragment of eIF4GI (1:2000; kindly donated by S. J. Morley, University of Sussex, UK) followed by peroxidase-labelled donkey anti-rabbit IgG (1:2500; Amersham). Detection onto X-ray film was achieved by using chemiluminescence reagents (Pierce).

PV infection of HeLa cells resulted in the rapid 2A protease-induced cleavage of eIF4GI, as detected by Western blot analysis of cell extracts (Fig. 1A). The intact protein (Mr 220000) was lost progressively with the concomitant appearance, within 2 h of infection, of the C-terminal cleavage product, with an apparent Mr of 120000. By 4 h, complete loss of the intact protein was apparent. The cleavage of eIF4GI occurred before any noticeable morphological changes of the cells, such as rounding up, blebbing or detachment from the dish (data not shown). Similarly, FMDV infection of BHK cells resulted in rapid L protease-induced cleavage of eIF4GI to generate similar-sized cleavage products of eIF4GI (Fig. 1C), as observed previously (Medina et al., 1993).

The presence of the broad-spectrum tripeptide caspase inhibitor Z-VAD-FMK added at 50 and 200 µM had no effect on the extent or the kinetics of eIF4GI cleavage in PV-infected cells (Fig. 1A). Likewise, Z-DEVD-CMK (200 µM), a modified tetrapeptide with higher specificity for members of the caspase-3 subfamily of caspases, did not modify eIF4GI cleavage in PV-infected cells (Fig. 1B). Similarly, the rapid cleavage of eIF4GI in FMDV-infected BHK cells was unaffected by the presence of Z-VAD-FMK (Fig. 1C). The concentrations of inhibitor used have been shown previously to result in efficient blocking of caspase activity (Chow et al., 1995; Jones et al., 1998). This was confirmed by showing (Fig. 2A) that Z-VAD-FMK (200 µM) completely prevented cleavage of eIF4GI in HeLa cells induced by staurosporine (1 µM) or recombinant human TRAIL (250 ng/ml, Apo-2 ligand) (MacFarlane et al., 1997; a gift from A.E. Willis, University of Leicester, UK). The cleavage products of eIF4GI induced by these treatments had approximate Mr of 150000 and 80000 and hence migrated very differently when resolved by SDS–PAGE from those fragments generated by virus infection. These results suggested that caspases do not play any detectable role in picornavirus-induced eIF4GI cleavage.

In order to examine the properties of the viral proteases individually, PV 2A and FMDV Lb were expressed transiently within BHK cells. Plasmids pAA802 and pLb (encoding PV 2A and FMDV Lb, respectively, under the control of a T7 promoter) have been described previously (Kaminski et al., 1990; Medina et al., 1993). These plasmids (2 µg) were transfected into BHK cells (35 mm dishes) using Lipofectin (8 µl; Life Technologies) and Optimem (Gibco-BRL) after incubation for 1 h with the recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase (Fuerst et al., 1986). After incubation for 20 h in the presence or absence of Z-VAD-FMK (200 µM), cell extracts were prepared and samples were analysed for eIF4GI cleavage as outlined previously. Western blot analysis of cell extracts showed evidence of eIF4GI cleavage induced by both proteases to the characteristic cleavage product corresponding to a fragment of Mr 120000 (Fig. 2B). Cleavage of eIF4GI occurred independently of the presence of the caspase inhibitor Z-VAD-FMK (200 µM) (Fig. 2B), further supporting the observation described above that caspases are not involved in picornavirus protease-induced cleavage of eIF4GI.

Finally, we examined the role of caspase-3 in PV-induced cleavage of eIF4GI by utilizing MCF-7 cells, which do not express caspase-3 due to a deletion mutation within the procaspase-3 gene (Jänicke et al., 1998). If caspase-3 plays a major role in PV-induced cleavage of eIF4GI, then no cleavage of this protein would be expected within PV-infected MCF-7

Fig. 2. FMDV L and PV 2A proteases induce the cleavage of eIF4GI within cells in the presence of Z-VAD-FMK. (A) HeLa cells were treated with Z-VAD-FMK (200 µM) prior to (and during) infection with PV as detailed in Fig. 1 or prior to (and during) treatment with TRAIL (250 ng/ml) or staurosporine (Stauro) (1 µM) for 4 h as indicated. Extracts were analysed as described in Fig. 1. The full-length and Ct fragments of eIF4GI induced during PV infection are indicated as before. The eIF4GI fragments generated after TRAIL or staurosporine treatment are indicated with asterisks. (B) The plasmids encoding FMDV L (pLb) or PV 2A protease (pAA258 and pAA802) were transfected into vTF7-3-infected BHK cells as described in the text. Cells were incubated in the absence or presence of Z-VAD-FMK (200 µM) as indicated. After 20 h, cell extracts were prepared and analysed as in Fig. 1. Note that in BHK cells, a species of intermediate mobility between eIF4GI and Ct is observed, which is lost when the full-length protein is completely cleaved; presumably this is a non-specific breakdown product of eIF4GI (see also Fig. 1C). A minor background band migrating just ahead of Ct is also observed.
cells. The cells were infected with PV as before and cleavage of eIF4GI was examined. As shown in Fig. 3, eIF4GI was still cleaved efficiently in PV-infected MCF-7 cells, indicating no significant involvement or requirement for caspase-3 in this event. The rate of cleavage did appear to be slower than that seen in HeLa cells, but we believe that this is due to a less-efficient infection process in this cell type. The eIF4GI cleavage product was indistinguishable in size from that generated in PV-infected HeLa cells (data not shown). Taken together, these data confirm that caspases play no role in the PV 2A- or FMDV-induced cleavage of eIF4GI.

Induction of apoptosis in HeLa cells with either staurosporine or TRAIL led to the production of different-sized eIF4GI cleavage products (M₁, of about 150,000 and 80,000) compared with those seen in picornavirus infection (Fig. 2A). This is consistent with the findings of Morley et al. (1998) and Clemens et al. (1998), who reported that induction of apoptosis in BJAB and Jurkat cells leads to the production of major p76 and transient p150 C-terminal fragments. These fragments are generated by the action of caspase-3 (Bushell et al., 1999). This shows that the activation of caspasas either through the death receptor or through the mitochondrial pathway of apoptosis (Green & Reed, 1998; Ashkenazi & Dixit, 1999) does not yield the same cleavage product of eIF4GI that is formed during picornavirus infection. Although some viruses can trigger apoptosis, our results are consistent with previous observations that, at least in the case of PV, the cleavage of eIF4GI does not involve caspases and this cleavage is mediated separately from the trigger for apoptosis (Agol et al., 1998; Carthy et al., 1998).

In conclusion, it is clear that the inhibition of protein synthesis during picornavirus infection, resulting from eIF4GI cleavage, does not involve Z-VAD-FMK-sensitive proteins. If the model of a cellular protease being required for eIF4GI cleavage is correct, then another protein(s) must be implicated. Further work will be required to elucidate the nature of this protease and to identify the mechanism by which this occurs.

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References


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