Dengue virus M protein contains a proapoptotic sequence referred to as ApoptoM

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The induction of apoptotic cell death is a prominent cytopathic effect of dengue (DEN) viruses. One of the key questions to be addressed is which viral components induce apoptosis in DEN virus-infected cells. This study investigated whether the small membrane (M) protein was involved in the induction of apoptosis by DEN virus. This was addressed by using a series of enhanced green fluorescent protein-fused DEN proteins. Evidence is provided that intracellular production of the M ectodomains (residues M-1 to M-40) of all four DEN serotypes triggered apoptosis in host cells such as mouse neuroblastoma Neuro 2a and human hepatoma HepG2 cells. The M ectodomains of the wild-type strains of Japanese encephalitis, West Nile and yellow fever viruses also had proapoptotic properties. The export of the M ectodomain from the Golgi apparatus to the plasma membrane appeared to be essential for the initiation of apoptosis. The study found that anti-apoptosis protein Bcl-2 protected HepG2 cells against the death-promoting activity of the DEN M ectodomain. This suggests that the M ectodomain exerts its cytotoxic effects by activating a mitochondrial apoptotic pathway. The cytotoxicity of the DEN M ectodomain reflected the intrinsic proapoptotic properties of the nine carboxy-terminal amino acids (residues M-32 to M-40) designated ApoptoM. Residue M-36 was unique in that it modulated the death-promoting activity of the DEN M ectodomain. Designing the ApoptoM-activated signalling pathways leading to apoptosis will provide the basis for studying how the M protein might play a key role in the fate of the flavivirus-infected cells.

INTRODUCTION

Mosquito-borne flaviviruses such as dengue (DEN), Japanese encephalitis (JE), Saint Louis encephalitis (SLE), West Nile (WN) and yellow fever (YF) viruses may cause epidemic disease outbreaks in humans. Infected patients may exhibit a wide range of acute diseases, from nonspecific febrile illness to severe haemorrhagic manifestations (DEN and YF) or encephalitic syndromes (JE, SLE and WN). Flaviviruses (family Flaviviridae) are single-stranded, enveloped RNA viruses (Chambers et al., 1990; Rice, 1996). The virion consists of three structural proteins: C (core protein), M (membrane protein) and E (envelope protein) (Chambers et al., 1990; Rice, 1996). The virion is first assembled as an immature particle, in which pM (the intracellular precursor of M) is noncovalently associated with E in a heterodimeric complex. Late in virus morphogenesis, pM is processed by subtilisin-like proteases to generate the mature M protein (Chambers et al., 1990; Rice, 1996). The M protein (7–9 kDa) consists of a 40 amino acid long ectodomain followed by the transmembrane-anchoring region including two transmembrane domains (TMDs) (Chambers et al., 1990; Rice, 1996). Three-dimensional imaging of the structure of the DEN virion, showing the location of the M protein with respect to the E homodimer, was recently carried out (Kuhn et al., 2002). Several studies have shown that the M ectodomain induces a neutralizing antibody response (Bray & Lai, 1991; Vazquez et al., 2002).

All four serotypes of DEN virus (DEN-1, DEN-2, DEN-3 and DEN-4) and JE, Langat, SLE, WN and YF viruses have been reported to trigger apoptosis in host cells (Després et al., 1996, 1998; Duarte dos Santos et al., 2000; Jan et al., 2000; Liao et al., 1997; Marianneau et al., 1997, 1998; Parquet et al., 2001, 2002; Prikhod’ko et al., 2001; Su et al., 2002; Xiao et al., 2001; Courageot et al., 2003; Catteau et al., 2003). Apoptosis is an active process of cell death involving a number of distinct morphological changes (Hengartner, 2000; Kimura et al., 2000). Apoptosis is induced via the activation of intracellular signalling systems, a number of which converge on mitochondrial membranes to induce...
their permeabilization (Hengartner, 2000; Desagher & Martinou, 2000; Ferri & Kroemer, 2001). Members of the Bcl-2 protein family have been shown to exhibit both anti-apoptotic and proapoptotic activities (Adams & Cory, 2001). For example, increased levels of Bcl-2 lead to cell survival whereas excess of Bax is associated with apoptosis (Yang et al., 1997; Jürgensmeier et al., 1998).

The intracellular production of viral proteins has been shown to be essential for the induction of apoptosis (Després et al., 1996; Duarte dos Santos et al., 2000; Prikhod’ko et al., 2001, 2002). However, potent death-promoting activity has yet to be demonstrated for particular DEN proteins. Here, we showed that the DEN M ectodomain can trigger apoptosis in host cells of various origins. Deletion analysis demonstrated that the nine carboxy-terminal amino acids of the DEN M ectodomain were necessary and sufficient for the induction of apoptosis.

### METHODS

**Cell lines and viruses.** Mouse neuroblastoma Neuro 2a and human hepatoma HepG2 cells were cultured as previously described (Duarte dos Santos et al., 2000). The human epithelial 293A cell line was purchased from Quantum Bioprobe. The monkey kidney HeLa cell line was generously provided by F. Delebecque (Pasteur Institute). The 293A and COS-7 cell lines and the human epithelial HeLa cell line were cultured in DMEM supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine.

The South American strains of DEN-1 virus FGA/89 (GenBank accession no. AF226687) and BR/90 (S64849) have been reported elsewhere (Després et al., 1993). DEN-1 virus strains FGA/89 and BR/90, DEN-2 virus strain Jamaica (M20558), DEN-3 virus strain H-87 (NC 001475), DEN-4 virus strain H-241 (NC 002640), JE virus strain Nakayama (JE virus strain SA[V], D90194) and WN virus strain IS-98-ST1 (AF481864) were produced in cultured *Aedes pseudoscutellaris* AP61 mosquito cells, as previously described (Després et al., 1993). YF virus strain 17D-204 Pasteur (X15062) was produced in human SW13 cells (Després et al., 1987).

**Plasmids.** pCI-neo, pEGFP-N1 and pEYFP-Golgi were purchased from BD Clontech BioSciences. Viral RNA was extracted from purified flavivirus or infected cell lysates using the RNA-plus reagent (Quantum Bioprobe). The RNA was reverse transcribed using the Titan One-Step RT-PCR kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. All constructs were verified by automated sequencing.

The BR/90 cDNA encoding residues C-95 to C-114 (amino acid residues are numbered as for DEN-1 virus; Després et al., 1993) was introduced into *NheI*/*Smal*-digested pEGFP-N1, the eukaryotic expression vector containing the gene encoding *enhanced green fluorescent protein* (EGFP). The resulting plasmid, pC95–114-EGFP, encodes the prM translocation signal followed by six vector-specified residues, EPFPVAT, fused in-frame with the amino terminus of EGFP.

Synthetic oligonucleotide primers containing recognition sites for *BsrGI* (5’ primer) and *NotI* (3’ primer) were used to amplify specific sequences of the flavivirus genome encoding the full-length M ectodomain (residues M-1 to M-74) (Table 1). We constructed pC95–114-EGFP-M1–40 by digesting the RT-PCR products with *BsrGI* and *NotI* and introducing the resulting fragment into *BsrGI/NotI*-digested pC95–114-EGFP, such that the full-length M was directly fused in-frame with the carboxy-terminal end of EGFP. We constructed pC95–114-EGFP-M1–40 by amplifying flavivirus cDNAs encoding the M ectodomain (residues M-1 to M-40) by PCR using pC95–114-EGFP-M1–74 as a template and a set of 3’ primers containing a stop codon (TGA) followed by a *NotI* restriction site. The PCR products were introduced into pC95–114-EGFP, such that the flavivirus M ectodomains were produced as fusions with EGFP.

To construct a series of mutants with deletions in the DEN-2 M ectodomain (M1–40/DEN-2), we generated PCR fragments using pC95–114-EGFP-M1–40/DEN-2 or pC95–114-EGFP-M9–40/DEN-2 as a template and primers containing recognition sites for *BsrGI* and *NotI* and a stop codon (TGA) (see Table 1). The PCR products encoding mutant proteins were inserted into pC95–114-EGFP downstream from the EGFP gene. Mutant protein C95–114-EGFP-M9–40/YF was generated.

### Table 1. Synthetic oligonucleotide primers

<table>
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<th>M</th>
<th>5’ primer</th>
<th>3’ primer</th>
<th>Strain</th>
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<tr>
<td>DEN-1</td>
<td>5’-gacaaagttctgctgtctctgtgacaaactctggcttgctagc-3’</td>
<td>5’-cacagaagctctacatagcatgagcactctgcc-3’</td>
<td>FGA/89</td>
</tr>
<tr>
<td>DEN-2</td>
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<td>5’-atattcctaaaggcccaatgattcagagc-3’</td>
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</tr>
<tr>
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<td>5’-tgatttccagcagacatcctcgctctctctcctgacc-3’</td>
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<td>5’-ttttggcagtacatcaatgggcg-3’</td>
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</tr>
<tr>
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<td>5’-agtcgacacggaccattgctatgctctctgtgacacccg-3’</td>
<td>IS-98-ST1</td>
</tr>
<tr>
<td>YF</td>
<td>5’-agacagactgctgtagctgtctccatttacatgctgcc-3’</td>
<td>5’-tgacagatcagtggcgttagctccccatgtcgcc-3’</td>
<td>17D-204</td>
</tr>
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**Mutants**

| M1–30/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M1–30/DEN-2 |
| M1–20/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M1–20/DEN-2 |
| M9–40/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M9–40/DEN-2 |
| M9–40/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M9–40/DEN-2 |
| M1–20/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M1–20/DEN-2 |
| M9–40/DEN-2 | 5’-ttttggcagtacatccggtacgagc-3’ | 5’-ttttggcagtacatccggtacgagc-3’ | M9–40/DEN-2 |

using pC95–114-EGFP-M1–40/YF-17D as a template and the 3′ primer 5′-AGAGTGCGGGCGCGAACATCGGGTTGCTCCTACCAACCTCT-3′ extended by 20 nucleotides to include a stop codon (TGA) followed by a NotI restriction site. Mutant protein C95–114-EGFP-M1–40/YF-17D (T34, I36, L71, H99) was generated using pC95–114-EGFP-M1–40/YF-17D as a template and the 3′ primer 5′-AGAGTCGCCGCGC- CAAATCAGGGTTGCTCCAGGTTCATGTCTCAACCTTTTGGAGT GC-3′ extended by 21 nucleotides to include a stop codon (TGA) followed by a NotI restriction site. Mutant protein C95–114-EGFP-M1–40/NotI (F9) was generated using pC95–114-EGFP-M1–40/NotI as a template and the 3′ primer 5′-TAGCGCTATGGCTGACGCTATCACG-3′ extended by 14 nucleotides to include a stop codon (TGA) followed by a NotI restriction site.

To construct pGal1–80-EGFP-M1–40/NotI, a 0.9 kb fragment containing the entire EGFP-M1–40/NotI fragment was excised from pC95–114-EGFP-M1–40/NotI with BamHI and NotI. This fragment was inserted into BamHI/NotI-digested pYEFG-Golgi, such that EGFP-M1–40/NotI was fused in-frame with the amino-terminal region of β1,4-galactosyltransferase (GalT). To construct pCR-CD721–136, total RNA from the spleens of BALB/cByJ mice was reverse transcribed to generate cDNA, which was used as template for PCR. An RT-PCR fragment encoding the endodomain of CD72 was amplified by the transmembrane domain of mouse CD72 glycoprotein (nt 1–445) was generated, using the following synthetic primers: 5′-TGGCTGAGGAATGACAGCTTAAATTATGGC-3′ corresponding to nt 1–31 of the 5′ end of the CD72 cDNA and 5′-TATTGGTGCCCTC CCAAATCTCTGGTCCC-3′ corresponding to nt 416–445 of the 3′ end of the CD72 cDNA. The RT-PCR product was directly inserted into pCR 2.1 TOPO (TOPO TA cloning kit, Invitrogen) according to the manufacturer’s instructions to give pCR-CD721–136.

We generated pCD721–118-EGFP-M1–40/NotI by amplifying the cDNA encoding the amino-terminal region of CD72 by PCR, using pCR-CD721–136 as a template and the following primers: 5′-GAGGGCCGCTAGGCTATGGTCGACCATCAG-3′ corresponding to the 5′ end of the CD72 gene and extended by 11 nucleotides to include a Nhel restriction site and 5′-AGACACOGCGGCTAGAAGACTCCAGGCG-3′ corresponding to nt 387–402 at the 3′ end of the CD72 gene and extended by 14 nucleotides to include a Smal restriction site. The PCR product was digested with Nhel and Smal and inserted between the Nhel and Smal sites of pC95–114-EGFP-M1–40/NotI to generate pCD721–118-EGFP-M1–40/NotI.

To construct pC95–114-EGFP-M1–40/NotI-KDEL, pGal1–80-EGFP-M1–40/NotI-KDEL, and pCD721–118-EGFP-M1–40/NotI-KDEL, PCR fragments containing the DEN-1 M ectodomain (M1–40/NotI) followed by the KDEL motif were generated with the 3′ primer 5′-TAAAGGCGGCGCTACAAACTGTCTTTTTGGGGGTTCACAAAGC CCAAATCTGGTCCC-3′ corresponding to the KDEL sequence and extended by 12 nucleotides to include a stop codon (TGA) followed by a NotI restriction site.

**Transient transfection of cells.** Cells were placed in Permanox plates and transfected with 6 μg of plasmid per 10^6 cells in the presence of FuGENE 6 transfection reagent (Roche Molecular Biochemicals), according to the manufacturer’s instructions. The fusion proteins were detected by monitoring the autofluorescence of EGFP. Images were processed on a computer, using RS Image 1.07, Simple PCI 5.1, Adobe Photoshop and PowerPoint software.

**Establishment of HepG2 cell clones overexpressing bcl-2.** pZipBcl-2, which contains the sequence encoding human Bcl-2, was generously provided by J. M. Hardwick (Johns Hopkins University, Baltimore, MD, USA). The cDNA encoding human Bcl-2 was inserted into pCI-neo to generate pCI-Bcl-2. We established cell clones that stably produced the Bcl-2 protein by transfecting HepG2 cells with pCI-Bcl-2 in the presence of DOTAP liposomal transfection reagent (Roche Molecular Biochemicals), according to the manufacturer’s instructions. The transfected cells were selected on medium containing G418 neomycin (France Biochem). Cell lines stably producing Bcl-2 protein were cloned from single cells by limiting dilution. Western blots were performed with rabbit anti-human Bcl-2 protein (kindly provided by R. Mahieux, Pasteur Institute). Indirect immunofluorescence assays were performed with mouse antibodies specific for the human Bcl-2 protein (kindly provided by E. Guellemard, Pasteur Institute).

**In situ detection of apoptotic cells.** The cells were fixed with 3.2% paraformaldehyde (PFA) in PBS for 20 min. We investigated the nuclear changes associated with apoptotic cell death by incubating fixed cells with 0.1 μg Hoechst 33258 (Sigma) ml^{-1} in 0.1% citrate buffer (pH 6.0) for 10 min at room temperature. Cells were considered to be apoptotic if they exhibited margined chromatin and nuclear condensation. At least 200 EGFP-expressing cells from three independent cell chambers were used to quantify apoptosis. Apoptosis-induced DNA breaks were detected by the deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method as previously described (Despres et al., 1996). Nuclear TUNEL assay was performed with Cy3-conjugated streptavidin (Jackson Immunoresearch).

**Immunofluorescence.** For immunofluorescence analysis, cells were plated on glass cover slips. Cells were fixed with 3.2% PFA and permeabilized with 0.1% Triton X-100 in PBS for 4 min. Rabbit polyclonal antibody to calnexin (SPA-865) was obtained from Stressgen Biotechnologies. Labelling experiments used Cy3-conjugated species anti-IgG antibodies. Cells were observed with an Axiovert 200M fluorescence microscope (Zeiss). Images were processed on a computer, using Simple PCI 5.1, Adobe Photoshop and Powerpoint software.

**Flow cytometry analysis of early apoptosis.** Apoptotic assays were carried out by surface staining with the Ca^{2+}-dependent phosphatidylinerine (PS)-binding protein Annexin V. Transfected HeLa cells were labelled by incubation with Annexin V-APC (BD Pharmingen Biosciences) and 5 μg propidium iodide (PI) (Sigma) ml^{-1} in a HEPES-based buffer (140 mM NaCl, 2.5 mM CaCl_{2}, 10 mM HEPES, pH 7.4) for 15 min on ice according to the manufacturer’s instructions. The stained cells were analysed in a FACScalibur (Becton Dickinson) using CellQuest 3.3 software.

**Immunoblots.** Following Western blotting, cell lysates were separated on a 15% SDS-PAGE gel and then transferred onto a PVDF membrane (Roche Molecular Biochemicals). Membranes were probed with the primary antibody in blocking buffer overnight at 4°C. Primary antibody binding was detected by incubation with alkaline phosphatase-coupled secondary antibody (BioSys). NBT/BCIP reagents were used to detect bound secondary antibodies.

**RESULTS**

**Expression of the M ectodomain leads to apoptosis**

To examine the role of M in the induction of apoptosis by DEN-1 virus, we inserted the FGA/89 cDNA encoding the M protein into a mammalian expression vector under the control of the human cytomegalovirus IE promoter. We constructed EGFP-fused M protein by fusing the DEN-1 cDNA gene sequence immediately downstream from the
reporter gene encoding EGFP (Fig. 1). The EGFP-fused M proteins contained either the complete M protein, including the both TMDs (residues M-1 to M-74), or only the M ectodomain (residues M-1 to M-40) (Fig. 1). The sequence encoding the internal signal sequence (C95–114), which is located at the junction of the DEN-1 C and prM proteins and directs the translocation of prM into the lumen of the endoplasmic reticulum (ER) (Chambers et al., 1990; Rice, 1996), was inserted upstream from sequences encoding the EGFP-fused DEN proteins (Fig. 1). The EGFP-fused E proteins including either the stem alone (residues E-392 to E-439) or the stem–anchor region (residues E-392 to E-487) of the E protein served as controls (Fig. 1).

We evaluated the ability of EGFP-fused DEN proteins to induce apoptosis by means of transient transfection experiments with HeLa cells. After 15 h of transfection, transiently transfected HeLa cells were assayed for EGFP production by direct fluorescence analysis. Upon transfection with pEGFP-N1, we observed autofluorescence of EGFP in more than 50% of the HeLa cells (data not shown). We found that the production of C95–114-EGFP-M1–40/DEN-1, which includes the M ectodomain, resulted in cell death (Fig. 2A). Approximately 15% of M1–40/DEN-1-expressing HeLa cells showed condensation and margination of nuclear chromatin after 25 h of transfection, with a peak of 20% at 30 h, as assessed by Hoechst 33258 staining (Fig. 2B). Transfected HeLa cells expressing the EGFP-fused E-stem also exhibited nuclear condensation, but the number of dead cells was not greatly increased relative to the negative control (5% of C95–114-EGFP-E392–439/DEN-1 versus 3% of C95–114-EGFP; \( P=0.05 \)) (Fig. 2A).

To confirm that apoptosis occurred in transfected HeLa cells producing the M ectodomain of DEN-1 virus, apoptotic DNA fragmentation was assessed by the nuclear TUNEL assay. We observed apoptotic nuclear fragmentation in more than 15% of C95–114-EGFP-M1–40/DEN-1-expressing cells after 25 h of transfection (Fig. 2C). The proportion of apoptotic cells determined by the TUNEL method correlated well with that determined by counting cells with nuclei displaying apoptotic morphology. As production of the full-length M protein or the stem–anchor region of the E protein did not result in cell death (Fig. 2A), the cytotoxicity of the M ectodomain was not due to an overexpression artifact after transfection. To exclude the possibility that EGFP contributes to the death-promoting activity of the EGFP-fused M1–40/DEN-1 protein, we constructed the fusion protein C95–114-M6–40/DEN-1, consisting of residues M-6 to M-40 of the DEN-1 ectodomain directly fused to the signal sequence (Fig. 1). Upon transfection with plasmid expressing C95–114-fused M6–40/DEN-1, approximately 10% of HeLa cells were apoptotic after 25 h of transfection. Thus, the M ectodomain (hereafter referred to as ecto-M) of DEN-1 virus can trigger HeLa cells to undergo apoptosis in the absence of other viral components.

**Transport of the M ectodomain through the secretory pathway leads to apoptosis**

Since the EGFP-M1–40/DEN-1 fusion (Fig. 1) did not induce apoptosis (Fig. 2A), it appeared that the death-promoting activity of ecto-M was abolished if the prM translocation sequence was deleted. This result suggests that the transport of ecto-M through the secretory pathway plays a key role in the initiation of apoptosis.

To monitor the distribution of ecto-M in relation to cellular organelles possibly involved in induction of apoptosis, we examined the localization of C95–114-EGFP-M1–40/DEN-1 and C95–114-EGFP-M1–40/DEN-1-KDEL, the latter consisting of the ER retrieval KDEL motif fused to the carboxy-terminal.
end of the M ectodomain. The KDEL sequence is present in several luminal ER proteins and is recognized by a specific receptor that mediates retrograde transport between the Golgi apparatus and the ER (Pelham, 1996). Immuno- labelling experiments revealed extensive colocalization of C95–114-EGFP-M1–40/DEN-1-KDEL with the cellular marker for the ER (anti-calnexin) in the perinuclear region, indicating that the ER retrieval sequence promotes the retention of ecto-M within the ER (Fig. 3A, left). In contrast, C95–114-EGFP-M1–40/DEN-1 was more dispersed along the secretory pathway. We investigated whether the presence of the M ectodomain in the ER was sufficient to trigger apoptosis by assessing the cytotoxicity of C95–114-EGFP-M1–40/DEN-1-KDEL. Expression of KDEL motif-tagged ecto-M did not cause an increase in the number of TUNEL-positive cells relative to the negative control (Fig. 3B), indicating that the ER retrieval sequence may prevent ecto-M-mediated cell death. This finding is consistent with the observation that the presence of the anchor region (C95–114-EGFP-M1–74 fusion protein) abolished the death-promoting activity of the M ectodomain (Cocquerel et al., 1999).

To investigate whether the Golgi localization of ecto-M is required for the induction of apoptosis, we constructed GalT1–80-EGFP-M1–40/DEN-1 and GalT1–80-EGFP-M1–40/DEN-1-KDEL fusion proteins containing the amino-terminal region of human β1,4-GalT. This region contains the membrane-anchoring signal peptide that targets the protein to the trans-medial region of the Golgi apparatus (Grabenhorst & Conradt, 1999). Upon production of GalT1–80-EGFP-M1–40/DEN-1, the autofluorescence of EGFP was readily detected in the Golgi apparatus of transfected HeLa cells (Fig. 3A, right). As observed by confocal microscopy, trans-Golgi-located α-mannosidase II and GalT1–80-EGFP-M1–40/DEN-1 were colocalized in the same Golgi subcompartment (data not shown). Unlike C95–114-EGFP-M1–40/DEN-1, neither GalT1–80-EGFP-M1–40/DEN-1 nor GalT1–80-EGFP-M1–40/DEN-1-KDEL caused an increase in the number of TUNEL-positive cells relative to the negative control (Fig. 3B).

Thus, our studies at this point suggested that the exit of ecto-M from the Golgi apparatus was required for the induction of apoptosis. To investigate this issue, we engineered a fusion protein, CD721–118-EGFP-M1–40/DEN-1, containing the cytosolic tail of a type II integral membrane glycoprotein, CD72 (Ying et al., 1995), in place of the ER targeting signal of prM. Residues CD721 to CD72118 encompass the membrane-anchoring signal peptide that targets the glycoprotein to the plasma membrane (PM). The PM, and to a lesser extent the Golgi apparatus, was clearly labelled in transfected HeLa cells producing CD721–118-EGFP, indicating that the CD72 translocation signal mediates the engagement of a transport pathway at the cell surface (data not shown). Both the Golgi apparatus and the cell surface were clearly labelled in HeLa cells producing CD721–118-EGFP-M1–40/DEN-1, whereas only the ER was stained in HeLa cells producing CD721–118-EGFP-M1–40/DEN-1-KDEL (Fig. 3A, right). Upon transfection with pCD721–118-EGFP-M1–40/DEN-1, we observed apoptotic nuclear fragmentation in more than 15% of fusion protein-expressing HeLa cells after 30 h of transfection (Fig. 3B). In contrast, production of CD721–118-EGFP or CD721–118-EGFP-M1–40/DEN-1-KDEL did not result in cell death. Taken together, these results indicate...
suggest that the export of ecto-M from the Golgi apparatus to the plasma membrane is essential for the initiation of apoptosis. Replacement of the prM translocation sequence by the CD72 membrane-anchoring signal peptide preserved the death-mediating activity of EGFP-fused M\(^{1-40/DEN-1}\) (Fig. 3B). Thus, ecto-M may exert its cytotoxic effects by activating an apoptotic signalling pathway that does not require a soluble form.

Fig. 3. Subcellular localization of the M ectodomain. Transfected HeLa cells producing fusion proteins were detected by monitoring the autofluorescence of EGFP (A) or analysed for apoptosis (B). (A) EGFP-positive cells were subjected to indirect immunofluorescence staining with anti-calnexin antibodies conjugated to Cy3. Overlap of C\(^{95-114}\)EGFP-M\(^{1-40}\) (M\(^{1-40}\)) or C\(^{95-114}\)EGFP-M\(^{1-40}\)-KDEL (M\(^{1-40}\)-KDEL) with the ER marker is represented by the yellow regions (left). Intracellular distribution of GalT\(^{1-80}\)-EGFP-M\(^{1-40/DEN-1}\) (GalT\(^{1-80}\)) and CD72\(^{1-118}\)-EGFP-M\(^{1-40/DEN-1}\) (CD72\(^{1-118}\)) without (-KDEL) or with (+KDEL) ER retrieval sequence KDEL (right). Scale bar, 10 \(\mu m\). (B) Nuclear DNA nicks of transfected cells were monitored by the TUNEL assay after 30 h of transfection. C\(^{95-114}\)EGFP, GalT\(^{1-80}\)EGFP and CD72\(^{1-118}\)EGFP served as negative controls (white bars). Fusion proteins were compared statistically with their respective negative controls as described in the legend to Fig. 2: not significant (n.s., \(P>0.05\)) or significant (*\(P<0.05\); **\(P<0.01\); ***\(P<0.001\)).
The M ectodomains of JE, WN and YF viruses have proapoptotic properties

As the DEN-1 M ectodomain induced apoptosis, we investigated whether the M ectodomains of other DEN virus serotypes and of other apoptosis-inducing flaviviruses, such as wild-type strains of JE, WN and YF viruses, also cause cell death. Production of the various EGFP-fused M ectodomains was confirmed by Western blotting (data not shown). All flavivirus M ectodomains induced apoptosis after 25 h of transfection (Fig. 4A), suggesting that the proapoptotic properties of ecto-M are conserved among apoptosis-inducing flaviviruses. The M ectodomains of DEN-1 and DEN-2 viruses were the most potent inducers of apoptosis.

Comparison of the genomes of the YF vaccine strains 17D and French neurotropic virus (FNV) with the parental and other wild-type YF viruses revealed a common difference at position M-36: the leucine residue at this position in the wild-type YF viruses (M1-40/YF.wt) was replaced by a phenylalanine (M1-40/YF.17D) during attenuation (Monath, 1999; Hahn et al., 1987). Unlike EGFP-fused M1-40/YF.wt, C95-114-EGFP-M1-40/YF.17D did not trigger apoptosis in transfected HeLa cells (Fig. 4B). Thus, the L36→F substitution observed in vaccine strains abolishes the death-promoting activity of the YF M ectodomain.

The DEN M ectodomains induce apoptosis in tumour and transformed cells

As DEN virus induces apoptosis in mouse neuroblastoma Neuro 2a and human hepatoma HepG2 cells (Després et al., 1996; Duarte dos Santos et al., 2000; Jan et al., 2000; Lin et al., 2000; Marianneau et al., 1997; Su et al., 2001), we tested the ability of the DEN M ectodomain to cause death in these susceptible cell lines. We showed that transfected Neuro 2a cells and HepG2 cells producing C95-114-EGFP-M1-40/DEN-1 or C95-114-EGFP-M1-40/DEN-2 underwent apoptosis after 30 h of transfection (Fig. 5), suggesting that DEN ecto-M induces apoptosis in tumour cells of various origins.

Transformed fibroblasts from monkey kidney COS-7 and

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**Fig. 4.** The M ectodomains from apoptosis-inducing flaviviruses have proapoptotic properties. HeLa cells were transfected with constructs encoding C95-114-EGFP (control, white bar), C95-114-EGFP-M1-40/DEN-1 (DEN-1), C95-114-EGFP-M1-40/DEN-2 (DEN-2), C95-114-EGFP-M1-40/DEN-3 (DEN-3), C95-114-EGFP-M1-40/DEN-4 (DEN-4), C95-114-EGFP-M1-40/JE (JE), C95-114-EGFP-M1-40/WN (WN) or C95-114-EGFP-M1-40/YF.wt (YF) (A), or with plasmids encoding C95-114-EGFP (control, white bar), C95-114-EGFP-M1-40/YF.wt (M1-40/YF.wt) or C95-114-EGFP-M1-40/YF.17D (M1-40/YF.17D) (B). Transfected HeLa cells were stained with Hoechst 33258 after 25 h of transfection and examined for changes in nuclear morphology. Fusion proteins were compared statistically with their respective controls as described in the legend to Fig. 2: not significant (n.s., *P*>0.05) or significant (*P*<0.05; **P*<0.01; ***P*<0.001).

**Fig. 5.** The DEN M ectodomain induces apoptosis in cells of various origins. Tumoral Neuro 2a and HepG2 cell lines and transformed 293A and COS-7 cell lines were transfected with plasmids encoding C95-114-EGFP-M1-40/DEN-1 (hatched bar) or C95-114-EGFP-M1-40/DEN-2 (grey bar). Transfected cells were stained with Hoechst 33258 and examined for chromatin condensation. The percentages of fusion protein-expressing cells with apoptotic nuclei after 30 h of transfection are indicated. Fusion proteins were compared statistically with C95-114-fused EGFP (white bar) as described in the legend to Fig. 2: not significant (n.s., *P*>0.05) or significant (*P*<0.05; **P*<0.01; ***P*<0.001).
human embryonic kidney 293A cells line displays an anti-apoptosis activity (Fearnhead et al., 1997; Tsai et al., 2000). COS-7 cells contain an integrated copy of the complete early region of simian virus 40 (SV40) DNA (Gluzman, 1981) and 293A cells express adenovirus 5 (AdS) early regions E1A and E1B (Louis et al., 1997). We assayed the death-promoting activity of ecto-M in both types of cell line. The transfected COS-7 cells that produced C95–114-EGFP-M1–40/DEN-1 or C95–114-EGFP-M1–40/DEN-2 underwent apoptosis after 30 h of transfection (Fig. 5). In contrast, cell death was not observed in transfected 293A cells expressing the EGFP-fused ecto-M (Fig. 5). Transiently transfected 293A cells producing C95–114-EGFP-M1–40/DEN-1 or C95–114-EGFP-M1–40/DEN-2 were still observed after 72 h of transfection (data not shown). Thus, 293A cells are protected against the death-promoting activity of DEN ecto-M.

**The apoptotic effect of the M ectodomain is blocked by Bcl-2**

We investigated whether the overexpression of Bcl-2 protected HepG2 cells against the apoptotic effects of M1–40/DEN-2 by establishing permanent HepG2 cell lines that stably over-expressed human Bcl-2, by transfection with pCI-Bcl-2. Western blot analysis (Fig. 6A) and an indirect immunofluorescence assay (Fig. 6B) showed that HepG2/bcl-2#5, a clone stably expressing bcl-2, overproduced human bcl-2. In these experiments, the HepG2/ne#1 cell clone served as a negative control. Comparison of HepG2/bcl-2#5 cells with HepG2/ne#1 cells showed that the overexpression of Bcl-2 did not affect the intracellular synthesis of the fusion proteins in transfected cells (data not shown). We then investigated the effect of bcl-2 on M1–40/DEN-2-induced apoptosis by monitoring changes in nuclear morphology (Fig. 6C). After 30 h of transfection, 7% of M1–40/DEN-2-expressing HepG2/ne#1 cells underwent apoptosis. In contrast, less than 2% of M1–40/DEN-2-expressing HepG2/bcl-2#5 cells were apoptotic at this time. After 48 h of transfection, M1–40/DEN-2-expressing cells were still detected among transfected HepG2/bcl-2#5 cells (data not shown). Thus, M ectodomain-induced apoptosis is inhibited by over-expression of Bcl-2.

**A nine residue sequence is required for the induction apoptosis by the M ectodomain**

We tried to identify the amino acid residues critical for the death-promoting activity of ecto-M more precisely by using a series of fusion proteins consisting of EGFP fused to truncations from both ends of the 40 amino acid ectodomain of the DEN-2 M protein. The amino acid sequences of the mutant proteins are given in Fig. 7(A). The apoptotic effects of the mutant proteins were assessed in HeLa cells after 25 h of transfection. Cell death was not observed in transfected HeLa cells expressing only the first 30 amino acids of the ecto-M (Fig. 7B), indicating that the amino-terminal part of the DEN-2 M ectodomain is not required for the induction of apoptosis. The production of mutant proteins containing residues M-30 to M-40 induced apoptotic changes in nuclei (Fig. 7B), leading to the suggestion that the last 11 amino acids are involved in the induction of apoptosis.

With a view to identifying the minimal sequence of the DEN-2 M ectodomain responsible for the induction of apoptosis, we engineered a construct encoding the residues M-32 to M-40 fused to EGFP (Fig. 7A). After 25 h of transfection, 13% of transfected HeLa cells producing the mutant protein C95–114-EGFP-M32–40/DEN-2 exhibited chromatin condensation (Fig. 7B, M32–40/DEN-2). To further examine the requirement of the nine residue sequence for apoptosis induced by ecto-M, the rate of early apoptosis was investigated by flow cytometry using the Annexin V affinity assay, which detects phosphatidylserine translocated to the outer layer of the cell membrane. The fusion proteins C95–114-EGFP and C95–114-EGFP-M1–30/DEN-2 were used as negative controls (data not shown). In three independent experiments, the transfected HeLa cells producing C95–114-EGFP-M32–40/DEN-2 displayed a significantly higher fraction of EGFP-positive cells labelled with Annexin V-APC than did cells producing C95–114-EGFP-M1–40/DEN-2 (Fig. 7C, inset squares). Thus, the nine residue sequence was able to induce apoptosis in HeLa cells as efficiently as ecto-M. These results indicate that segment 32-32ETWLRHP is responsible for the death-promoting activity of DEN-2 M ectodomain.

We investigated whether the nine residue sequence of the DEN-2 ecto-M is potent in triggering apoptosis by introducing the substitutions R34→T, F36→I, V37→L and N39→H into the EGFP-fused M1–40/YF.17D that had lost its cytoxicity (Fig. 8A). In transfected HeLa cells, expression of mutant protein C95–114-EGFP-M1–40/YF.17D (T348, I346, L377, H369) resulted in cell death (Fig. 8B), indicating that residues M-34, M-36, M-37 and M-39 are critical for induction of apoptosis by ecto-M. We evaluated the effect of the F36 substitution on the death-promoting activity of DEN ecto-M by generating a fusion protein, C95–114-EGFP-M1–40/YF.17D (T348, I346, L377, H369), with a phenylalanine residue in position 36 of the DEN-2 M ectodomain (Fig. 8A). In transfected HeLa cells, the resulting mutant protein C95–114-EGFP-M1–40/YF.17D (F36) induced apoptosis less efficiently than the parental fusion protein (Fig. 8B). Thus, the overall apoptosis-inducing activity of the M ectodomain reflected the intrinsic proapoptotic properties of residues M-32 to M-40 and the substitution of a leucine (YF ecto-M) or an isoleucine (DEN-2 ecto-M) for the phenylalanine in position M-36 can affect these properties.

**DISCUSSION**

No biological function has yet been assigned to the flavivirus membrane (M) protein. This report indicates for the first time that the intraluminal ectodomain of the DEN M protein has proapoptotic properties. Remarkably, the M ectodomain has a great potential for apoptosis induction in transformed and tumour cells of various origins such as Neuro 2a and HepG2 cells. Deletion mutant analysis...
showed that the nine carboxy-terminal amino acids of the DEN M ectodomain (residues M-32 to M-40, hereafter referred to as ApoptoM) are necessary and sufficient for the induction of apoptosis. The discovery of ApoptoM brings to light a role for the small membrane M protein in DEN virus-induced cytopathic effects. Detailed comparison indicated that ApoptoM of the four serotypes of DEN were more than 75% identical. Searches of nucleotide and protein databases showed that the nine residue sequence responsible for the cytotoxic effect of the M ectodomain displayed no obvious similarity to any known cellular protein.

The M ectodomains of wild-type strains of JE, WN and YF viruses also have proapoptotic properties, suggesting that the 40 amino acid ectodomain of M may play an important role in flavivirus-induced cytopathic effects. Viscerotropic YF virus causes damage to liver cells in humans and hepatocytic apoptosis has been observed in infected livers (Marianneau et al., 1998; Xiao et al., 2001). Two live attenuated vaccine strains, 17D and French neurotropic virus (FNV), are known to have lost the ability to cause viscerotropic disease (Wang et al., 1995). Little is known about the molecular determinants of virulence in wild-type YF virus. Interestingly, all YF vaccine strains studied to date include a common amino acid leucine to phenylalanine substitution at position M-36 (Monath, 1999; Hahn et al., 1987). We found that this substitution abolishes the death-inducing activity of the YF M ectodomain, suggesting that residue M-36 may play a crucial role in the attenuation of viscerotropic YF virus. Mutant YF viruses produced by infectious clone technology will elucidate the potential role of residue M-36 in YF virus pathogenicity. The I36→F substitution results in a reduction of the cytotoxicity of the DEN-2 M ectodomain. Thus, residue M-36 may be essential for the efficient induction of apoptosis by the DEN and YF M ectodomains.

Transformed human 293A cells were resistant to the apoptotic effects mediated by the M ectodomain. The embryonic kidney 293A cell line expresses the Ad5 E1A and E1B genes (Louis et al., 1997). The transforming activities of these genes are based on their ability to interact with the transcription factor p53, which is the central component in a complex network of apoptotic-signalling pathways (Burns & El-Deiry, 1999; White, 2001). The viral oncogene E1B encodes two proteins, E1B 55K and E1B 19K, which block intracellular signalling as part of p53-dependent proapoptotic pathways (White, 2001). COS-7 cells, which undergo apoptosis in response to M ectodomain production, possess an anti-apoptosis activity independent of p53 activity (Tsai et al., 2000). The cellular concentration of p53, one of the target genes for NF-κB, has been reported to increase during the early stages of DEN virus infection (Jan et al., 2000). We have undertaken experiments to determine whether the death-promoting activity of the M ectodomain requires p53 activation.
Apoptosis induced by flavivirus infection is efficiently prevented by the anti-apoptosis protein Bcl-2 (Liao et al., 1997, 1998; Su et al., 2001). The overexpression of Bcl-2 also protected HepG2 cells against the death-promoting activity of the M ectodomain. Bcl-2 probably regulates apoptosis by controlling the export of mitochondrial cytochrome c.

**Fig. 7.** The carboxy-terminal amino acids of the M ectodomain constitute a proapoptotic sequence. Transfected HeLa cells were assayed for apoptotic nuclear fragmentation after 25 h of transfection (B) or for the early stage of apoptosis after 20 h (C). (A) Amino acid sequence alignments for mutant proteins, the names of which are shown on the right. (B) HeLa cells were stained with Hoescht 33258 and examined for chromatin condensation. C25–114-fused EGFP (control, white bar) served as a negative control. Statistical analysis for fusion proteins was carried out by comparison with the control as described in the legend to Fig. 2: not significant (n.s., $P > 0.05$) or significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (C) The rate of early apoptosis was analysed by Annexin V binding, as assessed by flow cytometry analysis. Apoptosis was defined as EGFP-positive cells that bound Annexin V-APC but excluded propidium iodide (PI). For each sample, data from 10 000 EGFP-positive cells were collected. The percentages of M1–40- and M32–40-expressing cells labelled with Annexin V are indicated (inset squares).
and caspase activation (Adams & Cory, 2001; Desagher & Martinou, 2000). It has been suggested that the overproduction of Bcl-2 or of the Ad5 E1B 19K protein, a functional homologue of Bcl-2, prevents apoptosis by modulating the activities of the proapoptotic factor Bax, which is induced by p53 (Lomonosova et al., 2002). Bax is located predominantly in the cytosol, but is translocated to the mitochondria early in apoptosis, leading to the release of apoptogenic factors (Adams & Cory, 2001; Kuwana et al., 2002). A steady increase in the amount of Bax has been reported to occur in response to flavivirus infection (Parquet et al., 2001, 2002). A steady increase in the amount of Bax has been reported to occur in response to flavivirus infection (Parquet et al., 2001, 2002). It is therefore of great interest to consider the role of the factor Bax in apoptosis induced by the M ectodomain.

In summary, we have provided evidence that a nine residue sequence designated ApoptoM is directly involved in induction of apoptosis by the M protein. This finding raises the question of whether initiation of apoptosis may result from the interaction of ApoptoM with organelle-specific signalling molecules that elicit death pathways (Ferri & Kroemer, 2001). Such interaction might occur at the same time as the prM precursor glycoprotein is processed in the exocytotic pathway of the trans-Golgi network, resulting in M and a ‘pr’ fragment. Although M is not normally known to undergo proteolysis, an alternative explanation is that apoptosis-signalling pathways are activated through mechanisms that involve cleavage of ApoptoM from the M ectodomain during transport in the late secretory pathway.

The data presented here suggest an important role for the small M protein in flavivirus-induced apoptosis. During the late stage of apoptosis, the plasma membrane disassembles into membrane-enclosed vesicles (apoptotic bodies) that are generally eliminated by macrophage-like cells. The mechanisms of host defence involving phagocytosis of apoptotic bodies associated with the production of cytokines may cause local tissue injury. Our future experiments will assess the role of ApoptoM in the pathophysiological manifestations consecutive to flavivirus infection.
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