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A Novel Family of Viral Death Effector Domain-containing Molecules That Inhibit Both CD-95- and Tumor Necrosis Factor Receptor-1-induced Apoptosis*

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Molluscum contagiosum virus proteins MC159 and MC160 and the equine herpesvirus 2 protein E8 share substantial homology to the death effector domain present in the adaptor molecule Fas-associated death domain protein (FADD) and the initiating death protease FADD-like interleukin-1β-converting enzyme (FLICE) (caspase-8). FADD and FLICE participate in generating the death signal from both tumor necrosis factor receptor-1 (TNFR-1) and the CD-95 receptor. The flow of death signals from TNFR-1 occurs through the adaptor molecule tumor necrosis factor receptor-associated death domain protein (TRADD) to FADD to FLICE, whereas for CD-95 the receptor directly communicates with FADD and then FLICE. MC159 and E8 inhibited both TNFR-1- and CD-95-induced apoptosis as well as killing mediated by overexpression of the downstream adaptors TRADD and FADD. Neither viral molecule, however, inhibited FLICE-induced killing, consistent with an inhibitory action upstream of the active death protease. These data suggest the existence of a novel strategy employed by viruses to attenuate host immune killing mechanisms. Given that bovine herpesvirus 4 protein E1.1 and Kaposi's sarcoma associated-herpesvirus protein K13 also possess significant homology to the viral inhibitory molecules MC159, MC160, and E8, it may be that this class of proteins is used ubiquitously by viruses to evade host defense.

Cell suicide is a defense mechanism employed by host cells to inhibit viral replication and persistence. As a consequence, viruses have evolved numerous strategies to attenuate apoptosis (1). For example, the Epstein-Barr virus (EBV) encodes BHRF1, a homolog of the mammalian anti-apoptosis molecule bcl-2, and the cowpox virus encodes a serpin-like protein, CrmA, that blocks apoptosis by inhibiting proteases belonging to the caspase family.

Molluscum contagiosum virus (MCV) is the only poxvirus family member still associated with human disease (2). It usually causes asymptomatic cutaneous neoplasms that can spontaneously regress. However, with the advent of immunocompromised populations, particularly those afflicted with acquired immunodeficiency syndrome, MCV infection has become a clinical challenge (3). Unfortunately, due to the inability to grow the virus in tissue culture cells and the lack of a suitable animal model, little is known about host-virus relationships (4). Equine herpesvirus 2 (EHV2) is a member of the γ-herpesvirus subfamily that also includes herpesvirus saimiri, EBV, Kaposi's sarcoma-associated herpesvirus (KSHV), and bovine herpesvirus 4 (5, 6). Although EHV2 is ubiquitously distributed and has been implicated as a pathogen in immunosuppressed states, its mode of evading the host immune response is uncertain. However, the recent availability of the MCV and EHV2 genome sequences has begun to identify genes that suggest potential pathogenic mechanisms (7, 8).

MCV, surprisingly, does not encode many of the immunoregulatory molecules present in other poxviruses, especially those that antagonize the host cytokine-mediated inflammatory response. These include CrmA and a soluble TNFR-like[1] molecule (7). In contrast, EHV2 encodes an interleukin-10-like factor that may attenuate the host immune response (8). Regardless, MCV and EHV2 do not encode previously identified inhibitors of apoptosis (1). Instead, MCV and EHV2 encode novel members of an emerging family of molecules characterized by the presence of a death effector domain (DED) originally identified in signaling molecules engaged by the death receptors TNFR-1 and CD-95 (7, 8).

Both TNFR-1 and CD-95 contain a stretch of approximately 60–80 amino acids within their cytoplasmic domains termed the death domain. Upon activation the receptor death domains bind to corresponding death domains within the adaptor molecules TRADD (for TNFR-1) and FADD (for CD-95) (9–12). Utilizing the same mechanism, TRADD is able in turn to recruit FADD to the TNFR-1 signaling complex (13). FADD appears to play a central role as a conduit for death signals from both receptors as dominant negative versions that retain the death domain but lack the amino-terminal segment effectively attenuate both TNFR-1- and CD-95-induced killing (14). Since it is likely that the amino-terminal domain of FADD functions to engage downstream components of the death pathway, it has been termed the DED (14). The importance of this domain was dramatically underscored by the discovery of its presence within the prodomain of the death protease FLICE (15–17). It appears that the DED of FADD binds to the corresponding DED motif within the FLICE prodomain and thereby recruits this death protease to the receptor signaling complex. There-

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[1] The abbreviations used are: TNFR-1, tumor necrosis factor receptor 1; FADD, Fas-associated death domain protein; FLICE, FADD-like interleukin-1β-converting enzyme; TRADD, tumor necrosis factor receptor-associated death domain protein; MCV, molluscum contagiosum virus; EHV2, equine herpesvirus 2; KSHV, Kaposi's sarcoma-associated herpesvirus; DED, death effector domain.

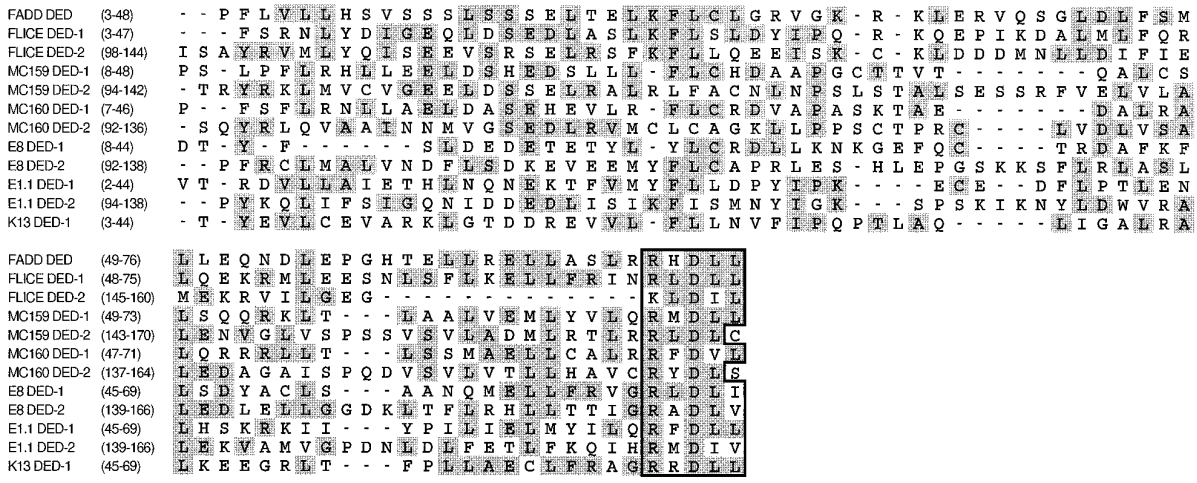

FIG. 1. **Sequence alignment of viral DED-containing molecules.** The DED motifs contained within the adaptor molecule FADD and the initiating caspase FLICE were aligned to DED-like motifs present in the molluscum contagiosum virus proteins MC159 and MC160, bovine herpesvirus 4 protein E1.1, equine herpesvirus 2 protein E8, and the Kaposi's sarcoma-associated virus protein K13. The conserved module RXDLL is *boxed*.

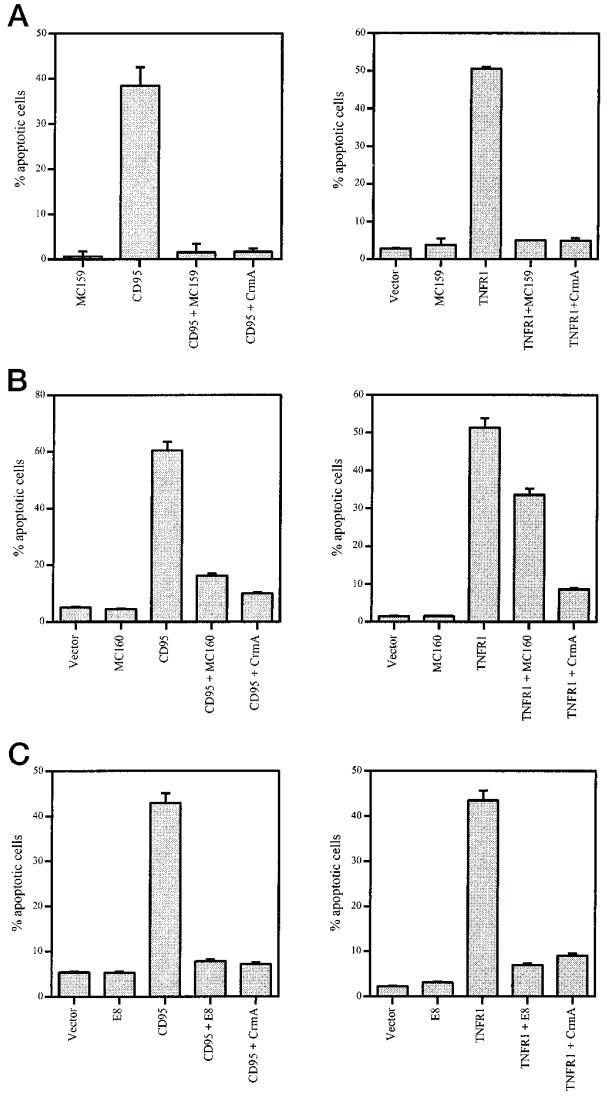

FIG. 2. **MC159, MC160, and E8 inhibit TNFR-1- and CD-95-induced apoptosis.** Overexpression of MC159 (*A*), MC160 (*B*), and E8 (*C*) inhibit CD-95- and TNFR-1-induced cell death. 293 or 293-EBNA cells were cotransfected with indicated plasmid together with the reporter construct pCMV β-galactosidase. Cells were fixed and stained 24–30 h following transfection. The data shown are the percentage of blebbing blue cells as a function of total number of blue cells counted.

fore, a homophilic binding mechanism involving DEDs is responsible for assembly of the receptor death signaling complex. Disruption of such a complex by DED-containing viral gene products could potentially abrogate propagation of the death signal.

MATERIALS AND METHODS

Cell Lines and Expression Vectors—Human embryonic kidney 293, 293T, and 293-EBNA cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Mammalian expression vectors encoding TNFR-1, CD-95, FADD, FLICE, MC159, MC160, E8, and CrmA were cloned into pcDNA3 (Invitrogen). The expression vector for TRADD was kindly provided by Dr. David Goeddel (Tularik, Inc.).

In Vitro Binding Assay—Full-length FADD and truncated N-FADD were expressed as GST-fusion proteins as described previously (18). [^{35}S]Methionine-radiolabeled MC159 was obtained by *in vitro* transcription/translation using the TNT T7-coupled reticulocyte lysate system (Promega). Binding reactions were performed as described previously (18).

Transfection, Coimmunoprecipitation, and Western Analysis—Transient transfections of 293T cells were performed as described previously (19). Cells were harvested 40 h following transfection, immunoprecipitated with the indicated antibodies, and analyzed by immunoblotting.

Cell Death Assay—For CD-95, TRADD, and FLICE killing, experiments were performed in 293-EBNA cells and in 293 cells for TNFR-1 and FADD killing. cDNAs encoding putative apoptosis inducers (0.5–0.8 μg) and potential inhibitors (2.5 μg) were cotransfected in each experiment together with the reporter plasmid pCMV β-galactosidase. Cells were fixed and stained 24–30 h following transfection. The percentage of apoptotic cells was determined by calculating the fraction of round membrane-blebbed blue cells as a function of total blue cells. All assays were evaluated in duplicate and the mean and standard deviation calculated.

RESULTS AND DISCUSSION

E8 and MC159 Are Members of the DED Family—MCV encodes two closely related proteins: MC159 and MC160 (6). The NH_2 termini of the 241-amino acid protein MC159 and the 371-amino acid protein MC160 contain two motifs homologous to DEDs present at the NH_2 terminus of FADD and repeated in tandem within the prodomain of FLICE (7). Interestingly, the DED motif is also present within EHV2-encoded protein E8 (171 amino acids), Kaposi's sarcoma-associated herpesvirus-encoded protein K13 (139 amino acids), and bovine herpesvirus 4-encoded protein E1.1 (182 amino acids) (Fig. 1) (20, 21). Unlike the MCV proteins, full-length E8, K13, and E1.1 encode only DED motifs. K13 encodes one complete and one incomplete DED, whereas E8 and E1.1 encode two DEDs. Each DED

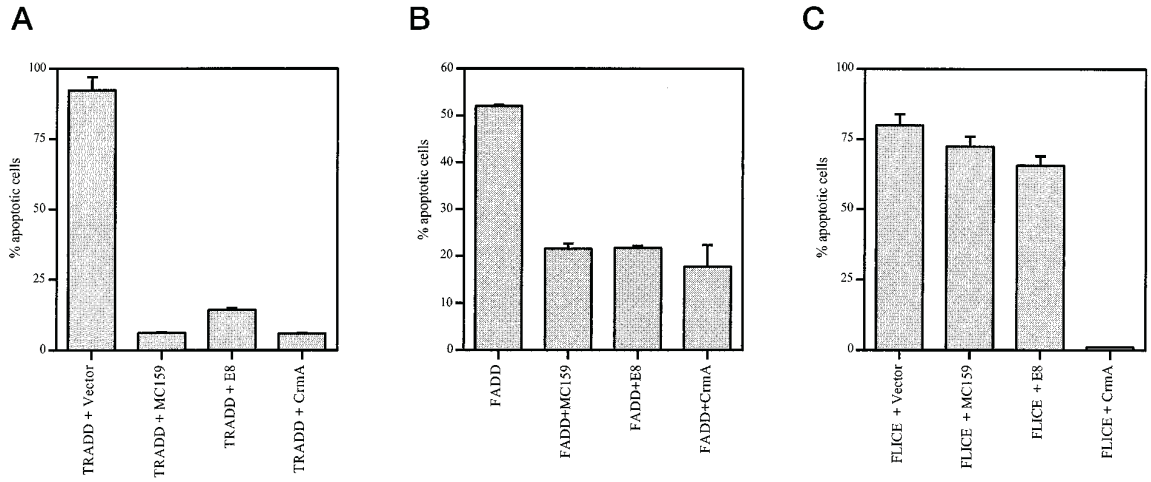

FIG. 3. **MC159 and E8 inhibit both TRADD and FADD but not FLICE killing.** MC159 and E8 inhibit TRADD (*A*) and FADD (*B*) killing but fail to inhibit FLICE killing (*C*). 293 or 293-EBNA cells were cotransfected with indicated plasmid together with the reporter construct pCMV β-galactosidase. Cells were fixed and stained 24 h following transfection. The data shown are the percentage of blebbing blue cells as a function of total number of blue cells counted.

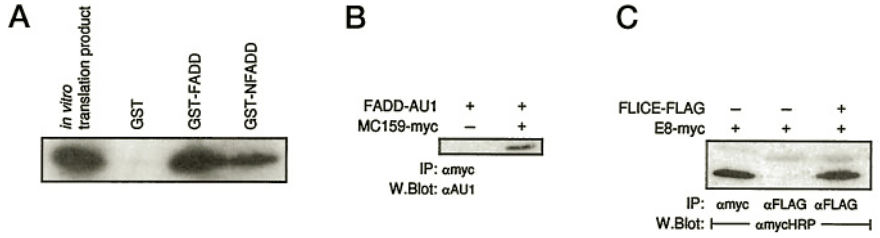

FIG. 4. **MC159 binds FADD, whereas E8 binds FLICE.** *A*, interaction of radiolabeled *in vitro* translated MC159 with GST-FADD and GST-N-FADD immobilized onto glutathione-Sepharose beads. 2 μl of ^{35}S-labeled translation reaction was directly loaded as a control, while 5 μl was used in each binding reaction. *B*, MC159 binds FADD. 293T cells were cotransfected with Myc-tagged MC159 and AU1-tagged FADD. 40 h following transfection, cells lysates were immunoprecipitated and immunoblotted with the indicated antibodies. *C*, E8 binds FLICE. 293T cells were cotransfected with Myc-tagged E8 and FLAG-tagged FLICE.

of these viral proteins contains a highly conserved module R*X*DL/I(L) (*X* is any amino acid) that is also conserved in the DEDs of FADD and FLICE. It appears that many other herpesviruses also encode DED-like molecules. Examples include the herpesvirus saimiri protein VG71 and human herpesvirus 6 protein U15 (22, 23).

E8 and MC159 Inhibit TNFR-1- and CD-95-induced Apoptosis—The presence of DEDs within E8, MC159, and MC160 suggests that these viral proteins might potentially antagonize the FADD-FLICE interaction and thereby attenuate TNFR-1- and CD-95-mediated apoptosis. Indeed, overexpression of MC159 significantly inhibited TNFR-1- and CD-95-induced cell death (Fig. 2*A*). The degree of inhibition was substantially greater than that achieved with the catalytically inactive dominant-negative version of FLICE (data not shown) and comparable in potency with CrmA. MC160 also inhibited TNFR-1- and CD-95-induced cell death (Fig. 2*B*), as did E8 (Fig. 2*C*).

E8 and MC159 Inhibit TRADD and FADD Killing but Not FLICE Killing—Additional studies were undertaken to delineate the point at which MC159 and E8 were exerting their inhibitory effect on the TNFR-1- and CD-95-induced death pathways. As shown in Fig. 3, both MC159 and E8 significantly blocked both TRADD and FADD killing, suggesting that these inhibitors must function downstream of these adaptor molecules. In contrast, MC159 and E8 did not inhibit FLICE-induced death, suggesting that they must act upstream of active FLICE. The overexpression of FLICE zymogen results in autoactivation to the active protease that is potently inhibited by the viral serpin CrmA (Fig. 3*C*).

MC159 Binds FADD, whereas E8 Binds FLICE—Binding studies were undertaken to investigate the potential mechanism utilized by E8 and MC159 to attenuate TNFR-1- and CD-95-induced cell death (Fig. 4). Radiolabeled *in vitro* translated MC159 was precipitated with various GST-fusion proteins immobilized onto glutathione-Sepharose beads, including GST-FADD, GST-NFADD containing only the NH_2-terminal DED (amino acid residues: 1–82), or GST alone (Fig. 4*A*). As expected from the homophilic binding nature of DEDs, MC159 strongly bound GST-FADD and GST-NFADD, but not GST alone.

To demonstrate the association of the viral inhibitory molecules with FADD or FLICE *in vivo*, 293 cells were transiently transfected with expression constructs encoding epitope-tagged versions of the respective molecules (Fig. 4). Consistent with the *in vitro* binding results, MC159 precipitated with FADD (Fig. 4*B*), but not with FLICE (data not shown). Conversely, E8 strongly associated with FLICE (Fig. 4*C*), but not with FADD (data not shown). This binding specificity of MC159 and E8 suggested that distinct mechanisms were employed by these two inhibitors. MC159 binds to FADD and presumably blocks its interaction with FLICE. The reverse is probably true for E8 in that it binds FLICE and inhibits its interaction with FADD. However, when FLICE is overexpressed (upon transfection), the binding of E8 is unable to overcome the propensity of this caspase to autoactivate (Fig. 3*C*). Therefore, once FLICE is active, E8 has no inhibitory influence. Regardless, either mechanism would disrupt the assembly of the receptor·FADD·FLICE signaling complex and abrogate activation of downstream caspases. Further studies will be needed to substantiate these proposed mechanisms.

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