Equine Herpesvirus-2 E10 Gene Product, but Not Its Cellular Homologue, Activates NF-κB Transcription Factor and c-Jun N-terminal Kinase*

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Margot Thome‡, Fabio Martinon‡, Kay Hofmann§, Verena Rubio‡, Véronique Steiner‡, Pascal Schneider‡, Chantal Mattmann‡, and Jürg Tschopp‡

From the ‡Institute of Biochemistry, University of Lausanne, and the §Swiss Cancer Research Institute (ISREC), BIL Biomedical Research Center, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland

We have previously reported on the death effector domain containing E8 gene product from equine herpesvirus-2, designated FLICE inhibitory protein (v-FLIP), and on its cellular homologue, c-FLIP, which inhibit the activation of caspase-8 by death receptors. Here we report on the structure and function of the E10 gene product of equine herpesvirus-2, designated v-CARMEN, and on its cellular homologue, c-CARMEN, which contain a caspase-recruiting domain (CARD) motif. c-CARMEN is highly homologous to the viral protein in its N-terminal CARD motif but differs in its C-terminal extension. v-CARMEN and c-CARMEN interact directly in a CARD-dependent manner yet reveal different binding specificities toward members of the tumor necrosis factor receptor-associated factor (TRAF) family. v-CARMEN binds to TRAF6 and weakly to TRAF3 and, upon overexpression, potently induces the c-Jun N-terminal kinase (JNK), p38, and nuclear factor (NF)-κB transcriptional pathways. c-CARMEN or truncated versions thereof do not appear to induce JNK and NF-κB activation by themselves, nor do they affect the JNK and NF-κB activating potential of v-CARMEN. Thus, in contrast to the cellular homologue, v-CARMEN may have additional properties in its unique C terminus that allow for an autonomous activator effect on NF-κB and JNK. Through activation of NF-κB, v-CARMEN may regulate the expression of the cellular and viral genes important for viral replication.

The ability to undergo apoptosis is an inherent property of all somatic cells. Apoptosis can be triggered by a variety of stimuli, such as ligands of the tumor necrosis factor family, cytotoxic drugs, growth factor starvation, and infectious agents such as viruses. Viruses are faced with the need to productively replicate within a receptive intracellular environment and thus have evolved strategies to counteract the death signaling machinery (1, 2). Documented viral apoptosis inhibitors include proteins that have a bcl-2-like function or those that directly interfere with caspase activity. We and others (3–5) have recently characterized a family of viral gene products, collectively called v-FLIPs,† that are produced by many γ-herpesviruses and by the molluscum contagiosum virus. The striking structural feature of the viral FLIPs is the presence of a tandem repeat of a so-called death effector domain (DED). The DED has structural homology to a motif called the death domain, which is present in the cytoplasmic part of death receptors such as Fas. The death domain of Fas interacts with the C-terminal death domain of FADD, which in turn induces the binding of the N-terminal DED of FADD to the DED-containing prodomain of caspase-8. The recruitment and activation of caspase-8 by Fas result in the processing of downstream caspases and the induction of apoptosis (6). The DED-containing v-FLIPs interfere with the FADD-caspase-8 interaction and hence block apoptosis (3). Thus, the DED and the death domain are important protein-protein interaction modules implicated in cell death signaling.

Recently, the existence of a third related protein-protein interaction domain, designated the caspase-recruiting domain (CARD), was revealed (7). The prototype CARD is found in Apaf-1, a mitochondrial protein related to the Caenorhabditis elegans protein CED-4 (8). Activation of Apaf-1 by cytochrome c leads to the recruitment of the CARD-containing caspase-9 and to subsequent apoptosis (9). Additional CARD-dependent protein interactions have been identified. The CARD-containing adaptor molecule RAIDD recruits caspase-2 to the tumor necrosis factor receptor 1 (10), whereas the CARD-containing kinase CARDIACKRIP-2 may mediate both the recruitment and activation of caspase-1 by CD40 and the activation of JNK and NF-κB (11, 12). The respective recruiting/regulatory proteins of other CARD-containing caspases (caspase-4, -5, -7, -11, and -12), are currently not known. Other CARD-containing proteins, i.e. ARC (13), c-IAP1, and c-IAP2 (14), are involved in the regulation of apoptosis.

We have previously reported on a gene product from equine herpesvirus type 2 (EHV-2), designated E10, that comprises a CARD motif (7). Because the E8 gene, which is found in the same region of the EHV-2 genome, encodes the potent apoptosis inhibitor v-FLIP, we undertook a study to investigate whether E10 may also be implicated in the modulation of apoptosis.

† The abbreviations used are: FLIP, FLICE inhibitory protein; CARD, caspase-recruiting domain; CARMEN, CARD-containing molecule enhancing NF-κB; DED, death effector domain; EHV-2, equine herpesvirus-2; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; NF, nuclear factor; TRAF, tumor necrosis factor receptor-associated factor; ORF, open reading frame; DN, dominant negative; HA, hemagglutinin; EST, expressed sequence tag; VSV, vesicular stomatitis virus.

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**Experimental Procedures**

Cloning of Viral, Human, and Mouse CARMEN cDNA—The complete ORF of viral E10 was amplified from viral EHV-2 DNA (a kind gift of A. J. Davison, Glasgow, United Kingdom). The amplified E10 ORF is longer than the originally published E10 ORF (after resequencing of the respective region of the viral genome, we had to correct the originally reported E10 sequence (15) that predicted a shorter ORF). EST clones encoding a related human and mouse CARD-containing protein were identified in the dbEST database at the National Center for Biotechnology Information (EST clones 703916 and 574273) by performing a data base search using a generalized profile method (16) based on the sequence homology among other CARD-containing proteins (7).

Northern Blot Analysis—Northern blot analysis was performed by procedures in Northern blot kit (CLONTECH), according to the manufacturer's instructions, with a 32P-labeled antisense RNA probe encompassing the complete ORF of murine CARMEN cDNA.

Expression Vectors—cDNAs encoding CARMEN proteins or fragments thereof were amplified by standard polymerase chain reaction methods using Pwo polymerase (Boehringer Mannheim) and specific primers containing suitable restriction sites on the 5'- and 3'-end on cDNA templates from the following sources. Full-length human CARMEN (amino acids 2–233), a deletion construct encompassing the N-terminal CARD (amino acids 2–89, h-CARMEN-NT) or lacking the CARD (amino acids 85–233, h-CARMEN-CT), was amplified on EST clone 703916; full-length murine CARMEN (amino acids 2–233) was amplified on EST clone 574273; and full-length v-CARMEN was amplified on EHV-2 DNA. An internal BamHI site at nucleotide 412 of v-CARMEN was used for the construction of a C-terminally truncated v-CARMEN encompassing the N-terminal CARD (amino acids 1–139, v-CARMEN-NT). A deletion construct lacking the CARD (amino acids 109–311, v-CARMEN-CT) was amplified on cloned full-length v-CARMEN. Amplified products were cloned into pCRblunt (Invitrogen) and sequenced. Subcloning into expression vectors derived from pCR-3 (Invitrogen) yielded expression constructs with an N-terminal FLAG, hemagglutinin (HA), or vesicular stomatitis virus (VSV) tag. For the yeast two-hybrid interaction assay, sequenced constructs of v-CARMEN, h-CARMEN, and their above described N-terminally truncated versions were subcloned into pGBluc-9 or pGAD10 (CLONTECH), yielding GALA-DNA-binding proteins or GALA-activation-domain fusion proteins, respectively.

The following expression plasmids were obtained from the indicated sources: NF-κB-Blue (V. Jongeneel, Lausanne, Switzerland); FLAG-tagged JNK (C. Widmann, Denver, CO); HA-tagged p38 (J. S. Gutkind, NIRI, Bethesda, MD); ERK-1 (J. Pouysségur, Nice, France); dominant negative (DN) GTP TRAF2 (amino acids 266–501) (H. Wajant, Stuttgart, Germany); DN TRAF6, wild type, and DN IKK2 (S. Whiteside and A. Israel, Paris, France); v-19 ras (E. Reichmann, Lausanne, Switzerland).

Transfection, Immunoprecipitation, Mitogen-activated Protein Kinase Assays—These techniques were performed essentially as described before (11, 17, 18). Activation of p38 and ERK-1 was determined using phosphospecific antibodies that detect the active, phosphorylated form of the respective mitogen-activated protein kinases (New England Biolabs).

Two-hybrid Interaction Assay—Protein-protein interactions were analyzed by cotransferring a plasmid encoding a v-CARMEN-GALA-DNA-binding (GALA-DB) fusion protein with plasmids encoding various v-CARMEN-GALA-activation-domain fusion protein constructs (2.5 μg of each plasmid) into the Saccharomyces cerevisiae strain Y190 and subsequent filter lift assays for colony development according to the manufacturer's instructions (CLONTECH, yeast protocols handbook). Controls were performed by using the respective vectors without insert.

**Results and Discussion**

To study the possible physiological role of the viral E10 gene product, designated v-CARMEN (for CARD-containing molecule enhancing NF-κB, see below), we assessed its effect on transformations that have been described for other CARD-containing proteins such as modulation of apoptosis and activation of NF-κB. Expression constructs encoding v-CARMEN and a number of proapoptotic proteins were transfected into 293T cells, and the presence of apoptotic cells was subsequently analyzed. Although overexpression of v-CARMEN was very weakly cytotoxic, expression of Fas, DR-3/TRAMP, TRAIL-R1, or TRAIL-R2 lead to extensive cell death, which was neither inhibitory nor enhanced by the coexpression of v-CARMEN (data not shown). Stable transfection of Jurkat cells with v-CARMEN or a construct lacking the CARD motif did not alter the cells' susceptibility to Fas or staurosporine-induced apoptosis (data not shown).

Next we tested whether v-CARMEN was able to stimulate activation of JNK by coexpressing v-CARMEN with JNK in 293T cells. Activation of JNK leads to its phosphorylation by upstream kinases, which can be detected by antibodies specifically recognizing the phosphorylated but not the unphosphorylated form of JNK. Compared with the vector control, v-CARMEN potently induced JNK activation (Fig. 1A). Activation of the kinase appeared to be dependent on the CARD motif, because overexpression of the C-terminal part of v-CARMEN (lacking the CARD) did not result in increased JNK activation, whereas a C-terminally truncated construct encompassing the CARD (v-CARMEN-NT) was able to induce some JNK activation. v-CARMEN-induced JNK activation was not inhibited by dominant negative versions of TRAF2 or apoptosis signal-regulating kinase 1 (ASK 1) (data not shown), indicating that v-CARMEN acts downstream or independently of these mediators of tumor necrosis factor-induced JNK activation (19). v-CARMEN also activated the stress kinase p38 in a CARD-dependent manner (Fig. 1B). In contrast, no activation of the MAP kinase ERK-1 was seen by the overexpression of the viral protein (Fig. 1C).

The ability of v-CARMEN to activate an NF-κB luciferase reporter plasmid was next investigated. Expression of v-CARMEN in 293T cells resulted in a dose-dependent activation of the reporter gene, reaching an approximately 10-fold increase in luciferase activity as compared with the vector control (Fig. 1, D and E). Induction of NF-κB activity by v-CARMEN was almost as strong as that seen with the adaptor protein MyD88 (Fig. 1D), which links interleukin-1R and toll receptors to NF-κB signaling pathways (17, 20–22). Similar to the requirement for JNK activation, the initiation of NF-κB signals by v-CARMEN was dependent on the presence of the CARD motif. Activation of NF-κB is dependent on the formation of a multiprotein complex, which is comprised of TRAFs, the NF-κB-inducing kinase (NIK), IKK1, IKK2, the NF-κB essential modulator (NEMO), IκBα, IκBβ, and the IKK-complex-associated protein (ICAP) (23). Dominant negative versions of some of these proteins can block NF-κB-activating signals triggered by upstream receptors. Indeed, DN IKK2, but not DN TRAF2 or DN TRAF6, inhibited v-CARMEN-mediated NF-κB activation, indicating that the overexpression of the viral protein initiated a signal cascade downstream of TRAFs but upstream of IKK2 in the NF-κB signaling complex (Fig. 1F and data not shown).

CARDs are known to serve as protein-protein interaction motifs (9, 10). It was therefore likely that v-CARMEN exerted its JNK and NF-κB activating effects through association with another CARD-containing protein. In this regard, by screening public data bases with a CARD sequence profile (7, 16) we discovered the presence of a novel CARD protein with high homology to the CARD motif of v-CARMEN, which we call c-CARMEN (Fig. 2A). v-CARMEN corresponds to a 311-amino acid protein with an N-terminal CARD motif followed by a C-terminal, glycine-rich extension of approximately 200 amino acid residues. After the resequencing of this region of the viral genome, we had to correct the original E10 sequence deposited in the data base, which predicted a shorter ORF corresponding to only 210 amino acids (15). The predicted protein size of the corrected 311-amino acid v-CARMEN (34 kDa) agrees well with the 36-kDa protein detectable after expression in 293T cells (see Fig. 1). c-CARMEN (the cellular homologue of v-
CARMEN) consists of an N-terminal CARD followed by a C-terminal extension of approximately 130 amino acids with a predicted molecular size of 26 kDa. The CARD of v-CARMEN shows 47 and 46% sequence identity with mouse and human c-CARMEN, respectively, but only approximately 20–25% sequence identity with other CARD-containing proteins (Fig. 2).

Little sequence identity is found in the C-terminal extensions of the viral and the cellular CARMEN, the latter of which lacks the glycine-rich regions. Neither of the C-terminal extensions has significant homology to protein sequences currently found in public databases.

Northern blot analysis revealed that murine CARMEN (m-CARMEN) was expressed in all mouse tissues as a single transcript of approximately 2.4 kilobases (Fig. 3), suggesting that the protein is of functional relevance in many cell types.

The potential interaction of c-CARMEN with other CARD-containing proteins was examined by coexpression of the different FLAG-tagged CARD proteins with HA-tagged human...
CARMEN (v-CARMEN) in 293T cells followed by co-immunoprecipitation studies. Using this technique, an interaction was noted only between v-CARMEN and h-CARMEN, whereas caspase-1, -2, -4, -9, RAIDD, or CARDIAK did not interact with h-CARMEN (Fig. 4A). We also did not detect an interaction with the CARD motif of Apaf-1, cIAP-1, Apaf-1, ARC, CED-3, and CED-4 (7). For each block of aligned sequences, black boxes indicate >50% amino acid sequence identity and gray shading indicates >50% sequence similarity through conservative amino acid substitutions.

A truncated version of v-CARMEN lacking the CARD motif (v-CARMEN-CT) did not interact with either human or mouse c-CARMEN, indicating that binding was mediated through the CARD motif (Fig. 4B). An analysis of this interaction in the yeast two-hybrid system confirmed a direct and CARD-dependent interaction of v-CARMEN with h-CARMEN (Table I). Interestingly, c-CARMEN of human and mouse origin also formed CARD-dependent homodimers in 293T cells (Fig. 4B). We were unable to detect homodimers of v-CARMEN in 293T cells, although a homodimeric interaction of v-CARMEN was detected in the yeast two-hybrid system (Table I).

Interestingly, the interaction between v-CARMEN and its cellular homologue lead to an altered electrophoretic mobility of the c-CARMENs (Fig. 4B). Human and murine CARMEN migrated slightly slower by SDS-polyacrylamide gel electrophoresis in the presence of v-CARMEN, suggesting that the cellular homologue may undergo a post-translational modification such as phosphorylation upon interaction with the viral homologue. Whether this is because of the JNK/IKK kinase-inducing activity of v-CARMEN (potentially recruiting kinases to the complex of viral/cellular CARMEN) remains to be seen.

Because v-CARMEN was a potent activator of the JNK and NF-κB signaling pathways, we anticipated a similar function for the cellular homologue. However, overexpression of either full-length c-CARMEN or of domains thereof was not capable of activating these signaling pathways in 293T cells (Fig. 5, A and B, and data not shown). c-CARMEN and its N- or C-terminal deletion constructs also did not inhibit NF-κB or JNK signals induced by v-CARMEN or by triggering the receptors for tumor necrosis factor or interleukin-1 (data not shown), indicating that the cellular homologue is not essential to these pathways.

**FIG. 2.** Panel A, structure and predicted amino acid sequence of viral, human, and mouse CARMEN. CARMENs contain a highly homologous CARD motif followed by an unrelated region of approximately 130–200 amino acids. Panel B, the CARD motif of CARMENs is aligned to the CARDs of RAIDD, caspase-1, -2, -4, -5, -9, -11, and -12, c-IAP1, c-IAP2, Apaf-1, ARC, CED-3, and CED-4 (7). For each block of aligned sequences, black boxes indicate >50% amino acid sequence identity and gray shading indicates >50% sequence similarity through conservative amino acid substitutions.

**FIG. 3.** Tissue distribution of murine CARMEN transcripts. A Northern blot of various mouse tissues (CLONTECH) was probed with a 32P-labeled antisense RNA fragment covering the complete murine CARMEN-coding region, and the blots were subsequently hybridized with a β-actin probe.

CARMEN (h-CARMEN) in 293T cells followed by co-immunoprecipitation studies. Using this technique, an interaction was noted only between v-CARMEN and h-CARMEN, whereas caspase-1, -2, -4, and -9, RAIDD, or CARDIAK did not interact with h-CARMEN (Fig. 4A). We also did not detect an interaction with the CARD motif of Apaf-1, cIAP-1, or cIAP-2 (data not shown). A truncated version of v-CARMEN lacking the CARD motif (v-CARMEN-CT) did not interact with either human or mouse c-CARMEN, indicating that binding was mediated through the CARD motif (Fig. 4B). An analysis of this interaction in the yeast two-hybrid system confirmed a direct and CARD-dependent interaction of v-CARMEN with h-CARMEN (Table I). Interestingly, c-CARMEN of human and mouse origin also formed CARD-dependent homodimers in 293T cells (Fig. 4B). We were unable to detect homodimers of v-CARMEN in 293T cells, although a homodimeric interaction of v-CARMEN was detected in the yeast two-hybrid system (Table I).

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We considered that the difference in signaling by v-CARMEN versus c-CARMEN might be because of their ability to interact with specific TRAF molecules, because members of the TRAF family of adaptor proteins have been shown to be implicated in the activation of transcription through JNK- and NF-κB-dependent pathways (reviewed in Ref. 24). We therefore tested the ability of c-CARMEN and v-CARMEN, and CARD-deletion mutants thereof, to bind to specific members of the TRAF family by coexpression of the respective proteins in 293T cells. The result of the co-precipitation study shown in Fig. 5 revealed a clear difference in the TRAF binding specificity of c-CARMEN and v-CARMEN; human CARMEN bound specifically to TRAF1 and TRAF5, whereas the v-CARMEN had a clear binding preference for TRAF6 and, to a smaller degree, for TRAF3 (Fig. 5, C and D).

EHV-2 is a slow growing, cytopathogenic γ-herpesvirus, which appears to be ubiquitous in the equine population (15). However, its precise role as a pathogen remains uncertain. In infected horses, the virus can induce chronic pharyngitis associated with lymphoid proliferation (25). The virus is persistent in peripheral blood lymphocytes, suggesting that it copes well with apoptotic processes occurring in the host. We have previously identified its DED-containing E8 gene product (v-FLIP) as a potent inhibitor of the death receptor-signaling pathways (3). Moreover, EHV-2 also has a predicted bcl-2 gene homo-
logue in its genome (2). Although its biological activity has not yet been verified, it is predicted that the gene product of this viral bcl-2 homologue inhibits apoptotic signals originating from the Apaf-1/caspase-9 pathway. In this report we have characterized the EHV-2 protein E10, designated v-CARMEN, as a potent inducer of the NF-κB and JNK pathways. Cellular gene products induced by the NF-κB transcription factor are known to inhibit apoptosis by a variety of means (26, 27). Thus, in addition to the E8-FLIP and the bcl-2 homologue, the E10/CARMEN gene product of EHV-2 may provide the virus with a third anti-apoptotic mechanism. In addition, NF-κB induction by v-CARMEN may serve to control the expression of viral genes with an NF-κB promoter in the course of viral replication (28).

We have identified a cellular homologue and binding partner of v-CARMEN as c-CARMEN, which is highly homologous to the viral protein in its N-terminal CARD motif. v-CARMEN and c-CARMEN interact directly in a CARD-dependent manner yet reveal different binding specificities toward members of the TRAF family. When overexpressed in 293T cells, v-CARMEN is a potent inducer of the JNK, p38, and NF-κB transcriptional pathways, whereas c-CARMEN or truncated versions thereof do not appear to activate these pathways by themselves nor do they affect the JNK and NF-κB activating potential of v-CARMEN, c-CARMEN may therefore act upstream of v-CARMEN in an as yet unidentified NF-κB/JNK-activating pathway. Alternatively, c-CARMEN may interfere with NF-κB/JNK activation only upon a post-translational regulatory event, such as phosphorylation, or upon interaction with a third component lacking in our cellular system. Finally, we cannot exclude the possibility that c-CARMEN mediates yet another NF-κB/JNK-unrelated function of v-CARMEN and that the observed CARD-dependent NF-κB/JNK activation through v-CARMEN is mediated by an as yet unidentified CARD-containing protein. Interestingly, mutation of a novel gene (Bcl10) identical to c-CARMEN was recently reported to be associated with B-cell lymphomas of mucosa-associated lymphoid tissue and other tumor types (29). Further study of the molecular mechanism of v-CARMEN function may help in the understanding of the physiological role of its cellular homologue and its potential implication in tumor development.

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