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CrmE, a Novel Soluble Tumor Necrosis Factor Receptor Encoded by Poxviruses

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Cytokines and chemokines play a critical role in both the innate and acquired immune responses and constitute prime targets for pathogen sabotage. Molecular mimicry of cytokines and cytokine receptors is a mechanism encoded by large DNA viruses to modulate the host immune response. Three tumor necrosis factor receptors (TNFRs) have been identified in the poxvirus cowpox virus. Here we report the identification and characterization of a fourth distinct soluble TNFR, named cytokine response modifier E (CrmE), encoded by cowpox virus. The *crmE* gene has been sequenced in strains of the orthopoxviruses cowpox virus, ectromelia virus, and camelpox virus, and was found to be active in cowpox virus. *crmE* is expressed as a secreted 18-kDa protein with TNF binding activity. CrmE was produced in the baculovirus and vaccinia virus expression systems and was shown to bind human, mouse, and rat TNF, but not human lymphotoxin α, conjugates of lymphotoxins α and β, or seven other ligands of the TNF superfamily. However, CrmE protects cells only from the cytolytic activity of human TNF. CrmE is a new member of the TNFR superfamily which is expressed as a soluble molecule that blocks the binding of TNF to high-affinity TNFRs on the cell surface. The remarkable finding of a fourth poxvirus-encoded TNFR suggests that modulation of TNF activity is complex and represents a novel viral immune evasion mechanism.

The complex interaction between pathogens and hosts has been determinant for the development of the vertebrate immune system, leading to immunocompetent hosts. Pathogens such as viruses have also developed specific strategies to counteract the host immune response. Molecular mimicry of cytokines and cytokine receptors is a common immune evasion strategy adopted by large DNA viruses (poxviruses and herpesviruses) (3, 28, 36, 39, 40). Characterization of these immunomodulatory proteins is not only revealing new aspects of viral pathogenesis, but is also providing valuable tools to study the immune system and to identify new strategies of immune modulation.

Poxviruses are large, complex DNA viruses that express several secreted proteins that modulate the host immune response and virus virulence (3, 28, 36, 39, 40). These include soluble cytokine receptors or binding proteins for tumor necrosis factor (TNF), interleukin-1β (IL-1β), alpha/beta interferon (IFN-α/β) and IFN-γ, CC chemokines, IL-18, and granulocyte and monocyte colony-stimulating factor and IL-2 (3, 28, 36, 38, 39, 44). Poxviruses also secrete homologs of humoral immune regulators, such as the viral IL-10 and vascular endothelial growth factor, encoded by orf virus, and the viral CC chemokine, encoded by *Molluscum contagiosum* virus (7, 21, 39). Some of these viral proteins seem to have been acquired from the host and modified during virus evolution to confer an advantage for virus replication, survival, or transmission.

TNF and lymphotoxin α (LTα, or TNF-β) have numerous physiological activities, being important cytokines in orchestrating the early defense against infection (12). Both TNF and LTα bind to the p55 and p75 cellular TNF receptors (TNFRs), inducing receptor oligomerization which triggers intracellular signaling. These molecules belong to the complex TNF ligand and receptor families that are structurally defined and function in the regulation of the immune and inflammatory responses and programmed cell death (42).

Viral TNFRs (vTNFRs) were identified in members of the poxvirus family by sequence similarity to the extracellular domain of cellular TNFRs, but lacked both the membrane anchor and cytoplasmic domains and were predicted to be secreted (19, 34, 41). The first vTNFR characterized was the T2 protein from the leporipoxviruses Shope fibroma virus and myxoma virus (34, 41). T2 is a secreted protein with TNF binding activity (34) and is important for myxoma virus virulence (41). The biochemical properties of T2 have been well characterized (7, 28). Determination of the complete genomic sequence has shown that T2 is the only vTNFR encoded by myxoma and Shope fibroma viruses (13, 43).

Interestingly, the orthopoxvirus cowpox virus (CPV) encodes three vTNFRs: cytokine response modifier B (CrmB) (20), CrmC (35), and CrmD (22). The TNF binding domains of CrmB, CrmC, and CrmD have sequence similarity, but they show different ligand specificities. CrmB and CrmD (48 and 46 kDa, respectively) are secreted proteins that bind both TNF and LTα, while CrmC (25 kDa) is specific to TNF. *crmB* is expressed at early times postinfection (p.i.), whereas *crmC* and *crmD* are expressed at late times p.i., after viral DNA replication. These genes were described for CPV strain Brighton Red (CPV-BR), but it is known that at least *crmD* is truncated in other CPV strains (22).

vTNFRs are also found in other orthopoxviruses. The study of vTNFRs in 15 vaccinia virus (VV) strains showed that only strains Lister, USSR, and Evans encode secreted TNFR activity (2). In the Lister strain, CrmC (designated A53R) is an active protein whereas CrmB is truncated. Interestingly, the VV strains encoding vTNFRs also express membrane-bound

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TNFR activity, which is not found in CPV-BR, CPV strain elephantpox (EP), or camelpox virus (2). In variola (smallpox) virus, *crmB* (termed G2R ORF) is the only vTNFR gene predicted to be active. The *crmC* and *crmD* genes are deleted from all variola virus strains sequenced to date (1, 24, 33). CrmD was the only vTNFR identified in ectromelia virus (EV) strain Moscow (22).

Here we describe a new vTNFR, named CrmE, encoded by CPV. The CrmE gene was found in various orthopoxviruses but only was functional in CPV-EP. We show that CrmE is a secreted protein that binds human, mouse, and rat TNF in vitro but that is effective in protecting cells only against human TNF. The properties of CrmE have been compared to those of known vTNFRs. We have also expressed CrmD from EV in eukaryotic cells.

MATERIALS AND METHODS

Reagents. Recombinant human ^{125}I-TNFα (45.4 μCi/μg) was supplied by NEN Life Science Products. Recombinant human (2×10^7 U/mg), murine (10^7 U/mg), and rat (5×10^6 U/mg) TNF, human LTα (2×10^7 U/mg), human cluster defined 40 ligand (CD40L; 50% effective dose [ED_{50}], 10 ng/ml), TNF-related apoptosis-inducing ligand (TRAIL; ED_{50}, 10 ng/ml), receptor activator of NF-κB (RANK; ED_{50}, 10 ng/ml), B-cell activating factor (BAFF; ED_{50}, 10 ng/ml), 4-1BB ligand (4-1BBL; ED_{50}, 10 ng/ml), TNF weak apoptosis inducer (TWEAK; ED_{50}, 10 ng/ml), and human macrophage inflammatory protein-1α (MIP-1α; ED_{50}, 50 ng/ml) were provided by PeproTech. Recombinant human, murine, and rat TNF and human LTα (ED_{50}, 0.02 to 0.05 ng/ml) for binding assays and human glucocorticoid-induced TNFR superfamily-related protein (GITR; ED_{50}, 2 μg/ml), LTα1/β2 (ED_{50}, 40 ng/ml), and LTα2/β1 (ED_{50}, 3 ng/ml) were provided by R&D Systems.

Cells, viruses, and viral DNA preparations. The growth of BSC-I, TK$^-$143B, U937, and L929 cells and the sources of VV and CPV strains, provided by Geoffrey L. Smith (Sir William Dunn School of Pathology, Oxford University), have been described elsewhere (5, 8). EV strain Hampstead (original stock from Keith Dumbell) was obtained from John Williamson (St. Mary's Hospital, Imperial College School of Medicine, London, United Kingdom), and the plaque-purified Moscow 3-P2 (Moscow) was obtained from Mark L. Buller (School of Medicine, St Louis University) (15). VV, CPV, and EV were propagated in BSC-I cells, and viral genomic DNA was prepared as described previously (16). The growth of *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPVs expressing VV Western Reserve (WR) protein B15R (AcB15R), VV Lister A53R (AcA53R), CPV-BR CrmC (AcCrmC), and CPV-BR CrmB (AcCrmB) in *Spodoptera frugiperda* (Sf) 21 insect cells has been described previously (2, 5). The recombinant VVs expressing VV Lister protein A53R (vA53R), CPV-BR CrmC (vCrmC), and CPV-BR CrmB (vCrmB) have been described previously (2).

DNA sequencing. Oligonucleotides were designed based on the sequence of CPV strain GRI90 ORF K3R (32) and were used to amplify by PCR, with *Taq* DNA polymerase, the cognate genes from viral DNA preparations from other orthopoxviruses. Oligonucleotides K3R2 (5′ CGGACGCGATATATTCCGAC ATGG 3′) and K3R5 (5′ GTATATTATATTTCATTATTAGGAGG 3′) were used for CPV-EP and camelpox virus, whereas K3R2 and K3R6 (5′ GATGAT TAAAAGTTAGGGAGGGGATG 3′) were used for EV strains. For CPV-BR, only a fragment of this gene was amplified by PCR with oligonucleotides K3R2 and K3R4 (5′ GGAGACAATAACTATTCGAGTCAC 3′). PCR products were then sequenced by the DNA Sequencing Service of the Department of Biochemistry (Cambridge University). The sequence data were analyzed using Genetics Computer Group (GCG) computer programs.

Construction of recombinant baculoviruses. CPV-EP *crmE* and EV Hampstead *crmD* genes were amplified by PCR using *Pfu* DNA polymerase, virus DNA as the template, and oligonucleotides corresponding to the 5′ and 3′ ends of the open reading frames (ORFs) which provided *Bam*HI and *Not*I/*Xho*I sites, respectively. CPV-EP *crmE* was amplified by PCR with oligonucleotides K3R8 (5′ CGCGGATCCGCTAGCATGACGAAAGTTATCATCATCTTAG 3′) and K3R9 (5′ CGCGCGGCCGCTCTTGTCATTGGTTTACATTGATC 3′), and EV-Hampstead *crmD* was PCR amplified with oligonucleotides CrmD7 (5′ CGC GTTTAAACGGATCCATGATGAAGATGACACCATCATA 3′) and CrmD9 (5′ CGCCTCGAGATCTCTTTCACAATCATTTGGTGG 3′). The resultant fragments were cloned into *Bam*HI and *Not*I/*Xho*I-digested pBac1 (Gibco), creating plasmids pMS3 (CPV-EP *crmE*) and pMS1 (EV Hampstead *crmD*). The DNA sequences of the inserts were confirmed not to contain mutations. Recombinant baculoviruses were produced as described previously (6) and were termed AcCrmE (CPV-EP CrmE) and AcCrmD (EV Hampstead CrmD).

Construction of recombinant VVs. The CPV-EP *crmE* gene was amplified by PCR with virus DNA as the template, *Pfu* DNA polymerase, and oligonucleotides K3R8 and K3R10 (5′ CGCGCGGCCGCTCTTGTCATTGGTTTACATT GATC 3′) containing *Bam*HI/*Kpn*I restriction sites. The DNA fragment was cloned into *Bam*HI/*Kpn*I-digested pMJ601 (14), provided by B. Moss (National Institutes of Health, Bethesda, Md.), creating plasmid pMS10 (CPV-EP *crmE*). The DNA sequence of the insert was confirmed not to contain mutations. The recombinant VV was produced as described previously (2) and termed vCrmE (CPV-EP CrmE).

Metabolic labeling of proteins and electrophoretic analysis. BSC-I or Sf cells were infected with orthopoxviruses or baculoviruses, respectively, at 10 PFU per cell. Cultures were pulse-labeled with 150 μCi of [^{35}S]methionine (1,200 Ci/mmol; Amersham)/ml and with 150 μCi of [^{35}S]cysteine (600 Ci/mmol; NEN)/ml in methionine- and cysteine-free medium in the absence of serum. Cells or media were dissociated in sample buffer, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels, and visualized by fluorography with Amplify (Amersham). Possible glycosylation of CrmE was determined by metabolic labeling of proteins in the presence of tunicamycin (10 μg/ml) and monensin (1 μM).

TNF binding assays. BSC-I or Sf cells were infected with orthopoxviruses or baculoviruses at 10 PFU per cell in serum-free medium, and supernatants were harvested at 2 or 4 days p.i., respectively, and prepared and inactivated as described previously (5, 8). The binding medium was RPMI with 0.1% bovine serum albumin (BSA) and 20 mM HEPES (pH 7.5). Soluble binding assays with human ^{125}I-TNF were performed at room temperature by precipitation of the ligand-receptor complexes with polyethylene glycol (PEG) and filtration (2). Nonspecific binding precipitated with binding medium alone or in the presence of excess unlabeled TNF was subtracted. In the competition assays with U937 cells and the assays of binding to infected BSC-I cells, human ^{125}I-TNF was added and bound ^{125}I-TNF was determined by phthalate oil centrifugation (2).

Inhibition of TNF or LTα cytotoxicity in L929 cells. Cytolytic assays were performed with mouse L929 cells as targets, TNF or human LTα as the cytotoxic agent, and recombinant CrmE as the inhibitor. TNF or human LTα was added to cells in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in the presence of actinomycin D (1 μg/ml), which potentiates the TNF cytolytic activity. Cell death was assessed 12 h after addition of TNF or human LTα by staining with crystal violet indicator as described previously (35). Percent cytotoxicity was calculated as $(OD_{medium} - OD_{TNF})/OD_{medium}$, where OD is optical density.

Nucleotide sequence accession numbers. The EMBL accession numbers of the sequences reported here are AJ272008 (CPV-EP *crmE*), AJ272007 (CPV-BR *crmE*), AJ272005 (EV Hampstead *crmE*), AJ272006 (EV Moscow *crmE*), and AJ272009 (camelpox virus *crmE*).

RESULTS

Identification and DNA sequence of a novel vTNFR gene present in several orthopoxviruses. The DNA sequence of the right-hand end of the CPV-GRI90 genome showed the presence of an ORF, designated K3R, predicted to encode a polypeptide with sequence similarity to cellular TNFRs, but distinct from known CPV TNFRs (32). Cognate genes in other orthopoxviruses, including other CPV strains, camelpox virus, and EV, were amplified by PCR and sequenced. CPV, so named because it was isolated from lesions on infected cattle, causes sporadic infections in cows, humans, cats, and a wide range of zoo animals, but its natural reservoir may be wild rodents. CPV-BR has been extensively used in research, and CPV-EP was isolated from an elephant in a zoo (4, 18). Camelpox virus has a narrow host range and causes a natural infection in camels (18). EV is a natural mouse pathogen that causes a severe disease with a high mortality rate known as mousepox, and has been isolated from outbreaks in laboratory mouse colonies. The most studied EV isolates are EV Hampstead, the first EV to be isolated (in 1930), and EV Moscow,

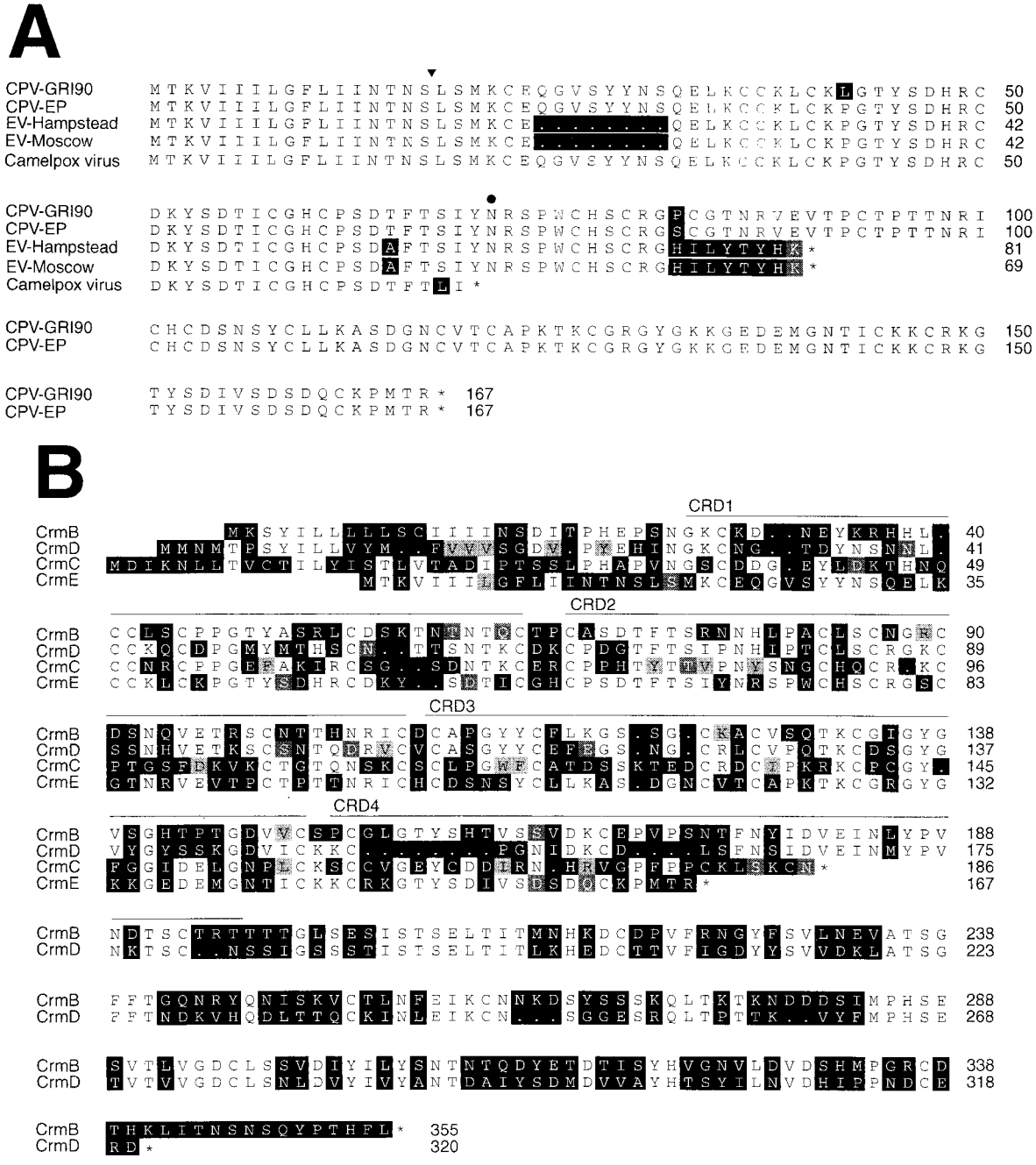

FIG. 1. Sequences of vTNFRs. Shown are pairwise alignments of the predicted amino acid sequences of CrmE in different orthopoxviruses (A) and of vTNFRs identified in CPV-BR (CrmB, -C, and -D) and CPV-EP (CrmE) (B). Solid backgrounds represent differences, and shaded backgrounds represent regions of high similarity. Dots and stars indicate deletions and stop codons, respectively. Solid circles and triangles show predicted N-glycosylation and signal peptide cleavage sites, respectively. The positions of CRDs are indicated. The accession numbers of the sequences are as follows: Y15035 (CPV-GRI90 *crmE*), AJ272008 (CPV-EP *crmE*), AJ272005 (EV Hampstead *crmE*), AJ272006 (EV Moscow *crmE*), AJ272009 (camelpox virus *crmE*), Q85308 (CPV-BR *crmB*), U87234 (CPV-BR *crmD*), and U55052 (CPV-BR *crmC*).

isolated in 1947 (17, 18). The viral species of all orthopoxviruses used in this study were confirmed by a diagnostic test based on the PCR amplification of the A-type inclusion body gene followed by restriction enzyme analysis (25; N. A. Bryant and A. Alcami, unpublished data).

The novel vTNFR gene, named *crmE*, was predicted to be active in CPV-EP but inactive, because of mutations that introduce stop codons or frameshifts, in camelpox virus and EV strains Hampstead and Moscow (Fig. 1A) and in CPV-BR. EV CrmE is a natural truncated protein with 81 amino acids (80% identical to the CPV-EP protein), showing a 17-amino-acid N-terminal signal peptide, one N-glycosylation site, and a molecular mass of approximately 9 kDa, which was predicted not to constitute an active TNFR. A truncated version of the myx-

oma T2 protein containing only the first two cysteine-rich domains (CRDs) is mainly retained inside the cell and lacks TNF binding activity (30). For CPV-BR, only the 5′ region of the *crmE* gene could be amplified by using various combinations of specific oligonucleotides, suggesting a different sequence at the 3′ region of the gene. The sequence of this 312-bp fragment (with 98.0 and 96.8% nucleotide sequence identity to CPV-EP and EV Hampstead, respectively) predicted a truncated CrmE polypeptide of 8 amino acids due to the presence of 5 stop codons within the first 80 amino acids of the ORF.

The analysis of the flanking regions of the *crmE* ORF did not enable prediction of the temporal class of *crmE*. In fact, *crmE* lacks both the early termination and the late promoter consensus sequences (T_5NT and TAAAT, respectively) flanking the ORF. The sequence of CPV-EP *crmE* was predicted to encode a 18-kDa polypeptide with a pI of 8.11 and with one N-glycosylation site. The presence of a 17-amino-acid N-terminal signal peptide and the absence of other hydrophobic regions suggested that the protein is secreted (Fig. 1A). The predicted amino acid sequence of CrmE was related to those of cellular TNFRs (with 27.5 and 31.1% amino acid identity to the TNFRs p55 and p75, respectively) and CPV-encoded vTNFRs (with 42.5, 32.9, and 37.1% amino acid identity to CrmB, CrmC, and CrmD, respectively). The alignment of CrmE with members of the vTNFR family showed that the ligand binding region, particularly the location of the CRDs, was well conserved, suggesting that this molecule may function as a vTNFR (Fig. 1B). The predicted CrmE and CrmC polypeptides are shorter than CrmB and CrmD, which contain a C-terminal region with no sequence similarity to the TNFR family members.

Characterization of the CrmE protein. The expression of CrmE from CPV-EP under the control of strong promoters in both baculovirus and VV expression systems showed the presence of an 18-kDa protein, as predicted from the amino acid sequence, that was efficiently secreted from infected cells (Fig. 2). Wild-type VV-WR and AcNPV were included as negative controls, and a recombinant baculovirus expressing the VV-WR IL-1βR (AcB15R) was used as a control secreted protein. The comparison of the molecular mass of CrmE with those of the previously expressed CPV-BR CrmB, CPV-BR CrmC, and VV Lister A53R (2), and with that of EV Hampstead CrmD, expressed in eukaryotic cells for the first time, confirmed that CrmE is a distinct member of the vTNFR family. The EV Hampstead CrmD showed a molecular mass of 46 kDa, similar to that of the CPV-encoded CrmD (22). Treatment of vCrmE-infected cells with tunicamycin had no effect on CrmE expression or size, while treatment with monensin impaired protein secretion (data not shown). This result suggested that CrmE is not highly N glycosylated, but that O glycosylation is important for its correct folding and secretion.

TNF binding activity, specificity, and affinity of CrmE. Secreted TNF binding activity was determined in a soluble binding assay with human ^{125}I-TNF. The recombinant baculovirus and VV expressing CrmE produced high levels of secreted vTNFR activity, compared to those of the other vTNFRs already described and of EV CrmD, expressed in the baculovirus system for the first time (Fig. 3). AcNPV or VV-WR expressed no vTNFR activity, whereas CPV-EP and EV Hampstead infections produced soluble TNF binding activity (2, 37). Secreted TNF binding activity was not detected in insect cells infected with a recombinant baculovirus expressing the truncated EV *crmE* ORF (data not shown).

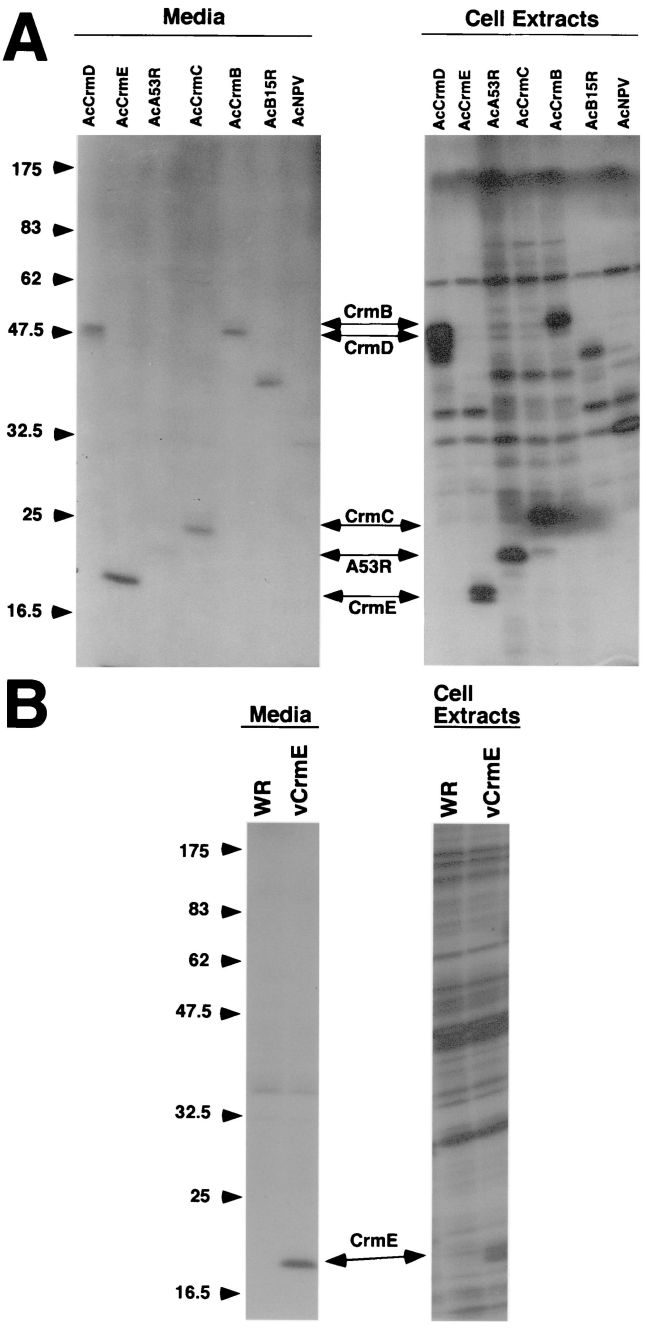

FIG. 2. Expression of vTNFRs in the baculovirus and VV expression systems. (A) Sf cells infected with AcNPV or the indicated recombinant viruses were pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine from 26 to 29 h p.i. (B) BSC-I cells were infected with VV-WR or vCrmE and pulse-labeled from 4 to 8 h p.i. In both panels A and B, proteins present in cells and media were analyzed by SDS-PAGE and visualized by fluorography. The positions of the expressed proteins in supernatants and cell extracts are indicated. Molecular masses (in kilodaltons) are shown.

vTNFR activity at the surfaces of cells infected by VV strain Lister, but not by CPV or camelpox virus, has been described

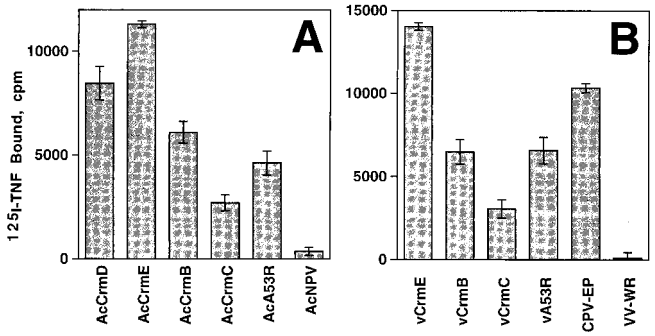

FIG. 3. TNF binding activity of vTNFRs expressed in the baculovirus (A) or VV (B) system. Supernatants from Sf (100 μl, corresponding to 2×10^5 cells) or BSC-I (50 μl, corresponding to 2.5×10^5 cells) cell cultures infected with the indicated recombinant baculovirus or VV, respectively, were incubated with 200 pM human ^{125}I-TNF. Levels of bound ^{125}I-TNF were determined by precipitation with PEG and filtration. The background radioactivity precipitated with PEG in the presence of binding medium has been subtracted. The specific ^{125}I-TNF binding of duplicate samples (means ± standard deviations) is shown.

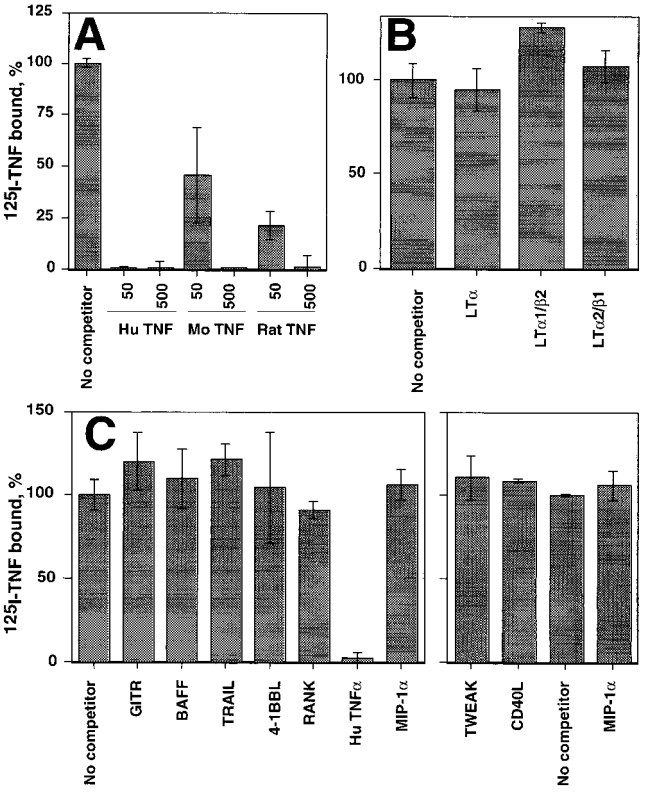

FIG. 4. TNF binding specificity of CrmE. Supernatants (2 μl, corresponding to 3.8×10^3 cells) from BSC-I cells infected with recombinant VV expressing CrmE were incubated with 150 pM human ^{125}I-TNF in the absence (no competitor) or in the presence of the indicated fold excess of unlabeled human (Hu), mouse (Mo), or rat TNF (A), a 500-fold excess of cold human LTα or the conjugate LTα1/β2 or LTα2/β1 (B), or a 500-fold excess of human GITR, CD40L, BAFF, TWEAK, TRAIL, 4-1BBL, RANK, or MIP-1α (C). Levels of bound ^{125}I-TNF were determined by precipitation with PEG and filtration. The percent specific ^{125}I-TNF binding of duplicate samples (means ± standard deviations) is calculated relative to binding in the absence of competitor, which was 4,828 cpm (A and B) or 3,606 cpm (C).

previously (2). Assays of ^{125}I-TNF binding to cells infected with vCrmE showed that CrmE does not confer TNFR activity at the cell surface (data not shown).

To determine the binding specificity of CrmE for human TNF and LT, for TNF from other species (mouse and rat), and for other ligands of the TNF superfamily, binding of human ^{125}I-TNF to recombinant CPV-EP CrmE expressed in the VV system was performed in the presence of excess unlabeled TNF, LT, or other TNF-related ligands. Figure 4A shows that CPV-EP CrmE expressed in the VV system bound human, mouse, and rat TNF, binding the human ligand more efficiently. In contrast, CrmE bound human LTα or conjugates of LTα with LTβ (LTα1/β2 or LTα2/β1) very poorly, as shown in Fig. 4B. Secreted LTα is known to complex with membrane-associated LTβ, generating two types of heterodimers, LTα1/β2 or LTα2/β1. The recombinant conjugates used (provided by R&D Systems) were produced by expressing the mature LTα and the extracellular domain of LTβ in Sf cells and purifying the noncovalently linked heterotrimers from the supernatant. Finally, CrmE was also shown not to bind several other members of the TNF ligand superfamily: GITR, CD40L, BAFF, TWEAK, TRAIL, 4-1BBL, and RANK (Fig. 4C). The same result was observed for CrmE expressed from a recombinant baculovirus and, for the competition with TNFs and LTα, with supernatants from CPV-EP-infected cultures (data not shown).

Biological activity of CrmE. The TNF inhibitory mechanism of CrmE was investigated. The specific binding of human ^{125}I-TNF to U937 cellular receptors was inhibited in the presence of recombinant CrmE, expressed in the baculovirus system, but not by supernatants containing the recombinant VV soluble IL-1βR (AcB15R) (Fig. 5). This indicated that CrmE blocks the interaction of TNF with the high-affinity cellular TNFRs.

In order to address the biological significance of CrmE, L929 cells were exposed to different doses of human, mouse, and rat TNF, and human LTα, preincubated or not with supernatants containing recombinant CrmE produced in the VV system (Fig. 6). Interestingly, we found that CrmE is biologically efficient only against human TNF, despite its ability to bind TNFs from different species in soluble binding assays. This experiment was repeated using increasing doses of supernatant with similar results, except with rat TNF, for which high doses of CrmE had a protective effect in L929 cells (data not shown). These results suggested that CrmE has low affinity for mouse and rat TNF and for human LTα.

DISCUSSION

We have identified a novel vTNFR, named CrmE, in orthopoxviruses, and we provide the sequence of the *crmE* gene in five orthopoxviruses, including two strains each of CPV and EV, as well as camelpox virus. From the sequence analysis, the new vTNFR was predicted to be active only in CPV-EP and CPV-GRI90. CrmE encoded by EV strains Hampstead and Moscow is a natural truncated version of the protein containing the first two CRDs and was predicted to lack activity. In preliminary studies we have failed to detect TNF binding ac-

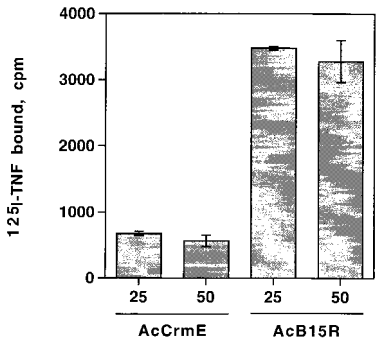

FIG. 5. Competition of TNF binding to U937 cells. Different amounts of medium (25 or 50 μl, corresponding to 5×10^4 or 1×10^5 cells, respectively) from cultures of Sf cells infected with the indicated recombinant baculoviruses were incubated with 200 pM human ^{125}I-TNF for 1 h at 4°C. U937 cells were added and incubated for 2 h at 4°C, and the amount of radioactivity bound to cells was determined by phthalate oil centrifugation. The specific ^{125}I-TNF binding of duplicate samples (mean ± standard deviation) is shown.

tivity in insect cells infected with a recombinant baculovirus in which the EV Hampstead *crmE* ORF was transcribed under the control of the strong polyhedrin promoter, under conditions in which we can detect TNF binding activity for all other vTNFRs (unpublished data). The crystal structure of the extracellular domain of the cellular TNFR p55 complexed with LTα showed that the first three CRDs interact with the ligand (9). Studies on the myxoma virus T2 protein confirmed the essential role of these CRDs in TNF binding (30).

CrmE is an 18-kDa secreted protein that binds human, mouse, and rat TNF but does not bind human LTα, alone or conjugated with LTβ, or seven other ligands of the TNF superfamily; but it inhibits the biological activity of human TNF only. There is a precedent in another vTNFR, the myxoma virus T2 protein, that binds TNFs from several species in vitro but shows high species specificity for rabbit TNF in biological assays (31, 34). The ability of CrmE to completely block human TNF-mediated cellular lysis, even when it is present in low doses, indicates high affinity for human TNF and suggests that the in vivo function of CrmE is the blockade of TNF activity in the infected host. We demonstrate that the mechanism of action of CrmE is the inhibition of binding of TNF to high-affinity cellular receptors.

The finding that *crmE* did not produce membrane-bound TNFR activity after expression from VV was consistent with the lack of membrane-bound activity encoded by CPV-EP (2). The expression of membrane-bound TNF binding activity by VV Lister suggests the existence of either a fifth orthopoxvirus vTNFR or a mechanism to anchor soluble TNFRs at the cell surface, which would be present in VV Lister but not in CPV or VV-WR. In VV Lister, *crmC* seems to be the only known vTNFR identified (2).

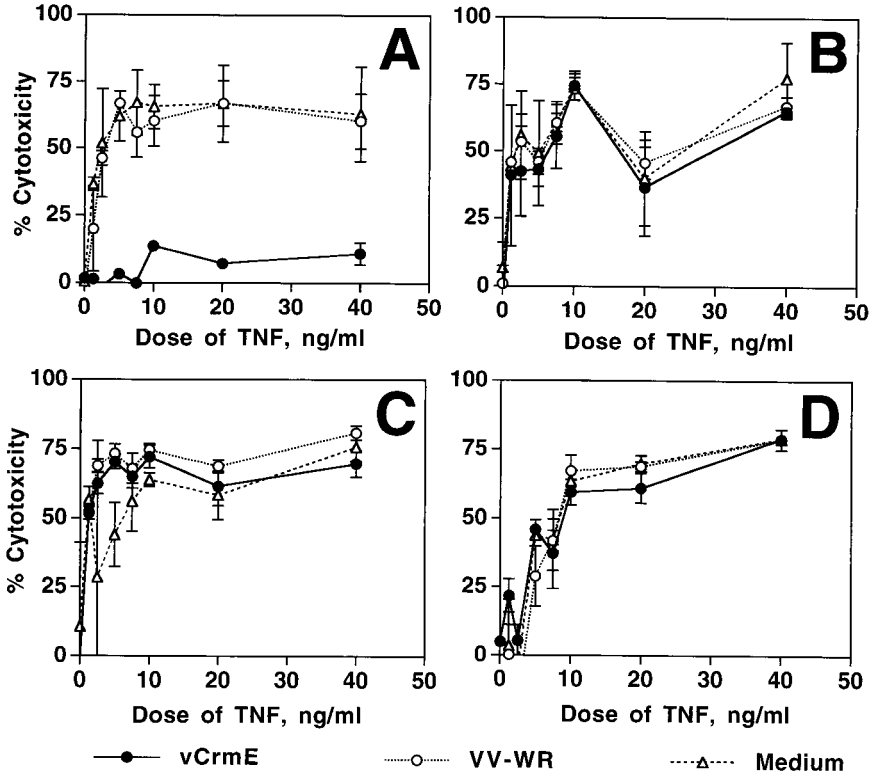

FIG. 6. Biological activity of CrmE. Shown is the effect of CrmE on the cytolysis of mouse L929 cells induced by human (A), mouse (B), or rat (C) TNF or by human LTα (D). Crystal violet staining was used to determine, in triplicate samples, the cell viability after 12 h of treatment with TNF or LTα alone or in the presence of binding medium (Medium) or supernatants from BSC-I cells infected with VV-WR or recombinant VV expressing CrmE (vCrmE). The amount of supernatant was 10 μl, corresponding to 2.5×10^3 cells. Percent cytotoxicity was calculated as described in Materials and Methods.

The expression of different vTNFRs in the same virus is somehow enigmatic, since viruses have limited coding capacity in their genomes. The four vTNFRs encoded by CPV are clearly different: they show distinct molecular sizes, are produced at different times during infection, and have different specificities for TNF. It is therefore possible that vTNFRs function in distinct ways and at different stages of the immune response, helping viruses to better escape from the host immune response. Interestingly, virulent poxviruses associated with high mortality encode only one vTNFR and have preference for a particular vTNFR. For example, variola virus encodes CrmB, EV encodes CrmD, and myxoma virus encodes T2 (a CrmB homolog). This suggests that expression of different vTNFRs may not be directly associated with increased virus virulence but instead may contribute to establishing an equilibrium between the virus and the host. There is precedent for a poxvirus soluble receptor for IL-1β that attenuates the severity of infection in a mouse model (5). Poxviruses like CPV, although not highly virulent, may be more successful and persist in the population. A role for poxvirus soluble vTNFRs as virulence factors has been demonstrated only for the myxoma virus T2 (41). Further characterization of other vTNFRs and identification of their unique properties will clarify their roles in virus virulence.

The existence of various vTNFRs may be related to the adaptation of these molecules against a particular host during virus evolution. CPV shows a broad host range and produces all known vTNFRs. In contrast, EV is a mouse pathogen with a narrow host range and may have lost the ability to express *crmE* because, as shown here, it preferentially binds human TNF. Finally, we cannot exclude the possibility that some vTNFRs bind other ligands of the TNF superfamily and thus act in a different, but complementary, way. Binding of other members of the TNF superfamily to CrmC (35) and CrmE (this report) has been tested, but so far none of them have been found to interact with these vTNFRs.

Mechanisms of interference with the TNF system are not exclusive to the poxvirus family. They exist in herpesviruses as well: herpes simplex virus type 1 uses a member of the TNFR family, the herpesvirus entry mediator (26), to enter the cell, and the human cytomegalovirus encodes a homolog of the herpesvirus entry mediator (11). Interference with TNF signaling is illustrated by the betaherpesvirus-encoded vFLIP and the Epstein-Barr virus-encoded LMP1, a protein that interacts with TRAFs (27). Proteins encoded by the adenovirus E3 region block the apoptotic function of TNF (23), and human immunodeficiency virus uses the NF-κB cascade induced by TNF signaling to enhance transcription (29). A soluble vTNFR has been predicted from sequence similarity in lymphocystis disease virus (7), a large DNA iridovirus that infects fish, consistent with the conservation of TNF throughout evolution (10).

The expression of a fourth vTNFR by poxviruses represents a novel immune evasion strategy and emphasizes the critical role of TNF in antiviral immune responses. CrmE is a new member of the TNFR superfamily and adds even more complexity to the TNF system. Receptors and ligands of the TNF superfamily may be both expressed at the cell surface and secreted. Soluble versions of TNFRs are shed from the cell surface as a mechanism to control the activities of their ligands. Understanding the functions and the mechanisms of action of various vTNFRs may provide alternative strategies to effectively block the activity of TNF in vivo, which may be applied to modulate an overreactive immune response in a number of human disease conditions such as septic shock, allergy, and rheumatoid arthritis. The remarkable finding that poxviruses have evolved four distinct soluble TNFRs, which may be expressed simultaneously in the same virus, suggests that modulation of TNF ligands by soluble receptors is complex.

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