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Characterization of a Myxoma Virus-Encoded Serpin-Like Protein with Activity against Interleukin-1β-Converting Enzyme

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A genomic library of myxoma virus (MV) DNA, a leporipoxvirus that causes myxomatosis, was constructed and screened by in vitro transcription-translation. A clone was selected on the basis of its strong reactivity with MV antiserum. Analysis of the corresponding DNA sequence and the deduced amino acid sequence revealed an open reading frame coding for a 34-kDa protein with strong homologies to members of the serpin superfamily. The gene encoding this new protein, called *serp2*, was localized on the MV genome. Interestingly, this gene is deleted in an attenuated strain. We constructed a baculovirus vector to produce recombinant Serp2 protein and raised specific antisera that allowed the characterization of Serp2 expression during the MV cycle. The biological relevance of this new serpin from MV was monitored, and it was shown that Serp2 could inhibit human interleukin-1β-converting enzyme activity.

Myxoma virus (MV) is a leporipoxvirus responsible for myxomatosis, a disease that causes severe losses of the European rabbit (*Oryctolagus cuniculus*). After a silent period of about 10 days following contamination, the primary site of intradermal inoculation evolves as a lesion characterized by tissue degeneration and necrosis. Soon after, generalized lesions occur in the areas of the skin, head, and genitals and gram-negative infections develop in the nasal and conjunctival mucosae (7, 8). Death is the most common outcome, because of extreme weakness and secondary infections of the respiratory tract.

The genome of poxviruses is a linear double-stranded DNA molecule with covalently closed hairpin termini and terminal sequences which are repeated and inverted (terminal inverted repeats). Being very large (163 kb for MV [34]), the genome encodes all the enzymes required for replication and transcription of its DNA. The genes encoding these enzymes are located in the central portion of the genome.

The genes responsible for virulence and host range—which are usually nonessential—are located near termini (6, 53, 54, 56). Some viral proteins help circumvent the host immune response: homologs of the tumor necrosis factor receptor (15, 40, 51), the interleukin-1β (IL-1β) receptor (1, 43), the gamma interferon receptor (44, 55), a complement control protein (16, 25), homologs of the epidermal growth factor (28, 50), and a membrane-bound receptor-like protein called M11L (11, 27), as well as serpins. These members of the serine protease inhibitor superfamily are known to regulate immune and inflammatory responses in mammals. They have been found both in orthopoxviruses and in leporipoxviruses (for a review, see reference 49). Orthopoxviruses contain three serpin genes, *SPI-1*, *SPI-2*, and *SPI-3*. SPI-1 is implicated in host restriction (3), whereas SPI-3 is an inhibitor of cell-cell fusion during vaccinia virus and cowpox virus infection (20, 48, 57). SPI-2 inhibits IL-1β-converting enzyme (ICE), a cysteine proteinase which generates the proinflammatory cytokine IL-1β by cleavage of its precursor, pro-IL-1β (18, 33). A fourth serpin, called SERP-1, with a strong antiinflammatory effect, has been discovered in the MV (21, 24, 52).

In this paper we describe the cloning and characterization of a member of the serpin superfamily encoded by the MV. The gene was mapped and sequenced. The kinetics of expression of the protein were determined. The biological relevance of Serp2 was investigated, and it could be demonstrated that Serp2 specifically binds to ICE, thus inhibiting the cleavage of pro-IL-1β by the protease.

MATERIALS AND METHODS

Cells and viruses. The wild-type strain T1 and the attenuated strain SG33 of MV were grown on rabbit kidney cells (RK13), in Dulbecco's minimum essential medium supplemented with 10% calf serum. The baculovirus BakPak6 (Clonetech) and the recombinant virus BakSerp2 were propagated in the insect cell line *Spodoptera frugiperda* (Sf9). These cells were maintained in Hink's medium supplemented with 10% fetal calf serum.

Construction and screening of an expression library. Viral DNA from the wild-type strain T1 was purified by electroelution following pulse-field gel electrophoresis. The purified DNA was partially digested with *Sau*IIIA restriction enzyme (Promega). Fragments approximately 1.5 to 3 kb in length were cloned at the *Bam*HI site of the Bluescript phagemid expression vector (pSK+; Stratagene) and used to transform competent HB101 *Escherichia coli*. Recombinant plasmids were screened by coupled in vitro transcription-translation with either the T3 or the T7 phage promoter (TNT-coupled transcription-translation; Promega). Clones, whose translation gave rise to proteins, were defined as positive and screened by immunoprecipitation of the encoded protein with a serum produced in rabbits that had recovered from infections with the wild-type virus.

Sequencing and computer analysis of DNA sequences. A recombinant plasmid was purified by the alkali lysis method (35), and sequencing was performed as described previously (36) with an automate (Pharmacia). The entire sequence was determined with synthetic oligonucleotide primers. DNA sequence data were analyzed with the BLAST program (GenBank) on a Macintosh personal computer. Edited sequences were analyzed with the GAP program of the University of Wisconsin Genetics Computer Group software package (SWISS-PROT, release 33, March 1996).

Southern blot analysis. The wild-type strain T1 and the attenuated strain SG33 DNAs were digested with either *Eco*RI or *Pst*I (Promega), transferred from pulse-field electrophoresis gels to nylon membranes, and hybridized to viral probes radiolabelled by random priming with [α-^{32}P]dATP (Amersham Corp.). Autoradiographs were exposed for 3 days.

Expression in a baculovirus system and production of an antiserum. The *serp2* gene was cloned in the BakPak6 expression vector, under the control of the polyhedrin promoter, by conventional methods (29). Recombinant baculoviruses were screened for the expression of Serp2, and after cloning, a good recombinant candidate was selected and called BakSerp2. Sf9 cells overexpressing Serp2 were rinsed twice in Hink's medium without serum and freeze-thawed three times. Broken cells were treated with a mixture of antiproteinases and centrifuged at

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3,000 × *g* for 15 min. The supernatant was ultracentrifuged at 30,000 × *g* for 2 h and analyzed for the presence of Serp2 by Western blot (immunoblot) analysis using anti-MV antibodies. The estimated amount of Serp2 in the extract was approximately 200 μg/ml, representing 20% of total proteins.

One hundred micrograms of Serp2 was injected without adjuvant into 2-month-old rabbits to produce Serp2 antiserum. Rabbits were injected three times at 1-month intervals. Blood samples were collected 2 weeks after the last immunization.

Expression of Serp2 in the MV. RK13 cell monolayers were infected with 10 PFU of the T1 or the SG33 strain of MV. Proteins were pulse-labelled for 45 min at various times postinfection (p.i.) with both [^{35}S]methionine and [^{14}C]valine (Amersham Corp.), after which the proteins were precipitated with Serp2 antiserum. Intracellular viral proteins were also labelled 3 to 10 h p.i., immunoprecipitated with Serp2 antiserum, and compared with proteins present in the supernatant that had been prepared under the same conditions. The glycosylation of Serp2 was determined by labelling T1-infected cells 3 to 10 hours p.i. with [^{14}C]*N*-acetylglucosamine and immunoprecipitating them with MV antiserum or Serp2 antiserum.

Assays for the inhibition of ICE. Human ICE was produced in *E. coli*. Human pro-IL-1β was produced by in vitro translation of a cDNA under the control of a T7 promoter. Cytoplasmic extracts from BakSerp2- and BakPak6-infected cells were preincubated for 15 min at room temperature with 700 ng of human ICE, after which in vitro-translated pro-IL-1β was added. Incubation was performed for 15 min at 30°C in 10 mM Tris–0.1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS)–5 mM dithiothreitol, (pH 7.5). The pro-IL-1β and its derivative were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16% gels) and exposed for 3 days.

For the fluorometric assay, 100 ng of human ICE was incubated at 37°C with cytoplasmic extracts from BakSerp2- and BakPak6-infected cells and with the synthetic peptide substrate Suc-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin (Bachem) in a total volume of 40 μl. The cleavage kinetics of the synthetic substrate were monitored on a fluorometric microplate reader (Fluostar, BMG) as previously described (18).

Binding of Serp2 to ICE. BakSerp2-infected cytoplasmic extracts (20 μl) were incubated for 1 h at 30°C with various amounts of human ICE in the presence of 5 mM dithiothreitol. Samples were run under nonreducing conditions and transferred to a nylon membrane, and Serp2 was revealed with MV antiserum.

Nucleotide sequence accession number. The nucleotide sequence and the deduced amino acid sequence have been deposited in GenBank under accession number U60474.

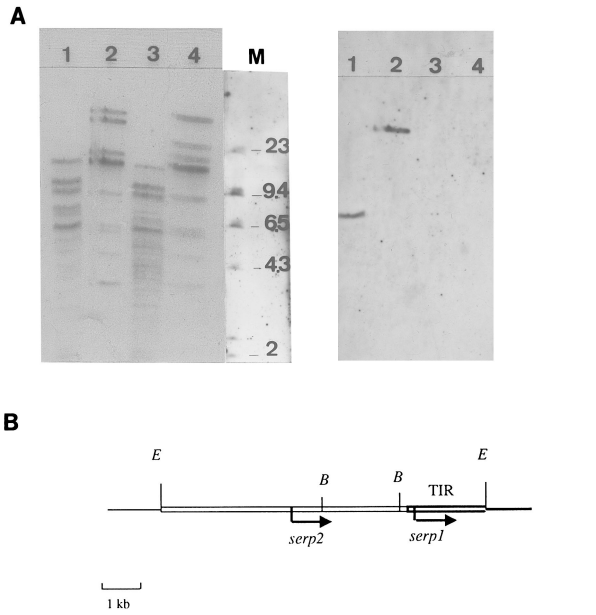

FIG. 1. Mapping of *serp2*. (A) DNAs from T1 (lanes 1 and 2) and SG33 (lanes 3 and 4) were digested with either *Hin*dIII (lanes 1 and 3) or *Eco*RI (lanes 2 and 4). The membrane was hybridized to a total T1 MV DNA probe (left gel) or the viral fragment extracted from p177 (right gel). Lane M, DNA molecular mass markers; sizes are indicated in kilobases. (B) Schematic map of the right end of the MV genome (strain T1), indicating the localization of *serp2*. *E*, *Eco*RI sites; *B*, *Bam*HI sites. The *Eco*RI F fragment is marked with a double line. The lines marking the terminal inverted repeat (TIR) are in bold. The location of *serp1* is indicated.

RESULTS

Selection of a recombinant clone and localization on the genome. With transcription beginning at the T3 promoter, a clone derived from the genomic library gave rise to a protein of an apparent molecular mass of 34 kDa, producing a strong signal with MV antiserum. The corresponding plasmid was used as a probe to map the gene (Fig. 1). Southern hybridization with a total T1 probe revealed differences in the restriction patterns of wild-type T1 and attenuated SG33 virus DNAs (Fig. 1A). The *Hin*dIII-B fragment, which is 34.5 kb long in the T1 strain (34), was shortened to ca. 23.5 kb in the SG33 strain. The double bands corresponding to the *Eco*RI E and *Eco*RI F fragments (respectively, 8.6 and 8.4 kb) in the T1 DNA pattern became a single band in the analysis of the SG33 strain, the *Eco*RI F fragment having disappeared. Southern hybridization with the fragment excised from the plasmid revealed that the gene encoding the 34-kDa immunogenic protein was present in viral fragments *Hin*dIII B and *Eco*RI F of MV T1 in single copies (Fig. 1B) but was deleted from the attenuated strain SG33.

The 34-kDa polypeptide belongs to the serpin superfamily. The plasmid containing a 1.5-kb viral fragment was purified, and the insert was sequenced. Analysis of the nucleotide sequence indicated an open reading frame corresponding to the size of the observed protein. This open reading frame was complete, with typical poxvirus AT-rich transcription initiation signals, indicating that the insert carries all the coding information for the viral protein (Fig. 2). The deduced amino acid sequence corresponded to a 333-amino-acid protein, with a predicted molecular mass of 38 kDa and with one potential glycosylation site (at position 87) and two cysteine residues (Fig. 2).

The hydrophobicity profile is shown in Fig. 3. There is no clear hydrophobic or hydrophilic sequence indicating that this protein contains a signal sequence or a membrane anchor domain. Data bank analysis revealed 34.5% identity with CrmA (31), 34.8% identity with horse leukocyte elastase inhibitor (19), 33.1% identity with swinepox virus K1R (23), 32.3% identity with rabbitpox virus SPI-1 (3), and 35.2% identity with vaccinia virus SPI-2 (42); all are members of the serine protease inhibitor superfamily. Only 28.7% identity was revealed with SERP-1 (52), the first serpin identified in MV, which is known to down-regulate the inflammatory response (22). This new protein was called Serp2.

Table 1 summarizes the comparison analysis of Serp2 with other poxvirus serpins.

Comparison of the putative reactive center of Serp2 with the carboxy-terminal region of other serpins did not reveal a clearly conserved motif at the key point (Fig. 4). It could, however, be seen that, provided the P-14 residue of Serp2 was a threonine, the putative P-1–P′-1 residues could be either Asp-Phe or Phe-Gly.

Cloning and production into baculovirus. In order to obtain a good yield of the Serp2 protein, the *serp2* gene was cloned in the BakPak6 expression vector, under the control of the polyhedrin promoter, by conventional methods (29). The best moment for harvesting Serp2 was 3 days p.i. when Serp2 was trapped inside Sf9 cells (data not shown). The estimated yield of Serp2 production was 100 μg/10^9 Sf9 cells, with Serp2 representing 20% of the total BakSerp2-expressed proteins. The presence of anti-Serp2 antibodies was revealed by immunoprecipitation of T1 viral proteins radiolabelled with [^{35}S]methionine (Fig. 5). An MV viral band of 33 to 34 kDa was reactive with the anti-Serp2 antiserum. Conversely, antibodies directed against a protein produced in BakSerp2-infected Sf9 cells were

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1    GATCAAATTTAAAATCCTTAATAACCTAACCAACAAGGATTTAGTTTTTTTAAATGAAAATAAAGTAGAGTATAA

                M  E  L  F  K  H  F  L  Q  S  T  A  S  D  V  F  V  S  P  V
76   TGTATAAGTGCGATTATGGAGCTTTTCAAGCATTTCCTCCAATCGACAGCGTCGGACGTCTTCGTTTCTCCCGTA

     S  I  S  A  V  L  A  V  L  L  E  G  A  K  G  R  T  A  A  Q  L  R  L  A  L  E
151  TCCATTTCAGCGGTTCTCGCCGTATTATTGGAAGGGGCAAAGGGACGCACTGCCGCGCAATTACGTCTCGCCCTA

        P  R  Y  S  H  L  D  K  V  T  V  A  S  R  V  Y  G  D  W  R  L  D  I  K  P
226  GAGCCGAGATATTCCCATTTGGATAAAGTAACCGTCGCATCCAGAGTATATGGCGACTGGAGACTGGATATAAAA

        T  F  M  Q  V  V  R  D  R  F  E  L  V  N  F  *N  H  S  P  E  K  I  K  D  D
301  CCCACGTTTATGCAGGTGGTACGTGATCGATTTGAACTGGTAAACTTCAATCACAGTCCTGAAAAGATTAAGGAC

        I  N  R  W  V  A  A  R  T  N  N  K  I  L  N  A  V  N  S  I  S  P  D  T  K
376  GATATCAACCGATGGGTGGCGGCCAGAACAAATAATAAAATACTCAACGCCGTAAATAGTATCAGTCCTGACACT

        L  L  I  V  A  D  I  Y  F  E  V  A  W  R  N  Q  F  V  P  D  I  T  I  E  G
451  AAACTCCTCATCGTCGCAGACATCTACTTTGAAGTGGCGTGGAGAAACCAATTTGTGCCCGACATCACGATAGAG

        E  F  W  V  T  K  D  V  S  K  T  V  R  M  M  T  L  S  D  D  F  R  F  V  D
526  GGGGAGTTCTGGGTTACCAAAGATGTGTCGAAGACGGTTCGAATGATGACATTGAGCGACGACTTCAGATTCGTG

        V  R  N  E  G  I  K  M  I  E  L  P  Y  E  Y  G  Y  S  M  L  V  I  I  P  D
601  GACGTGAGGAACGAGGGCATAAAAATGATAGAACTCCCGTACGAGTACGGGTACTCCATGCTGGTTATCATTCCG

        D  L  E  Q  V  E  R  H  L  S  L  M  K  V  I  S  W  L  K  M  S  T  L  R  Y
676  GACGACTTAGAACAGGTCGAACGACATCTGTCGCTTATGAAGGTTATATCATGGCTGAAAATGTCCACGTTGCGA

        V  H  L  S  F  P  K  F  K  M  E  T  S  Y  T  L  N  E  A  L  A  T  S  G  V
751  TACGTACATCTCTCGTTTCCCAAGTTCAAGATGGAAACCTCTTACACGCTAAACGAGGCGTTGGCGACATCGGGC

        T  D  I  F  H  P  N  F  E  D  M  T  D  D  K  N  V  A  V  S  D  I  F  H  K
826  GTAACAGATATCTTCGCACATCCTAACTTCGAGGACATGACCGATGACAAAAACGTGGCCGTGTCGGACATCTTT

        A  Y  I  E  V  T  E  F  G  T  T  A  A  S  C  T  Y  G  C  V  T  D  F  G  G
901  CACAAGGCCTACATAGAAGTGACCGAGTTCGGTACGACAGCTGCATCTTGTACGTACGGATGTGTTACAGACTTT

        T  M  D  P  V  V  L  K  V  N  K  P  F  I  F  I  I  K  H  D  D  T  F  S  L
976  GGTGGTACGATGGATCCCGTCGTATTAAAGGTGAACAAGCCGTTCATTTTCATCATCAAACACGACGACACGTTT

        L  F  L  G  R  V  T  S  P  N  Y  -
1051 TCGTTACTATTCCTGGGTAGAGTCACTTCTCCCAATTACTAAATATGGATATCTTTAATCATTTAAATAGCATTA

1126 ACCCACGCACACGTTTTTGGTTCTCTCCTGTATCCGTTAGCTATGCATTATCTGTATGTTGTACGGGTAATGTTC

1201 CGTCGGATTACGTATCCTCCACAGTCGTTGTAAAAAACAAAGTATACATAAACGCGTTTAAACAGTCCCCCGTAC
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FIG. 2. Sequence of *serp2*. The predicted open reading frame corresponding to the 33- to 34-kDa protein is shown with amino acids in single-letter code above the corresponding DNA sequence. Symbols: −, stop codon; ∗, potential glycosylation site.

present in the MV T1 antiserum (Fig. 5). There was no cross-reaction between the proteins produced in BakPak6-infected cells and MV antiserum.

Expression of the MV *serp2* gene. The kinetics of expression of Serp2 in the MV T1 strain were determined by pulse labelling of T1 proteins at various times from 2 h p.i. to 15 h p.i., after which the proteins were immunoprecipitated with Serp2 antiserum. Serp2 was produced as early as 2 h p.i. and throughout the viral cycle (Fig. 6A). Several other proteins precipitated with Serp2 antiserum. One of these was a protein with a molecular mass of ca. 46 kDa which, since its amount decreased during the infectious cycle, was probably actin. Two others had molecular masses of 36 and 32 kDa, the latter probably being a cleavage product of Serp2. The band corresponding to a protein with a molecular mass of ca. 70 kDa was a dimer of Serp2, and its amount decreased as the amount of β-mercaptoethanol in the sample buffer increased (data not shown). In order to determine whether Serp2 was associated with cells or secreted in the supernatant, intracellular T1 proteins were labelled 3 to 10 h p.i., immunoprecipitated with

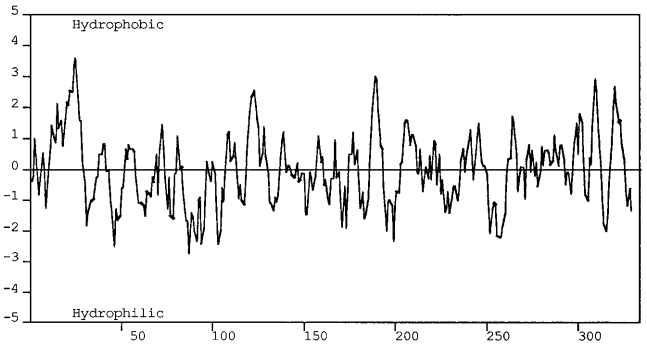

FIG. 3. Hydrophobicity profile of Serp2 determined by the Kyte and Doolittle hydropathicity plotting method. Vertical lines on the horizontal axis indicate blocks of 50 amino acids.

TABLE 1. Overall comparison of Serp2 with other serpins of poxviruses

Serpin[a]	% Similarity to Serp2	% Identity to Serp2
VV SPI-1	48.76	28.88
RBP SPI-1	57.62	32.31
VV SPI-2	58.41	35.16
RBP SPI-2	57.49	34.86
CPV CrmA	57.32	34.45
VV SPI-3	48.76	28.88
SWP SPI-7	54.89	33.12
MV SERP-1	51.83	28.66

[a] VV, vaccinia virus; CPV, cowpox virus; RBP, rabbitpox virus; SWP, swinepox virus.

Serp2	275	IEVT**E**FG**T**TAASC**T**YGCVTDFGGTMDPVVLKVNK**PF**IFI**IK**H**H**DDTFSL**LF**	324
CrmA	284	IDVN**E**EY**T**EAAAA**T**CALVADCASTVTN-EFCADH**PF**IYV**IR**H**H**VDG-KI**LF**	333
SPI-1	302	IDVN**E**EY**T**EASAV**T**GVFMTNFSMVYRTKVY-INH**PF**MYMIK-DNTGRI**LF**	349
HLEI	324	VDLN**E**EG**T**EAAAA**T**AGTIM-LAMLMPEENFNADH**PF**IFF**IR**H**H**NPSANI**LF**	372
Serp1	299	IEAD**E**RG**T**TASSD**T**AITLIPRNA-LTAIV--ANK**PF**MFL**IY**H**H**KPTTTV**LF**	345

FIG. 4. Comparison of carboxy-terminal regions of serine protease inhibitors in and around the reactive center. The alignment includes cowpox virus 38K (CrmA), rabbitpox virus SPI-1, MV Serp1, and horse leukocyte elastase inhibitor. Also aligned is the sequence for Serp2. Identical amino acids for the five sequences are in boldface type. The P-1–P′-1 residues are underlined.

Serp2 antiserum, and compared with proteins present in the supernatant that had been prepared under the same conditions. A quantitative comparison was possible as the cells and the supernatant had the same volume. Figure 6B indicates that Serp2 is an intracellular protein.

The proteins produced in T1-infected cells were labelled with [^{14}C]*N*-acetylglucosamine (Fig. 7) to investigate a post-translational glycosylation. Whereas glycosylated proteins could be recognized by MV antiserum (Fig. 7, lane 1), Serp2 did not appear when the proteins were precipitated with Serp2 antiserum (lane 2). This indicates that Serp2 is probably not glycosylated after translation. We also tested Serp2 produced in baculovirus on a concanavalin A-Sepharose column. The protein was not retained (data not shown), indicating that the only potential glycosylation site defined in the primary sequence was probably not used.

Biological relevance of Serp2 as a protease inhibitor. Extracts of soluble proteins from the cytoplasm of BakSerp2-infected cells were examined for their ability to inhibit the processing of human pro-IL-1β by human ICE. Figure 8 shows that extracts from cells infected by the recombinant baculovirus expressing Serp2 inhibit processing whereas the same amount of proteins from a non-recombinant baculovirus does not. As shown in Fig. 9, the extract containing Serp2 was able to inhibit the cleavage of a synthetic peptide representing the cleavage site in pro-IL-1β.

We also incubated Serp2 with decreasing amounts of ICE and analyzed the formation of a complex under nonreducing conditions (Fig. 10). Serp2 was revealed with MV antiserum. As the concentration of ICE was lowered, the amount of the complex between Serp2 and ICE (upper band) decreased. A cleavage product of Serp2, whose amount decreased when the amount of ICE decreased (lower band), could be seen. The specificity of the binding of Serp2 to ICE was assessed by testing the ability of Serp2 to bind trypsin, antithrombin III, urokinase, and elastase, by a method described elsewhere (21). In conditions under which SERP-1 binds to some of these serine proteinases and is cleaved by them, no effect on Serp2 could be seen (data not shown).

DISCUSSION

We have shown that MV encodes a new serpin-like protein, which we have called Serp2. Serp2 is expressed as early and late proteins, is intracellular, and is not glycosylated. In vitro experiments indicate that Serp2 is able to inhibit the processing of pro-IL-1β into its active form and to bind to human ICE. The inhibition experiments were performed on crude extracts of Serp2 expressed in a baculovirus system. The activity of Serp2 on the enzyme was determined by comparison with

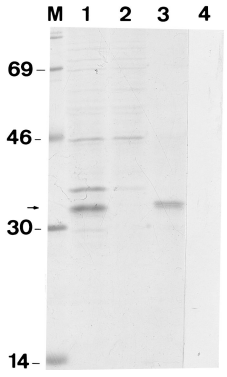

FIG. 5. SDS-PAGE of Serp2 produced in MV and in BakSerp2. In lane 1, MV T1 proteins were labelled and immunoprecipitated with Serp2 antiserum. The band at 33 to 34 kDa indicated by an arrow corresponds to Serp2. Lane 2 contains the negative control with MV SG33 proteins, *serp2* being deleted in this strain. In lane 3, BakSerp2 proteins were labelled and immunoprecipitated with MV T1 antiserum. A single band appears at 33 to 34 kDa. Lane 4 contains BakPak6 proteins labelled and immunoprecipitated with MV antiserum. Lane M contains molecular mass markers, with sizes indicated in kilodaltons.

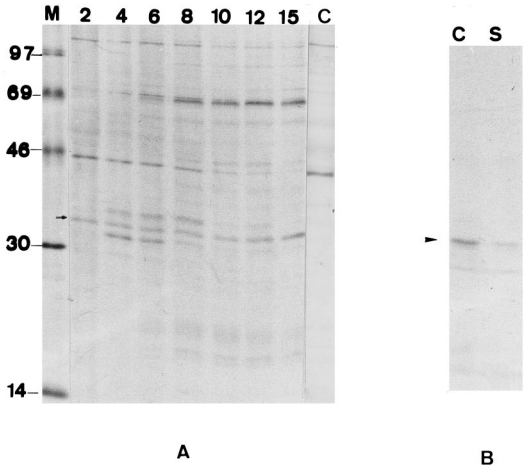

FIG. 6. Expression of MV-Serp2. (A) Pulse-labelling of MV T1 proteins at the times indicated above the lanes in hours p.i. Intracellular proteins were precipitated with anti-Serp2 antiserum. Serp2 is indicated by an arrow. Lane C contains the control (mock-infected cells) and lane M contains molecular mass markers (in kilodaltons). (B) Comparison of Serp2 associated with cells (lane C) and in the supernatant (lane S). MV T1 proteins were labelled from 3 to 10 h p.i. The same amount of total proteins was deposited in each lane.

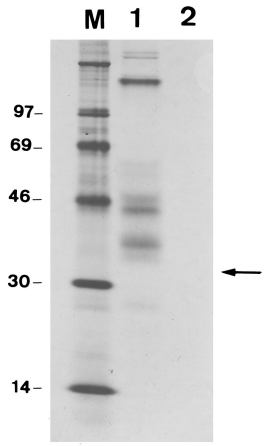

FIG. 7. SDS-PAGE analysis of proteins produced in MV T1 and immunoprecipitated with MV antiserum (lane 1) or Serp2 antiserum (lane 2). The viral proteins were labelled 3 to 10 h p.i. with [^{14}C]*N*-acetylglucosamine. The band corresponding to the expected size of Serp2 is absent in lanes 1 and 2 (arrow). Lane M, molecular mass markers. Sizes are indicated in kilodaltons.

extracts of baculoviruses not expressing Serp2 (negative controls).

Serp2 belongs to a superfamily whose members are mainly extracellular proteins that regulate the proteolytic cascades of blood coagulation, complement activation, and inflammatory responses (5). Apart from eukaryotes, serpins have been found in baculoviruses and poxviruses. The latter viruses have developed sophisticated means of evading immune defenses of the infected host. This leads to a decrease in the level of immunologic response efficiency, which, in the particular case of myxomatosis, results in the development of gram-negative infection of the respiratory tract.

The immunodepression responsible for this pathogenic scheme is attributed to several proteins, which either mimic targets for host cytokines or directly counteract them (reviewed in references 2, 24, 30, and 41). Serpins probably do play their parts in this scheme. They have been identified in orthopoxviruses as well as in leporipoxviruses, and precise functions have been attributed to some. SPI-1 from the rabbitpox virus has been shown to inhibit apoptosis in restrictive infected cells (4). However, SPI-1 mutants are not markedly attenuated (17, 47). Deletion mutants in other serpin genes show that serpins are virulence factors (20, 21, 22, 52, 57). SERP-1 has been shown to inhibit in vitro the serine proteinases human plasmin, urokinase, and tissue plasminogen activator, as well as C1s (21). Because of its antiinflammatory activity, SERP-1 has been successfully used in the prevention of atherosclerosic plaque development (49).

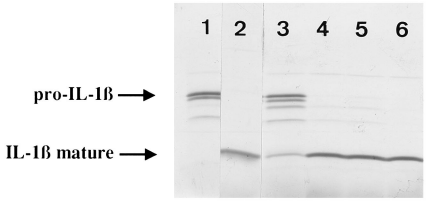

FIG. 8. Inhibition of ICE by Serp2. Various amounts of cytoplasmic extracts containing Serp2 were incubated for 15 min at room temperature with 700 ng of human ICE. Then 3 μl of in vitro-translated human pro-IL-1β was added, and the incubation was continued at 30°C for 15 min. Lane 1, pro-IL-1β alone; Lane 2, pro-IL-1β incubated with ICE; lanes 3 and 4, pro-IL-1β incubated with ICE that had been preincubated with 6 or 0.6 μl of a baculovirus extract containing Serp2; lanes 5 and 6, pro-IL-1β incubated with ICE that had been preincubated with 6 or 0.6 μl of a nonrecombinant baculovirus extract. The amounts of total proteins were the same in lanes 3 to 5 and 4 to 6.

The mechanism by which Serp2 inhibits ICE is not new and has been well documented in cowpoxvirus, in which CrmA (SPI-2) has been shown to inhibit ICE (31, 32). This is the second example of what has been called cross-class interaction (18), because of the fact that a serpin inhibits a nonserine proteinase. Apart from the sequence homology between Serp2 and CrmA, there are other similarities between the two proteins: both are intracellular, not glycosylated, and expressed at an early stage of infection. However, CrmA is not expressed at late stages whereas Serp2 is present throughout the viral cycle. The need for both serpins to be expressed early is obvious, since by inhibiting ICE, they block the synthesis of active IL-1β at an early stage. The fact that Serp2 is also expressed as a late protein might reflect another biological activity of the protein. It is thus possible that Serp2, apart from its anti-ICE activity, possesses an as-yet-unknown extra target.

A serpin having several targets would not be novel in poxviruses: SERP-1 binds to and inhibits a number of proteinases (21). Even CrmA has been shown to bind to granzyme B as well as to ICE (32). The granzymes are a family of serine proteinases present in the granules of cytotoxic T lymphocytes and natural killer cells. Among them, granzyme B is responsible for the most rapidly induced cell death (38).

It is well documented that the reactive center of serpins, extending over about 20 amino acids near the C terminus, is characterized by two residues, P-1 and P′-1, which are involved in the interaction with the catalytic apparatus of the serine protease (10, 12, 13). The P-1 residue determines the specificity of the target. Both granzyme B and ICE recognize Asp in the substrate site (14, 26, 39). The sequence Cys-Val-Thr-Asp-Phe of Serp2 seems suitable for human ICE. It has to be compared with the Leu-Val-Ala-Asp-Cys of CrmA (18). The relative affinity of Serp2 and CrmA for ICE has to be determined by a competitive binding of the proteins.

In our experiments we have shown that Serp2 can bind to and inhibit the activity of human ICE. It is difficult to ascertain whether this effect demonstrated in vitro reflects a physiological reality. Since Serp2 was not purified, we have only a poor idea of its concentration in the samples. It was therefore not possible to determine its level of affinity for ICE. It is also possible that the real target of Serp2 is not ICE but a member of the ICE family.

On the other hand, MV is a virus with a narrow host range; one explanation for this lies in the specificity of the virokines and the viroceptors encoded by MV for the cytokines of its natural host. It is likely that Serp2 inhibits rabbit ICE more efficiently than human ICE. Binding assays using purified Serp2 with ICEs of different origins and with ICE-like proteins will tell us more about the real target of this serpin.

Current hypotheses suggest that members of the ICE family are able to cause apoptosis as well as activate inflammation in animal organisms. CrmA has been shown to prevent ICE-mediated apoptosis in a cell culture system (9). On the other hand, granzyme B, another target of CrmA, has been proposed to be an essential component of the Ca^{2+}-dependent cytolysis pathway (38). Granzyme B and ICE were shown to compete for binding to CrmA, indicating that they probably react with the same inhibitory site (32). Moreover, CrmA can block Fas-induced apoptosis (45), which is a Ca^{2+}-independent component of cytotoxic T-lymphocyte killing. CrmA seems to block Fas-mediated killing, in which ICE and ICE-like molecules

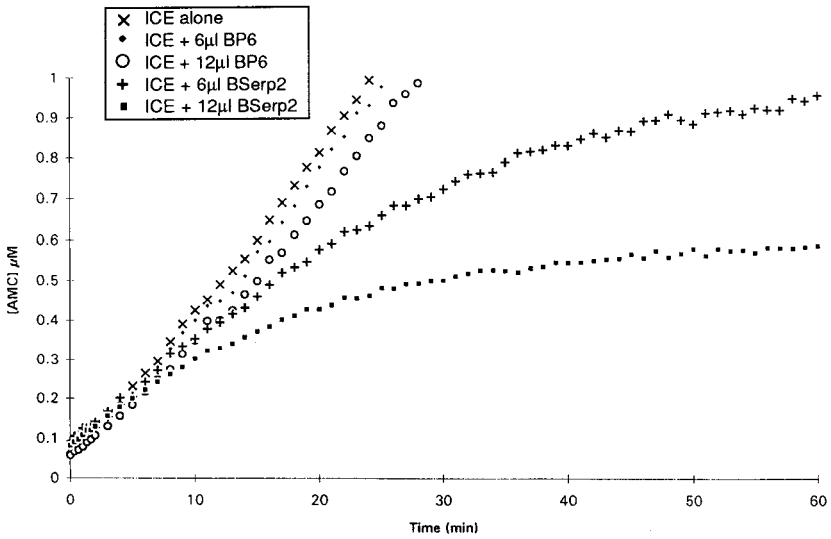

FIG. 9. Assay on a synthetic substrate. Extracts of baculovirus-infected cells were added to assay buffer containing 100 ng of ICE and the synthetic substrate (10 µl of a 200 µM stock) in a thermostated cuvette at 37°C to give a volume of 40 µl. Product formation due to substrate hydrolysis by ICE was observed as a function of time. BP6, nonrecombinant baculovirus; BSerp2, recombinant baculovirus expressing Serp2.

play a role, in a more efficient way than it does in the granzyme B-based pathway (46).

Thus, an attractive idea is that cowpox virus has developed the ability of dampening apoptosis, whether it is initiated inside or outside the infected cell (32).

It is far too early to assert whether Serp2, like CrmA, is involved in anti-T-lymphocyte-mediated cytotoxicity, which is part of the mechanism by which poxviruses combat immune surveillance.

It is interesting to note that SG33, which is naturally deleted in the region containing *serp2*, is a highly attenuated strain of MV. It is used in France to immunize rabbits against myxomatosis and induces only a mild primary lesion at the site of injection when administered to 2-month-old rabbits (37). The action of Serp2, by reducing the secretion of active IL-1β, may modify the ability of infected cells to generate an IL-1β response to infection. Specific knockout of *serp2* will ascertain its involvement in diminishing the inflammatory response against MV infection. Most probably, as was suggested for CrmA, viral inhibition of IL-1β alone will not be sufficient to circumvent all the inflammatory processes.

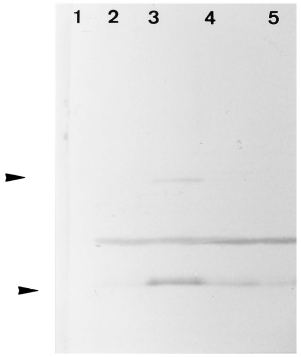

FIG. 10. Western blot analysis of a native polyacrylamide gel of ICE, Serp2, and the complex. Cells (20 µl) infected with a baculovirus expressing Serp2 were incubated for 1 h at 30°C with ICE in 5 mM dithiothreitol. Lane 1, 1 µg of ICE alone; lane 2, Serp2 alone; lane 3, Serp2 and 1 µg of ICE; lane 4, Serp2 and 200 ng of ICE; lane 5, Serp2 and 100 ng of ICE. Serp2 was revealed with MV antiserum.

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