JOURNAL OF VIROLOGY, June 2007, p. 6117–6121 Vol. 81, No. 11
0022-538X/07/$08.00+0 doi:10.1128/JVI.02679-06

Sequential Autoprocessing of Marek's Disease Herpesvirus Protease Differs from That of Other Herpesviruses[▽]

S. Laurent,[1]* C. Blondeau,[1] M. Belghazi,[3] S. Remy,[1] E. Esnault,[1] P. Rasschaert,[1] and D. Rasschaert[1,2]

Equipe Télomérase et Lymphome Viro-induit, UPR INRA 1282 IASP-213, INRA de Tours, 37380 Nouzilly, France[1]*; UFR Sciences et Techniques de l'Université François Rabelais de Tours, 37200 Tours, France*[2]*; and Service de Spectrométrie de Masse pour la protéomique, Unité PRC, INRA de Tours, 37380 Nouzilly, France*[3]

Received 5 December 2006/Accepted 14 March 2007

Herpesviruses encode a unique serine protease essential for viral capsid maturation. This protease undergoes autoprocessing at two sites, R and M, at the consensus sequence (V, L, I)$_{P3}$-X$_{P2}$-A$_{P1}$/S$_{P1'}$ (where X is a polar amino acid). We observed complete autoprocessing at the R and M sites of Marek's disease virus (MDV) protease following production of the polyprotein in *Escherichia coli*. Site-directed mutagenesis confirmed the predicted sequence of the R and M sites, with the M site sequence being nonconsensual: M$_{P3}$-N$_{P2}$-A$_{P1}$/S$_{P1'}$. Mutagenesis and expression kinetics studies suggested that the atypical MDV M site was cleaved exclusively by the processed short protease, a feature making MDV unique among herpesviruses.

Marek's disease virus (MDV), the causative agent of a type of T lymphoma in chickens, is a herpesvirus classified as gallid herpesvirus 2 in the *Mardivirus* genus of the *Alphaherpesvirinae* subfamily. Marek's disease is one of the most important and widespread infectious diseases of chickens, despite the availability of antitumor vaccines (11).

As with other herpesviruses, MDV virions contain multiple layers, which are assembled in several stages. The spherical precursor capsid, the procapsid, consists of four proteins surrounding an internal scaffold. It forms a major, transient intermediate in the capsid maturation pathway in vivo (14). The scaffold is composed of the products of two overlapping genes (*UL26* and *UL26-5* in herpes simplex virus type 1) encoding proteins with identical C termini. The N-terminal part of UL26 corresponds to one of the two proteases produced by herpesviruses. During capsid maturation, the *UL26*-encoded protease precursor undergoes autoprocessing, releasing the N-terminal protease domain by cleavage at the R site. This domain undergoes further autoprocessing and

TABLE 1. Primers used for pET construction

Primer	Sequence (5′–3′)	Direction[a]	Construct
862	CCATGGATCCGGCCGACCATCCATCGGTG	F	pET-8B6
863	CTCGAGTGCTTGCAAATACTTTTGTCCAG	R	pET-8B6
864	GGATCCGATGAACACTCAATCTTCTCGCCC	F	pET-FD6
865	CTCGAGAGCATTCATGGTATCCCGTG	R	pET-FD6
904	GCTCTAGATCATGATGAATCCGGCCGACCATCC	F	pET-UL26
905	GCTCTAGACTCGAGTTATTGATGCGCCATCATTTG	R	pET-UL26
T7	TTAATACGACTCACTATA	Fm	S116D A234R L232M
859	GAATTCTGGACGGTTCTATTAGCGATTGC	Rm	S116D A234R L232M
914	TCCCCCGGTGCTAGGCGTCGTGAGGACAGGTCGACCGATGGAAGATAATT	IR	S116D
913	TATATATAATTACAAATTATCTTCCATCGGTCGACCTGTCCTCACGACGC	IF	S116D
910	GTCATTGAATCGGTCAATGCCGTAAAAGAAGATCTTTGCAAATACTTTTG	IR	A234R
909	CTGGTATAACTGGACAAAAGTATTTGCAAAGATCTTCTTTTACGGCATTG	IF	A234R
M200	CCGTAAAAGATGATGCTTGCAACATCTTTTGTCC	IR	L232M
M199	GGTATAACTGGACAAAAGATGTTGCAAGCATC	IF	L232M
902	GACCTTGCTATCACAGGG	Fm	A638R M636V
905	GCTCTAGACTCGAGTTATTGATGCGCCATCATTTG	Rm	A638R M636V Sc
912	TCACTGGTGCGGTGTATACCAGCTACGGCAGATCTATTCATGGTATCCCG	IR	A638R
911	GCATCGAGCAATCACGGGATACCATGAATAGATCTGCCGTAGCTGGTATA	IF	A638R
M102	TGGTGCGGTGTATACCAGCTACGGCGCTAGCGTTAACGGTATCCCGTGAT	IR	M636V
M101	GCATCGAGCAATCACGGGATACCGTTAACGCTAGCGCCGTAGCTGGTATA	IF	M636V
M122	TCATGAATGTCATCTTTTACGGCATTGACCGA	Fm	Sc

[a] F and R, forward and reverse primers, respectively; IF and IR, internal forward and internal reverse primers, respectively, used for overlapping PCR to introduce mutations; Fm and Rm, forward and reverse primers used with IF and IR, respectively, to introduce mutations.

* Corresponding author. Mailing address: Equipe Télomérase et Lymphome Viro-induit, UPR INRA 1282 IASP-213, INRA de Tours, 37380 Nouzilly, France. Phone: 33 247427682. Fax: 33 247427774. E-mail: slaurent@tours.inra.fr.

▽ Published ahead of print on 21 March 2007.

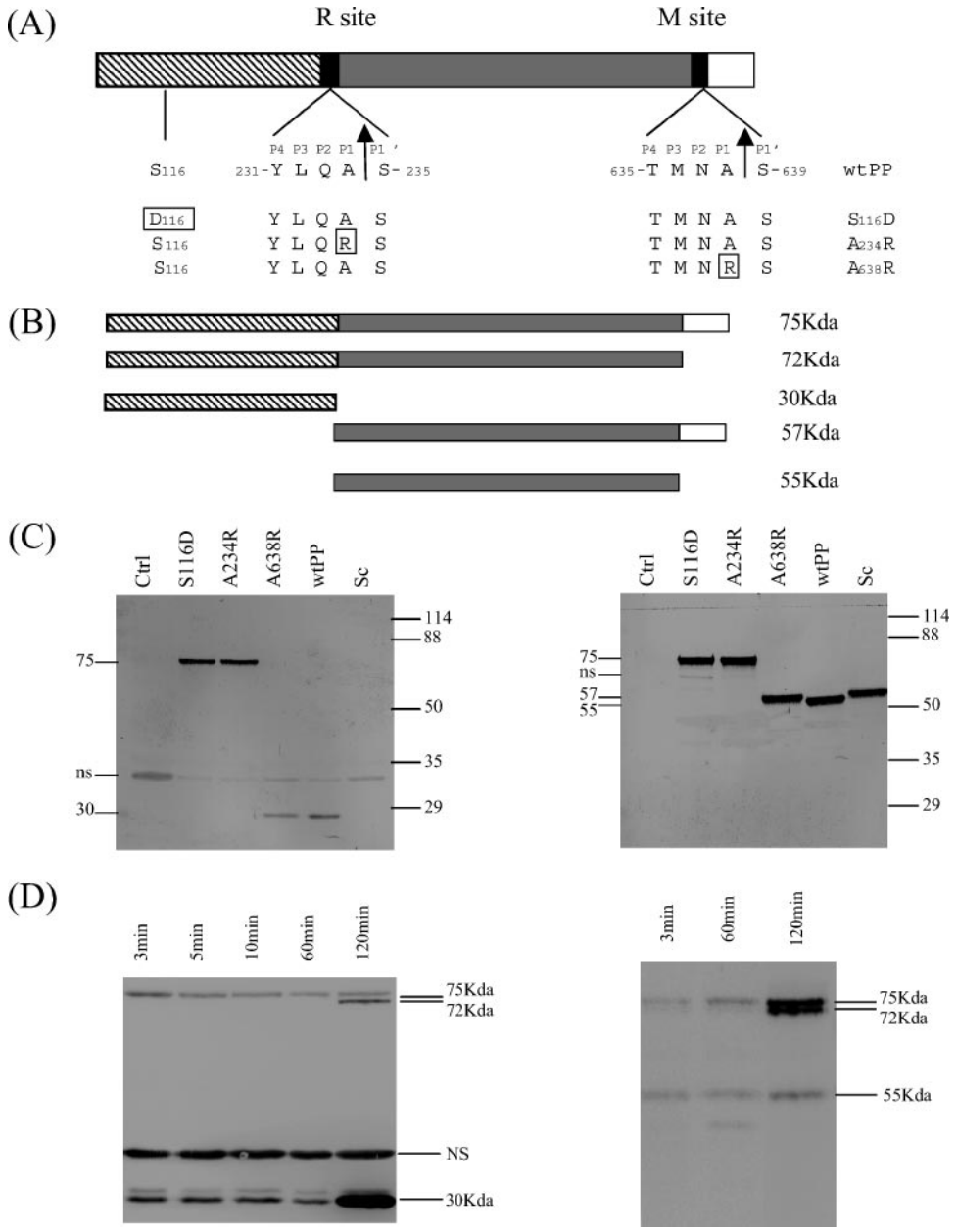

FIG. 1. Comparison of autoprocessing of the wild-type MDV protease precursor and that of precursors with mutations affecting the catalytic and cleavage sites (R and M). (A) Schematic representation of the protease polyprotein, with its R and M cleavage sites indicated. Hatched and gray boxes indicate the protease part of the protein and the matured scaffold protein, respectively. A part of the amino acid sequences of all constructs, with each mutation being framed by a rectangle, is indicated below the scheme. (B) Diagram of cleavage products, with their calculated molecular masses shown. (C) Western blots of proteins corresponding to the constructs expressed in *E. coli* 3 h after induction (with 1 mM IPTG [isopropyl-β-D-thiogalactopyranoside]). Two blots, corresponding to the same sodium dodecyl sulfate-polyacrylamide gel (1), were probed with the antiprotease MAb 8B6 (left) and the anti-scaffold protein MAb FD6 (right). The molecular mass of the marker and that of the immunodetected polypeptide are indicated to the right and the left of each blot, respectively. ns, nonspecific band from *E. coli*; Ctrl, negative control (*E. coli*). (D) Kinetics of hydrolysis of wtPP. Samples of the wtPP expressed in *E. coli* were collected at various times after induction with 1 mM IPTG and analyzed by Western blotting. (Left) Analysis of five time points of the kinetic assay. The blot was probed with the antiprotease MAb 8B6. (Right) Analysis of three time points of the kinetic assay. The blot was probed with the anti-scaffold protein FD6.

then cleaves the *UL26-5*-encoded protein at an additional site (M), near the C terminus, releasing a 25-amino-acid peptide that tethers the scaffold to the capsid shell. This step coincides with genome packaging; it is the final step in capsid maturation and is therefore a key stage in capsid assembly (21).

The herpesvirus protease, which belongs to a new class of serine proteases with a specific Ser-His-His catalytic triad (18), is highly conserved (up to 90%) among members of one herpesvirus subfamily (12). A naturally occurring cleavage sequence has been identified for the R and M sites: (V, L, I)-X-A/S, where X is a polar amino acid.

The catalytic triad (His_{49}-Ser_{116}-His_{135}) and the cleavage

TABLE 2. Identification of the C-terminal ends of MDV protease polypeptides by mass spectrometry

Construct	Molecular mass (kDa)	Proteolysis agent	No. of peptides	Protein coverage (%)	C-terminal end of polypeptide[a]
wtPP	30	Trypsin	12	73	223-MAGITGQKYLQA-234
	55	Lys-C	3	5	627-ESIEQSRDTMNA-639
A638R mutant	30	Trypsin	10	63	223-MAGITGQKYLQA-234
	57	Trypsin	4	12	646-TSDAGVDVFINQMMAHQ-663
S116D mutant	75	Trypsin	9	24	646-TSDAGVDVFINQMMAHQ-663
A234R mutant	75	Trypsin	15	27	646-TSDAGVDVFINQMMAHQ-663
A234R M636V mutant	75	Trypsin	8	15	646-TSDAGVDVFINQMMAHQ-663
	72	Lys-C	4	7	627-ESIEQSRDTVNA-639
L232M mutant	75	Trypsin	17	29	646-TSDAGVDVFINQMMAHQ-663
M636V mutant	30	ND[b]	ND	ND	ND
	55	ND	ND	ND	ND

[a] Underlining represents the catalytic site R or M cleaved after P1. Numbers indicate amino acid residues.
[b] ND, not determined.

sites R (232-L_{P3}-Q_{P2}-A_{P1}/$S_{P1'}$-235) and M (636-M_{P3}-N_{P2}-A_{P1}/$S_{P1'}$-639) were identified by aligning the predicted amino acid sequence of the polyprotein of MDV with the analogous polyproteins of alphaherpesviruses. Surprisingly, the predicted M site does not match the consensus sequence, differing at M_{P3}. We characterized the MDV protease cleavage sites by production of the wild-type polyprotein (wtPP) of MDV RB1B and a series of mutants with modified catalytic and cleavage sites by use of a pET-14 expression system (Novagen).

The wtPP gene was amplified by PCR using the primers listed in Table 1 and the PBS library, as previously described (8). It was inserted into the appropriate restriction sites of pET-14 to generate pET-UL26, which was used as a template for PCR mutagenesis and as a subcloning vector for mutated DNA fragments. Mutations were introduced at appropriate locations, employing the overlapping PCR technique (6) with the primers listed in Table 1. Western blot immunodetection of the proteins was carried out with monoclonal antibodies (MAb) against the protease (8B6) and the scaffold protein (FD6) by use of a passive blotting protocol that allows the analysis of two identical symmetrical immunoblots (1). MAb were produced as previously described (15), using His-Bind resin-purified polypeptides produced in the pET-22 system (Novagen) (Table 1) as an antigen.

We assessed the ability of the MDV polyprotein to undergo autoprocessing in *Escherichia coli* by comparing the MDV wtPP with an S116D mutant harboring mutations in the active site of the enzyme, predicted to result in an absence of catalytic activity (4, 17, 19) (Fig. 1A). As expected, no cleavable product was detected with the S116D mutant; this is consistent with an absence of processing at the R and M sites (Fig. 1B and C). By contrast, with the wtPP, products, most likely cleaved at R, were detected with the antiprotease MAb 8B6 or the anti-scaffold protein MAb FD6.The comparison between the products of cleavage revealed by FD6 and the Sc control (where Sc represents construction of the scaffold protein from the predicted R to the carboxyl end) seems to indicate that the product of scaffolding could correspond to a total cleavage on the level of the predicted sites R and M, considering the observed shift of migration between the wild type and the Sc control (predicted carboxyl fragment of 2,655 Da removed). Autoprocessing of the MDV polyprotein seems to occur in the *E. coli* expression system, which is consistent with findings for other herpesviruses (Fig. 1B and C) (3, 16).

To confirm the positions of the predicted R and M cleavage sites, we generated two mutants, the A234R and A638R mutants. As with the wtPP, R cleavage products were immunodetected for the A638R mutant (Fig. 1C). Two different scaffold peptides were detected for the wtPP and the A638R mutant, possibly corresponding to scaffold proteins cleaved in M and not cleaved at M, respectively (Fig. 1B), and comparison of the A638R mutant with the scaffold control seems to confirm this. These results are therefore consistent with the predicted site acting as a genuine atypical M site.

The Western blot profile obtained with the A234R mutant was identical to that for the S116D mutant, with one immunoreactive band of 75 kDa (Fig. 1B and C). Surprisingly, no polyprotein cleaved at M (72-kDa band) was detected, whereas such polyproteins are generally detected with other herpesviruses (Fig. 1B) (5, 7, 8, 9, 16, 19).

We therefore carried out liquid chromatography-mass spectrometry experiments to confirm the peptide profiles obtained. Protein bands were excised manually from electrophoresis gels stained with Coomassie blue, subsequently reduced and alkylated, and finally subjected to in-gel proteolysis with trypsin or Lys-C. All digested proteins were sequenced by nano-liquid chromatography–tandem mass spectrometry (Q-TOF-Ultima Global equipped with a nano-electrospray ionization source, coupled with Cap LC nanoHPLC; Waters Micromass). Data were interpreted using ProteinLynx global server software (Waters Micromass). All immunoreactive polypeptides were clearly identifiable, with a coverage of 5 to 73% (Table 2), confirming our identification of the R and M sites. We also confirmed that the 75-kDa product corresponds to the uncleaved protein. Thus, MDV seems to differ from other herpesviruses in that invalidation of the R site prevents cleavage of the M site, indicating

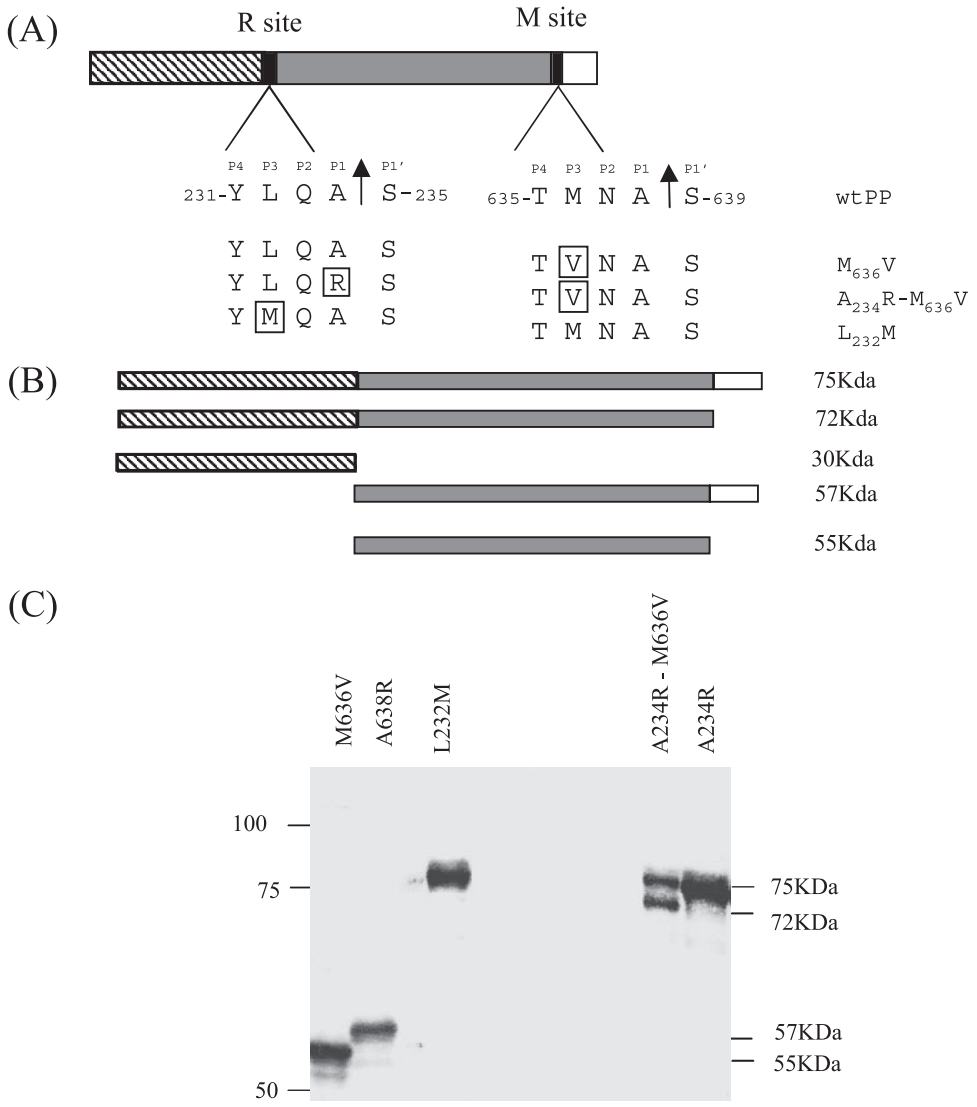

FIG. 2. Mutation of the P3 amino acid of cleavage sites R and M of the MDV protease precursor. (A) Schematic representation of the protease polyprotein, with its R and M cleavage sites indicated. Hatched and gray boxes indicate the protease part of the protein and the matured scaffold protein, respectively. A part of the amino acid sequences of all constructs, with each mutation being framed by a rectangle, is indicated below the scheme. (B) Diagram of cleavage products, with their calculated molecular masses shown. (C) Western blots of proteins corresponding to the constructs expressed in *E. coli* 3 h after induction (with 1 mM IPTG [isopropyl-β-D-thiogalactopyranoside]). The blot was probed with the anti-scaffold protein MAb FD6. The molecular mass of the marker and that of the immunodetected polypeptide are indicated to the right and the left of the blot, respectively.

that the MDV M site may be cleaved exclusively by the processed short protease domain.

To confirm this hypothesis, we carried out a kinetic study with the wtPP construct, collecting protein lysate samples at various times after induction (Fig. 1D). The R site was rapidly cleaved, as both the processed short protease and the scaffold protein were detectable 3 min after induction. While the 75-kDa uncleaved polyprotein was immunodetected in all samples, the 72-kDa polyprotein cleaved at M was not detectable until 2 h after induction. Given the strong signal seen at this time in the immunoblot revealed by 8B6, a high concentration of processed short protease seems to be necessary for cleavage of the M site of the polyprotein. However, scaffold protein cleavage in M occurs early (i.e., 3 min postinduction), demonstrating a very rapid cleavage of the M site of this processed substrate.

The lack of M site cleavage when the R site was not cleavable may be due to insufficient recognition of the atypical MDV M site by the full-length protease. We tested this hypothesis by replacing the nonconsensual M_{P3} in the MDV M site with the consensual V_{P3} of other alphaherpesviruses. First, we confirmed that this modified MDV M site was fully cleaved in the M636V mutant (Fig. 2), demonstrating that the M636V substitution did not prevent M cleavage. We then introduced the M636V mutation into the mutant with the invalidated R site (the A234R mutant) to generate the A234R M636V mutant. Two bands, at 75 kDa and 72 kDa, were detected on

Western blots, suggesting that both the full-length precursor and the polyprotein cleaved at the M site were present, as confirmed by mass spectrometry (Table 2; Fig. 2). Thus, the M site was recognized by the full-length protease once the methionine at P3 had been replaced with a valine residue, confirming the importance of the P3 position in the herpesvirus cleavage site, as demonstrated in previous studies for herpes simplex virus type 1 (9, 10) and cytomegalovirus (13, 19).

We further investigated the impact of having a methionine residue in the P3 position by introducing M_{P3} in the R site (the L232M mutant). Similar Western blot results were obtained for the S116D, A234R, and L232M mutants, with no cleavage at either the R or the M site, demonstrating that a methionine residue at P3 of the R or M site abolishes recognition by the full-length protease (Fig. 2). Furthermore, these data confirmed that R site cleavage was required for M site cleavage, as no 72-kDa product was observed.

Interestingly, these sequential cleavages seem to occur in the order opposite those in other herpesviruses (5, 7, 19). However, in good agreement with observations for the cytomegalovirus protease (20) the full-length protease seemed to have only weak activity, and the lack of cleavage at the M site when the R site was not cleavable may be due to a combination of the weak activity of the polyprotein and poor recognition of the atypical MDV M site by the full-length protease.

As the MDV M site seemed to be cleaved only in *trans* by the processed short protease, sequential cleavage seems to be necessary, according to the model proposed for Epstein-Barr virus, with three steps and an inactive full-length protease activated by an unknown factor, involving the release of the active short protease (2). Our results also expand the previously described herpesvirus consensus cleavage site from (V, L, I)-X-A/S to (V, L, I, M)-X-A/S.

This work was supported by grants from the Ligue Nationale Contre le Cancer-Comité du Cher and the Région Centre, France.

We thank S. Trapp for helpful remarks on the manuscript.

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