Core Protein of Pestiviruses Is Processed at the C Terminus by Signal Peptide Peptidase

Manuela Heimann,1 Gleyder Roman-Sosa,1 Bruno Martoglio,2 Heinz-Jürgen Thiel,1 and Till Rümenapf1*

Institut für Virologie (FB Veterinärmedizin), Justus Liebig Universität Giessen, Frankfurter Str. 107, D-35392 Giessen, Germany, and Novartis Institutes for BioMedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland

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The core protein of pestiviruses is released from the polyprotein by viral and cellular proteinases. Here we report on an additional intramembrane proteolytic step that generates the C terminus of the core protein. C-terminal processing of the core protein of classical swine fever virus (CSFV) was blocked by the inhibitor (Z-LL)2-ketone, which is specific for signal peptide peptidase (SPP). The same effect was obtained by overexpression of the dominant-negative SPP D265A mutant. The presence of (Z-LL)2-ketone reduced the viability of CSFV almost 100-fold in a concentration-dependent manner. Reduction of virus viability was also observed in infection experiments using a cell line that inducibly expressed SPP D265A. The position of SPP cleavage was determined by C-terminal sequencing of core protein purified from virions. The C terminus of CSFV core protein is alanine255 and is located in the hydrophobic center of the signal peptide. The intramembrane generation of the C terminus of the CSFV core protein is almost identical to the processing scheme of the core protein of hepatitis C viruses.

Pestiviruses are small, enveloped RNA viruses that account for important diseases in farm animals, e.g., classical swine fever (also known as hog cholera) and bovine viral diarrhea. Pestiviruses constitute one genus within the Flaviviridae and contain a message sense RNA of about 12.3 kilobases. The genome is translated into a single polyprotein that is processed by cellular and viral proteases into 12 mature proteins. The virion consists of four structural proteins, the core protein and the glycoproteins E2ms, E1, and E2 (19). The core protein of all virions is a small protein rich in basic amino acids and locates at or near the N terminus of the polyprotein (11). In the pestivirus polyprotein, the core protein is located between the N-terminal protease Npro and the glycoprotein E2ms. Npro generates the N terminus (Ser169) of the core protein by autocatalytic cleavage of the polyprotein (15, 18). Core protein is followed by E2ms, whose N terminus (Asp208) is generated by signal peptidase (signalase, or SP) (16).

Two different mechanisms of release of core protein from the polyprotein have been described for members of the Flaviviridae. Members of the genus Flavivirus (e.g., yellow fever virus [YFV]) employ the virally encoded NS2B-3 protease to generate the C terminus of core protein. The NS2B-3 protease of YFV cleaves the core protein precursor at a tribasic consensus sequence at the N terminus of the preM signal peptide (1). For hepatitis C virus (HCV), signal peptide peptidase (SPP) was recently determined to cleave within the C-terminal domain of core protein (7). Thus far, three different C termini have been proposed for HCV core protein. N-terminal sequencing of HCV core-dihydrofolate reductase fusion proteins revealed cleavage after Leu179 or Leu182 (4). In a recent report, Phe177 was identified as the C terminus of HCV core protein by using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (8). All proposed C termini of HCV core protein locate within the central hydrophobic domain of the signal peptide that conducts the translocation of E1 to the endoplasmic reticulum (4). Evidence for the involvement of the intramembrane protease SPP in the processing of HCV core protein came from inhibitor studies using peptidomimetic (carboxybenzoyl-Leu-Leu)2-ketone [(Z-LL)2-ketone] and overexpression of the SPP D265A loss-of-function mutant (9, 20). Analysis of the SPP cleavage site revealed that helix-breaking or -bending residues are a prerequisite for SPP cleavage to occur. These residues were identified in the HCV sequence by site-directed mutagenesis of either Ser183Cys184 (7) or Ile175Phe176 (9). SPP is an aspartyl protease of the GXXGD type and is related to preselinin (20). Several SPP-like proteins that operate at different subcellular localizations have been identified or functionally characterized (5). SPP promotes intramembrane proteolysis to release biologically important peptides that are incorporated, for example, as histocompatibility E (HLA-E) epitopes into polymorphic major histocompatibility complex class I molecules (2). The experiments described here show that processing of classical swine fever virus (CSFV) core protein is conducted by SPP and that inhibition of SPP results in a reduced virus yield.

MATERIALS AND METHODS

Viruses, cells, antibodies, and immunoblotting. CSFV Alfort (14) was propagated on SK6 cells (3) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C under 5% CO2. For detection of core protein, an anti-D1 polyclonal antiserum (18) and monoclonal antibody (MAb) GRS-M4 (unpublished) were used. The FLAG epitope was detected with monoclonal antibody M2 (anti-FLAG) from Sigma. Immunoblot analysis of bacterial lysates

1 Corresponding author. Mailing address: Institut für Virologie, FB Veterinärmedizin, Justus Liebig Universität Giessen, Frankfurter Str. 107, D-35392 Giessen, Germany. Phone: 49-641-9938356, Fax: 49-641-9938359. E-mail: Till.H.Ruemenapf@vetmed.uni-giessen.de.

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or CSFV-infected cells was done essentially as described elsewhere (6). Peroxidase-conjugated anti-rabbit- or anti-mouse secondary antibodies were from Dianova. The signal was revealed using chemiluminescence and exposure to Kodak Biomax film (Sigma, Munich, Germany).

**Bacterial expression of C-terminally truncated core protein.** CSFV cDNA encoding Npro-core was amplified using oligonucleotides co64 (CGGGATCCATGGATGTAAGGATCTT), which adds a BamHI restriction site to the 5’ end of the Npro gene, and nucleotides that introduce an XhoI site downstream of an opal codon at positions analogous to GluA211 (co207, AACTCGAGTCACCAAGCCAACAG CAG), ValA260 (co210, AACTCGAGTCACCCGCAAGCCAGGGC), Ala265 (co211, AACTCGAGTCACCAGCGAACAGGCTTTC), and Trp264 (co212, AACTCGAGTCACGAAGCCAGGGCTTTC). The PCR products were treated with BamHI and XhoI and ligated into BambHI/XhoI-digested plasmid pGEX6p1 (Amersham, Freiburg, Germany) in frame with a glutathione-S-transferase (GST) gene. The resulting plasmids p680 to p685 were transformed into Rosetta pLyS cells (Novagen, Darmstadt, Germany). Protein expression was initiated by addition of 1 mM isopropyl-thiogalactoside (Alexis, Gruenberg, Germany) at an optical density of 0.8 for 1 h at 37°C. Cells were boiled in 1% sodium dodecyl sulfate (SDS), subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using Tris-Tricine buffers (17), and analyzed by immunoblot analysis using anti-D1+ antisera at a dilution of 1:10,000.

**Mammalian cell translation.** SP6 transcripts were synthesized from cDNA encoding Npro-core-EF1 α from CSFV and positioned downstream of a SP6 promoter and the 56-nucleotide (nt) untranslated region of Sindbis virus (15). The plasmid was linearized with Xbal and subjected to transcription with SP6 RNA polymerase (Novatec, Kelsterbach, Germany). Uncapped transcripts were added to rabbit reticulocyte lysate (Promega, Mannheim, Germany) which was supplemented with canine microsomal membranes (Promega, Mannheim, Germany) and [35S]methionine (Amersham, Freiburg, Germany). Translation reactions were initiated by addition of cycloheximide, reaction products were diluted with phosphate-buffered saline (PBS), and microsomes were pelleted at 100,000 × g for 15 min in a TLA 45 rotor (Beckman, Munich, Germany). The microsomal pellet was washed once with ice-cold PBS and subjected to SDS-PAGE in Tris-Tricine gels. Gels were fixed in a solution containing 10% acetic acid and 40% methanol and were dried before exposure to Kodak Biomax X-ray film (Sigma, Munich, Germany).

**Inhibition of core C-terminal processing.** Multiple tissue culture plates containing 5 × 10⁶ SK6 cells were infected with CSFV Alfort at a multiplicity of 3. At 12 h postinfection (p.i.), (Z-LL)-keteone (from B.M.) was dissolved in dimethyl sulfoxide (DMSO) and added to the translation reaction mixture at a concentration of 10 μM. Translation reactions were terminated by addition of cycloheximide, reaction products were diluted with phosphate-buffered saline (PBS), and microsomes were pelleted at 100,000 × g for 15 min in a TLA 45 rotor (Beckman, Munich, Germany). The microsomal pellet was washed once with ice-cold PBS and subjected to SDS-PAGE in Tris-Tricine gels. Gels were fixed in a solution containing 10% acetic acid and 40% methanol and were dried before exposure to Kodak Biomax X-ray film (Sigma, Munich, Germany).

**Expression of dominant-negative signal peptide peptide.** (i) Construction of wt SPP and SPP D264A expression plasmids. The coding regions of human myc wild-type (wt) SPP and D264A mutant SPP (20) were ligated via HindIII (filled in) and EcoRI sites into BamHI-digested plasmid pTRE (BD Clontech, Heidelberg, Germany) carrying a tetracycline-responsive element (12). A FLAG tag was introduced between the sixth and fifth from last amino acid of the signal peptidase II (SPase II) recognition site (Hankins et al., 1979). The resulting plasmids p927 and p928 were cotransfected with pEE-pac into SK6 TET-on (tetracycline-inducible expression) and SK6T cells using Superfect reagent (Qiagen, Hilden, Germany). SK6T cells constitutively express the rTA activator and were established by transfection of plasmid pCAGGS-rTA-Neo (12) and selection with 1 mg/ml G418 (Calbiochem, Darmstadt, Germany). SK6T cell clones expressing human wt and D264A mutant SPPs were first selected with 5 mg/ml paromycin (Alexis, Gruenberg, Germany) and then identified by immunohistochemistry using the anti-FLAG monoclonal antibody M2 (Sigma, Munich, Germany) after induction of the cells with 2 mg/ml doxycycline (Dox; MP Biochemicals, Eschwege, Germany) for 24 h.

**RESULTS**

Inhibitor of signal peptide peptidase blocks processing of CSFV core protein. The core protein of CSFV requires processing at the N and C termini in order to be released from the polyprotein. These cleavages are performed by the virally encoded autoprotease Npro and the host cellular SP between CSFV C1106/Se/160 and Al4267/Asp260, respectively. Because the apparent molecular mass of a bacterially expressed core protein that terminates at Al4267 exceeded the apparent molecular mass of core protein from CSFV virions, a further proteolytic step was suspected (Fig. 1, lanes 1 and 2). Bacterially expressed core proteins with defined C termini (Al4267, ValC4268, AlaC4275, TrpC4274, AlaC4273) were expressed as GST-Npro-core fusions in E. coli. The autoproteolytic activity of Npro leads to the release of core protein. Crude bacterial lysates (lanes 1, 3, 4, 5, and 7) along with pelleted CSFV (lanes 2 and 6) were subjected to SDS-PAGE and immunoblot analysis. Polyclonal anti-D1+ serum detects epitopes within the core protein as well as the Npro moiety of the GST-Npro fusion. While core protein with a C terminus at Al4267 (lane 1) has a higher apparent molecular mass, core proteins ending around TrpC4274 comigrate with the mature core protein from the virus. A light gray line is laid through the core protein bands from virions (lanes 2 and 5) to allow easier detection of mass differences.
core protein with C termini around tryptophan 254 almost comigrated with core protein from CSFV virions in immunoblot analysis (Fig. 1, lanes 2 to 7). This result indicated that mature CSFV core protein is about 12 to 14 amino acids smaller than the core precursor protein. A precise determination of the core protein’s borders was not possible by the comigration approach, because it was not clear whether the additional processing step affected the N and/or C terminus. Recently a cleavage that occurs within the membrane-spanning C-terminal domain of the core protein of HCV (7) has been identified. As the processing protease, the novel aspartic protease SPP has been proposed (20). Important evidence for this finding came from the use of (Z-LL)2-ketone and the protease inhibitor II (Prot.inh.II) (lane 5), or ALLN (lane 6) was added for 10 h. All inhibitors were dissolved in DMSO and were used at concentrations of 10 μg/ml. Cells were lysed and subjected to SDS-PAGE and immunoblot analysis. Core protein from concentrated CSFV virions (equal to 106 FFU) is shown in lane 1. For detection of core protein, an anti-D1 polyclonal antiserum was employed which also detects Npro (visible at the upper margin). Detection of the weak signals (chemiluminescence) required extended exposure times (1 h).

membranes was core protein observed, which in the enriched microsome fraction is resolved into a doublet with apparent molecular masses of 14 kDa and 16 kDa. In the presence of 10 μM (Z-LL)2-ketone, the 16-kDa band (core+) is the predominant form of the core protein (Fig. 2, lanes 4 and 5). Both smaller and larger core protein bands are visible in the crude lysate (Fig. 2, lanes 2 and 3), but the resolution of the SDS-PAGE was massively compromised by high concentrations of hemoglobin. Enrichment of the microsomal membranes by centrifugation improved the separation of protein bands in the 12- to 20-kDa molecular mass range (Fig. 2, lanes 4 and 5) but led to underrepresentation of the mature core protein.

Previous experiments had shown that CSFV core protein is easy to detect in material from concentrated virions by immunoblotting but rather difficult to find in CSFV-infected cells. Because there was reason to believe that core protein is unstable in CSFV-infected cells, the effects of (Z-LL)2-ketone and a selection of proteasome inhibitors on the processing and stability of core protein were studied (Fig. 3). SK6 cells were infected at a multiplicity of 3 with CSFV Alfort. At 12 h postinfection, (Z-LL)2-ketone (Fig. 3, lane 2) and the proteasome inhibitors MG132, lactacystin, proteasome inhibitor II, and calpain inhibitor I (ALLN) were added for 10 h. Core protein was detected in the virus pellet (Fig. 3, lane 1) and cell lysates by Western blotting using anti-D1 polyclonal antiserum (18). In the cell lysates no significant differences in the quantities were apparent, while the sizes of the core protein differed (Fig. 3, lanes 2 to 7). Core protein from cell lysates treated with lactacystin, ALLN, or DMSO (as a control) (Fig. 3, lanes 4, 6, and 7, respectively) comigrated with core protein of pelleted virus. The presence of (Z-LL)2-ketone (Fig. 3, lane 2) or proteasome inhibitor II (Fig. 3, lane 5) resulted in an increased molecular mass. MG132 gave rise to the simultaneous appearance of processed and unprocessed forms of core protein (Fig. 3, lane 3), a picture similar to that observed after cell-free translation.

Inhibition of SPP activity affects CSFV viability. Processing of CSFV core protein can apparently be blocked by (Z-LL)2-ketone.
ketone and certain inhibitors of proteasomal degradation. It was therefore straightforward to examine the importance of C-terminal processing of core protein for the release of infectious CSFV. Pilot experiments revealed that MG132 and proteasome inhibitor II were not suitable for virus viability studies, because they exerted strong cytotoxicity. In contrast, (Z-LL)2-ketone significantly reduced the viability of CSFV but did not inhibit Teasome inhibitor II were not suitable for virus viability studies, because they exerted strong cytotoxicity. In contrast, (Z-LL)2-ketone left the monolayer intact even at high concentrations and thus allowed determination of growth kinetics after infection with CSFV. SK6 cells were infected with CSFV at a multiplicity of 3. At 6 h after infection, (Z-LL)2-ketone was added at concentrations of 10 μM, 50 μM, or 250 μM. As a control, no inhibitor (w/o) or 1% DMSO was added to the culture medium. The culture medium was removed at 12, 24, and 72 h after transfection and replaced with fresh medium [with or without (Z-LL)2-ketone]. Virus titers were determined for each time point by infectious center assays on SK6 cells. The graph shows means from three independent experiments.

**Cleavage of core protein is blocked by the dominant-negative D265A mutation of SPP.** Inhibition of SPP by (Z-LL)2-ketone significantly reduced the viability of CSFV but did not abrogate generation of infectious virus (20). For this purpose, SK6 cell lines that inductively expressed human D265A mutant and wt SPPs, respectively, were constructed. The SPP genes were modified by introduction of a C-terminal FLAG tag and were cloned into a tetracycline (TET-on)-controlled plasmid (pTRE). These plasmids were transfected into SK6T cells, which encode the tetracycline activator. SPP-expressing cell clones were identified by immunohistochemistry using the anti-FLAG antibody M2 after selection with puromycin.

The effect of expression of D265A dominant-negative mutant SPP on core protein processing was analyzed by infecting SK6T–SPP wt (Fig. 5, lanes 3 and 4) and SK6T–SPP D265A (Fig. 5, lanes 5 and 6) cells with CSFV at a multiplicity of 3. SK6T cells served as a control (Fig. 5, lanes 1 and 2). Induction of SPP expression was initiated by addition of Dox simultaneously with the infection. Cells were lysed 24 h after infection, and the processing of core protein and expression of SPP were analyzed by immunoblotting using monoclonal antibodies GRS-5H4 (anti-CSFV core protein) and M2 (anti-FLAG). FLAG-SPP expression clearly increases with induction. Only in induced SK6T cells expressing SPP D265A does the uncleaved 16-kDa core precursor prevail (core+) (lane 6); in all other cells, C-terminal processing of core protein occurs.

**FIG. 5. Processing of core protein is blocked by expression of the dominant-negative SPP D265A mutant.** Tetracycline-inducible SK6 cells (SK6T) (lanes 1 and 2) or SK6T cell lines expressing FLAG-tagged wt SPP (lanes 3 and 4) or SPP D265A (lanes 5 and 6) were infected with CSFV at a multiplicity of 3. SPP expression was induced by addition of Dox (5 μg/ml) at the time of infection (lanes 2, 4, and 6). Cells were lysed 24 h after infection and subjected to SDS-PAGE. Immunoblot analysis was performed to detect the presence of mature core protein, the borders of mature core protein were determined. To precisely define the SPP cleavage site in the pestivirus polyprotein, the borders of mature core protein were determined. To this end a recombinant CSFV that carried a FLAG tag at the N terminus of the core protein was constructed. Viable virus
can therefore be assigned to Ala255.

The intramembrane cleavage occurs C-terminally of the signal peptide (Fig. 8) The C terminus of CSFV core protein is evident from the increased molecular mass (lanes 2 and 4) and the reactivity with MAb M2 (lane 4). (B) Purification of core protein by immunoaffinity chromatography. A total of 10^11 FFU of CSFVv585 was harvested from infected 38A1D cells and subjected to SDS-PAGE and immunoblot analysis using an anti-D1 plus polyclonal antibody (lanes 1 and 2) or monoclonal antibody M2 (anti-Flag) (lanes 3 and 4). The presence of the FLAG epitope in the structural core protein is evident from the increased molecular mass (lanes 2 and 4) and the reactivity with MAb M2 (lane 4).

**DISCUSSION**

Three lines of evidence strongly suggest that SPP is responsible for the maturation of core protein. These are (i) the blocking of core protein processing by (Z-LL)_2-ketone, (ii) the inhibition of the C-terminal cleavage by overexpression of D265A SPP, and (iii) the determination of an intramembrane cleavage site. The determination of the C terminus of core protein is the most important result of our study, because it describes the final processing product that is incorporated into CSFV particles. C-terminal sequencing was unambiguous and revealed a C terminus at alanine 255. It was also important to show that the N terminus of core protein remains unchanged after the release by Npro. According to these data, FLAG-core protein was acetylated (8). Acetylation (42.03 Da) of the N-terminal serine residue of insect cell-expressed HCV core protein consists of 95 amino acids and has a calculated average molecular mass of 10,863.31 Da (22). This is very close to the molecular mass determined by MALDI-TOF MS [10,895.7 Da (M + H^+)]. The deviation of 32.4 Da accounts for less than one amino acid and may indicate that the core protein is posttranslationally modified. It was recently reported that the N-terminal serine residue of insect cell-expressed HCV core protein is acetylated (8). Acetylation (42.03 Da) of the N-terminal serine of CSFV core protein would increase the calculated mass to 10,905.33 Da. The deviation from the observed mass (10,895.7 Da) accounts for less than the mass of a carbon atom.

The intramembrane cleavage occurs C-terminally of the se-
The participation of SPP in C-terminal processing of CSFV core was further confirmed by the use of the dominant-negative D265A SPP mutant. Overexpression of this enzymatically inactive mutant (20) but not of wild-type SPP in tetracycline-inducible SK6 cell lines led to an accumulation of unprocessed core protein after infection with CSFV. Apparently, only high concentrations of D265A SPP were able to inhibit CSFV core protein processing after induction, while low-level basal expression in noninduced SK6 cells had no apparent effect (Fig. 6). Because the dominant-negative effect of SPP relies on the competition between active and inactive enzymes for the substrate, the relatively low expression levels may account for the missing inhibitory effect of D265A SPP on HCV core protein cleavage in 293T cells (9). McLauchlan et al. have put forward the idea that blocking SPP might “have the potential to affect the HCV life cycle and reduce any impact of HCV on associated disease” (7). This idea could be confirmed for CSFV, because inhibition of SPP by different approaches reduced the release of infectious virus as much as 100-fold. Nevertheless, we were surprised that even high concentrations of (Z-LL)2-ketone or expression of D265A SPP did not result in a more pronounced effect. The strongest titer reductions by (Z-LL)2-ketone in water may be critical for bioavailability. The titer at the top shows the expans of the signal peptide of CSFV and YFV. The SP cleavage site is indicated by white arrowheads; black arrowheads mark the processing sites of SPP in the polyproteins of CSFV and HCV. The gray arrowhead indicates the NS2B-3 processing site at the C terminus of YFV. For HCV three different C termini of core protein, Phe177, Leu179, and Leu182 (indicated by arrowheads 1, 2, and 3, respectively), have been proposed (4, 8). Interestingly, the position analogous to the SPP-generated C terminus of CSFV core protein, Ala255, would be Leu178 in the HCV sequence.

**FIG. 8.** C-terminal alignment of the amino acid sequences (single-letter code) of the C-terminal core-glycoprotein (Eɛ, E1, preM) junction from different members of the Flaviviridae. The arrow at the top shows the expans of the signal peptide of CSFV and YFV. The SP cleavage site is indicated by white arrowheads; black arrowheads mark the processing sites of SPP in the polyproteins of CSFV and HCV. The gray arrowhead indicates the NS2B-3 processing site at the C terminus of YFV. For HCV three different C termini of core protein, Phe177, Leu179, and Leu182 (indicated by arrowheads 1, 2, and 3, respectively), have been proposed (4, 8). Interestingly, the position analogous to the SPP-generated C terminus of CSFV core protein, Ala255, would be Leu178 in the HCV sequence.

A signal peptide peptidase that degrades the signal peptide was identified in 2002 by affinity purification using the diazirine-containing derivative of (Z-LL)2-ketone, TBL4K (20). Its inhibitory effect on the processing of HCV core protein supported the claim that SPP is responsible for the cleavage. Interestingly, the peptidomimetics MG132 and proteasome inhibitor II (Z-LLF-aldehyde) apparently bind to SPP and inhibit its function.

The participation of SPP in C-terminal processing of CSFV core was further confirmed by the use of the dominant-negative D265A SPP mutant. Overexpression of this enzymatically inactive mutant (20) but not of wild-type SPP in tetracycline-inducible SK6 cell lines led to an accumulation of unprocessed core protein after infection with CSFV. Apparently, only high concentrations of D265A SPP were able to inhibit CSFV core protein processing after induction, while low-level basal expression in noninduced SK6 cells had no apparent effect (Fig. 6). Because the dominant-negative effect of SPP relies on the competition between active and inactive enzymes for the substrate, the relatively low expression levels may account for the missing inhibitory effect of D265A SPP on HCV core protein cleavage in 293T cells (9). McLauchlan et al. have put forward the idea that blocking SPP might “have the potential to affect the HCV life cycle and reduce any impact of HCV on associated disease” (7). This idea could be confirmed for CSFV, because inhibition of SPP by different approaches reduced the release of infectious virus as much as 100-fold. Nevertheless, we were surprised that even high concentrations of (Z-LL)2-ketone or expression of D265A SPP did not result in a more pronounced effect. The strongest titer reductions by (Z-LL)2-ketone were observed early after infection. The inhibition of SPP by (Z-LL)2-ketone is probably incomplete, which would allow accumulation of small amounts of correctly processed core protein over time. Also, the limited solubility of (Z-LL)2-ketone in water may be critical for bioavailability. The titer reduction observed with the dominant-negative SPP D265A mutant displays different kinetics. Here the virus release remains steady at 20- to 30-fold-reduced levels over 72 h. Due to induced expression, an accumulation of the inactive enzyme can be postulated. Thus, the dominant-negative effect is increasingly stronger within the observation window, which counteracts the accumulation of processed (functional) core protein. In accordance with this assumption, induction of D265A SPP 12 h before CSFV infection showed an even stron-
ger inhibitory effect (data not shown). Interestingly, addition of (Z-LL)₂-ketone to CSFV-infected D₂₆₅A SPP-expressing SK6T cells did not reduce virus titers below the level observed with D₂₆₅A SPP (not shown).

Intramembrane cleavage is an unusual processing mechanism that has thus far not been observed in viral (poly)protein biosynthesis other than in C-terminal core protein processing of pestiviruses and hepaciviruses. The usage of SPP in polyprotein cleavage therefore defines yet another common feature of pestiviruses and hepaciviruses. The usage of SPP in polyprotein biosynthesis other than in C-terminal core protein processing is a cellular receptor for bovine viral diarrhea virus. J. Virol. 78:4760–4768.

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