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Identification of the Active-Site Residues of the L Proteinase of Foot-and-Mouth Disease Virus

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The foot-and-mouth disease virus (FMDV) leader (L) protein is involved in autocatalytic cleavage at the L/P1 junction and in the cleavage of translation initiation factor p220, a subunit of the cap-binding protein complex. It has been suggested that this proteinase has homology to the papain-like family of cysteine proteinases, and from this information, we have investigated the active-site residues by introducing specific mutations into the L gene. Mutations of Cys-23 to Ala or His-120 to Leu resulted in enzymes that lacked *cis* activity at the L/VP4 cleavage site, *trans* activity on a truncated L-P1 substrate, and p220 cleavage activity. Mutations of Cys-23 to Ser or His-110 to Leu resulted in enzymes that retained some or all *cis* activity and had reduced p220 cleavage. These mutations were introduced separately into a full-length FMDV cDNA, and RNA transcripts derived from these cDNAs were translated in a cell-free system and transfected into cells. The C23S mutant inefficiently cleaved at the L/P1 junction and within P1, and virus obtained from transfected cells reverted to wild type. The H110L mutant cleaved the L/P1 junction almost as well as the wild-type enzyme, and virus recovered from transfected cells retained the mutation and displayed wild-type viral protein synthesis and host shut-off kinetics.

Foot-and-mouth disease virus (FMDV) virion RNA is translated into a polyprotein which is processed, during its synthesis, into four primary cleavage products, L, P1, P2, and P3. Initiation of translation of FMDV RNA begins at either of two in-frame AUG codons, approximately 1,100 bases from the 5′ end of the RNA, resulting in the synthesis of two L proteins, Lab and Lb, differing at their amino termini by 28 amino acids (24). Lb, the smaller of the two proteins, is the major species synthesized in infected cells and in a cell-free system. Both proteins autocatalytically cleave themselves from the viral polyprotein at the L/P1 junction (26) and cleave p220, a subunit of the cap-binding protein complex eIF-4, involved in the initiation of translation at the 5′ end of capped mRNAs (3, 14, 20). Cleavage of this component results in the shut-off of most host cell protein synthesis (5), but FMDV RNA translation, which occurs by a cap-independent mechanism, is not impaired.

Analysis of amino acid sequence data has suggested that the L protein is a thiol protease related to the papain family of proteases (7). We have shown that E-64, a specific inhibitor of thiol proteases, including the papain family of cysteine proteases (10), blocks the autocatalytic cleavage of L-P1 in a cell-free system and that an uncharged analog, E-64d, blocks autocatalytic and p220 cleavage in infected cells (15). Furthermore, we demonstrated that E-64d blocks virus assembly and, as a consequence, reduces virus yield, suggesting that this or a related compound might be an effective agent against foot-and-mouth disease.

We have inserted the Lb gene into a plasmid under the control of a T7 promoter and demonstrated that the expressed protein has *cis* and *trans* cleavage activity at the L/P1 junction and p220 cleavage activity (21). Essentially identical results were also obtained by Kirchweger et al. (14). To determine if the sequence similarity between L and the papain proteases represents a functional similarity, we expressed L proteins with mutations at residues thought to form part of the catalytic dyad (7) and examined the enzymatic activity of the mutant proteins expressed in either an in vitro transcription-translation system or an *Escherichia coli* system. Mutations that produced enzymes with partial or complete autocatalytic activity and reduced p220 cleavage activity were introduced into a full-length cDNA, and RNA transcripts were translated in vitro and transfected into susceptible cells. In this communication, we describe the results of these experiments.

MATERIALS AND METHODS

Plasmids. Plasmid pLb-T7 contains the complete wild-type (WT) Lb gene inserted in the vector pT7-7, and pLb-VP4′-3D′ contains the Lb gene fused in frame to portions of the VP4 and 3D genes (21). Plasmid $pRMC_{35}$ is a full-length infectious clone of FMDV serotype A12 119ab (22). Plasmid pRM-ΔL contains the full-length FMDV genome with an internal deletion in the L gene (21) (previously called $pRMC_{35}\Delta L$).

Site-directed mutagenesis. Mutant Lb genes were constructed by overlap extension PCR by standard techniques (11). Each mutant gene had a unique *Nde*I site, followed by an in-frame start codon (ATG) at the 5′ end and a termination codon (TAG) followed by a *Bam*HI restriction site at the 3′ end. The mutated PCR products were digested with *Nde*I and *Bam*HI and ligated into *Nde*I- and *Bam*HI-digested pT7-7 vector. Plasmids containing the mutant Lb genes were identified and sequenced through the amplified region by the dideoxy chain termination method (25) with Sequenase Version 2.0 (U.S. Biochemicals). Mutant Lb plasmids are designated by the single-letter code for the WT amino acid followed by the codon number and the mutated amino acid (e.g., Cys at position 23 to Ala is C23A).

To produce plasmids for *cis* cleavage assays, each Lb mutant in pT7-7 was excised with *Eco*RI and *Afl*II and inserted into pLb-VP4′-3D′ digested with the same enzymes, and pLb-VP4′-3D′ derivatives of each Lb mutant were confirmed by sequence analysis.

Construction of full-length cDNA clones with mutations in the L gene. To construct a full-length cDNA with the C23S mutation, pLbC23S-T7 was digested with *Eco*RI and *Afl*II, and the fragment harboring the mutation was inserted into similarly digested pTRP12 (27). The resulting plasmid was digested with *Xba*I

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and *Afl*II, and the fragment containing the L gene was ligated to a similarly digested subgenomic clone containing bases 716 to 5464 of the FMDV genome. This plasmid was digested with *Eco*RI, and the fragment containing the mutant L gene was ligated to the appropriate fragment from a similarly digested, alkaline phosphatase-treated full-length cDNA containing a deletion in the P1 region, pRM-ΔP1 (18). Full-length cDNAs derived from this construct, pRM-LC23S, were checked by restriction enzyme analysis, and the Cys-to-Ser substitution was confirmed by sequencing.

Substitution of mutation H110L into a full-length cDNA was done by insertion of the *Sal*I-*Afl*II fragment from pLbH110L-T7 into a similarly digested subgenomic clone containing bases 716 to 5464 of the FMDV genome. The resulting plasmid was digested with *Eco*RI, and the fragment containing the mutant L gene was ligated to the appropriate fragment from *Eco*RI-digested, alkaline phosphatase-treated pRM-ΔP1. Full-length clones derived from this construct, pRM-LH110L, were checked as described above.

Expression of WT and mutant Lb in *E. coli*. WT and mutant pLb-T7 plasmids were expressed in BL21(DE3)/pLysE cells, and supernatant and pellet fractions were prepared (21). The amount of WT and mutant Lbs was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining, and equivalent amounts of each enzyme were used in the assays.

In vitro transcription, translation, and cleavage assays. Transcripts from *Bam*HI-linearized WT and mutant pLb plasmids were synthesized with T7 RNA polymerase and the mMESSAGE, mMACHINE capping kit (Ambion), while transcripts from *Not*I-linearized plasmids pRM-ΔL, pRM-LC23S, and pRM-LH110L were synthesized with the T7 MEGAscript kit (Ambion). Approximately 0.5 to 1 μg of RNA from transcription reaction mixes or 1 μg of FMDV virion RNA was used in a rabbit reticulocyte lysate in vitro translation system as previously described (1, 27). To measure *trans* cleavage activity, unlabeled translation products prepared from WT or mutant pLb-T7 transcripts or *E. coli* lysates from cells transformed with mutant or WT plasmids (described above) were incubated with the radiolabeled translation products of transcripts from pRM-ΔL, and the products of the reaction were analyzed by SDS-PAGE. *cis* cleavage was assayed by SDS-PAGE analysis of the radiolabeled translation products of WT or mutant transcripts of plasmid pLb-VP4′-3D′.

p220 cleavage assay. p220 cleavage activity was assayed in vitro as previously described (21). Briefly, postmitochondrial HeLa cell cytoplasmic extracts (S10) were incubated at 30°C overnight with L WT or mutants expressed either in *E. coli* or in cell-free translation reactions, and the integrity of p220 was determined by Western (immunoblot) analysis.

Transfection and characterization of L mutant viruses. RNA transcripts were transfected into baby hamster kidney cells (BHK-21) by using lipofectin, and the specific infectivities of each RNA were determined by plaque assay (22). T7 transcripts were also introduced into cells by electroporation (19), and supernatants from transfected cells (pass 1) were used to produce virus stocks (pass 2). RNA extracted from pass 2 virus was used as a template for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Superscript; Gibco/BRL) and random hexamers. The cDNA corresponding to the L gene was amplified with the appropriate primers by PCR, and the PCR products were directly sequenced with the Sequenase PCR product sequencing kit (USB/Amersham). Cells transfected by electroporation were radiolabeled with [^{35}S]methionine, and the culture fluids were examined for virus assembly by sedimentation through 10 to 50% (wt/vol) sucrose gradients as previously described (19). L mutant viruses were designated A12, for the FMDV serotype, followed by the nomenclature used for mutant Lb plasmids, i.e., A12-LH110L.

Immunoprecipitation. Immunoprecipitation of [^{35}S]methionine-labeled in vitro translation reactions, transfected cells, or infected cells was performed as previously described (15). The monoclonal antibody 2PD11, which recognizes conformational determinants on the viral capsid of serotype A12, has been described previously (2, 9).

Molecular modeling. At present, neither X-ray crystallographic structures nor nuclear magnetic resonance models exist for L proteinase. Since no such models are currently available, we developed a theoretical model for L constrained by site-specific mutation information. It is recognized, of course, that no one has yet succeeded in predicting the correct three-dimensional structure of a protein de novo from sequence information alone. Therefore, the theoretical model that we describe here should not be construed as a prediction of the "correct" structure of L. Rather, it should be considered as a low-resolution working model for generating ideas and testing hypotheses. Working three-dimensional models have recently been used successfully to predict the functionality of proteins whose three-dimensional structure cannot be obtained from either X-ray crystallographic or two-dimensional nuclear magnetic resonance spectroscopy (6, 16, 23).

All molecular modeling calculations were performed with Tripos Sybil software (Tripos Associates, St. Louis, Mo.) in vacuum (no waters were added) in order to maintain a reasonable calculation time. Each potential model was energy minimized and equilibrated at 300 K by using molecular dynamics. This procedure employed a Kollman force field with electrostatics (Amber), with a united-atom approach with essential hydrogens, a nonbonded cutoff of 8 Å (0.8 nm), and a dielectric constant that varied with distance. We assume here that equilibrium has been reached when the radius of gyration and root mean square fluctuations of all atoms attain nearly constant values. The final dynamic working model of L was energy minimized. The three-dimensional coordinates of this model are available in the Brookhaven Protein Data Bank format upon request through electronic mail from Tkumosinski@arserrc.gov.

RESULTS

Construction of mutant Lb genes. Alignment of the amino acid sequence of putative thiol proteases encoded by a number of positive-strand RNA viruses suggested that the L protein of FMDV is related to cellular papain-like proteases which contain essential Cys and His residues as part of their catalytic domain (7). This analysis predicted Cys-23 of FMDV L as the putative catalytic residue, but because of weak sequence similarity around the catalytic His of viral papain-like proteases, His residues at positions 81, 110, and 120 of L were suggested as candidates. To determine the role of these residues in the proteinase activity of Lb, mutations were introduced individually at these sites by overlap extension PCR (11), the mutant genes were inserted into the expression vector pT7-7 under the control of a T7 promoter (Fig. 1), and the expressed proteins were assayed for enzymatic activity. From the results obtained from these experiments (Fig. 2 to 4), three-dimensional molecular modeling was performed, assuming a structural homology between L and papain and utilizing the known three-dimensional structure of papain (12). These studies suggested that Glu-48 and Asp-136 might also be involved in the enzymatic activity of Lb, and mutations were introduced individually at these sites. Figure 1 shows the positions of the mutations introduced in the Lb gene.

Processing of the L-P1 precursor by WT and mutant Lb. To determine if the mutant Lbs were able to cleave at the L/P1 junction in *trans*, crude bacterial extracts containing the Lb proteins were incubated with radiolabeled in vitro translation products of pRM-ΔL-derived transcripts. This plasmid contains a deletion in the L gene which results in a truncated L protein (ΔL) that cannot cleave itself at the L/P1 junction (21). An S10 extract from FMDV-infected bovine kidney (LF-BK) cells, bacterial extracts containing WT Lb, and mutants E48Q and H81L were able to completely process ΔL-P1-2A to P1-2A (Fig. 2, lanes 13, 4, 7, and 8, respectively). Bacterial extracts containing Lb mutant D136N had almost WT activity, while H110L extracts had somewhat reduced activity (lanes 11 and 9). Extracts containing mutants C23A, C23S, and H120L (lanes 5, 6, and 10), an extract from bacteria transformed with vector alone (lane 3), and an S10 extract from mock-infected LF-BK cells (lane 12) were completely inactive in this *trans* cleavage assay. The band migrating slightly faster than P1-2A in lanes 5, 6, and 10 is also present at essentially the same intensity in control lanes 3 and 12 as well as after incubation of substrate with WT L in the presence of L inhibitor E-64 (data not shown). Essentially identical results were obtained when unlabeled in vitro translation products from RNA transcripts of WT and mutant Lbs were incubated with radiolabeled translation products of pRM-ΔL transcripts (data not shown).

Autocatalytic cleavage by WT and mutant Lbs. The initial processing at the L/P1 junction probably occurs in *cis*, while subsequent cleavage can occur in *cis* or in *trans*. To examine the ability of the Lb mutants to process in *cis*, mutant Lbs were introduced into plasmid pLb-VP4′-3D′. This plasmid contains the complete Lb gene, 33 codons of VP4, and 163 codons of 3D. Transcripts from plasmids linearized at the end of the 3D gene were translated in a cell-free system and examined by PAGE for *cis* processing. Translation of WT Lb and mutants H81L and H110L resulted in the appearance of only Lb, demonstrating that cleavage at the L/VP4 junction was complete (Fig. 3, lanes 2, 6, and 7). Translation of mutants C23S, E48Q,

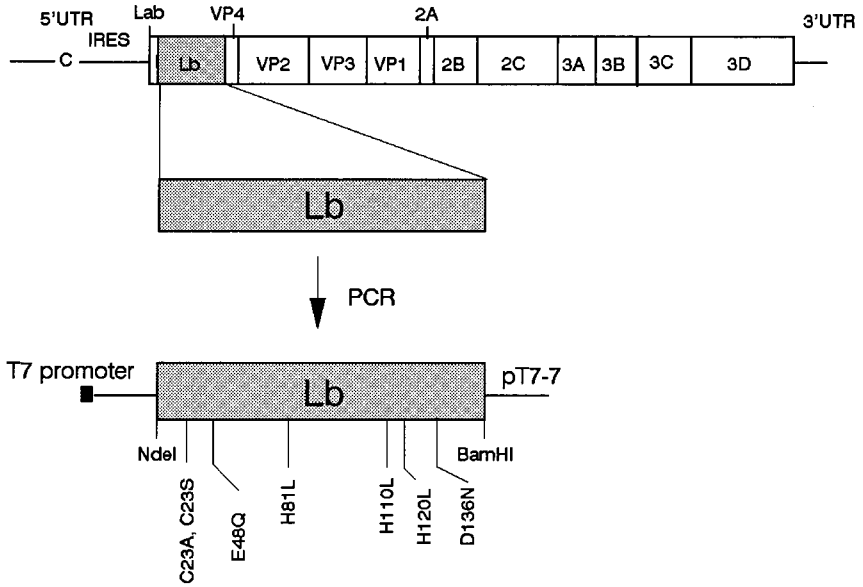

FIG. 1. Schematic representation of the FMDV genome and construction of mutant Lb genes. The FMDV genome, including the 5′ untranslated region (UTR) that contains the polycytidylic acid tract (C), the internal ribosome entry site (IRES), and the protein-coding region, and the 3′ UTR are indicated. All constructs are under the control of a T7 RNA polymerase promoter. Individual mutations introduced into the Lb gene are indicated.

and D136N resulted in the appearance of mainly Lb-VP4′-3D′, but limited processing at the L/VP4 site occurred to release Lb for all three mutants (lanes 4, 5, and 9). The limited cleavage activity of mutants E48Q and D136N was confirmed by immunoprecipitation of Lb from translation reaction mixes with L antiserum (data not shown). The Lb protein containing a mutation at either C23A or H120L was completely inactive in autocatalytic cleavage (lanes 3 and 8).

p220 cleavage. To assay for p220 cleavage by WT and mutant Lbs, crude extracts of *E. coli*-produced Lb were incubated with HeLa S10 as a source of p220. Western blot analysis with polyclonal serum against p220 revealed that WT Lb as well as mutants E48Q and H81L completely degraded intact p220 (Fig. 4, lanes 2, 5, and 6). Incubation with mutants C23S and H120L resulted in no degradation of p220 (lanes 4 and 8), while mutants H110L and D136N demonstrated some cleavage activity (lanes 7 and 9). Mutant C23A demonstrated some cleavage activity, but a densitometer scan showed that it was two to six times less active than D136N or H110L (data not shown). Furthermore, in independent experiments, mutant C23A displayed no p220 cleavage activity. Essentially identical results were obtained when in vitro translation products from RNA transcripts of WT or mutant Lbs were incubated with a source of p220 (data not shown).

Effect of mutant Lb genes on viral replication. The finding that certain Lb mutations reduced p220 cleavage more than

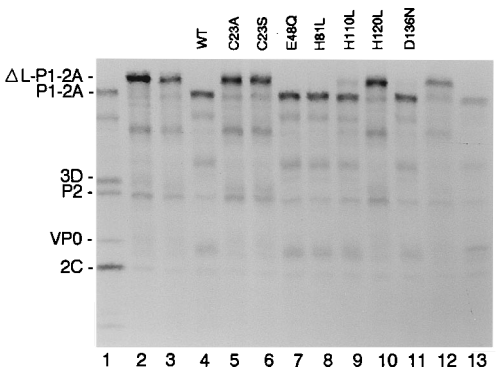

FIG. 2. Cleavage of the L/P1 junction in *trans* by WT and mutant Lb proteinases. pRM-ΔL-derived transcripts were translated in a cell-free system in the presence of [^{35}S]methionine and incubated overnight at 30°C with extracts of *E. coli* transformed with vector alone (lane 3), WT Lb (lane 4), mutants C23A, C23S, E48Q, H81L, H110L, H120L, and D136N (lanes 5 to 11, respectively) or with S10 extracts from mock-infected or FMDV-infected LF-BK cells (lanes 12 and 13, respectively) and examined by SDS-PAGE on a 15% gel. Lanes 1 and 2 are cell-free translations programmed with FMDV virion RNA and pRM-ΔL-derived transcripts, respectively.

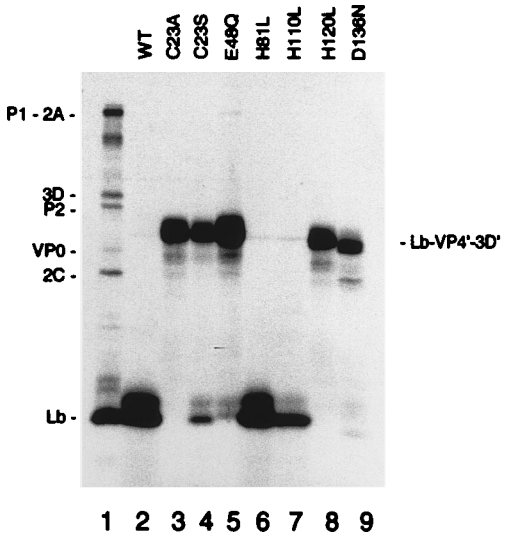

FIG. 3. Autocatalytic cleavage activity of WT and mutant Lb proteinases. RNA transcripts generated from WT pLb-VP4′-3D′ (lane 2) or its mutagenized derivatives C23A, C23S, E48Q, H81L, H110L, H120L, and D136N (lanes 3 to 9, respectively) were translated in a cell-free system, and the radiolabeled products were examined by SDS-PAGE on a 15% gel. Lane 1 is a cell-free translation programmed with FMDV virion RNA.

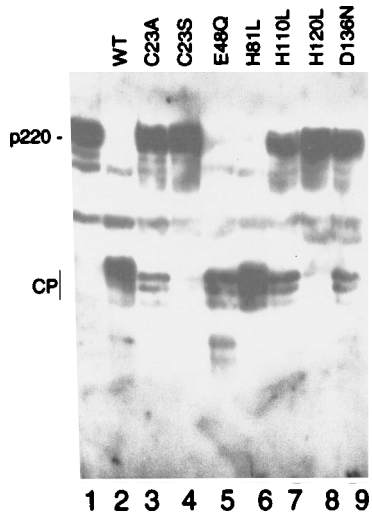

FIG. 4. Cleavage of p220 by WT and mutant Lb proteinases. HeLa S10 was incubated overnight at 30°C alone (lane 1) or with extracts of *E. coli* expressing WT Lb (lane 2) or mutants C23A, C23S, E48Q, H81L, H110L, H120L, or D136N (lanes 3 to 9, respectively). Samples were separated on a 7.5% minigel, and Western blot analysis was performed. The positions of p220 and p220 cleavage products (CP) are indicated.

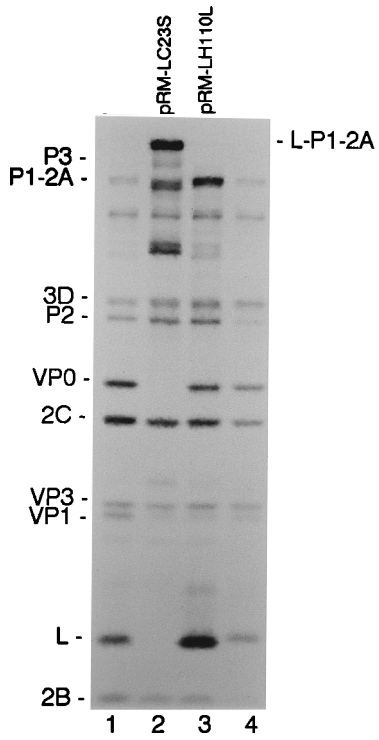

FIG. 5. Translation of full-length transcripts containing mutations in the L gene. Transcripts from cDNA clones pRM-LC23S (lane 2) and pRM-LH110L (lane 3) were translated in a cell-free system, and the radiolabeled products were examined by SDS-PAGE on a 15% gel. Lanes 1 and 4 are cell-free translations programmed with $pRMC_{35}$-derived transcripts and FMDV virion RNA, respectively.

L/P1 cleavage suggested that viruses containing these mutations could exhibit reduced ability to shut off host cell protein synthesis. To evaluate this possibility, two mutations (H110L, which exhibited greatly reduced p220 cleavage and nearly normal L/P1 cleavage, and C23S, which exhibited undetectable p220 cleavage and reduced L/P1 cleavage) were inserted separately into plasmids containing the full-length cDNA, to produce plasmids pRM-LH110L and pRM-LC23S, respectively. Translation of pRM-LC23S-derived transcripts resulted in the synthesis of a protein larger than P1-2A (L-P1-2A), drastically reduced amounts of P1-2A, and no Lb or VP0 (Fig. 5, lane 2). The high-molecular-weight protein was immunoprecipitated with L and bovine convalescent-phase antiserum and is presumably L-P1-2A (data not shown). Translation of pRM-LH110L-derived transcripts resulted in the synthesis of essentially the WT pattern of proteins, including normal amounts of P1-2A and L (Fig. 5, lane 3). Small amounts of a protein comigrating with L-P1-2A were also present in some translations. The degree of processing at the L/P1 junction by the translation products of the mutant full-length clones was essentially the same as found in the *cis* cleavage assay (compare Fig. 5, lanes 2 and 3, with Fig. 3, lanes 4 and 7).

RNA transcripts from both full-length mutant cDNAs as well as the WT infectious cDNA were transfected into BHK cells by lipofectin, and specific infectivities were determined by plaque assay. Similar specific infectivities, i.e., 3.4×10^4 to 3.6×10^4 PFU/μg, were obtained from cells transfected with $pRMC_{35}$- and pRM-LH110L-derived transcripts, but no plaques were detected in cells transfected with pRM-LC23S-derived RNA. Transcripts from pRM-LH110L produced plaques that were slightly smaller than those obtained with $pRMC_{35}$-derived transcripts. To examine viral protein synthesis in cells transfected with mutant transcripts, 5 to 10 μg of RNA was electroporated into cells. We have previously shown that electroporation is a highly efficient method of transfection and can be used to examine viral replication in the absence of a complete infectious cycle (19). [^{35}S]methionine-labeled supernatants from cells electroporated with $pRMC_{35}$-, pRM-LC23S-, or pRM-LH110L-derived transcripts were immunoprecipitated with bovine convalescent-phase serum. Viral replication occurred in these transfected cells, since nonstructural proteins 3D and 3C were present (Fig. 6). In support of these results, nonstructural proteins 3D, 2C, and 2B were immunoprecipitated from cell lysates of these transfected cells (data not shown). In cells transfected with $pRMC_{35}$- and pRM-LH110L-derived transcripts, viral structural proteins VP3, VP1, VP2, and VP4 were immunoprecipitated with convalescent-phase serum (Fig. 6, lanes 1 and 3) or monoclonal antibody 2PD11, which reacts with a conformational epitope present on virus and other capsid intermediates (data not shown) (2, 9). Sucrose gradient analysis demonstrated that virus particles were assembled in these transfected cells (data not shown). In cells transfected with pRM-LC23S-derived transcripts, viral structural proteins VP3, VP1, a band migrating slightly slower than VP0, and a higher-molecular-weight band were all precipitated from the supernatant with bovine convalescent-phase serum (Fig. 6, lane 2), 2PD11, and L antiserum (data not shown). Virus particles were not present in these transfected cells (data not shown).

The supernatants from the electroporated cells were used to infect BHK cells, and RNA extracted from this blind passage was used as a template for reverse transcription-PCR. Under these passage conditions, viable, plaque-forming virus was obtained from cells transfected with pRM-LC23S-derived transcripts, but the mutant Ser codon of the L gene had reverted to a Cys codon. The Cys codon obtained in this revertant, TGC, differed from the original mutant Ser, AGC, and the WT Cys codon, TGT, demonstrating that it was a true revertant. Virus derived from the first blind passage of material harvested from

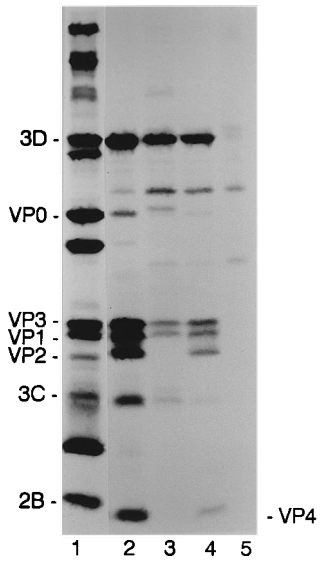

FIG. 6. Immunoprecipitation of the supernatant from cells electroporated with RNA transcripts. BHK-21 cells were electroporated with RNA transcripts from plasmids $pRMC_{35}$ (lane 2), pRM-LC23S (lane 3), or pRM-LH110L (lane 4) or mock transfected (lane 5) and radiolabeled overnight. The supernatants were immunoprecipitated with bovine convalescent-phase serum, and the products were examined by SDS-PAGE on a 15% gel. Lane 1 represents the in vitro translation products of FMDV virion RNA.

cells transfected with the pRM-LH110L-derived RNA retained the mutant Leu codon.

To examine the ability of mutant virus A12-LH110L to shut off host cell protein synthesis, either BHK-21 or LF-BK cells were infected with this virus, examined for cytopathic effects, and radiolabeled with [^{35}S]methionine at various times postinfection. The appearance of cytopathic effect was somewhat delayed compared with that in WT-infected cells, but the shutoff of host protein synthesis, the initiation of viral protein synthesis, and the cleavage of p220 occurred essentially at the same time in WT and mutant virus-infected cells (data not shown).

DISCUSSION

Amino acid residues critical for the enzymatic activity of FMDV Lb have been identified by site-directed mutagenesis (Table 1). These studies confirmed the prediction of Gorbalenya et al. (7) that the Lb protein is related to cellular papain-like proteases. Cys-23 forms a portion of the catalytic site, although we found that substitution of Cys-23 with Ser, C23S, resulted in an enzyme that retained some autocatalytic activity, suggesting that the hydroxyl group of Ser can partially substitute for the sulfhydryl group of Cys. Similar results have been obtained with poliovirus 3C proteinase (13, 17) and FMDV 3C (8). We also demonstrated that His-120, one of three His residues suggested by Gorbalenya et al. (7), is required for the catalytic activity of Lb.

Although papain-like proteinases may contain only two residues that are required for catalytic activity, structural studies have suggested that neighboring acidic amino acid chains may be important for substrate binding (3). Utilizing our findings that Cys-23 and His-120 are critical while His-81 has no role in Lb enzymatic activity and the known three-dimensional structure of papain (12), we performed molecular modeling studies on Lb. Molecular dynamic calculations constraining the distance of Cys-23 and His-120 to a value comparable to the catalytic dyad of papain were performed. The resulting three-dimensional working model is shown as a ribboned backbone structure in Fig. 7A. Also shown are the ball and stick side chain structures of Cys-23, Thr-27, Glu-48, His-110, Glu-119, His-120, and Asp-136. From this structure, residues 27, 48, and 136 are predicted to be near or involved in the active site. We mutated Glu-48 and Asp-136 to polar, uncharged residues to evaluate their involvement in enzymatic activity. Both mutants had reduced activity in L/P1 cleavage, but E48Q had WT activity in *trans* cleavage and p220 cleavage, while D136N had reduced activity in both *trans* assays. Strebel and Beck (26) demonstrated that mutation of FMDV C1 Lb at Thr-27 to Ile also altered enzymatic activity at the L/P1 junction. Since WT FMDV O1K L has an Ala at amino acid 27 (26), substitution of the bulky side chain of Ile in a residue close to the active site presumably has an adverse effect on enzymatic activity, supporting our working model.

Our studies showed that mutations at a second of the potential catalytic His residues, His-110, partially reduced cleavage of p220, indicating that a change at this position could alter the activity of the enzyme. To accommodate this information and allow alignment of L onto papain necessitated the elimination of 36 residues (amino acids 56 to 91) from papain (note that Lb is actually 36 residues shorter than papain). Visual inspection clearly showed that residues 56 to 91 (unlabeled balls and sticks, Fig. 7B) could easily be deleted from the structure without affecting the active-site residues. The resulting Lb protease model was energy minimized and equilibrated via molecular dynamic calculations. This structure is represented in Fig. 7C and exists as two domains, with the active-site residues Cys-23 and His-120 on each domain oriented toward the interface which defines the active site for the enzyme. In

TABLE 1. Characterization of L mutants[a]

Mutant	Codon change	Polyprotein cleavage (*cis*)	Polyprotein cleavage (*trans*)	p220 cleavage (*trans*)	Virus recovery
Wild type		++++	++++	++++	Yes
C23A	TGT→GCT	−	−	−/+	ND[b]
C23S	TGT→AGC	+	−	−	Reverted[c]
E48Q	GAG→CAA	+	++++	++++	ND
H81L	CAC→CTG	++++	++++	++++	ND
H110L	CAT→CTC	++++	++	+	Yes
H120L	CAC→CTC	−	−	−	ND
D136N	GAC→AAC	+	+++	+	ND

[a] The results represent averages of at least three independent experiments. Characterization of the p220 cleavage activity of L WT and mutants is based on densitometry scans and is a summary of all the independent experiments. ++++, level of WT activity; −, inactive enzyme.
[b] ND, not determined; this mutation was not transferred to a full-length cDNA.
[c] The revertant Cys codon, TGC, differed from the WT Cys codon, TGT.

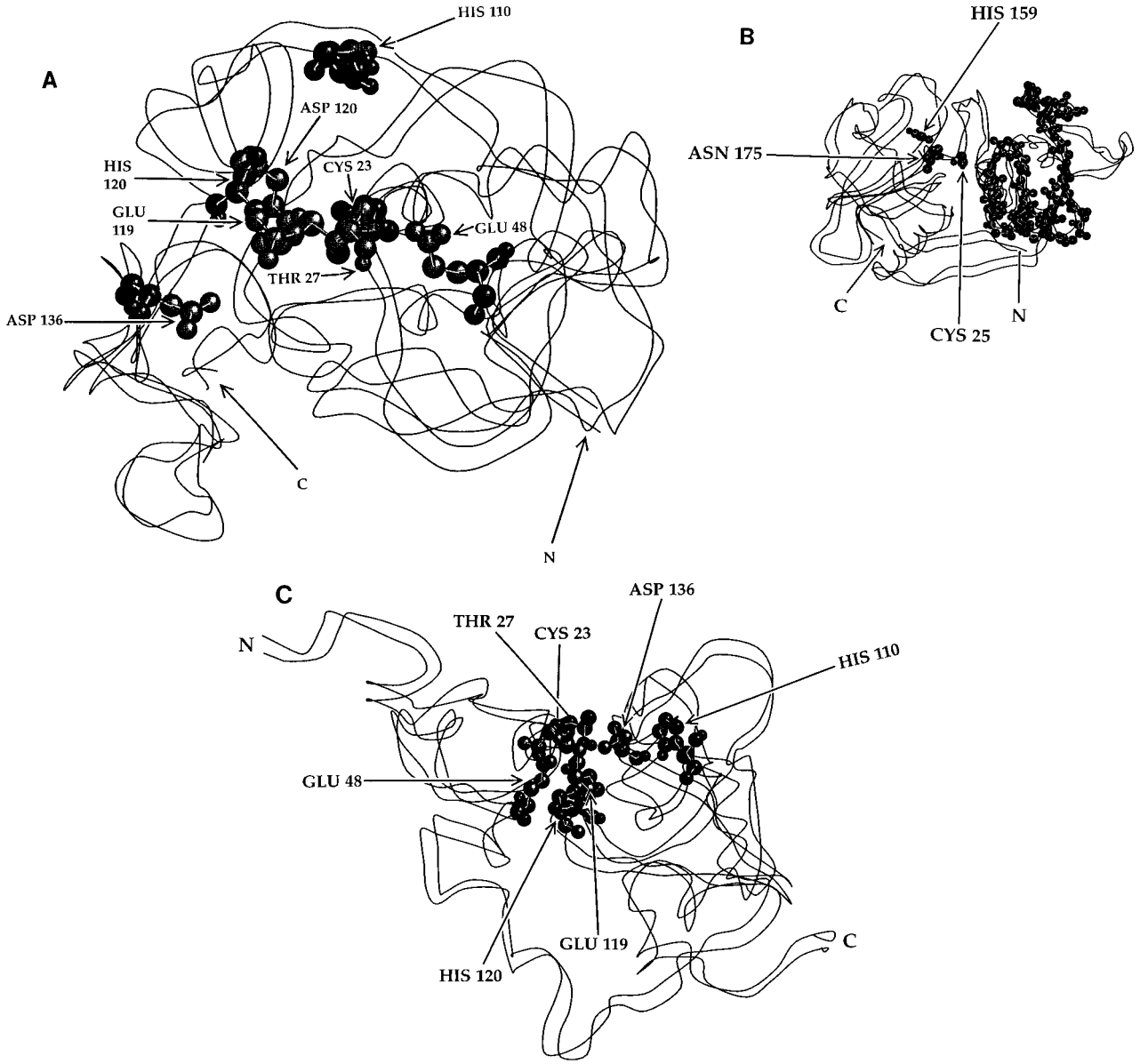

FIG. 7. Molecular model of FMDV Lb. (A) Ribboned backbone, width 2, of equilibrated and energy-minimized predicted three-dimensional model of Lb. Balls and sticks represent side chains of Cys-23, Thr-27, Glu-48, His-110, Glu-119, His-120, and Asp-136. (B) Ribboned backbone structure of papain. The unlabeled balls and sticks represent the portion of papain that was deleted in order to allow alignment of Lb onto papain. (C) Ribboned backbone structure of Lb after alignment onto papain, 60 ps of molecular dynamics equilibration, and further energy minimization. The labeled side chains of Cys-23, Thr-27, Glu-48, His-110, Glu-119, His-120, and Asp-136 are represented as balls and sticks. In each panel, N and C refer to the amino and carboxy termini of the protein. respectively.

this structure, the side chain residues of Cys-23, His-120, Glu-48, His-110, Glu-119, and Asp-136 form a hydrogen-bonded network. The Glu-48, His-110, and Asp-136 may be linked to proteolysis by increasing the substrate binding. This is in agreement with in vitro studies, but in vivo the disruption of this hydrogen-bonded network in A12-LH110L does not limit proteolysis. Nevertheless, this three-dimensional working model is an acceptable structure at present and can be used to further examine the amino acid residues of Lb that are involved in catalytic activity and substrate binding.

Mutants C23S and H110L exhibited a differential reduction in p220 cleavage, a phenotype that could result in viral attenuation in cell culture and in vivo. We introduced these mutations individually into genome-length cDNA. Transcripts harboring these mutations displayed the expected phenotype in in vitro translation and were transfected into BHK-21 cells. Comparison of the specific infectivities of RNAs carrying these mutations demonstrated that RNA with the H110L mutation was nearly wild type, whereas pRM-LC23S-derived RNA did not produce plaques in transfected monolayers. Evaluation of first-cycle viral protein synthesis in cells transfected with pRM-LH110L-derived RNA by electroporation revealed that all of the expected viral proteins were synthesized and that virus recovered from these cells maintained the H110L mutation. This virus is not attenuated in tissue culture, suggesting that the ability to partially cleave p220 is sufficient to generate a WT phenotype. Cells transfected with pRM-LC23S-derived RNA produced the expected P2 and P3 products but showed altered

P1 products, consistent with an impaired ability to cleave the L/P1 junction. Specifically, these cells produced WT VP3 and VP1, no VP2 or VP4, what appeared to be proteins that migrated more slowly than VP0, and a higher-molecular-weight protein. Interestingly, these three proteins were immunoprecipitated with a monoclonal antibody that recognized capsid intermediates and with L antiserum; similar products have been observed in cells infected with WT FMDV and treated with the papain proteinase inhibitor E-64 or E-64d (15). Following a blind passage in BHK-21 cells, virus was recovered from cells transfected with pRM-LC23S-derived RNA, but had regained a Cys codon at this position, further indicating that Cys-23 is essential for efficient processing of the L/P1 junction. Although we were able to observe some cleavage at this site by the mutant Lb in in vitro assays, efficient cleavage may be absolutely required for production of viable virus, since capsid intermediates that do not contain an accurately cleaved VP4 molecule could fail to assemble and could complex with correctly cleaved intermediates to remove structural proteins from the virion assembly pathway.

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