Mutagenesis of the Catalytic and Cleavage Site Residues of the Hypovirus Papain-Like Proteases p29 and p48 Reveals Alternative Processing and Contributions to Optimal Viral RNA Accumulation

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ABSTRACT

The positive-stranded RNA genome of the prototypic virulence-attenuating hypovirus CHV-1/EP713 contains two open reading frames (ORF), each encoding an autocatalytic papain-like leader protease. Protease p29, derived from the N-terminal portion of ORF A, functions as a suppressor of RNA silencing, while protease p48, derived from the N-terminal portion of ORF B, is required for viral RNA replication. The catalytic and cleavage site residues required for autoproteolytic processing have been functionally mapped *in vitro* for both proteases but not confirmed in the infected fungal host. We report here the mutagenesis of the CHV-1/EP713 infectious cDNA clone to define the requirements for p29 and p48 cleavage and the role of autoproteolysis in the context of hypovirus replication. Mutation of the catalytic cysteine and histidine residues for either p29 or p48 was tolerated but reduced viral RNA accumulation to ca. 20 to 50% of the wild-type level. Mutation of the p29 catalytic residues caused an accumulation of unprocessed ORF A product p69. Surprisingly, the release of p48 from the ORF B-encoded polyprotein was not prevented by mutation of the p48 catalytic and cleavage site residues and was independent of p29. The results show that, while dispensable for hypovirus replication, the autocatalytic processing of the leader proteases p29 and p48 contributes to optimal virus RNA accumulation. The role of the predicted catalytic residues in autoproteolytic processing of p29 was confirmed in the infected host, while p48 was found to also undergo alternative processing independent of the encoded papain-like protease activities.

IMPORTANCE

Hypoviruses are positive-strand RNA mycoviruses that attenuate virulence of their pathogenic fungal hosts. The prototypic hypovirus CHV-1/EP713, which infects the chestnut blight fungus *Cryphonectria parasitica*, encodes two papain-like autocatalytic leader proteases, p29 and p48, that also have important functions in suppressing the RNA silencing antiviral defense response and in viral RNA replication, respectively. The mutational analyses of the CHV-1/EP713 infectious cDNA clone, reported here, define the requirements for p29 and p48 cleavage and the functional importance of autoproteolysis in the context of hypovirus replication and exposed an alternative p48 processing pathway independent of the encoded papain-like protease activities. These findings provide additional insights into hypovirus gene expression, replication, and evolution and inform ongoing efforts to engineer hypoviruses for interrogating and modulating fungal virulence.

Many single-stranded RNA viruses that infect hosts within all kingdoms comprising the Eukaryota have evolved gene expression strategies that employ proteolytic processing of encoded polyproteins (1–4). This includes members of the family *Hypoviridae* that infect and attenuate virulence of the fungal pathogen responsible for chestnut blight, *Cryphonectria parasitica*. The two open reading frames (ORFs) encoded by the 12.7-kb genome of prototypic hypovirus CHV-1/EP713 contain N-terminal leader papain-like protease domains. Protease p29 is released from the N-terminal portion of a 69-kDa precursor polypeptide, p69, encoded by ORF A to form p29 and p40 (5, 6). Cell-free translation studies indicated that p29 processing occurs during translation (5) and identified p29 residues Cys162 and His215 as essential for autocatalytic cleavage between Gly248 and Gly249 (7). Processing of baculovirus-expressed p69 to form p29 and p40 and a requirement for Cys162 was also demonstrated in Sf9 insect cells (8).

Protease p48 is released from the N-terminal portion of CHV-1/EP713 ORF B-encoded polypeptide predicted to be in excess of 200 kDa and to contain the viral polymerase and helicase domains (6). Using a combination of cell-free translation and *Escherichia coli* expression studies, Shapira and Nuss (9) identified p48 resi-

dues Cys341 and His388 as catalytic residues essential for autocatalytic cleavage between Gly418 and Ala419. Phylogenetic analysis (10) underscored similarities between the hypovirus proteases and the papain-like plant potyvirus-encoded helper component proteases (HC-Pro) noted by Choi et al. (5) and led to the proposal that p29 and p48 are the products of an intragenomic duplication event and subsequent divergence (9, 10).

Like many viral leader proteases, p29 and p48 also serve important functional roles. Although the p29 protease is not essential for viral replication (11), it serves as a suppressor of the *C. parasitica* antiviral RNA silencing response to increase viral RNA accumulation (12–15). Unlike p29, p48 is not dispensable for viral

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replication. However, when supplied *in trans*, p48 can rescue a mutant virus lacking the p48 coding region, Δ p48 (16). Surprisingly, once rescued, the Δ p48 mutant virus continued to replicate in the absence of p48. Thus, p48 appears to have the unusual property of being required for the initiation but not the maintenance of viral RNA replication.

Although both p29 and p48 have been detected in cell extracts prepared from infected mycelium by immunological methods, processing has not been studied in the infected fungal host. Given the important functional roles played by the hypovirus leader proteases, we initiated, and report here, a mutational analysis using the CHV-1/EP713 infectious cDNA clone to define the requirements for p29 and p48 cleavage and the functional importance of autoproteolysis in the context hypovirus replication.

MATERIALS AND METHODS

Fungal strains and growth conditions. The *C. parasitica* strains used in the present study were maintained on potato dextrose agar (PDA; Difco) as previously described (15). Wild-type (WT) *C. parasitica* strain EP155 (ATCC 38755) and the isogenic strain EP713 (ATCC 52571) infected with hypovirus CHV-1/EP713 have been described by Chen and Nuss (17). The RNA silencing mutant strain containing a disruption of the Dicer gene *dcl2* (Δ dcl2) was generated in the study described in Segers et al. (18). Fungal cultures used for nucleic acid and protein preparations were grown for 7 or 10 days in potato dextrose broth (PDB; Difco) at 22 to 24°C.

Mutagenesis and modification of CHV-1/EP713 infectious cDNA clone. (i) **Point mutations of protease catalytic and cleavage site residues.** Point mutations were introduced into the p29 and p48 protease domains using a QuikChange II XL site-directed mutagenesis (Stratagene) protocol but employing the Phusion high-fidelity polymerase (New England BioLabs) in place of PfuUltra high-fidelity polymerase. Mutagenesis of the p29 coding domain was performed using plasmid pRFL4 that contains the full-length CHV-1/EP713 cDNA proceeded by the T7 polymerase promoter and flanked at each end by NotI sites cloned into a modified pTZ19 vector (US Biochemicals, Cleveland, OH) (described by Zhang et al. [19]). Using appropriate primer sets (Table 1), individual point mutations were introduced at either Cys162 or His215 to generate plasmids pRFL4-p29(C162S) and pRFL4-p29(H215S), respectively. The pRFL4-p29(C162S) construct then served as the template DNA for additional mutagenesis to generate a double mutant, pRFL4-p29(C162S:H215S).

Point mutations were introduced for p48 catalytic and cleavage site residues using a subcloned p48 coding domain. Nucleotides (nt) 392 to 4182 of plasmid pRFL4 were amplified by using the primers 391-ecoR1-FW (5'-GCATGAATTCAGTGAATTCGAGCTCGGTACC-3') and 4182-xbaI-RV (5'-CGCTCTAGAGGTATTATCTTTGGCCCTTG GC-3'), digested using EcoRI (New England BioLabs) and XbaI (New England BioLabs) restriction enzymes, and ligated into pUC19 to yield plasmid pUC-p48. Using appropriate primer sets (Table 1) individual point mutations were introduced at Cys341, His388, and Gly418 in pUC-p48. The Cys341 mutant was used to subsequently generate the Cys341:His388 double mutant, which was subsequently used to generate the Cys341:His388:Gly418 triple mutation. The mutated pUC-p48 plasmids were sequenced to confirm all mutations and digested with restriction enzymes MreI (Fermentas, Glen Burnie, MD) and NheI (New England BioLabs). The resulting fragments were used to replace the corresponding fragment in wild-type pRFL4 cDNA. The designations given to these plasmids were as follows: pRFL4-p48(C341S), pRFL4-p48(H388S), pRFL4-p48(C341S:H388S), pRFL4-p48(G418R), pRFL4-p48(C341S:G418R), pRFL4-p48(H388S:G418R), and pRFL4-p48(C341S:H388S:G418R).

(ii) **Construction of the Δ p29-p48 triple mutant.** The p29 coding domain was deleted from the p48 triple mutant plasmid pRFL4-p48(C341S:H388S:G418R) as follows. A primer set (AscI-F and 1.8KR

[Table 1]) was designed to amplify nt 1 to 1836 from plasmid pLDST- Δ p29 (20) that contains the CHV-1/EP713 viral RNA sequence lacking 88% of the p29 coding domain. The resulting amplified fragment contained only the first 25 amino acids of p29, which were shown to be essential to viral replication, as well as an introduced AscI site at the 5' terminus. Nucleotides 1836 to 4000 containing the p48 catalytic and cleavage site mutations were then amplified (primers 1.8KF and 4KR) from plasmid pRFL4-p48(C341S:H388S:G418R). The two fragments were stitched together by a second round of PCR using the primers AscI-F and 4KR to create the final Δ p29-p48 triple mutant fragment, which was subsequently cloned into a PCR-blunt vector (Zero-Blunt PCR cloning kit; Invitrogen, Carlsbad, CA). Individual isolates were sequenced to confirm that the point and deletion mutations were maintained, and the resulting plasmid, Δ p29-p48(C341S:H388S:G418R)-blunt, was digested with restriction enzymes AscI (Fermentas) and NheI. The resulting fragment was used to replace the corresponding fragment in plasmid pRFL4, containing the wild-type cDNA clone of CHV-1/EP713 virus. Individual isolates were sequenced to confirm that the desired deletion was still present and no additional point mutations were accidentally generated. The resulting plasmid was designated pRFL4(Δ p29)-p48(C341S:H388S:G418R).

(iii) **Construction of CHV-1/EP713 virus containing a 6 $\times$ His tag within the p48 coding domain.** Overlapping PCR was used to introduce a 6 $\times$ His epitope tag between amino acid residues 14 and 15 within the p48 coding region of wild-type RFL4 and the triple mutant RFL4-p48(C341S:H388S:G418R) viral cDNAs. Using the primer set 1KF/p48-6 $\times$ His-R and p48-6 $\times$ His-F/5KR (Table 1), PCR was performed with plasmid pRFL4 as the template to generate two fragments, containing overlapping ends. The two fragments were stitched together by a second round of PCR using the primers 1KF and 5KR to create the final fragment containing the 6 $\times$ His sequence. The resulting fragment was ligated into a PCR-blunt vector, and individual isolates were sequenced to confirm the insertion sequences. The resulting plasmids containing the 6 $\times$ His tag [p48(His)blunt and p48(C341S:H388S:G418R)(His)blunt] were digested with the restriction enzymes MreI and NheI, and the resulting fragments were used to replace the corresponding fragment in plasmid pRFL4, containing a wild-type cDNA clone of CHV-1/EP713 virus. Individual isolates were sequenced to confirm that the desired deletion was still present and that no additional point mutations were accidentally generated. The resulting plasmids were termed pRFL4-p48(His) and pRFL4-p48(C341S:H388S:G418R)(His). In addition, a 6 $\times$ His tag was introduced into the pRFL4-p48(C341S), pRFL4-p48(H388S), and pRFL4-p48(G418R) mutant viral cDNAs using the methods outlined above.

Transfection of *C. parasitica* spheroplasts with mutant CHV-1/EP713 viral transcripts and characterization of recovered mutant virus RNA. Infection of fungal strains with hypovirus CHV-1/EP713 and mutant viruses was initiated by electroporation-mediated transfection of fungal spheroplasts with synthetic transcripts generated *in vitro* from SpeI-linearized viral cDNA clones using methods previously described by Suzuki and Nuss (21) and Chen et al. (22). Surviving spheroplasts were cultured on osmotic solid regeneration media for 7 to 10 days to allow cell wall regeneration and then transferred to PDA plates for phenotypic characterization and analysis.

cDNA clones of the viral replicative form double-stranded RNA isolated from mutant virus-infected strains were generated through the use of a Monstscript first-strand cDNA synthesis kit (Epicentre Biotechnologies, Madison, WI). In accordance with the manufacturer's protocols, primer 12.5KR (Table 1) was used to prime cDNA synthesis starting at the 3'-terminal end of the viral RNA. PCR was subsequently performed using primer set 1KF-5KR to generate a 4-kb product that was sequenced (Macrogen, Inc., Rockville, MD) to confirm that no changes in sequence had been introduced into the mutated p48 coding domain or flanking regions. The sequencing primers included 1KF, 2KF, 3KF, 4KF, 2KR, 3KR, 4KR, and 5KR (Table 1). Sequence analysis of the mutated viral p29 coding domain and flanking regions was performed on a 3-kb fragment amplified

TABLE 1 Primers used in this study

Name	Sequence (5'–3')	Nucleotide region	Note
NotIF and KF/KR primers			
NotIF	ggatccgcggccgcGCCTATGGGTGGTCTACA	1–14	The NotI sequence is in lowercase; the CHV-1/EP713sequence is in uppercase
1KF	ATCTCTCGGCCATTGTAG	979–996	
2KF	CACGACCCGGTTGAGGTC	1976–1993	
3KF	GGAAATGGATCTATGGTC	2976–2993	
4KF	CCTCCGCGATTTGTGGGA	3976–3993	
1KR	CTACAATGGCCGAGAGAT	979–996	
2KR	GACCTCAACCGGGTCGTG	1976–1993	
3KR	GACCATAGATCCATTTC	2976–2993	
4KR	TCCCACAAATCGCGGAGG	3976–3993	
5KR	CGGGATCAAGAGCGTGGA	4976–4993	
12.5KR	CTTTGTTGTTCTTCCC	12688–12705	
p29 protease primers			
p29-162S-F	GGGCAGGGCTACT <u>TCGT</u> TATCTCTCGGCC	963–990	The Cys-to-Ser point mutation site is underlined
p29-162S-R	GGCCGAGAGATAC <u>GAG</u> TAGCCCTGCCC	963–990	The Cys-to-Ser point mutation site is underlined
p29-215S-F	GACCATGTTTAT <u>TCCG</u> TGGTCGTCGAC	1122–1149	The His-to-Ser point mutation site is underlined
p29-215S-R	GTCGACGACCAC <u>GGA</u> AATAACATGGTC	1122–1149	The His-to-Ser point mutation site is underlined
p48 protease primers			
p48-341S-F	CGAAGAGGGTCGA <u>AGCT</u> TTCGAGCTCTTGTC	3128–3158	The Cys-to-Ser point mutation site is underlined
p48-341S-R	GAACAAGAGCTCGA <u>AGCT</u> TTCGACCCTCTTCG	3128–3158	The Cys-to-Ser point mutation site is underlined
p48-388S-F	GTGACCAATGCGTG <u>AGC</u> ATTGTCGCTGGTGAAACC	3268–3302	The His-to-Ser point mutation site is underlined
p48-388S-R	GGTTTACCAGCGACAAT <u>GCT</u> CACGCATTGGTCAC	3268–3302	The His-to-Ser point mutation site is underlined
p48 cleavage site primers			
p48-418R-F	GCCTGACATCCTCGTT <u>AGGG</u> GCTGAAGAAGGTTCAAGTCGC	3595–3634	The Gly-to-Arg point mutation site is underlined
p48-418R-R	GCGACTGAACCTTCTTCAGC <u>CCCT</u> AACGAGGATGTCAGGC	3595–3634	The Gly-to-Arg point mutation site is underlined
Δp29 primers			
AscI-p29-F	atcgatggcgcgcGCCTATGGGTGGTCTACATAGG	1–22	The AscI site is in lowercase; the CHV1/EP713 sequence is in uppercase
1.8KF	CGACGCAAAGATTCAAGTCATAGGG	1812–1836	
1.8KR	CCCTATGCACTGAATCTTTGCGTCG	1812–1836	
p48-6×His tag primers			
p48-His-F	catcatcaccatcacca <u>GGTAGCGGT</u> CAGGTCATGGACGGGCCAACATGG	2402–2425	The 6×His tag is in lowercase; the GSG linker is underlined; the p48 is in uppercase
p48-His-R	gtggtgatggtgatgatg <u>ACCGCTACCT</u> GTTTCGCCACACTTCAATAGGTCG	2378–2401	The 6×His tag is in lowercase; the GSG linker is underlined; the p48 is in uppercase

FIG 1 Diagram of the CHV-1/EP713 viral genome indicating the positions of p29 and p48 catalytic and cleavage site residues. The p29 essential catalytic residues were mapped by *in vitro* studies to Cys162 and His215 with cleavage at the Gly248/Gly249 dipeptide (5, 7, 8). Similar analyses identified Cys341 and His388 as the essential p48 catalytic residues with self-cleavage occurring between Gly418 and Ala419 (9). The asterisk (*) denotes the location of a 6×His tag placed after amino Thr14. The catalytic residues of both p29 and p48 were mutated to serine and Gly418 at the p48 cleavage dipeptide was changed to an Arg, mutations previously shown to prevent autoproteolysis *in vitro* (7, 9).

with primers Not1F, 1KF, 1KR, 2KF, 2KR, 3KF, and 3KR (Table 1). Sequence analysis was performed using DNASTAR Lasergene 10 (Madison, WI) software.

Viral RNA and protein extraction and analysis. Cultures used for viral RNA and protein analysis were grown in 200 ml of PDB for 7 days. Mycelium was harvested by filtration through Miracloth (Calbiochem, La Jolla, CA), frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle.

(i) Viral RNA extraction and quantification. Mycelial powder was resuspended in 4 ml of RNA extraction buffer (100 mM Tris-HCl [pH 8.0], 200 mM NaCl, 4 mM EDTA, 2% sodium dodecyl sulfate), and total nucleic acid was extracted sequentially twice with phenol-chloroform and once with chloroform and precipitated by the addition of 2 volumes of ethanol. The extracted nucleic acid samples were treated with RQ1 DNase I (Promega, Madison, WI) to eliminate fungal chromosomal DNA, followed by two rounds of phenol-chloroform extractions, ethanol precipitation, and resuspension in double-distilled water. Total RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The relative levels of viral RNA accumulation for wild-type and mutant viruses were measured by semiquantitative real-time reverse transcriptase PCR (RT-PCR) as described by Suzuki and Nuss (21).

Calculations of viral RNA levels were performed with the comparative threshold cycle method ($\Delta\Delta C_T$) normalized against the 18S rRNA levels (21). The relative viral RNA levels were reported as a percentage of the value for CHV-1/EP713-infected extracts, with standard deviations indicated by error bars, based on three replicates each for three independent infected colonies cultured in parallel.

(ii) Total protein extraction and Western blot analysis. Total protein extracts for Western blot analysis were prepared according to the method of Parsley et al. (23) using TEDS-C extraction buffer (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM dithiothreitol, 150 mM NaCl, 1% [wt/vol] CHAPS). Immunoblot analysis of relative p29 and p48 protein levels was performed as described previously (6) with rabbit antisera raised against recombinant hypovirus CHV-1/EP713 proteins expressed from map positions nt 496 to 898 (α p15) for p29 and nt 2364 to 4070 (α B1) for p48. The antigen used to generate antibody α B1 included all of the p48 coding region and extended 150 codons past the p48 Gly418-Ala419 cleavage dipeptide. Commercial antibodies were used for immunoblot analysis of p48 proteins containing the 6×His epitope tag (Thermo Scientific).

RESULTS

Mutation of the p29 catalytic residues. The cysteine and histidine residues shown to be essential for p29-mediated autoproteolysis when translated in cell extracts (7) or expressed in Sf9 insect cells (8) were mutated in the CHV-1/EP713 infectious cDNA clone to determine whether they are also required for p29 processing and

for viral replication in the infected host (Fig. 1). Transfection of *C. parasitica* strain EP155 spheroplasts with viral transcripts containing the Cys162 mutation [CHV-1/p29(C162S)], the His215 mutation [CHV-1/p29(H215S)], or the double mutation [CHV-1/p29(C162S:H215S)] all resulted in productive infections, as judged by the expression of typical infection symptoms (Fig. 2A).

Deletion of 88% of the p29 coding domain (Δ p29 virus) was shown previously to reduce viral RNA accumulation by more than 50% (21). Based on real-time RT-PCR analysis, point mutations of p29 Cys162 and His215 also resulted in a similar level of reduction in viral RNA accumulation (Fig. 2B). The recovered mutant viral RNAs were also analyzed by RT-PCR amplification and sequencing to confirm that the mutations were still present and that no compensatory mutation had been generated within the ORF A coding domain.

Western blot analysis with p29-specific antibody failed to detect p29 or precursor proteins in extracts prepared from strain EP155 infected with the p29 mutant viruses. Consequently, the p29 mutant viruses were introduced into the *C. parasitica* Δ dcl2 strain in which the host antiviral RNA silencing response is disrupted by deletion of the Dicer 2 gene *dcl2* (18). The active RNA silencing pathway degrades hypovirus RNA and promotes viral recombination-mediated production of internally deleted defective interfering (DI) RNAs and the instability of sequence tags introduced into viral coding domains (14, 15). Thus, the Δ dcl2 strain serves as a useful experimental tool because of the increased accumulation of CHV-1/EP713 RNA and proteins, the absence of DI RNAs, and the stability of recombinant viruses. Disruption of the antiviral RNA silencing response pathway in the Δ dcl2 strain also results in a very severe slow-growth phenotype when infected by CHV-1/EP713. As shown in Fig. 2A, a similar colony morphology and growth phenotype was observed upon infection by the CHV-1/EP713 wild-type and p29 protease mutant viruses.

Western blot analysis with anti-p29 antibody showed that the processed p29 protein found in extracts from the CHV-1/EP713-infected Δ dcl2 strain was replaced by the unprocessed p69 protein in p29 protease mutant virus-infected cell extracts (Fig. 3). A significant difference was also observed in the relative accumulation of p29 in the WT-infected strain and p69 in the mutant virus-infected strains; 50-fold less of the WT virus-infected cell extract was required to give a Western blot signal similar to that for the p29 mutant virus extracts (Fig. 3). This difference is much greater than the difference in viral RNA accumulation levels for the wild-

FIG 2 Mutagenesis of p29 catalytic residues in a CHV-1/EP713 infectious cDNA clone. (A) Colony morphology of *C. parasitica* infected with CHV-1/EP713 p29 mutants. Synthetic RNA transcripts derived from cDNA plasmids containing mutations to p29 catalytic residues Cys162 and His215 were transfected into EP155 and $\Delta dcl2$ spheroplasts. Mutation of the p29 catalytic residues did not significantly alter EP155 or $\Delta dcl2$ colony morphology or pigmentation compared CHV-1/EP713-infected isolates. (B) Real-time RT-PCR measurement of mutant p29 viral RNA accumulation. Accumulation of p29 mutant viral RNA in infected fungal strain EP155 was measured as described in Materials and Methods. Viral RNA levels are reported as percentages of the value for CHV-1/EP713-infected extracts, with standard deviations indicated by the error bars, based on three replicates each for three independent infected colonies cultured in parallel.

FIG 3 Western blot analysis of ORF A processing. Protein extracted from $\Delta dcl2$ strain-infected isolates was analyzed by Western blotting with a polyclonal antibody to detect p29 (α p15). The CHV-1/EP713-infected $\Delta dcl2$ strain (lane 2) displayed a large accumulation of viral protease p29. Mutation of either p29 catalytic residue Cys162 (lane 3), residue His215 (lane 4), or both catalytic residues Cys162 and His215 (lane 5) resulted in an apparent absence of mature p29 and a buildup of unprocessed p69. In addition, mutation of the catalytic residues resulted in a large decrease in total viral protein levels. To avoid overloading p29 isolated from CHV-1/EP713-infected isolates, total protein levels were diluted 50-fold compared to protein extracted from isolates infected with the p29 protease mutant viruses, as reflected in the differences in signals for the actin control lanes at the bottom. Lane 1 contained protein extract from the uninfected $\Delta dcl2$ strain.

type and p29 mutant viruses (Fig. 2B) and suggests that p69 may be less stable than p29. The combined results confirm that Cys162 and His215 are required for p29 processing in the infected host. However, the catalytic residues are not required for viral replication but do contribute to optimal viral RNA accumulation.

Mutation of p48 protease residues. Shapira and Nuss (9) reported that Cys341 and His388 were essential for autoproteolytic release of p48 from the precursor polypeptide in cell-free translation studies. Similar to the results for the p29 protease mutant viruses, viral transcripts containing mutations of these residues independently or in combination were all found to establish a productive infection in the EP155 and $\Delta dcl2$ strains (Fig. 4A). Infection of strain EP155 with the C341S mutant virus derived from plasmid pRFL4-p48(C341S) resulted in phenotypic changes indistinguishable from those caused by wild-type CHV-1/EP713. Infection with the H388S mutant [from plasmid pRFL4-p48(H388S)] and double mutant [from plasmid pRFL4-p48(C341S:H388S)] consistently resulted a slightly different colony morphology characterized by fewer aerial hyphae and increased orange pigmentation.

The effect of the p48 protease mutations on viral RNA accumulation was examined by real-time RT-PCR. Mutant virus RNAs were

FIG 4 Mutagenesis of p48 catalytic residues in the CHV-1/EP713 infectious cDNA clone. (A) Colony morphology of EP155 and $\Delta dcl2$ strains transfected with RNA transcripts corresponding to wild-type CHV-1/EP713 and p48 protease mutant viruses CHV-1/p48(C341S), CHV-1/p48(H388S), and CHV-1/p48(C341S:H388S). Infection of strain EP155 with CHV-1/p48(C341S) produced a fungal phenotype similar to CHV-1/EP713, including a white phenotype and a reduction in sporulation. Infection of EP155 with CHV-1/p48(H388S) and CHV-1/p48(C341S:H388S) resulted in further reduction of aerial hyphae; however, there was increased fungal pigmentation compared to CHV-1/EP713 infection. Infection of the $\Delta dcl2$ strain with the p48 protease mutant viruses produced a phenotype similar to that of CHV-1/EP713-infected colonies. (B) Real-time RT-PCR quantification of viral RNA accumulation for p48 catalytic residue mutant viruses. Viral RNA accumulation levels were measured as described in Materials and Methods and are reported as percentages of the value obtained for CHV-1/EP713-infected EP155 fungal extracts, with standard deviations indicated by the error bars, based on three replicates each for three independent infected colonies cultured in parallel.

found to accumulate to substantially lower levels than wild-type virus RNA, with the double mutant virus showing the largest decrease; a greater than 80% reduction (Fig. 4B). In addition, viral RNA was screened by RT-PCR and sequenced to confirm that the protease mutations, C341S and H388S, were still present and that the virus had not reverted or generated a compensatory mutation to allow for virus replication. Thus, transcripts derived from the p48 protease mutant plasmids were able to establish an infection when introduced into *C. parasitica* spheroplasts, indicating that the catalytic residues are not essential for virus replication.

Similar to the results for detection of p29 related proteins, p48-related proteins were not detectable by Western blotting in extracts prepared from strains EP155 infected with the p48 mutant viruses. Western analysis of extracts prepared from the $\Delta dcl2$ strain infected with the p48 mutant viruses detected a strong signal at the migration position of p48 (Fig. 5), indicating that p48 processing occurs in the infected host even when both Cys341 and His388 are mutated.

Mutation of the p48/ORF B cleavage site does not prevent p48 processing. Given the discrepancy in results for *in vitro* and *in*

vivo processing of p48 Cys341 and His388 mutants reported above, we next sought to determine whether the G418R mutation at the p48 cleavage site would prevent processing in the infected cell as had previously been reported in cell-free translation studies (6). The G418R

FIG 5 Western blot analysis of extracts from p48 mutant virus-infected $\Delta dcl2$ strain with anti-p48 antibody (α B1). Despite mutation of the p48 catalytic residues, a signal with a similar migration position was observed for extracts isolated from the $\Delta dcl2$ strain infected with full-length CHV-1/EP713 (lane 1) and infected with p48 protease mutant viruses [lane 2, CHV-1/p48(C341S); lane 3, CHV-1/p48(H388S); lane 4, CHV-1/p48(C341S:H388S)]. The WT p48 protein was diluted 10-fold compared to mutant p48 before loading the gel. The p48 protein was not detected in protein extracts from an uninfected $\Delta dcl2$ isolate (lane 5).

FIG 6 Mutagenesis of the p48 cleavage site. (A) Colony morphology of EP155 and $\Delta dcl2$ isolates infected with p48 cleavage site mutant viruses. The EP155 and $\Delta dcl2$ fungal strains were transfected with RNA transcripts derived from p48/ORF B cleavage site mutant virus cDNA plasmids. Fungal colonies were photographed after culturing on PDA plates for 7 days. Infection of EP155 with the CHV-1/p48(G418R) mutant virus resulted in a hypovirulent phenotype similar to wild-type CHV-1/EP713. Infection of EP155 with a CHV-1 virus containing a mutation to the protease residues and the cleavage site residue results in an increase in fungal pigmentation. When the p48 mutant viruses were introduced into $\Delta dcl2$ spheroplasts, the infected fungal isolates displayed a faster growth rate and increased pigmentation compared to CHV-1/EP713 infection. (B) Western blot analysis of extracts isolated from the $\Delta dcl2$ strain infected with 6 $\times$ His-tagged WT and mutant p48 viruses. Lane 1, untagged CHV-1/p48; lane 2, CHV-1/p48(His); lane 3, CHV-1/p48(C341S)(His); lane 4, CHV-1/p48(H388S)(His); lane 5, CHV-1/p48(G418R)(His); lane 6, CHV-1/p48(C341S:H388S:G418R)(His).

mutation was introduced individually and in combination with either one or both of the p48 protease mutants. Upon transfection of RNA transcripts into EP155 and $\Delta dcl2$ spheroplasts through electroporation, all mutant viruses were replication competent and caused a decrease in pigmentation and sporulation (Fig. 6A). Mutant CHV-1/EP713 isolates (i.e., C341S:G418R, H388S:G418R, and C341S:H388S:G418R) displayed more pigmentation than WT CHV-1/EP713. This is likely to be the result of a decrease in viral RNA accumulation by the mutant viruses.

Western blot analysis on total protein extracts from the cleavage site mutant virus-infected strain $\Delta dcl2$ indicated that p48/ORF B processing was still occurring (Data not shown). The antibody used in these Western blot analyses was a polyclonal antiserum made against a protein that consisted of all of p48 and 150 codons past the putative p48 cleavage site (G418/A419). To confirm the immune blot analysis results using independent monoclonal detection antibodies, epitope tags were introduced into the p48 coding domain just after Thr14 in the following mutant viruses; CHV-1/p48(C341S), CHV-1/p48(H388S), CHV-1/p48(G418R) and CHV-1/p48(C341S:H388S:G418R), as well as in wild-type CHV-1/EP713. Immunoblot analysis with a monoclonal anti-6 $\times$ His antibody (Pierce Scientific) confirmed processing of the epitope-tagged p48 for each of the p48 mutant viruses (Fig. 6B). As observed with the anti-p48 polyclonal antisera, accumulation of the tagged p48 protein was considerably lower in extracts from p48 mutant virus-infected mycelia than from WT virus-infected mycelia.

Processing of mutant p48 occurs in the absence of protease p29. One possible explanation for catalytic/cleavage site mutant

p48/ORF B processing is that protease p29 has the ability to process ORF B and is responsible for the low level of viral replication that is observed. To investigate this possibility, the p29 coding domain, with the exception of the first 25 amino acids, which are essential for viral replication, was deleted from the plasmid encoding the CHV-1/p48(C341S:H388S:G418R) mutant virus to generate a CHV-1/ Δ p29-p48(C341S:H388S:G418R) mutant virus. Corresponding synthetic RNA transcripts were generated and electroporated into $\Delta dcl2$ spheroplasts. As shown in Fig. 7, the CHV-1/ Δ p29-p48(C341S:H388S:G418R) mutant virus was replication competent, and mutant p48/ORF B processing was observed. These results indicate that processing of the mutant p48 protein is independent of the virus-encoded papain-like protease activities and suggests that p48 processing can also occur through an alternative nonviral, host-dependent pathway.

DISCUSSION

The hypovirus papain-like leader proteases p29 and p48 have been studied extensively both *in vitro* and *in vivo*, but the requirements for and importance of autoproteolysis had not been examined in the context of virus infection. *In vitro* expression analysis and mutagenesis clearly demonstrated autocatalytic activity for both proteins and identified essential catalytic residues and cleavage dipeptides (5, 8, 9). Important functional roles in suppression of the host RNA silencing antiviral defense response and viral RNA replication have been established for p29 (12–15) and p48 (16), respectively. We report here that the essential autocatalytic and cleavage site residues required for p29 and p48 cleavage *in vitro* were dispensable for viral replication but contributed to optimal

A

CHV-1/ Δ p29-p48(C341S:H388S:G418R)

Δ dcl2

B

FIG 7 Protease p29 not responsible for p48 cleavage. (A) Phenotype of a Δ dcl2 fungal isolate transfected with RNA transcripts derived from a CHV-1/ Δ p29-p48(341S:388S:418R) mutant virus cDNA plasmid. The infected fungal phenotype is similar to one exhibited by CHV-1/EP713 infection (Fig. 2A). (B) Western blot analysis of proteins extracted from uninfected Δ dcl2 strain (lane 1) and Δ dcl2 strain infected with CHV-1/EP713 (lane 2) or the CHV-1/ Δ p29-p48(C341S:H388S:G418R) mutant virus (lane 3). The CHV-1/EP713-infected Δ dcl2 protein extract was diluted 10-fold compared to the extract from the mutant p48 virus extract. Protein p48 was detected by using a polyclonal antibody against p48 (α B1).

viral RNA accumulation. Surprisingly, processing of p48 continued despite mutations of the essential catalytic and cleavage site residues and elimination of p29.

The dispensability of p29 and p48 autoproteolytic activity for hypovirus replication was unexpected given the reported requirement of autoproteolysis by the HC-Pro leader protease for potyvirus genome RNA replication (24). Phylogenetic analysis predicted a common ancestry for hypoviruses and the plant potyviruses (10). Moreover, alignment of HC-Pro, p29, and p48 amino acid sequence revealed striking similarities in the sequences flanking the essential Cys and His catalytic residues, the nature of cleavage sites and the distance between the catalytic and cleavage site residues (7). HC-Pro and p29 also share similar functional roles as suppressors of RNA silencing. However, unlike the results reported here for p29 and p48, HC-Pro active-site mutations were lethal.

Although leader proteases play pivotal roles in the infection cycle of numerous positive-strand RNA viruses, the degree to which viral replication is dependent on the autocatalytic activity varies considerably. Coronavirus murine hepatitis virus (MHV) remained replication competent, but accumulated to a much lower titer, following mutagenesis of the catalytic cysteine residue and cleavage sites of leader protease PLP1 (25, 26). Mutation of the catalytic cysteine for PL2^{Pro} of human coronavirus 229E (HCoV229E) was found to be lethal, while the autoproteolytic

activity for the paralogous PL1^{Pro} was dispensable (27). Mutation of the catalytic residues of the papain-like protease domain, PCP α , of the nsp1 α protein of the arterivirus porcine reproductive and respiratory syndrome virus, completely blocked subgenomic RNA synthesis but did not negatively affect genome RNA replication. In contrast, similar mutations of the protease domain, PCP β , in the adjacent nsp1 β , completely blocked viral RNA replication (28). A pattern similar to that observed for HCoV229E was reported for the tandem leader proteases of the closterovirus *Grapevine leafroll-associated virus 2*, where the autocatalytic cleavage by L2, but not L1, was found to be essential for viral genome RNA replication (29). The differences in the dispensability of autocatalytic activity for viral replication likely reflect the genetic flexibility and functional diversity of viral papain-like leader proteases.

The roles of Cys162 and His215 in p29 self-processing, previously identified *in vitro*, were confirmed in the infected host in the present study. The apparent processing of p48, even after mutation of the catalytic residues and cleavage site and in the absence of p29, was unexpected (Fig. 5, 6B, and 7). We are unaware of any similar reports for other virus-encoded papain-like viral leader protease. Extensive computer-assisted searches failed to detect similarities between any CHV-1-encoded proteins and serine, cysteine, and acidic or picornavirus 3C-like proteases (10). This raises the possibility that the mutant p48 undergoes alternative processing via a nonviral, host-dependent pathway. Examples of cellular processing of viral proteins include Kex2 processing of the L-A virus-encoded yeast killer toxin (30) and furin-mediated maturation of surface proteins of positive- and negative-strand RNA viruses and retroviruses (see, for example, references 31, 32, 33, and 34). Efforts to identify the mutant p48 cleavage site have been unsuccessful and are actively being pursued.

Although autocatalysis by p29 and p48 is dispensable for viral replication, both activities are required for optimal viral RNA accumulation. This observation fits with the suggestion offered by Denison et al. (25) for the PLP1 leader protease of MHV that autocatalysis evolved after the functions of the cleavage products to increase replication efficiency. Combined with the amino acid sequence similarities and different functional roles for p29 and p48, the following series of events is envisioned. A portion of the p48 coding domain played an essential function role in the replication of an ancestral hypovirus and was released from a larger protein through a cellular processing pathway. The cellular pathway may have involved multiple steps, required intracellular transport, or resulted in lower accumulation of processed p48. The acquisition of virus-autonomous cotranslational autoproteolytic processing of p48 resulted in increased efficiency of viral RNA replication. The p48 protease then underwent duplication to form the p29 progenitor. Through divergent evolution, p29 gained the auxiliary function of suppressing the RNA silencing antiviral defense response but retained the autoproteolytic activity. Under this scenario, the RNA silencing suppression function of p29 would be dispensable, whereas the replication function of p48 would remain essential, and mutation of the p29 catalytic residues would eliminate p29 processing, whereas p48 processing would continue through the alternative cellular pathway. It is conceivable that both the autocatalytic release and the alternative cellular processing of p48 operate during hypovirus infection and that disruption of the former reduces viral RNA replication efficiency while disruption of the latter is lethal. Such a dependence

on a cellular processing pathway could serve as a host range determinant, a possibility that warrants further investigation.

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