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Short Communication

Further *in vitro* characterization of mouse hepatitis virus papain-like proteinase 1: Cleavage sequence requirements within PP1a

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Proteolytic processing of the mouse hepatitis virus strain A59 (MHV-A59) replicase gene product, pp1a, results in polypeptides p28, p65, p50, and p240 in infected cells. Based on previously identified p28 and p65 cleavage sites, a p50 cleavage site was proposed to occur between Ala-1262 and Ala-1263. Results of mutagenesis and *in vitro* cleavage assays show that PLP-1 was able to cleave in *trans* when the proposed p50 cleavage sequence replaced the p28 cleavage sequence. Mutagenesis was also used to investigate cleavage between Gly-904 and Val-905, a cleavage site predicted to produce a precursor of p65, p72, that was detected in cells infected with MHV strain JHM, but not with MHV-A59. No cleavage could be detected using substrate that carried both the p65 site and the predicted p72 cleavage sequence. Thus, it appeared that PLP-1 could recognize the proposed p50 sequence but not the predicted p72 site under the *in vitro* conditions used. *Journal of NeuroVirology* (2002) **8,** 143–149.

Keywords: proteolytic processing; viral papain-like proteinase

Mouse hepatitis virus (MHV) is an enveloped virus that belongs to the Coronaviridae family. The genome of MHV is a 31-kb positive-sense RNA. In infected cells, the 5′ end of the genome (gene 1) is translated to produce an RNA-dependent RNA polymerase. This viral-encoded RNA polymerase transcribes negative-sense template RNA, from which a nested set of six 3′-coterminal subgenomic mRNAs, encoding the structural and other nonstructural genes, are transcribed (de Vries *et al*, 1997). Gene 1, which is 21.7 kb, contains two open reading frames (ORFs), ORF1a and ORF1b. Sequence analysis has led to the proposal that ORF1a gene product, pp1a, contains two papain-like proteinase (PLP, also referred to as PCP; Baker *et al*, 1989) domains, an X domain of unknown function adjacent to PLP-1, and a poliovirus 3C-like proteinase domain (3CLpro) flanked by two hydrophobic domain regions (HD1 and HD2; Figure 1A). Transcriptional readthrough of the ORF1a termination codon via a frameshift mechanism results in translation of ORF1b and synthesis of pp1ab. It has been proposed that proteolytic processing of pp1ab releases the RNA polymerase, putative helicase, NTPase, and zinc-binding domains (Lee *et al*, 1991). Similar genome organizations and replication strategies among members of the Arteriviridae and Coronaviridae families led to their classifcation under the order Nidovirales (de Vries *et al*, 1997; Snijder and Meulenberg, 1998). Inhibition of proteolytic processing during coronaviruses or arteriviruses infection results in reduced viral RNA synthesis, indicating the important roles played by proteinase in viral maturation and infectivity (Kim *et al*, 1995; de Vries *et al*, 1997; Liu *et al*, 1997; Herold *et al*, 1998; Lim and Liu, 1998; Snijder and Meulenberg, 1998; Shi *et al*, 1999).

Previous studies with MHV strain A59 (MHV-A59)-infected cells demonstrated that pp1a is processed from the amino terminus to produce p28, p65, and p290; p290 is further processed to produce

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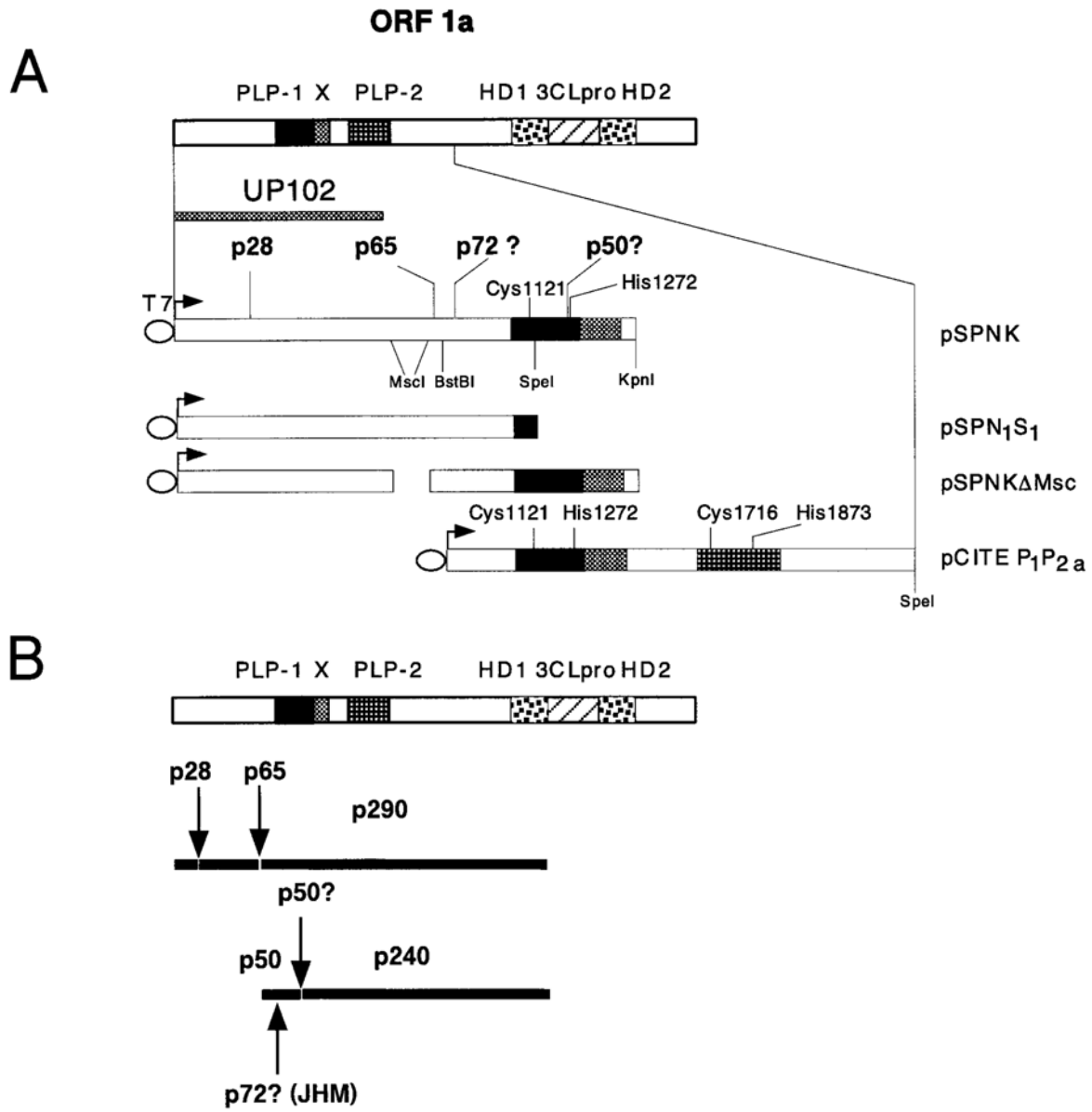

Figure 1 Functional domains of MHV-A59 gene 1, ORF1a plasmids, and proteolytic processing scheme of A59 pp1a. (**A**) The functional domains of ORF 1a: papain-like proteinases 1 and 2 (PLP-1 and PLP-2), X domain (X), poliovirus 3C-like proteinase domain (3CLpro), and hydrophobic domains 1 and 2 (HD 1 and HD 2), are shown. The region in ORF1a that was used to raised rabbit antiserum UP102 (directed against amino acids Met-1 to Gln-592; Denison *et al*, 1995) is shown. Plasmid constructs $pSPN_1S_1$ (Teng *et al*, 1999), pSPNKΔMsc (Bonilla *et al*, 1995), and pCITE P_1P_{2a} (Teng *et al*, 1999) are indicated. The locations of the cleavage sites P28 (between Gly-247 and Val-248, Table 1), p65 (between Ala-832 and Gly833), and the predicted cleavage sites for p72? (between Gly-904 and Val-905 in MHV-JHM sequence) and p50? (between Ala-1262 and Ala-1263) are indicated on $pSPN_1S_1$. The catalytic residues of PLP-1, Cys-1121 and His-1272, and the catalytic residues of the predicted PLP-2 domain, Cys-1716 and His-1873 (analogous to the catalytic residues Cys-1715 and His-1872 in MHV-JHM PLP-2; Gorbalenya and Snijder, 1996, Kanjanahaluethai and Baker, 2000), are also shown. (**B**) MHV-A59 ORF1a (with its functional domains) and *in vivo* proteolytic processing products (Denison and Perlman, 1986; Denison *et al*, 1992, 1995; Weiss *et al*, 1994). The locations of the p28 and p65 cleavage sites, and the predicted cleavage sites for p50? (Bonilla *et al*, 1997) and p72? (JHM)(Schiller *et al*, 1998) are indicated. (MHV-A59 p290 is believed to extend from the carboxyl terminus of p65 site to the carboxyl terminus of HD1, Denison *et al*, 1992, 1995.)

p50 (amino terminal product) and p240 (carboxyl terminal product; Figure 1B) (Denison *et al*, 1992, 1995; Weiss *et al*, 1994). The electrophoretic mobility of p290 is consistent with the predicted molecular weight (278 kDa) of the polypeptide encoded between the C-terminus of p65 and the C-terminus of HD1 (Figure 1). A role for PLP-1 in the production of p28 and p65 has been demonstrated *in vitro* using various ORF1a cDNA clones (Baker *et al*, 1989, 1993; Bonilla *et al*, 1995, 1997; Dong and Baker, 1994; Hughes *et al*, 1995). The cleavage sites for p28 and p65 have been identified between Gly-247 and

Table 1 Sequence comparisons of MHV cleavage sites[a]

			P5	*P4*	*P3*	*P2*	*P1*	*P1′*	*P2′*	*P3′*	*P4′*	*P5′*[b]	
A59	p28	243[c]	K	G	Y	R	G	V	K	P	I	L	252
JHM	p28	243	R	G	Y	R	G	V	K	P	I	L	252
A59	p65	828	R	F	P	C	A	G	K	K	V	E	837
JHM	p65	828	R	F	P	C	A	G	K	K	V	V	837
Consensus sequence			K/R	X	X	X	A/G	A/G/V	X				
Predicted sites[d]													
A59	p50	1253	K	V	F	R	A	A	C	A	V	D	1267
JHM	p72	900	C	K	E	H	G	V	I	G	T	K	909

[a]Bonilla *et al*, 1994; Lee *et al*, 1991.
[b]Cleavage occurs between P1 and P1′ residues.
[c]Amino acid residues in pp1a.
[d]Cleavage proposed to occur between P1 and P1′ residues. Bonilla *et al*, 1997; Schiller *et al*, 1998.

Val-248 and between Ala-832 and Gly-833, respectively (Figure 1A and Table 1; Bonilla *et al*, 1997; Dong and Baker, 1994; Hughes *et al*, 1995). In contrast, p50 production has not been detected under *in vitro* conditions (Baker *et al*, 1989; Denison and Perlman, 1986; Bonilla *et al*, 1995, 1997); this has made the further study of the p50 polypeptide difficult. It could be that under the *in vitro* conditions used, the polypeptide substrate adopted a conformation that prevented PLP-1 access to the p50 site. This would be similar to the observation that p65 production was not detected *in vitro* unless an ORF1a deletion construct was used as template (Bonilla *et al*, 1995). We (Bonilla *et al*, 1997) proposed a consensus recognition sequence for PLP-1 cleavage (Table 1) based on sequence comparisons and *in vitro* mutagenesis studies with the p28 and p65 cleavage sites in MHV-A59 and MHV strain JHM (MHV-JHM). We further predicted that in MHV-A59 pp1a, the sequence from Lys-1258 to Ala-1263 conforms to this consensus, with cleavage between Ala-1262 and Ala-1263 (see Figure 1A and Table 1). Cleavage at this predicted site (and at the carboxyl terminus of p65) would result in a 47.4-kDa polypeptide, which has similar molecular mass to p50 detected in MHV-A59-infected cells (Denison *et al*, 1992). Because the proposed cleavage site is located between the catalytic Cys-1121 and His-1272 residues of PLP-1, we (Bonilla *et al*, 1997) speculated that cleavage at this site could provide a mechanism for PLP-1 to autoregulate its activity *in vivo*. At present, there is no direct evidence for this type of autoregulation in the replication of any coronavirus; thus this remains an hypothesis to be tested.

Interestingly, in spite of similar amino acid sequences between MHV-A59 and MHV-JHM (Weiss *et al*, 1994), the proteolytic processing patterns of pp1a are not identical between these two strains. In MHV-JHM-infected cells, pp1a is processed to produce p28, p65, and p250 (with similar gel mobility to p290 in MHV-A59), which was further proteolyzed to release p210 (with similar electrophoretic mobility to p250 in MHV-A59) and p40. The model of processing of the MHV-JHM pp1a, however, maps the end of p210 to the N-terminus of HD1 (Kanjanahaluethai and Baker, 2000). Furthermore, it is not clear whether p40 in MHV-JHM pp1a has similar origin as p50 in MHV-A59 pp1a (Schiller *et al*, 1998), nor more generally, whether these differences in the two proposed schemes are indeed strain-specific or simply due to differences in apparent molecular weights assigned to these polypeptides. In addition, processing of MHV-JHM pp1a results in production of p72, identified to be a precursor of p65; which is not detected in MHV-A59-infected cells (Figure 1B; Gao *et al*, 1996). Schiller *et al* (1998) proposed that p72 resulted from cleavage between Gly-247 and Val-248 (at the amino terminus) and between Gly-904 and Val-905 (at the carboxyl terminus; Table 1). The authors further suggested that substitution of Gly-904 in MHV-JHM pp1a by an Asp in MHV-A59 pp1a results in lack of p72 production in MHV-A59-infected cells. The proteinase responsible for p72 production was not identified, but the predicted site is similar to a PLP-1 cleavage site (Table 1) (Schiller *et al*, 1998). Recently, MHV-JHM PLP-2 has been demonstrated to cleave at the amino terminus of HD1, however, there are no data indicating a role for PLP-2 in p50 or p72 production (Kanjanahaluethai and Baker, 2000).

Does the predicted p50 sequence results in PLP-1-mediated cleavage when placed in the position of the p28 cleavage site?

Because p50 could not be detected by *in vitro* studies (Baker *et al*, 1989; Bonilla *et al*, 1995, 1997; Denison and Perlman, 1986), we investigated the proposed p50 cleavage sequence (Table 1; Bonilla *et al*, 1997) by introducing this sequence into the p28 site. The rationale for this approach is the observation that exchanging the P2, P1, and P1′ residues of the p28 and p65 cleavage sequences with each other still results in cleavage at the mutated sites (Bonilla *et al*, 1997). Therefore, if the p50 cleavage sequence is recognized by PLP-1, then introducing this sequence into a more accessible region (the p28 site) might allow cleavage

to occur. Mutagenesis was performed using the PCR-based QuickChange kit (Stratagene) according to the manufacturer's protocol and as described previously (Teng *et al*, 1999). To introduce the proposed p50 sequence into the p28 sequence, mutagenic primers were used to mutate $pSPN_1S_1$, which encodes pp1a sequence from Met-1 to Lys-1160 and could not undergo *cis* cleavage to produce p28 (Figure 1A; Teng *et al*, 1999). $pSPN_1S_1$ G247A/V248A has the P1 and P1′ residues of the p28 sequence replaced by the corresponding residues of the proposed p50 sequence (Table 1). $pSPN_1S_1$ G244V/G247A/V248A contains an additional mutation at the P4 location. $pSPN_1S_1$ Y245F/G247A/V248A has the P3, P1, and P1′ residues of the p28 site replaced by those of the proposed p50 sequence. $pSPN_1S_1$ G244V/Y245F/G247A/V248A has the P4, P3, P1, and P1′ residues of the p28 site replaced. The constructs were used in *in vitro* transcription/translation with the TnT coupled reticulocyte lysate system (Promega) as described previously (Bonilla *et al*, 1995, 1997). Polypeptides translated from constructs described here were used as substrates and incubated with enzyme translated from pCITE P_1P_{2a} (Teng *et al*, 1999), which encodes both PLP domains (cleavage in *trans*). For negative control, enzyme was translated from pCITE P_1P_{2a} H1272P, which is the same as pCITE P_1P_{2a} except the PLP-1 domain contains an inactivating H1272P mutation (Teng *et al*, 1999).

Figure 2 shows that substitution of the P1 and P1′ residues (with or without substitution of the P3 residue) of the p28 cleavage sequence by the corresponding residues of the proposed p50 sequence, results in p28 production (lanes 3 and 4, 7 and 8), comparable to that observed with substrate translated from wild-type $pSPN_1S_1$ (lanes 1 and 2). The absence of p28 production when substrates were incubated with polypeptides carrying inactive PLP-1 (odd numbered lanes indicated with minus signs in Figure 2) show that the observed cleavage was catalyzed by PLP-1. (Active PLP-2, also encoded in pCITE P_1P_{2a}, was present in all reactions and was apparently unable to mediate cleavage when PLP-1 was inactivated.) Preliminary results also show that *in vitro* transcription and translation of a mutant construct, which has the P2 and P1′ residues of the p65 site replaced by the corresponding residues of the proposed p50 sequence (Table 1), produced similar proteolytic products as the wild-type construct (data not shown), suggesting that the proposed p50 sequence (Bonilla *et al*, 1997) may function to mediate cleavage when placed in the p65 cleavage site as well as the p28 cleavage site positions.

Surprisingly, additional substitution at the P4 position of the p28 site (Gly-244; Table 1) by the corresponding residue of the proposed p50 sequence (Val; Table 1) abolished p28 production (Figure 2, lanes 5 and 6, 9 and 10). The results imply that PLP-1 can recognize and cleave at the -Ala-Ala-dipeptide, the proposed P1 and P1′ residues of the p50 sequence, provided the P4 residue is not a Val. Because mutagenesis studies of the p28 and p65 sites did not point to strict conservation at their P4 residues (Gly and Phe, respectively), the reason for the observed inhibition remains unclear. It could be that under our *in vitro* conditions, the G244V substitution (in addition to the substitutions at the P1 and P1′ residues) specifically reduced cleavage at the mutated cleavage site. Incidentally, previous studies on the p28 site also show that the G244V mutation resulted in lower level of p28 when compared to the G244D mutation (Hughes *et al*, 1995). Alternatively, the presence of a Val at the P4 position could prevent unregulated cleavage (by PLP-1) at the natural p50 site *in vivo*. In support of this proposal, replacing the P2 to P1′ residues of the proposed p50 sequence with the corresponding residues of the p28 cleavage sequence did not result in cleavage at the mutated region (results not shown), indicating that other factors, such as change in substrate conformation induced by a P4 Val, determine PLP-1 access to the proposed p50 site. If this were the case, then viral and/or host factors, not present in our *in vitro* conditions, would be required to maintain the substrate in a conformation that favors cleavage at the p50 site.

Our data, to date, do not prove that the proposed p50 cleavage site is utilized during processing of pp1a *in vivo*. We must consider possible explanations for our results. For example, it is possible that the observed cleavage products derived from mutant constructs in Figure 2 (lanes 4 and 8) arise from cleavage at nearby undefined sites (Table 1; Bonilla *et al*, 1997); the observed cleavage could be due to a relaxed specificity of PLP-1 at the p28 and p65 sites under our *in vitro* conditions (i.e., PLP-1-mediated cleavage could tolerate substitutions at the p28 and p65 sites by an -Ala-Ala-dipeptide). However, if such nonspecific cleavages within pp1a were to occur *in vivo*, then that could disrupt the regulation of proteolytic processing, thus compromising viral RNA synthesis and virus maturation. In results not presented, introducing the P2 to P1′ residues of the p28 sequence, shown to play important roles in cleavage specificity (Baker *et al*, 1993; Hughes *et al*, 1995), into a random location within pp1a (just upstream of the p28 cleavage site in a construct in which the authentic p28 site is inactivated), did not create a new cleavage site, providing further support to the assumption that cleavage by PLP-1 is both sequence- and location-specific. Thus, to determine under what conditions, and if so, cleavage at the p50 site may indeed occur, further experiments must be carried out. These would include immunoprecipitation experiments using antisera specific against sequences within the PLP-1 domain, the isolation and characterization of p50 from infected cells, and mutagenesis studies (e.g., with additional substitutions at the P4 Val residue in the proposed p50 cleavage site).

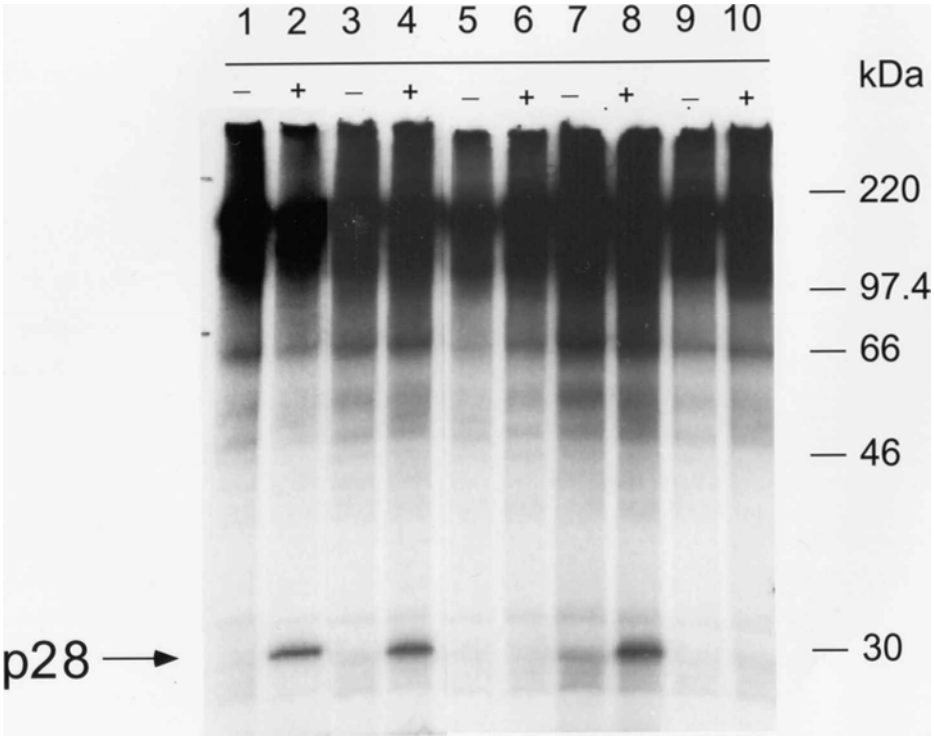

Figure 2 PLP-1 cleaves at the predicted p50 sequence in the p28 sequence location. Substrates were transcribed and translated *in vitro* from pSPN$_1$S$_1$ (with the sequence 243 -K-G-Y-R-G↓V-K- 249) (lanes 1 and 2), pSPN$_1$S$_1$ G247A/V248A (243 -K-G-Y-R-A↓A-K- 249) (lanes 3 and 4), pSPN$_1$S$_1$ G244V/G247A/V248A (243 -K-V-Y-R-A/A-K- 249) (lanes 5 and 6), pSPN$_1$S$_1$ Y245F/G247A/V248A (243 -K-G-F-R-A↓A-K- 249) (lanes 7 and 8), and pSPN$_1$S$_1$ G244V/Y245F/G247A/V248A (243 -K-V-F-R-A/A-K- 249) (lanes 9 and 10). (The arrow within the sequences indicates that cleavage does occur with the addition of active PLP-1, whereas the slash indicates cleavage does not occur in the presence of active PLP-1.) Substrates were then incubated with inactive PLP-1 transcribed and translated *in vitro* from pCITE P$_1$P$_{2a}$ H1272P (−lanes), or with active PLP-1 transcribed and translated *in vitro* from pCITE P$_1$P$_{2a}$ (+lanes). The cleavage products were electrophoresed on an SDS-12.5% polyacrylamide gel. The molecular masses of marker proteins are indicated on the right. The location of p28 is indicated. In lane 7, the protein band with mobility similar to p28 migrates faster than the authentic p28 and is not a cleavage product.

The putative JHM p72 cleavage recognition sequence is not cleaved by PLP-1 when introduced into A59 pp1a.

Previously we reported that *in vitro* transcription and translation of pSPNKΔMAG D904G, which contains the predicted p72 cleavage sequence of MHV-JHM (with the D904G mutation), but has the p65 cleavage site dipeptide of MHV-A59 deleted; did not result in production of p72 (Teng *et al*, 1999). In this work we further examined the proposed p72 site by constructing pSPNKΔMsc D904G, which introduced the D904G mutation into MHV-A59 pp1a while retaining a functional p65 site. The construct was transcribed and translated *in vitro* as described before (Bonilla *et al*, 1995, 1997). Aliquots were withdrawn at indicated times and the reactions quenched with 4 mM leupeptin (Denison and Perlman, 1986). Results in Figure 3A show that when pSPNKΔMsc D904G was transcribed and translated *in vitro*, p28, p43, and p70 (resulting from cleavage at wild-type p28 and p65 sites) were detected. When rabbit antiserum UP102 (directed against pp1a Met-1 to Gln-592; Denison *et al*, 1995) was used to immunoprecipitate the cleavage products transcribed and translated *in vitro* from pSPNKΔMsc D904G, only p28, p43, and p70 were detected (Figure 3B). There is no evidence for additional proteolytic product resulting from cleavage at the mutated site such as the predicted 77-kDa polypeptide, which would have been immunoprecipitated as well. Results in Figure 3C show that substitutions of the P2, P1, and P1′ residues of the p65 site with the corresponding residues of the predicted p72 sequence (Table 1) did not result in cleavage at the mutated p65 site (lane 3), in contrast to the wild-type construct (lane 1; Bonilla *et al*, 1995) and similar substitutions by the p28 sequence (lane 2; Bonilla *et al*, 1997).

Gao *et al* (1996) initially suggested that p72 synthesis was mediated by 3CLpro, with cleavage between Glu-894 and Ser-895. Although cleavage between a Glu and a Ser has been reported for equine arterivirus virus, an arterivirus, this cleavage site has not been reported for MHV 3CLpro (Gorbalenya and Snijder, 1996; de Vries *et al*, 1997; also see Liu *et al*, 1997; Piñón *et al*, 1997; and van Dinten *et al*, 1999). Subsequently, Schiller *et al* (1998) suggested that the p72 cleavage site occurs between Gly-904 and Val-905, similar to the p28 cleavage site (Table 1).

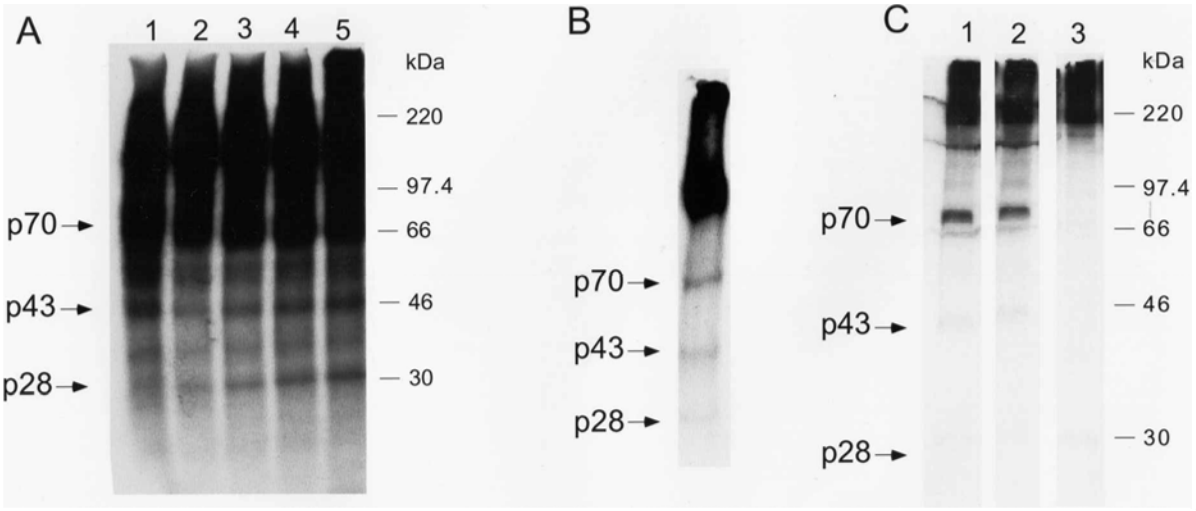

Figure 3 The predicted p72 cleavage site is not cleaved in polypeptide derived from A59 pp1a. (A) *Cis* cleavage time course of pSPNKΔMsc D904G (with the sequence 900 -C-K-E-H-G/V-I- 906, slash indicates cleavage does not occur). Aliqouts were withdrawn at 30 min (lane 1), 45 min (lane 2), 60 min (lane 3), 75 min (lane 4) and 90 min (lane 5) and quenched with 4 mM leupeptin (Denison and Perlman, 1986). The products were separated on an SDS-12.5% polyacrylamide gel. Molecular masses of marker proteins are indicated on the right side. Locations of p28, p43, and p70 are indicated by arrows. (B) Immunoprecipitation of *cis* cleavage reactions. Substrate transcribed and translated *in vitro* from pSPNKΔMsc D904G was immunoprecipitated with UP102 (Denison *et al*, 1995) according to Bonilla *et al* (1995). Cleavage products were separated on an SDS-12% polyacrylamide gel. Locations of p28, p43, and p70 are indicated by arrows. (C) Replacing the p65 cleavage site by the proposed p72 sequence did not result in cleavage. *Cis* cleavage reactions were carried out with substrates transcribed and translated *in vitro* from pSPNKΔMsc (with the sequence 828 -F-R-P-C-A↓G-K- 834, arrow indicates cleavage occurs; lane 1)(Bonilla *et al*, 1997), pSPNKΔMsc C831R/A832G/G833V (828 -F-R-P-R-G↓V-K- 834; lane 2)(Bonilla *et al*, 1997), and pSPNKΔMsc C831H/A832G/G833V (828 -F-R-P-H-G/V-K- 834; lane 3). Cleavage products were separated on an SDS-12.5% polyacrylamide gel. Molecular masses of marker proteins are indicated on the right. Locations of p28, p43, and p70 are indicated by arrows.

However, in the putative p72 cleavage sequence the P5 residue is a Cys instead of a basic residue, and does not conform to the proposed consensus sequence (Table 1). Nevertheless, our experimental results cannot rule out the possibility that strain specific differences allow cleavage at the p72 site within MHV-JHM pp1a. Possibly, under *in vivo* conditions, MHV-JHM pp1a adopts a conformation that enables PLP-1 to access the proposed p72 site. It is also possible that the PLP-1 of JHM has the ability to cleave at the proposed p72 site while the PLP-1 encoded by A59 does not; this seems unlikely as the PLP-1 domain is highly conserved between the two strains of virus (Lee *et al*, 1991; Bonilla *et al*, 1994). Currently, our experimental results support neither the proposed p72 sequence nor its proposed location.

Conclusions

In conclusion, the *in vitro* data presented here showed that substitutions of the P2 to P1′ residues of the p28 or p65 site by the corresponding residues of the predicted p50 cleavage site (Bonilla *et al*, 1997) can support cleavage at the mutated p28 and p65 sites, in agreement with our proposed p50 cleavage sequence. As the predicted p50 site (Table 1) is located between the catalytic residues of PLP-1 (Cys-1121 and His-1272), the virus can regulate *in vivo* proteinase activity (hence viral genome replication) through self inactivation of PLP-1 (Bonilla *et al*, 1997). On the other hand, the lack of p50 production in cells infected with MHV-JHM hints that MHV-JHM may regulate PLP-1 activity differently from MHV-A59. It is possible that differences in proteolytic processing of pp1a between MHV-A59 and MHV-JHM could contribute to differences in the biology of these two strains. However, to study the full effects of these processing events *in vivo*, we must await methods to introduce the various mutations into the genome (Yount *et al*, 2000). It is hoped that integration of the *in vivo* and *in vitro* data can help us to gain greater insight into the complex proteolytic processing events of coronavirus, and providing us a guide to rational antiviral drug design.

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