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The rubella virus nonstructural protease recognizes itself via an internal sequence present upstream of the cleavage site for *trans*-activity

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Summary. The substrate requirement for rubella virus protease *trans*-activity is unknown. Here, we analyzed the cleavability of RV P200-derived substrates varying in their N-terminal lengths (72–475 amino acids) from the cleavage site by the RV protease *trans*-activity. Only substrates with at least 309 amino acid residues N-terminal to the cleavage site were able to undergo cleavage. Further, rubella sequence was found to be necessary in the N-terminal region of the substrate, whereas a heterologous sequence C-terminal to the cleavage site was tolerated. These results demonstrated a requirement for residues located between amino acids 994–1102 of the RV P200 polyprotein, besides its cleavage site for RV protease *trans*-activity. This region overlaps with the starting site of the essential *cis*-protease activity of RV P200 polyprotein. This is a novel observation for a viral protease of the family *Togaviridae*.

Introduction

A number of positive-stranded RNA viruses employ viral nonstructural polyprotein processing as a strategy for genome expression by encoding their own (viral) proteases [5, 7, 14]. Thus, in order to achieve the expression of multiple proteins from a single message that are essential for regulation of viral replication and biogenesis, proteolysis of the viral polyprotein precursor is an essential event in most of the positive-strand RNA viruses [14, 16]. This is in contrast to the mRNAs of their eukaryotic host cells, which mostly code for single proteins [16]. In alphaviruses and rubella virus, members of the family *Togaviridae*, two

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polyproteins are expressed: the nonstructural polyprotein, directly expressed from the genomic RNA, and another polyprotein, expressed from a sub-genomic mRNA synthesized during the viral infection [16].

Rubella virus is an enveloped virus of the genus *Rubivirus* in the family *Togaviridae* [2]. The virion has a 40S (9762 nucleotide long) single-stranded, positive-sense, polyadenylated RNA genome, which serves as the mRNA following a series of events that lead to the uncoating of the viral particle upon entry into cells [1, 2, 4]. In an infected cell, RV mRNA first translates into a P200 nonstructural polypeptide [2]. Newly synthesized P200 often undergoes *cis*-cleavage at NH_2-SRGG$^{1301/1302}$G-COOH into two mature nonstructural proteins, P150 and P90, by a protease domain contained within the C-terminal region of P150 [3, 9, 11, 18]. The viral P200 nonstructural polyprotein contains four conserved functional domains that are involved in viral RNA synthesis and replication. They are sequentially located from the N terminus to the C terminus, as methyltransferase, protease, helicase, and RNA-dependent RNA polymerase domains based on bioinformatic analysis [4, 6]. The first two motifs are located on the N and C termini of P150, and the latter two are on the N and C termini of P90 [5, 13].

Rubella virus P150-protease was identified to be a papain-like-cysteine protease [5]. It utilizes divalent cations and also demonstrates *trans*-cleavage activity on homologous substrates *in vitro* [9] as well as *in vivo* [17]. Further characterization of RV protease revealed that it exhibits zinc-binding activity as integral to its protease activity, thus suggesting that it is a novel viral metalloprotease [9, 10]. Within the C-terminal half of P150, Cys-1152 and His-1273 are the catalytic sites for this protease [3, 11]. Domains of RV protease required for *cis*- and *trans*- activity are mapped [8, 11]. These studies have thus far focused on defining the catalytic sites, active domains, and *cis*- and *trans*-activity requirements of RV protease [8–11, 17].

Experimental evidence suggests that P200 is actively involved in the synthesis of viral negative-strand RNA, and its cleavage into P150 and P90 has been suggested to switch the complex to initiate positive RNA synthesis [8]. This leads to the logical hypothesis that, in the replication complex, those P200 molecules engaged in negative-strand RNA synthesis must remain as P200 (i.e., lose their *cis*-protease activity) as long as they are required to participate in negative-strand RNA synthesis. Subsequently, the P150-protease *trans*-activity cleaves these P200 molecules to signal the replication complex to switch to positive-strand RNA synthesis mode. However, besides the presence of the cleavage site, what the internal sequence requirements of the P200 and its intermediaries for RV protease *trans*-cleavage activity are, and how the *cis*-activity of P200 is regulated to maintain optimal negative RNA synthesis have not been identified.

In this report, we analyzed the substrate features that are required of the RV protease *trans*-activity by using a truncated version of RV P200 (amino acid residues 827–1548) that was previously shown to function as protease but had lost its *cis*-activity due to a substitution, G1301S, in the cleavage site, and a series of P200-derived substrates that lack protease activity due to an amino acid

substitution, C1152S, in the catalytic dyad [8]. Our analysis identified a region in the substrate corresponding to residues 994–1102 of the P200 polyprotein that is required for the protease *trans*-activity, and this region overlaps with the starting site of the essential *cis*-protease activity of RV P200 polyprotein. Potential implications of the requirement of the *cis*-activity region of P200 substrates for the RV protease *trans*-activity are discussed.

Materials and methods

Plasmid construction

Plasmids capable of expressing functional RV protease and P200-derived substrates in mammalian cells were constructed as follows: Appropriate cDNA segments representing various RV genomic regions were PCR-amplified and inserted into a mammalian expression plasmid, pcDNA4-His/Max-C, driven by a CMV promoter. This plasmid also contains an in-frame Xpress epitope tag for easy identification of the expressed protein by anti-Xpress monoclonal antibody in immunobloting analysis (Invitrogen Inc, CA). Two independent DNA preparations for each plasmid were sequenced to ensure authenticity of the plasmids used in the study. RV *trans*-cleavage active protease-encoding cDNA was amplified by PCR with a pair of primers (Table 1), using a previously well-established infectious RV cDNA template, pBRM33-G1301S, which contains an active protease domain (catalytic site, C1152) but lacks the *cis*-cleavage capability due to a substitution at residue G1301 within the cleavage site (NH_2-SRG**G**/G-COOH) to S1301 (NH_2-SRG**S**/G-COOH) [8]. The amplified DNA product was cloned at the BamHI and EcoRI sites in the expression plasmid described above. In this construct, in-frame with the protease sequence C-terminus, a green fluorescent protein (GFP) ORF was also inserted, so that the expressed protease would have a higher molecular weight to distinguish it from its substrate in immunoblot analysis. This expression plasmid was designated as pRVP (Fig. 1A).

To evaluate the *trans*-cleavage activity of RV protease on P200-derived substrates, a series of RV P200-related polypeptide expression plasmids that express protein with different N-terminal lengths from the cleavage site (72, 199, 309, and 475 residues) were constructed. This was achieved by using a previously well-established RV cDNA template, pBRM33-C1152S. The template contains RV cDNA with the cleavage site (N′-SRG**G**/G-C′) unmodified but lacks the protease activity due to a substitution at the catalytic activity residue (C1152S) of the enzyme [8]. Desired DNA segments of pBRM33-C1152S template with the same C-terminus (amino acid position 1548) but varying in length at the N-terminal end were amplified using appropriate sets of primers (Table 1) and cloned at the BamHI and EcoRI sites of the plasmid vector described above. The plasmids were designated, pRVS-827–1548, pRVS-994–1548, pRVS-1102–1548, and pRVS-1228–1548, producing proteins with 475-, 309-, 199-, and 72-residue N-terminal lengths from the cleavage site, respectively (Fig. 1B).

To test whether replacement of P200-related sequences C-terminal to the cleavage site by non-rubella sequences affect RV protease *trans*-activity, the plasmid pRVS-GFP, which contains PCR-amplified RV protease substrate sequences representing polypeptide residues 827–1306 (residues 1302–1306 represent the C-terminal side of the cleavage site), fused at its C-terminus to GFP ORF in-frame, was created (Fig. 1B). Similarly, to test whether a heterologous sequence on the N-terminal side of the cleavage site affects substrate recognition by the protease *trans*-activity, GFP ORF was fused in-frame at the N-terminus of the rubella sequence in the plasmid pRVS-1102–1548 to create pRVS-GFP-1102–1548 (Fig. 1B). The template for the PCR in both cases was pBRM33-C1152S.

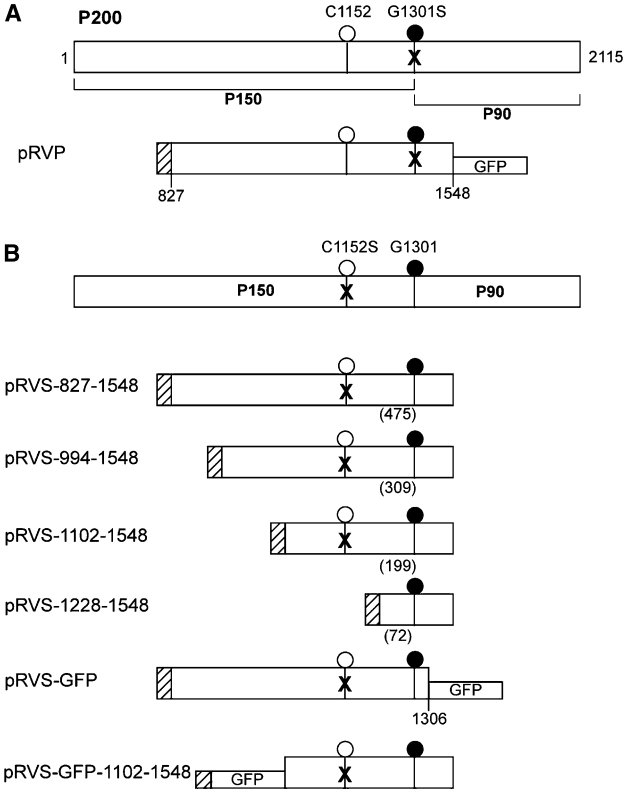

Fig. 1. Schematics of *trans*-active protease and substrate encoding expression plasmid constructs. (**A**) Shows pBRM33-G1301S-derived P200 that was used as a PCR-template to clone the *trans*-active protease region. The P150 and P90 domains of P200 are indicated. The protease catalytic residue, *Cys* at amino acid 1152 (C1152) and a cleavage site substitution mutation (*Gly* to *Ser*) at P150 C-terminal residue, 1301 (G1301S, represented by 'X' in the P150 box and by a black circle on the top) are as shown. The mutation eliminates the *cis*-activity of the protease, leaving its *trans*-activity intact [8]. Numbers (1–2115) on the side represent amino acid residues of P200. The *trans*-active protease domain spans from amino acid position 827 to 1301 [8]. Expression plasmid, pRVP contains this protease domain in-frame flanked by an N-terminal Xpress epitope tag (hatched box) and a C-terminal GFP ORF. (**B**) Shows pBRM33-C1152S-derived P200 used as a PCR-template to produce a series of deletion substrates. The P150 and P90 domains on P200 were as shown. To serve as homologous substrates, the protease activity was eliminated due to a substitution at the catalytic residue, *Cys*-1152 to a *Ser* [8] (C1152S, represented by 'X' and by an open circle on the top) but with an intact cleavage site at the P150 C-terminal residue, 1301 (G1301) as shown. Expression plasmids containing RV P200-related cDNA pRVS-827–1548, pRVS-994–1548, pRVS-1102–1548, and pRVS-1228–1548 (numbers denote position of amino acid residues within the P200 polyprotein representing the N and C termini of the substrate) used in the study were as illustrated. These plasmids express proteins with varying N-terminal lengths from the cleavage site (475, 309, 199 and 72 aa respectively as shown in parentheses under each construct box). Cleavage in the substrate occurs at amino acid residue G1301. In pRVS-GFP, the RV cDNA insert was amplified from position 827–1306 (5 residues upstream of the cleavage site) and a GFP-ORF was fused at its C-terminus, whereas in pRVS-GFP-1102–1548, the GFP-ORF was fused at the N-terminus of the 199 amino acid residue-substrate. All plasmids shown here contain an in-frame N-terminal Xpress epitope tag (hatched box) for immunoblot identification of the proteins

Table 1. Primers used in this study

Primer designation	Nucleotide position in RV genome	Sequence*
5′-827	2519–2538	5′-AATGGATCCATGGACCACCGCCCGGCTGC-3′
5′-994	3023–3040	5′-CTGGATCCCCTCCGACCGAGCCCCTC-3′
5′-1102	3347–3364	5′-CTGGATCCATGTGCGGGAGTGACATG-3′
5′-1228	3728–3749	5′-ATAGGATCCGTGTGGGTCGGCTCCGAG-3′
3′-1306	3976–3959	5′-AGAGAATTCGGCGGCACAAGTGCCACC-3′
3′-1548	4701–4684	5′-TATGAATTCGCCTACATGGATGCAGGC-3′

*Nucleotide sequence representing BamHI in 5′-primers and EcoRI in 3′-primers were underlined

Cell transfections and immunoblot analysis

RV-protease, and substrate plasmids were expressed in human embryonic kidney (HEK) 293T cells (ATCC, VA) by transient transfection using Lipofectamine-2000 reagent as per supplier's protocol (Invitrogen, CA). Following a 24 h incubation of cells at 37 °C in a CO_2 (5%) incubator, cell lysates were prepared and subjected to polyacrylamide gel electrophoresis (either 10% or, 4–20% gradient) with 0.1% sodium dodecyl sulphate, followed by immunoblot analysis as described [12]. Substrates as well as the protease all had an Xpress epitope tag at their N-terminus to detect the N-terminal side of the cleavage products.

Results

RV protease trans-activity recognizes substrate N-terminal length from the cleavage site

We evaluated the protease *trans*-cleavage activity on a series of RV P200-derived polypeptides in which the protease catalytic site at amino acid position 1152 was mutated (C1152S) to serve as substrate (i.e. to lack protease activity, but retain the cleavage site). HEK 293T cells were co-transfected with pRVP (Protease, Fig. 1A), and each of the substrate expression plasmids, pRVS-827–1548, pRVS-994–1548, pRVS-1102–1548, and pRVS-1228–1548. Each substrate plasmid expressed RV P200-derived polypeptides of different N-terminal lengths (72–475 residues) from the cleavage site (Fig. 1B). Following transfection, cell lysates were prepared, and the proteins were subjected to immunoblot analysis. The substrates as well as the protease all had an Xpress epitope tag at their N-terminus. Our analysis of RV protease substrates revealed that homologous polypeptides of N-terminal 475- and 309-residue length from the cleavage site, expressed from pRVS-827–1548 and pRVS-994–1548 (Fig. 2, lanes 2 and 4, 80 and 67 kDa, respectively), were able to undergo cleavage when co-expressed along with RV protease (Fig. 2, lanes 1, 3, and 5, 110 kDa) with an expected size of the N-terminal cleaved products of 55- and 37-kDa size (with additional Xpress epitope residues), respectively (Fig. 2, lanes 3 and 5). However, sub-

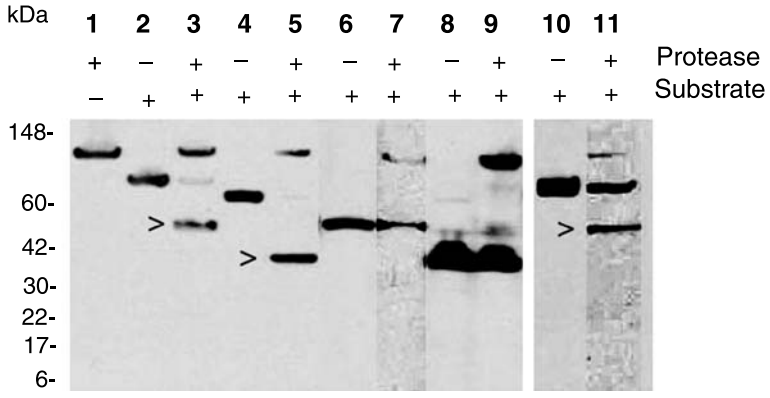

Fig. 2. Immunoblot analysis of RV protease *trans*-cleavage activity on various substrates. HEK 293T cells were transfected with either individual plasmids representing the protease (pRVP) and various substrates (pRVS-827–1548, pRVS-994–1548, pRVS-1102–1548, pRVS-1228–1548, and pRVS-GFP) or, co-transfected each substrate plasmid in combination with the protease plasmid. Cell lysates were prepared and subjected to immunoblot analysis following separation of the proteins on a 4–20% PAGE. Anti-Xpress epitope antibodies were used to probe the membrane. Symbols (+) or (−) above each lane indicate the presence or absence of the protease and the substrate in that lane. Numbers on left indicate molecular weight markers represented in kDa and on top, represent lanes of the immunoblot performed on the transfected-cell lysates. *1* represents protease alone, *2* indicates 475-substrate alone, and *3* represents protease and 475-substrate cotransfection. *4* shows 309-substrate and *5* represents protease and 309-substrate cotransfection. *6*, 199-substrate and *7*, protease and 199-substrate cotransfection. *8* represents 72-substrate and *9*, protease and 72-substrate cotransfection. *10* represents protease alone and *11*, protease and RVS-GFP-substrate together. *7* and *11* shown are longer exposures of the same blot to film. Note that substrates generated from pRVS-827–1548 and pRVS-994–1548 were cleaved by the protease (“>” identifies the cleaved products of 475 and 309 residues respectively in *3* and *5*), whereas, 199- and 72-residue (from the cleavage site) representing substrates expressed from pRVS-1102–1548 and pRVS-1228–1548 were not cleaved by the protease (*7* and *9*). Also note that the protease was able to cleave the RVS-GFP substrate with a GFP on the C-terminal side of the cleavage site to a lesser extent (<50%), as a significant portion of the substrate remained uncleaved

strates of N-terminal 199- and 72-residue length from the cleavage site (plus the Xpress epitope residues), expressed from pRVS-1102–1548 and pRVS-1228–1548, respectively (Fig. 2, lanes, 6 and 8, 55- and 37-kDa), did not undergo cleavage when co-expressed along with the protease (Fig. 2, lanes 7 and 9) as evidenced by the lack of Xpress-tagged N-terminal cleavage products detected in the immunoblot analysis. (If the substrates were cleaved, the expected sizes of the N-terminal products would be 25 kDa and 14 kDa, respectively.) This clearly suggests that amino acid residues residing between residues 200–308 in the substrate are required in order for the substrate to be targeted by the *trans*-activity of the protease.

RV protease trans-activity tolerates heterologous amino acid sequence C-terminal to the cleavage site in the substrate

We extended our analysis to identify any homologous sequence requirement of the substrate on the C-terminal side of the cleavage site. To address this, we utilized the plasmid pRVS-GFP, which expresses an RV protease homologous substrate representing amino acids 827–1306 (residues 1302–1306 represent the C-terminal side of the cleavage site), fused at its C-terminus to a GFP ORF in-frame (Fig. 1B). When this plasmid was co-transfected along with RV protease plasmid (pRVP) into HEK 293T cells, we observed that the substrate (Fig. 2, lane 10, 80-kDa protein) was cleaved by the protease at the cleavage site, based on the expected molecular weight of the cleaved N-terminal product of 55 kDa (Fig. 2, lane 11). This clearly suggests that the protease was able to cleave the RVS-GFP substrate with a GFP on the C-terminal side of the cleavage site, but to a lesser extent (<50%), as a significant portion of the substrate was reproducibly observed to be

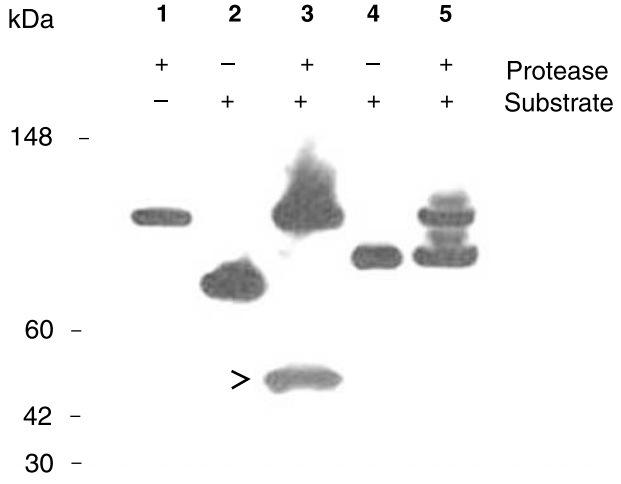

Fig. 3. Immunoblot analysis of RV protease *trans*-cleavage activity on a shorter homologous substrate extended at its N-terminus with a GFP. HEK 293T cells were transfected with either individual plasmids representing the protease (pRVP), a positive control substrate (pRVS-827–1548, generates 475 amino acid residue-substrate) and the shorter homologous substrate (pRVS-GFP-1102–1548) or, co-transfected with each substrate plasmid in combination with the protease plasmid. Cell lysates were prepared and subjected to immunoblot analysis following separating the proteins on a 4–20% PAGE. Anti-Xpress epitope antibodies were used to probe the membrane. Numbers on top represent lanes of the immunoblot and numbers on left indicate molecular weight markers represented in kDa. Symbols (+) or (−) above each lane indicate the presence or absence of the protease and the substrate in that lane. *1* is pRVP-transfected cell lysate expressing the protease alone, *2* is pRVS-transfected cell lysate expressing the positive control substrate only, and *3* represents pRVP and pRVS cotransfection, wherein the substrate was cleaved by protease *trans*-activity (cleaved product was shown by ">"). *4* represents the N-terminal extended (by GFP) 199-substrate expressed following pRVS-GFP-199 transfection, and *5* represents the protease and substrate together expressed in cells cotransfected with pRVP and pRVS-GFP-199. Note that protease did not cleave the N-terminal extended 199-substrate

left uncleaved in three independent experiments (as illustrated in Fig. 2, lane 11, middle band).

RV protease trans-activity requires an internal sequence located upstream of the cleavage site in the substrate

The fact that 309-substrate was cleaved and 199-substrate was not suggested that *trans*-protease processing of the substrate either requires a minimum N-terminal length from the cleavage site in the substrate, or it recognizes an internal amino acid sequence within the 309-substrate that is lacking in the 199-substrate. If indeed an internal domain within the 309-substrate is being recognized by the protease, then this sequence should be present in the N-terminal region of 309-substrate. To verify the above hypothesis, we utilized plasmid pRVS-GFP-1102–1548 (Fig. 1), which expresses a chimeric substrate in which the 199-substrate was extended at its N-terminus by GFP to compensate for the length (increased from 199 to 479 amino acids) along with the protease plasmid, pRVP. Immunoblot analysis (Fig. 3) demonstrated that when the protease (Lane 1) and a positive control 475-substrate (Lane 2) were coexpressed, the substrate did undergo cleavage (Lane 3). Although the N-terminal length was increased in the chimeric GFP-199-substrate (Lane 4), it still failed to undergo cleavage when coexpressed with the protease (Lane 5), suggesting that it is not the N-terminal length from the cleavage site that is required of the substrate, but in fact it is the internal sequence present within the substrate that is recognized by the protease for *trans*-processing. The internal recognition domain identified in this report corresponds to amino acid

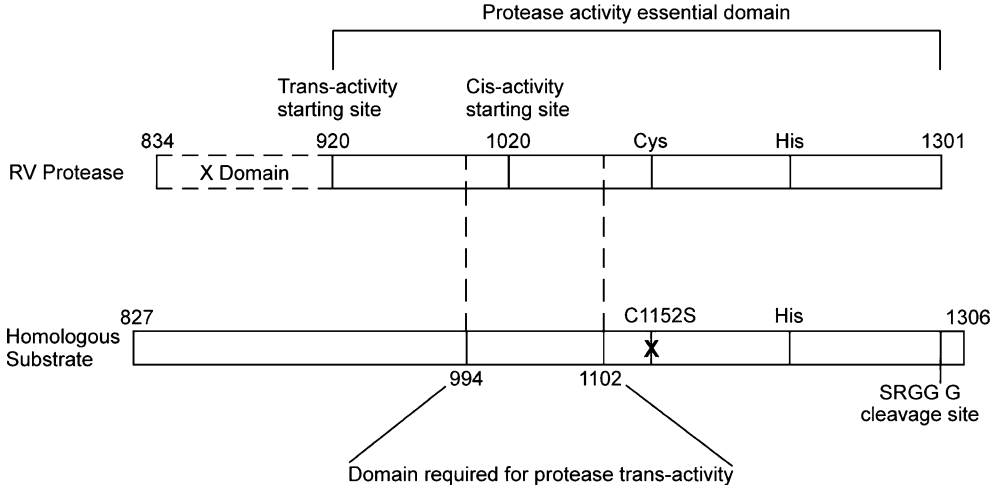

Fig. 4. Schematic illustration of RV protease recognition domain on P200 substrate. Numbers indicate amino acid positions on the RV P200 polyprotein. RV protease *trans*- and *cis*-activity starting sites, the catalytic residues, and the X domain (proline-rich, conserved in M-group PCPs) important for the protease *trans*-cleavage activity as shown on the top are adapted from Liang et al. [8]. In the bottom panel, the protease substrate P200 is depicted with the location of the newly identified domain required for protease *trans*-activity (this report), which overlaps with the protease *cis*-activity starting site of P200

position 994–1102 of RV P200. This region overlaps with the N-terminal site of the essential *cis*-protease activity region on the P200 described by Liang et al. [8]. The location of the substrate recognition region (this report) relative to the essential *cis*-activity domain is illustrated in Fig. 4. At this time we could not refine the recognition region further, as plasmids with deletions in this region of RV cDNA could not be rescued in bacteria.

Discussion

In this report, we defined the minimal substrate (template) for proteolytic processing *in trans* by the rubella virus nonstructural protease using an epitope-tagged protein expression system. Results presented with RV protease (which has been functionally demonstrated to be similar to P150 activity [8]) demonstrated that the protease *trans*-activity recognizes a region N-terminal to the cleavage site in the P200-derived substrates. Thus, the *trans*-protease activity requires not only specific amino acids present at or proximal to the cleavage site, but also sequences upstream at a distance from the cleavage site. We also demonstrated that, on the C-terminal side of the cleavage site, heterologous residues unrelated to RV are somewhat tolerated by the protease, but we routinely observed (in three separate experiments) that the efficiency of the protease *trans*-activity on this type of substrate was less than 50% (illustrated in Fig. 2, lane 11). This clearly suggests that the P90 domain residing on the C-terminal side of the cleavage site in P200 does influence the *trans*-activity of the protease. In another positive-stranded RNA virus, mouse hepatitis virus (MHV), one of the two virus-encoded proteases, PLP-1 (PCP), demonstrates a homologous substrate length requirement on the C-terminal side of the cleavage site and the cleavage efficiency increases with increasing substrate and enzyme polypeptide length, although in this case, the protease recognition sequence on the substrate was not identified [15]. The RV protease *trans*-activity-associated P200 internal sequence requirement, as identified here, is unique, and this is the first such report for the viruses that belong to the family *Togaviridae*.

In this study, we mapped the regions of the protease substrate required for *trans*-activity, which is reciprocal to the Liang et al. study wherein they mapped the essential *cis*- and *trans*-activity regions of the protease itself and also demonstrated that the X domain (proline-rich, conserved in all M-group PCPs) present in the RV protease is important for the protease *trans*-activity [8]. They further speculated that this proline-rich X domain could serve as a protein-protein interaction domain that enhances the opportunity to meet its *trans*-cleavage substrate [8]. However, in this report, we clearly demonstrate by deletion analysis that the X domain in the substrate is dispensable for recognition by RV protease *trans*-activity and the actual recognition region is located downstream of the X domain, overlapping with the N-terminal starting-site (a term coined by Liang et al. [8]) of the essential *cis*-activity domain of the substrate. Our results show that RV P150-associated protease *trans*-activity requires a specific region within the P200 that represents P150 itself (illustrated in Fig. 4). Taken together, our studies and those of Liang et al. [8] advance the field in enhancing our understanding of the molecular

determinants that define the rubivirus protease *trans*-activity requirements that are essential for RV replication.

In this report, we have shown that RV protease *trans*-activity demonstrates substrate specificity by requiring an internal sequence within the region that is N-terminal to the cleavage site. Identification of a region in the P200-related sequence-containing substrate (we utilized polypeptides from amino acid positions 827 to1548 of P200 or shorter) that is important for RV protease *trans*-activity suggests that this region may offer a specific fold or a conformation to the P200 substrate to facilitate cleavage by the protease. Since most protease-substrate interactions involve transient binding of the protease to its cognate substrate, it is conceivable that RV protease *trans*-activity on homologous substrates also involves transient binding of protease to the substrate, and such binding perhaps could occur within the sequence that is required on the substrate for protease *trans*-activity. We attempted to perform coimmunoprecipitation experiments to establish the protease-substrate binding following cotransfection of cells with both plasmid constructs, but failed to obtain reproducible results, perhaps due to the transient nature of the interaction. However, if this binding truly occurs in the viral infection cycle, then, as RV protease is recognizing itself as substrate in the essential *cis*-activity region for the *trans*-activity (illustrated in Fig. 4), it is tempting to speculate that the *trans*-activity may be regulating the *cis*-activity of P200. This process could explain how P200 remains as P200 in the replication complex to initiate viral negative-strand RNA synthesis. As discussed in the introduction, previous experimental evidence suggested that P200 initiates the synthesis of viral negative-strand RNA, and its cleavage into P150 and P90 plays a critical role in switching the replication complex to the positive RNA synthesis mode [8]. In this context, our results leads us to speculate that perhaps the P150-protease *trans*-activity requirement of the P200 sequence within its essential *cis*-activity region (illustrated in Fig. 4) could transiently stall the P200 *cis*-protease activity, provided that P150 binds to P200 in this region, to allow the negative-strand RNA synthesis to occur. This certainly is a verifiable future experimental direction to capture viral events that further enhance our understanding of the rubivirus proteases.

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