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A cysteine-rich metal-binding domain from rubella virus non-structural protein is essential for viral protease activity and virus replication

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Abstract

The protease domain within the RUBV (rubella virus) NS (non-structural) replicase proteins functions in the self-cleavage of the polyprotein precursor into the two mature proteins which form the replication complex. This domain has previously been shown to require both zinc and calcium ions for optimal activity. In the present study we carried out metal-binding and conformational experiments on a purified cysteine-rich minidomain of the RUBV NS protease containing the putative Zn²⁺-binding ligands. This minidomain bound to Zn²⁺ with a stoichiometry of ≈ 0.7 and an apparent dissociation constant of < 500 nM. Fluorescence quenching and 8-anilino-1-naphthalene-sulfonic acid fluorescence methods revealed that Zn²⁺ binding resulted in conformational changes characterized by shielding of hydrophobic regions from the solvent. Mutational analyses using the minidomain identified residues Cys¹¹⁷⁵, Cys¹¹⁷⁸, Cys¹²²⁵ and Cys¹²²⁷ were required for the binding of Zn²⁺. Corresponding mutational analyses using a RUBV replicon confirmed that these residues were necessary for both proteolytic activity of the NS protease and viability. The present study demonstrates that the CXXC(X)₄CXC Zn²⁺-binding motif in the RUBV NS protease is critical for maintaining the structural integrity of the protease domain and essential for proteolysis and virus replication.

Keywords

conformational change; fluorescence; protease; rubella virus (RUBV); zinc-binding protein

INTRODUCTION

RUBV (rubella virus), an important human pathogen causing German measles, is a simple, plus-strand RNA virus that is the sole member of the genus Rubivirus in the Togaviridae family of animal viruses. The RUBV genome encodes five proteins which are expressed from two ORFs (open reading frames): the two proteins involved in viral RNA replication that are translated from one [termed the 'NS (non-structural) protein' or 'NS-ORF'] and the three structural proteins that form the virus particle from the second. Proteolysis is a

common feature in expression of the proteins from both ORFs, as the primary translation product of the NS-ORF is cleaved into its two component proteins by a protease embedded in one of these proteins, whereas the structural-protein ORF translation product is processed co-translationally by the cellular enzyme signalase. The two replicase proteins, P150 and P90, are situated at the N- and C-terminal ends of the NS-ORF precursor respectively, and the embedded NS protease is at the C-terminus of P150 immediately upstream from the cleavage site. Other than cleaving the NS-ORF precursor, the NS protease has no known cellular targets [1].

Previous studies in our laboratories have shown that the RUBV NS protease is a papain-like protease with a catalytic dyad comprising Cys¹¹⁵² and His¹²⁷³ (P150 has 1301 residues) [2]. We recently showed that the NS protease contains a linear EF-hand Ca²⁺-binding motif that plays a structural role in stabilizing the protease at physiological temperatures [3]. To our knowledge, this is the only example of Ca²⁺ playing such a role in a viral protease. We also showed that Zn²⁺ is required for optimal proteolytic activity [4,5]. However, the exact Zn²⁺-binding ligands and the role Zn²⁺ plays in protease activity have yet to be identified. On the basis of our model structure of the RUBV NS protease, a cluster of cysteine residues (Cys¹¹⁶⁷, Cys¹¹⁷⁵, Cys¹¹⁷⁸, Cys¹²²⁵ and Cys¹²²⁷) are arranged in close proximity in space and could potentially form a Zn²⁺-binding pocket. Structural Zn²⁺-binding sites are generally co-ordinated by four cysteine residues and histidine residues as ligands, although there are no histidine residues nearby in this cysteine cluster. In the present study we expressed and purified a minimal metal-binding domain from the NS protease, RUBCa (comprising amino acids 1143–1252 of P150), which contains the EF-hand Ca²⁺-binding loop as well as all of the putative cysteine residues involved in Zn²⁺ binding. We used the well-folded RUBCa minidomain to study Zn²⁺ binding and the resulting changes in minidomain structure with various spectroscopic methods and, furthermore, took a site-directed-mutagenesis approach to identify the ligands involved in Zn²⁺ binding. Mutants were also introduced into a RUBV infectious cDNA replicon to correlate Zn²⁺ binding with NS protease activity and viability.

MATERIALS AND METHODS

Plasmid construction and site-directed mutagenesis

RUBCa, the minidomain containing the Zn²⁺- and Ca²⁺-binding motifs of the RUBV NS protease (amino acids 1143–1252 of P150) was expressed using the pGEX-2T expression vector [3]. A series of cysteine-to-serine substitution mutations were created in this vector. These mutations were also introduced into the RUBV replicon RUBrep-HA/GFP (where HA is the haemagglutinin epitope and GFP is green fluorescent protein) [6]. Transfection of Vero cells with replicon transcripts, determination of viability and assay of *in vivo* NS protease activity were performed as previously described [3].

Protein expression and purification

The RUBCa minidomain and its cysteine-to-serine mutants were expressed as a GST-fusion protein in *Escherichia coli* BL21(DE3) as previously described [3]. The proteins were purified by following the protocols for GST-fusion protein purification [7] using glutathione-Sepharose 4B beads (GE Healthcare). The GST tag was subsequently cleaved with thrombin, followed by further purification by gel-filtration (Superdex 75) and cation-exchange chromatography (Hitrap SP; GE Healthcare). The molecular mass of RUBCa was confirmed by MALDI-TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/417/bj4170477add.htm>) in the Advanced Biotechnology Core Facilities of Georgia State University. The concentration of RUBCa was measured by its A₂₈₀ using an absorption coefficient of 19 630 M⁻¹ · cm⁻¹.

calculated as described previously [8]. Metal elements in the purified protein were screened by ICPMS (inductively coupled plasma MS) at the Chemical Analysis Laboratory at the University of Georgia.

Determination of protein-bound amounts of zinc by the colorimetric PAR [4-(2-pyridylazo)resorcinol] assay

The PAR colorimetric assay, with slight modification, was used to determine the amount of protein-bound Zn^{2+} as previously described [9]. Protein samples were extensively dialysed against Chelex-100-(Bio-Rad)-pretreated water to remove background metal ions and then digested in a total volume of 100 μl with 100 $\mu\text{g/ml}$ protease K (Sigma) in Chelex-100-treated HSD buffer (50 mM Hepes/KOH, 200 mM NaCl and 5 mM dithiothreitol), pH 7.0, at 56°C for 30 min. Subsequently, an identical volume of HSD containing 5 mM iodoacetamide and 200 μM PAR (Sigma) was added. Absorbance over the range 300–600 nm was measured using an RF-1501 UV spectrometer (Shimadzu). HSD containing 0–15 μM standard ZnCl_2 solution was used to create a standard curve (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/417/bj4170477add.htm>). Carbonic anhydrase, which contains a single Zn^{2+} -binding site, was used as positive control.

Fluorescence measurements and determination of Zn^{2+} binding constant

Fluorescence emission spectra were measured on a PTI lifetime fluorimeter at 25°C. For intrinsic tryptophan fluorescence, spectrum measurements were carried out at protein concentrations of 2–5 μM in 10mM Tris, 100mM KCl and 5mM DTT (dithiothreitol), pH 7.4, with slit widths of 4 and 8 nm for excitation and emission respectively. The emission spectra were collected from 300 to 400 nm with an excitation wavelength of 282 nm. ANS (8-anilinonaphthalene-1-sulfonic acid) fluorescence emission spectra were recorded from 400 to 600 nm, with an excitation wavelength of 390 nm. Protein samples (2 μM) were added to the solution containing 40 μM ANS, 10 mM Tris, 100 mM KCl and 5 mM DTT at pH 7.4. For metal titration, aliquots of 5–10 μl of zinc stock solution (20–80 μM) were gradually added into the sample solution. The apparent Zn^{2+} binding constant was obtained by fitting the acquired data to the following equation:

$$f = \frac{([P]_T + [M]_T + K_d) - \sqrt{([P]_T + [M]_T + K_d)^2 - 4[P]_T \cdot [M]_T}}{2 \cdot [P]_T} \quad (1)$$

where f is the fractional change of fluorescence intensity, K_d is the dissociation constant for zinc, and $[P]_T$ and $[M]_T$ are the total concentrations of protein and Zn^{2+} respectively.

Acrylamide quenching of intrinsic tryptophan fluorescence

Tryptophan fluorescence quenching experiments were carried out at 25°C by adding 10–20 μl aliquots of 4 M acrylamide to the sample solution containing 2 μM protein. The excitation wavelength was set at 282 nm and the emission spectra were acquired from 300 to 400 nm. The integrated area of fluorescence intensity between 300 and 400 nm was used for data analysis by fitting to a modified Stern–Volmer equation:

$$\frac{F_0}{F} = (1 + K_{sv} \cdot [Q]) \cdot e^{[Q] \cdot V} \quad (2)$$

where K_{SV} is the dynamic or collisional quenching constant, V is the static quenching constant, $[Q]$ is the concentration of added acrylamide, and F_0 and F are the integrated fluorescence intensity in the absence and presence of acrylamide respectively.

Homology modelling of the protease domain

The homology modelling of the RUB NS protease domain was constructed using the comparative structure modelling programs SWISSMODEL and MODELLER [10]. The leader protease of FMDV (foot-and-mouth-disease virus; PDB code: 1qmy), which shares over 15% sequence similarity and a highly similar secondary-structural arrangement (see Supplementary Figure 3 at <http://www.BiochemJ.org/bj/417/bj4170477add.htm>), serves as the best available template for structure modelling [11,12]. Energy minimization was subsequently performed using SYBYL. The secondary-structure prediction was carried out by using the program PredictProtein [13]. The metal-binding site was further modified on the basis of the Zn^{2+} binding sites in the proteinase with four cysteine liganding residues such as found in SARS-CoV (severe-acute-respiratory-syndrome-associated coronavirus) [34] by fixing the backbone atoms and allowing free rotation of sulfur atoms on the side chain using the software DeepView, and energy minimization was subsequently carried out using SYBYL.

RESULTS

Zn^{2+} binding by the RUBCa minidomain

Our previous studies demonstrated the requirement of Zn^{2+} for proteolytic activity of the RUBV NS protease *in vitro* and further narrowed down the potential Zn^{2+} -binding sequences to within residues 1143–1301 of the P150 replicase protein (Figure 1) [4,5]. On the basis of its primary- and secondary-structure similarity (see Supplementary Figure 3) to the well-characterized leader protease of FMDV (PDB code 1qmy), we built a model structure for the RUBV NS protease by homology modelling [3]. This model proved accurate in our previous study on the linear EF-hand Ca^{2+} -binding domain (residues 1197–1225) within RUBV NS protease [3]. As shown in Figure 1(B), Cys¹¹⁵² and His¹²⁷³ form the active site of this papain-like protease. A cluster of cysteine residues (Cys¹¹⁶⁷, Cys¹¹⁷⁵, Cys¹¹⁷⁸, Cys¹²²⁵ and Cys¹²²⁷), which is conserved in all genotypes of RUBV [14] and located ≈ 20 Å (1 Å = 0.1 nm) away from the active site, was found to be arranged in close proximity in space and could potentially form a Zn^{2+} -binding pocket (Figure 1B). Binding of Zn^{2+} was studied using the RUBCa minidomain spanning residues 1143–1252 of P150, including the putative Zn^{2+} -binding pocket; use of a larger polypeptide proved impossible, because of solubility problems [3].

Using MBP (maltose-binding protein) fusions, we previously demonstrated the binding of Zn^{2+} ions to various sub-regions of the NS protease domain with a radioactive-⁶⁵ Zn^{2+} blotting assay [5]. Although MBP itself did not bind Zn^{2+} , we were not able to rule out the possibility that an artificial Zn^{2+} binding pocket might be formed by residues from both the MBP tag and the RUBV NS protease domain. In addition, the ⁶⁵ Zn^{2+} blotting does not provide quantitative estimation of Zn^{2+} binding constants. With the tagless RUBCa minidomain in hand, we were able to confirm that the protease domain itself bound Zn^{2+} . The purified minidomain was first subjected to metal-element screening by using ICP-MS, which revealed that it bound 0.6 mol of zinc and 0.7 mol of calcium ion per mol of protein simultaneously. The amount of minidomain-bound Zn^{2+} was further confirmed with an independent colorimetric assay. In this latter assay, after purifying the RUBCa minidomain and removing background metal ions with Chelex 100 resin, the Zn^{2+} content was determined with the metal indicator PAR, which detects protein-bound Zn^{2+} released by protease K digestion. The released Zn^{2+} forms a $Zn-(PAR)_2$ complex accompanied by a

large increase in intensity at 500 nm ($\Delta\epsilon=6.6\times10^4\text{ M}^{-1}\cdot\text{cm}^{-1}$ at pH 7.0) within 3 ms [15] (see Supplementary Figure 2). The amount of RUBCa-bound Zn^{2+} ions ($[\text{Zn}^{2+}]/[\text{P}]$) was found to be 0.69 ± 0.03 . Thus the bacterially expressed cysteine-rich RUBCa minidomain from the RUB NS protease was capable of binding Zn^{2+} ions by itself.

Zn^{2+} -induced conformational changes in RUBCa revealed by fluorescence spectroscopy

We next asked whether the binding of Zn^{2+} ions would lead to induction of conformational changes using fluorescence spectroscopy. The RUBCa minidomain contains three aromatic tryptophan residues, one of which, Trp^{1231} , is in close proximity ($\approx 5\text{ \AA}$) to the proposed cysteine-rich zinc-binding pocket (Figure 1B). In comparison with fully exposed tryptophan (as seen in L-tryptophan solution) or highly buried tryptophan residues [as seen in domain 1 of the cell-adhesion molecule CD2 (cluster of differentiation 2)], the tryptophan residues in RUBCa were relatively well protected (Figure 2 and Table 1), indicating the structural integrity of the minidomain. Since aromatic residues are highly sensitive to local or global conformational changes, we took advantage of this feature to probe Zn^{2+} -induced conformational changes by monitoring intrinsic tryptophan fluorescence. As shown in Figure 3(A), with the gradual addition of Zn^{2+} ions to the minidomain, the emission maximum blue-shifted from 339 nm to 335 nm with a concomitant increase of fluorescence intensity by at least 15%, indicating that Zn^{2+} ions caused conformational changes and led to the shielding of aromatic residues from the solvent.

To gain quantitative information on the extent of Zn^{2+} -induced conformational changes, we then carried out an experiment to measure fluorescence quenching by acrylamide in the presence and absence of Zn^{2+} (Figure 2). The binding of Zn^{2+} to RUBCa provided protection against the quenching by acrylamide. In the presence of Zn^{2+} , the dynamic quenching constant, K_{SV} , decreased by $\approx 40\%$ from $10.5\pm0.4\text{ M}^{-1}$ to $6.3\pm0.2\text{ M}^{-1}$ (Table 1).

Zn^{2+} -induced conformational change in the RUBCa mini-domain was further confirmed by monitoring ANS fluorescence. The anionic amphiphilic ANS was used as a hydrophobic probe to examine the conformational properties of proteins. As shown in Figure 3(B), on the addition of RUBCa, the emission peak of ANS fluorescence blue-shifted from 510 to 500 nm and the intensity increased by $\approx 30\%$, suggesting that some of the hydrophobic regions of the purified RUBCa were exposed to the solvent and thus accessible to ANS. The addition of increasing amounts of Zn^{2+} caused a red shift (by 2 nm) and $\approx 10\%$ decrease of the fluorescence intensity (Figure 3B). Taken together, these data indicate that the binding of Zn^{2+} induced significant conformational changes in RUBCa by reducing the exposure of hydrophobic regions to the surface.

By monitoring tryptophan intensity change, a dissociation constant of $202\pm74\text{ nM}$ for Zn^{2+} was obtained by assuming a 1:1 binding ratio (inset to Figure 3A). Consistent with this result, an apparent dissociation constant of $468\pm321\text{ nM}$ was obtained by monitoring the ANS fluorescence intensity change as a function of Zn^{2+} concentration (inset to Figure 3B).

Mapping cysteine residues involved in Zn^{2+} binding by site-directed mutagenesis

The Zn^{2+} content of the RUBCa minidomain containing the WT (wild-type) sequence or one or more cysteine-to-serine substitution mutations (Figure 1A) were compared using the PAR colorimetric assay. As shown in Figure 4(A), except for the WT RUBCa ($[\text{Zn}^{2+}]/[\text{P}] = 0.69\pm0.03$) and the mutant C1167S ($[\text{Zn}^{2+}]/[\text{P}] = 0.67\pm0.05$), all of the other cysteine-to-serine mutants [C1175S, C1178S, C1225S, C1227S, C1167S/C1175S, C1175S/C1178S, treble mutant (C1167S/C1175S/C1178S) and C1225S/C1227S] showed a significantly lower metal to protein ratio ($[\text{Zn}^{2+}]/[\text{P}] = 0.08\pm0.29$). With the quintuple mutant (when all five of the cysteine residues were replaced by serine), the minidomain failed to bind Zn^{2+} .

Thus Cys¹¹⁷⁵, Cys¹¹⁷⁸, Cys¹²²⁵ and Cys¹¹²⁷, but not Cys¹¹⁶⁷, were involved in the coordination of Zn²⁺ in the binding pocket.

Effects of cystine-to-serine mutations on NS protease activity and virus replication

We further introduced the individual or combined cysteine-to-serine substitution mutations into RUBrep-HA/GFP, a replicon in which the RUBV structural protein ORF is replaced with a GFP reporter gene. Upon transfection of *in vitro* replicon transcripts into Vero culture cells, the transcripts were translated to produce the NS-ORF precursor which is proteolytically cleaved if it contains a functional NS protease. Cleavage is detected through the presence of an HA epitope tag in P150 by Western blotting using anti-HA antibodies [3,6]. As shown in Figure 4(B), NS protease cleavage of the precursor (termed P200) to P150 was efficient in WT RUBrep-HA transfected cells as well as in RUBrep-HA/GFP-C1167S transfected cells (the other cleavage product, P90, was not detected by anti-HA antibodies). Vero cells transfected with RUBrep-HA/GFP constructs with single cysteine-to-serine mutations at positions 1175, 1178, 1225, 1227, similar to the construct with a C1152S substitution at the catalytic site that served as the negative control, were unable to undertake cleavage (that is, only the P200 precursor was detected). In addition, cleavage in Vero cells transfected with RUBrep-HA/GFP constructs with combined cysteine-to-serine mutations (C¹¹⁶⁷S/C¹¹⁷⁸S, C¹¹⁷⁵S/C¹¹⁷⁸S, the treble mutant, C¹²²⁵S/C¹²²⁷S and the quintuple mutant) was abolished (Figure 4B). These findings correlated exactly with the effects of these mutations on Zn²⁺ binding in that mutants unable to bind Zn²⁺ lacked protease activity, whereas the Cys¹¹⁶⁷ mutant able to bind Zn²⁺ exhibited protease activity.

After transfection of *in vitro* replicon transcripts into cells, the replicon is capable of replication, as evidenced by production of the GFP reporter gene, but not cell-to-cell spread [3,6]. As expected, Vero cells transfected with transcripts containing mutations that compromised the protease activity showed no GFP fluorescence, indicating an inability to replicate (Table 2). Although the mutant C1167S was able to undertake cleavage of the precursor P200, it exhibited no fluorescence signal at all when transfected into Vero cells (see Supplementary Figure 4 at <http://www.BiochemJ.org/bj/417/bj4170477add.htm>). It seems likely that Cys¹¹⁶⁷ is not directly required for the Zn²⁺-dependent cleavage process, but is essential for the efficient replication of RUBV.

DISCUSSION

The importance of Zn²⁺ in the life cycles of viruses has been well recognized. Viral Zn²⁺-binding proteins include proteins that interact with nucleic acids and are important in transcription and/or replication, such as UL52 (unique long region 52) of HSV-1 (herpes simplex virus type 1), a member of a helicase–primase complex [16], the nucleocapsid of retroviruses (HIV-1 and murine-leukaemia virus) [17,18], the V protein of Sendai virus involved in RNA editing [19], helicases of nidoviruses such as SARS-CoV and equine-arteritis virus [20,21], the NS5A replicase component of hepatitis C virus (HCV) [22–24], and several viral proteases, such as the papain-like cysteine protease 2 of SARS-CoV [25], nsp1 (NS protein 1) papain-like cysteine protease of equine-arteritis virus (a nidovirus) [26], the leader protease of encephalomyocarditis virus [27] and the NS3 (NS protein 3) protease of HCV [28].

In our previous study [3], a model of the RUBV NS protease, based on the FMDV leader papain-like cysteine protease with which it shares reasonable (albeit distant) homology, revealed a putative cysteine-rich Zn²⁺-binding pocket with no histidine residues present in close proximity. This pocket was present in the previously characterized RUBCa minidomain, which we purified away from the GST expression tag, discovering that it bound Zn²⁺ with apparent nanomolar affinity.

Our previous study [3] reported that this minidomain also contains an EF-hand Ca^{2+} -binding motif that is required for structural stability and optimal protease activity. The requirement for both Ca^{2+} and Zn^{2+} as cofactors in a protease is reminiscent of thermolysin, a thermostable metalloprotease produced by the Gram-positive bacteria *Bacillus thermoproteolyticus*. The only bound Zn^{2+} in thermolysin is directly involved in the enzymatic activity, whereas the binding of four Ca^{2+} ions is required for thermostability [29,30]. In the present study we showed that the minidomain is well folded in the absence of Zn^{2+} , which is different from the situation in zinc-finger proteins, which require Zn^{2+} binding for folding. Our conformational analyses clearly demonstrate that the bound Zn^{2+} plays a structural role by maintaining structural integrity and shielding some hydrophobic regions from bulk solvent. Site mutagenesis of the putative Zn^{2+} -binding ligands identified Cys¹¹⁷⁵, Cys¹¹⁷⁸, Cys¹²²⁵ and Cys¹²²⁷, but not Cys¹¹⁶⁷, as co-ordinating ligands, and this corresponds exactly to protease activity *in vivo*, which requires the four earlier cited residues, but not C¹¹⁶⁷.

We previously reported that mutagenesis of Cys¹¹⁷⁵, Cys¹¹⁷⁸ and Cys¹²²⁷ results in the loss of $^{65}\text{Zn}^{2+}$ -binding activity, whereas mutagenesis of Cys¹¹⁶⁷ does not [4,5], a finding similar to that in the present study, but mutagenesis of Cys¹²²⁵ did not reduce $^{65}\text{Zn}^{2+}$ binding, in contrast with the results of the present study. In our previous study [3], the mutagenesis and $^{65}\text{Zn}^{2+}$ binding experiments were performed using MBP fusions in which Cys¹¹⁶⁷, Cys¹¹⁷⁵ and Cys¹¹⁷⁸ are present in one fusion while Cys¹²²⁵ and Cys¹²²⁷ are present on a different one, which would destroy the Zn^{2+} -binding pocket identified in the present study. In our previous study [3], Cys¹¹⁷⁵, Cys¹¹⁷⁸ and Cys¹²²⁷ mutants were found to lose *in vitro* protease activity (using a coupled transcription/translation system), a finding similar to that obtained in the present study. In the previous study, a Cys¹¹⁶⁷ mutant lost activity (this mutant retained $^{65}\text{Zn}^{2+}$ binding), whereas a Cys¹²²⁵ mutant retained *in vitro* protease activity [5], a result in contrast with the *in vivo* protease activity obtained in the present study.

The discrepancy with these previously obtained results could arise for several reasons. First, in our previous studies [3], the expressed NS protease regions were never successfully separated from the MBP tag, and thus we could not unambiguously rule out the contribution of Zn^{2+} binding by the fusion tag. Additionally, identification of Zn^{2+} -binding ligands was done using site-directed mutagenesis and a $^{65}\text{Zn}^{2+}$ blotting assay in which the MBP–NS protease region fusions were not in solution (although subjected to renaturing conditions following blotting). It should also be noted that binding using the $^{65}\text{Zn}^{2+}$ binding assay was assessed as ‘all or none’ by visual inspection, whereas the quantitative PAR assay used in the present study revealed that the decrease in Zn^{2+} binding exhibited by the single mutants was in the range of 50–65%, potentially leading to a false negative result (that is, identifying a mutant as retaining Zn^{2+} binding when it did not). Thirdly, the *in vitro* coupled transcription–translation assay was carried out under aerobic, non-reducing conditions that may not truly reflect the reducing environment of cell-based assays. Given the high numbers of cysteine residues in the NS protease (six in the less-than-100-amino acids region between residues 1152 and 1227), a reducing environment is more favourable for Zn^{2+} binding and protein folding by preventing formation of mismatched disulfide bonds. It is to be noted that, in the *in vitro* assay, cleavage of the P200 precursor was only partially complete, whereas, in the *in vivo* assay, cleavage was nearly complete. Given this, we consider that cell-based assay carried out in the present study is more reliable than the *in vitro* assay.

In summary, site-directed mutagenesis analyses carried out on the bacterially expressed minidomain within the RUBV NS protease have defined a CXXC(X)₄₈CXC Zn^{2+} -binding motif that is required for protease activity. On the basis of conformational studies on the minidomain, Zn^{2+} plays a structural role in protease activity. Given the necessity of post-

translational processing of the P200 precursor by this protease in the formation of replication complexes, it is not surprising to find that mutation of any of these residues impairs replication of a RUBV replicon. Interestingly, a nearby cysteine residue, Cys¹¹⁶⁷, is necessary neither for Zn²⁺ binding nor protease activity, but is still required for replication. Tetrahedral Zn²⁺ binding sites have been found that play structural roles in a number of other proteases of positive-sense RNA viruses [31-33], and the requirement of Zn²⁺ ion as a structural cofactor in these proteases is not a rare scenario. The spacing of the ligand residues of the RUBV NS protease Zn²⁺-binding site CXXC(X)₄₈CXC is reminiscent of the CXXC(X)₃₁CXC spacing of Zn²⁺-binding ligands in the PLpro (papain-like protease) of SARS-CoV [33,34]. Mutational analyses have revealed that the four cysteines in SARS-CoV involved in the binding of Zn²⁺ are also crucial for the proteolytic activity of PLpro. The crystal structure of SARS-CoV PLpro further confirms the mutational analyses and reveals that the Zn²⁺ ion is co-ordinated with four cysteine residues residing on two β -hairpins by adopting a tetrahedral geometry [34]. The four cysteine residues are located at or close to the termini of anti-parallel β -strands. Similar to the Zn²⁺-binding site in SARS-CoV PLpro, our results suggest that the Zn²⁺-binding site in the protease domain of RUBV are also formed by four cysteine residues. However, the four cysteine residues in RUBV protease are flanked by helices instead of β -strands. By fixing the backbone atoms and allowing free rotation of sulfur atoms on side chain in our model structure of the RUBV protease domain, the minimal distance between two sulfur atoms of proposed cysteine ligands falls into the range of 3.5–6.0 Å, which is in good agreement with the average value of 4.0 Å in SARS-CoV PLpro. Further structural determination of the RUBV protease domain will unambiguously reveal the detailed co-ordination properties of Zn²⁺-binding pocket of RUBV protease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

ANS	8-anilinonaphthalene-1-sulfonic acid
DTT	dithiothreitol
FMDV	foot-and-mouth-disease virus
GFP	green fluorescent protein
HA	haemagglutinin
ICP-MS	inductively coupled plasma MS
MBP	maltose-binding protein
NS	non-structural
ORF	open reading frame

PAR	4-(2-pyridylazo)resorcinol
PLpro	papain-like protease
quintuple mutant	one in which all five of the cysteine residues are replaced by serine
RUBV	rubella virus
SARS-CoV	severe-acute-respiratory-syndrome-associated coronavirus
triple mutant	C1167S/C1175S/C1178S
WT	wild-type

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Figure 1. Cysteine-rich metal-binding domain within the RUBV NS protein P150

(A) Domain architecture of the RUBV NS proteins and cysteine-to-serine mutations used in the present study. The NS-ORF within the context of the RUBV genome is shown at the top. Motifs present within the NS proteins include: M, methyltransferase; G, proline-rich domain; X, a region with unknown function that belongs to the Appr-1 (ADP-ribose-1"-monophosphatase) processing-enzyme family; P, protease domain; H, helicase, R, RNA-dependent RNA polymerase. In the expanded RUBCa peptide, potential residues involved in the binding of Zn^{2+} ions are labelled. Residues Cys¹¹⁵² (orange star) as well as His¹²⁷³ constitute the catalytic dyad of the protease domain, and EF is an EF-hand Ca^{2+} -binding motif in the protease domain. A series of cysteine (yellow circles)-to-serine (white circles) substitution mutations made in the study are shown. (B) Modelled structure of the RUBV NS protease with the catalytic dyad Cys¹¹⁵² and His¹²⁷³. The EF-hand Ca^{2+} -binding motif is shown in blue. The enlarged area shows the cysteine residues forming a potential Zn^{2+} -binding cluster. Cysteine residues are represented in 'ball-and-stick' format, and a tryptophan residue nearby (W¹²³¹) is shown in purple.

Figure 2. Stern–Volmer plot for the quenching of intrinsic tryptophan fluorescence of the minidomain by acrylamide

The minidomain RUBCa in the absence (apo-RUBCa) or presence [Zn(II)-RUBCa] of 100 μ M ZnCl₂ was titrated with increasing amounts of acrylamide. L-Tryptophan (L-Trp) and domain 1 of CD2 (CD2) were used as reference for fully exposed or well-buried tryptophan residues.

Figure 3. Zn^{2+} -induced conformational changes of the minidomain RUBCa revealed by fluorescence spectroscopy

(A) Tryptophan fluorescence spectra of $4 \mu\text{M}$ RUBCa in the absence (broken line) or presence (continuous line) of $20 \mu\text{M Zn}^{2+}$ in 10 mM Tris, 100 mM KCl and 5 mM DTT at pH 7.4. The inset shows the relative integrated intensity between 320 and 400 nm plotted as a function of Zn^{2+} concentration. (B) Fluorescence spectra of $40 \mu\text{M}$ ANS alone (bold continuous dark-grey line) and ANS with $4 \mu\text{M}$ RUBCa in the absence (broken line) or presence (thinner continuous line) of $20 \mu\text{M Zn}^{2+}$ in 10 mM Tris, 100 mM KCl and 5 mM DTT at pH 7.4. The inset shows the relative intensity plotted as a function of Zn^{2+} concentration. The peak positions of the emission spectra are indicated by arrows.

Figure 4. Effects of cysteine-to-serine mutations on Zn^{2+} binding and the enzymatic activity of the protease domain

(A) Zn^{2+} to protein molar ratio ($[Zn^{2+}]/[P]$) of the minidomain and its cysteine-to-serine mutants determined by the colorimetric PAR assay ($n = 2$ or 3). Carbonic anhydrase (CA), which contains a single Zn^{2+} -binding site, was used as positive control. Abbreviation: C5S, quintuple mutant. (B) P200 cleavage assay in transfected Vero cells. Vero cells were transfected with transcripts from the WT replicon construct RUBrep-HA/GFP or its mutants containing single or combined cysteine-to-serine substitutions, all of which expressed an HA-epitope-tagged P150. At 6 h after transfection, the P200 precursor and P150 product were probed with anti-HA antibodies after SDS/PAGE. The other product, P90, does not appear, because it does not contain the HA epitope. Cells transfected with a replicon containing a C1152S catalytic site mutation that failed to cleave the P200 precursor served as a negative control. Abbreviations: C3S, treble mutant; C5S, quintuple mutant.

Table 1

Quenching of the intrinsic tryptophan fluorescence by acrylamide in the absence ($-Zn^{2+}$) or presence ($+Zn^{2+}$) of $100\ \mu M\ Zn^{2+}$

The results (means $\pm$ S.E.M., $n = 2$ or 3) were obtained by fitting the curve in Figure 2 with a modified Stern–Volmer equation, taking into account both dynamic and static quenching processes (eqn 2). L-Tryptophan and the domain 1 of CD2 (CD2.D1) are used as representatives of fully exposed tryptophan or mostly buried tryptophan in solution respectively.

Sample	$\lambda_{\text{emission}}\ (\text{nm})$		$K_{SV}\ (M^{-1})$		$V\ (M^{-1})$	
	$-Zn^{2+}$	$+Zn^{2+}$	$-Zn^{2+}$	$+Zn^{2+}$	$-Zn^{2+}$	$+Zn^{2+}$
L-Tryptophan	355	355	20.9 ± 0.9	20.7 ± 0.6	0.67 ± 0.10	0.66 ± 0.12
CD2.D1	315	315	2.0 ± 0.1	1.9 ± 0.2	0.43 ± 0.06	0.44 ± 0.03
RUBC _a	339	335	10.5 ± 0.4	6.3 ± 0.2	0.63 ± 0.06	0.99 ± 0.05

Table 2
Effects of cysteine-to-serine mutations within the RUBV NS protease domain on zinc binding, protease activity and virus replication

Abbreviation: ND, not determined.

Mutation	[Zn ²⁺]/[P] [*]	Protease activity [‡]	Virus replication [§]
WT	0.69±0.03 0.6 [†]	+	+
C1152S [¶]	ND	–	–
C1167S	0.67±0.05	+	–
C1175S	0.08±0.02	–	–
C1178S	0.25±0.05	–	–
C1167S/C1175S	0.28±0.03	–	–
C1175S/C1178S	0.29±0.04	–	–
Treble mutant	0.21±0.01	–	–
C1225S	0.15±0.02	–	–
C1227S	0.26±0.02	–	–
C1225S/C1227S	0.24±0.03	–	–
Quintuple mutant	0.01±0.00	–	–

^{*} Data obtained from zinc-binding PAR assay results (n = 2–3) on bacteria-expressed and purified tag-less minimal metal binding domain in RUB NS protease.

[†] ICP-MS measurement (n = 1).

[‡] Data from the Western-blotting results using the construct RUBrep-HA/GFP and its cysteine-to-serine mutants.

[§] Data from the presence or absence GFP fluorescence expressed in Vero cells transfected with RNA transcripts of the replicon construct RUBrep-HA/GFP and its cysteine-to-serine mutants.

[¶] The active-site mutation was used as negative control for protease activity assay.