Formation of transient dimers by a retroviral protease

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Retroviral proteases have been shown previously to be only active as homodimers. They are essential to form the separate and active proteins from the viral precursors. Spumaretroviruses produce separate precursors for Gag and Pol, rather than a Gag and a Gag–Pol precursor. Nevertheless, processing of Pol into a PR (protease)–RT (reverse transcriptase) and integrase is essential in order to obtain infectious viral particles. We showed recently that the PR–RT from a simian foamy virus, as well as the separate PRshort (protease) domain, exhibit proteolytic activities, although only monomeric forms could be detected. In the present study, we demonstrate that PRshort and PR–RT can be inhibited by the putative dimerization inhibitor cholic acid. Various other inhibitors, including darunavir and tipranavir, known to prevent HIV-1 PR dimerization in cells, had no effect on foamy virus protease in vitro. 1H–13N HSQC (heteronuclear single quantum coherence) NMR analysis of PRshort indicates that cholic acid binds in the proposed PRshort dimerization interface and appears to impair formation of the correct dimer. NMR analysis by paramagnetic relaxation enhancement resulted in elevated transverse relaxation rates of those amino acids predicted to participate in dimer formation. Our results suggest transient PRshort homodimers are formed under native conditions but are only present as a minor transient species, which is not detectable by traditional methods.

Key words: cholic acid, foamy virus, NMR, paramagnetic relaxation enhancement (PRE), protease, spin label, transient dimer.

INTRODUCTION

The virus family of retroviridae consists of the two subfamilies orthoretrovirinae and spumaretrovirinae or FVs (foamy viruses). Retroviruses create viral proteins by producing large polyprotein precursors, derived from the three genes gag, pol and env. gag encodes the structural proteins (e.g. capsid, matrix and nucleocapsid protein), pol harbours the ORFs (open reading frames) for the viral enzymes [PR (protease), RT (reverse transcriptase) and integrase] and env encodes the surface and transmembrane proteins, which are localized in the viral lipid envelope and are essential for binding to the cellular receptors. The Gag and Pol polyprotein precursors are processed by the viral PR during virion maturation [1].

FVs differ in several aspects from orthoretrovirinae, e.g. FVs synthesize separate Gag and Pol precursors whereas in the case of orthoretrovirinae a Gag and a Gag–Pol precursor are formed. In FVs the PR domain, which is located at the N-terminus of the Pol precursor protein, is not cleaved off from the RT. Only the C-terminal integrase is removed from Pol, thus leading to a mature PR–RT enzyme [2–5]. In contrast, in orthoretroviruses the PR is created by autoprocessing of the Gag–Pol precursor protein and is subsequently present as a separate enzyme [6,7].

PRs from retroviruses are members of the well-characterized family of aspartic PRs [8,9]. This group also includes cellular mammalian PRs such as chymosin and pepsin. In contrast with the cellular proteases, which are monomers with distinct N- and C-terminal domains, retroviral PRs are homodimers [10]. In order to create the active site of retroviral PRs each subunit of the homodimer contributes one catalytic aspartic residue located in the conserved motif Asp-Thr/Ser-Gly. Moreover, the flap and the C- and N-termini are important for formation of the active dimer [7].

In orthoretroviruses the viral genomic RNA and the precursor proteins are packaged to form the viral particles. Therefore regulation of the activity of retroviral PRs during the viral life cycle is absolutely required to avoid premature processing of the precursors. If untimely cleavage of the polyproteins happened before virus assembly, this would lead to incomplete uptake of various viral proteins as not all of them harbour independent packaging signals.

For HIV-1 it has been shown that regulation of PR activity in the Gag–Pol precursor protein is modulated by the N-terminal flanking transframe region sequence [11–16], whereas the C-terminal RT domain does not significantly influence the PR activity of the precursor [17,18]. The presence of the N-terminal extension leads to the formation of weak dimers with low PR activity. Once the N-terminal region is cleaved off, stable and active PR dimers can be formed [19]. This type of regulation cannot take place with the FV PR as there is no Gag–Pol precursor and thus no N-terminal extension of the PR. How FV PRs are activated is still unknown. Nevertheless, dimerization, in order to form the catalytic centre, also appears to be a prerequisite for FV PR activity.

We have demonstrated recently that PRshort (the separate PR domain) of SFVmac [SFV (simian foamy virus) from macaques], as well as the full length PR–RT, exhibit proteolytic activity. Nevertheless, both enzymes appear as monomeric protein species when analysed by size-exclusion chromatography or analytical ultracentrifugation [20]. Furthermore, determination of the solution structure of PRshort by NMR also corroborated the existence of a monomeric protein [20]. We thus postulated that...
PRshort, as well as the PR domain of PR–RT, have to form weak transient dimers that are only present under certain conditions and are only populated to a low fraction and are not detectable by the methods and/or conditions applied previously.

Thus we set out to analyse the monomer/dimer status of PRshort by PRE (paramagnetic relaxation enhancement) analysis, a NMR method exquisitely suited for detecting the presence of minor species, in our case the postulated transient dimer [21–23].

A transient state is characterized by an equilibrium between a locally populated short-lived state in high-dynamic exchange with the ground state. The fast exchange of the different states causes an averaging of observable properties. If the locally populated transient state does not contribute significantly to the observed parameters it remains undetected. This could be the reason why several techniques for apparent molecular mass determination of PRshort could not detect a dimer.

PRE relies on the fact that the nuclear spins can be influenced by an unpaired electron of a paramagnetic molecule in close proximity, i.e. less than approx. 20 Å (1 Å = 0.1 nm) away. Depending on the distance, the interaction of the nuclear spin with the unpaired electron can enhance transverse relaxation rates up to several decades; a paramagnetic centre of a nitroxy group located approx. 8 Å from a given amide proton adds approx. 1800 Hz to the transverse relaxation rate, typically in the range 30–50 Hz in the diamagnetic state. Therefore even a low fraction of transient states contributes significantly to the observed population averaged rate and allows the detection of these states. In the case of transient interactions between different molecules (e.g. dimer formation) the interaction can be detected elegantly by placing the nuclear spins observed by NMR spectroscopy and the paramagnetic centres on different molecules [21–23].

In the present study, we show for the first time by biochemical and NMR analyses, using a PR inhibitor and PRE, that indeed a transient SFVmac PR homodimer is formed.

EXPERIMENTAL

Gene expression and protein purification

Expressions and purifications of 15N-labelled and unlabelled SFVmac PRshort and PR–RT and the PR substrate GB1 (immunoglobulin-binding domain B1 of streptococcal protein G)–GFP (green fluorescent protein) were performed as described previously [28,29].

PR inhibition assay

The proteolytic activities of SFVmac PR–RT and PRshort were tested as described previously [20] in buffer [50 mM Na2HPO4/NaH2PO4, pH 6.4, containing 3 M NaCl and 0.5 mM DTT (dithiothreitol]) for 2 h at room temperature (20 °C) using substrate and enzyme concentrations of 10 and 5 μM respectively.

The inhibitor tipranavir was dissolved in 5 μM DMSO and added to the PRshort solution at an approx. 5-fold molar excess over the basic ε-amino groups of PRshort. The solution was incubated using an end-over-end shaker at room temperature for 1 h followed by 1 h at 4 °C. Buffer exchange was performed by dialysing the solution against buffer (50 mM Na2HPO4/NaH2PO4, pH 7.4, containing 300 mM NaCl) using a Vivaspin concentrator with a molecular mass cut-off of 5000 (Sartorius). The efficiency of the labelling procedure was analysed by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) at the Zentrale Bioanalytik, Universität Köln, Germany, and indicated a mixture of different PRshort species containing one to all nine labelled lysine residues (results not shown).

Spin labelling of PRshort

Spin labelling of the ε-amino groups of lysine residues was performed essentially as described previously [28,29]. Freeze-dried SFVmac PRshort was dissolved in 10 mM NaHCO3, pH 9.2, to a final concentration of 9.4 mg/ml. Oxy1-1-NHS (1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate N-hydroxysuccinimide ester; Toronto Research Chemicals) was dissolved in 100 % DMSO and added to the PRshort solution at an approx. 5-fold molar excess over the basic ε-amino groups of PRshort. The solution was incubated using an end-over-end shaker at room temperature for 1 h followed by 1 h at 4 °C. Buffer exchange was performed by dialysing the solution against buffer (50 mM Na2HPO4/NaH2PO4, pH 7.4, containing 300 mM NaCl) using a Vivaspin concentrator with a molecular mass cut-off of 5000 (Sartorius). The efficiency of the labelling procedure was analysed by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) at the Zentrale Bioanalytik, Universität Köln, Germany, and indicated a mixture of different PRshort species containing one to all nine labelled lysine residues (results not shown). The labelled species were almost equally distributed with a slightly increased peak for the doubly labelled species. Owing to the solvent-exposed position of all lysine residues in PRshort we assume a random distribution of the spin label in cases of incomplete labelling. The fractional presence of completely labelled PRshort in the mass spectrum showed that all lysine residues are accessible for labelling. The reaction was not driven further to completion in order to avoid large influences of the spin label on the PR structure or on dimer formation.

Antiviral activity of cholic acid was analysed essentially as described previously [27]. In brief, HEK-293T cells [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were infected with pCMV52V2, SFV-1 and NL4-3. The supernatants were removed 16 h after transfection and replaced by fresh pre-warmed medium containing cholic acid at final concentrations from 0.125 to 2 mM or a DMSO solvent control. All cholic acid titrations were performed in independent triplicate assays. Cells were harvested 48 h after infection [27], washed twice with PBS and lysed with 200 μl sample buffer [27], and Gag expression was analysed by Western blotting. Infectious viral titres were determined on indicator cells, which expressed the LacZ gene from an LTR (long terminal repeat) promoter responsive to the respective viral transactivator.
NMR measurements

NMR experiments were recorded on Bruker Avance 600 MHz, 700 MHz (equipped with a CryoProbe) and 800 MHz (equipped with a CryoProbe) spectrometers at a sample temperature of 298 K.

NMR samples contained 100–200 μM 15N-labelled SFVmac PR short in 50 mM Na2HPO4/NaH2PO4, pH 7.4, containing 100 mM NaCl and 1 mM DTT. Samples used for PRE measurements did not contain DTT to avoid reduction of the spin label. Cholic acid was added to the desired concentration from a stock solution in DMSO. Addition of the same amount of free DMSO to the PR solution did not result in any chemical shift changes. This verifies the absence of DMSO binding to PR short. Resonance assignments were taken from the literature [20,24]. PREs of amide protons were determined using a two-point measurement with an HSQC (heteronuclear single quantum coherence)-based experiment [30]. The dissociation constant for cholic acid was determined by fitting the chemical shift changes to a two-state model during successive addition of cholic acid in a series of HSQC experiments.

RESULTS

In vitro inhibition of PR activity by cholic acid

We have shown previously by prevalent methods that FV PR behaves like a monomeric protein [20]. However, dimerization is a prerequisite for functional retroviral PRs. As we were able to detect proteolytic activities with FV PR, we postulated that a low population of the protein is present as a dimer. Thus potential protease dimerization inhibitors should be able to inhibit proteolytic activity.

For HIV-1, triterpenes and steroids have been shown to inhibit PR activity [31]. Molecular modelling studies with HIV-1 PR suggested that these substances work by inhibiting dimerization or formation of the correct, and thus active, dimer [32]. The structures of retroviral PRs are very similar, even if their primary sequences exhibit large differences [7,33]. The monomer structure of SFVmac PRshort reveals high structural homology with the HIV-1 PR monomer, as well as to the monomeric subunits of the homodimer, even though the similarity on the amino acid level is only approx. 29% [20,34,35]. Therefore we tested whether the steroid inhibitors cholic acid, lithocholic acid and betulinic acid, which have been shown to inhibit HIV-1 PR activity [32], are also able to inhibit PR activity of SFVmac PR short and of full length PR–RT. The IC50 values for in vitro HIV-1 PR inhibition by these substances were approx. 350 μM for cholic acid, 10 μM for lithocholic acid and 2.5 μM for betulinic acid [32].

The proteolytic activities of PR short and the full length PR–RT were tested with a GB1–GFP fusion protein, a substrate used previously, harbouring the natural SFVmac PR cleavage site of the Pol precursor between the GB1 and GFP domains [20]. As we have shown previously that, similar to other FV PRs, SFVmac PR short is only active at high salt concentrations, the pH optimum for PR activity was determined using NaCl concentrations of 3 M [20]. Our results indicated the highest cleavage activity at pH 6.4 (results not shown), thus these conditions were used for further analyses.

Our results using cholic acid (Figure 1) show that substrate cleavage can be inhibited in vitro at increasing cholic acid concentrations, implying that this HIV-1 PR inhibitor is functional with SFVmac PR short as well as PR–RT. Quantification of the cleavage products yielded comparable IC50 values for cholic acid, approx. 0.6 mM for SFVmac PR short and 0.75 mM for PR–RT.

Figure 1 PR short and PR–RT inhibition by cholic acid

Inhibition of 5 μM SFVmac PR–RT (○) or PR short (●) by increasing concentrations of cholic acid. The IC50 values for PR–RT (749 ± 32 μM) and PR short (815 ± 70 μM) were determined. The curves show the best fit to the data using the equation given in the Experimental section.

Testing the inhibitory effect of cholic acid against SFVmac in tissue culture assays was prevented by the toxicity of the substance for the cells at concentrations higher than 500 μM. The IC50 values for PR–RT using lithocholic acid was approx. 1 mM, whereas the IC50 of betulinic acid could not be determined due to enzyme precipitation upon addition (results not shown).

As the IC50 values obtained with the steroid derivatives are relatively high, we tested additional substances known to inhibit the PR activity of HIV-1 at much lower concentrations than cholic acid. Two non-peptidyl inhibitors, namely darunavir and tipranavir, are used for the treatment of HIV infections in patients. They have been shown to inhibit HIV-1 replication by blocking the formation of active PR dimers at the stage of PR maturation [36–38]. However, they fail to dissociate mature PR dimers [36]. These inhibitors prevent HIV-1 PR dimerization at concentrations as low as 0.01 μM [36]. As the largest portion of FV PR–RT is monomeric, these inhibitors appeared to be good candidates to prevent FV PR dimerization. However, neither of these substances was able to inhibit the proteolytic activity of PR–RT at concentrations up 100 μM in our assays (results not shown).

Furthermore, the peptidomimetic HIV-1 PR inhibitor indinavir, an active-site transition state analogue, was also not capable of inhibiting FV PR–RT at concentrations up to 100 μM. Thus we used cholic acid for further analysis.

Cholic acid binds in the putative dimerization interface of SFVmac PR

To determine the inhibitor-binding interface and to confirm the integrity of the three-dimensional structure of PR short after inhibitor addition, we analysed PR short in the absence and presence of increasing concentrations of cholic acid by observing chemical shift perturbations in 1H-15N HSQC experiments (Figure 2A). Addition of cholic acid to 15N-labelled PR short, up to a protein/inhibitor ratio of 1:150, led to gradual chemical shift changes in the 1H-15N HSQC spectra, characteristic for complex formation in the fast-exchange regime of the NMR time scale. Residues showing chemical shift perturbation upon addition of cholic acid were found in (or sequentially close to) the active-site loop (e.g. Trp23, Ala27, Thr28, Thr30 and Val31), the flap region (Ile43, Thr45, Met48, Lys49, Thr50, His51 and Gln57) and in the C-terminal region (Leu53, Met54, Lys56 and Leu60). All of these regions contribute to the known dimer interface for retroviral PRs, e.g. the essential intermonomeric antiparallel β-sheet involving the N- and C-terminals, as well as contacts close to the active site and the flap region [7,33,34] (Figures 2B–2D).

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From the chemical shift changes obtained upon addition of cholic acid, the dissociation constant \( K_d \) could be determined assuming a two-state binding model. Exemplified titration curves for some of these residues are shown in Figure 2(B). The \( K_d \) values determined for all of the amino acids analysed were in the range of \( 5.3 \pm 0.9 \text{ mM} \). For residues spatially close to the N- and C-terminus (Leu\(^{10}\) and Met\(^{146}\)) slightly weaker affinities were observed (\( K_d \) of 10 mM). Comparison of the dimensions of the interaction surface, defined by strongly shifting residues, with the size of cholic acid reveals a region significantly larger than cholic acid (Figure 2D). The chemical shift changes observed may arise from direct interaction of amino acid residues with cholic acid or by subtle structural changes (e.g. side chain rotations) transmitted to regions farther away from the exact binding site. Therefore determining a more detailed location of the binding site on SFVmac PRshort is difficult. Taken together, our results suggest that the inhibitor impairs the formation of an active PRshort dimer which in turn hinders the ability of PR to catalyse substrate cleavage.

**Specific transient dimerization detected by PRE**

Electron–nuclear spin interactions that result in a dramatic enhancement of transverse relaxation rates of protons close to a paramagnetic centre [22, 23, 40], could be assigned to residues from the N- and C-terminal regions (Figure 3A) (e.g. Leu\(^7\), Leu\(^8\), Lys\(^{96}\) and Leu\(^{146}\)), from the active-site loop (e.g. Trp\(^{23}\), Asp\(^{24}\), Ser\(^{25}\), Thr\(^{28}\) and Ile\(^{39}\)) and from the flap region (e.g. Lys\(^{49}\) and Gln\(^{56}\)). All of these regions are known to contribute to the dimer formation of active retroviral PRs [7, 41]. These results clearly demonstrate an interaction of spin-labelled SFVmac PRshort. The transversal relaxation rates, \( \Gamma_2 \), after the addition of spin-labelled PRshort indicated that the residues affected are located in the putative dimerization region of the PRshort monomer (Figure 3B). Amid protons of residues far from the dimerization interface do not exhibit significant changes in the transversal relaxation rates, demonstrating that the transient dimerization of SFVmac PR is structure-specific (Figure 3C).

Despite the large distance between Gln\(^{67}\) and Lys\(^{70}\) and the dimerization interface, significant \( \Gamma_2 \) values were observed. These residues are located in the \( \beta \)-sheet strand 6 and the preceding loop. Inspection of the dimeric structure of HIV-1 PR reveals that this region is close to the intermonomeric \( \beta \)-sheet [34]. Therefore short distances to residues of the C-terminal region of the other monomer carrying spin-labelled lysine residues can be expected.

To support the hypothesis of the role of cholic acid as a dimerization inhibitor, the inhibitor was added to the mixture of \( ^{15}\text{N} \)- and spin-labelled PRshort (Figure 4). Chemical shift changes
Transient retroviral protease dimers

Figure 3 Determination of the dimer interface

(A) Overlay of 1H-15N HSQC spectra of 15N-labelled PRshort recorded in the absence (black) or presence (red) of equimolar amounts of PRshort labelled at lysine residues with the paramagnetic spin label oxyl-1-NHS. (B) The PREs of 15N-labelled PRshort after the addition of the spin-labelled PRshort. The relevant regions of the protein are indicated at the top of the Figure. (C) Three-dimensional structure representing the hypothetical SFVmac PR dimer. The structure is based on the crystal structure of HIV-1 PR (PDB code 3HVP). The left half of the molecule represents the 15N-labelled monomer with colour coded PREs upon addition of spin-labelled PR. Residues with PREs > 20 Hz are coloured red, with PREs > 10 Hz are coloured orange. Spin-labelled lysine residues are highlighted in green on the right-hand monomer subunit.

Figure 4 PREs in the presence of cholic acid

The PREs of 15N-labelled PRshort after the addition of cholic acid to the mixture. Numbers indicate the sequence position and the relevant regions of the protein are labelled at the top of the figure.

observed for the 15N-labelled PRshort are virtually identical with the values found in the previous titration experiments presented in Figure 2 where 15N-labelled PRshort is titrated with cholic acid. These results confirm the binding of cholic acid. The titration curves reveal saturation with cholic acid to an extent of approx. 70%. The PRE rates observed for the mixture of 15N-labelled and spin-labelled PRshort in the presence of cholic acid (Figure 4) show slightly reduced values (approx. 80% of the original values), but are still present for all regions, indicating that formation of the correct dimer is impaired, but not dimerization as such.

In summary the results of the present study reveal for the first time the transient nature of the SFVmac PRshort dimer.

DISCUSSION

The active site of retroviral PRs is composed of residues from two monomeric subunits. Therefore dimerization is a prerequisite for PR activity [7,33,34].

The NMR structure of the PR domain from SFVmac, as well as analytical ultracentrifugation and size-exclusion analyses, revealed previously that the protein is a monomer in solution.
[20]. This is in strong contrast with HIV-1 PR, where several mutations were necessary to obtain the monomeric form [42–44]. Despite the exclusive detection of this apparent monomeric state for SFVmac PRshort under prevalent experimental conditions, proteolytic activity could be observed [20]. Thus a small fraction of an active dimeric species was hypothesized to exist. To test this hypothesis, we analysed SFVmac PR activity in the presence of several HIV-1 PR inhibitors, which had been suggested to impair PR dimerization. Indinavir, a peptidomimetic HIV-1 PR inhibitor, which binds to the active site, was also tested for comparison, but did not have any impact on FV PR activity. Tipranavir and darunavir, which inhibit HIV-1 PR at concentrations as low as 0.01 μM by preventing dimerization in cells, showed no inhibitory effect on SFVmac PR. This could be due to the assay conditions which included 3 M NaCl (the inhibitors might not be able to bind to the PR in high-salt buffers). Furthermore, deviations in the dimeric interfaces of HIV-1 and FV PRs, which are based on low sequence homologies, obviously result in dissimilar monomer/dimer states of the two proteins, indicating that these differences are too large to allow for the inhibitors to bind to FV PR.

Of the steroid derivatives suggested to inhibit HIV-1 PR dimerization cholic acid and lithocholic acid were unable to impair FV PR activity. However, as cholic acid showed lower IC50 values, of 0.6–0.75 mM, this inhibitor was used for further analyses. Our results have shown that SFVmac PR activity is reduced in the presence of cholic acid (Figure 1), and NMR titration experiments (Figure 2) revealed the interaction sites of cholic acid with SFVmac PRshort.

The mode of action of cholic acid as a dimerization inhibitor for HIV-1 PR was proposed by an in silico study that suggested a binding site of cholic acid between the active-site loop and the intermonomeric β-sheet of the PR [32]. This is consistent with the large number of chemical shift perturbations of SFVmac PRshort observed in our titration experiments for residues in the N- and C-terminal regions as well as for residues in the active-site loop. In addition, we observed strong chemical shift changes in the flap region, which is located at the opposite site of the protein (Figure 2). This region is flexible in solution and forms the gate for the substrate [7,41]. The chemical shift changes observed could be explained by large structural rearrangements throughout the protein. Cholic acid binds between the intermonomeric β-sheet and the active-site loop.

Alternatively, a second independent binding site could exist between the active-site loop and the flap region, either close to or in the active-site cavity. However, we were unable to directly deduce whether there was a second binding site from the titration curves (Figure 2), as the Kd values for cholic acid were too weak and the inhibitor concentration could not be increased due to solubility problems. More than one binding site for an HIV-1 PR inhibitor has been detected previously by high-resolution crystallography [45].

Our PRE measurements using spin-labelled SFVmac PRshort clearly show the presence of intermonomeric contacts even in the presence of cholic acid (Figure 4). The reduction of the PREs after the addition of cholic acid is smaller than expected for the case of complete suppression of dimer formation, implying that cholic acid only hinders the concerted interaction of regions necessary to form the correct dimer interface. Thus the action of cholic acid should be better characterized as impairing the formation of the active dimer rather than completely preventing dimerization.

The existence of specific contacts between different monomers can be shown explicitly by the observation of PREs on the 15N-labelled SFVmac PRshort after mixing with the spin-labelled PR species (Figure 3). The PREs were detected on HSQC signals corresponding to the monomeric protein, demonstrating that the interaction is in fast exchange and transient. This is in agreement with the recent observation of transient events during N-terminal autoprocessing of HIV-1 PR [19]. The observed monomer/dimer equilibrium probably regulates PR activity, as for retroviruses the flap region processing of the Gag–Pol or, in the case of FVs, the Pol precursor protein is essential for virus maturation. Premature autoprocessing of the precursors is detrimental for the virus, as uptake of all viral proteins necessary for formation of the infectious virus particle would be prevented.

For HIV-1 PR it has been shown that autoprocessing of the N-terminus of the PR at the Gag–PR junction is essential for the regulation of activity and occurs via an intramolecular first-order cleavage of the precursor, whereas processing at the C-terminus of the PR does not appear to be important [12,46,47]. However, this regulatory mechanism cannot be responsible for PR activation in FV, as no Gag–Pol precursor exists and FVs already comprise the N-terminus of the Pol precursor. Thus, as yet unknown, regulatory mechanisms for the activation of the PR appear to be important during the life cycle of FVs.

AUTHOR CONTRIBUTION

Birgitta Wöhrl conceived and co-ordinated the study. Maximilian Hartl conducted the majority of the experiments together with Kristian Schweimer and Martin Reger. Jochen Bodem designed and performed the cell culture assays. Kristian Schweimer and Stephan Schwarzinger designed the NMR experiments. Kristian Schweimer, Maximilian Hartl, Martin Reger and Stephan Schwarzinger analysed the NMR data. Paul Rösch and Stephan Schwarzinger provided conceptual input and suggestions for the completion of the manuscript. Birgitta Wöhrl, Kristian Schweimer and Maximilian Hartl wrote the paper.

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