Probing protein interactions in the membrane-containing virus PRD1

Sari Mattila,1 Hanna M. Oksanen2 and Jaana K. H. Bamford1

1Centre of Excellence in Biological Interactions, Department of Biological and Environmental Science and Nanoscience Center, PO Box 35, University of Jyväskylä, 40014 Jyväskylä, Finland
2Department of Biosciences and Institute of Biotechnology, PO Box 56, University of Helsinki, 00014 Helsinki, Finland

PRD1 is a Gram-negative bacteria infecting complex tailless icosahedral virus with an inner membrane. This type virus of the family Tectiviridae contains at least 18 structural protein species, of which several are membrane associated. Vertices of the PRD1 virion consist of complexes recognizing the host cell, except for one special vertex through which the genome is packaged. Despite extensive knowledge of the overall structure of the PRD1 virion and several individual proteins at the atomic level, the locations and interactions of various integral membrane proteins and membrane-associated proteins still remain a mystery. Here, we demonstrated that blue native PAGE can be used to probe protein–protein interactions in complex membrane-containing viruses. Using this technique and PRD1 as a model, we identified the known PRD1 multiprotein vertex structure composed of penton protein P31, spike protein P5, receptor-binding protein P2 and stabilizing protein P16 linking the vertex to the internal membrane. Our results also indicated that two transmembrane proteins, P7 and P14, involved in viral nucleic acid delivery, make a complex. In addition, we performed a zymogram analysis using mutant particles devoid of the special vertex that indicated that the lytic enzyme P15 of PRD1 was not part of the packaging vertex, thus contradicting previously published results.

INTRODUCTION

Among the prokaryotic viruses infecting either archaea or bacteria, close to half of the described virus morphotypes contain lipid as a structural component (reviewed by Atanasova et al., 2015). Membrane-containing viruses are the most characteristic virus types among the eukaryote-infecting viruses. One type of virus is the icosahedral internal membrane-containing morphotype. This type of virus is found infecting organisms of all domains of life, exemplified by bacteriophages PRD1, PM2 and P23-77 (Abrescia et al., 2004, 2008; Jaatinen et al., 2008), archaeal viruses SH1, Sulfolobus turreted icosahedral virus and Haloarcula hispanica icosahedral virus 2 (Jääniloja et al., 2008; Jaakkola et al., 2012; Veesler et al., 2013), and Paramecium bursaria chlorella virus 1 and mimivirus of eukaryotes (Xiao et al., 2005; Zhang et al., 2011).

Bacteriophage PRD1 is one of the best-known internal membrane-containing icosahedral viruses. It infects a variety of Gram-negative bacteria harbouring incompatibility P, N or W group plasmids (Olsen et al., 1974). PRD1 is the type member of the family Tectiviridae (for review, see Grahn et al., 2006; Oksanen & Bamford, 2012). The linear dsDNA genome of PRD1 (~15 kb) has inverted terminal repeats and covalently linked terminal proteins at the 5′ ends (Bamford & Mindich, 1984; Bamford et al., 1983, 1991; Savilahti & Bamford, 1986). The internal membrane consists of both protein and phospholipids in ~1:1 mass ratio (Davis et al., 1982). Eighteen out of the 31 genes encode structural proteins either associated with the membrane (half of the protein species) or with the capsid shell (Fig. 1). The major capsid protein P3 (43 kDa) is a trimer organized on the pseudo T=25 lattice forming the icosahedral capsid (Abrescia et al., 2004). The minor capsid protein P30 is necessary for particle formation as a tape-measure protein defining the dimensions of the virus particle (Abrescia et al., 2004; Rydman et al., 2001). At least 11 of the 12 fivelfold vertices are occupied by a receptor-recognizing complex formed by monomeric receptor-binding protein P2 (64 kDa), trimeric spike protein P5 (34 kDa), pentameric penton protein P31 (14 kDa) and internal membrane protein P16 (13 kDa) (Rydman et al., 1999; Jaatinen et al., 2004). One of the fivelfold vertices is different (special vertex), where the DNA-packaging machinery exists (Gowen et al., 2003; Strömsten et al., 2003). This special vertex complex consists of the integral membrane proteins P20 (5 kDa) and P22 (6 kDa) as well as packaging efficiency factor P6 (18 kDa) and packaging ATPase P9 (26 kDa) (Karhu et al., 2007; Strömsten et al.,
et al. (1982a, 1982b; Strömsten et al., 2003). In PRD1, the numerous suppressor-sensitive mutants have been invaluable in studying protein interactions and assigning functions to the corresponding proteins, e.g. revealing mechanisms for virus genome packaging (Karhu et al., 2007; Strömsten et al., 2005) or entry (Bamford & Bamford, 2000; Grahn et al., 2002a, b). PRD1 is the first and the only virus with an internal membrane so far solved by X-ray crystallography at the atomic level (~4 Å resolution; Abrescia et al., 2004; Cockburn et al., 2004). Other approaches, such as cryo-electron microscopy, cryo-electron tomography, X-ray crystallography of structural proteins, antibody labelling and biochemical virion dissociation experiments, have also provided insights into virion organization and subunit interactions (Benson et al., 1999; Caldentey et al., 1993; Gowen et al., 2003; Peralta et al., 2013; San Martin et al., 2002). However, these approaches have only provided information about interactions of soluble proteins and their possible membrane association; they could not be used to study the inner parts of the virion. Thus, the interactions of the majority of the membrane proteins and membrane-associated proteins still remain unresolved.

In this study, we applied blue native (BN)-PAGE for the analysis of protein complexes and membrane proteins and their interactions in bacteriophage PRD1. The presence of lytic enzyme P15 in the packaging-deficient mutant virus particles was also analysed by zymogram assay in order to unravel its localization and interactions in the virion.

RESULTS AND DISCUSSION

BN-PAGE as a tool to separate complexes of dissociated virus particles

BN-PAGE, combined with the solubilization of the membrane in mild conditions, is a method capable of preserving weak protein–protein interactions. Originally, it was used in the separation of mitochondrial protein supercomplexes (Schägger & von Jagow, 1991), but was later also employed in a variety of applications, such as determination of oligomeric states and native masses and identification of protein–protein interactions (Wittig et al., 2006). For viruses, BN-PAGE has been used for studying protein interactions in extracts of hepatitis C virus-infected cells as well as in measles virus particles (Brindley & Plemper, 2010; Stapleford & Lindenbach, 2011). Prior to BN-PAGE analysis, samples are usually treated with non-ionic detergents to obtain solubilized membrane protein complexes. One widely used mild detergent, digitonin, even preserves some intact supercomplexes (Wittig et al., 2006). N-dodecyl-β-maltoside (DDM) is a stronger detergent and it delipidates proteins more efficiently than digitonin, leading to more disrupted samples. Although SDS is an anionic detergent, it has been used in low concentrations prior to BN-PAGE (Klodmann et al., 2011). BN-PAGE is based on the ability of Coomassie brilliant blue of the virus (Bamford & Mindich, 1982; Mindich et al., 1982a, b; Strömsten et al., 2003). In PRD1, the numerous suppressor-sensitive mutants have been invaluable in studying protein interactions and assigning functions to the corresponding proteins, e.g. revealing mechanisms for virus genome packaging (Karhu et al., 2007; Strömsten et al., 2005) or entry (Bamford & Bamford, 2000; Grahn et al., 2002a, b). PRD1 is the first and the only virus with an internal membrane so far solved by X-ray crystallography at the atomic level (~4 Å resolution; Abrescia et al., 2004; Cockburn et al., 2004). Other approaches, such as cryo-electron microscopy, cryo-electron tomography, X-ray crystallography of structural proteins, antibody labelling and biochemical virion dissociation experiments, have also provided insights into virion organization and subunit interactions (Benson et al., 1999; Caldentey et al., 1993; Gowen et al., 2003; Peralta et al., 2013; San Martin et al., 2002). However, these approaches have only provided information about interactions of soluble proteins and their possible membrane association; they could not be used to study the inner parts of the virion. Thus, the interactions of the majority of the membrane proteins and membrane-associated proteins still remain unresolved.

In this study, we applied blue native (BN)-PAGE for the analysis of protein complexes and membrane proteins and their interactions in bacteriophage PRD1. The presence of lytic enzyme P15 in the packaging-deficient mutant virus particles was also analysed by zymogram assay in order to unravel its localization and interactions in the virion.

RESULTS AND DISCUSSION

BN-PAGE as a tool to separate complexes of dissociated virus particles

BN-PAGE, combined with the solubilization of the membrane in mild conditions, is a method capable of preserving weak protein–protein interactions. Originally, it was used in the separation of mitochondrial protein supercomplexes (Schägger & von Jagow, 1991), but was later also employed in a variety of applications, such as determination of oligomeric states and native masses and identification of protein–protein interactions (Wittig et al., 2006). For viruses, BN-PAGE has been used for studying protein interactions in extracts of hepatitis C virus-infected cells as well as in measles virus particles (Brindley & Plemper, 2010; Stapleford & Lindenbach, 2011). Prior to BN-PAGE analysis, samples are usually treated with non-ionic detergents to obtain solubilized membrane protein complexes. One widely used mild detergent, digitonin, even preserves some intact supercomplexes (Wittig et al., 2006). N-dodecyl-β-maltoside (DDM) is a stronger detergent and it delipidates proteins more efficiently than digitonin, leading to more disrupted samples. Although SDS is an anionic detergent, it has been used in low concentrations prior to BN-PAGE (Klodmann et al., 2011). BN-PAGE is based on the ability of Coomassie brilliant blue
(CBB) dye to bind hydrophobic proteins and provide them with a negative net charge, but not to interfere with most of the protein–protein interactions. However, some weaker interactions might be disrupted, which can be occasionally overcome using lower concentrations of CBB (Neff & Dencher, 1999). In BN-PAGE, protein samples covered with CBB are run in non-denaturing gel electrophoresis, leading to the separation of proteins and protein complexes according to their mass. The separated protein complexes can be resolved into their subunits in second-dimension denaturing SDS-PAGE and identified using Western blotting or MS.

Here, the purified PRD1 particles were treated with mild detergents, i.e. digitonin or DDM, to obtain protein complexes. Preliminary screenings were carried out using detergent concentrations between 0.01 and 10 % (w/v) as recommended previously (Reisinger & Eichacker, 2007). The PRD1 membrane is very resistant to various conditions, such as heating and basic pH (Caldentey et al., 1993). Also, e.g. Triton X-100 (1 %) is not able to solubilize the membrane entirely at room temperature (Luo et al., 1993a). Consequently, we combined the heat treatment with the detergent treatments when using digitonin or DDM. We obtained the largest amount of visible protein complexes in the BN-PAGE gel when 70 °C was used (data not shown for other temperatures). This is consistent with the notion that the virus capsid consisting of the trimeric major capsid protein P3 is very stable and dissociates completely only at 75 °C (Caldentey et al., 1993). In addition to mild detergents, we used the strong ionic detergent SDS, as it is known that the PRD1 membrane can be dissolved by SDS treatment even at room temperature, leaving the P3 protein capsid intact (Luo et al., 1993a).

The final experimental conditions were chosen based on the repeatability of the dissociations and maximizing the amount of protein complexes. The treated samples were analysed using a 5–16 % BN-PAGE gradient gel (the first-dimension gel) to separate detached protein complexes (Fig. 2a). Treatment with 0.1 % SDS (20 min, on ice) resulted in six visible complexes having masses of ~100–700 kDa (Fig. 2b). Treatment with 1 % digitonin or 2 % DDM at 70 °C produced nine (~70–680 kDa) and 16 (~30–900 kDa) protein complexes, respectively (Fig. 2b). We are aware of the mass estimation problems with soluble protein markers in BN-PAGE (Wittig et al., 2010), and therefore we used the estimations here only to differentiate between complexes and to have some rough values of their masses.

**Production of new polyclonal antisera against lytic enzyme P15**

To identify the protein species in different complexes, the analysis was performed using denaturing second-dimension SDS-PAGE gels combined with Western blotting. We utilized monoclonal and polyclonal antibodies recognizing PRD1 structural proteins (P2, P3, P5, P6, P7/P14, P9, P11, P16, P22, P31), and produced a new polyclonal antibody against P15 to be able to detect it in the formed subviral complexes. For this, gene XV was cloned, and the protein was expressed and purified for antibody production. Sequencing of the cloned gene revealed a point mutation (Trp6 to Leu), which was assessed not to affect antibody production, because polyclonal antibodies recognize several epitopes of the protein. The new antibody recognized specifically the viral protein P15 by Western blotting (Figs

![Fig. 2.](http://vir.sgmjournals.org)
S1–S3, available in the online Supplementary Material). In the sequential labelling, antibody detecting P15 was used first. The specificity was also tested with highly purified PRD1 Sus232 mutant (amber mutation in gene XV) particles. No signal was obtained by Western blotting (data not shown). Antibodies detecting P8, P18, P20, P30, P32 and P34 were not available for Western blotting, and so these protein species were not included in the analysis.

Receptor-recognizing vertex complex can be resolved by BN-PAGE after SDS disruption

Interpretation of the second-dimension results was occasionally quite challenging, because the complexes were not always separated clearly, which is typical for native gels. However, we were able to determine the composition of most of the complexes. The second-dimension data are shown in Figs S1–S3 and summarized in Table 1. Some of the structural proteins were detected in several complexes. Many proteins were not found in any of the multiprotein complexes obtained, but found as monomers or homomultimers (see below).

<table>
<thead>
<tr>
<th>SDS</th>
<th>Digitin</th>
<th>DDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>372–694 kDa</td>
<td>412–680 kDa</td>
<td>398–876 kDa</td>
</tr>
<tr>
<td>P2</td>
<td>P2</td>
<td>P2</td>
</tr>
<tr>
<td>P5</td>
<td>P5</td>
<td>P5</td>
</tr>
<tr>
<td>P16</td>
<td>P6</td>
<td>116–203 kDa</td>
</tr>
<tr>
<td>P31</td>
<td>P9</td>
<td>P2</td>
</tr>
<tr>
<td>110 kDa</td>
<td>113–206 kDa</td>
<td>P5</td>
</tr>
<tr>
<td>P2</td>
<td>P2</td>
<td>P7</td>
</tr>
<tr>
<td>P5</td>
<td>P5</td>
<td>P16</td>
</tr>
<tr>
<td>P11</td>
<td>P16</td>
<td>P31</td>
</tr>
<tr>
<td>75 kDa</td>
<td>P7/P14</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Proteins identified in the complexes by Western blotting in the second-dimension SDS-PAGE gels after different disruptions: SDS (treatment with 0.1 % SDS, on ice, 20 min), digitonin (treatment with 1 % digitonin, 70 °C, 10 min) and DDM (treatment with 2 % DDM, 70 °C, 10 min)

Complexes of similar protein composition are grouped (see Figs 2 and S1–S3).

SDS Digitin DDM

372–694 kDa 412–680 kDa 398–876 kDa

P2 P2 P2

P5 P5 P5

P16 P6 116–203 kDa

P31 P9 P2

110 kDa 113–206 kDa P5

P2 P2 P7

P5 P5 P16

P11 P16 P31

75 kDa P7/P14

Monomers (<66 kDa)

P3 P2 P2

P6 P6 P6

P9 P9 P9

P7/P14 P7/P14 P11

P11 P11 P16

P15 P16 P22

P16 P22 P31

P22 P31

P31

Spread through the whole gel

P3 P3 P3

P11 P11

After treatment with SDS (0.1 %), the complexes with estimated masses of 372 and 398 kDa in the first-dimension gel included at least proteins P2, P5, P31 and P16 (Fig. 3). The receptor-recognizing vertex of PRD1 contains a P2 monomer (64 kDa), P5 trimer (3 × 34 kDa) and P31 pentamer (5 × 14 kDa). In addition, there are five copies of protein P16 (13 kDa) linking the P2–P5–P31 complex to the underlying membrane (Abrescia et al., 2004). The P2–P5–P31–P16 complex has a theoretical molecular mass of 301 kDa, which is approximately the same as of those obtained by 0.1 % SDS treatment (estimated as 372 and 398 kDa complexes; Fig. 2).

In addition to those complexes, using 0.1 % SDS, we obtained several other protein complexes with higher molecular masses (~600 kDa and over), but with similar protein composition (Table 1, Figs 3 and S1). Major capsid protein P3 was detected in each of these complexes. It is possible that the peripentonal P3 trimers surrounding the vertex structures were present in these complexes in varying amounts, which would explain why complexes with different sizes were detected. It is known that SDS treatment of the PRD1 procapsid (Sus1 mutant particles missing protein P9) detaches the peripentonal trimers from the receptor-recognizing vertex structure leaving the rest of the P3 shell intact (Butcher et al., 1995; Luo et al., 1993a). This released complex is the same as that observed here by BN-PAGE. In addition, the transformation of the membrane to a tube for DNA delivery is accompanied by decapping of some of the vertex structures, including peripentonal P3 trimers (Peralta et al., 2013).

We also obtained smaller complexes containing P2, P5 and P16 by treatment with digitonin or DDM, which may represent further dissociated versions of the vertex structure (Table 1, Figs S2 and S3). Following DDM treatment, P31 seemed to be present in some amounts in these complexes. After SDS treatment, some minor amounts of P2 appeared at ~140 kDa in the second dimension, as well as significant amounts of P5 (Table 1, Figs 3 and S1). These results are in accordance with the model suggesting that P5 and P2 proteins interact with each other as separate spikes, and that P5 attaches to the virus particle via P31 (Huiskonen et al., 2007). Under most conditions, P16 and P31 appeared partly as monomers (Table 1, Figs S1–S3). Interestingly, no monomeric spike protein P5 was detected in any of the disruptions.

Major capsid protein P3 and major membrane protein P11 were detected in almost every protein complex

In certain cases, one protein species could be found in all of the complexes. The major capsid protein P3 was detected in several complexes in every disruption condition tested (Table 1, Figs S1–S3). It is the major protein species in the virion, most probably having a wide interaction network with several other structural proteins. Notably, the major capsid protein P3 was never seen as a monomer in the
milder disruption conditions (Table 1, Figs S2 and S3). The smallest units were >100 kDa, indicating that P3 always appeared in some bigger complexes or at least as a trimer. It has previously been shown that the P3 trimer is very stable (Caldentey et al., 1993; Mindich et al., 1982a). A fraction of major capsid protein P3 (43 kDa; Stewart et al., 1993) was found to be monomeric after treatment with SDS, known to be a strong detergent.

Aggregation factor P11 is the major membrane protein and also appeared over a wide mass range in the BN-PAGE gels (Table 1, Figs S1–S3). It seemed to exist at least partly in some of the complexes with P2 and P5, although we cannot draw any specific conclusions. However, it has been proposed that P11 covers the membrane vesicle, making the vesicular particles insoluble without the capsid shell (Bamford & Mindich, 1982). This might explain why it was found associated with so many different protein species. In addition, the membrane protein P16 was associated with several protein complexes after digitonin and DDM disruption (Table 1, Figs S2 and S3). This can be broadly explained by its association with vertex structures and major capsid protein P3.

Components of the packaging vertex dissociate readily

We interpreted proteins smaller than the 66 kDa marker detected in second-dimension SDS-PAGE gels as monomers. Certain proteins, such as P6 and P9, we found mostly in that area, indicating that they detached as monomers in all of the tested conditions. Proteins P6 and P9 are part of the DNA-packaging machinery located at the special vertex, and the packaging enzyme P9 is the last protein to be incorporated to the forming virion (Strömsen et al., 2003, 2005). In addition, the proposed locations of P6 and P9 indicate localization on the surface of the virion (Gowen et al., 2003; Strömsen et al., 2003), which might explain their release in the presence of detergents. In digitonin disruption, P6 and P9 were occasionally seen in the complexes of ≥412 kDa (Table 1, Fig. S2). However, the special vertex protein P22 was always monomeric. Thus, no conclusions about packaging vertex protein interactions could be drawn.

Proteins P7 and P14 appear to form a heteromultimer attached to the virus membrane

P7 and P14 were found as monomers in SDS and digitonin treatment (Table 1, Figs S1 and S2). However, a specific protein complex obtained by 2 % DDM treatment at 70 °C had an estimated mass of ~75 kDa (Figs 2 and S3, Table 1). Protein P7 has previously been detected in cross-linking experiments in a complex having a mass of similar range (Luo et al., 1993b). Here, the obtained complex included at least proteins P7 (27 kDa) and P14 (15 kDa) (Fig. 4a). In addition, protein P11 might take part in this complex, but it was detected through the whole gel, so specific conclusions could not be drawn (Fig. S3, Table 1). The mass of the complex indicated that P7 and P14 formed a heteromultimeric complex, as predicted previously (Fig. 4b) (Rydman & Bamford, 2000). Taking into account that mass estimations based on BN-PAGE analyses are only approximates, it is possible that the 75 kDa complex consisted of more than one P7 or P14 unit.

Both proteins P7 and P14 are encoded by the gene VII (Fig. 4c) (Hänninen et al., 1997). The full-length gene product P7 includes the transglycosylase domain at its N terminus and the predicted transmembrane helix at the C terminus (Rydman & Bamford, 2000). The 3’ end of the gene encodes protein P14, and thus most probably both P7 and P14 are membrane proteins. According to Davis et al. (1982), the copy number of protein P7 is ~20 copies per virion. Based on Western blots of purified virus using the mAb detecting both P7 and P14, the copy number of P14...
may be very similar (Fig. 4a, the control on the left). There could be two copies of both proteins for each of the vertices, as suggested by Rydman & Bamford (2000). Our data are in accordance with this estimation, because two P7 and two P14 proteins would form a complex of ~84 kDa.

**Zymogram analysis suggests that P15 is not part of the special vertex**

Using BN-PAGE, P15 could be detected only after 0.1 % SDS treatment and only as a monomer (Table 1, Fig. S1). It is possible, that the interaction of P15 with other structural proteins in PRD1 is so weak that it cannot be detected by BN-PAGE. No conclusions about its interactions or location could be drawn. We utilized zymogram analysis to test whether P15 could be part of the special vertex of the virion, as suggested previously (Rydman & Bamford, 2002). Zymogram analysis relies on protein PAGE, where the peptidoglycan cast in the separation gel is used as a substrate for proteins with peptidoglycan-hydrolysing activity (Bernadsky et al., 1994). The proteins first separated in the denaturing gel are renaturated and their lytic activity can be then observed as clear zones after staining the gel. We conducted experiments using purified WT particles and two P20-deficient mutant particles, Sus526 and Sus400 (amber mutation in gene XX; Strömsten et al., 2003). In addition, the ATPase P9 and the packaging-complex-associated protein P6 were missing from the mutant particles. As reported previously (Rydman & Bamford, 2000), the zymogram assay revealed that the PRD1 virion contained two lytic proteins, P7 and P15 (Fig. 5). Both mutant particles had similar lytic peptidoglycan-degrading activities as the WT particles (Fig. 5). This contradicts previously published results (Rydman & Bamford, 2002) and questions the localization of P15 as a part of the special vertex. The absence of P7 causes delayed DNA synthesis and virion liberation (Rydman & Bamford, 2000), but does not prevent virus entry. Rydman & Bamford (2002) speculated that P15 might replace P7. If this is the case, they would probably localize similarly in the virus capsid, and P15 might function in both cell lysis and cell penetration.

**CONCLUSIONS**

Viral membranes, for which the lipids are acquired from the host cell cytoplasmic membrane, can reside either outside or inside a proteinaceous capsid, or be the only protecting layer for the genome, as seen in the pleomorphic archaeal and bacterial viruses (Dybvig et al., 1985; Pietilä et al., 2012). Common for all virus morphotypes is that the viral lipid bilayer is supplemented with virus-specific transmembrane or membrane-associated proteins. In addition, the membrane organization and protein–protein interactions in the membranes are not typically symmetrically organized. Although the structure of the icosahedral
PRD1 virus with an internal membrane has been solved at 4 Å resolution (Abrescia et al., 2004; Cockburn et al., 2004), only one viral transmembrane protein was visualized in the X-ray electron density map. Various methods are needed to probe the complex structures of membrane-containing viruses, especially those techniques that could solve asymmetrical protein–protein interactions, including interactions with the membranes. One approach is BN-PAGE combined with screening different dissociation conditions. We have illustrated here that this technique is suitable for detecting membrane protein interactions in a rigid virus with strong protein–protein interactions. The feasibility of BN-PAGE was demonstrated by showing that we could identify the whole PRD1 vertex structure formed of four protein species: P2, P5, P31 and P16 (Fig. 3). P16 is the transmembrane protein linking the vertex structure to the virus membrane (Abrescia et al., 2004; Jaatinen et al., 2004). Although different mild detergents were tested, only SDS treatment was able to detach the vertices from the virus particle, allowing the analysis of its subunits by electrophoresis. The mass estimation of the complex formed of P2, P5, P31 and P16 obtained by BN-PAGE is in agreement with the theoretical mass of the vertex structure. Using DDM treatment of PRD1 particles, we could also demonstrate that transmembrane protein P7 with lytic activity and transmembrane protein P14 possibly interact together in a complex, in which they might have equal copy numbers (Fig. 4). These proteins function during virus entry at the peptidoglycan digestion and DNA translocation step (Grahn et al., 2002a). To further probe the location of lytic enzymes in the virion, we utilized zymogram analysis of P20-deficient mutant virus particles (Fig. 5) and analysed whether the lytic enzyme P15 is absent in the particles without the special vertex, as suggested previously (Rydman & Bamford, 2002). Our analysis proposes that the special vertex does not include protein P15; thus, it is still unclear where the lytic enzymes of PRD1 reside in the virion.

**METHODS**

**Production and purification of PRD1 particles.** PRD1 WT was grown on *Salmonella enterica* sv. *Typhimurium* LT2 DS88, and PRD1 mutants Sus232 (amber mutation in gene KV), Sus400 (amber mutation in gene XX) and Sus526 (amber mutation in gene XX) were grown on suppressor strains DB7156, PSA and DB7154, respectively (Mindich et al., 1976, 1982b; Strömsten et al., 2003; Winston et al., 1979). Bacteria were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) with appropriate antibiotics at 37 °C. For production of WT and mutant PRD1 particles, DS88 cells grown to a cell density of 1 × 10⁹ c.f.u. ml⁻¹ were infected at m.o.i. 7. Cells containing Sus232 mutant particles missing lytic enzyme P15 were collected by centrifugation (Sorvall SLA-3000 rotor, 5000 r.p.m., 15 min, 4 °C) 2 h after infection and disrupted with a French press (Thermo; 1000 p.s.i., twice). For disruption, cells were resuspended in 20 mM potassium phosphate buffer (pH 7.4, 1/80 of the original volume). Before polyethylene glycol (PEG) precipitation, buffer was added to restore the volume. Phage particles were precipitated by 10 % (w/v) PEG-6000/0.5 M NaCl and purified by 5–20 % (w/v) rate zonal sucrose gradient centrifugation (defined as 1 × virus; Beckman SW28 rotor, 24 000 r.p.m., 1 h, 15 °C). For zymogram analysis and P15 antibody specificity tests, material was further purified by 20–70 % equilibrium sucrose gradient centrifugation (defined as 2 × virus; Beckman SW28 rotor, 22 000 r.p.m., 17–19 h, 15 °C) (Bamford & Bamford, 1990, 1991). Purified particles were collected by differential centrifugation (Beckmann 45Ti rotor, 33 000 r.p.m., 3 h, 5 °C; Beckmann 70Ti rotor, 33 000 r.p.m., 3 h or 40 000 r.p.m., 1 h 45 min, 5 °C) and suspended in 50 mM BisTris/HCl buffer (pH 7.0, 1 × virus) or 20 mM potassium phosphate buffer (pH 7.2, 2 × virus). Protein concentration was determined by the CBB method using BSA as a standard (Bradford, 1976).

**Detergent treatment of virus samples.** Purified WT 1 × virus (1 mg ml⁻¹) was incubated in 50 mM BisTris/HCl, 500 mM 6-aminohexanoic acid and 1 mM EDTA, pH 7.0 (Schägger & von Jagow, 1991) containing 0.01–1.0 % (w/v) SDS for 20 min on ice. Treatments with DDM (0.5–2.5 %, w/v) or digitonin (0.1–2.5 %, w/v) were carried out by incubation at 70 °C for 10 min. Optimized detergent concentrations were 0.1 % SDS, 2 % DDM and 1 % digitonin.

**Gel electrophoresis.** After detergent treatments, glyceral and CBB G-250 were added to a final concentration of 5 and 0.5 % (w/v), respectively. Samples were loaded onto a linear 5–16 % (w/v) acrylamide gradient gel prepared as described previously (Schägger & von Jagow, 1991). The first-dimension gels for complex separation were run at 4 °C for at least 16 h at 100–500 V (SE400 vertical unit gel electrophoresis system; GE Healthcare). Current was limited to 15 mA. Molecular masses of the protein complexes were determined using Quantity One 1-D analysis software (Bio-Rad) and a HMW Native Marker (GE Healthcare). One complex (680 kDa, 1 % digitonin treatment) was added manually because the program did not recognize it. Vertical gel slices from the first-dimension gel were placed on the second-dimension gel (SE400 vertical unit gel electrophoresis system; GE Healthcare). Second-dimension SDS-PAGE was performed using 16 % (w/v) acrylamide gels as described previously (Ollkonen & Bamford, 1989). Agarose (0.5 %, w/v) in the gel running buffer was laid on top of the gel slice to support the control sample (20–30 μg purified 1 × PRD1 absorbed in a slice of ~8 mm width).
Whatman paper) and to seal the gel cassette. Gels were run at 50–200 V for 16–24 h. Current was limited to 15 mA. To test P15 antibody specificity, SDS-PAGE was performed as described except that the current was limited to 25 mA. PRD1 WT and Sus323 sample series of 40, 20, 10 and 5 µg were used in the analysis.

**Zymogram analysis.** Peptidoglycan sacculus was obtained from *Escherichia coli* DH5α cells (Hanahan, 1983) as described previously (Ryden & Bamford, 2002). Zymogram analysis was used to detect muramyl activity of proteins as described by Bernadsky et al. (1994). Peptidoglycan preparation was added to a final concentration of 15% (v/v) to the separation gel of the SDS-16% (w/v) polyacrylamide gel (Olkkonen & Bamford, 1989). After electrophoresis, the gels were rinsed and incubated for 30–60 min in distilled water at 4°C. The gels were incubated for 60 min in renaturation buffer (25 mM potassium phosphate buffer, pH 7.2, 0.2% Triton X-100) with gentle agitation at 4°C and incubated in fresh renaturation buffer for 48–72 h at 4°C. Zymograms were stained with 0.1% (w/v) methylene blue 0.01% (w/v) KOH at room temperature for 1 h and destained with distilled water. Virus concentrations were optimized with quality One 1-D analysis software (Bio-Rad) using P3 protein on gels as a standard. An aliquot of 5 µg 2× virus or lysozyme control was used in zymogram analysis.

**Glutathione S-transferase (GST)-P15 cloning and recombinant P15 protein purification.** Standard molecular biology techniques were used in DNA manipulations (Sambrook et al., 1989). A derivative of expression plasmid pGEX-4T-3 (GE Healthcare), in which a TEV cutting site was included, was used for cloning. PCR was used to amplify the gene sequence using the PRD1 genome as a template. Primers hybridizing to the target area were designed: 5’ primer PRD1_P15F (ATATATGAAAGACACCATGGGACAATATACACTTT-Nco) and 3’ primer PRD1_P15R (GGGATATTATGATATCAGGGC caricat aTACATTCTT-GGATATTTATCAGGGC) containing a Ncol site and a restriction enzyme site (Sambrook et al., 1989). Amplified fragments were inserted between the Ncol and NheI sites of plasmid pGEX-4T-3 TEV and transformed into *E. coli* HB101 (Bolivar, 1979; Boyer & Roulland-Dussioix, 1969). The sequence of the insert was determined (ABI Prism 3130d; University of Jyväskylä). Plasmid pSPM1 was transformed into *E. coli* BL21-Gold (Carstens & Waesche, 1999), resulting in the GST-P15-producing strain BL21-Gold (pSPM1).

For production of GST-P15, an overnight culture of BL21-Gold (pSPM1) cells was diluted 1/20 and grown at 28°C for production of GST-P15, an overnight culture of BL21-Gold (pSPM1) (18 ml) was used to amplify the gene with a TEV cutting site was included, was used for cloning. PCR was used to amplify the gene sequence using the PRD1 genome as a template. Primers hybridizing to the target area were designed: 5’ primer PRD1_P15F (ATATATGAAAGACACCATGGGACAATATACACTTT-Nco) and 3’ primer PRD1_P15R (GGGATATTATGATATCAGGGC cacratat cA TACATTCTT-GGATATTTATCAGGGC) containing a Ncol site and a restriction enzyme site (Sambrook et al., 1989). Amplified fragments were inserted between the Ncol and NheI sites of plasmid pGEX-4T-3 TEV and transformed into *E. coli* HB101 (Bolivar, 1979; Boyer & Roulland-Dussioix, 1969). The sequence of the insert was determined (ABI Prism 3130d; University of Jyväskylä). Plasmid pSPM1 was transformed into *E. coli* BL21-Gold (pSPM1). For production of GST-P15, an overnight culture of BL21-Gold (pSPM1) cells was diluted 1/20 and grown at 28°C until the OD 550 reached 0.75 (Clorich; Selecta). Protein production was induced with 1 mM IPTG and the temperature was reduced to 18°C. After overnight growth the cells were collected (Sorval SLA-3000 rotor, 5000 r.p.m., 10 min, 4°C) and suspended in 1/100 of the original volume using PBS (pH 7.3) containing 1 mM DTT/5% (v/v) glycerol. Cells were stored at −80°C.

Concentrated cell suspension (100 ×) of BL21-Gold (pSPM1) (18 ml) was thawed and buffer (PBS/1 mM DTT/5% glycerol) was added to a final volume of 40 ml to purify recombinant protein P15. After disruption of cells with a French press (Thermo) at 2000 p.s.i. (twice), Pefabloc SC (Roche) was added to a concentration of 1 mM. The cell debris were removed by centrifugation (Sorvall SS-34 rotor, 20000 r.p.m., 30 min, 4°C) after which protein solution was filtered (Sartorius Minisart; 0.45 µm). For purification, GST-P15 was bound to an affinity column at 20°C (Glutathione Sepharose 4 Fast Flow matrix; GE Healthcare). After washing the column with PBS containing 1 mM DTT, GST-P15 was eluted with 40 mM reduced glutathione in PBS containing 1 mM DTT, filtered and the buffer was changed (Amicon Ultra-15 Millipore; Ultracel-10K) to 20 mM HEPES/1 mM EDTA/150 mM NaCl/1 mM DTT (pH 7.9). The GST-tag was cleaved with ProTEV protease (Promega; 0.3 U µl−1) at 8°C overnight. P15 was purified by gel filtration (GE Healthcare; HiLoad 26/60; Superdex; 1 ml min−1; 20 mM HEPES/1 mM EDTA/150 mM NaCl/1 mM DTT, pH 7.9). P15-containing fractions were pooled and concentrated (Millipore; Amicon Ultra-15, Ultracel-10K). The protein purity (~90%) was estimated by SDS-PAGE using 15% (v/v) acrylamide gel (Olkkonen & Bamford, 1989) and the concentration was determined by the Bradford assay using BSA as a standard (Bradford, 1976). The identity of the purified protein was confirmed by MS analysis with a high-performance nano LC-(H)ESI Orbitrap Elite Hybrid Mass Spectrometer (Thermo Scientific; University of Helsinki) and its activity was tested by zymogram analysis. Polyclonal sera against protein P15 were raised in rabbit using purified P15 as antigen (Inbiolabs).

**Antibodies and Western blotting.** Western blotting was performed by transferring the proteins from the SDS-PAGE gels onto PVDF membranes (Millipore), followed by visualization with peroxidase-conjugated secondary antibodies (Dako) and chemiluminescent detection using a Super Signal West Pico (Thermo) system. Specific rabbit antiserum recognizing PRD1 proteins P2 (Grahn et al., 1999), P6 (Karhu et al., 2007), P3 (Ryden et al., 2001), P5 (Hänninen et al., 1997), P9, P22 (Strömsten et al., 2003), P15 (this study) and P31 (Ryden et al., 1999) as well as mAbs 7N41 (for P7 and P14), 11T25 (for P11) and 16A201 (for P16) (Hänninen et al., 1997) were used as primary antibodies for sequential labelling.

**ACKNOWLEDGEMENTS**

This work was supported by the Finnish Centre of Excellence Program of the Academy of Finland (Virus Research 2006–2011, 1129684; Biological Interactions 2012–2017, 252411), Academy of Finland grant to J. K. H. B. (251106), and Ellen and Artturi Nyssönen Foundation grant to S. M. We also thank the University of Helsinki and Academy of Finland (grants 271413 and 272853) for the support to the EU ESFRI Instruct Centre for Virus Production (ICVIR). We thank Professor Jari Ylänne for kindly providing plasmid pGEX-4T-3 TEV, Dr Ulla Penttikäinen for advice on sequencing and cloning of gene XV, and Mr Petri Papponen and Mrs Irene Hellkala for skilled technical assistance.

**REFERENCES**


Bamford, J. K. & Bamford, D. H. (2000). A new mutant class, made by targeted mutagenesis, of phage PRD1 reveals that protein P5...


