Triatoma virus structural polyprotein expression, processing and assembly into virus-like particles

Rubén Sánchez-Eugenia,1 Fernando Méndez,2 Jailson F. B. Querido,1,3,4 Marcelo Sousa Silva,4,5 Diego M. A. Guérin,1,6 and José F. Rodríguez2

1Unidad de Biofísica (CSIC, UPV/EHU), Barrio Sarriena S/N, 48940 Leioa, Bizkaia, Spain
2Departamento de Biología Molecular y Celular, Centro Nacional de Biotecnología-CSIC, Darwin 3, 28049 Madrid, Spain
3Fundación Biofísica Bizkaia, Barrio Sarriena S/N, 48940 Leioa, Bizkaia, Spain
4Centro de Malária e Outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira, 100, 1349-008 Lisboa, Portugal
5Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, Brazil
6Departamento de Bioquímica y Biología Molecular, Facultad de Ciencia y Tecnología, Universidad del País Vasco (EHU), Barrio Sarriena S/N, 48940 Leioa, Bizkaia, Spain

In contrast to the current wealth of structural information concerning dicistrovirus particle structure, very little is known about their morphogenetic pathways. Here, we describe the expression of the two ORFs encoded by the Triatoma virus (TrV) genome. TrV, a member of the Cripavirus genus of the Dicistroviridae family, infects blood-sucking insects belonging to the Triatominae subfamily that act as vectors for the transmission of Trypanosoma cruzi, the aetiological agent of the Chagas disease. We have established a baculovirus-based model for the expression of the NS (non-structural) and P1 (structural) polyproteins. A preliminary characterization of the proteolytic processing of both polyprotein precursors has been performed using this system. We show that the proteolytic processing of the P1 polyprotein is strictly dependent upon the coexpression of the NS polyprotein, and that NS/P1 coexpression leads to the assembly of virus-like particles (VLPs) exhibiting a morphology and a protein composition akin to natural TrV empty capsids. Remarkably, the unprocessed P1 polypeptide assembles into quasi-spherical structures conspicuously larger than VLPs produced in NS/P1-coexpressing cells, likely representing a previously undescribed morphogenetic intermediate. This intermediate has not been found in members of the related Picornaviridae family currently used as a model for dicistrovirus studies, thus suggesting the existence of major differences in the assembly pathways of these two virus groups.

INTRODUCTION

The Dicistroviridae family clusters a group of small picornavirus-like viruses harbouring positive-strand (+) ssRNA genomes characterized by the possession of two non-overlapping cistrons or ORFs (Mayo, 2002). This family belongs to the Picornavirales order, and currently encompasses 15 characterized species grouped in two genera, Aparavirus and Cripavirus (Le Gall et al., 2008). Some dicistroviruses, e.g. Acute bee paralysis virus, Acute bee paralysis virus, are highly pathogenic to beneficial arthropods (Genersch & Aubert, 2010; de Miranda et al., 2010; Wertheim et al., 2009). Other family members, e.g. Drosophila C virus, have been used as models for characterizing antiviral defence mechanisms in insects (Kingsolver et al., 2013). Additionally, some dicistroviruses like Solenopsis invicta virus-1 and Triatoma virus (TrV), the object of the present report, are highly relevant for human health (Bonning & Miller, 2010).

A salient feature of dicistroviral genomes is the presence of two independent internal ribosome entry sites (IRES) located immediately upstream of each cistron (Czbener et al., 2005). A schematic representation showing the major elements of the Dicistroviridae genome is presented in Fig. 1. TrV ORF 1 is located at the 5'-genome end and encodes a polyprotein with an estimated molecular size of 204 kDa, known as NS, harbouring the non-structural virus proteins, i.e. helicase, protease and RNA-dependent RNA polymerase (RdRp) (Czbener et al., 2000). Positions of the NS cleavage sites have been experimentally mapped only in Plautia Stali intestine virus (PSIV), another member of the Dicistroviridae family, and it turned out that the
processing gives rise to nine different products comprising one helicase, one protease, multiple VPgs (genome-linked virus proteins), one RdRp and other unidentified proteins (Nakashima & Ishibashi, 2010; Nakashima & Nakamura, 2008; Nakashima & Shibuya, 2006). TrV ORF 2 occupies the 3′-genome region and encodes a polyprotein, known as P1, with a predicted M, of 95.5 kDa. P1 is the precursor of the TrV structural proteins, i.e. VP0 (37.3 kDa), VP1 (29.7 kDa) and VP2 (28.4 kDa). VP0 is further processed releasing the VP3 (31.8 kDa) and VP4 (5.5 kDa) polypeptides (Agirre et al., 2011; Czibener et al., 2000).

TrV infects several insect species belonging to the Triatominae subfamily (Hemiptera: Reduviidae) commonly known as triatomines or kissing bugs (Marti et al., 2013; Muscio et al., 1988). Triatomines are blood-sucking insects acting as the chief vectors for the transmission of Trypanosoma cruzi, a protozoan responsible for the Chagas disease (American Trypanosomiasis) (Grayson, 2010).

TrV is transmitted both horizontally and vertically causing lethal and sublethal effects. This, together with the narrow host range of the virus that precludes the infection of mammalian species (Querido et al., 2013), has raised a growing interest in the use of TrV as a promising biological agent to control the spreading of Chagas disease vectors (Susevich et al., 2012; Bonning & Miller, 2010; Czibener et al., 2000; Muscio et al., 1997).

The capsid structures of two dicistroviruses, TrV and Cricket paralysis virus (CrPV), have been characterized at the atomic level (Squires et al., 2013; Tate et al., 1999). These capsids are naked icosahedrons (T = 3 symmetry) built by 60 copies of each of the three major structural proteins VP1–3 (all folded with a jelly roll core). Both TrV and CrPV capsids are quite similar (Squires et al., 2013), with the location of the VP4 peptide being the main structural difference between them (Tate et al., 1999; Squires et al., 2013). Characterization of the TrV capsid disassembly and genome release allowed us to propose a novel RNA-release mechanism for this virus (Agirre et al., 2013; Snijder et al., 2013) that differs from those proposed for members of the Picornaviridae family, i.e. Human Rhinovirus 2 (Garriga et al., 2012; Hewat et al., 2002) and Poliovirus (Bostina et al., 2011; Levy et al., 2010).

Characterization of TrV gene expression has been largely hampered by the lack of cell lines supporting virus replication. To overcome this experimental limitation we have generated two recombinant baculoviruses (rBVs) independently expressing the TrV ORFs. Data presented here show that the proteolytic processing of the P1 polyprotein is strictly dependent upon coexpression of the NS polyprotein, and that coexpression of both TrV cistrons leads to the assembly of virus-like particles (VLPs) akin to natural TrV empty capsids (Agirre et al., 2011, 2013). We also demonstrate that the unprocessed form of the P1 polypeptide assembles into quasi-spherical structures larger than VLPs produced in NS/P1-coexpressing cells. Results presented here provide an important insight into the dicistrovirus molecular and structural biology, and open a new venue for the characterization of the TrV assembly pathway.

**RESULTS**

**Expression of recombinant TrV ORFs 1 and 2 in insect cells**

In order to assess the expression of TrV-encoded polypeptides, two rBVs, BV_NS and BV_P1, containing TrV ORF 1 and 2 respectively, were generated as described in the Methods section.

As a first approach to assess the expression TrV ORFs 1 and 2, H5 monolayers grown in glass coverslips were infected using an m.o.i. of 0.3 p.f.u. per cell of either WT BV (BV_WT), BV_NS or BV_P1. Some cultures were coinfected with both BV_NS and BV_P1 also using an m.o.i. of 0.3 p.f.u. of each virus. The m.o.i. used for these experiments was to allow a proper scrutiny of the specificity of the used sera by having both infected and uninfected, as well as single and double infections, in every sample under analysis. At 48 h post-infection (p.i.), cultures were processed for immunofluorescence (IF) using sera against the RdRp and VP1 domains of the NS and P1 precursor polypeptides, respectively.

As shown in Fig. 2(a, b), cells infected with either BV_NS or BV_P1 exhibited specific IF signals that in both cases were strictly restricted to the cytoplasm of rBV-infected cells. It is noteworthy that coinfected cultures showed the presence of both RdRp- and VP1-specific IF signals in a significant number of cells (Fig. 2c), thus showing that both protein precursors are efficiently coexpressed at a
single cell level. The specificity of the anti-RdRp and -VP1 polyclonal sera used for these experiments was further confirmed by the absence of significant IF signal in cells infected with the BV_WT control virus.

To further characterize the expression of both TrV ORFs 1 and 2, a series of Western blot analyses were performed. For this, H5 cultures infected with BV_WT, BV_NS or BV_P1 at an m.o.i. of 2 p.f.u. per cell, or coinfected with both BV_NS and BV_P1 using a m.o.i. of 2 p.f.u. per cell of each virus, were harvested at 48 h p.i., and the corresponding extracts subjected to SDS-PAGE followed by Coomassie blue staining or Western blotting against the RdRp or VP1 polypeptides, respectively. As shown in Fig. 3(a), specific NS- or P1-derived polypeptides were not readily detectable in Coomassie blue-stained gels, thus suggesting a moderate expression of both recombinant polypeptides in rBV-infected cells. However, Western blotting performed with the anti-RdRp serum revealed the presence of three immunoreactive bands with estimated Mr of ~110, 90 and 63 kDa in samples from cells infected with BV_NS or coinfected with BV_NS and BV_P1 (Fig. 3b). The Mr of the smallest product is comparable to that reported (62 kDa) for the PSIV RdRp (Le Gall et al., 2008), thus possibly corresponding to the mature form of the TrV RdRp polypeptide. It seems likely that the two larger products might correspond to NS processing intermediates. A band matching the Mr of the full-length NS precursor was not detected. These data indicate that the NS protease domain efficiently directs the self-cleavage of its precursor polypeptide.

The anti-VP1 serum identified a major polypeptide with an electrophoretic mobility of ~95 kDa, akin to that expected for the unprocessed form of the P1 polypeptide (Czibener et al., 2000), in extracts from cells infected with BV_P1 (Fig. 3c). The faint ladder of faster-migrating polypeptide also detected in this sample might represent P1 degradation products (Fig. 3c). It is noteworthy that the anti-VP1 serum recognized two protein products in extracts from cells coinfected with BV_NS and BV_P1; a major species corresponding to a polypeptide of ~29 kDa, similar to the mature VP1 polypeptide found in natural TrV capsids (Agirre et al., 2011; Czibener et al., 2000), and a minor one, comigrating with the intact P1 product detected in cells infected with BV_P1 alone, thus likely corresponding to the unprocessed form of the P1 protein (Fig. 3c). This observation indicates that the NS-encoded protease specifically cleaves the P1 product rendering a VP1 polypeptide of the expected Mr.

**Proteolytic processing of NS and P1 precursor polypeptides**

To further characterize the processing of TrV precursor polypeptides, an immunoprecipitation (IP) analysis was performed using extracts from 35S-methionine-labelled rBV-infected cells. Two sets of H5 monolayers were infected with BV_WT, BV_NS or BV_P1 at an m.o.i. of 2 p.f.u. per cell, or coinfected with both BV_NS and BV_P1 using a m.o.i. of 2 p.f.u. per cell of each virus. At 48 h p.i. cells were labelled for 1 h with 35S-methionine. The first culture set was collected immediately after the labelling period whilst the second one was further incubated in medium containing a 10-fold excess of cold methionine for 12 h. Samples were processed for IP analysis.

As shown in Fig. 4(a), no obvious differences were detected between the total radioactive polypeptide patterns from BV_WT- and BV_NS-infected cells. However, a distinctive band of a ~95 kDa was clearly detectable on extracts from cells infected with BV_P1 or coinfected with BV_NS and BV_P1. In agreement with Western blot results, the IP performed with the anti-RdRp serum specifically recognized three polypeptides with electrophoretic mobilities of ~110, 90 and 63 kDa, respectively, in extracts from both cultures.
infected with BV_NS either alone or coinfected with BV_P1 (Fig. 4b). Additionally, IPs carried out with this serum also rendered several faster-migrating bands. These polypeptides might represent either NS-derived degradation products or minor NS processing polypeptides. The anti-RdRp serum also rendered a weak protein band of ~95 kDa in extracts from cells infected with BV_P1, suggesting a possible cross-reaction of this serum with the P1 precursor.

As expected, the anti-VP1 serum specifically recognized a major 95 kDa protein band in extracts from BV_P1-infected cells (Fig. 4c) collected both immediately after the radioactive pulse or following the 12 h chase period. This band was also found in the sample corresponding to BV_NS/BV_P1-coinfected cells harvested immediately after labelling, but not in that collected after the chase. Interestingly, both samples from BV_NS/BV_P1-coinfected cells showed the presence of new ~29 kDa protein akin to that previously detected by Western blotting (Fig. 3c).

**TrV VLP assembly**

The results described above suggested the possibility that the coexpression of both TrV cistrons might lead to the assembly of TrV-derived VLPs. To explore this hypothesis, H5 cultures were infected with BV_WT, BV_NS or BV_P1, or coinfected with BV_NS and BV_P1, fixed at 48 h p.i., and used for thin-section electron microscopy (TEM) analysis. As shown in Fig. 5(a), the cytoplasm of ~40 % cells coinfected with BV_NS and BV_P1 contained isometric particles with a morphology akin to that expected for TrV particles. Some (~10 %) of the analysed cells contained large accumulations formed by hundreds of tightly packed particles (Fig. 5b). Unexpectedly, the cytoplasm of BV_P1-infected cells also contained quasi-spherical assemblages resembling VLPs (Fig. 5c). However, their diameter was conspicuously larger than that of the isometric structures detected in NS/P1-coexpressing cells. None of these assemblages were detected in BV_NS- or BV_WT-infected cells (not shown).

To further characterize the detected assemblages, extracts from cells infected with BV_NS, BV_P1, or coinfected with both rBVs were subjected to sucrose gradient fractionation. As expected, samples from BV_NS-infected cells did not contain NS-derived polypeptides, thus indicating that products released upon NS processing do not assemble into particulate structures (data not shown). In contrast, high-density fractions from cells infected with BV_P1 contained a single protein band of ~95 kDa, identical to the full-length P1 polypeptide detected in extracts from BV_P1-infected cells (Fig. 6a). The identity of this protein was checked by anti-VP1 Western blot analysis.

High-density fractions from gradients from cells coinfected with BV_NS and BV_P1 contained five bands of ~38, 35.5, 33, 29 and 27 kDa (Fig. 6b), that likely correspond to

**Fig. 3.** SDS-PAGE and Western blot analysis of NS and P1 expression in H5 cells. Samples from cells infected with BV_NS (NS) or BV_P1 (P1) or coinfected with both viruses (NS+P1) were subjected to SDS-PAGE followed by Coomassie blue staining (a) or Western blotting using anti-RdRp (b) or anti-VP1 (c) antibodies. Samples from WT baculovirus-infected cells (BV-WT) and from purified TrV particles (TrV) were used as controls for these experiments. Positions of Mr markers are indicated.
the VP0, VP3, VP1 and VP2 polypeptides, respectively. The identity of the 29 kDa product was confirmed to be VP1 by Western blot analysis (Fig. 6b). The 35.5 kDa product might correspond to PP3, a non-canonical TrV polypeptide previously detected in native TrV empty particles (Agirre et al., 2011).

Selected gradient fractions from P1 or NS/P1 samples were pooled and subjected to ultracentrifugation to concentrate the particulate material, and visualized by TEM. As shown in Fig. 6(c), samples from BV_P1-infected cells contained a population of quasi-spherical particles with a diameter of $52.7 \pm 4.9$ nm. In contrast, samples from BV_NS/BV_P1-coinfected cells contained isometric particles with a diameter of $30.6 \pm 1.5$ nm (Fig. 6d) and an icosahedral morphology indistinguishable from that of previously described native TrV empty capsids ($31.1 \pm 1.8$ nm of diameter; Fig. 6e) (Agirre et al., 2011). Average yields of particles purified from cells infected with BV_P1 or coinfecte
d with BV_P1 and BV_NS were of $\sim 65 \mu g/10^{-7}$ cells.

**DISCUSSION**

Despite the current wealth of dicistrovirus particle structure data, the assembly pathways of this virus group remain poorly understood. This is largely due to the lack of reliable molecular tools facilitating the characterization of critical events such as the processing of virus-encoded polyproteins, and the assembly of structural polypeptides. Repeated attempts to express different versions of the TrV P1 precursor polypeptide using a variety of prokaryotic vectors failed due to aggregation of P1-derived products. To overcome this problem, we decided to explore the feasibility of using rBV expression vectors.

**TrV protein expression and proteolytic processing in rBV-infected cells**

Although the described NS and P1 recombinant genes are placed under the transcriptional control of the strong BV polyhedrin promoter, data presented in this report reveal that the level of expression of the TrV-derived polyproteins is somewhat lower than expected. Indeed, attempts to enhance NS and P1 expression levels, e.g. increasing the m.o.i. of infecting rBVs and extending the length of the infection, did not significantly improve the accumulation of recombinant TrV-derived products. The reasons underlying the moderate expression levels of both polyproteins are unknown at this point. Certainly, it would be interesting to determine whether recombinant TrV genes
containing their corresponding IRES sequences might boost expression levels.

Western blot and pulse–chase IP-analyses of radiolabelled polypeptides showed that BV-mediated TrV ORF 1 expression led to accumulation of three NS-derived products of 110, 90 and a 63 kDa, likely corresponding to the mature RdRp. IP data also show that after their release from the precursor polyprotein, these three polypeptides are not further processed. Interestingly, a product with the molecular size expected for the full-length NS precursor was not detected by Western blot or by IP analyses. This suggests that the recombinant NS polyprotein undergoes a swift self-proteolytic processing event preventing the accumulation of the precursor.

Western blot and IP data revealed that expression of the TrV ORF 2 led to the synthesis of a 95 kDa product that, according to its size, represents the unprocessed form of the P1 precursor. In the absence of ORF 1, the P1 polyprotein remains unprocessed. However, in cells coexpressing the ORF 1, the P1 precursor is efficiently processed rendering a ~29 kDa product that, as described above, likely represents the VP1 structural polypeptide found in purified TrV virions (Agirre et al., 2011), hence evidencing the accuracy of the processing performed by the 3C-protease expressed in this system.

Data presented here show that the described rBV expression vectors are a useful tool to systematically characterize the series of proteolytic processing events undergone by both TrV precursor polyproteins. Indeed, additional antibodies against other NS and P1 protein domains might shed further light on their proteolytic processing.

**Assembly of TrV VLPs**

Electron microscopy (EM) analysis of thin sections of cells coexpressing the NS and P1 by EM revealed the presence of
virus-like assemblies within the cell cytoplasm, thus matching the subcellular localization of the VP1 and RdRp polypeptides, as determined by confocal laser scanning microscopy. Application of a sucrose gradient-based protocol to extracts of NS/P1-coexpressing cells led to the isolation of icosahedral VLPs indistinguishable from bona fide TrV particles when observed under EM. SDS-PAGE analysis of VLP-containing gradient fractions revealed that the protein composition of the purified VLPs was akin to that of natural empty TrV particles containing the VP0–VP3 polypeptides (Agirre et al., 2011). The TrV VLP protein pattern also showed a high proportion of the VP0 precursor along with a minor relative abundance of the putative mature VP3 product. The presence of the putative VP3 polypeptide in the VLPs suggested that the VP0→VP4+VP3 TrV maturation step (Agirre et al., 2011) is not directly associated with the encapsidation of the virus genome. However, the possibility that ssRNA molecules, potentially mimicking the virus genome, might be incorporated during the assembly of TrV VLPs cannot be completely ruled out at this point.

The absence of non-isometric assemblages and the P1 precursor form in purified VLP preparations suggests that the low amount of unprocessed precursor remaining in initial infected-cell extracts might be completely processed by the C3 protease, also present in the extracts, during the purification procedure.

Interestingly, TrV VLPs contained a minor polypeptide band exhibiting an electrophoretic mobility akin to that of the PP3 protein found in both purified TrV virions and native empty particles (Agirre et al., 2011). This finding

Fig. 6. Characterization of particles isolated from P1- and P1/NS-expressing cells. Fractions collected from sucrose gradients performed with samples from cells infected with BV_P1 (a) or coinfected with BV_NS and BV_P1 (b) were subjected to SDS-PAGE followed by silver staining and anti-VP1 Western blot analysis, respectively. Positions of M, markers are indicated. Analysed fractions were pooled, concentrated, spotted onto carbon-coated grids and negatively stained. Particles from cells infected with BV_P1 (c) or BV_NS and BV_P1 (d) are shown. A TEM image of naturally occurring empty capsids of TrV (e) is shown for comparison. Insets show higher magnification (×2) details of the original images.
constitutes an additional indication that the PP3 product is not an adventitious contaminant, and that it might play a role during particle assembly. Proteomic analysis might be instrumental in establishing the PP3 origin.

Whilst this manuscript was under revision, a report describing the generation of VLPs from Israeli acute paralysis virus (IAPV), another member of the Dicistroviridae family, was published. IAPV-derived VLPs were generated in insect cells infected with an rBV expressing a chimeric gene formed by the fusion the IAPV ORF 2 in-frame that corresponds to its cognate 3C-like protease from ORF 1 (Ren et al., 2014).

Unprocessed P1 precursor particle assembly

Members of the Picornaviridae family have been commonly used as a model for understanding dicistrovirus molecular and structural biology. Picornavirus particle assembly and maturation has been extensively characterized using different virus models, e.g. Foot-and-mouth disease virus (Goodwin et al., 2009), Enterovirus 71 (Hu et al., 2003), Poliovirus (Hellen & Wimmer, 1992) and Rhinovirus (Palmenberg, 1990). Picornaviruses also possess (±)ssRNA genomes but, unlike dicistroviruses, they harbour a single ORF encoding all the structural and non-structural polypeptides (Tuthill et al., 2010). In most picornaviruses, genome translation gives rise to a single polypeptide that is self-processed by the 2A-protease domain releasing a series of polypeptides, including the P1 capsid precursor polypeptide. The 3C-protease processes P1 releasing the VP0, VP3 and VP1 structural polypeptides. Processed structural proteins remain associated forming a protein complex that acts as a monomer for the self-assembly of the 12 pentameric subunits that build the virus capsid (Goodwin et al., 2009; Li et al., 2012). However, if the P1 capsid precursor is not processed into individual proteins, large-sized particles are not formed (Goodwin et al., 2009). It has been generally shown that P1 cleavage precedes and is necessary for the assembly of the pentameric subunits (Tuthill et al., 2010).

Unexpectedly, data presented here show that the unprocessed form of the TrV P1 precursor gives rise to non-isometric assemblages accumulating in the cell cytoplasm. These assemblages, not detected in samples from TrV-infected specimens, are conspicuously larger than natural TrV VLPs. SDS-PAGE analysis demonstrated that the purified P1-particles are exclusively built by the 95 kDa P1 precursor polypeptide, thus indicating that, in contrast to what has been found for members of the Picornaviridae family, the unprocessed TrV form of the P1 polypeptide self-assembles into a coherent large-sized structure. At this point it is not feasible to determine whether P1-assemblages represent a true TrV morphogenetic intermediate or a dead-end structure. Indeed, the three-dimensional reconstruction of this novel structure might shed some light on this intriguing question. Additionally, structural analysis of P1-assemblages following incubation with recombinant TrV C3-protease might also help in further understanding their role during TrV assembly. These experiments are currently under way.

Finally, TrV VLPs described here constitute a promising addition to the existing arsenal of VLP platforms currently used for recombinant vaccine generation (Rodriguez & Guérin, 2014).

METHODS

Cells and viruses. Trichoplusia ni H5 insect cells (Invitrogen) were used for rBV infections. H5 cells were grown in TC-100 medium (Gibco) containing 10% FCS. rBVs were grown and titered as previously described (Martinez-Torrecuadrada et al., 2000).

Recombinant baculoviruses (rBVs). A cDNA fragment corresponding to the TrV genome region encoding the P1 polyprotein (nucleotides 6109–8715 from TrV full-length RNA; NCBI reference sequence: NC_003783) was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: (sense) 5'-CCGGAATTCATGCCTCCGTTAAATGAATATG, incorporating an EcoRI restriction site (underlined) and ATG initiation codon (bold); and (anti-sense) 5'-ATAAAGATTCGCGGCGGCCCCAATGAGTGC, incorporating a NotI restriction site (underlined) and a TAG termination codon (bold). A cDNA fragment encoding the NS polyprotein (nucleotides 549–5933 from TrV full-length RNA) was generated by RT-PCR using the following primers: (sense) 5'-ATAAAGATTCGCGGCGGCCCCAATGAGTGC, incorporating a NotI restriction site (underlined) and an ATG initiation codon (bold); and (anti-sense) 5'-CCGCTCGAATCTCATGCACTTGCG, incorporating an XhoI restriction (underlined) site and a TGA termination codon (bold). The resulting PCR products were cloned into pFastBac1 plasmid (Invitrogen) and the resulting plasmids, pFastBac1 and pFastBac1-NS, were analysed to assess the correctness of the cloned TrV sequence and used to generate the corresponding bacmids by transforming DH10Bac Escherichia coli strain. Recombinant bacmids were selected, purified and transfected into H5 cells following the Bac-to-Bac protocol as recommended by the manufacturer (Invitrogen). The resulting rBVs were termed BV_P1 and BV_NS, respectively. Additionally, an empty rBV, BV_WT, was generated using a bacmid produced following transformation of DH10Bac cells with pFastBac1. BV_WT was used as a control for some experiments.

Purification of TrV-derived VLPs. H5 monolayers were infected with BV_P1 or coinfected with BV_P1 and BV_NS at an m.o.i. of 2 p.f.u. of each virus per cell. At 48 h.p.i., cells were harvested by centrifugation and resuspended in lysis buffer [50 mM Tris/HCl pH 7, 500 mM NaCl, 1 mM MgCl2 and 0.25 % (v/v) Igepal CA-630 (Sigma-Aldrich)] supplemented with complete protease inhibitor cocktail (Roche). Cells were lysed by incubation in lysis buffer for 20 min at 4 °C followed by three freeze–thaw cycles, and sonication. Cell lysates were centrifuged at 15 000 g for 20 min to pellet cell debris, and supernatants centrifuged through a 20 % (w/v) sucrose cushion in NMT buffer at 100 000 g for 4 h at 4 °C. The resulting pellets were resuspended in 1 ml of NMT buffer (50 mM Tris/HCl pH 7, 10 mM NaCl and 1 mM MgCl2) and then loaded on the top of 35 ml discontinuous 10–30 % (w/v) sucrose gradients. The subsequent centrifugation was carried out at 100 000 g for 2.5 h at 4 °C. Gradients were fractionated into 1.5 ml aliquots. Fractions enriched in TrV structural proteins were collected, dialysed against NMT buffer, and loaded onto a Sephacryl S-500 HR (GE Healthcare) column equilibrated with gel filtration buffer (50 mM Tris pH 7.5 and 150 mM NaCl). Fractions enriched in structural proteins were concentrated by centrifugation at 180 000 g for 3 h at 4 °C. The
resulting pellets were resuspended in NMT buffer. Purification steps were monitored by SDS-PAGE followed by silver staining and by anti-VP1 Western blot analysis.

**Preparation of specific anti-VP1 and anti-RdRp polyclonal sera.** For the preparation of antibodies against P1 and NS, two different regions of these polypeptides were selected, cloned and overexpressed in the *E. coli* BL21(DE3). Polyclonal antibodies were prepared by immunizing BALB/c mice and New Zealand white rabbits with purified protein inclusion bodies following a previously described protocol (Querido et al., 2013).

**Confocal laser scanning microscopy (CLSM).** H5 cells seeded for the preparation of antibodies against P1 and NS, two sera. Preparation of specific anti-VP1 and anti-RdRp polyclonal VP1 Western blot analysis. were monitored by SDS-PAGE followed by silver staining and by anti-RdRp monoclonal antibodies. Samples were visualized by epifluorescence. Fluorescent signals detected by confocal laser scanning microscopy were recorded separately by using appropriate filters.

**SDS-PAGE and Western blot analyses.** SDS-PAGE and Western blot analyses were carried out to analyse the expression and processing of both the P1 and NS precursors and to assess VLPs purification steps. Samples were loaded on 12 % SDS-polyacrylamide gels. Gels were either stained with Coomassie blue (Sigma) or electroblotted to nitrocellulose membranes (Whatman). After blocking with 5 % (w/v) milk in TBST buffer [10 mM Tris pH 7.5, 150 mM NaCl and 0.05 % (v/v) Tween 20 (Sigma-Aldrich)], membranes were incubated with primary antibody. Coverslips were repeatedly washed in PBS, and incubated with the appropriate secondary antibodies: goat anti-rabbit coupled to Alexa-488 (green), or goat anti-mouse coupled to Alexa-594 (red), with the appropriate secondary antibodies: goat anti-rabbit coupled to Alexa-488 (green), or goat anti-mouse coupled to Alexa-594 (red), diluted in PBS supplemented with 1 % FCS for 45 min at 37 °C. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) diluted in PBS for 30 min at room temperature. Finally, coverslips were dehydrated with ethanol and mounted with ProLong antifade reagent (Invitrogen). Samples were visualized by epifluorescence. Fluorescent signals detected by confocal laser scanning microscopy were recorded separately by using appropriate filters.

**Immunoprecipitation (IP) analysis.** H5 cell monolayers grown in p100 dishes were infected with the described rBVs at an m.o.i. of 2 p.f.u. per cell of each virus. At 48 h.p.i., monolayers were washed twice with PBS, and incubated for 30 min with methionine-free TC-100 medium (Genaxxon Bioscience) for 1 h. Following this methionine-starving step, cultures were incubated with methionine-free TC-100 medium supplemented with 1.85 × 10^6 Bq of [35S]methionine ml⁻¹ for 1 h at 28 °C. After this period, cultures were either harvested and stored at −80 °C until processing or washed twice with warmed PBS and further incubated in TC-100 medium supplemented with a 10-fold excess of methionine for 12 h before harvesting. Cell pellets were resuspended in radioimmunoprecipitation (RIPA) buffer (50 mM Tris/HCl pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1 % NP-40, 1 % sodium deoxycholic acid, 0.1 % SDS). Samples were then subjected to IP using the anti-RdRp and -VP1. IP reactions were performed using Protein A Sepharose CL-4B (GE Healthcare) as previously described (Rodriguez et al., 1989). The resulting samples were subjected to 12 % SDS-PAGE gels followed by autoradiography.

**ACKNOWLEDGEMENTS**

This work was supported by the Spanish Ministry of Economy and Competitiveness (AGL2011-24758), awarded to J. F. R. S.-E. and supported by a predoctoral fellowship from the Basque Government and F. M. by an FPI fellowship from the Spanish Ministry of Economy and Competitiveness. D. M. A. G. was supported by the Basque Government (MV-2012-2-41, AE-2012-1-44, S-PE12FB001) and by the MICINN (BFU2012-36241), Spain. We thank Antonio Varas and Dr Artiz Durana for their interest and excellent technical support as well as the Scientific Microscopy Service of the UPV/EHU (SGiker) for the use of EM facilities.

**REFERENCES**


