Short Communication

Identification of the Borna disease virus (BDV) proteins required for the formation of BDV-like particles

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Borna disease virus (BDV) is an enveloped virus with a non-segmented, negative-strand RNA genome that has an organization characteristic of Mononegavirales. However, based on its unique genetics and biological features BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*. Here, the use of a reverse genetic approach to identify the viral proteins required for packaging of BDV RNA analogues (MG) into infectious virus-like particles (VLPs) was described. Plasmids encoding individual BDV proteins under the control of a RNA polymerase II promoter were co-transfected with a plasmid that allows for intracellular synthesis of a BDV MG mediated by the cellular RNA polymerase I. Clarified lysates from transfected cells were passaged onto fresh cells that were previously transfected with plasmids expressing the minimal BDV *trans*-acting factors L, N and P required for RNA synthesis mediated by the BDV polymerase. Reconstitution of BDV MG-specific packaging and passage of infectious VLP was monitored by expression of the chloramphenicol acetyl transferase reporter gene present in the BDV MG. BDV M and G, in addition to L, N and P, were sufficient for the passage of chloramphenicol acetyl transferase activity, which could be blocked by BDV neutralizing antibodies to G, indicating that VLP infectivity was fully mediated by BDV G. Passage of BDV MG was abrogated by omission of either M or G.

BDV causes central nervous system (CNS) disease that is frequently manifested by behavioural abnormalities (Ikuta *et al.*, 2002; Pletnikov *et al.*, 2002; Rott & Becht, 1995). Evidence indicates that the natural host range of BDV, as well as its prevalence and geographical distribution are very broad (Hatalski *et al.*, 1997; Ikuta *et al.*, 2002; Richt *et al.*, 1997; Richt & Rott, 2001; Rott & Becht, 1995; Staeheli *et al.*, 2000). Moreover, serological data and molecular epidemiological studies indicate that BDV can infect humans, and it might be associated with certain neuropsychiatric disorders (Billich *et al.*, 2002; Carbone, 2001; Planz *et al.*, 2002; Richt *et al.*, 1997; Richt & Rott, 2001; Rott & Becht, 1995; Staeheli *et al.*, 2000).

BDV is an enveloped virus with a non-segmented, negative-strand (NNS) RNA genome. Its genome (ca 8·9 kb), the smallest among known NNS RNA viruses, has an organization similar to that of other mononegaviruses (de la Torre, 1994; Schneemann *et al.*, 1995). Six major open reading frames (ORFs) are found in the BDV genome sequence (de la Torre, 1994; Schneemann *et al.*, 1995). Based on their positions in the viral genome (3′-N-p10/P-M-G-L-5′), together with their biochemical and sequence features, as well as recent functional studies using reverse genetic approaches (Kawaoka, 2004) these polypeptides are the counterparts of the nucleoprotein (N), phosphoprotein (P) transcriptional activator, matrix (M) protein, surface glycoprotein (G) and L polymerase, respectively, found in other NNS RNA viruses (Conzelmann, 2004; Tordo *et al.*, 1992). The p10 ORF starts 49 nt upstream from P within the same mRNA and p10 overlaps, but in a different frame, with the 71 N-terminal amino acids of P. Notably, BDV has the property, unique among known animal NNS RNA viruses, of a nuclear site for the replication and transcription of its genome (Briese *et al.*, 1992; Cubitt & de la Torre, 1994). In addition, BDV uses a remarkable diversity of strategies, including RNA splicing, for the regulation of its genome expression (Cubitt *et al.*, 2001; de la Torre, 1994; Jehle *et al.*, 2000; Schneemann *et al.*, 1995; Tomonaga *et al.*, 2000). Based on its distinct genetic and biological features among known NNS RNA viruses, BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*, within the order Mononegavirales.

As with other negative-strand RNA viruses, the template of the BDV polymerase is exclusively a nucleocapsid (NC) consisting of the genomic RNA tightly encapsidated by the virus N protein. This NC associated with the viral polypeptides of the polymerase complex forms a ribonucleoprotein (RNP) complex active in transcription and replication, which is

also the minimum unit of infectivity (Conzelmann, 1998, 2004; Garcia-Sastre & Palese, 1993; Tordo *et al.*, 1992). Thus, generation of biologically active synthetic virus from cDNA will require *trans*-complementation by all viral proteins involved in virus replication and transcription. For a variety of negative-strand RNA viruses, systems have been developed which permit the encapsidation, transcription, replication and packaging of synthetic genomic RNA analogues into virus-like particles (VLPs) in cells expressing all the required viral polypeptides from plasmid (Kawaoka, 2004). These VLPs are budded into the extracellular space and can infect new cells, where they will replicate if the required *trans*-acting viral proteins are also expressed. These systems have facilitated the investigation of the viral *cis*-acting sequences and proteins required for genome packaging, as well as maturation and budding of VLPs. Moreover, it has allowed the generation and rescue of infectious viruses entirely from cloned cDNAs for members of several different families of negative-strand RNA viruses (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Kawaoka, 2004; Neumann *et al.*, 2002).

Recently, we (Perez *et al.*, 2003b) and others (Schneider *et al.*, 2003) have documented the establishment of a reverse genetic system for intracellular reconstitution of BDV replication and transcription. Similarly to other NNS RNA viruses examined (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Neumann *et al.*, 2002), BDV L, N and P constituted the minimal viral *trans*-acting factors required for MG expression (Perez *et al.*, 2003b; Schneider *et al.*, 2003). Notably, of the two isoforms of the BDV N (Np40 and Np38) found in BDV-infected cells, only Np40 was competent in promoting BDV MG replication and expression (Perez *et al.*, 2003b; Schneider *et al.*, 2003). The polypetide of 10 kDa encoded by the p10 ORF and present in BDV-infected cells (Wehner *et al.*, 1997) was not required for RNA synthesis mediated by the BDV polymerase, but rather it exhibited a strong inhibitory effect in both RNA replication and transcription of the BDV MG (Perez *et al.*, 2003b; Schneider *et al.*, 2003). Several negative-strand RNA viruses code accessory proteins that are not strictly required for RNA synthesis mediated by the virus polymerase, but they contribute to the regulation of a variety of steps in the virus life cycle (Neumann *et al.*, 2002). Interestingly, the accessory C proteins of the rhabdovirus vesicular stomatitis virus (VSV) and several paramyxoviruses are encoded, as p10, by the P gene and are expressed via RNA editing or from additional ORFs overlapping the P gene (Nagai, 1999). These C proteins have been implicated in different activities including virus assembly, virulence and viral countermeasures of the interferon induced antiviral stage, as well as regulation of RNA synthesis by the virus polymerase (Nagai, 1999; Neumann *et al.*, 2002). Whether BDV p10 might have similar kind of functions remains to be determined.

Here, we have examined the requirements of viral proteins for production of BDV infectious VLPs. For most negative-strand RNA viruses, this process is assumed to depend on the interaction between the RNP core and the virus-encoded transmembrane glycoproteins (G) (Lyles *et al.*, 1992; Mebatsion *et al.*, 1999). The M protein is thought to play an essential role in this interaction. Moreover, budding of rabies virus and VSV does not require strictly the presence of G, suggesting an intrinsic budding activity of the M protein (Justice *et al.*, 1995; Mebatsion *et al.*, 1999; Takada *et al.*, 1997). Nevertheless, G can significantly enhance budding (Mebatsion *et al.*, 1996; Robison & Whitt, 2000).

Using a pseudotype approach based on a recombinant VSV in which the gene for green fluorescent protein is substituted for the VSV G protein gene (VSVΔG*) (Takada *et al.*, 1997), we have shown that BDV G is sufficient to mediate receptor recognition and cell entry (Perez *et al.*, 2001). Based on this observation and the evidence accumulated with other NNS RNA viruses, we hypothesized that also for BDV, the viral M and G proteins were sufficient, to direct the assembly of VLPs. To test this hypothesis, we transfected 293T cells with the minimal viral *trans*-acting factors (N, P and L) together with plasmids expressing M and G, as well as the BDV MG, and examined whether G-containing BDV infectious VLPs could be generated. For these studies, we used a plasmid (hPol I-MG) that allowed for intracellular synthesis of a BDV MG RNA directed by the human polymerase I present in 293T cells. To generate this construct, the previously described BDV MG (pol I-MG) (Perez *et al.*, 2003b) was subcloned into a plasmid containing the human RNA polymerase I promoter (Fodor *et al.*, 1999). We first determined transfection conditions that allowed for co-expression of M and G without significantly affecting levels of BDV MG expression. For this we transfected 293T cells (1×10^6/M6 well) with hPol I-MG, together with the indicated combination of plasmids expressing N (pC-N), P (pC-P), L (pC-L), p10 (pC-p10), and various amounts of plasmids expressing M and G. The pC-P construct used for these experiments only contains the P ORF and hence it cannot direct expression of p10. Sixty hours later cell extracts (CE) were prepared and assayed for CAT activity as described previously (Perez & de la Torre, 2003). Previously, we have shown that levels of MG-derived CAT activity correlates well with levels of RNA synthesis mediated by the virus polymerase (Perez *et al.*, 2003b). As previously reported BDV L, N and P were sufficient for efficient BDV MG replication and expression (Fig. 1, lane 1). Moreover, we observed that in cells co-transfected also with 0·2 μg of each plasmid expressing M and G, levels of BDV MG expression remained unaffected (Fig. 1, lane 2). Previously, we (Perez *et al.*, 2003b) and others (Schneider *et al.*, 2003) have shown that low amounts of pC-p10 (60 ng plasmid per 1×10^6 cells) completely inhibited BDV MG expression. Consistent with this we observed that addition of 100 ng pC-p10 to the transfection mix caused a very strong inhibitory effect on BDV MG expression (Fig. 1, lane 3). Notably, this inhibitory effect was released in cells co-transfected also with M and G

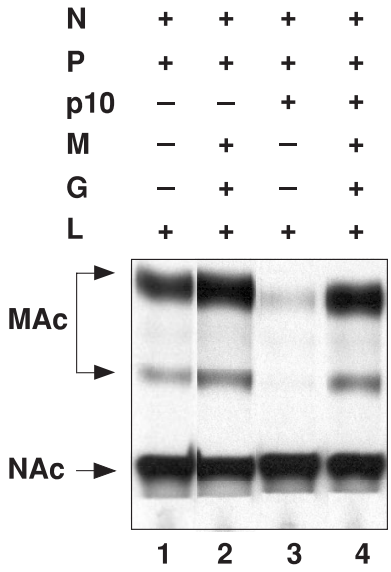

Fig. 1. Co-expression of M and G does not affect BDV MG expression. 293T cells (1×10^6/M6 well) were transfected using Lipofectamine 2000 (LF2000) (2·5 μg LF2000 μg^{-1} DNA) with hpol I-MG (0·5 μg) and the indicated combination of plasmids: N (0·5 μg), P (40 ng), p10 (0·1 μg), M (0·2 μg), G (0·2 μg) and L (0·5 μg). Sixty hours later CE were prepared and assayed for CAT activity as described previously (Perez & de la Torre, 2003).

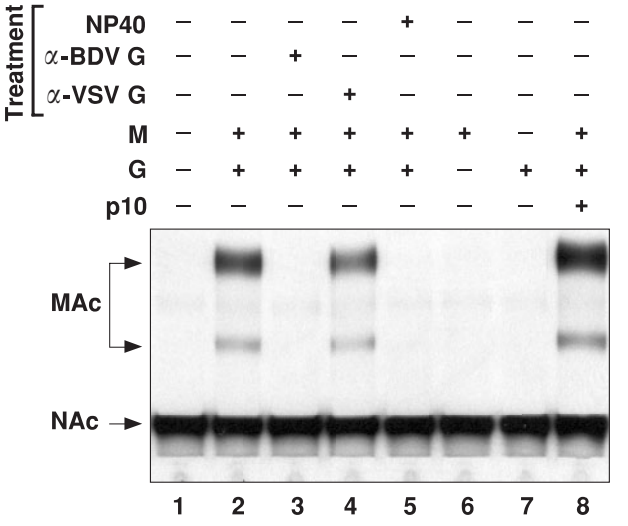

Fig. 2. Generation of BDV infectious VLPs. 293T cells (1×10^6 cells/M6 well) were transfected as in Fig. 1 with hPol I-MG (0·5 μg), L (0·5 μg), N (0·5 μg), P (40 ng), and the indicated plasmid combinations. Seventy-two hours after transfection whole CE were prepared by ultrasonication and VLP collected by ultracentrifugation through a 20 % sucrose cushion. VLP-containing pellets were resuspended in Optimem containing 1 % fetal bovine serum. Aliquots of VLPs were subjected to the treatments indicated on the top of Fig. 2: (i) NP40 (0·05 % for 20 min in ice), (ii) serial dilutions of the nt RbAb to BDV G (60 min at 30 °C) and (iii) the nt mouse monoclonal Ab to VSV G (60 min at 30 °C). Untreated samples were incubated for 60 min at 30 °C in the presence of 10 % heat inactivated normal rabbit serum. Treated and untreated VLP-containing samples were used to infect cells previously transfected with L, N and P. Sixty hours later CE were prepared and assayed for CAT activity as described previously (Perez & de la Torre, 2003).

(Fig. 1, lane 4). The reason for this finding remains to be determined, but it would suggest that in the presence of M and G additional interactions among viral, or viral and cellular, proteins take place and prevent p10 from exerting its inhibitory effect on RNA synthesis mediated by the virus polymerase. It is worth noting that we have observed a similar situation with the arenavirus small RING finger Z protein (Lee *et al.*, 2002). Thus, the very powerful inhibitory of the arenavirus Z on RNA synthesis mediated by the virus polymerase was dramatically diminished in the presence of the virus surface G (Perez *et al.*, 2003a).

We then examined the viral protein requirements for the formation of BDV infectious VLPs. For this we transfected 293T cells with the indicated combination of plasmids (Fig. 2), using established conditions (Fig. 1) that allowed for co-expression of M and G and good levels of BDV MG expression (Fig. 1). Seventy-two hours later whole-cell extracts were prepared by ultrasonication and VLPs collected by ultracentrifugation through 20 % sucrose cushion. VLP-containing pellets were resuspended in Optimen containing 1 % fetal bovine serum, and aliquots treated as indicated (Fig. 2), and then used to infect 293T cells previously transfected with N, P and L. Sixty hours later CE were prepared and assayed for CAT activity. Consistent with the lack of cell-free virus associated with BDV infections, only clarified whole-cell lysates, but not supernatants, of transfected cells were capable of passaging the CAT reporter gene activity (not shown). Both M and G were required for the passage of reporter gene activity (Fig. 2, lanes 2, 6 and 7), suggesting that these two viral gene products are required for efficient assembly and passaging of BDV VLPs. The requirement of G for the formation of infectious VLPs was expected based on its established role in virus receptor recognition and cell entry (Perez *et al.*, 2001). M is not required for replication and expression of BDV MG (Perez *et al.*, 2003b; Schneider *et al.*, 2003). Hence, the requirement of M for production of infectious VLPs reflects, most likely, a role of M in BDV particle assembly. Nevertheless, we cannot currently rule out a possible participation of M in early steps of virus entry. Using RT-PCR we detected BDV MG RNA associated with infectious particles present in clarified extracts prepared from cells transfected with M and G in addition to L, N and P plasmids (not shown). It should be noted that this finding, however, did not allow us to distinguish between MG RNA associated with bona fide BDV VLPs, or with BDV RNP. The low level of VLP production may be related to the BDV intrinsic feature of an

extremely low level of virion production in virus-infected cells [0·05 to 0·1 focus-forming units (FFU) per cell] (Danner *et al.*, 1978; Danner & Mayr, 1979). This, in turn, jeopardizes a direct biochemical characterization of BDV VLPs obtained via reverse genetics.

We conducted several control experiments to confirm that the CAT activity detected in the passage was mediated by G-containing VLPs. Thus, incubation of clarified CE with a neutralizing (nt) rabbit serum (RbAb) to BDV G, but not with a nt monoclonal Ab (I1) to VSV G (Lefrancois & Lyles, 1982), abrogated CAT activity in the passage (Fig. 2, lanes 3 and 4). In addition, treatment of clarified CE with the non-ionic detergent NP40 (Fig. 2, lane 5), but not with RNase (not shown), prevented the passage of reporter gene activity. To verify the specificity of the rabbit serum to BDV G with neutralizing activity used for these studies we performed neutralization assays using bona fide BDV, or as a control VSV, virions (Fig. 3). The RbAb to BDV G neutralized BDV but not VSV. Conversely, the monoclonal Ab I1 neutralized VSV but not BDV. The RbAb to BDV G did not have any significant effect on the infectivity associated with BDV RNP (Fig. 3). These findings further support our conclusion that the passage of reporter gene activity was mediated by G-containing VLPs and not by RNP complexes or free CAT RNA molecules. Passage of clarified CE onto non-transfected cells did not result in detectable levels of CAT activity in the passage (not shown), indicating that BDV VLPs enclosed an MG encoding CAT RNA rather than a CAT enzyme.

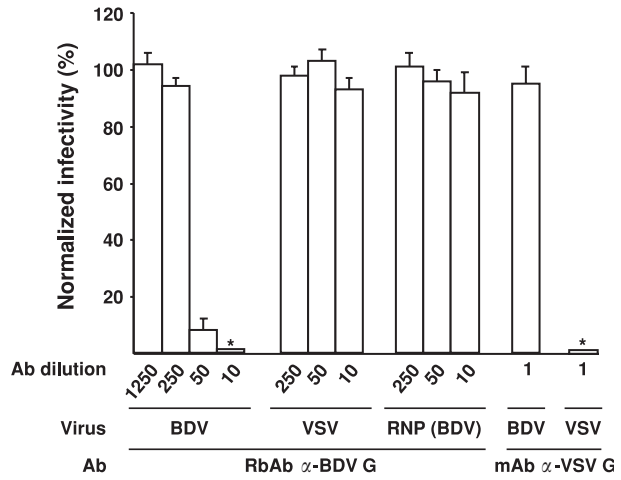

Fig. 3. Specificity of the nt RbAb to BDV G. BDV (250 FFU) or VSV (250 p.f.u.), or the amount of BDV infectious RNP (Cubitt & de la Torre, 1994) corresponding to 250 FFU, were treated with the indicated serum dilutions (60 min at 30 °C). Control samples were incubated in the presence of 10 % heat inactivated normal rabbit serum (60 min at 30 °C). After the treatments, BDV infectivity was determined using an immunofocus assay (Cubitt & de la Torre, 1994), and VSV by standard plaque assay. Infectivity associated to BDV RNP was assessed by transfecting cells (Cubitt & de la Torre, 1994) and determining FFU using an immunofocus assay. The asterisk indicates a normalized virus infectivity below 1 %.

The establishment of a BDV MG rescue system (Perez *et al.*, 2003b; Schneider *et al.*, 2003) together with the findings reported here opens the possibility for future studies aimed at the investigation of the molecular interactions between viral and viral–cell proteins required for the formation of infectious BDV particles, as well as studies aimed at defining amino acid residues within BDV G involved in receptor recognition and cell entry. Moreover, the reported findings provide the foundations for the rescue of infectious BDV from plasmid DNA. The ability in the future to generate predetermined specific mutations within the BDV genome and analyse their phenotypic expression *in vivo*, will significantly contribute to the elucidation of the molecular mechanisms underlying BDV–host interactions, including the basis of BDV persistence in the CNS and associated disease.

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