Short Communication

Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin

P. L. Delputte, S. Costers and H. J. Nauwynck

Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Correspondence
H. J. Nauwynck
hans.nauwynck@UGent.be

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Heparan sulphate and sialoadhesin were previously identified on porcine macrophages as receptors for porcine reproductive and respiratory syndrome virus (PRRSV). In this study, the exact role and cooperation of heparan sulphate and sialoadhesin during PRRSV attachment and internalization was analysed. It was observed that both heparan sulphate and sialoadhesin mediate PRRSV attachment and that only these two receptors are involved in attachment. Analysis of attachment kinetics of PRRSV to macrophages revealed that early attachment is mediated mainly via an interaction with heparan sulphate, followed by a gradual increase in interaction with sialoadhesin. By using wild-type CHO and CHO deficient in heparan sulphate expression, it was shown that heparan sulphate alone is sufficient to mediate PRRSV attachment, but not entry, and that heparan sulphate is not necessary for sialoadhesin to function as a PRRSV internalization receptor, but enhances the interaction of the virus with sialoadhesin.

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae*, which is grouped together with the *Coronaviridae* and the *Roniviridae* in the order of the *Nidovirales* (Enjuanes *et al.*, 2000; Mayo, 2002). A characteristic for the *Arteriviridae* is that they all share a marked *in vivo* tropism for cells of the monocyte/macrophage lineage (Plagemann & Moennig, 1992; Snijder & Meulenberg, 1998). *In vivo*, PRRSV infects a subpopulation of differentiated macrophages, which can be identified by expression of sialoadhesin (Sn) (Duan *et al.*, 1998; Vanderheijden *et al.*, 2003). Besides differentiated macrophages, porcine testicular germ cells (spermatids and spermatocids) show limited susceptibility to PRRSV infection (Sur *et al.*, 1997). *In vitro*, porcine alveolar macrophages (PAM), some cultivated peripheral blood monocytes and the African green monkey kidney cell-line MA-104, and cells derived thereof, such as Marc-145 and CL-2621, can be used to grow the virus (Kim *et al.*, 1993; Voicu *et al.*, 1994; Duan *et al.*, 1997).

At present, two PRRSV receptors are identified on porcine macrophages, the *in vivo* target cell. Heparan sulphate was identified as a receptor on macrophages for both European and American strains, and the viral matrix protein on itself, or as a complex with GP_5, was identified as a heparin-binding protein (Delputte *et al.*, 2002). The macrophage-specific protein Sn was identified as a receptor that mediates PRRSV internalization of European and American strains (Vanderheijden *et al.*, 2003). mAb 41D3, specific for Sn, is able to block infection completely, and cells that are not permissive for PRRSV infection internalize the virus upon expression of a recombinant Sn (Duan *et al.*, 1998; Vanderheijden *et al.*, 2003). Although these cells can internalize the virus, they are not productively infected. The virus apparently remains in the endosome and the viral genome is not released in the cytoplasm, indicating that a cellular factor, which is essential for infection, is lacking in these cells (Vanderheijden *et al.*, 2003).

In this study, we investigated how exactly heparan sulphate and Sn cooperate in PRRSV attachment, and if other receptors are involved in PRRSV attachment. We also investigated what the role is of heparan sulphate and Sn during PRRSV internalization, and if heparan sulphate is needed for Sn-mediated PRRSV internalization.

The role of heparan sulphate and Sn in PRRSV attachment to PAM was evaluated by performing flow cytometric attachment studies at 4 °C as described previously (Delputte *et al.*, 2002). At this temperature, only attachment and no internalization can occur. PRRSV strain Lelystad virus (Wensvoort *et al.*, 1991) grown on Marc-145 cells was used in the experiments. Virus was purified by ultracentrifugation and biotinylated as described earlier (Delputte *et al.*, 2002). Binding was studied in the presence of free heparin, which was shown to interfere with attachment to heparan sulphate (Delputte *et al.*, 2002), and/or protein G Sepharose purified mAb 41D3, which blocks attachment

to Sn (Duan *et al.*, 1998; Vanderheijden *et al.*, 2003). Biotinylated virus attached to the cells was visualized with FITC-labelled streptavidin. Flow cytometric analysis was done on 10 000 events for each sample, and three parameters were stored for further analysis: forward light scattering (FSC), sideward light scattering (SSC) and green fluorescence (FL-1). The median fluorescence intensity (MFI) was calculated using CellQuest software and the relative MFI was calculated according the following formula: relative MFI = 100 × {1–[(MFI when no inhibitor is present–MFI when inhibitor is present)/MFI when no inhibitor is present]}.

Heparin and mAb 41D3 clearly reduced PRRSV attachment in a dose-dependent manner, with a maximum reduction of 83 and 50 %, respectively (Fig. 1). When both heparin and mAb 41D3 were present during virus attachment, 99 % reduction of attachment could be observed at a concentration of 2500 μg heparin ml^{-1} and 10 μg mAb 41D3 ml^{-1}, which was considered to be a full block of attachment. Isotype matched control mAb 13D12 (Nauwynck & Pensaert, 1995) had no effect on PRRSV attachment at a concentration of 10 μg ml^{-1} (data not shown). In a previous study, no effect was observed using 2500 μg heparin ml^{-1} on infection of PAM with the heparin insensitive pseudorabies virus gC^{null} mutant, indicating that this concentration of heparin does not negatively affect PAM (Delputte *et al.*, 2002). Together, these data show that heparan sulphate and Sn mediate PRRSV attachment via different mechanisms, since the effect of heparin and mAb 41D3 on attachment is additive.

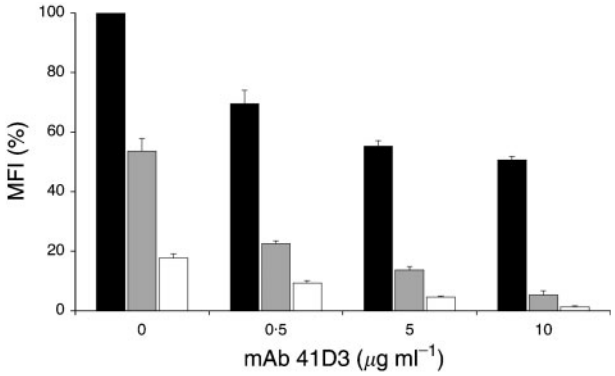

Fig. 1. Effect of heparin and Sn-specific mAb 41D3 on PRRSV attachment to macrophages. Macrophages were incubated for 1 h at 4 °C with biotinylated PRRSV in the presence of different concentrations of Sn-specific mAb 41D3, in combination with 0 μg heparin ml^{-1} (black bars), 250 μg heparin ml^{-1} (grey bars) or 2500 μg heparin ml^{-1} (open bars). Cells were then washed to remove unbound virus, incubated with FITC-labelled streptavidin, and the MFI was determined by flow cytometry. Data represent means ± standard deviation of three independent experiments.

Since PRRSV attachment was fully blocked using both heparin and mAb 41D3, we concluded that heparan sulphate and Sn are the only two receptors involved in PRRSV attachment to PAM.

Next, we investigated the kinetics of PRRSV attachment to PAM at 4 °C, by washing PAM at different times after PRRSV attachment with either heparin, to remove virus attached to heparan sulphate, with mAb 41D3 to remove virus attached to Sn, or with heparin and mAb 41D3 to remove virus attached both to heparan sulphate and Sn. PAM were incubated for 5 min at 4 °C with biotinylated virus and then washed to remove unbound virus. At different times thereafter, cells were washed with 2500 μg heparin ml^{-1}, 25 μg mAb 41D3 ml^{-1} or 2500 μg heparin ml^{-1} together with 25 μg mAb 41D3 ml^{-1}. Shortly after PRRSV binding to PAM, 80 % of the virus could be removed by washing with heparin, while washing with PBS did not reduce the amount of virus bound at any time point (Fig. 2). Initial virus attachment was sensitive to washing with heparin, indicating that virus was mainly bound to heparan sulphate, and apparently not to Sn. Sensitivity to washing the cells with heparin gradually decreased with time, and at 90 min, virus could no longer be removed, indicating that the virus became associated with another receptor. To investigate the involvement of Sn in the heparin resistant binding to PAM, cells were washed with both heparin and mAb 41D3. Doing so, virus could efficiently be removed from the cell surface at all

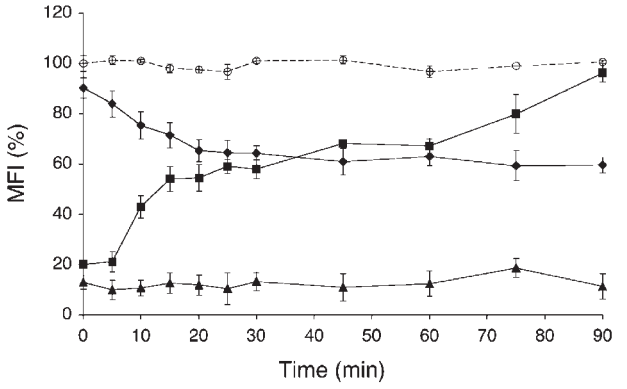

Fig. 2. Kinetics of PRRSV attachment to macrophages. Macrophages were incubated at 4 °C with biotinylated PRRSV for 5 min, washed with PBS to remove unbound virus, and then further incubated at 4 °C for the indicated times. Cells were then washed with PBS alone (open circles), with 2500 μg heparin ml^{-1} (squares) to remove virus attached to the macrophages via heparan sulphate, with 25 μg mAb 41D3 ml^{-1} to remove virus attached to Sn (diamonds) or with 2500 μg heparin ml^{-1} and 25 μg Sn-specific mAb 41D3 ml^{-1} (triangles) to remove virus attached either to heparan sulphate or to Sn. Data represent means ± standard deviation of three independent experiments.

times, indicating that virus which is resistant to washing with heparin is attached to Sn. Washing the cells with mAb 41D3 alone only slightly reduced virus attachment early after virus addition, further sustaining the hypothesis that virus is attached to the heparan sulphate receptor at that time. At 90 min after virus addition, a maximum of 40 % of the virus is removed by washing with mAb 41D3, and since our data also indicate that at that time point all the virus is attached to Sn, we conclude that at least some of the virus that is removed from Sn by washing with mAb 41D3 attaches again to the heparan sulphate receptor. Together, these data show that virus attaches first to heparan sulphate followed by an interaction with Sn, making PRRSV binding resistant to washing with heparin, but sensitive to washing with both heparin and mAb 41D3.

Previous reports have shown that PRRSV can attach to several cell lines, but that these cannot be infected (Therrien *et al.*, 2000). Since most cells contain heparan sulphate on their cell surface, but not the macrophage restricted protein Sn, we hypothesized that heparan sulphate accounts for the observed attachment. To evaluate PRRSV attachment to cells that are not susceptible to PRRSV and that do not express Sn, and to assess the potential role of heparan sulphate in this attachment, wild-type CHO K1, CHO PGS A745 (deficient in glycosaminoglycan synthesis) and CHO PGS D667 (deficient in heparan sulphate synthesis) (Esko *et al.*, 1988) were incubated with PRRSV for 1 h at 4 °C. The cells were then extensively washed to remove unbound virus, freeze–thawed to release attached virus, the supernatant was titrated on Marc-145 cells, and the absolute values of the virus titre were set relative to that of the CHO K1 cells. The virus titre of the lysate of CHO cells lacking heparan sulphate (CHO PGS D667) or all glycosaminoglycans (CHO A745) was reduced by 85 ± 7 or 83 ± 6 %, respectively, compared with wild-type CHO K1 cells (Fig. 3a). To evaluate the role and necessity of heparan sulphate during Sn-mediated PRRSV internalization, CHO K1, PGS A745 and PGS D667 were transfected with plasmid pcDNA3.1/pSn, containing the porcine Sn cDNA (Vanderheijden *et al.*, 2003) and used 24 h after transfection for internalization studies. Cells were incubated for 1 h at 37 °C with PRRSV, washed to remove unbound virus and fixed with methanol for 10 min at −20 °C. Virus particles were then stained with the PRRSV nucleocapsid-specific mAb P3/27 and FITC-labelled goat-anti-mouse IgG as described (Vanderheijden *et al.*, 2003) and Sn

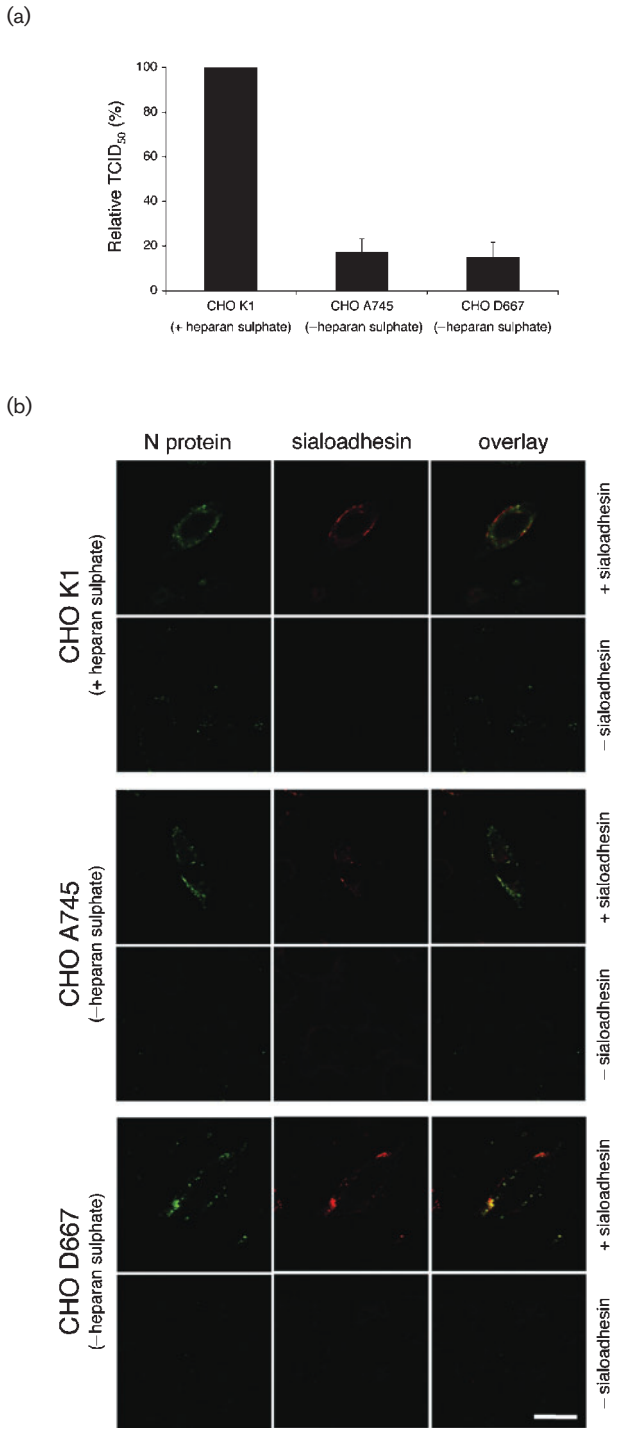

Fig. 3. (a) Analysis of PRRSV attachment to wild-type CHO cells expressing heparan sulphate (CHO K1) or to mutant CHO cells deficient in heparan sulphate (CHO A745 and CHO D667). Cells were incubated with PRRSV for 1 h at 4 °C and washed to remove unbound virus. Cells were then freeze–thawed twice to release bound virus, the amount of virus attached to the cells was determined by titration on macrophages, and the absolute values of the virus titre were set relatively to that of the CHO K1 cells. Data represent means ± standard deviation of three independent experiments. (b) Analysis of PRRSV attachment to and internalization in CHO cells and CHO cells expressing recombinant Sn. CHO cells expressing heparan sulphate (CHO K1) and deficient in heparan sulphate (CHO A745 and CHO D667) were transfected with Sn cDNA. The cells were incubated with PRRSV for 1 h at 37 °C. Cells were then fixed and stained to detect both PRRSV N protein (green fluorescence) and Sn (red fluorescence) as described earlier. Images represent one confocal section through the middle of the cell. Bar, 8 μm.

was stained with biotinylated mAb 41D3, followed by TxR-labelled streptavidin. Cells were analysed by confocal microscopy with a Leica TCS SP2 laser-scanning spectral confocal system. Fig. 3(b) shows clear attachment and internalization in CHO cells expressing recombinant Sn. Both heparan sulphate-expressing CHO K1 cells, and CHO A745 and D667 cells, deficient for heparan sulphate, were capable of internalizing PRRSV upon expression of recombinant Sn. In non-transfected CHO K1 cells, some PRRSV attachment, but no internalization, was observed, while virus attachment was not detected in the CHO A745 and D667 cells that did not express recombinant Sn. The absence of virus attachment to non-transfected CHO A745 and D667 cells can probably be explained by the lack of the heparan sulphate receptor on these cells. Together, these data clearly show that heparan sulphate alone can mediate low levels of virus attachment but no internalization, that Sn expression is sufficient for both PRRSV attachment and internalization and that heparan sulphate is not essential for internalization.

In conclusion, we propose a model for PRRSV infection of macrophages, based on our results and on previous findings (Duan *et al.*, 1998; Nauwynck *et al.*, 1999; Delputte *et al.*, 2002; Vanderheijden *et al.*, 2003). PRRSV first binds to heparan sulphate glycosaminoglycans. This interaction does not lead to virus internalization, but is thought to concentrate virus particles at the surface for subsequent binding to one or more receptors involved in virus internalization, a mechanism already proposed for other viruses (reviewed by Bernfield *et al.*, 1999). This first, unstable interaction is then followed by an interaction with Sn, which mediates internalization of the virus via a mechanism of clathrin-mediated endocytosis (Nauwynck *et al.*, 1999; Vanderheijden *et al.*, 2003). PRRSV attachment to heparan sulphate is not necessary for the subsequent interaction of the virus with Sn, but enhances virus binding to Sn, resulting in enhanced internalization and infection. Sn was previously described as a sialic acid-binding lectin (Crocker *et al.*, 1994) and, in a previous study, we found that sialic acid removal from the PRRS virion surface almost completely blocks infection, and reduces virus attachment up to 50 % (Delputte & Nauwynck, 2004). These data indicate that virus attachment to Sn is probably not mediated by a viral protein, but by sialic acids present on one or more viral glycoproteins. Upon Sn-mediated virus internalization, virus is transported to endosomes, and acidification of the endosomal compartment is needed to achieve infection (Nauwynck *et al.*, 1999). Although cells expressing recombinant Sn can bind and internalize the virus, the fusion between the endosomal membrane and the viral envelope does not occur, since disaggregation of internalized virus, which is observed in macrophages and Marc-145 cells, does not occur in recombinant Sn expressing cells (Vanderheijden *et al.*, 2003). Consequently, infection does not, or only very rarely occurs in recombinant Sn expressing cells. Thus, we propose that upon internalization of the virus, the presence of one or more macrophage-specific factors is essential to allow fusion between the endosomal membrane and the viral envelope, release of the viral genome in the cytoplasm and consequent infection.

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