Short Communication

Porcine, murine and human sialoadhesin (Sn/Siglec-1/CD169): portals for porcine reproductive and respiratory syndrome virus entry into target cells

Wander Van Breedam, Mieke Verbeeck, Isaura Christiaens, Hanne Van Gorp and Hans J. Nauwynck

Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

Porcine sialoadhesin (pSn; a sialic acid-binding lectin) and porcine CD163 (pCD163) are molecules that facilitate infectious entry of porcine reproductive and respiratory syndrome virus (PRRSV) into alveolar macrophages. In this study, it was shown that murine Sn (mSn) and human Sn (hSn), like pSn, can promote PRRSV infection of pCD163-expressing cells. Intact sialic acid-binding domains are crucial, since non-sialic acid-binding mutants of pSn, mSn and hSn did not promote infection. Endodomain-deletion mutants of pSn, mSn and hSn promoted PRRSV infection less efficiently, but also showed markedly reduced expression levels, making further research into the potential role of the Sn endodomain in PRRSV receptor activity necessary. These data further complement our knowledge on Sn as an important PRRSV receptor, and suggest – in combination with other published data – that species differences in the main PRRSV entry mediators Sn and CD163 do not account for the strict host species specificity displayed by the virus.

The porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major disease problems in the swine industry today. The virus specifically infects swine and there is currently no evidence that any other species is susceptible to PRRSV infection. Within its host, PRRSV shows a narrow cell tropism, with macrophages being major target cells (Duan et al., 1997a, b; Teifke et al., 2001). As transfection of the viral RNA genome in otherwise non-permissive cells results in productive infection, it was postulated that the restricted cell tropism is linked with the presence of specific entry mediators in target cells (Kreutz, 1998; Meulenberg et al., 1998). PRRSV entry in alveolar macrophages has already been extensively studied and a number of entry mediators have been identified (Van Breedam et al., 2010a). Two of these molecules appear to be crucial for efficient infection of porcine alveolar macrophages: sialoadhesin (Sn/Siglec-1/CD169) and CD163. Sn mediates efficient binding and internalization of the virus (Delputte et al., 2007; Van Breedam et al., 2010b; Vanderheijden et al., 2003), whereas CD163 is most likely involved in genome release (Calvert et al., 2007; Van Gorp et al., 2008). For CD163, it was reported that not only the porcine variant of this protein, but also several homologues originating from other mammalian species, display a PRRSV entry mediator activity (Calvert et al., 2007). In addition, the CD163 endodomain was found to be dispensable for its functionality as a PRRSV entry mediator (Lee & Lee, 2010; Van Gorp et al., 2010). Although the PRRSV GP2 and GP4 glycoproteins were identified as potential interaction partners for CD163 (Das et al., 2010), the exact functioning of this molecule in PRRSV infection remains unclear. This stands in sharp contrast to our current knowledge on Sn. Ample data have shown that sialic acids on the virion surface interact with the N-terminal sialic acid-binding domain of Sn, upon which the virus–receptor complex is internalized via a process of clathrin-mediated endocytosis (Delputte & Nauwynck, 2004; Delputte et al., 2007; Nauwynck et al., 1999; Van Breedam et al., 2010b; Vanderheijden et al., 2003). Unlike for CD163, however, the importance of an intact Sn endodomain has not been investigated and neither has it been evaluated whether Sn homologues from other mammalian species display PRRSV entry mediator activity. To complement our knowledge on Sn, this study aimed to evaluate whether Sn homologues from other mammalian species also show PRRSV entry mediator activity, and to assess the importance of a functional sialic acid-binding ectodomain and an intact endodomain for their role as virus receptors.

†These authors contributed equally to this paper.

The GenBank/EMBL/DDBJ accession numbers for porcine, murine and human sialoadhesin are AF509585, NM_011426 and NM_023068, respectively, and the accession number for pCD163 is EU016226.
Sn is an evolutionarily conserved type I transmembrane glycoprotein mainly expressed on macrophages and belongs to a family of sialic acid-binding lectins called Siglec. Sn has a large ectodomain consisting of an N-terminal V-set Ig-like domain followed by 16 C2-set Ig-like domains, a transmembrane region that spans the membrane once and a short endodomain that lacks the typical tyrosine-based signalling motifs found in most other Siglec (Crocker & Redelinghuys, 2008; Crocker et al., 2007; Klaas & Crocker, 2012). Different studies have focused on the molecular basis of sialic acid binding by Sn. The sialic acid-binding site was mapped to the N-terminal V-set domain (Nath et al., 1995), and site-directed mutagenesis, nuclear magnetic resonance and crystallography studies enabled the identification of the amino acid residues that are critical for its sialic acid-binding activity. In particular, the R^{116} residue within the N-terminal domain of Sn seems to be essential, as it forms a salt bridge with the carboxylate group of sialic acid ligands (Crocker et al., 1999; May et al., 1997; Vinson et al., 1996).

**Non-sialic acid-binding Sn mutants**

<table>
<thead>
<tr>
<th>Pig</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSnRE</td>
<td>mSnRE</td>
<td>hSnRE</td>
</tr>
<tr>
<td>FW 5'-GCCGCTCTCCTCCTAAGTTCAATGGAGGCACGC'</td>
<td>FW 5'-CACTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
<tr>
<td>RV 5'-GCCGCTCTCCTCCTCCTAAGTTCAATGGAGGCACGC'</td>
<td>RV 5'-CACTCTGAGATGCTGATGCTAGC'</td>
<td>RV 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
<tr>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
</tbody>
</table>

**Sn endodomain deletion mutants**

<table>
<thead>
<tr>
<th>Pig</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSnRE</td>
<td>mSnRE</td>
<td>hSnRE</td>
</tr>
<tr>
<td>FW 5'-GCCGCTCTCCTCCTAAGTTCAATGGAGGCACGC'</td>
<td>FW 5'-CACTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
<tr>
<td>RV 5'-GCCGCTCTCCTCCTCCTAAGTTCAATGGAGGCACGC'</td>
<td>RV 5'-CACTCTGAGATGCTGATGCTAGC'</td>
<td>RV 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
<tr>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
<td>FW 5'-GAGCTCTGAGATGCTGATGCTAGC'</td>
</tr>
</tbody>
</table>

**Percentage Sn-positive cells (relative)**

- Porcine
- Murine
- Human

**MFI (relative)**

- Porcine
- Murine
- Human

W. Van Breedam and others

1956

Journal of General Virology 94
The cDNA encoding porcine Sn (pSn/pSnWT; GenBank accession number AF509585) was previously cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen) (Vanderheijden et al., 2003). A pcDNA1 vector (Invitrogen) encoding murine Sn (mSn/mSnWT; GenBank accession number NM_011426) was generously provided by Dr Paul R. Crocker. A cDNA encoding human Sn (hSn/hSnWT; GenBank accession number NM_023068) was synthesized by Invitrogen (GeneArt) and cloned into the pcDNA3.1(–) vector (Invitrogen). To obtain non-sialic acid-binding mutant forms of Sn (SnRE), R116E mutations were introduced into the N-terminal V-set domain of pSnWT, mSnWT and hSnWT via site-directed mutagenesis (QuickChange II; Stratagene). To obtain mutant forms of Sn that lack the endodomain (Sn-endo), site-directed mutagenesis was used to introduce stop codons flanking the predicted transmembrane region of pSnWT, mSnWT and hSnWT. Sequencing confirmed the presence of the correct mutations (Fig. 1a). The specific primers used for site-directed mutagenesis are described below, equal amounts of vector DNA were used in all conditions.

To evaluate expression of porcine, murine and human SnWT, SnRE and Sn-endo, adherent cultures of porcine kidney cells (PK-15) were transiently transfected with the Sn-encoding constructs using Lipofectamine (Invitrogen; manufacturer’s instructions were followed) and cultured for 24 h to allow protein expression. Subsequently, cells were fixed with paraformaldehyde, permeabilized with Triton X-100 (total expression) or not permeabilized (surface expression), and stained with Hoechst 33342 (nuclei) and Sn-specific mAbs (pSn: mAb 41D3 (Duan et al., 1998; Vanderheijden et al., 2003); mSn: mAb 3D6 (Abcam); hSn: mAb 24F6). Stained cells were analysed using (confocal) fluorescence microscopy (Fig. 1c). pSnWT, mSnWT and hSnWT were detected in permeabilized as well as non-permeabilized transfected cells, indicating that these molecules are expressed on the cell surface. Although similar observations were made for the SnRE and Sn-endo proteins, their expression levels appeared to differ from the respective SnWT. To confirm and quantify differences in surface expression levels, a fluorescence-based flow cytometric analysis was performed. Adherent cultures of PK-15 cells were transiently transfected with the Sn-encoding constructs using Lipofectamine and cultured for 48 h. Subsequently, cells were washed and trypsinized to obtain single-cell suspensions. After trypsin inactivation, cells were kept at 4°C and stained for Sn surface expression using Sn-specific mAbs (pSn: mAb 41D3; mSn: mAb SySy94; hSn: mAb 24F6). Dead cells were stained using propidium iodide. Flow cytometric analysis confirmed the microscopy data, and revealed that the SnRE mutants were surface-expressed at equal or higher levels and Sn-endo mutants at lower levels than their respective SnWT (Fig. 1d).

To evaluate the sialic acid-binding capacity of porcine, murine and human SnWT, SnRE and Sn-endo, standard erythrocyte-binding assays were performed (Crocker et al., 1994; Delputte et al., 2007; Kelm et al., 1994). Briefly, adherent cultures of PK-15 cells were transiently transfected with the Sn-encoding constructs using Lipofectamine and cultured for 24 h. Subsequently, cells were treated for 1 h at 37°C with 10 μM m-1 Vibrio cholerae sialidase (Roche) to remove cis-inhibiting sialic acids from the cell surface, washed and incubated with human erythrocytes for 1 h at 37°C. Finally, unattached erythrocytes were removed through washing and erythrocyte binding was analysed via light microscopy (Fig. 2). Cells expressing an SnWT or an Sn-endo efficiently bound erythrocytes, showing that these proteins display sialic acid-binding capacity. Sialidase pretreatment of erythrocytes precluded binding, confirming the sialic acid dependency of the interactions (data not shown). Cells expressing an SnRE did not bind erythrocytes, proving that these proteins had lost their lectin activity.

Finally, infection assays were performed to evaluate the functionality of porcine, murine and human SnWT, SnRE
and Sn-endo as PRRSV entry mediators. Adherent cultures of PK-15 cells were transiently co-transfected with equal amounts of the Sn-encoding constructs and an expression vector encoding porcine CD163 (pCD163). A variant of pCD163 (GenBank accession number EU016226) has previously been cloned into the pcDNA3.1D/V5-HisTOPO vector (Invitrogen) (Van Gorp et al., 2008). As controls, cell cultures were transiently transfected with the pCD163-encoding vector or one of the Sn-encoding constructs alone (i.e. not co-transfected); non-transfected cultures were also included in the experiment. At 24 h post-transfection, cells were inoculated with equal amounts of PRRSV Lelystad virus (Wensvoort et al., 1991) and incubated for 1 h at 37 °C. The virus suspension was then removed, cells were washed five times with medium (the final wash solution was collected for titration) and cells were further incubated in medium. At 24 h post-inoculation (p.i.), the cell supernatants were collected for titration and cells were fixed with methanol. PRRSV nucleocapsid-specific immunofluorescence staining (mAb 13E2) was performed to visualize virus-infected cells (Van Breedam et al., 2011). Hoechst 33342 was used to visualize cell nuclei and infection efficiency was evaluated via fluorescence microscopy (Fig. 3a). To quantify virus production, cell supernatants collected at 24 h p.i. were titrated on MARC-145 cells as described by Van Gorp et al. (2008) (Fig. 3b). The final wash solution collected at 1 h p.i. was titrated to estimate the residual virus after removal of the inoculum. In line with previous studies on pSn, no virus-positive cells were detected in non-transfected cell cultures and cell cultures transfected with an Sn-encoding construct alone (data not shown). In cell cultures transfected with the pCD163-encoding vector alone (pCD163+), only a few virus-positive cells were detected and production of new virions was limited. In pCD163+pSnWT+, pCD163+mSnWT+ and pCD163+hSnWT+ cells, the number of virus-positive cells was markedly higher and the titrations revealed a much more efficient production of new PRRSV virions. These data indicate that mSnWT and hSnWT, just like pSnWT, can promote PRRSV infection of target cells by binding and shuttling the virus to the correct endosomal compartment for pCD163-mediated genome release. The receptor functionality of Sn is directly linked with its sialic acid-binding capacity: in pCD163+pSnRE+, pCD163+mSnRE+ and pCD163+hSnRE+ cells, the number of virus-positive cells was clearly lower than in the cell cultures co-expressing pCD163 and the respective SnWT variants. In line with this, virus production was significantly reduced (P<0.001) to levels approximating those observed for pCD163+ cells. Endodomain deletion also had a significant effect. In pCD163+pSn-endo+, pCD163+mSn-endo+ and pCD163+hSn-endo+ cells, the number of virus-positive cells was lower than in the corresponding pCD163+SnWT+ cells and virus production was significantly reduced (P<0.001 or P<0.01). Nevertheless, infection and virus production levels were higher than those observed for pCD163+ cells, indicating that the Sn-endo molecules retained a capacity to promote infection. The exact reason behind the reduced infection and virus production in pCD163+Sn-endo+ cells compared to pCD163+SnWT+ cells remains unknown. One possible explanation may be that the observed reduction is merely a reflection of the reduced expression levels of the Sn-endo mutants (see above). In this scenario, internalization of Sn is not strictly dependent on the Sn endodomain and may involve certain co-factors that drive ligand-induced internalization. Alternatively, it is possible that the Sn-endo mutants have lost their capacity to efficiently internalize ligands, but can still function as attachment factors for the virus and thus promote viral infection by concentrating infectious virions at the target cell surface. At present, however, the exact mechanisms of ligand-induced signalling by and internalization of Sn have not been elucidated, and further research is necessary to characterize these.

In conclusion, we showed that, apart from pSn, also mSn and hSn have PRRSV receptor activity. To our knowledge, this is the first study that identifies homologues of pSn as functional PRRSV entry mediators. These data, combined with the data published by Calvert et al. (2007), suggest that species differences in Sn and CD163 do not account for the strict host species specificity displayed by PRRSV. Sialic acid-binding capacity appears to be crucial for the functionality of the different sialoadhesins as PRRSV receptors. Specific involvement of the Sn endodomains remains unclear. Future research will be aimed at elucidating the ligand-induced internalization process of Sn.
Acknowledgements

The authors thank C. Boone, N. Dennequin, D. Helderweirt, L. Sys and C. Vanmaercke for excellent technical assistance. Study design: W. V. B. Experimental work: W. V. B., M. V., I. C., H. V. G. Data analysis: W. V. B., M. V., I. C., H. V. G., H. J. N. Authorship: W. V. B. This work was supported by the Special Research Fund (BOF) of Ghent University, the Flemish Institute for the Promotion of

Fig. 3. Evaluation of the functionality of porcine, murine and human SnWT, SnRE and Sn-endo as PRRSV entry mediators. PK-15 cells, transiently transfected with a pCD163-encoding vector alone (pCD163 +) or in combination with an Sn-encoding vector (pCD163 + Sn+), were inoculated with PRRSV Lelystad virus. At 1 h p.i., the inoculum was removed, cells were washed and washing fluids were replaced with medium. At 24 h p.i., cell supernatants were collected and cells were fixed with methanol. To analyse PRRSV infection, cells were analysed using fluorescence microscopy after staining with Hoechst 33342 (nuclei; blue) and mAb 13E2 (PRRSV nucleocapsid protein; green). Representative images are shown in (a). Bar, 250 μm. The absolute number of infected cells in each condition was determined (for each condition, a total surface area of 113 mm² was analysed) and the results are displayed in the images as mean ± SEM of six independent experiments. To evaluate virus production, cell supernatants collected at 24 h p.i. were titrated [detection limit 0.8 TCID₅₀ ml⁻¹ (log₁₀)] and results are displayed in the graphs in (b) (black bars). To estimate residual virus after removal of the inoculum, (final) washing fluids collected at 1 h p.i. were also titrated (white bars). Values represent mean ± SEM of six independent experiments. To determine statistically significant differences, virus production by pCD163 + SnRE + or pCD163 + Sn-endo + cells was compared with virus production by pCD163 + SnWT + cells using one-way ANOVA and Dunnett’s multiple comparison test. **, P<0.01; ***, P<0.001.
Innovation by Science and Technology (IWT-Flanders) and the European Union (Seventh Framework Programme; project no. 245141).

References


