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Short communication

Analysis of the integrin β_3 receptor for pathogenic orthohantaviruses in rodent host species

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ABSTRACT

Host reservoir specificity of pathogens is complex and may depend on receptor variability. For pathogenic orthohantaviruses, integrin β_3 had been previously identified as entry receptor and the presence of aspartic acid residue at position 39 (D39) in human integrin β_3 was described to be a prerequisite for infection of primate cells with Hantaan virus (HTNV). However, the role of integrin β_3 in orthohantavirus infection of host animals is not completely understood. Therefore, we analyzed the nucleotide sequence of the integrin β_3 gene of *Myodes glareolus* and *Apodemus agrarius,* the hosts of Puumala virus (PUUV) and HTNV, respectively. Sequence analysis in tissue samples demonstrated that the amino acid residue D39 is not present in integrin β_3 of these natural orthohantavirus hosts. Furthermore, we analyzed the transcription and protein expression levels of integrin β_3 in the renal cell line BVK168 generated from the PUUV host, bank vole. Transcription level of integrin β_3 was 100-fold lower in BVK168 cells than in Vero E6 cells and integrin β_3 expression was not detectable in BVK168 cells. However, despite the absence of amino acid residue D39 and no detectable integrin β_3 expression, BVK168 cells are susceptible to infection with both PUUV and HTNV. These results indicate that the mechanism of orthohantaviral entry in rodent species does not correspond to the requirements that were described for the entry in primate cells in vitro.

Rodents are the main reservoir of orthohantaviruses that cause diseases in humans. Although different species of rodents and orthohantaviruses co-circulate sympatrically, each orthohantavirus species is mostly associated with a specific host (Heyman et al., 2009; Vaheri et al., 2013). Nevertheless, analysis of wild-trapped animals demonstrates that spill-over infections and host sharing exist for some orthohantaviruses (Klingström et al., 2002; Laenen et al., 2018; Nemirov et al., 2002; Popugaeva et al., 2012; Schmidt-Chanasit et al., 2010).

To examine the host specificity of Old World orthohantaviruses, we analyzed if the renal tubular epithelial cell line BVK168 (Essbauer et al., 2011), established from the natural PUUV host, bank vole (*Myodes glareolus*), is susceptible to infection with PUUV and with HTNV, which is normally carried by the striped field mouse (*A. agrarius)*. BVK168 cells were inoculated with PUUV or HTNV and the infection was monitored by detection of the viral nucleocapsid N protein (Reuter and Krüger, 2018) via immunofluorescence and Western blot analysis (Fig. 1). N proteins of both PUUV and HTNV were detected in infected BVK168 cells. At day 14 post infection 58.36% ± 3.50% and 73.16% ± 11.06% of BVK168 cells were infected with PUUV and HTNV, respectively. In conclusion, a renal cell line established from the PUUV host, bank vole, is susceptible to infection not only with the host-associated PUUV but also “heterologous” HTNV.

Previously, several receptors have been identified to be involved in orthohantavirus infection (Choi et al., 2008; Gavrilovskaya et al., 1999; Krautkrämer and Zeier, 2008; Raftery et al., 2014). In vitro cell culture experiments demonstrated that pathogenic orthohantaviruses causing hantaviral cardiopulmonary syndrome (HCPS) or hemorrhagic fever with renal syndrome (HFRS) preferentially use β_3 integrins for entry (Gavrilovskaya et al., 1999, 1998). Therefore, we analyzed the integrin β_3 nucleotide sequence of bank vole. We used RNA isolated from BVK168 cells. RNA was reversely transcribed into cDNA using random hexamer oligonucleotides. Amplification of full-length integrin β_3 was performed using oligonucleotides targeting 5´-AAGGGCGGCGGACAG GATG-3´ and 5´-TGTCCTCTGTAAACATGATGGCAG-3´. The products

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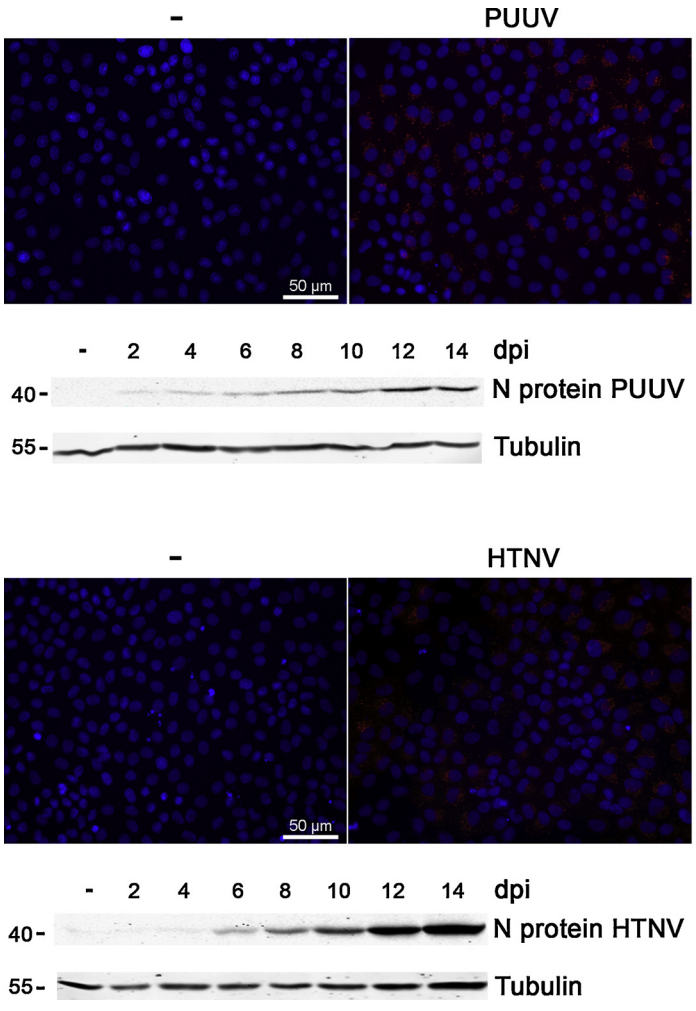

Fig. 1. Infection of bank vole cell line BVK168 with PUUV and HTNV. Stocks of PUUV strain Vranica and HTNV strain 76–118 propagated and titrated on Vero E6 cells were used. BVK168 cells were infected with PUUV and HTNV using a multiplicity of infection (MOI) of four. Infection of BVK168 cells was quantified via immunofluorescence with the monoclonal anti-PUUV N protein (clone A1C5) or anti-HTNV N protein (clone B5D9) antibody (Progen). Three independent experiments were performed. Shown is mean ± standard deviation (SD). Western blot analysis was performed with rabbit polyclonal anti-PUUV N protein or anti-HTNV N protein antibody.

Table 1
Percentage identity of cds and amino acid sequences of integrin β_3 of primate and rodent species.

	M. glareolus	*A. agrarius*	*M. musculus*	*C. sabaeus*	*H. sapiens*
	nucleotide identity (%)				
M. glareolus	–	91.92	91.07	87.45	87.49
A. agrarius	95.30	–	94.63	86.78	86.95
M. musculus	95.04	97.20	–	86.61	86.52
C. sabaeus	90.48	90.86	90.48	–	97.89
H. sapiens	90.86	91.12	90.61	99.24	–
	amino acid identity (%)				

Accession numbers for cds identity: *C. sabaeus*, XM_008012292.1; *H. sapiens*, NM_000212.2; *M. glareolus*, KF155167.1; *A. agrarius*, KX237565.1; *M. musculus*, NM_016780.2.

were sequenced (GATC Biotech) and the complete coding sequence (cds) of integrin β_3 (*ITGB3*) from *M. glareolus* cell line BVK168 and the deduced amino acid sequence were deposited in GenBank, accession numbers KF155167 and AHN82190. Comparison of bank vole integrin β_3 revealed 87% identity in the nucleotide sequence and 91% identity in the deduced amino acid sequence with human integrin β_3. The identity of cds and amino acid sequences of full-length integrin β_3 of different primate and rodent species is shown in Table 1. The amino acid identity between the species varies from 90% to 97% and does not allow any conclusion concerning the susceptibility of the species.

β subunits of integrins contain an N-terminal plexin-semaphorin-integrin (PSI) domain (Bork et al., 1999; Xiao et al., 2004). This PSI domain of integrin β_3 was described to be crucial for orthohantaviral entry (Raymond et al., 2005). Aspartic acid at position 39 (D39) in the PSI domain of human integrin β_3 was identified to be required for entry of HTNV and New York-1 virus (NY-1V) in primate cells and was absent from the PSI domain of *Mus musculus* as a non-susceptible species (Matthys et al., 2010; Raymond et al., 2005). Despite the highly specific requirement of D39 within the PSI domain for entry of NY-1V and HTNV in primate cells, we found this amino acid residue to be replaced by asparagine in the bank vole sequence (Fig. 2A).

To exclude that the PSI domain of integrin β_3 obtained from bank vole renal cell line BVK168 differs from that in bank vole animals, we sequenced samples obtained from renal tissue of *M. glareolus* (Fig. 2B). Bank voles were collected in Bavaria (Germany) using Sherman live traps. Animals were sacrificed according the German Animal Protection Law. Species determination and monitoring of orthohantavirus infection was done as described elsewhere (Essbauer et al., 2011). In brief, animals were classified morphologically and confirmed by sequencing of partial mitochondrial cytochrome *B* gene (data not shown here). Orthohantavirus infection was monitored serologically and after amplification of partial PUUV S segment by RT-PCR and subsequent sequencing. The sequences of the integrin β_3 PSI domain of six animals were analyzed and no differences as compared to the sequence of the BVK168 cell line were observed. Since orthohantaviral N antigen is mostly found in the lung of infected animals (Essbauer et al., 2006; Korva et al., 2009), we also analyzed the PSI sequence present in pulmonary tissue. PSI sequences obtained from BVK168 cell line as well as renal and lung tissue samples revealed no differences in the amino acid sequence.

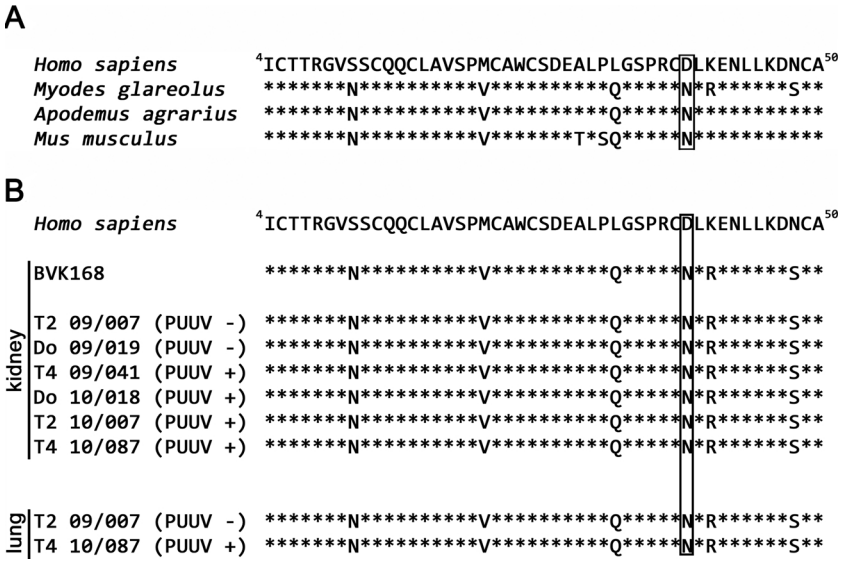

Fig. 2. Comparison of amino acid sequences of the PSI domain of integrin β_3 of different mammals (A) and kidney and lung tissue samples of PUUV-infected and non-infected bank voles (B). Sequence of *A. agrarius* was derived from lung tissue. GenBank accession numbers: *H. sapiens:* NP_000203; *M. glareolus:* AHN82190; *A. agrarius*: ANW48447; *M. musculus*: NP_058060; *ITGB3* cds PSI domain from lung and kidney tissues from PUUV-infected and non-infected *M. glareolus*: KT989779. Boxed: aspartic acid (D39) and asparagine (N39) in human and in rodent integrin β_3 sequence, respectively.

Dubois et al. observed differences between bank voles from nephropathia epidemica endemic and non-endemic regions concerning gene expression and susceptibility to PUUV infection (Dubois et al., 2017, 2018). Therefore, we analyzed the sequences derived from kidney and lung tissue of PUUV-infected and non-infected animals; no differences were observed between these animals (Fig. 2B).

We also sequenced the region encoding for the PSI domain of integrin β_3 from the natural HTNV host *A. agrarius* trapped in Riems (Germany) (Fig. 2A). Analysis of integrin β_3 PSI domain from lung of striped field mouse revealed that two amino acid substitutions exist between bank vole and striped field mouse (R41K, S48N). However, the amino acid residue D39, which was postulated to be crucial for orthohantaviral entry in primates, is also not present in integrin β_3 from *A. agrarius*.

To further examine the role of integrin β_3 in bank vole infection, we analyzed transcription and expression levels of integrin β_3 in BVK168 cells (Fig. 3). Vero E6 cells from African Green Monkey (*Chlorocebus sabaeus*), which are commonly used in orthohantavirus research, served as positive control. The integrin β_3 protein sequence of African Green Monkey (GenBank accession: XP_008010483) is 99% identical to the sequence of the human subunit and contains the amino acid residue D39 in the PSI domain.

Relative transcription levels of integrin β_3 in BVK168 and Vero E6 cells were analyzed by RT-PCR and by quantitative real-time RT-PCR using the GoTaq® qPCR Master Mix (Promega). For amplification of integrin β_3 cds (*ITGB3*), the following oligonucleotides were used: 5´-ATCTCCCCACCAGAGGCCCTC-3´ and 5´-CCTCATTGAAGCGGGTCACC TGG-3´. Amplification of a fragment of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) transcripts was used as reference (5´-TTCTTTG CTGACCTGCTGGA-3´ and 5´-TGTATTTGGCTTTTCCAGTTTCACT-3´). Results showed high transcription levels of integrin β_3 in Vero E6 cells. In contrast, transcription levels of integrin β_3 in BVK168 cells were 100-fold lower than in Vero E6 cells (Fig. 3A and B).

To detect integrin β_3 from primate and bank vole origin by Western blot, we used rabbit anti-integrin β_3 antibody EPR2417Y (Abcam), which recognizes a C-terminal epitope of integrin β_3. To test this

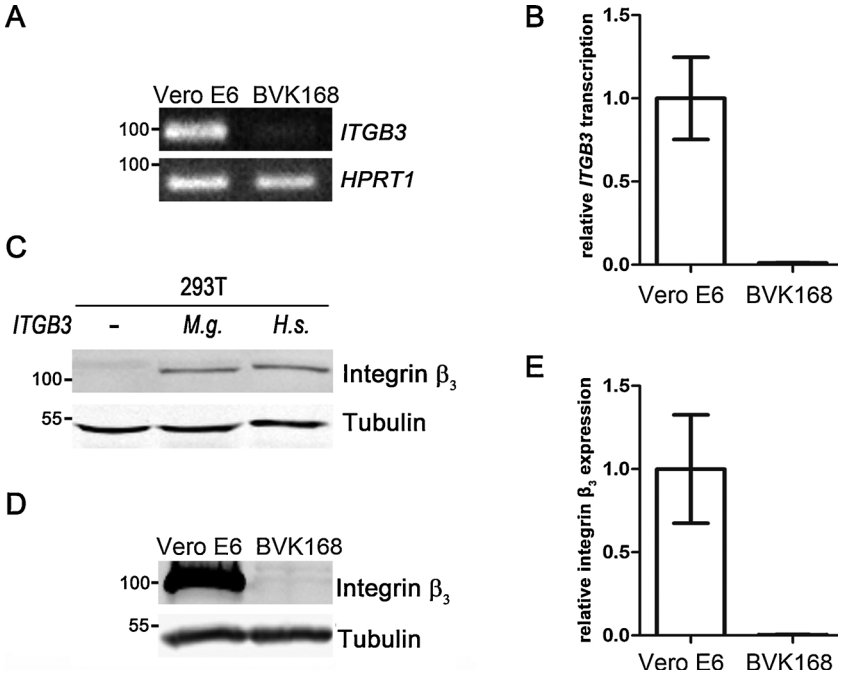

Fig. 3. Transcription and expression of integrin β_3 in BVK168 cell line. Transcription of ITGB3 and HPRT1 (GenBank accession number of partial HPRT1 cds from *M. glareolus* cell line BVK168: KU094073) were analyzed in Vero E6 and BVK168 cells by (A) RT-PCR and (B) quantitative real-time RT-PCR. The averaged values of duplicate reactions in three independent experiments were used to calculate relative gene expression by the ΔΔCt method. The transcription in Vero E6 cells was set to 1. Shown are mean and SD of three independent experiments. (C) 293T cells were transfected with plasmid encoding full-length integrin β_3 of *H. sapiens* (H.s.) or *M. glareolus* (M.g.). Recombinant primate and bank vole integrin β_3 was detected with antibody EPR2417Y (Abcam) by Western blot. Tubulin served as control for equal loading. (D) Endogenous integrin β_3 expression in cell lysates. Tubulin was detected as loading control. One representative blot of three independent experiments was shown. (E) The relative levels of integrin β_3 protein were quantified via band intensities from Western blot with Odyssey Li-Cor software and normalized for tubulin. The expression level in Vero E6 cells was set to 1. Shown are the mean and SD of three independent experiments.

antibody for its ability to recognize bank vole integrin β_3, we transfected 293T cells with plasmid encoding full-length integrin β_3 from *Homo sapiens (Origene)* or from *M. glareolus*. Full-length coding sequence of integrin β_3 of *M. glareolus* was amplified from BVK168 cells with oligonucleotides 5´-CCGCGATCGCACAGGATGCGAGCGC-3´ and 5´-CGCTCGAGTTAGGTGCCCCGGTAGG-3´ and cloned into pCMV6-Entry (Origene) at *Sgf*I and *Xho*I sites. 293T cells were transfected with empty vector, or with plasmids encoding full-length human or bank vole integrin β_3 using Metafectene (Biontex). One day post transfection, cells were lysed and equal amounts of total protein were analyzed for the expression of recombinant integrin β_3 proteins with rabbit anti-integrin β_3 antibody EPR2417Y by Western blot. This antibody detects recombinant bank vole integrin β_3 as shown by the specific band in lysates of transfected 293T cells (Fig. 3C). As revealed by the comparison with the lysate of 293T cells transfected with human integrin β_3, the antibody detects the primate and rodent integrin β_3 with the same sensitivity. Thereafter, lysates of Vero E6 and BVK168 cells were analyzed for expression of endogenous integrin β_3 via Western blot with antibody EPR2417Y (Fig. 3D and E). Integrin β_3 protein was detected in Vero E6 cells. In contrast, no protein was detectable in lysates of BVK168 cells. Our results demonstrate that PUUV and HTNV are able to infect rodent cells independently from the presence of D39 residue and from substantial expression of integrin β_3.

For orthohantaviruses, the knowledge about receptor engagement in different cell types and host species is limited. Possible host-specific variances in the viral replication cycle may be responsible for the pathogenicity in certain infected species in contrast to the life-long persistence and shedding in chronically infected host species animals (Voutilainen et al., 2015). Since hantaviral particles are found especially in the lung of infected animals, differences in the entry mechanism may also result in a change of organ tropism between species.

The entry via integrin β_3 of pathogenic hantaviruses in primate cells was often discussed to play a role in the pathogenesis of HFRS and HCPS in vivo (Gavrilovskaya et al., 2002). However, a mechanistic link between entry via integrin β_3 and human disease still remains elusive. Several studies demonstrate that the entry of orthohantaviruses seems to be more complex and may vary between virus species, cell types and host species. In vitro studies with pseudovirions revealed that cell tropism of orthohantaviruses does not correlate with the expression of integrin $\alpha_v\beta_3$ on the surface (Higa et al., 2012). In addition, neutrophils were infected in vitro by orthohantaviruses in the absence of integrin β_3 expression (Raftery et al., 2014). Recently, protocadherin-1 was identified to be involved in the entry of HCPS-causing New World orthohantaviruses (Jangra et al., 2018). These data and our findings presented here indicate that orthohantaviruses may use alternative receptors and that differences between entry in primate and rodent cells exist. Further studies will be necessary to clarify the role of receptors that mediate entry in rodent host cells.

Ethical approval

All procedures performed in studies involving animals were in accordance with the German Animal Welfare Act.

Declarations of interest

The authors declare that they have no competing interests.

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