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Tubulins interact with porcine and human S proteins of the genus *Alphacoronavirus* and support successful assembly and release of infectious viral particles

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ABSTRACT

Coronavirus spike proteins mediate host-cell-attachment and virus entry. Virus replication takes place within the host cell cytosol, whereas assembly and budding occur at the endoplasmic reticulum-Golgi intermediate compartment. In this study we demonstrated that the last 39 amino acid stretches of *Alphacoronavirus* spike cytoplasmic domains of the human coronavirus 229E, NL63, and the porcine transmissible gastroenteritis virus TGEV interact with tubulin alpha and beta chains. In addition, a partial co-localization of TGEV spike proteins with authentic host cell β-tubulin was observed. Furthermore, drug-induced microtubule depolymerization led to changes in spike protein distribution, a reduction in the release of infectious virus particles and less amount of spike protein incorporated into virions. These data demonstrate that interaction of *Alphacoronavirus* spike proteins with tubulin supports S protein transport and incorporation into virus particles.

1. Introduction

Coronaviruses (CoVs) are positive single-stranded RNA viruses. They infect birds and mammals, especially their respiratory and gastrointestinal systems. Due to high mutation and recombination rates in coronaviruses frequent host-shifting events from animal-to-animal and animal-to-human have occurred (Chinese, 2004; Guan et al., 2003; Lau et al., 2005; Woo et al., 2009). Bats were identified as a natural reservoir during the severe acute respiratory syndrome (SARS) outbreak in China in the year 2002–2003 (Ge et al., 2013; Li et al., 2005). Similarly, a strain closely related to human CoV 229E was found in bats in Ghana (Corman et al., 2015; Pfefferle et al., 2009). Thus, it is speculated that several human and animal CoVs originated from bats (Liu et al., 2015; Shi and Hu, 2008; Woo et al., 2009).

The CoV spike (S) glycoprotein, a key determinant for host range, is necessary for receptor binding and membrane fusion (Graham and Baric, 2010). The S protein contains a large ectodomain, a transmembrane domain, and a C-terminal cytoplasmic tail. The cytoplasmic domain consists of a cysteine-rich and a charge-rich region and mediates S incorporation into virions resulting in infectious virus particles (Bosch et al., 2005; Godeke et al., 2000). Some CoVs like the transmissible gastroenteritis virus (TGEV), as well as the HCoVs NL63 and 229E contain a tyrosine-based sorting signal within their charge-rich region which – in the case of the TGEV S protein – was shown to be important for intracellular retention (Schwegmann-Wessels et al., 2004).

Free movement of virus particles through the host cell cytoplasm is restricted. The cytosol is highly viscous and contains structural barriers like organelles as well as cytoskeletal elements (Leopold and Pfister, 2006; Luby-Phelps, 2000; Verkman, 2002). Therefore, diffusion of virus-sized particles is very unlikely and the arrival at specific cellular regions or compartments is nearly impossible (Sodeik, 2000). Consequently, many viruses use the

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cytoskeleton network of the host cell as a transport system to reach the compartment where replication processes take place or to find their way out of the cell (Leopold and Pfister, 2006; Ploubidou and Way, 2001; Radtke et al., 2006). Furthermore, it is known that the cytoskeleton plays a crucial role during virus attachment, internalization, endocytosis, transcription, replication, assembly, exocytosis as well as cell-to-cell spread. For this purpose viruses rearrange cellular filaments and use them as tracks (Radtke et al., 2006). Regarding TGEV, the actin-binding protein filamin A is a putative interaction partner of the TGEV S protein (Trincone and Schwegmann-Wessels, 2015) and in the case of TGEV-infected IPEC-J2 cells actin filaments were shown to be important for viral replication and release (Zhao et al., 2014). Additionally, an interaction of TGEV nucleocapsid protein with the type 3 intermediate filament vimentin was shown to be required for viral replication as well (Zhang et al., 2015). Another major component of the dynamic cytoskeletal matrix is represented by microtubules. Those polarized structures are built of α/β-tubulin heterodimers and are important for cell shape, transport, motility, and cell division (Heald and Nogales, 2002; Nogales, 2000). Several viruses are known to interact with tubulin or their molecular motors like kinesin or dynein (Biswas and Das Sarma, 2014; Han et al., 2012; Hara et al., 2009; Henry Sum, 2015; Hsieh et al., 2010; Hyde et al., 2012). CoVs like a demyelinating strain of mouse hepatitis virus (MHV) use microtubules for neuronal spread and the feline infectious peritonitis virus (FIPV) is transported via microtubules towards the microtubule organizing center (Biswas and Das Sarma, 2014; Dewerchin et al., 2014). For TGEV an up-regulation of microtubule-associated α- and β-tubulin was detected in swine testis (ST) cells after infection (Zhang et al., 2013). In the study presented here, we analyzed the interaction of tubulin with the last 39 amino acid stretches of the S protein cytoplasmic tail of *Alphacoronaviruses* like TGEV, HCoV NL63, and HCoV 229E. Our results show that tubulins interact with the cytoplasmic domain of α-CoVs spike proteins. Reduced release of infectious virus particles as well as differentially distributed S proteins was observed after drug-induced tubulin depolymerization. Therefore, tubulin may help the S protein to be properly transported, localized, and assembled into virions.

2. Material and methods

2.1. Cell lines and virus strains

Human embryonic kidney cells (HEK-293) were used for co-immunoprecipitation via GFP Trap® pull down assay and ST cells were used for immunofluorescence analysis and plaque assay. Both cell lines were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Hypsignathus monstrosus kidney cells (HypNi/1.1) (Kuhl et al., 2011) and Pipistrellus pipistrellus kidney cells (PipNi/1) (Muller et al., 2012) (provided by Marcel Müller) were used for immunofluorescence analysis and were grown in DMEM supplemented with 5% fetal calf serum.

The Purdue strain of TGEV (PUR46-MAD, provided by L. Enjuanes) was propagated in ST cells. After 24 h of incubation at 37 °C the supernatants were harvested, centrifuged and after addition of 1% fetal calf serum stored at −80 °C.

2.2. Plasmids

Fusion proteins of the last 39 amino acid (aa) stretches of TGEV-S, SARS-CoV-S, HCoV-NL63-S, and HCoV-229E-S cytoplasmic domains with GFP were constructed (S-39aa-GFP-CT and S-39aa-GFP-NT) by using Invitrogen/Life Technologies Gateway cloning (Table 1).

Table 1
Last 39 amino acid stretches of coronavirus cytoplasmic domains linked to GFP.

TGEV	CCCSTGCCGCIGCLGSCCHISCSRRQFENYEPIEKVHVH
HCoV NL63	CLSTGCCGCCNCLTSSMRGCCDCGSTKLPYYEFEKVHVQ
HCoV 229E	LCCCSTGCCGFFSCFASSIRGCCESTKLPYYDVEKIHIQ
SARS-CoV	CCMTSCCSCLKGACSCGSCCKFDEDDSEPVLKGVKLHYT

Transmissible gastroenteritis virus (TGEV), human coronavirus (HCoV), severe acute respiratory syndrome coronavirus (SARS-CoV).

Full length TGEV S wildtype was fused to GFP named TGEV Swt-GFP. The full length mutant of TGEV S where the tyrosine at position 1440 is exchanged by an alanine fused to GFP is named TGEV S Y/A-GFP. Tubulins C-terminally tagged with an HA (YPYDVPDYA) peptide (TUBB2-HA, TUBB4A-HA, TUBB6-HA, TUBA4A-HA) were used for co-immunoprecipitation. As compartment markers a GFP-tagged ERGIC-53 and galactosyltransferase were used (Winter et al., 2008). Full length TGEV M cDNA was transfected for co-localization studies. Full length TGEV nucleocapsid (N) cDNA and empty GFP plasmid served as negative control.

2.3. GFP Trap® pull down assay and SDS-PAGE

To determine interaction partners of the S protein cytoplasmic domain, HEK-293 cells were seeded on 10 cm dishes and transfected with empty GFP vector or with S-39aa-GFP-CT/NT fusion protein one day later by using Lipofectamine® 2000 (Life Technologies) following the manufacturer's instructions.

For co-immunoprecipitation, the cells were additionally co-transfected with the tubulin candidates tagged with the HA-peptide by using Polyethylenimine (PEI 1 μg/μl, Polysciences). DNA (24 μg) was mixed with 3 ml Opti-MEM (Life Technologies) and incubated for 5 min. Following this, 20 μl of PEI was added and incubated for 15 min. Then this mixture was added dropwise to the cells and incubated overnight.

HEK-293 cells were lysed in NP-40 lysis buffer in the presence of complete™ (protease inhibitor cocktail, Roche Diagnostics, Mannheim) for 30 min on ice followed by centrifugation for 10 min at 20,000*g* and 4 °C. ChromoTek GFP-Trap® which consists of agarose beads coated with antibodies derived from alpaca against GFP was used for both, the general screening method as well as specific interaction studies between tubulins and S proteins. The purification was done as described in the manufacturer's protocol. For the interaction experiments, the NaCl concentration of the washing buffer was increased from 150 mM to 300 mM to avoid unspecific pull down of tubulins. Instead of 100 μl 2 × SDS sample buffer 25 μl 5 × SDS sample buffer was used. Cell lysates and eluates were subjected to SDS-PAGE. By a semi-dry technique (Kyhse-Andersen, 1984) the proteins in the gel were transferred to nitrocellulose membranes (GE Healthcare) which were subsequently blocked in 5% milk powder in TBS for one hour and then incubated with the first antibody overnight at 4 °C (rat-anti-HA antibody 1:300 provided by E. Kremmer; rat-anti-GFP antibody 1:1000, Chromotek). The next day, the membrane was washed 3 times with TBS-T for 10 min and treated with the secondary antibody (HRP-conjugated donkey-anti-rat antibody 1:10,000, Sigma-Aldrich) for 1–2 h. Afterwards, the membrane was washed 3 times in TBS-T for 10 min and once in TBS for 5 min. The blot was exposed to Millipore Immobilon™ Western Chemiluminescent HRP substrate (Fischer Scientific) and visualized in a GelDoc documentation system. Regarding the general screening for interaction partners of the S protein, the SDS gel was fixed and then stained with Coomassie solution. After destaining of the gel, visible bands were cut out, digested, and analyzed by mass spectrometry.

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