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Interaction between human BAP31 and Respiratory Syncytial Virus Small Hydrophobic (SH) protein.

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*Running title: Interaction between BAP31 and RSV SH protein

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ABSTRACT

The small hydrophobic (SH) protein is a short channel-forming polypeptide encoded by the human respiratory syncytial virus (hRSV). Deletion of SH protein leads to viral attenuation in mice and primates, and delayed apoptosis in infected cells. We have used a membrane-based yeast Two-Hybrid system (MbY2H) and a library from human lung cDNA to detect proteins that bind SH protein. This led to the identification of a membrane protein, B-cell associated protein 31 (BAP31). Transfected SH protein co-localizes with transfected BAP31 in cells, and pulls down endogenous BAP31. Titration of purified C-terminal endodomain of BAP31 against isotopically labeled SH protein in detergent micelles suggests direct interaction between the two proteins. Given the key role of BAP31 in protein trafficking and its critical involvement in pro- and anti-apoptotic pathways, this novel interaction may constitute a potential drug target.

ABBREVIATIONS. Cub, C-terminal fragment of ubiquitin; DMSO, Dimethyl sulfoxide; DPC: n-dodecylphosphocholine; EDTA, Ethylenediaminetetraacetic acid; EGFP, Enhanced Green Fluorescent Protein; HRSV, Human Respiratory Syncytial Virus; LB, Luria-Bertani Broth; M, Matrix protein; MbY2H, Membrane-based Yeast Two-Hybrid; Nub, N-terminal fragment of ubiquitin; SDS-PAGE, sodium Dodecyl Sulfate Polyacrymide Gel Electrophoresis; SH, Small Hydrophobic.

INTRODUCTION

The human respiratory syncytial virus (hRSV) is an enveloped pneumovirus in the paramyxoviridae family that causes lower respiratory tract disease in infants, elderly and immunocompromised populations worldwide (Dowell et al., 1996). Up to 64 million reported cases of hRSV infection and 160,000 deaths occur each year. Although the virus was identified almost half a century ago (Blount et al., 1956), there are still no vaccines or effective antiviral drugs available. hRSV can cause repeated reinfections throughout life, and its molecular mechanism of pathogenesis is not yet completely understood.

The hRSV genome comprises a nonsegmented negative-stranded RNA of ~ 15 kb that transcribes 11 proteins, including the three membrane proteins F, G, and small hydrophobic (SH). Proteins F and G are key factors during virus attachment, fusion and entry into host cells (Krusat and Streckert, 1997; Lamb, 1993). The role of SH protein is less clear; RSV lacking the *SH* gene (RSV Δ SH) caused formation of syncytia, and grew as well as the wild-type (WT) virus in cells, but it replicated 10-fold less efficiently than the WT in the upper respiratory tract, and was attenuated *in vivo* (Bukreyev et al., 1997; Fuentes et al., 2007; Jin et al., 2000; Karron et al., 1997; Whitehead et al., 1999). Overall, these results indicate involvement of SH protein in the pathogenesis of RSV infection.

Homologs of RSV SH protein are found in parainfluenza virus 5 (PIV5), mumps virus (MuV), and J paramyxovirus (JPV). In all these systems, SH protein seems to block or delay apoptosis in infected cells through inhibition of the TNF- α pathway (Fuentes et al., 2007; Li et al., 2011; Lin et al., 2003; Wilson et al., 2006a), although this may not be the only mechanism involved. It is thought that by delaying apoptosis the virus may evade host inflammatory responses and the premature death of the host cells.

The SH protein is a 64 (RSV subgroup A) or 65 (RSV subgroup B) amino acids long type II integral membrane protein, with C- and N-termini oriented lumenally/extracellularly and cytoplasmically, respectively. In infected cells, most SH protein accumulates at the membranes of the Golgi complex, but it has also been detected in the endoplasmic reticulum and plasma membranes (Rixon et al., 2004). During infection, the full-length unmodified form is the major species (Collins and Mottet, 1993),

although a truncated form (4.5 kDa) and glycosylated and phosphorylated forms have also been detected (Olmsted and Collins, 1989; Rixon et al., 2005). SH protein has a single predicted α -helical transmembrane (TM) domain (Collins and Mottet, 1993) which is highly conserved (Chen et al., 2000; Collins et al., 1990).

A pentameric oligomer is responsible for channel activity in planar lipid bilayers (Gan et al., 2008; Gan et al., 2012). The correct orientation of the TM monomers in the lipid bilayer was determined using site specific infrared dichroism (Gan et al., 2008) and NMR studies in dodecylphosphocholine (DPC) micelles (Gan et al., 2012). In the latter system, the single α -helical TM domain is flanked N-terminally by an α -helix (cytoplasmic), and C-terminally (lumenal) by an extended β -hairpin. Later refinement of this structure in bicelles, a closer membrane-mimic system, confirmed the presence of a cytoplasmic N-terminal helix, but the TM domain extended its helix in the lumenal domain until His-51 (Li et al., 2014). In the latter paper, channel activity measurements in black lipid membranes of purified SH protein demonstrated low or no ion selectivity and a mild inhibition at low pH.

Since the structure of SH protein in micelles and in bicelles has been determined reliably, a search for protein binding partners was performed with the membrane-based Yeast Two-Hybrid System, which is especially suitable for membrane proteins (Huang et al., 2005; Lentze and Auerbach, 2008; Wang et al., 2004). The system uses split-ubiquitin, where reassembly of N- and C-termini (Nub and Cub) leads to proteolytic cleavage, and release of transcription factor triggers the activation of a reporter system (Thaminy et al., 2004). An artificial transcription factor consisting of bacterial LexA protein and the *Herpes simplex* VP16 transactivator protein is fused to the Cub moiety (Cub-LexA-VP16 reporter cassette). As SH protein is type II, its N-terminal extramembrane α -helix should be in the cytosol.

The interaction with an important membrane protein, BAP31, was confirmed by co-localization and pull-down studies with both transfected and endogenous BAP31. In addition, the cytoplasmic domain of BAP31 was expressed and purified, and its interaction with SH protein in the presence of detergent was characterized using isotopically labeled SH protein. Future work in RSV infected cells should

determine the importance and effect of this interaction, and characterization of the protein-protein interaction has to be made using labeled BAP31 endodomain.

MATERIALS AND METHODS

Yeast two-hybrid screen. The assay used was a split-ubiquitin based yeast two-hybrid screen (Dualsystems Biotech, Switzerland) that is an adaptation of the ubiquitin-based split protein sensor (USPS) (Johnsson and Varshavsky, 1994). As RSV SH protein is a type II integral protein, pBT3-N was chosen as bait vector. *SH* was cloned into *Sfi I* restriction sites of pBT3-N, so that the cytoplasmic construct LexA-VP16-Cub was fused to the N-terminus of SH protein. Prey proteins from *Homo sapiens* adult total lung RNA (4.8×10^6 independent clones) were used to construct the library in the pPR3-N vector as a fusion to the N-terminal half of ubiquitin (NubG). Transformation of the cDNA library was performed according to the manufacturer's protocol. Upon interaction of a prey protein with the bait protein, the C- and N-terminal halves of ubiquitin form a functional enzyme with consequent translocation of the transcription factor LexA-VP16 into the nucleus. Transcription of LexAVP16 was assessed using a quantitative LacZ assay, and the strongest positive clones were identified and subsequently isolated. The sequences were then used for further studies.

Full length SH protein, or a truncated version, SH_{TR} (M1-L44) were tagged to Cub, whereas the proteins in the library were tagged to NubG. The human adult lung NubG-x cDNA library was transformed into yeast expressing LexA-VP16-Cub-SH. The transformants were grown on selective media with 3 mM 3-aminotriazole (3-AT) in SD-TLH, and 2 mM 3-AT in SD-TLHA. Positive clones were identified based on quantitative LacZ assays, and the clones with the highest expression were isolated. A total of 41 colonies with robust growth were picked up from the screening media.

BAP31 and SH protein co-localization. Full length SH protein was fused to Enhanced Green Fluorescent Protein (EGFP), and BAP31 was fused with Red Fluorescent Protein (RFP). Both constructs were transfected to mouse myoblast C2C12 cells and after 36 h post transfection, cells were visualized using a fluorescence microscope fitted with Photo metrics CoolSNAP^{HQ2} camera. The images were analyzed using Metamorph software (Molecular devices, CA, USA). In order to confirm the co-localization of BAP31 with SH protein, similar experiments were performed with human lung epithelial carcinoma A549 cells.

Pull down experiments of EGFP-SH and co-transfected or endogenous BAP31. HEK293T cells were transfected with a BAP31-His construct together with (i) EGFP or (ii) EGFP-SH. The lysate was incubated with Ni-NTA Agarose beads and washed at least three times with lysis buffer. The beads were boiled in SDS-PAGE loading buffer, resolved and immuno-blotted with either anti-His or anti-GFP, to detect BAP31 or GFP/EGFP-SH, respectively. Interaction of endogenous BAP31 with SH protein was performed using GFP-TRAP immuno-precipitation as suggested (Chromotek). Briefly, HEK293T cells were transfected either with EGFP or EGFP-SH. 36 hour post transfection, cells were lysed and GFP-TRAP-IP was performed. Endogenous BAP31 was detected using anti-BAP31 antibody (Abcam; ab37120).

Protein expression and purification of SH protein and BAP31 endodomain. The SH protein used in this work - strain S2 ts1C accession number NP_044594.1- (Tolley et al., 1996) was cloned, expressed, purified and isotopically labelled essentially as described previously (Gan et al., 2012). The C-terminal cytoplasmic domain (endodomain) of human BAP31 (UniProt: P51572, residues 124–242) corresponding to two consecutive coiled-coil forming domains, was cloned, expressed and purified as described previously (Quistgaard et al., 2013).

NMR Spectroscopy. NMR experiments were performed at 37 °C (310 K) using a Bruker Avance-II 700 NMR spectrometer equipped with a cryogenic probe. The NMR data were processed using TopSpin version 3.1 and analyzed using CARRA. ¹⁵N-labelled SH protein (0.6 mM) was reconstituted in 100 mM acetate buffer at pH 4.4 and 200 mM DPC. The titration experiments with BAP31 endodomain were performed with ¹⁵N-labeled SH protein in DPC micelles from SH protein/BAP31 endodomain molar ratio 1:0.5 to 1:2. Chemical shift perturbation (CSP) values were calculated using the formula $CSP = \sqrt{\Delta \delta H^2 + (0.23 * \Delta \delta N)^2}$.

RESULTS.

BAP31 was identified as a novel target for RSV SH protein using a yeast two-hybrid screen. To identify potential interacting partners of RSV SH protein, we performed a yeast two-hybrid analysis which can detect interactions involving both cytoplasmic and transmembrane domains. Initially, full length SH protein was expressed in plasmid pBT3-SH, i.e., as a fusion to the C-terminal half of ubiquitin (Cub) linked to the transcription factor LexA-VP16. However, when this plasmid was co-transformed with pOst1-NubI (wild-type N-terminal semi-ubiquitin) or with pPR3-N (mutant), cells grew only in SD-LT non-selective plate, indicating lack of SH protein expression in this system (not shown). Therefore, following experiments used a truncated form of SH protein, SH_{TR}, spanning the transmembrane domain and an intact cytoplasmic domain (residues M1-L44).

From a blast result (BLASTX), 16 proteins were identified. However, only six genes were expressed in-frame with the NubG moiety: ribosomal proteins L3, L7, S13, P450 cytochrome oxidoreductase, transaldolase 1, and only one membrane protein, B-cell receptor-associated protein 31 (BAP31). BAP31 is the most abundant membrane protein in the ER, and has three predicted TM domains and a large cytoplasmic C-terminal domain (endodomain). Because of BAP31 role in sorting of membrane proteins and in caspase-8 mediated apoptosis, we focused our efforts in confirming BAP31-SH interaction in the cellular environment.

BAP31 and SH protein co-localization in C2C12 and human lung carcinoma A549 cells. We next investigated the co-localization of SH protein and BAP31 when both proteins were transfected. It is known that BAP31 and SH protein localize to the ER and Golgi, respectively. However, SH protein can also be found in the ER (see above). Mouse myoblast C2C12 cells were transiently transfected with a vector expressing EGFP-tagged SH protein at the N-terminus (EGFP-SH), together with BAP31 tagged with RFP at the C-terminus (BAP31-RFP). The cells were analyzed by fluorescence microscopy 36 h after transfection, and showed that both BAP31 and SH protein constructs co-localize in a perinuclear pattern consistent with the ER (Fig. 1A). Since RSV is a human respiratory pathogen, we further analyzed the localization pattern of BAP31 and SH protein in lung epithelial

carcinoma A549 cells. Consistent with the result obtained in C2C12 cells, BAP31 and SH proteins were found to be co-localized in A549 cells as well (Fig. 1B).

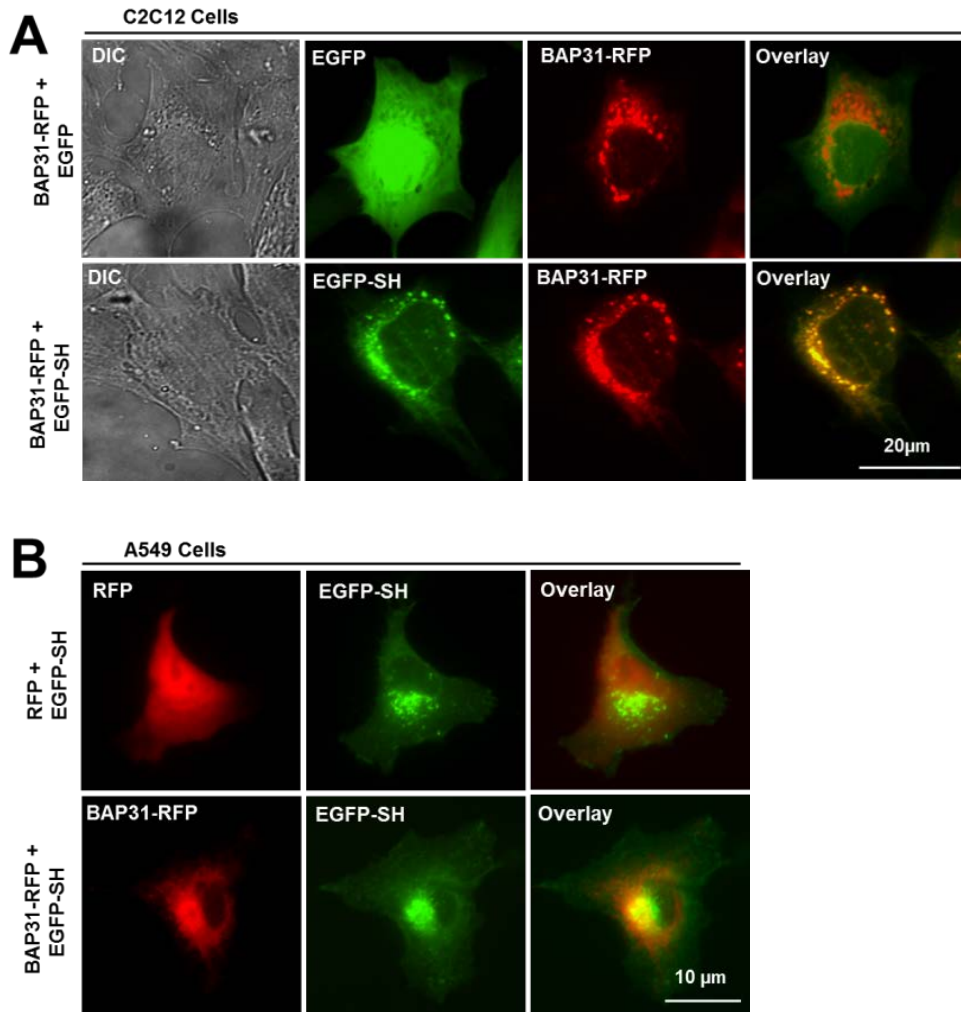


Figure 1. EGFP-SH protein co-localization with BAP31-RFP in C2C12 and human lung carcinoma A549 cells. (A) C2C12 myoblast cells transfected with plasmids pBAP31-RFP and either pEGFP (negative control) or pEGFP-SH; co-localization was detected with fluorescence microscopy; DIC, differential interference contrast microscopy; (B) human lung carcinoma A549 cells transfected with pEGFP-SH with pRFP (negative control) or pBAP31-RFP. EGFP-SH was found to be co-localized with BAP31-RFP.

Pull-down of BAP31 and SH protein. Since SH protein and BAP31 were co-localized, it was important to determine if they were associated in complexes. For these studies, a series of immunoprecipitation experiments were performed using HEK293T cells transiently transfected with expression vectors for EGFP-SH protein or EGFP alone, together with BAP31-His construct (cloned in pFIV-copGFP vector; System Biosciences). The cell lysate was prepared 36 h after transfection, and incubated with Ni-NTA Agarose beads to trap BAP31 and its binding partners. The beads were washed with lysis buffer, and boiled in SDS-PAGE loading buffer, resolved in SDS-PAGE, and immuno-blotted with either anti-His or anti-GFP, to detect BAP31 or GFP/EGFP-SH, respectively. Western blot analysis showed that BAP31-His (detected with anti-His antibody), EGFP-SH and EGFP (detected with anti-GFP antibody) expressed well in the cells (Fig. 2A, lysate). However, EGFP-SH protein, but not EGFP alone, was pulled down with BAP31-His (Fig. 2A, bound) indicating direct or indirect complex formation between SH and BAP31 proteins in the cell.

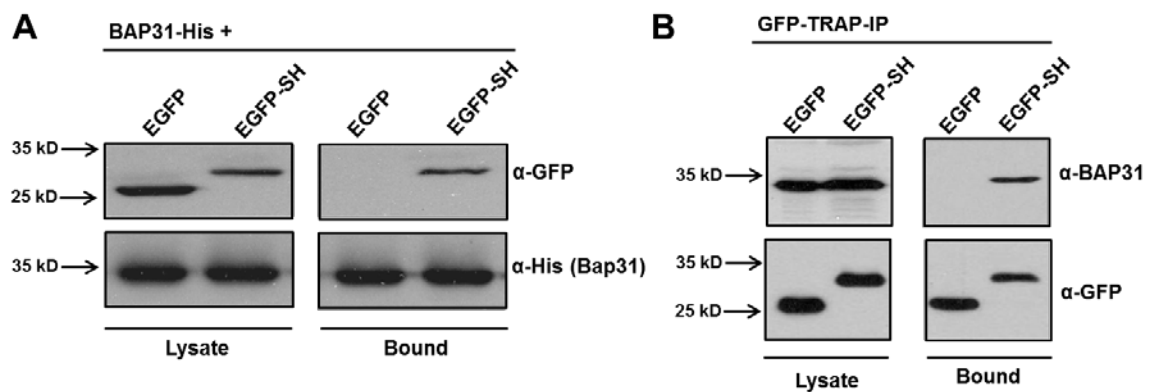


Figure 2. BAP31 interacts with SH protein. (A) Western Blot analysis corresponding to whole lysate (left) and bound fraction (right) after a BAP31-His pull down of EGFP alone or EGFP-SH. Detection was performed with anti-His antibody against BAP31 (lower panel) or anti-GFP antibody against EGFP-SH (upper panel); (B) Endogenous BAP31 was pulled down by EGFP-SH using a GFP-TRAP; BAP31 presence was detected using anti-BAP31 antibody.

In order to confirm the interaction between BAP31 and SH protein, a similar immuno-precipitation experiment was performed to trap endogenously expressed BAP31 (detected by anti-BAP31 antibody) using GFP-TRAP, which binds EGFP and EGFP-SH proteins expressed in HEK293T cells. While endogenous BAP31, EGFP and EGFP-SH were expressed (Fig. 2B, lysate), BAP31 was only present in the bound fraction in cells expressing EGFP-SH, but not when cells expressed EGFP alone (Fig. 2B, bound).

Binding site of BAP31 on SH protein. Since the SH protein construct used in the split ubiquitin experiment was truncated and only included the TM domain and the cytoplasmic domain, it is likely that interaction with BAP31 occurs in at least one of these two regions. To test if the interaction is direct, we attempted to confirm interaction at the extramembrane cytoplasmic domains between BAP31 and SH protein. Thus, we used purified full length SH protein and the endodomain (cytoplasmic) of BAP31 in solution NMR experiments. ¹⁵N-labeled SH protein in DPC detergent micelles was titrated with increasing proportion of purified water soluble non-labeled BAP31 endodomain. Addition of BAP31 endodomain caused a global reduction of signal intensities (Fig. 3A), suggesting binding to the micelles that incorporate SH protein, with a corresponding increase in correlation time and band broadening. Changes were more localized at some residues in the N-terminal extramembrane domain (Fig. 3B). Some residues in the TM domain were also affected, although the latter is probably due to allosteric effects since these residues are protected by the micelle environment (Gan et al., 2012) and the BAP31 endodomain used is water soluble. Three residues at the N-terminal helix showed pronounced intensity reduction, Ile-6, Ile-8, Ser-12 and Trp-15 (Fig. 3B). The fact that the bands did not shift suggests that the interaction between SH and BAP31 is probably in the intermediate exchange regime. Not only these residues are part of a cytoplasmically oriented N-terminal α -helix, but the residues most affected by this interaction seem to be located on the same side of the α -helix (Fig. 3C-D)(Gan et al., 2012; Li et al., 2014). Thus, this N-terminal helix of SH protein constitutes a likely binding site for BAP31, although it does not exclude that the TM domains of SH and BAP31 proteins interact. In fact, a SH double mutant was designed to disrupt that cytoplasmic

interaction S12A-W15A, but both co-localization and pull down results were the same (not shown), suggesting that the two proteins indeed also interact at the TM domain.

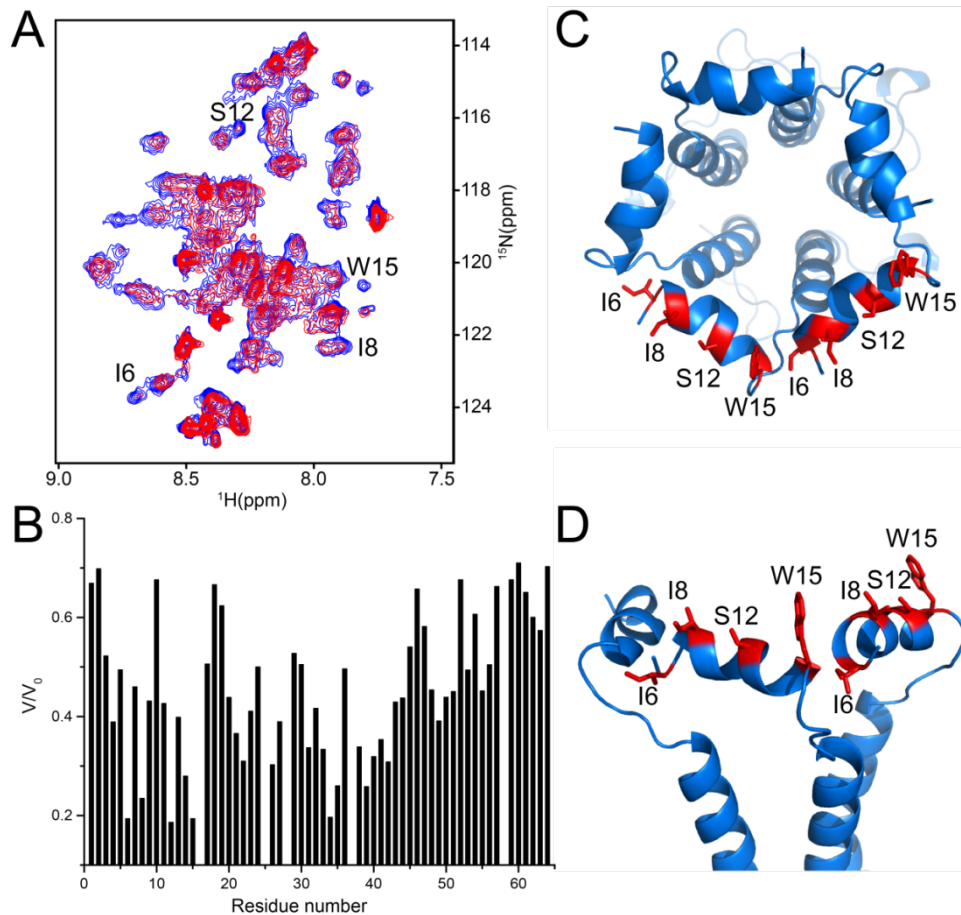


Figure 3. Interaction between SH protein and BAP31. (A) Result of the titration of ^{15}N -labeled SH protein by unlabeled BAP31 endodomain at a SH protein/BAP31 molar ratio of 1:1, monitored by $[\text{}^1\text{H}-^{15}\text{N}]$ -HSQC, with SH protein alone (blue), and after addition of BAP31 endodomain (red). Residues most affected are labeled; (B) Peak intensities expressed as ratio between peak volume after (V) and before (V_0) addition of unlabeled endodomain BAP31 to labeled SH protein. Side (C) and top (D) views of SH protein pentameric model in micelles (Gan et al., 2012), and BAP31 binding site. Residues that experience extreme line broadening are labeled in red, and their side chains are shown as line representations. Prolines and overlapping resonances are omitted from the analysis.

DISCUSSION

The ER stress response is essential for intracellular homeostatic balance, and unsettled ER stress can lead to apoptosis. SH protein in infected cells has been shown previously to have a protective effect, preventing or delaying apoptosis. The link between SH protein and apoptosis makes the identification of BAP31 as a binder of SH protein particularly intriguing. Indeed, in addition to its role in ER protein trafficking, sorting newly synthesized membrane proteins within the ER (Wang et al., 2008), BAP31 regulates apoptosis through an interaction with Bcl-2 or Bcl-XL and caspase-8 (Ng et al., 1997; Ng and Shore, 1998). A number of apoptotic pathways involve the cleavage of the endodomain of BAP31 by caspase-8 (Breckenridge et al., 2002; Breckenridge et al., 2003), to produce a p20BAP31 fragment that is a pro-apoptotic factor (Breckenridge et al., 2003; Rosati et al., 2010).

Cleavage and association to Bcl-2 require the formation of a complex at the ER membrane with CDIP1, a proapoptotic p53 target, so that apoptotic signals can be transduced from the ER to the mitochondria under ER stress (Namba et al., 2013). Further, crosstalk of apoptosis signals between mitochondria and ER are enhanced by association between BAP31 and mitochondrial fission factor Fis1 (Iwasawa et al., 2011).

In this context, our results suggest a possible link between the protective effects of RSV SH protein and BAP31, possibly through sequestering of the BAP31 endodomain by SH protein, thus preventing cleavage by caspase-8 and delaying apoptosis. It would be interesting to know if BAP31 interaction is also present for other SH proteins. Indeed, the cytopathic effect (CPE) produced by paramyxovirus parainfluenza virus 5 PIV5 (He et al., 2001; Lin et al., 2003; Wilson et al., 2006b), also a member of the *Paramyxoviridae* family, was blocked by SH protein or SH protein homologs from RSV A or B subgroups (Fuentes et al., 2007). Similarly, SH protein from PIV5 could be substituted by SH protein from mumps virus (Wilson et al., 2006b). However, these proteins have no apparent sequence homology, which argues against a protective mechanism involving specific protein-protein interactions such as that found with BAP31.

An alternative hypothesis is that the observed interaction may affect protein sorting or viral egress, and be confined to RSV. We note that the sequences of RSV SH proteins are particularly well conserved at the TM and cytoplasmic domain, where we propose the interaction with BAP31 takes place. Our identification of BAP31 using a membrane-associated yeast two hybrid analysis indicates that SH protein probably binds directly to BAP31, rather than through intermediary proteins, and this is also indicated by the NMR data. However, a larger complex of proteins involving SH protein and BAP31 is still possible. Lastly, it is interesting that using an identical yeast-based system used herein, the interaction of BAP31 with high risk human papillomavirus (HPV) 16 and 31 E5 protein was detected (Regan and Laimins, 2008). Like SH protein, E5 is also a viroporin (Wetherill et al., 2012). The interaction of E5 with BAP31 was proposed to take place through the last E5 C-terminal amino acids, although this has never been confirmed using biophysical assays using purified protein. In that case, it was proposed that HPV E5 targets BAP31 to support proliferative competence following differentiation. Although the role of the interaction between SH and BAP31 proteins is not clear, the results presented here support a direct interaction that may lead to sequestering of BAP31 from contacts with other cellular proteins. These possibilities should be further explored once the relevance of this interaction to the viral life cycle is studied in more detail.

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