Palmitoylation is required for intracellular trafficking of influenza B virus NB protein and efficient influenza B virus growth in vitro

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All influenza viruses bud and egress from lipid rafts within the apical plasma membrane of infected epithelial cells. As a result, all components of progeny virions must be transported to these lipid rafts for assembly and budding. Although the mechanism of transport for other influenza proteins has been elucidated, influenza B virus (IBV) glycoprotein NB subcellular localization and transport are not understood completely. To address the aforementioned properties of NB, a series of trafficking experiments were conducted. Here, we showed that NB co-localized with markers specific for the endoplasmic reticulum (ER) and Golgi region. The data from chemical treatment of NB-expressing cells by Brefeldin A, a fungal antibiotic and a known chemical inhibitor of the protein secretory pathway, further confirmed that NB is transported through the ER–Golgi pathway as it restricted NB localization to the perinuclear region. Using NB deletion mutants, the hydrophobic transmembrane domain was identified as being required for NB transport to the plasma membrane. Furthermore, palmitoylation was also required for transport of NB to the plasma membrane. Systematic mutation of cysteines to serines in NB demonstrated that cysteine 49, likely in a palmitoylated form, is also required for transport to the plasma membrane. Surprisingly, further analysis demonstrated that in vitro replication of NBC49S mutant virus was delayed relative to the parental IBV. The results demonstrated that NB is the third influenza virus protein to have been shown to be palmitoylated and together these findings may aid in future studies aimed at elucidating the function of NB.

INTRODUCTION

Influenza B virus (IBV) is an important respiratory pathogen that co-circulates with influenza A virus (IVA) (family Orthomyxoviridae; genus Influenzavirus B and Influenzavirus A, respectively) and contributes to influenza-related morbidity and mortality in humans (Palese & Shaw, 2007). IBV is an enveloped virus containing eight negative-sense RNA segments. RNA segment 6 of the IBV genome encodes two proteins; neuraminidase (NA) and a 100 aa glycoprotein designated NB, which is exclusive to IBV (Shaw et al., 1983). The function of NB remains unknown; however, a study by Hatta and Kawaoka (2003) showed that NB is dispensable for virus replication in vitro (Hatta & Kawaoka, 2003). They also found that NB knockout virus was attenuated in mice (Hatta & Kawaoka, 2003). As NB is highly conserved among IBVs, it is likely NB has a functional role in vivo (Hatta & Kawaoka, 2003).

NB is a type III integral membrane protein that transverses the membrane once, and contains an 18 aa N-terminal ectodomain, a 22 aa hydrophobic transmembrane domain and a 60 aa cytoplasmic tail (Shaw et al., 1983; Williams & Lamb, 1986). IBV assembles and buds at the apical portion of the plasma membrane of infected cells (Rodriguez Boulan & Pendergast, 1980; Rodriguez Boulan & Sabatini, 1978; Roth et al., 1983). As a result of this budding location, all structural and non-structural proteins as well as RNA segments must be brought to the assembly site in the apical plasma membrane (Barman et al., 2001). NB is expressed abundantly at the cell surface (Williams & Lamb, 1986) and is also a component of the virion, with 15–100 molecules being incorporated per virion (Brasnard et al., 1996). Although NB is transported to the plasma...
membrane, the transport mechanism is unknown. A study by Williams and Lamb (1988) showed that NB glycosylation is not required for NB transport to the plasma membrane, although it is modified by N-glycosylation—a process that occurs in the endoplasmic reticulum (ER) (Williams & Lamb, 1988). This suggests that NB is likely co-translated in the ER; however, NB lacks a cleavable N-terminal signal sequence (Williams & Lamb, 1986) common among proteins that are co-translated in the ER and subsequently transported through the Golgi en route to the plasma membrane (Osborne et al., 2005). Although the NB membrane-targeting signal is undetermined, it has been suggested that the transmembrane domain of NB acts as both a targeting signal for transport and an anchor for integration into the plasma membrane (Williams & Lamb, 1986). In comparison, IBV proteins NA and haemagglutinin (HA) contain characterized determinants for membrane trafficking in their transmembrane domains (Kundu et al., 1996; Lin et al., 1998). Among the determinants in HA are three critical cysteine residues located in the boundary region between the transmembrane and cytoplasmic tail domains. These cysteines undergo post-translational modification by the addition of palmitic acid (palmitoylation or S-acylation) through a thioether linkage (Veit et al., 1991). Studies have demonstrated that palmitoylation of these cysteines is not critical for transport of HA; however, they may play a role in HA-mediated membrane fusion. Studies examining the role of palmitoylation of HA have produced conflicting results; this may be due to the dynamic process of palmitoylation modification of proteins (Naeve & Williams, 1990; Simpson & Lamb, 1992; Steinhauer et al., 1991). NB has been shown to be palmitoylated; however, which cysteine residues act as palmitoylation acceptors and the role of palmitoylation of NB remain unknown (Brassard et al., 1996). Several cellular proteins have been shown to require palmitoylation for targeting to lipid raft domains within plasma membranes, such as B-cell tetraspanin CD81 (Cherukuri et al., 2004) and several proteins of the Src kinase family (Koeogl et al., 1994). It is possible that palmitoylation could play a role in the transport of NB to the plasma membrane, despite the fact that it does not play a role in the transport of other influenza proteins to the plasma membrane.

In this study, we demonstrated that NB localizes in the perinuclear region and utilizes the ER–Golgi secretory pathway and palmitoylation of cysteine 49 for efficient transport to the plasma membrane. Furthermore, we showed that the growth kinetics of a mutant virus with disruption of palmitoylation of cysteine 49 of NB was delayed relative to WT IBV in cell culture.

RESULTS

Subcellular localization of IBV NB

Native antibody specific to IBV NB was not available at the time of this study, so a construct in which an epitope tag was fused to the C terminus of NB was generated. The NB fusion constructs were detected with detergent treatment followed by immunofluorescent staining. COS-1 cells transfected with NB fusion constructs displayed a staining pattern at the plasma membrane indicating that the protein was localized at the plasma membrane (data not shown). The data indicate that fusion of an HA or Myc epitope tag to the C terminus did not alter NB localization as an integral membrane protein. This result could also be seen when a Golgi marker was used (Fig. 1b). The results of our experiment indicate that the NB subcellular localization is in the ER–Golgi/perinuclear region.

Two well-characterized markers for labelling ER and Golgi compartments, i.e. ERGIC-53 (ER–Golgi intermediate compartment) and Giantin (Golgi apparatus), were also used in this study. Our data showed that NB likely resides in the ER–Golgi region and is transported to the plasma membrane (Fig. 1a). To confirm this hypothesis, a protein co-localization experiment was performed. Fluorescent signals of NB were seen both in the perinuclear region and in the plasma membrane (Fig. 1a), similar to what was observed for the subcellular localization pattern displayed by the pNB-HA fusion protein. Also, NB co-localized with ERGIC-53, as indicated by the bright fluorescence observed in the perinuclear region (white arrows in the merged panel indicate co-localization). This indicated that NB is transported to this intermediate compartment. To demonstrate that NB is transported to the Golgi, we used an anti-Giantin antibody to label the Golgi apparatus. Fluorescence signals for NB were similar to what was seen previously (Fig. 1a), and co-localization was also observed for NB and the Giantin marker (white arrows in the merged panel indicate co-localization). Fig. 1(a, b) confirmed that NB is transported from the ER in an ER–Golgi compartment to the Golgi apparatus and ultimately to the plasma membrane.

To validate the above observation of protein localization and confirm precisely if NB was transported to the plasma membrane through the ER–Golgi secretory pathway, cells expressing pNB-HA were incubated in the presence or absence of Brefeldin A (BFA; 5 µg ml⁻¹), a fungal antibiotic and a known inhibitor of the ER–Golgi secretory pathway. BFA blocks the transport of proteins through the ER–Golgi to the plasma membrane by disrupting anterograde protein transport from the ER to the Golgi apparatus (Fujiiwara et al., 1988). In BFA-treated cells, NB was located in the perinuclear region, not the plasma membrane (Fig. 1c, merged panel). Restricted localization of NB in the perinuclear region by BFA treatment confirmed that NB utilized the ER–Golgi secretory pathway to reach the cell surface.

Identification of IBV NB membrane-targeting signal

To identify the protein domain responsible for NB trafficking to the cell membrane, a series of truncation mutants of NB were generated in which each of the
structural domains of NB was deleted in a systematic fashion. The localization of NB deletion mutants in transfected cells should allow for the identification of regions required for cellular transport. We focused our mutagenesis and construct generation on transmembrane and intracellular cytoplasmic domains of NB because numerous studies have shown these regions are responsible for protein sorting and targeting to the membranes for other proteins (Guerriero et al., 2008). To this end, a panel of deletion mutants spanning a portion of the N terminus, the entire transmembrane domain or the cytoplasmic domain of NB was generated (Fig. 2a). The mutants NBA10–18 and NBA81–100 showed a localization pattern similar to that of WT NB (Fig. 2b), indicating that aa 10–18 and 81–100 were not required for cellular transport. Note that a deletion mutant spanning aa 2–18 was also made; however, for an unknown reason, this construct did not express in transfected cells.

Conversely, mutants NBA19–40 and NBA41–80 showed a divergent localization pattern from that of WT NB (Fig. 2b). Over 50% (Table 1) of the cells expressing mutant NBA19–40 accumulated predominantly in the nucleus and perinuclear region, and showed less membrane staining, whilst 67% (Table 1) of the cells expressing mutant NBA41–80 localized in the perinuclear region with no membrane staining. These results indicated that the transmembrane domain of NB and aa 41–80 of NB are required for cellular transport of NB to the plasma membrane.

**Cysteine 49 in IBV NB is required for transport of NB to the plasma membrane**

Palmitoylation of M2 and HA of IVA virus has been shown to be important for cellular transport of these proteins. Palmitoylation occurs at cysteine residues within proteins...
and therefore we chose to first examine the effect of mutating systematically these cysteines in NB. There are seven cysteines in NB with one in the N-terminal ectodomain, two in the hydrophobic transmembrane domain and four in the first predicted helix (aa 41–80) in the cytoplasmic tail domain (Fig. 3a). A series of NB site-directed mutants were generated where selected cysteines were mutated to serines (Fig. 3a). All the mutants had a pattern similar to WT except for NBC49S, in which 70 % (Table 2) of the cells displayed a fluorescent pattern restricted to the perinuclear region only (Fig. 3b), similar to that seen for the truncation mutant NB Δ41–80 (67 %; Table 1).

**Table 1.** Cellular localization of NB truncation mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Total cells positive for TRITC (N)</th>
<th>Plasma membrane + perinuclear region [n (%)]</th>
<th>Nucleus [n (%)]</th>
<th>Perinuclear region only [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB/Myc</td>
<td>72</td>
<td>20 (28)</td>
<td>0</td>
<td>52 (72)</td>
</tr>
<tr>
<td>NBΔ10–18</td>
<td>24</td>
<td>12.5 (52)</td>
<td>0</td>
<td>11.5 (48)</td>
</tr>
<tr>
<td>NBΔ19–40</td>
<td>66</td>
<td>10 (15)</td>
<td>34 (52)</td>
<td>22 (33)</td>
</tr>
<tr>
<td>NBΔ41–80</td>
<td>49</td>
<td>0</td>
<td>16 (33)</td>
<td>33 (67)</td>
</tr>
<tr>
<td>NBΔ81–100</td>
<td>52</td>
<td>12 (23)</td>
<td>5 (10)</td>
<td>35 (67)</td>
</tr>
</tbody>
</table>
Table 1) (Fig. 2b). These data indicated that cysteine at position 49 in the first predicted helix of NB is required for the transport of NB to the plasma membrane and provides an explanation for why NBΔ41–80 has a restricted localization in Fig. 2b.

**IBV NB transport to the plasma membrane requires palmitoylation**

To determine the effect of palmitoylation on the transport of NB to the plasma membrane we utilized a pharmacological inhibitor of protein palmitoylation, called 2-bromopalmitate (2BP) (Webb et al., 2000). The majority of pNB-HA transfected cells (69%; Table 3) treated with 2BP displayed a fluorescent pattern restricted to the perinuclear region with no plasma membrane staining observed (Fig. 4) – a pattern similar to that seen when aa 41–80 of NB were deleted (Fig. 2b). This indicated that palmitoylation is required for the transport of NB to the plasma membrane.

**Palmitoylation of IBV NB is required for efficient growth of IBV in vitro**

Next, we evaluated the effect of NBC49S mutation on IBV replication by comparing the replication kinetics of parental IBV and NBC49S mutant virus stocks in cultures of MDCK cells. Virus stocks collected from transfected 293T/MDCK co-cultures with corresponding IBV reverse-genetics systems were analysed for their infectivity (TCID50) in MDCK cells. Fresh MDCK cells were then infected in parallel with either the WT or the NBC49S mutant at an m.o.i. of 0.01. Aliquots of the tissue culture supernatant were taken at periodic intervals and analysed by using the standard HA assay for the measurement of virus replication kinetics. Although no significant differences could be observed in peak viral titres of the viruses after 60 h, the initial replication of the NBC49S mutant viruses was delayed. For example, at 36 h post-infection, NBC49S mutant viruses had an HA titre 2 log2 lower than that of WT virus (Fig. 5). The effect was less pronounced at 48 h post-infection where only 1 log2 difference was observed between WT and mutant viruses. The NA enzymic activity assay performed at 32 h post-infection revealed no differences between rYam98 WT and NBC49S viruses, indicating that the delayed growth kinetics observed was not attributable to a defect in NA (Fig. S1, available in the online Supplementary Material). These data indicated that the effect of NBC49S mutation did not affect overall virus growth, but rather delayed growth at early time points.

**Fig. 3.** Localization of NB cysteine-to-serine mutants. (a) Schematic displaying structural domains of NB and positions of cysteines in NB. (b) Localization of cysteine-to-serine mutants. Cells were transfected with NB cysteine-to-serine mutant plasmid DNA. At 48 h post-transfection, cells were fixed in 4% formaldehyde and stained with anti-c-Myc conjugated to TRITC.
### DISCUSSION

IBV glycoprotein NB is a type III integral membrane protein that lacks an N-terminal leader signal sequence and is modified by N-glycans in the lumen of the ER. Here, we demonstrated that NB is transported through the ER–Golgi intermediate pathway to the plasma membrane utilizing its hydrophobic transmembrane domain presumably for integration into the ER membrane and palmitoylation of cysteine at position 49 in the cytoplasmic tail domain. Furthermore, both palmitoylation of cysteine 49 and an intact transmembrane domain of NB are required for the transport of NB to the plasma membrane.

We also demonstrated that NB co-localized with both Golgi and ER–Golgi intermediate specific markers. NB localization was restricted to the perinuclear region in the presence of a known chemical inhibitor of the ER-Golgi protein secretory pathway, BFA. From these results we conclude that NB is transported to the plasma membrane via the ER–Golgi secretory pathway. This finding confirmed a previous hypothesis that NB used the ER–Golgi secretory pathway and is not surprising as NB is modified by N-glycans – a process that occurs in the lumen of the ER (Williams & Lamb, 1986). Furthermore, this demonstrated that the route of transport for NB is the same as for the other IBV envelope proteins M2, HA and NA (Watanabe et al., 2003). Although NB has been shown to be dispensable for replication in vitro, it does play a role in viral fitness (Hatta & Kawaoka, 2003) in vivo, possibly through increasing viral replication efficiency or modulation of host viral immune responses. For the previous assumption, a common trafficking pathway for IBV envelope proteins serves to provide an environment that could facilitate necessary protein–protein interactions, promoting the production of a virus with improved fitness in the host.

We also found that the transmembrane domain of NB serves as a membrane-targeting signal. Although the exact mechanism by which the transmembrane domain serves as a localization signal is not known, it is likely that loss of the transmembrane domain prevents the incorporation of NB into the ER membrane, as it has been shown that NB lacks an N-terminal cleavable signal sequence (Williams & Lamb, 1986) – a sequence common among proteins destined for transport through the ER. It remains unclear how exactly the transmembrane domain is responsible for the incorporation of NB in the ER membrane.

The NB deletion mutant lacking the transmembrane domain (NBΔ19–40) showed no localization at the plasma membrane, but rather was found in the nucleus (Fig. 2b). NB lacks any apparent nuclear localization signals, so it is likely that the protein diffused passively into the nucleus, as NB mutants lacking the transmembrane domain would be significantly smaller than the 40 kDa molecular mass limitation for passive diffusion through the nuclear pores (Terry et al., 2007). The abundance of NB in the nucleus may also be explained by the overall positive charge of NB under physiological conditions (pI 8.5), which may allow NB to interact with the negatively charged DNA and could explain why so much NB is apparently trapped within the nucleus.

There are a total of seven cysteines in NB: one in the N-terminal ectodomain, two in the hydrophobic transmembrane domain and four in the first helix of the cytoplasmic tail domain. Due to the fact that NBΔ19–80 had restricted localization and it contains four out of the seven cysteines in the protein sequence of NB, we decided to examine the role of palmitoylation in the transport of NB to the plasma membrane.

Palmitoylation is a frequent post-translational modification of proteins in which a 16-carbon fatty acid called palmitate is added to internal cysteine residues. Palmitoylation has been shown to contribute to membrane association, protein sorting and stability of proteins.

### Table 2. Cellular localization of NB cysteine-to-serine mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Total cells positive for TRITC (N)</th>
<th>Plasma membrane + perinuclear region [n (%)]</th>
<th>Nucleus [n (%)]</th>
<th>Perinuclear region only [n (%)]</th>
</tr>
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<tbody>
<tr>
<td>NBC49S</td>
<td>53</td>
<td>16 (30)</td>
<td>0</td>
<td>37 (70)</td>
</tr>
<tr>
<td>NBC62S</td>
<td>58.5</td>
<td>44 (75)</td>
<td>0</td>
<td>14.5 (25)</td>
</tr>
<tr>
<td>NBC65S</td>
<td>65.5</td>
<td>49 (75)</td>
<td>0</td>
<td>16.5 (25)</td>
</tr>
<tr>
<td>NBC69S</td>
<td>76</td>
<td>49 (65)</td>
<td>1 (1)</td>
<td>26 (34)</td>
</tr>
</tbody>
</table>

### Table 3. Cellular localization of NB in the presence of palmitoylation inhibitor 2BP

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Total cells positive for TRITC (N)</th>
<th>Plasma membrane + perinuclear region [n (%)]</th>
<th>Nucleus [n (%)]</th>
<th>Perinuclear region only [n (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB/HA</td>
<td>167</td>
<td>135 (81)</td>
<td>0</td>
<td>32 (19)</td>
</tr>
<tr>
<td>NB/HA + 2BP</td>
<td>147</td>
<td>46 (31)</td>
<td>0</td>
<td>101 (69)</td>
</tr>
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</table>
Palmitoylation is a readily reversible process which may regulate the functional activities of integral membrane proteins (Bijlmakers & Marsh, 2003). This is applicable to NB because palmitoylation has also been shown to play an important role in the targeting of proteins to lipid rafts (Melkonian et al., 1999). Lipid raft microdomains are dynamic structures in cellular membranes that are rich in cholesterol and sphingolipids, and can serve as platforms for signal transduction as well as protein incorporation (Simons & Toomre, 2000). Several enveloped viruses, including influenza viruses, utilize these lipid rafts for budding and assembly from infected cells (Scheiffele et al., 1999; Takeda et al., 2003). Palmitoylation of NB has been reported previously; however, the specific cysteine residues that undergo palmitoylation have not been identified (Brassard et al., 1996). Localization of all but a single cysteine mutant showed a pattern similar to that of WT NB. Localization of the NBC49S mutant was restricted to the perinuclear region, displaying no localization at the plasma membrane. These data demonstrated that cysteine 49 in the first predicted helix of the cytoplasmic tail domain of NB is required for the transport of NB to the plasma membrane. WT NB in the presence of 2BP displayed a localization pattern restricted to the perinuclear region, similar to what is observed for NBA41–80 or NBC49S mutants, indicating cysteine at position 49 is likely palmitoylated.

Several viral and cellular integral membrane proteins are palmitoylated on cysteines that are either close to their transmembrane/cytoplasmic tail domain boundary region or located in their cytoplasmic tail domain (Bijlmakers & Marsh, 2003). Both IVA HA and M2 have cysteines that undergo palmitoylation located in their transmembrane domain/cytoplasmic tail domain boundary regions; however, palmitoylation of these cysteines is not required for their transport to the plasma membrane. IAV M2 has a single palmitoylation site, which does not require palmitoylation for virus replication; however, it does affect viral virulence in mice (Grantham et al., 2009).

**Fig. 4.** Localization of NB-HA in the presence of palmitoylation inhibitor 2BP. Cells were transfected with pNB-HA plasmid DNA. At 4 h post-transfection, cell culture medium was changed with either fresh medium (untreated) or fresh medium containing 100 μM 2BP. At 48 h post-transfection, cells were fixed in 4 % formaldehyde and stained with anti-HA conjugated to FITC. In the presence of palmitoylation inhibitor 2BP fluorescence is restricted to the perinuclear region, whereas untreated cells show a pattern indicative of the plasma membrane.

**Fig. 5.** Growth kinetics of WT and NBC49S mutant viruses in MDCK cells. MDCK cells grown in six-well plates were infected with either parental IBV (■) or the NBC49S mutant virus (●) at an m.o.i. of 0.01. Virus replication was monitored at various intervals following infection by measuring the HA titre in the culture supernatant, which was expressed as the log₂ reciprocal titre. The data shown represent the mean ± SD of two independent experiments.
NBC49S mutant virus had significantly delayed growth kinetics in vitro compared with WT, which suggests that efficient trafficking of NB to the surface is required for efficient growth of the virus in vitro, but this effect may also be true in vivo given the effect observed in the case of IVA M2 which is structurally analogous to NB. Although the reason for NBC49S virus delayed growth kinetics is unclear, it is possible that an accumulation of NB in the ER could induce either transient or permanent interactions between NB and other membrane proteins, such as M2, HA or NA. Along these lines, the HA titre could be lowered if HA is being sequestered within the cell.

Analysis of 3994 (all available sequences at the date of this publication) NB amino acid sequences from the NCBI Influenza Virus Resource demonstrated that 3967 (99 %) sequences contained a cysteine at position 49, indicating this cysteine is highly conserved at this position. This level of conservation likely indicates this position is important for NB function and/or virus infectivity (replication or virulence). Further studies are needed to elucidate the effect of NBC49S both in vitro and in vivo on virus growth and pathogenesis.

In summary, NB is transported through the ER–Golgi secretory pathway utilizing two important signals: the hydrophobic transmembrane domain and palmitoylation of cysteine 49 in the cytoplasmic tail domain of NB. Furthermore, palmitoylation of cysteine 49 is necessary for efficient IBV growth in vitro. These results may serve as valuable information in studies aimed at elucidating the role of NB in the replication, transmission and pathogenesis of IBV.

**METHODS**

**Plasmids.** PCR and cloning were performed with proofreading Vent DNA polymerase (New England Biolabs) under standard reaction conditions. The integrity of all PCR-derived DNA fragments was verified by sequencing. For expression of viral protein NB used in the study, the cDNA sequence encoding NB was amplified individually from B/Lec/40 (Dauber et al., 2004) by PCR and subcloned into eukaryotic expression vector pCAGGS (Matsuda & Cepko, 2004). For construction of NBA10–18, NBA19–40, NBA41–80 and NBA81–100 mutants, overlapping PCR in conjunction with primers excluding specific regions of NB was used.

Cysteine-to-serine point mutants were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and phosphorylated primers (sequences available from the authors upon request).

**Cells, transfection and antibodies.** COS-1 and MDCK cells were cultured in Dulbecco’s minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10 % FBS. mAb against the HA epitope was purchased from Sigma-Aldrich. mAb against c-Myc epitope conjugated to tetramethylrhodamine (TRITC) was purchased from Santa Cruz Biotechnology. Golgi apparatus staining was performed using polyclonal antibody against Giantin purchased from Covance, followed by incubation with anti-rabbit IgG conjugated to Alexa Fluor 594 (Invitrogen). Staining of the ER–Golgi intermediate was performed using rabbit anti-ERGIC-53/p58 conjugated to Cy3 (Sigma-Aldrich). For all transfections, cells were transfected with 1 μg DNA using TransIT-LT1 transfection reagent (Mirus Bio) following the manufacturer’s protocol.

**Immunofluorescence assay and confocal microscopy.** We conducted immunostaining using the anti-Giantin antibody specific to Giantin, a Golgi-resident protein, coupled with anti-rabbit IgG conjugated to Alexa Fluor 594 and ER–Golgi intermediate vesicles, anti-ERGIC53/p58 conjugated to Cy3. We also used the well-characterized chemical inhibitor BFA to block protein transport through the ER–Golgi to the plasma membrane. BFA inhibits normal cellular protein transport functions by disruption of anterograde protein transport from the ER to the Golgi apparatus (Fujiiwara et al., 1988).

COS-1 cells were transfected transiently with the pNB-HA expression plasmid and fixed at 48 h post-transfection. Cells were then stained with anti-HA conjugated to FITC and anti-ERGIC53/p58 or anti-Giantin. COS-1 cells grown on coverslips were transfected with NB expression plasmids (pNB-HA and pNB-Myc). At 24 or 48 h after transfection, cells were washed and fixed with 4 % formaldehyde (Electron Microscopy Sciences). Cells were then permeabilized with 0.2 % (v/v) Triton X-100 in PBS. Cells were blocked with 2 % BSA in PBS prior to incubation with the appropriate primary antibody conjugated directly with either anti-HA conjugated with FITC or anti-C-Myc conjugated with TRITC. Cells were finally washed briefly in 0.5 % BSA in PBS before being mounted on a slide with ProLong Gold antifade reagent containing DAPI dye (Invitrogen), which was used to stain the nuclei. Fluorescent imaging of fixed cells was done using a FluoView FV300 confocal system (Olympus) equipped with an IX81 microscope. Digital images were processed with Adobe Photoshop (version 6). All images were taken under similar experimental conditions (i.e. exposure time, magnification and intensification) and were subject to standardized processing.

For protein co-localization assays, cells expressing the pNB-HA protein were fixed and permeabilized with Triton X-100 prior to incubation with combinations of primary antibodies and Alexa Fluor 594-conjugated goat secondary antibody. For the pNB transport inhibition experiment, 5 μg BFA ml−1 (Sigma-Aldrich) was added to COS-1 cells 24 h post-transfection. BFA was maintained in cell culture throughout the experiment, which lasted 24 h on average. A similar procedure as described above was used to visualize either the pNB-HA protein or cellular markers (ERGIC-53 and Giantin).

**Palmitoylation inhibitor treatment.** A pharmacological inhibitor of protein palmitoylation, 2BP (Sigma-Aldrich), was used to address the effect of palmitoylation on transport of palmitoylated NB to the cell surface. COS-1 cells were transfected with pNB-HA as described above. At 4 h post-transfection, 100 μM 2BP was added and maintained throughout the period of the culture. Immunofluorescence assay using anti-HA/FITC antibody was performed.

**Virus growth kinetics and haemagglutination assay.** The protocol used for rescue of the parental IBV or NB C49S mutant virus was based on the protocol for generation of IBV (McCullers et al., 2005).

The haemagglutination assay was conducted following the WHO standard procedure. MDCK cells were infected in parallel with either the WT or the NBC49S mutant at an m.o.i. of 0.01. Aliquots of the tissue culture supernatant were taken at periodic intervals and analysed by using the haemagglutination assay for the measurement of virus replication kinetics. Virus replication experiments were conducted using two independent experiments, each performed in triplicate.
NA activity assay. NA activity was measured by using a standard fluorescent assay. The clarified supernatant and fluorogenic substrate MUNANA (Sigma) were added to a 96-well plate, mixed, and incubated at 37 °C for 1 h. After stopping the reaction by adding 50 mM glycine buffer, fluorescence was measured in a fluorometer with an excitation wavelength of 355 nm and an emission wavelength of 460 nm, and relative fluorescent units were recorded.

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