Association of Japanese encephalitis virus NS3 protein with microtubules and tumour susceptibility gene 101 (TSG101) protein

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Previously reported findings by our group showed that non-structural protein 3 (NS3) of Japanese encephalitis virus (JEV) was localized mainly in the JEV-induced convoluted membrane (CM), which has been proposed to originate from rough endoplasmic reticulum (rER), Golgi apparatus or the trans-Golgi network (TGN), and serves as a reservoir for viral proteins during virus assembly. Earlier findings indicated that NS3 of Kunjin virus interacts with microtubules. In addition, one of the Golgi-associated proteins, tumour susceptibility protein 101 (TSG101), associates with microtubules and is required for budding of retroviral particles. To clarify the association of NS3 with microtubules or with TSG101 during JEV assembly, we applied immunofluorescence, co-immunoprecipitation and immunoelectron microscopic methods. Virus infection, as well as transfection with an NS2B–NS3 expression plasmid, induced microtubule rearrangement. When cells were treated with colchicine, which interferes with microtubule polymerization, NS3 still associated with tubulin and TSG101. Furthermore, tubulin and TSG101 were co-localized with NS3 in the CM by immunogold labelling. Our observations indicate that microtubules and TSG101 associate with NS3, which is incorporated into the JEV-induced structure during JEV replication.

INTRODUCTION

Japanese encephalitis virus (JEV), an enveloped virus 50 nm in diameter, belongs to the family Flaviviridae. Its genome consists of a single positive-stranded RNA of approximately 11 kb. This viral RNA, encodes a long polyprotein consisting of three structural proteins (capsid, C; premembrane, PrM; and envelope, E) and seven non-structural proteins (NS1 to NS5), appearing as NH2-C–PrM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5–COOH (Rice, 1996). NS3 is a multifunctional protein with activities of serine protease, triphosphatase and RNA helicase, and plays important roles in JEV replication (Rice, 1996). The N-terminal one-third of NS3 in combination with NS2B is responsible for cleavage of the viral polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions (Murthy et al., 1999). The C-terminal portion of NS3 contains the DEXH helicase motif and RNA helicase activity has been demonstrated in JEV (Utama et al., 2000) and dengue virus (Li et al., 1999). NS3 is essential for virus assembly. It associates with viral RNA and NS5 protein in the replication complex (Chen et al., 1997) and has been implicated in hydrolysing ATP and by its triphosphatase activity to unwind the viral double-stranded RNA intermediate. Furthermore, recent data from complementation analysis on Kunjin virus revealed that full-length NS3 is necessary for viral RNA packaging (Liu et al., 2002).

Our previous studies indicated that NS3 is localized mainly in the membrane vesicle structure (Wang et al., 1998), also called the convoluted membrane (CM) (Leary & Blair, 1980). CM is found in the late stage of flavivirus replication (Leary & Blair, 1980) and is probably derived from ER, Golgi apparatus and trans-Golgi networks (TGN) because the marker proteins for these organelles were found in the CM (Mackenzie et al., 1999). CM is rich in viral NS3/NS2B proteins in Kunjin virus-infected cells (Westaway et al., 1997). Our previous studies showed that E protein of JEV was also localized in the CM (Wang et al., 1997, 1998). Therefore, we suggested that the CM could serve as a reservoir for viral proteins and is possibly associated with the cytoskeleton (Wang et al., 1998).

Kunjin virus NS3 has been reported to be associated with microtubules (Ng & Hong, 1989). Microtubules are known to be responsible for the intracellular trafficking of virus...
components (Beachy & Heinlein, 2000; Chu & Ng, 2002) as well as transportation of virions (Bose et al., 2001; Hollinshead et al., 2000). Evidence that the stability of microtubules is altered during infection and replication of various viruses has accumulated (Elliott & O’Hare, 1998; Parker et al., 2002; Ploubidou et al., 2000; Walter & Nowotny, 1999). Microtubule functions could also be affected by virus infection. Putting all these results together, it was still not known if JEV NS3 was associated with microtubules during JEV replication.

Tumour susceptibility protein 101 (TSG101) is localized in the Golgi apparatus during interphase and associated with spindle fibres during mitosis (Xie et al., 1998). This observation suggested an association between TSG101 and microtubules. TSG101 is essential for the formation of multivesicular bodies (MVB) in the endosomes (Dupré et al., 2001). TSG101 can interact with ubiquitylated proteins and incorporate into vesicles to form MVB in the lumen of late endosomes during the process of protein sorting. It has been also reported that, via the same mechanism as MVB formation, TSG101 can interact with late domain of Gag protein from human immunodeficiency virus type 1 (HIV-1) and with Ebola virus to facilitate virus budding (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). Since the CM is derived from rough endoplasmic reticulum (rER), Golgi apparatus and TGN, and associated with the cytoskeleton (Mackenzie et al., 1999; Wang et al., 1997, 1998), we could suppose that TSG101 could also associate with NS3, as well as microtubules, in JEV infection. In this study, we aimed to clarify the association of NS3 with microtubules and TSG101 by biochemical and morphological methods, and investigated the location of these proteins in JEV-infected cells during virus assembly.

**METHODS**

**Cells, reagents and antibodies.** BHK-21 and Vero cells were cultured in RPMI 1640 or DMEM, respectively, supplemented with 10% foetal bovine serum (Gibco). JEV neuronal virulence strain RP9, the NS2B3-expressing plasmid pNS2B3 and anti-NS3 monoclonal antibody were prepared as previously described (Chang et al., 1999; Chen et al., 1996; Wang et al., 1998). Colchicine, cytochalasin B, Cy3-conjugated goat anti-β-tubulin antibody and TRITC-phalloidin (a specific label for actin) were purchased from Sigma. Anti-tubulin and anti-TSG101 monoclonal antibodies were purchased from NeoMarkers and GeneTex respectively. Secondary antibodies for immunofluorescence were obtained from Jackson Immunoresearch Laboratories. Secondary antibodies for immunogold labelling and Western blotting were purchased from Amersham Biosciences. Rabbit antiserum against TSG101 was a gift from J. T. Cheng (Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan).

**Cell fractionation.** BHK-21 cells were infected with RP9 with m.o.i. of 1. At 24 h post-infection, the cells were lysed with 1% Triton X-100 in microtubule stabilizing buffer [MTSB; 100 mM PIPES (pH 6.9), 1 mM EGTA, 4% PEG 8000, protease inhibitor cocktail (Calbiochem)] by gentle pipetting, and the lysates were incubated on ice for 15 min. After micro-centrifugation, supernatant was washed twice with MTSB and subsequently resolved in 2× SDS sample buffer to obtain the detergent-insoluble fraction.

**Immunofluorescent staining.** Cells were mock-infected or infected with RP9 (m.o.i. 1) and incubated in culture medium containing 25 μM colchicine or 10 μM cytochalasin B for 8 h. The cells were fixed with methanol for 15 min at room temperature. The fixative was removed and the cells were washed with PBS several times. Subsequently, the cells were blocked for 1 h in blocking buffer (1% BSA, 0.1% NaN3 in PBS) and then incubated with the primary antibody diluted in blocking buffer (1:1000 for mouse anti-NS3 antibody or 1:100 for rabbit anti-TSG101 antibody) for 1 h at room temperature or overnight at 4°C. The cells were washed several times with PBS and then incubated with the fluorescent conjugated secondary antibody in blocking buffer (1:300 dilution) for another 1 h at room temperature. To label microtubules or microfilaments, the Cy3-conjugated anti-β-tubulin antibody in blocking buffer (1:100 dilution) or TRITC-conjugated phalloidin in PBS (1:1000 dilution) was applied to the cells after the secondary antibody incubation. Cells were mounted with Citifluor (Agar Scientific) and observed under an LSM510 confocal microscope (Zeiss). The samples were excited with 488 and 543 nm laser light and the emission signals were recorded using appropriate filters (505 to 530 nm band pass filter for FITC and 560 nm long pass filter for TRITC or Cy3) along with a 250 μm pinhole.

**Immunoprecipitation.** Cells infected with RP9 (m.o.i. 1) for 24 h were lysed in RIPA buffer (10mM Tris/HCl, pH 7.4, 0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitor cocktail. The lysates were rocked at 4°C for 30 min and then centrifuged (12000 g, 10 min) to remove the cell debris. Lysates were pre-cleared by incubation with protein A/G agarose beads (Santa Cruz) for 1 h at 4°C with inverted shaking. Subsequently, pre-cleared lysates, containing an equal amount of protein, were incubated with antibodies for 1 h and then with protein A/G agarose beads for another 1 h at 4°C under inverted shaking. After centrifugation (500 g, 5 min), pellets were washed five times with MTSB; 100 mM PIPES (pH 6.9), 1 mM EGTA, 4% PEG 8000, protease inhibitor cocktail and resuspended in 2× SDS sample buffer.

**Fig. 1.** NS3 is present in the detergent-insoluble fraction. JEV-infected BHK-21 cells were separated into detergent-soluble and detergent-insoluble fractions by 1% Triton X-100. Western blot analysis revealed that NS3 was restricted to the detergent-insoluble fraction as a 68-KDa protein. The numbers on the left indicates marker sizes (KDa). S, the detergent-soluble fraction; P, the detergent-insoluble fraction.
times with RIPA buffer and then boiled in 2× SDS sample buffer for 5 min. Proteins were size-separated in a 10% SDS-PAGE gel for further Western blot analysis.

**Electron microscopy preparation.** JEV-infected Vero cells (m.o.i. 1) were prepared for electron microscopic (EM) examination as described previously (Wang *et al.*, 1997). For immunogold labelling, thin sections were incubated with the primary antibody (1:100 for mouse anti-NS3 antibody; 1:50 for rabbit anti-TSG101 and rabbit anti-tubulin antibodies) for 1 h at room temperature or overnight at 4°C. The sections were washed with PBS, labelled with the colloidal gold-conjugated secondary antibody (1:25, 5 nm gold particles for NS3 and 15 nm gold particles for tubulin and TSG101) and then examined under a JEOL 1230 electron microscope.
**Western blot analysis.** Proteins were separated in a 10% SDS-PAGE gel and then transferred onto a Hybond C membrane. The membrane was washed with TBST (0.8% NaCl, 0.02% KCl, 0.05% Tween 20, 25 mM Tris/HCl pH 7.4) three times, blocked in blocking buffer (5% non-fat milk, 1% NP-40 in TBST) and incubated with anti-NS3 antibody (1:3000 dilution in blocking buffer) for 1 h at room temperature. Subsequently, the membrane was incubated at room temperature with the biotin-conjugated secondary antibody (1:2000) for 1 h and with streptavidin–HRP (1:2000) for a further 1 h. The NS3 band was visualized by the Super Signal Chemiluminescent-HRP substrate system (Pierce).

**Measurement of one-step growth curve.** BHK-21 cells were pretreated with 25 μM colchicine for 4 h and then infected with JEV (m.o.i. 5) for 1 h. Subsequently, cells were washed three times with PBS and incubated in culture medium containing 25 μM colchicine.
Colchicine was kept in the culture medium until cells were harvested at the stated post-infection time-points. The virus titres in the culture media were measured by plaque-forming assay as described previously (Wang et al., 1997).

RESULTS

NS3 is associated with microtubules and TSG101

To verify whether JEV NS3 is associated with the cytoskeleton, we separated JEV-infected cells into detergent-soluble and detergent-insoluble fractions with 1 % Triton X-100. Our examination indicated that NS3 was restricted to the detergent-insoluble fraction (Fig. 1) which suggests that NS3 is a cytoskeleton-associated protein.

From double labelling of NS3 and the cytoskeleton components, we found that NS3 was not labelled and the microtubules were shown spreading out from the perinuclear region in mock-infected cells (Fig. 2A). In JEV-infected cells, NS3 was co-localized with microtubules at the perinuclear cytoplasm (Fig. 2B). To further examine these co-localizations, we treated JEV-infected cells with colchicine, which induced microtubule rearrangements and caused the appearance of some microtubule aggregations in the cytoplasm surrounding the nucleus (Fig. 2C). However, localization of NS3 differed from that of microfilaments (Fig. 2D). When the association of actin microfilaments was disrupted by cytochalasin B treatment, the location of NS3 was apparently not affected (Fig. 2E). These results indicated that NS3 is associated with microtubules but not with actin microfilaments. TSG101 was also found co-localized with NS3 (Fig. 2F). TSG101 and NS3 moved to the same area in JEV-infected cells after treatment with colchicine (Fig. 2G). The results indicated that TSG101 was associated with NS3. These same phenomena were also observed in N18 and Vero cells (data not shown), indicating that the association between NS3, microtubules and TSG101 could be universal.

To clarify if NS3 was directly associated with microtubules without the involvement of other virus components, such as NS5 and RNA (Chen et al., 1997), a BHK-21 cell system permanently expressing NS3 was established by transfecting the cells with the NS2B–NS3 expression plasmid pJNS2B3 (Chang et al., 1999). Similar to what we found in JEV-infected cells, NS3 was also localized in the perinuclear region of NS3-expressing cells and, surprisingly, microtubules were co-localized with NS3 (Fig. 2H).

Microtubules were shown spreading out from the perinuclear region of JEV-infected cells (Fig. 2B and C, arrowheads) when compared with non-infected cells (Fig. 2B and C, arrows). However, as seen from our Western blot data, no significant difference in the amounts of tubulin or TSG101 expressed was detected between JEV-infected cells and mock-infected cells (Fig. 3). The condensing of microtubules in the perinuclear region of JEV-infected cells suggested that some unknown factors derived from JEV infection could induce rearrangement of microtubules. Staining of microtubules in NS2B–NS3-expressing cells presented the same pattern as in JEV-infected cells (Fig. 2H, arrowheads), indicating that either NS2B or NS3 is capable of inducing microtubule redistribution.

Further, we performed immunoprecipitation with antibody against either tubulin or TSG101, and then determined the presence of NS3 in the precipitate by Western blotting to verify the linkage between NS3 and tubulin or NS3 and TSG101. Analysis of the lysates from mock-infected and JEV-infected cells by Western blotting alone showed that NS3 was detected only in the JEV-infected cells (Fig. 4A, lanes 1 and 2). After immunoprecipitation with anti-NS3 antibody, NS3 was detected in the JEV-infected cell lysate, but not in the mock-infected lysate (Fig. 4A, lanes 3 and 4). In order to confirm the specificity of tubulin and NS3 interactions, immunoprecipitation with different amounts of anti-tubulin antibody (0, 2, and 2 µg respectively) was performed. The results indicated that co-precipitation of NS3 with tubulin by anti-tubulin antibody occurred, and the pull-down amount of NS3 increased in parallel with that of tubulin, i.e. when more anti-tubulin antibody was added (Fig. 4A, lanes 5–7). These observations indicated that NS3 was associated with tubulin. NS3 could also be co-immunoprecipitated by anti-TSG101 antibody (Fig. 4A, lane 8), suggesting that NS3 was associated with TSG101.

Fig. 2. Co-localization of NS3 with microtubules and TSG101. Double labelling of NS3 and cytoskeleton or TSG101 is shown for mock-infected (panel A), JEV-infected (panels B–G) and pJNS2B3-transfected BHK-21 cells (panel H). No NS3 was labelled and the microtubule distribution pattern (red) was shown in mock-infected cells (panel A). NS3 are labelled in green while microtubules are labelled in red. The merged image (in yellow) shows NS3 and microtubules co-localized mostly at the perinuclear regions (arrowheads, panel B). In JEV-infected cells in the presence of colchicine, microtubules (red) were condensed and shown as amorphous aggregations associated with NS3 (arrowheads, panel C). Note that some cells were not infected by JEV (arrows, panels B–C). Actin filaments or bundles (red) were found in the cytoplasm of JEV-infected cells, and no co-localization of NS3 (green) and actin was seen (panel D). In JEV-infected cells treated with cytochalasin B, the disrupted actin filaments are not associated with NS3 distribution (panel E). Co-localization of NS3 (green) and TSG101 (red) was observed (panel F). Colchicine disrupts the distribution patterns of both NS3 and TSG101 in the cytoplasm, but doesn’t affect the co-localization of NS3 and TSG101 (panel G). NS3 (green) was co-localized with microtubules (red) in pJNS2B3-transfected cells (arrowheads, panel H). Note that microtubules were more condensed at the perinuclear regions of cells with NS3 expression (arrowheads, panels B–C and H). Col, colchicine; Cyto B, cytochalasin B; MT, microtubules; MF, microfilaments. Bar, 10 µm.
There was no NS3 co-immunoprecipitated with anti-tubulin antibody in the mock-infected cell lysate (Fig. 4A, lane 9). Together, these results suggest that JEV NS3 is associated with microtubules and TSG101.

The same analyses were performed in the pJNS2B3-transfected cells expressing NS3. The mock-infected and JEV-infected cell lysates are presented here as the negative and positive controls respectively (Fig. 4B, lanes 1 and 2). In pJNS2B3-transfected cells, NS3 was detected in the cell lysate as a 68 kDa band (Fig. 4B, lane 3), indicating that NS2B–NS3 had been cleaved into NS2B and NS3 by its functional protease activity. NS3 could be co-immunoprecipitated by the anti-NS3 antibody (Fig. 4B, lane 4), but not by normal mouse IgG (Fig. 4B, lane 5). NS3 was also co-immunoprecipitated by the anti-tubulin antibody (Fig. 4B, lane 6) and the anti-TSG101 antibody (Fig. 4B, lane 7). These results indicated that the interaction of NS3 with microtubules and TSG101 did not necessarily require involvement of viral proteins other than NS2B.

**Tubulin and TSG101 are co-localized with NS3 in the CM**

The ultrastructural changes induced by JEV infection were seen more clearly in Vero cells than in BHK-21 cells, so we used the Vero cells to examine the ultrastructural changes after JEV infection and to investigate the localization of NS3, tubulin and TSG101 by immunogold labelling. Compared to the mock-infected cells (Fig. 5A), unique structures induced by JEV replication appeared in Vero cells (Fig. 5B). Electron-dense CM was found to connect with the distended rER and smooth membrane structure (SMS) was seen inside the rER. The JEV virions were localized in the vesicles of the SMS (Fig. 5C). We also found some JEV virions attaching to the CM in the junction of the CM and distended rER (Fig. 5D). This result suggested that JEV assembled at the junction of the CM and distended rER. By immunogold labelling, we found that tubulin was co-localized with NS3 in the CM, but not in the rER or SMS (Fig. 5E). TSG101 was also observed in the CM (Fig. 5F). These results indicated that both tubulin and TSG101 were co-localized with NS3 in the CM.

**JEV multiplication is not affected by colchicine treatment**

The plaque-forming unit assay was used to investigate the effect of colchicine on JEV replication. A one-step growth curve of JEV replication, in the absence or presence of colchicine, was plotted. A delay in JEV multiplication occurred in the first 18 h post-infection; however, colchicine treatment caused little decrease in virus titre after 18 h (Fig. 6).

**DISCUSSION**

**Location of flavivirus assembly**

In studies on Kunjin virus, viral structural proteins PrM, C and E, as well as viral RNA, were localized in the rER or around the CM (Mackenzie & Westaway, 2001; Mackenzie et al., 1996, 1998; Westaway et al., 1997). The authors proposed that the site of flavivirus assembly is in cisternae of rER. In this study, we found that the JEV virions attached to the edge of the CM or at the junction of the CM and distended rER in JEV-infected Vero cells (Fig. 5D). The same phenomenon was also observed in brain cells of JEV-infected mice (Wang et al., 1997). It is evident that JEV assembly possibly occurs at the junction of the CM and the distended rER.

**Microtubules might play important roles in the CM formation**

Our previous results indicated that the CM acts as the reservoir for viral proteins (Westaway et al., 1997; Wang et al., 1997, 1998). It receives viral proteins frequently transported from the rER and Golgi apparatus. Microtubules serve as trails for bi-directional vesicle transport between rER and Golgi apparatus (Thyberg & Moskalewski, 1999). Our results showed that virus infection and pJNS2B3 transfection induced microtubule redistribution (Fig. 2C, D, H). It may be deduced that NS2B or NS3 alone could be sufficient for inducing microtubule rearrangement. In our opinion, NS2B or NS3 might induce microtubule...
reorganization in order to facilitate the transport of other viral proteins from rER and Golgi apparatus to the CM during JEV multiplication. Furthermore, since tubulin exhibits the functions of a chaperone (Guha et al., 1998; Manna et al., 2001), it may help viral proteins to maintain their conformation in the CM.

**Fig. 4.** Immunoprecipitation (IP) with antibodies against tubulin, TSG101 and NS3, followed by Western blot analysis with anti-NS3 antibody to detect NS3 in JEV-infected cell lysates (A) and pJNS2B3-transfected cell lysates (B). Mock-infected (lane 1 in both A and B) and JEV-infected (lane 2 in both A and B) BHK-21 cell lysates, respectively, served as negative and positive controls. Mock-infected (lanes 3 and 9 in A), JEV-infected (lanes 4–8 in A) and pJNS2B3-transfected (lanes 3–7 in B) BHK-21 cell lysates were immunoprecipitated with (+) or without (−) the indicated antibodies against NS3, tubulin and TSG101. The precipitated proteins were separated in a 10% SDS-PAGE gel, transferred and then incubated with anti-NS3 antibody to detect NS3. Note that NS3 increased in parallel with the amount of anti-tubulin antibody used in IP (lanes 5–7 in A) and was not detected when normal mouse IgG was used for IP (lane 5 in B). Dense bands at approximately 50 kDa (●) are heavy chains of antibodies used in the immunoprecipitation. The numbers on the left are molecular sizes (kDa). Arrows indicate NS3.
Fig. 5. Ultrastructural observation in mock-infected (A) and JEV-infected Vero cells (B–F). CM and distended rER were clearly seen in JEV-infected Vero cells (B). JEV virions were localized in the SMS (arrowheads in C) and in the peripheral region of the CM (arrowhead in D, and double arrowheads in F). A JEV virion is also attached to the CM at the junction of the CM and distended rER (arrow in D). Immunogold labelling data revealed that tubulin (15 nm, arrow in E) and TSG101 (15 nm, arrow in F) were co-localized with NS3 (5 nm, arrowheads in E and F) in the CM. M, mitochondria; CM, convoluted membrane; rER, rough endoplasmic reticulum; SMS, smooth membrane structure. Bar, 500 nm (A, B), 200 nm (C–F).
Kunjin virus NS3 was first shown to be associated with microtubules by Ng & Hong (1989). Since then, no further data about the significance of microtubules in flavivirus replication has been reported. The envelope and capsid proteins of West Nile virus are transported from the perinuclear region to plasma membranes via microtubules, and microtubule disruption by vinblastine sulfate dramatically reduces extracellular virus titres (Chu & Ng, 2002). However, the extracellular Kunjin virus titre was not affected when infected cells were treated with nocodazole (Mackenzie & Westaway, 2001). These results suggested that microtubules are not significant in Kunjin virus assembly and maturation. In our current study, the one-step growth curve showed that JEV replication was slowed slightly when JEV-infected cells were treated with colchicine (Fig. 6). Nevertheless, the effect is not virologically significant. These results suggested that there might be some factors, probably NS2B or NS3, helping to act against the effects of the microtubule-disrupting drug during JEV replication.

**Possible roles of TSG101 in the CM during JEV replication**

This is the first study reporting that TSG101 is found in the CM. However, the role of TSG101 in JEV replication is unknown. The N-terminal portion of TSG101 is homologous to the ubiquitin-conjugating (E2) enzyme, which has been demonstrated to recognize ubiquitylated proteins and incorporate them into internal vesicles in the lumen of late endosomes (Dupré et al., 2001; Pornillos et al., 2002). TSG101 recognizes the PTAP sequence of the late domain of HIV-1 Gag protein and is essential for HIV-1 virion budding and release from cells (Garrus et al., 2001; VerPlank et al., 2001). We found it highly possible that the JEV virions were budded out from the border of the CM (Fig. 5D). Therefore, we thought JEV might utilize TSG101 for enclosing its envelope during assembly in the CM, just like the mechanism that mediates HIV-1 budding from the plasma membrane. However, neither the PTAP motif nor mono-ubiquitination was identified in the JEV viral proteins. TSG101 is also likely to regulate microtubule network dynamics. The coiled-coil domain of TSG101 interacts with stathmin (Li & Cohen, 1996), which has been shown to bind the α/β dimer of tubulin and then destabilize the microtubule structure (Cassimeris, 2002). Some of the microtubule-associated proteins are ubiquitylated (Murti et al., 1988); therefore, TSG101 might bind to an ubiquitylated microtubule-associated protein and regulate the stability of microtubules.

**Associations between NS3, microtubules and TSG101**

We disrupted microtubules using colchicine and found that the distribution of NS3, microtubules and TSG101 was consequently altered, but still overlapped (Fig. 2C and G). Co-localization of TSG101 and microtubules was also observed (data not shown). These results clearly elucidated the association of NS3 with tubulin and TSG101 in JEV-infected cells. Immunogold labelling of these proteins revealed that there is some distance between NS3 and tubulin (Fig. 5E), as well as NS3 and TSG101 (Fig. 5F). It suggested that NS3 was probably indirectly associated with tubulin and TSG101. It is likely that NS3 is associated with microtubules and TSG101 through microtubule-associated proteins.

**Conclusion**

The association of NS3 with tubulin and TSG101 was demonstrated and co-localizations of these three proteins in the CM were shown by morphological and biochemical methods. Our results also provide evidence for possible association between TSG101 and tubulin or microtubules in JEV assembly.

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