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# The dengue virus M protein localises to the endoplasmic reticulum and forms oligomers

Sook-San Wong [a,1], Gholamreza Haqshenas [a,2], Eric J. Gowans [a,3], Jason Mackenzie [b,*]

[a] Burnet Institute, 85 Commercial Road, Prahran, Victoria 3004, Australia
[b] Department of Microbiology, La Trobe University, Bundoora, Victoria 3086, Australia

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ABSTRACT

**The dengue virus membrane (M) protein is a key component of the mature virion. Here, we characterised the cellular behaviour of M using a recombinant protein construct to understand its inherent properties. Using confocal microscopy, we showed that M and its intracellular precursor, prM, localised to the endoplasmic reticulum. M protein was also detected on the cell surface and secreted, suggesting that M can enter the secretory pathway. In addition, cross-linking studies showed that M can form dimers and tetramers. These findings suggest that M behaves as a secretory protein analogous to the major envelope protein E.**


## 1. Introduction

Dengue virus (DENV) is a mosquito-borne human pathogen classified in the *Flaviviridae* family, within the flavivirus genus. Typical of all flaviviruses, the DENV genome is a single-stranded, (+) sense RNA molecule of approximately 11 kb. The viral genome has a single open-reading frame that encodes a large polyprotein that is subsequently cleaved by a combination of host and viral proteases to yield the three structural proteins (capsid, prM/membrane and envelope) and the seven non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B and 5) [1]. The membrane (M) and envelope (E) proteins form the external surface of the mature virus particle, while the uncleaved precursor of M, prM, is found as a prM–E heterocomplex on the immature virion [2]. Cellular furin cleaves prM during virus egress through the trans-*Golgi* network (TGN) allowing for structural rearrangements of the prM–E heterocomplex to the M–E homodimers on the mature particle [2,3]. This transition is pH-dependent and alterations in the intracellular pH results in the release of non-infectious prM–E containing virions [4].

M consists of 75 amino acids and at approximately 9 kDa is the second smallest of the dengue proteins [5]. Apart from its structural role in forming part of the prM–E complex, little is known about the cellular or biochemical properties of prM/M. The available literature showed that M is capable of inducing apoptosis in a sequence and localisation-dependent manner [6–8] and interacts with host proteins during the entry and assembly stage of the virus lifecycle [9–11]. This suggests that there are potential non-structural roles for prM/M during virus replication. Therefore, as part of our broader goal to understand the functional significance of M in the virus lifecycle, we studied the cellular properties of prM and M when expressed as individual proteins in mammalian cells.

## 2. Materials and methods

### 2.1. Plasmid constructs

Details of the construction of the plasmids used were described previously [12]. Briefly, plasmid pSVprM–E which encodes the structural gene regions (prM to E) of the DENV-2 New Guinea C strain [13] was used as template to amplify the prM/M genomic sequences for subsequent construction of plasmids Nmyc and PrMmyc. Nmyc has the myc-epitope (EQKLISEEDL) fused to the N-terminus of the

* Corresponding author. Fax: +61 3 9479 1222.
*E-mail addresses:* Sook-San.Wong@stjude.org (S.-S. Wong), gholamreza.haqshenas@monash.edu.au (G. Haqshenas), eric.gowans@adelaide.edu.au (E.J. Gowans), j.mackenzie@latrobe.edu.au (J. Mackenzie).
[1] Present address: St. Jude Children's Research Hospital, 262 Danny Thomas Pl, Memphis, TN 38105, USA.
[2] Present address: Department of Microbiology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, Victoria 3800, Australia.
[3] Present address: Discipline of Surgery (DX465702), Basil Hetzel Institute, The University of Adelaide, TQEH, Woodville Road, Woodville, SA 5000, Australia.

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DENV-2M. The signal sequence (residues 95–114 of the capsid protein), required for correct translocation of M in the endoplasmic reticulum, followed by the first four amino acids of the prM peptide (designated as sp-Pr*) was inserted upstream of the myc-epitope to preserve the signal sequence cleavage site. PrMmyc encodes the signal sequence, full-length prM but with the myc-epitope fused at the C-terminus instead (Fig. 1A). Three glycine residues were inserted between prM or M and the myc-epitope to create a flexible hinge for ease of myc-epitope detection. The constructs were cloned into the mammalian expression vector, pcDNA3.1 (Invitrogen) and confirmed correct by nucleotide sequencing.

### 2.2. Cell culture and transfection

Vero and 293T cells used in this study were propagated in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% foetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Transient transfections were performed on cells seeded overnight on glass coverslips in a 24-well plate. 293T cells were seeded at $2 \times 10^5$ cells per well while Vero cells were seeded at $8 \times 10^4$ cells per well. The cells were transfected with 2 μl of Lipofectamine 2000 (Invitrogen) per 1 μg of DNA according to the manufacturer's instructions. For secretion studies, transfected cells were incubated in OPTIMEM (GIBCO) reduced serum media for 48 h.

### 2.3. Immunofluorescence and confocal microscopy

For co-localisation studies, transfected Vero cells were fixed with 4% paraformaldehyde (PFA) for 20 min, then washed three times with PBS. For protein disulphide isomerase (PDI) staining, the cells were fixed in methanol/acetone (50:50) fixative. The cells were subsequently permeabilised with 0.1% Triton-X 100 (Sigma) for 3 min in PBS, washed and blocked for 1 h with PBS containing 3% FCS. After blocking, the cells were incubated for 1 h at room temperature with anti-myc MAb (Invitrogen) and antibodies to specific cellular markers. Antibodies for the following markers were used: anti-calreticulin (Stressgen) and anti-PDI (Abcam) as the endoplasmic reticulum (ER) markers; anti-giantin for *cis-medial* Golgi; anti-early-endosome antigen 1 (EEA-1; Abcam) for early endosomes; and Mitotracker Red CMXRos (Molecular Probes) for mitochondria. AlexaFluor-488 goat anti-mouse and AlexaFluor-568 donkey anti-rabbit were used as secondary detection antibodies (Molecular Probes). The stained cells were visualised with a Bio-Rad MRC1024 confocal microscope. Images were captured and analysed with LaserSharp 2000 software (Bio-Rad).

To detect the conformational specificity of PrMmyc, the flavivirus prM-specific monoclonal antibody, 2H2, was used (Chemicon). Cells were visualised with images captured at a magnification of 1800× on a Deltavision Deconvolution Microscope.

### 2.4. Western blot

To prepare the cell lysate, chilled cells were rinsed once with cold PBS then gently scraped into cold PBS containing complete protease inhibitor cocktail (Roche). The cells were then pelleted and subsequently resuspended in lysis buffer (6 M urea, 10% glycerol, 5% SDS, 500 μM DTT, 0.002% bromophenol blue, 62.5 mM Tris–HCl, pH 8.3, complete protease inhibitor cocktail). The lysates were analysed by reducing SDS–PAGE and the proteins transferred to PVDF membrane using a Trans-Blot Semi-Dry Transfer apparatus (Bio-Rad). Proteins of interest were detected using anti-myc, followed by anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:10,000, Amersham) and visualised by the enhanced chemiluminescence system (Amersham) as recommended by the manufacturer. To precipitate secreted proteins, culture supernatant was mixed with 2 volume of cold methanol, 0.5 volume of cold chloroform and 1 volume of water, vortexed and centrifuged at

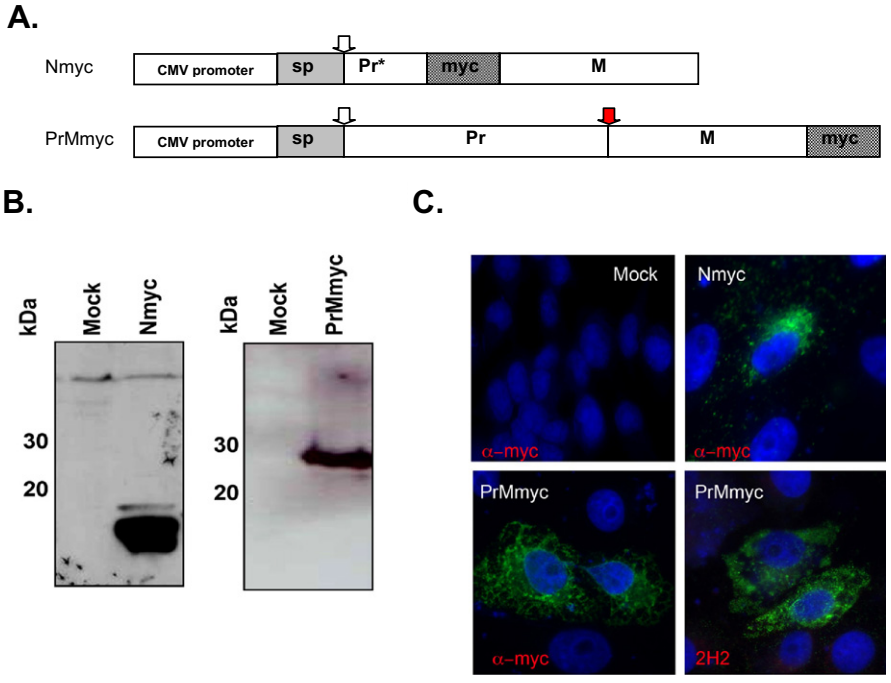


**Fig. 1.** Expression of recombinant tagged DENV-2 prM and M protein. (A) Schematic representation of the gene constructs used in this study. White arrow indicates the signal peptide cleavage site, while the red arrow indicates the furin cleavage site. (B) Detection of Nmyc (left) and PrMmyc (right) by Western blot in transfected 293T cell lysate with anti-myc. (C) Detection of Nmyc or PrMmyc (indicated in white) by IF in transfected Vero cells with anti-myc or MAb 2H2 (indicated in red). The myc or prM-specific signal is indicated by green fluorescence and the cell nuclei are stained blue with 4′,6-diaminodino-2-phenylindole (DAPI).

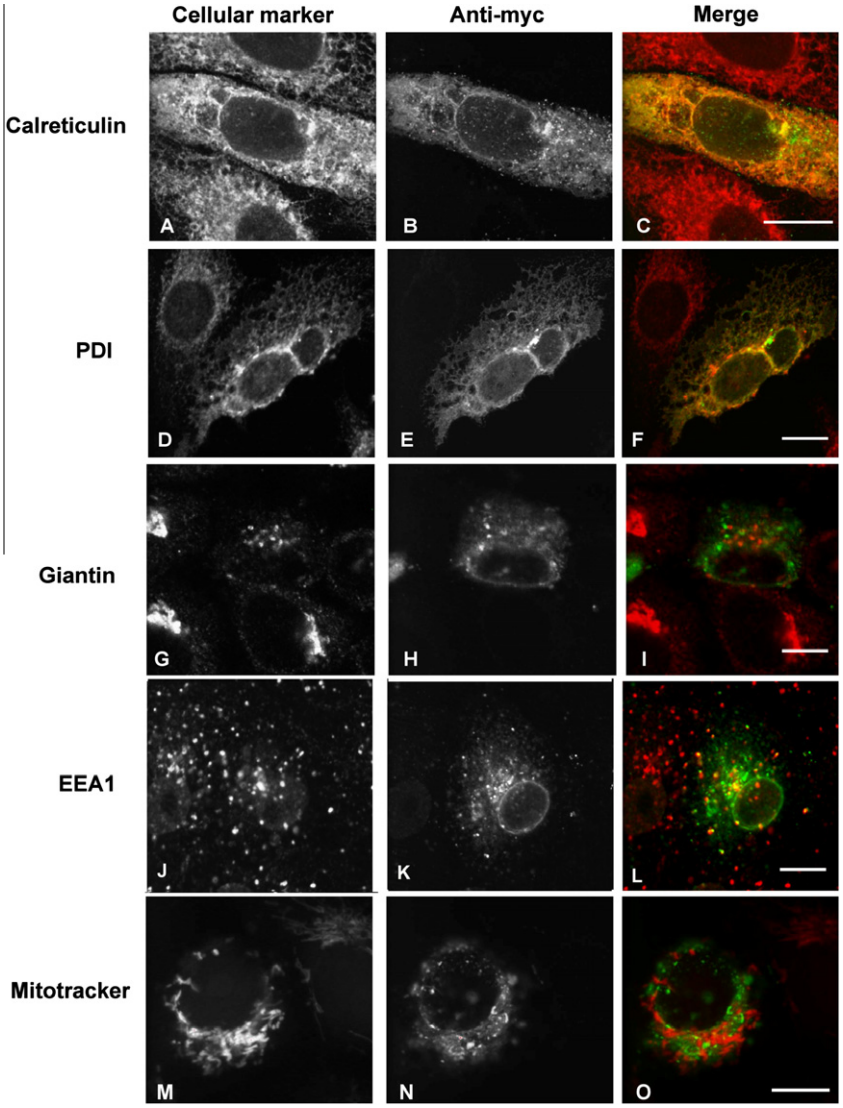


**Fig. 2.** Subcellular localisation of the M protein when expressed as an individual protein. Vero cells were transfected with Nmyc, fixed at 48 h post-transfection and dual-labelled with anti-myc (B, E, H, K and N) and antibodies specific to subcellular compartment marker proteins; calreticulin (A), PDI (D) giantin (G), EEA-1 (J) and Mitotracker™ (M). The merged fluorescence signals are shown in panels (C, F, I, L and O). Scale bar represents 10 μm.

13,000×*g* for 3 min at 4 °C. The resulting pellet was solubilised in Laemmli sample buffer (Bio-Rad). Equal volumes of protein samples were then loaded for SDS–PAGE.

### 2.5. Protein cross-linking assay

At 48 h post-transfection, 293T cells were rinsed and washed three times with cold PBS, then resuspended in PBS. The cells were treated with the cross-linking agent disuccinimidyl suberate (DSS, Pierce Endogen) at final concentrations ranging from 0 (untreated) to 4 mM of DSS for 30 min at room temperature. Mock-transfected cells were treated in parallel. The reaction was quenched with 20 mM Tris–HCl, pH 7.5. The cells were subsequently lysed by 3× freeze–thaw cycles and centrifuged at 10,000×*g* for 5 min at at 4 °C. The supernatant was collected as the soluble fraction and the pellet solubilised in the urea lysis buffer described above by 30 min incubation on ice interspersed with vigorous vortexing. The solubilised lysate was designated as the total membrane fraction and subjected to SDS–PAGE under non-reducing conditions and used for Western blotting as described above.

## 3. Results

### 3.1. Nmyc and PrMmyc localises to the endoplasmic reticulum

Since the intracellular localisation of a protein often correlates with its role, we investigated the subcellular localisation of M when expressed on its own. We constructed recombinant myc-epitope tagged prM and M proteins to facilitate their intracellular detection as there is currently no antibody for this purpose. PrMmyc was constructed to determine if the pr peptide is important in the subcellular localisation of M (Fig. 1A). The myc-epitope was fused to the C-terminus of PrM rather than at the N-terminus to avoid generating a Myc-tagged Pr fragment after furin cleavage at the prM/M junction.

By Western blotting, Nmyc was detected at its expected size (approximately 10 kDa) whereas PrMmyc was detected to be migrating slower than its predicted molecular weight of the 23 kDa (Fig. 1B). This suggests that PrMmyc was not cleaved by the host-furin. By immunofluorescence, PrMmyc was detected by both anti-myc and a conformational-dependent prM monoclonal antibody, 2H2 (Fig. 1C), indicating that the protein folded correctly.

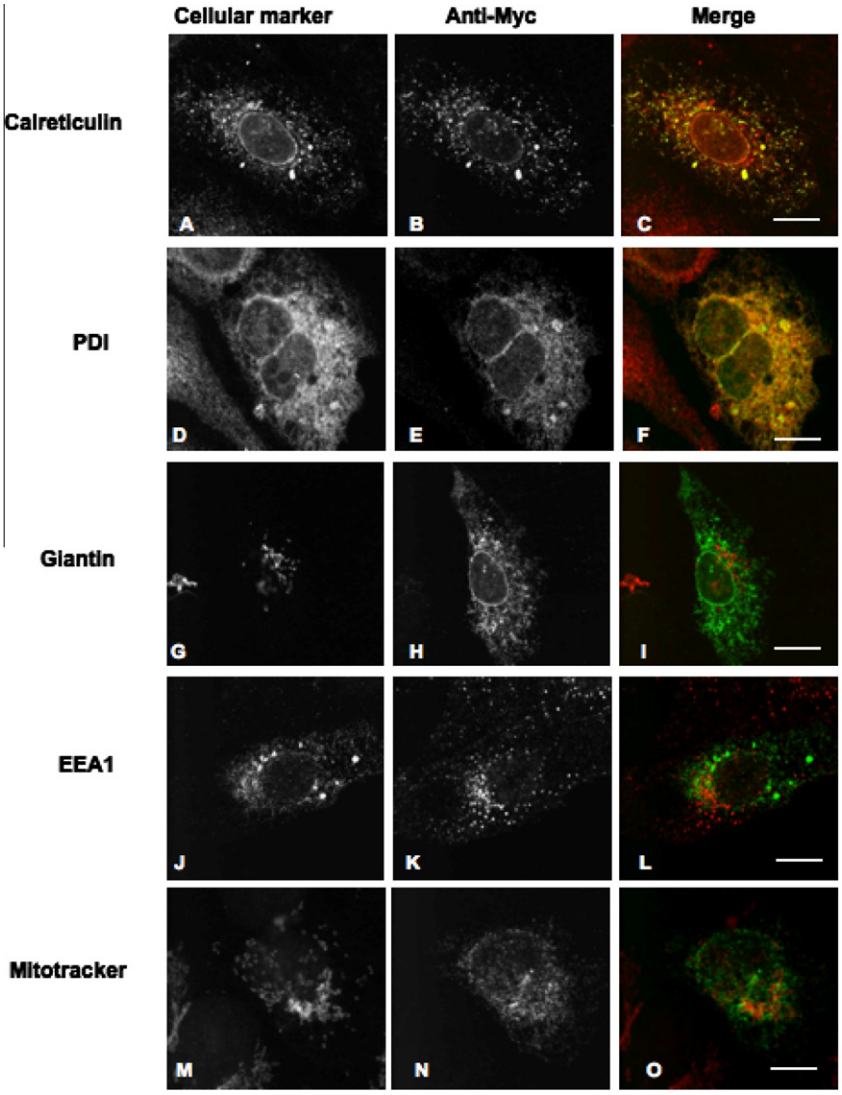


**Fig. 3.** Subcellular localisation of the prM protein when expressed as an individual protein. Vero cells were transfected with PrMmyc and dual-labelled with anti-myc and antibodies specific to subcellular compartment marker proteins; calreticulin (A), PDI (D) giantin (G), EEA-1 (J) and Mitotracker™ (M) and anti-myc (B, E, H, K and N). The merged fluorescence signals are shown in panels (C, F, I, L and O). Scale bar represents 10 μm.

Nmyc and PrMmyc were transiently expressed in Vero cells, fixed and immunostained with anti-myc antibodies and protein markers of different subcellular compartments (Figs. 2 and 3). We observed significant overlap of the Nmyc-fluorescence with calreticulin and PDI, indicating that Nmyc localised to the ER and its associated network (Fig. 2J–L). Partial co-localisation was also detected with EEA-1, an endosomal marker. No co-localisation was observed with either anti-giantin or Mitotracker™, markers associated with the Golgi apparatus and mitochondria respectively. Of note, Figs. 2H and 3N did not show a typical ER stain since the images were captured at a different focal plane compared to Fig. 2B and E to accommodate for the subcellular marker stains. The Golgi and Mitotracker™ typically stain more intensely in the perinuclear region.

Similarly, PrMmyc showed significant co-localisation with calreticulin and PDI (Fig. 3A–F), but not with any other subcellular markers. This suggests that the pr domain does not appear to alter the ER-localisation of M. The retention of PrMmyc within the ER corroborates the lack of prM cleavage, which normally occurs within the TGN.

### 3.2. Nmyc is also secreted

The above data showed that PrM and M localised to the ER and our previous topology study on M [12] showed that M was also detected on the cell surface. Since this implied that the proteins maybe treated as a secretory protein, we then investigated if Nmyc or PrMmyc were secreted from the transfected cells.

Nmyc was detected in the supernatant fraction and was significantly more abundant in the supernatant of transfected 293T cells compared to Vero cells (Fig. 4). PrMmyc was not detected in the culture supernatant of either cell line but this could be due to the overall low protein expression level as detected by the different band intensities in the cell lysates. Based on our observation that PrMmyc remains uncleaved when expressed and its' strict ER-localisation, it would imply that the protein does not enter the secretory pathway and is most likely not secreted. Thus, although Pr does not alter the ER-localisation of M, the co-expression of Pr with M may result in differential trafficking property of M.

### 3.3. Nmyc forms dimers that are associated with membrane fractions

In initial western blot experiments, we detected faint higher mw bands that may have represented dimers of Nmyc (approximately 20 kDa), suggesting that M might form oligomers. To further explore this, we added the lipid-soluble, cross-linking agent, DSS, to Nmyc-transfected 293T cell lysates to stabilise po-

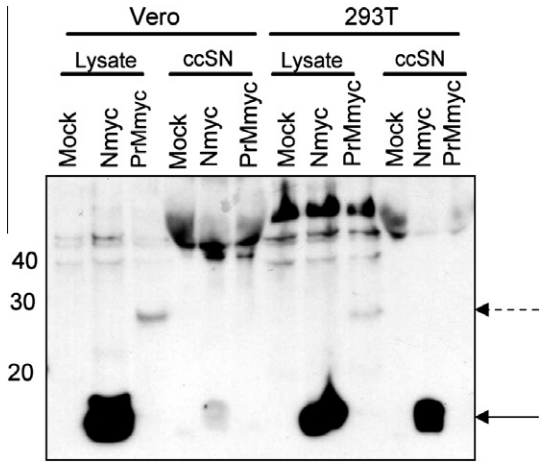


**Fig. 4.** Secretory profile of the Nmyc and PrMmyc. (A) To detect secreted proteins, cell lysates and total secreted proteins precipitated from culture supernatant (ccSN) of Nmyc and PrMmyc-transfected cells were collected at 48 h post-transfection and analysed by Western blot. The solid arrow indicates a band corresponding to the mw of Nmyc and the dashed arrow indicates a band corresponding to the size of PrMmyc.

tential oligomeric complexes. Since M is a hydrophobic protein and likely to be membrane-associated, cell lysates were subsequently processed to separate the soluble and membrane-associated proteins. Western blot of the soluble fraction revealed a band of approximately 10 kDa corresponding to the molecular weight of the monomer (Fig. 5A). In the membrane fraction, strong bands of approximately 20 kDa and a faint band at 40 kDa (arrows, Fig. 5B) were detected in addition to the 10 kDa bands. A faint band of ~20 kDa was also detected in the Nmyc-transfected sample not treated with DSS (Fig. 5, dashed arrow). This band was significantly fainter than the bands detected in the DSS-treated samples, suggesting that DSS stabilised the formation of the 20 kDa dimer. These bands were not present in the DSS-treated mock-transfected samples. Based on the corresponding increment of 10 kDa in the molecular weight of these protein species we suggest that they represent monomers (10 kDa), dimers (20 kDa) and tetramers (40 kDa) and not protein aggregates.

## 4. Discussion

In this study, we used a plasmid expression system as a model to study the cellular behaviour of the dengue virus prM/M proteins. This system is useful to understand the cellular behaviour of these proteins when expressed in isolation and how they may contribute to our current understanding of the flavivirus lifecycle. In using the recombinant myc-tagged-protein constructs, we noted that the protein expression level for PrMmyc was lower compared to Nmyc. Despite extensive optimisation on cell lines and transfection conditions, the PrMmyc expression level remained low, suggesting that the poor protein expression efficiency is intrinsic to the nature of the protein construct. The lower protein expression level could be due to prM being a larger protein and the C-terminal location of the myc-tag. A C-terminal myc-tagged M protein construct that we used in our previous study also showed a lower protein expression level compared to Nmyc [12], suggesting that the addition of the myc-tag at the C-terminal could affect the efficiency of protein expression. Although the protein expression level of Cmyc was decreased, it did not affect localisation pattern of M since we verified that the Cmyc construct also co-localised with the ER (data not shown).

In this and our previous study, we have shown that expression of M alone is sufficient for it to adopt its predicted topology [12] and localise to the endoplasmic reticulum. Similarly, we believe PrMmyc is also correctly folded since we successfully detected PrMmyc using the 2H2 monoclonal antibody in our study. The conformational specificity of this antibody is well established [15], Furthermore, Lorenz et al. showed that expression of the closely related tick-borne encephalitis virus (TBEV) PrM protein alone using a plasmid construct was sufficient for PrM to achieve its correct folding [14]. Therefore, the defective PrMmyc's cleavage to yield pr and a Mmyc fragment is not likely to be due to protein misfolding. It is also unlikely that the myc-epitope itself interfered with cleavage as it is located distal from the cleavage site. A possible explanation is that the PrMmyc protein was retained within the ER and did not enter the secretory pathway where cleavage via furin in the TGN could occur. The ER-localisation and the absence of PrMmyc in the endosomal and Golgi compartments support this explanation. This was previously observed for NS1, whereby the cell lysate form of NS1 only contained the high mannose species and not the complex glycans acquired during protein maturation through the Golgi [16]. Since M is able to localise to the ER and

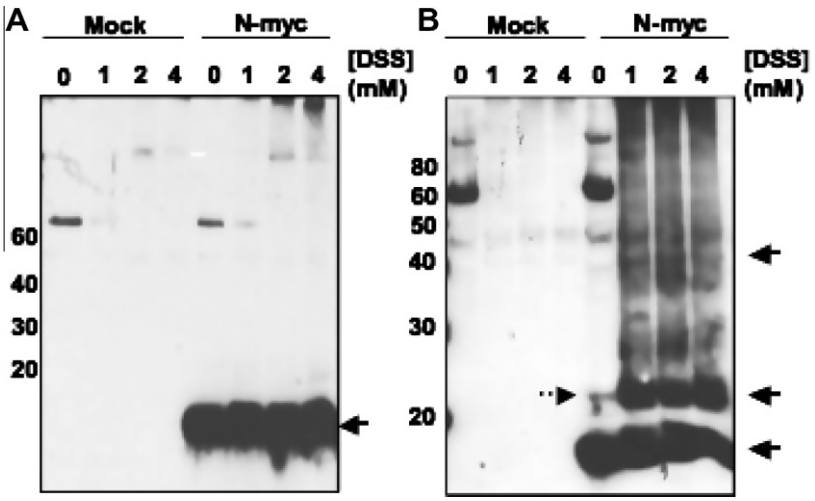


**Fig. 5.** Oligomerization analysis of M. 293T cells were transfected with Nmyc and treated with the lipid-soluble, non-cleavable cross-linking reagent, DSS, at 48 h post-transfection at concentrations ranging from 0 to 4 mM. The cell lysates were collected and separated into (A) the soluble and (B) total membrane protein-associated fraction. Solid arrows denote species corresponding to monomers, dimers and tetramers. Dashed arrow denotes dimers in the DSS-untreated sample.

the cell surface, and be secreted without the pr domain, this suggests that the pr domain may contain an ER-retention signal. The presence of a ER-localisation signal within the transmembrane domains (TMDs) has been reported in other flavivirus and hepacivirus M and E proteins [17–19], with potentially varying strengths. It is possible that pr may encode an ER-retention signal stronger than those in the TMDs of M. We were unable to conclusively determine if PrMmyc was secreted from cells due to the relatively low level of protein expression. However, as mentioned above, due to the absence of cleavage, it is likely not secreted. This suggests that the co-expression of E and the subsequent formation of the prM–E complex are important for the correct trafficking of these proteins in the context of the viral particle, during exit.

We showed that Nmyc forms higher order oligomers that are membrane-associated. Since we did not detect any M oligomers in the soluble fraction and had never detected M oligomers in the culture supernatant, we believe that M is secreted in the monomeric form. Formation of these oligomeric complexes appeared to be facilitated by its association to the cellular membranes and is mediated by weak interactions, as it was difficult to detect in the absence of the cross-linking reagent. Interestingly, the TBEV M protein was also detected as a dimer in the purified mature virus preparation and only after in vitro furin cleavage of prM in the immature virus preparation [20]. This suggests that the final structural and conformation of mature M may facilitate this dimerisation process. This could represent a novel mechanism by which the virus achieves and stabilises its final mature conformation. We did not find any dimerization motifs in M such as the GxxxG or Phe/Leu rich sequences that have been reported for the hepatitis C p7 protein [21,22]. It would be interesting for future experiments, to determine if M dimers could be detected in both the purified immature and mature virus preparation with M-specific antisera.

In conclusion, we have shown that the DENV M protein is localised to the ER and is secreted. M protein has the propensity to self-assemble into higher order oligomers that is consistent with the arrangement of the structural proteins on the flavivirus particle.

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