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

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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

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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 2×10^5 cells per well while Vero cells were seeded at 8×10^4 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

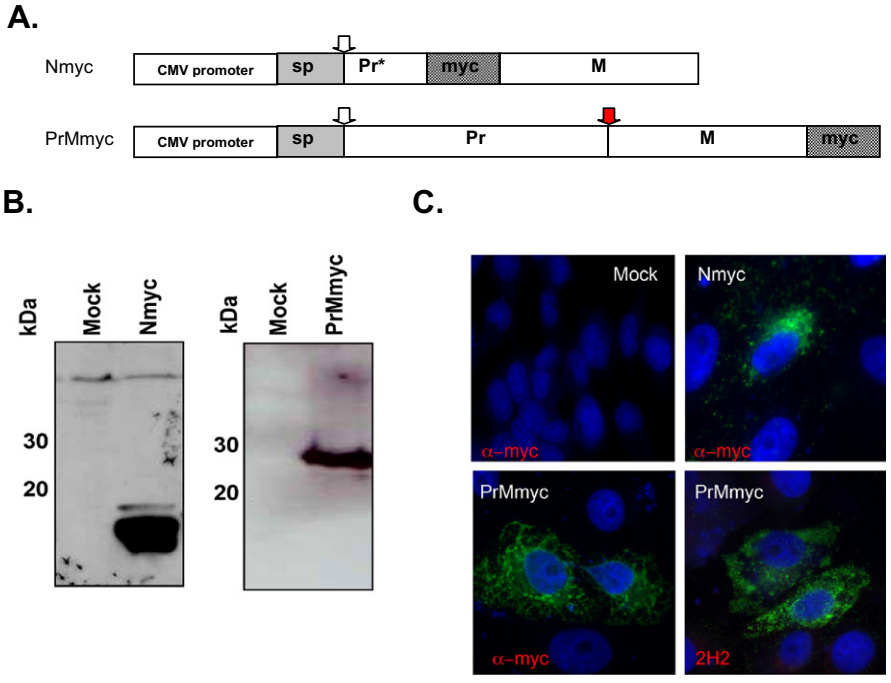

Fig. 1. Expression of recombinant tagged DENV-2 prM and M protein. (A) Schematic representation of the gene constructs used in this study. White arrow indicates the signal peptide cleavage site, while the red arrow indicates the furin cleavage site. (B) Detection of Nmyc (left) and PrMmyc (right) by 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).