ORIGINAL ARTICLE

Production and characterization of anti-dengue capsid antibodies suggesting the N terminus region covering the first 20 amino acids of dengue virus capsid protein is predominantly immunogenic in mice

Chunya Puttikhunt · Prapapun Ong-ajchaowlerd · Tanapan Prommool · Sutha Sangiambut · Janjuree Netsawang · Thawornchai Limjindaporn · Prida Malasit · Watchara Kasinrerk

Abstract We produced monoclonal and polyclonal antibodies to the capsid (C) protein of dengue serotype 2 virus (DV2 C). First, a maltose-binding protein fused to DV2 C protein (MBP-C) was overproduced in *E. coli*. The affinity-purified MBP-C protein was cleaved by factor Xa protease to obtain a recombinant DV2 C protein, which was then used for mouse immunizations. Two hybridoma cell lines producing anti-C Mabs as well as anti-C polyclonal antibody were successfully generated and characterized. Interestingly, all of the generated antibodies specifically recognized the first 20 amino acids of the DV2 C protein, as determined by peptide epitope mapping and via a recombinant DV2 C protein in which this region was deleted. The results suggested that this region is predominantly immunogenic in mice.

C. Puttikhunt and P. Ong-ajchaowlerd contributed equally to this work.

C. Puttikhunt (✉) · P. Ong-ajchaowlerd · T. Prommool · S. Sangiambut · P. Malasit
Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 12th Floor Adulyadej-Vikrom Building, Siriraj Hospital, Bangkok 10700, Thailand
e-mail: chunyapk@biotec.or.th

C. Puttikhunt · S. Sangiambut · P. Malasit
Division of Medical Molecular Biology, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

J. Netsawang
Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

T. Limjindaporn
Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand

Introduction

Infection of humans with dengue virus (DV) frequently results in a severe illness known as dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS), which continues to be a public health problem in tropical and subtropical regions. Four serotypes of DV (DV1–DV4) are classified in the genus *Flavivirus* of the family *Flaviviridae*. Capsid (C), one of the three structural proteins of DV, is a relatively small structural protein (approximately 15 kDa) containing highly conserved basic amino acids. Multiple copies of C interact with a single copy of viral RNA, resulting in the formation of a 30-nm-diameter nucleocapsid (NC), which is surrounded by a host-derived lipid bilayer associated with viral envelope (E) and prM/M transmembrane proteins. Cryo-EM images of both mature and immature DV demonstrate a well-organized icosahedral structure of the viral envelope, while a disordered structure of the internal

W. Kasinrerk
Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Sciences and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

W. Kasinrerk (✉)
Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
e-mail: watchara@chiangmai.ac.th

Present Address:
P. Ong-ajchaowlerd
Department of Virology, US Army Medical Component-Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

NC has been suggested to be loosely associated with the lipid bilayer of the virion [6, 22].

The C protein has been suggested previously to be a flexible molecule and to be largely alpha-helical in structure [5]. The secondary structure of recombinant C protein from DV2 and yellow fever virus as determined by NMR techniques shows that the flavivirus C proteins are predominately dimeric in solution and are composed of four alpha helices (α1–α4), in which the N terminus (residues 1–20) is conformationally labile or unstructured [3]. The first elucidated 3D structure of DV2 C dimer (residues 21–100) suggested possible mechanisms for its interactions with RNA and the viral membrane [9]. The domain responsible for homotypic interaction was further pinpointed by co-immunoprecipitation of residues 37–72, which overlap the internal hydrophobic region [20].

DV C protein is found not only in the cytoplasm, but also in the nucleus of infected cells [2, 10, 18, 19, 21]. The C protein region responsible for nuclear localization was determined to be a bipartite structure, RKeigrmlnilnRRRR, at amino acid residues 85–100, as determined using transfected cells expressing C protein [19]. Alanine substitution mutations in the C protein at amino acid residues 73, 74, 85 and 86 of DV2 mutants eliminated nuclear localization of C protein [17]. However, no correlation between nuclear localization and viral growth properties has been observed [17]. Interaction of C and a Fas-associated nuclear protein, Daxx, in liver cells, which was reported recently, may suggest an involvement of C protein in apoptosis caused by DV infection [8]. However, the significance of nuclear C protein remains unclear and requires further investigation.

One of the key reagents that are necessary for functional study of DV C protein is a specific antibody. So far, limited numbers of anti-C Mabs have been generated. In this study, we aimed to generate Mabs specific for the DV2 C protein, as well as anti-C polyclonal antibody (Pab), by immunization of mice with a recombinant DV2 C protein expressed in *E. coli*. The generated antibodies were characterized for their specificity for viral protein, dengue serotype and recognition epitope. Peptide mapping, as well as a reduced reactivity of anti-C antibodies to the deleted C protein, suggested that the N-terminal region consisting of the first 20 amino acids of DV2 C protein is predominantly immunogenic in mice.

Materials and methods

Construction of pMal/DV2C and pMal/DV2C(Δ1-20) plasmids

The gene encoding the capsid protein of DV2 strain 16681 (DV2C, 339 nt located at nt 100-438 of the complete DV2

genome; accession number M84727) was amplified from a previously constructed plasmid, pTrcHis/DV2C (Puttikhunt C, unpublished) by PCR using CBamHI(L) (5′-CGGG ATCCAATGACCAACGG AAAAAGGCG-3′) and CAP-SID(R) (5′-CCGCTCGAGTTACGCCATCACTGTTGGA AT-3′) oligoprimers. The DV2C PCR product was digested with *Bam*HI and *Xho*I restriction enzymes and then cloned into a pMal-c2 expression vector (New England Biolabs, USA) at *Bam*HI and *Sal*I sites, downstream from the *malE* gene, which encodes a maltose-binding protein (MBP). *E. coli* containing recombinant plasmid pMal/DV2C was selected. The pMal/DV2C plasmid was verified by nucleotide sequencing. To construct pMal/DV2C(Δ1-20), the primers C(21-113) *Bam*HI(L) (5′-CGGGATCCCGCGTG TCGACTGTGCAACAGCTG-3′) and CAPSID(R) were used to amplify the deleted DV2C gene (279 nt), which lacked the 60 nt encoding the first 20 amino acids of DV2 C protein from the pMal/DV2C plasmid.

Protein expression and purification of MBP-C and MBP-C(Δ1-20)

Overnight cultures of *E. coli* harboring the pMal/DV2C or pMal/DV2C(Δ1-20) plasmids were inoculated into fresh LB broth containing 0.2% glucose and 50 μg/ml ampicillin and incubated at 37°C in a shaking incubator. When the absorbance of the culture at 600 nm (A600) reached 0.6, 0.3 mM IPTG was added to induce the expression of the recombinant MBP-C or MBP-C(Δ1-20) fusion protein, and shaking was continued for an additional 3 h at 37°C. The bacterial cells were harvested by centrifugation at 7,000 rpm for 20 min and washed with 100 ml PBS buffer. The bacterial cells were resuspended in 40 ml of column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA), and sonicated on ice for cell breakage. The sonicated suspension was centrifuged at 10,000 rpm for 30 min to separate the soluble and insoluble protein fractions. The MBP-C or MBP-C(Δ1-20) protein in the soluble cell lysate was purified by amylose affinity chromatography. The bound proteins were eluted with 10 mM maltose elution buffer and measured by spectrophotometry at 280 nm (A280). The high-absorbance fractions were pooled together, and the concentration of purified protein was determined by a Bio-Rad protein assay. Purity of the obtained protein was verified by SDS-PAGE.

Recombinant MBP-C protein cleavage by factor Xa protease

Purified MBP-C proteins (10 μg), in a spectrum of pH values ranging from acidic to basic pH (pH 4–9), or MBP-C(Δ1-20) at pH 7.4 were incubated with 1% (w/w) of

factor Xa protease at 23°C for 10 h. The protein reactions were then centrifuged at 10,000 rpm for 10 min to separate the insoluble pellet from the soluble fraction and analyzed by SDS-PAGE.

Mouse immunization and Mab production

The C protein pellet, obtained by digestion of MBP-C with 1% factor Xa protease at pH 7.4, was mixed with complete Freund's adjuvant and used to immunize two 6-week-old BALB/c mice at doses of 100 μg. After 2 weeks, the following immunizations were done with a mixture of antigen and incomplete Freund's adjuvant for three additional doses at 2-week intervals: blood samples were collected before immunization (labeled as pre-immune samples) and at 2 weeks after each injection (labeled as bleed-1 to bleed-4) by tail bleeding. Mouse sera were separated and anti-C antibodies were detected either by Western blot analysis or indirect ELISA. When the target increment of antibody titer was detected, the mouse was killed. Fusion of mouse spleen cells and myeloma cells was then performed as described previously [13]. Hybridoma clones producing anti-dengue Mabs were selected by indirect ELISA, using plates coated with lysates from DV2-infected cells. Single-cell cloning of positive hybridomas was performed by limiting dilution to obtain hybridoma clones that produced anti-C Mabs.

Production of mouse ascitic fluid with anti-C Pab

The method was modified from Kurpisz et al. [7]. Briefly, BALB/c mice were immunized with insoluble C protein at doses of 100 μg at 2-week intervals for a total of three doses via intraperitoneal injection. After anti-C antibody was induced, the mice were then pretreated with pristane and injected with non-DV-related hybridoma cells to induce the formation of ascites. The mouse ascitic fluid containing anti-C Pab was collected and stored at −20°C.

Mouse ascitic fluid with anti-DV Pab

Anti-DV Pab in mouse ascitic fluid, obtained by immunization with inactivated DV2, was provided by Dr. Ananda Nisalak (Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand).

Indirect ELISA for detection of anti-C antibody

A 96-well ELISA plate was coated with DV2-infected or mock C6/36 cell lysate and stored at 4°C overnight. After blocking with 1% BSA-PBS, the diluted immunized mouse sera or hybridoma supernatants were added and incubated at

37°C for 1 h. After washing, HRP-conjugated anti-mouse immunoglobulins were added and incubated for 1 h. To visualize the reaction, o-phenylenediamine (OPD)–H₂O₂ substrate was added to develop color intensity, and absorbance at 492 nm was measured using an ELISA reader.

Western blot analysis

The protein samples were treated with or without β-mercaptoethanol with or without heating at 95°C before being subjected to 12% SDS-PAGE. The separated proteins were semi-dry blotted from the gel onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk/PBS blocking buffer for 1 h, and then reacted with anti-MBP Mab (Puttikhunt C, unpublished data) or anti-C antibodies for 1 h at room temperature. After washing, HRP-conjugated rabbit anti-mouse immunoglobulins were added and the membrane was incubated for 1 h in the dark. The enzymatic reaction was developed in a diaminobenzidine (DAB)–H₂O₂–NiCl₂ chromogenic substrate solution for 5 min to visualize the dark brown band of the antigen-antibody complex on the membrane.

Epitope mapping by peptide-coated ELISA

Five 20-mer oligosynthetic peptides spanning the DV2 C protein (aa 1–100 of 113-aa full-length C) were kindly provided by Dr. Juthathip Mongkolsapaya (Department of Immunology, Division of Medicine, Hammersmith Hospital, Imperial College, London, UK). The peptides used in this study were as follows: P1: NNQRKKAKNTPFNMLKRERN (aa 1–20); P2: RVSTVQQLTKRFSLGMLQGR (aa 21–40); P3: GPLKLFMALVAFLRFLTIPP (aa 41–60); P4: TAGILKRWGTIKKSKAINVL (aa 61–80) and P5: RGFRKEIGRMLNILNRRRRS (aa 81–100).

A 96-well microtiter plate (Maxisorp™ surface, Nunc, Denmark) was coated with 20 μg/100 μl of each C peptide in carbonate buffer, pH 9.6, in a moist chamber overnight. After blocking with 3% BSA in PBS, 20 μg of purified anti-C Mab or diluted anti-C Pab (in 100 μl) was added, followed by incubation at 37°C for 1 h. After washing, HRP-conjugated rabbit anti-mouse immunoglobulins (Dako, dilution 1:2,000) were added followed by incubation at 37°C for 1 h. The antigen–antibody complex was detected by the addition of OPD–H₂O₂ substrates. The color intensity was measured with an ELISA reader at 492 nm.

Indirect immunofluorescence assay (IFA)

A monolayer of dengue-virus-infected or mock-infected cells on coverslips was fixed with 4% paraformaldehyde for 20 min and washed twice with PBS. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for

10 min. After washing with 0.1% Triton X-100 in PBS, the treated cells were stained with anti-C Mab for 1 h at room temperature, followed by incubation with Cy3-conjugated rabbit anti-mouse immunoglobulins for 30 min in the dark. Fluorescent cytoplasmic staining was observed using confocal laser scanning microscopy (LSM510 META; Carl Zeiss).

Absorption of antibodies by MBP or MBP-C protein

DV2-C-immunized mouse sera (bleed-4) at a dilution of 1:50 were incubated with 5 μg/ml of MBP or MBP-C protein for 1 h at 37°C in an absorption buffer containing 20 mM Tris–HCl, pH 7.4, and 200 mM NaCl. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected and used as pre-absorbed antiserum for determination of its reactivity with DV2-infected cell lysates. The anti-C Pab obtained from mouse ascitic fluid (10 μl) was incubated with 300 μg of purified MBP in 200 μl absorption buffer for 1 h at 37°C. After centrifugation, the pre-absorbed anti-C Pab at 1:10,000 final dilution was used to determine its reactivity with recombinant MBP and MBP-fusion proteins.

Viruses

Prototype strains of each DV serotype, including the DV1-Hawaii strain, DV2-16681 strain, DV3-H87 strain, and DV4-H241 strain, were used to characterize the antibodies in this study. Cells were infected at a multiplicity of infection (MOI) of 0.1, and the viruses were propagated in a monolayer of C6/36 cells in L-15 culture media containing 1% fetal bovine serum (Gibco BRL) for 3–7 days at 28°C. The harvested culture supernatants containing the viruses were divided into aliquots and stored as virus stocks at −70°C.

Results

Expression and purification of recombinant MBP-C protein

To produce the recombinant DV2 C protein in *E. coli*, we cloned a gene encoding full-length DV2 C protein (113 amino acids) into a pMal-c2 expression vector, yielding the plasmid construct pMal/DV2C. *E. coli* harboring pMal/DV2C plasmid was induced by IPTG to express the recombinant MBP-C fusion protein, in which the DV2 C protein is located at the C terminus of the MBP. The majority of the expressed MBP-C protein was in the soluble fraction and was subjected to one-step purification by amylose affinity chromatography. The purified MBP-C

protein of approximately 60 kDa was analyzed by SDS-PAGE (Fig. 1). The production yield of MBP-C was about 8.3 mg per litre of *E. coli* culture.

Efficiency of MBP-C cleavage by factor Xa protease and solubility of C protein product are pH-dependent

To obtain isolated C protein, purified MBP-C was cleaved by factor Xa protease, with its recognition site (Ile–Glu–Gly–Arg) located between the MBP and C proteins. Cleavage of MBP-C protein by 1% factor Xa was first performed at pH 7.4 as recommended by the manufacturer. SDS-PAGE analysis indicated that MBP-C was almost completely cleaved, resulting in two cleavage products, soluble MBP (45 kDa) and precipitated C (15 kDa). To obtain the soluble C protein cleavage product, we then tried changing the pH of the reaction buffer for cleavage, from an acidic pH of 4.0 to a basic pH of 9.0. The cleavages at acidic to neutral pHs were preferable, as shown by the decreasing amounts of uncleaved MBP-C remaining after cleavage at pH 4.0, 7.4 and 8.0 (in lane W) compared to that at pH 8.5 and 9.0 (Fig. 2). This suggests that the efficiency of factor Xa cleavage was pH-dependent. However, the C protein cleavage product was completely precipitated at pH 4.0, similar to pH 7.4 (lane P), whereas soluble C protein was observed at the basic pH 8.5 and 9.0 (lane S). Cleavage at pH 8.0 resulted in C product in both forms. These results suggested that the solubility of the C protein product and the efficiency of MBP-C cleavage by factor Xa were pH-dependent in an inverse manner.

### Immunogenicity of insoluble C protein in mice

As the incomplete cleavage of MBP-C at basic pH caused a lower yield of soluble C protein product, which required further purification, we therefore chose an insoluble form
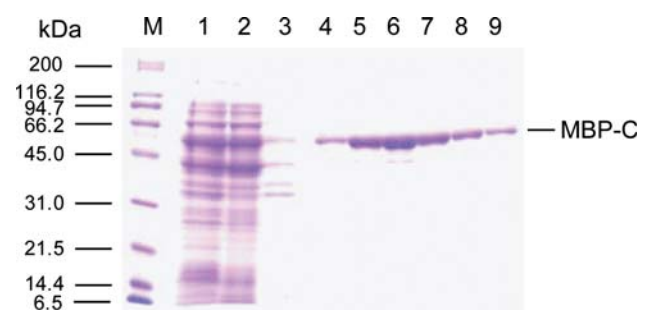
Fig. 1 SDS-PAGE of purified recombinant MBP-C protein by amylose affinity chromatography. *Lane 1* whole cell lysate of *E. coli* (pMal/D2C); *lane 2* soluble protein fraction of bacterial lysate; *lane 3* pellet protein fraction of bacterial lysate; *lanes 4–9* purified MBP-C protein fractions eluted from amylose affinity column; *lane M* broad-range standard protein markers. The protein molecular sizes (in kDa) are shown on the *left*. MBP-C is indicated on the *right*

Fig. 2 SDS-PAGE of purified MBP-C protein cleavage by factor Xa protease. MBP-C was cleaved by 1% factor Xa at 23°C for 3 h at the indicated pH values. The protein cleavage products (*W*) were centrifuged to separate the soluble protein fraction (*S*) from the precipitated protein fraction (*P*). *U* uncleaved MBP-C protein; *M* broad-range standard protein markers. The protein molecular sizes (in kDa) are shown on the *left*. The MBP-C, MBP and C proteins were indicated on the *right*
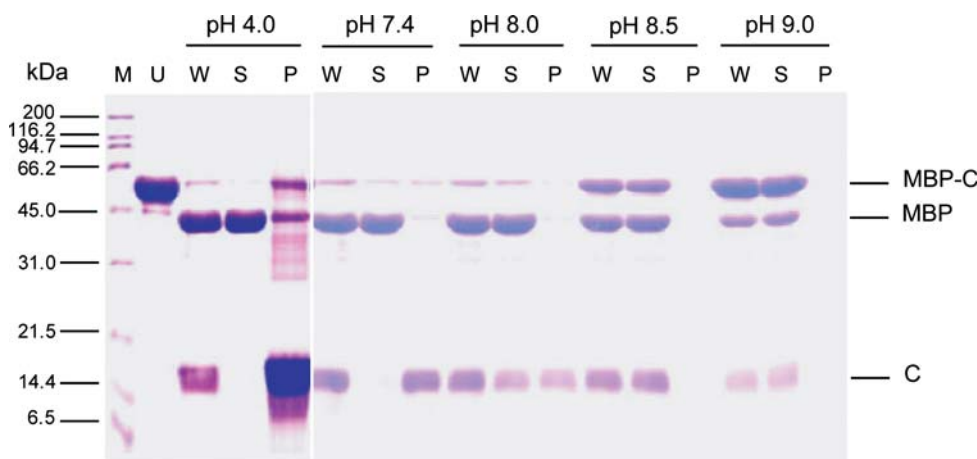

of C protein from MBP-C cleavage at pH 7.4 for mouse immunization. Two BALB/c mice were immunized with the insoluble C protein to generate anti-C antibody. Mouse sera collected 2 weeks after each immunization were tested for the presence of anti-C antibody by ELISA, using plates coated with lysates from DV2-infected cells (Fig. 3a). Mouse anti-C antibody was initially detected 2 weeks after the second injection (bleed-2), and its titer was significantly increased in the following bleeds. The Cap B mouse gave a higher titer than the Cap A mouse (Fig. 3a).

To determine the specificity of mouse antibody to DV2 C protein, the immunized mouse sera from the Cap B mouse were analyzed by Western blot analysis using a DV2-infected cell lysate. The immunized mouse sera reacted to a protein band of approximately 15 kDa, corresponding in size to the DV2 C protein (Fig. 3b). The intensity of the reactive band was first detected in bleed 2, and gradually increased in the following bleeds, corresponding to the ELISA result (Fig. 3b). No band was observed in pre-immune and bleed-1 sera.

The specificity of anti-C antibody for the DV2 C protein was further confirmed using pre-absorbed Cap B mouse serum (bleed-4) with MBP-C or MBP and re-analyzed by Western blot analysis. This shows that the mouse serum that was pre-absorbed with MBP-C lost its reactivity to the DV2 C protein (Fig. 3c, lane 2), whereas mouse serum that was pre-absorbed with MBP or not pre-absorbed still reacted with the DV2 C protein (Fig. 3c, lane 3 and 1, respectively). From the above results, we conclude that immunization with insoluble recombinant C protein in mice could produce an antibody that is specifically reactive to the DV2 C protein.

Generation of anti-C Mabs

To generate hybridoma clones producing anti-C Mabs, we performed fusion of Cap B mouse spleen cells and cells
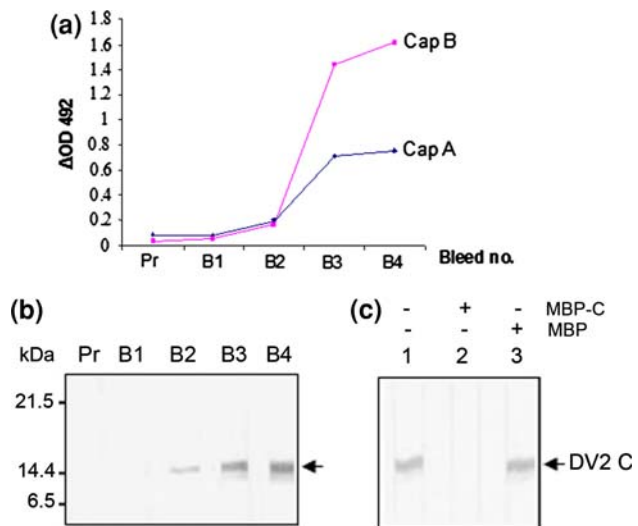
Fig. 3 Immunogenicity of C protein in BALB/c mice (Cap A and Cap B). **a** Detection of antibodies in sera of immunized mice by ELISA using plates coated with lysate from DV2-infected C6/36 cells. Cap A and Cap B mouse sera from pre-immune (Pr) and the bleed-1 to -4 (B1–B4) were diluted to 1:640. **b** Western blot analysis of Cap B mouse sera to DV2 C proteins. Mouse sera at 1:50 dilution were reacted with DV2-infected C6/36 cell lysate. Protein molecular sizes in kDa are shown on the *left*. **c** Western blot analysis of pre-absorbed immunized mouse sera to DV2 C protein. Cap B mouse sera (bleed-4) that were pre-absorbed with MBP-C (*lane 2*), MBP (*lane 3*) or reaction buffer (*lane 1*) were reacted with DV2-infected C6/36 cell lysates. D2V C protein is indicated on the *right*

from a myeloma cell line according to standard hybridoma technology. After screening and single-cell cloning, we obtained two hybridoma clones, named D2-C1 and D2-C2, for further characterization.

Characterization of anti-C Mabs

The immunoglobulin isotypes of anti-C Mabs were determined using an ELISA isotyping kit (Sigma-Aldrich, USA), and all were found to be of the IgG1 isotype. The specificity of anti-C Mabs for DV C protein of each DV

serotype was determined by Western blot analysis and IFA. Prototype strains of each DV serotype (DV1-Hawaii strain, DV2-16681 strain, DV3-H87 strain, DV4-H241 strain) were used in this study. In Western blot analysis, Mab D2-C1 specifically reacted with the 15-kDa C protein of DV2, whereas Mab D2-C2 reacted to that of both DV2 and DV4 (Fig. 4a). Concordant results were also obtained using IFA (Fig. 5a). These results suggest that Mab D2-C1 was DV2-specific, whereas Mab D2-C2 was cross-reactive with DV2 and DV4.

To determine the epitope type recognized by each Mab, DV2-infected cell lysates were treated with β-mercaptoethanol or left untreated and/or heated at 95°C, then reacted with both Mabs by Western blot analysis. As shown in Fig. 4b, both types reacted with the DV2 C protein under all treatment conditions, suggesting that they recognized a linear epitope of the C protein.
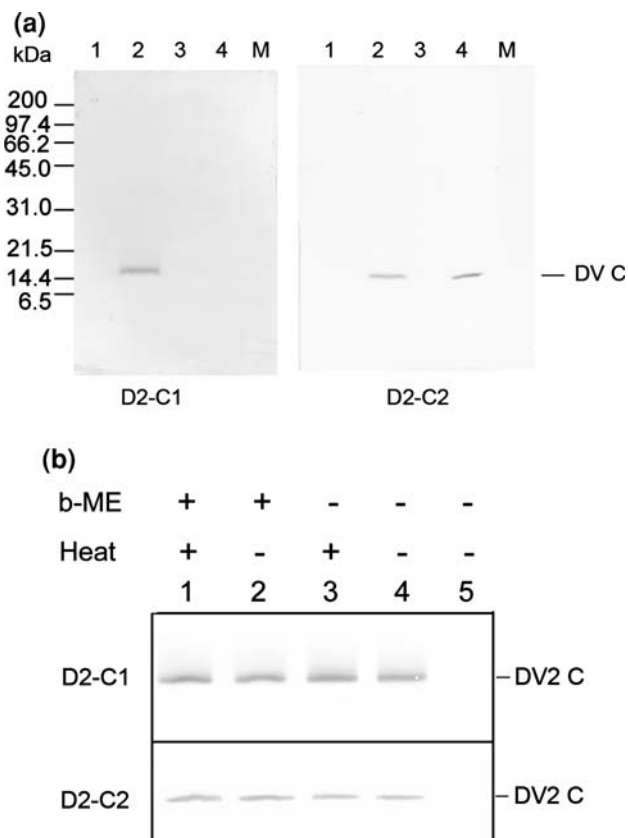
Fig. 4 Western blot analysis of anti-C Mabs. **a** Determination of serotype specificity. C6/36 cell lysates infected with DV1 to DV4 (*lanes 1–4*) and mock-infected cell lysates (*M*) were reacted with the indicated anti-C Mabs. **b** Type of DV2 C epitope recognized by anti-C Mabs. Mock (*lane 5*) or DV2-infected C6/36 cell lysates (*lane 1–4*) treated or non-treated with β-mercaptoethanol (b-ME) and/or heat at 95°C, as indicated above each lane, were reacted with the anti-C Mabs. Protein molecular sizes (in kDa) are shown on the *left*. DV2 C protein is indicated on the *right*

Additionally, we applied Mab D2-C1 to monitor the presence of DV2 C in the nucleus of DV2-infected BHK-21 and Vero cells at various time points postinfection (p.i.) by IFA. Mab D2-C1 reacted with DV2 C protein in the cytoplasm and nucleus in both cell types (Fig. 5b). DV2 C protein was clearly visible in the nucleolus from 8 h p.i. onward in BHK-21 cells, but this was delayed to 24–48 h p.i. in Vero cells. Similar results were obtained with Mab D2-C2 in infected cells of both types at 48 h p.i. (data not shown).

## Mapping of the DV2 C region recognized by anti-C Mabs

To identify the DV2 C protein region recognized by each of the anti-C Mabs, five 20-mer oligopeptides covering 100 amino acid residues of the DV2 C protein were used for epitope mapping by peptide-based indirect ELISA. We found that Mabs D2-C1 and D2-C2 were specifically reactive to peptide P1, the first peptide, covering the first 20 amino residues of DV2 C protein (Fig. 6a).

Characterization of mouse anti-C Pab

In parallel with the production of Mabs, Pabs to the DV2 C protein in mouse ascitic fluid were generated by immunization with the same antigen, insoluble C recombinant protein. The anti-C Pab reacted with the 15-kDa native DV2 C protein, similar to the generated Mab, in Western blot analysis (Fig. 7c). Epitope mapping by peptide-based indirect ELISA indicated that the Pab reacted strongly with the same peptide (P1) as the anti-C Mabs (Fig. 6b). Two possible factors may have contributed to this rather unlikely result. One is that other reactive epitopes on the DV2 C protein might have been disturbed or eliminated when presented as linear peptides used in the ELISA. Also, the region of amino acids 1–20 might be the predominant immunogenic site of the C protein.

To clarify this point, the MBP-C(Δ1-20) protein, which lacked the first 20 residues of the DV2 C protein was constructed, expressed and affinity-purified from *E. coli* harboring plasmid pMal-D2C(Δ1-20) (Fig. 7a-i). The MBP as well as two versions of MBP-fusion protein, MBP-C and MBP-C(Δ1-20), were used to examine the reactivity of all generated antibodies by Western blot analysis. As expected, the anti-MBP Mab reacted well with all three types of MBP proteins (Fig. 7a-iii), whereas the D2-C1 Mab reacted only with the MBP-C protein (Fig. 7a-ii). However, reactivity of anti-C Pab to both types of MBP fusion proteins as well as MBP alone was observed (Fig. 7a-iv). This result suggested the presence of anti-MBP antibody in the anti-C Pab mouse ascitic fluid, which might have been due to contamination with MBP in the recombinant DV2C

Fig. 5 Indirect immunofluorescence assay of anti-C Mabs. **a** Determination of serotype specificity. DV1 to DV4 or mock-infected C6/36 cells were stained intracellularly with the indicated anti-C Mabs (D2-C1 and D2-C2) or anti-E Mab (4G2) as a control antibody. **b** Nuclear localization of DV2 C protein. DV2-infected BHK21 (*upper panel*) or Vero cells (*lower panel*) at 8, 24, 32 and 48 h postinfection (as indicated above) were stained intracellularly with D2-C1 Mab, followed by Cy3-conjugated goat anti-mouse IgG antibody
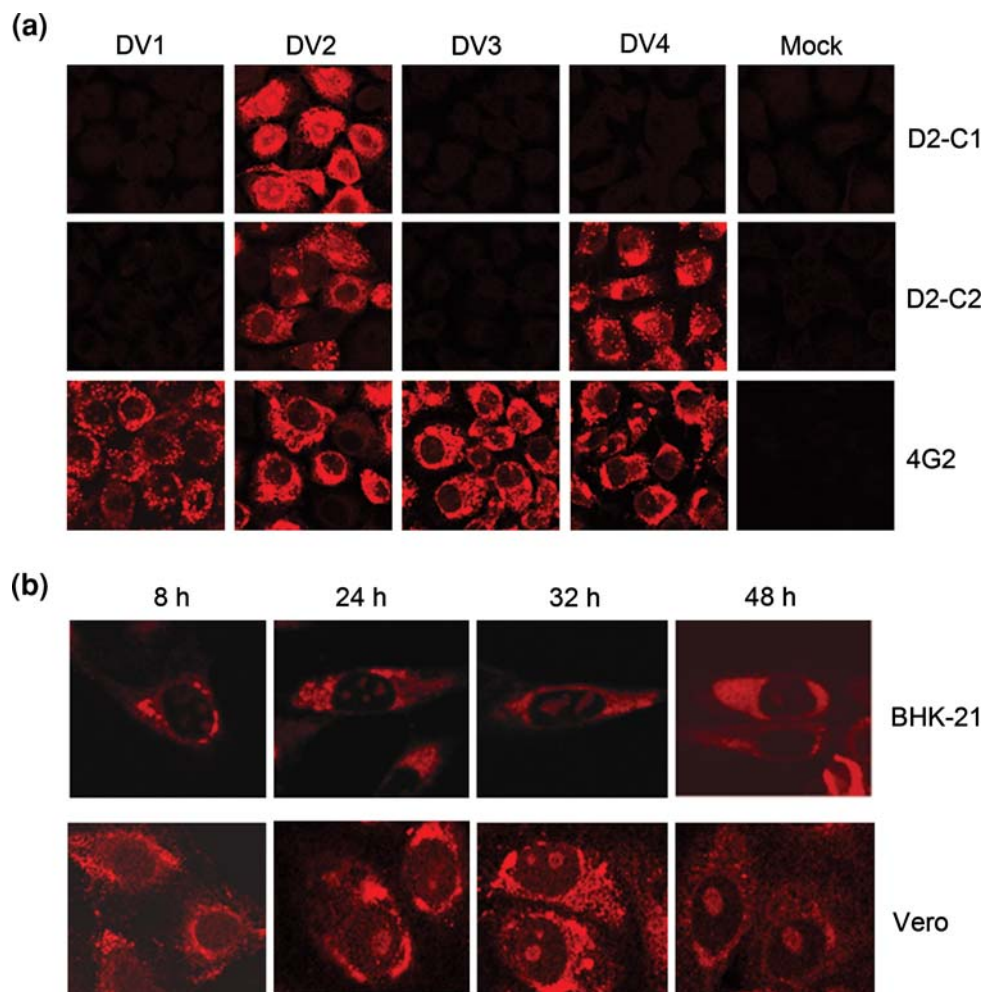
antigen preparation used for mouse immunization. To address this point, the mouse ascites were pre-absorbed with MBP to minimize the level of anti-MBP antibody. The pre-absorbed anti-C Pab was then reacted with MBP-C, MBP-C(Δ1-20) and MBP proteins (Fig. 7a-v). The results indicated that the pre-absorbed antibody did not react with MBP as expected (Fig. 7a-v, lane 3), whereas it could react much more strongly with MBP-C than with MBP-C(Δ1-20) (Fig. 7a-v, lane 1 and 2). In addition, we cleaved the same amount of MBP-C and MBP-C(Δ1-20) in parallel with factor Xa protease and separated the protein cleavage products, C and C(Δ1-20), by SDS-PAGE (Fig. 7b-i). Western blot analysis of those proteins demonstrated that mouse ascites anti-C Pab clearly reacted with the C protein, but had very weak reactivity to the C(Δ1-20) protein cleavage product (Fig. 7b-ii).

Additional experiments were performed to investigate the reactivity of anti-DV Pab, obtained from DV2 immunized mouse ascitic fluid, with the recombinant MBP-C, MBP-C(Δ1-20) and their cleavage products. The anti-DV Pab was first reacted with DV2-infected cell lysates by

Western blot analysis. The results showed that the antibody reacted with C as well as with other dengue proteins (i.e., E, prM, NS1, NS3), confirming the existence of antibody to DV C in the anti-DV Pab (Fig. 8a). The anti-DV Pab was then reacted with two recombinant MBP fusion proteins and their cleavage products. It is demonstrated that anti-DV2 Pab reacted with MBP fusion proteins and C cleavage products, but not with C(Δ1-20) (Fig. 8b), suggesting its specific reactivity with the first 20 amino acids region of C. Taken together, these results indicated that the N-terminal region consisting of 20 amino acids of DV2 C is predominantly immunogenic in mice.

Discussion

In comparison with prM and E structural proteins, few Mabs to DV C protein have been generated and reported to date. From previously published reports, only one or two hybridoma clones producing anti-C Mab have been obtained by immunization with whole virus particles
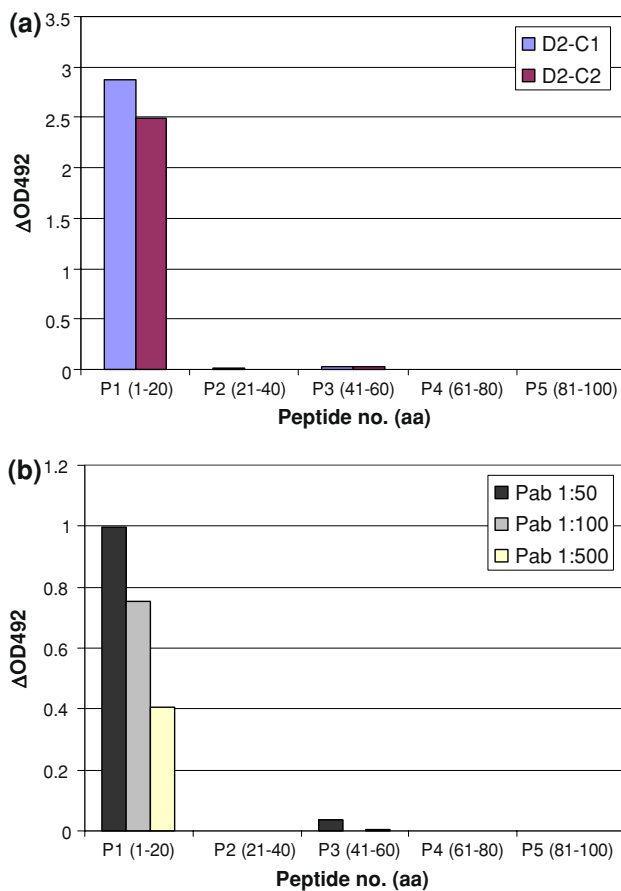
Fig. 6 Peptide mapping of generated anti-C antibodies by peptide-coated ELISA. Five 20-mer oligopeptides of D2V C protein (peptide no. P1–P5) were reacted with anti-C antibodies. The corresponding 20-amino-acid residue positions of the DV2 C protein are shown in parentheses. **a** Anti-C Mabs including D2-C1 and D2-C2. **b** Anti-C Pab at 1:50, 1:100 and 1:500 dilutions

[12, 16, 18, 19], whereas immunization with purified DV2 C protein resulted in 6 Mabs to DV C protein [2]. Immunization with purified recombinant DV4 C protein from a baculovirus expression system also induced a high titer of anti-C Pab in mice [10]. In contrast, whole-virus-particle immunization resulted in a high titer of mouse polyclonal antibodies to prM, E and NS1, but no or less reactivity to C protein (Fig. 8a). These observations may suggest a dependence of the immunogenicity of the DV C protein on its structure, and therefore, separating the C protein from viral particle is probably more effective for mouse immunization. In this study, we therefore used the purified recombinant DV2 C protein from *E. coli* as the source of an immunogen for the production of anti-DV C Mabs because of its simplicity and the fact that it is a well-established expression system. Expression of recombinant DV2 C as a soluble MBP-C fusion protein in this study demonstrates the advantage of MBP in making the protein more soluble [4, 14].

Our data suggest that the optimal cleavage of MBP-C by factor Xa protease occurs over a wide range of pH (from acidic to neutral). The inefficient cleavage of MBP-C observed under basic conditions may be involved in structural alterations in the MBP-C protein, which causes low accessibility of the protease to its recognition site, similar to a previous observation of inappropriate cleavage of an MBP fusion with poliovirus 2C protein (MBP-2C) [15].

Although the MBP-C was soluble, the C protein was precipitated after cleavage by factor Xa at acidic or neutral pH. This phenomenon was similar to that observed with a recombinant human papillomavirus 16 oncoprotein E6 that is expressed in *E. coli* as a soluble MBP-E6 protein [11]. The authors of the aforementioned study demonstrated, by several optical and immunochemical approaches, that the soluble MBP-E6 behaved as multimeric aggregates composed of folded, active MBP moieties fused to misfolded E6 polypeptide chains. Solubility of MBP-E6 could be achieved by burying the majority of the hydrophobic misfolded E6 moieties (hydrophobic chains) inside and exposing MBP moieties to the solvent (polar heads), analogous to lipid micellar structures. Therefore, after removal of MBP by factor Xa cleavage, E6 was precipitated [11]. Comparing the results to those of our study, the soluble MBP-C was found to be oligomeric based on size-exclusion chromatography (data not shown). The C proteins may possibly be expressed as misfolded intermediates, which bury the two hydrophobic parts (internal and C terminus) inside and expose the MBP carrier to the solvent, resulting in a soluble MBP-C protein. Without the aid of MBP as cleaved by factor Xa, the misfolded C protein thus would be precipitated. MBP-C cleavage at basic pH may somehow cause rearrangement of the C protein folding, thus allowing the solubility of the C protein cleavage product as observed in this study. However, a soluble C(Δ1-20) protein cleavage product (Fig. 7b) was obtained from the cleavage of MBP-C(Δ1-20) by factor Xa at pH 7.4. This may be related to the structural significance of the first 20 amino acid region of the C protein.

In this study, we demonstrated the capability of C protein in a precipitated form to induce antibodies to DV2 C protein in mouse sera, using indirect ELISA with lysates from infected cells and Western blot analysis. The specificity of the antibody for the DV2 C protein could be further confirmed by the disappearance of the C protein band on a Western blot when using a mouse serum that was pre-absorbed with MBP-C, but not MBP protein (Fig. 3c). These results suggest that although the protein was precipitated, its antigenic determinants still existed, supporting previous reports that immunization with purified C protein could significantly induce anti-C antibody production in mice [2, 10].
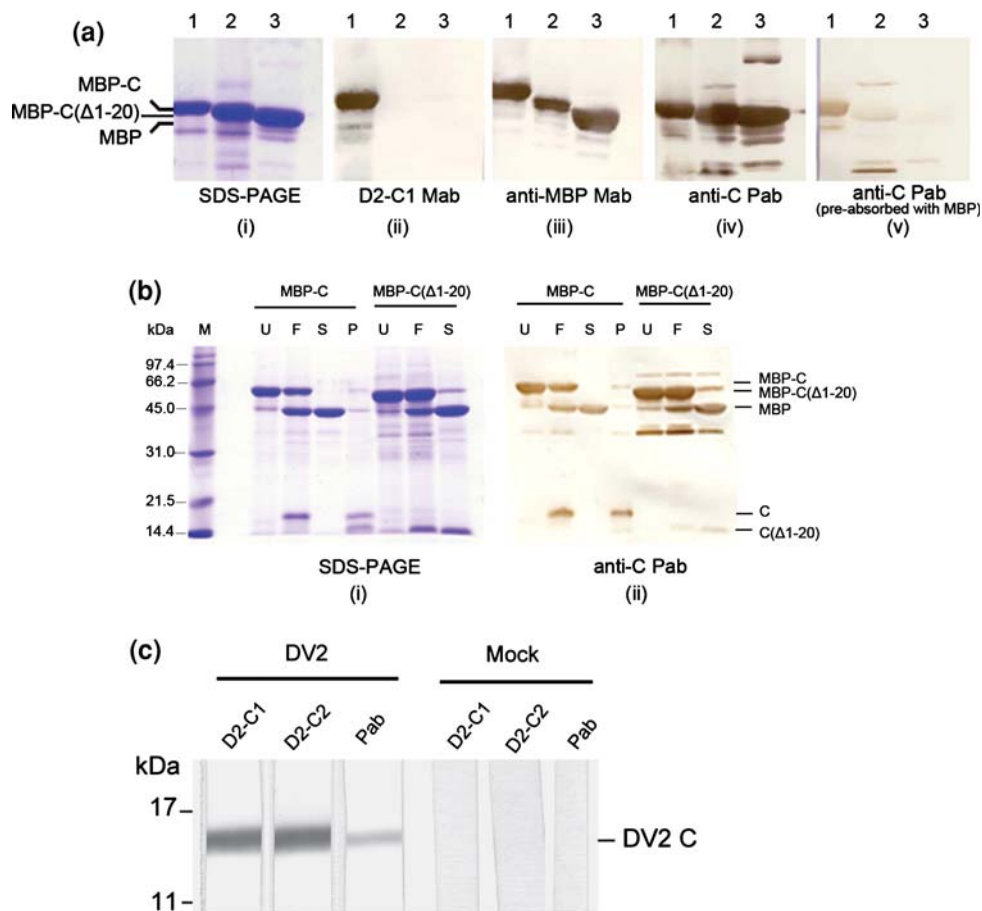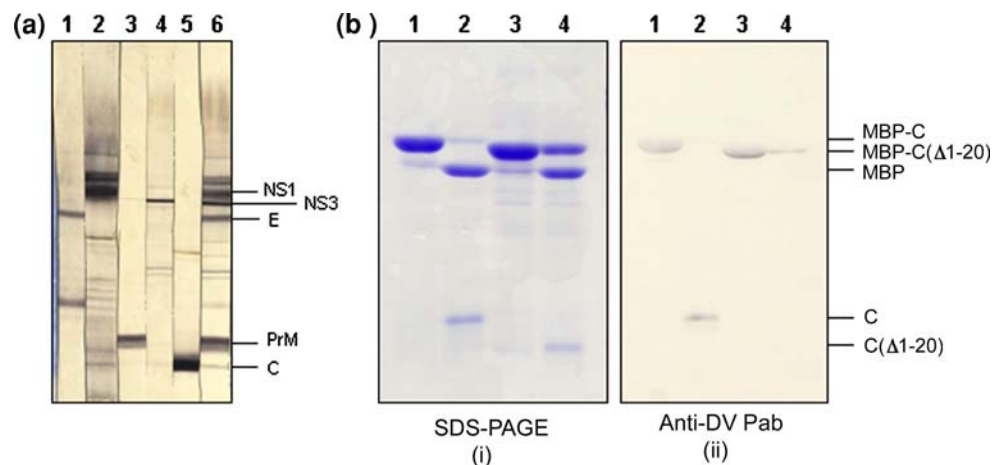
Fig. 7 Reactivity of anti-C Pab by Western blot analysis. **a** Purity of purified MBP-C (*lane 1*), MBP-C(Δ1-20) (*lane 2*) or MBP (*lane 3*) protein as determined by (*i*) SDS-PAGE and their reactivity against various antibodies; (*ii*) D2-C1 Mab, (*iii*) anti-MBP Mab, (*iv*) anti-C Pab or (*v*) anti-C Pab pre-absorbed with MBP by Western blot analysis. **b** The purified MBP-C or MBP-C(Δ1-20) was digested with factor Xa protease at pH 7.4 for 19 h and centrifuged to separate the supernatant (*lane S*) and pellet (*lane P*) fractions. The protein samples were analyzed by (*i*) SDS-PAGE followed by (*ii*) Western blot analysis with anti-C Pab (at dilution 1:1,000). *Lane U* proteins before addition of factor Xa protease; *lane F* proteins immediately after the addition of factor Xa protease (at 0 h); *lane M* broad-range standard protein markers. The protein molecular sizes (in kDa) are shown on the *left*. **c** Western blot analysis of anti-C antibodies to DV2 C. DV2-infected or mock-infected C6/36 cell lysates were reacted with anti-C Mabs D2-C1 and D2-C2 or anti-C Pab (at dilution 1:500)

In the attempt to generate anti-C Mabs, we were finally able to obtain two hybridoma lines, D2-C1 and D2-C2. Characterization of the generated D2-C1 and D2-C2 Mabs indicated the capability to detect DV C protein in the nucleus of infected cells, in common with other reported Mabs [2, 10, 17–19]. Concerning the serotype specificity, only the D2-C1 Mab is DV2-specific, whereas D2-C2 is cross-reactive with DV2 and DV4, which is similar to most of the previously reported anti-C Mabs [2, 18]. An anti-C Mab reported by Tadano et al. [18] is cross-reactive between DV4 and DV1, while six anti-C Mabs generated by Bulich and Aaskov [2] are cross-reactive with DV1, DV2 and DV4. From epitope mapping results, both of the generated Mabs recognize the first 20 amino acids of DV C protein, but according to their serotype-specific properties, they might recognize different epitopes. Amino acid alignment of DV C proteins of the four serotypes indicated

some different residues within amino acids 1–20 between DV2 and DV4, which may associated with the actual binding site for both antibodies. Interestingly, epitope mapping of two anti-C Mabs as well as anti-C Pab located within the first 20 amino residues of DV2 C was similar to results obtained with previously generated anti-C Mabs [2]. These concordant results, together with the evidence that the reactivity of anti-C antibodies to MBP-C(Δ1-20) was reduced, may suggest that this region is the predominant immunogenic determinant of the DV2 C protein in mice. The immunogenic determinant of DV2 C suggested in this study is unique and different from that found in a previous report in which the C-terminal alpha-4 helix exposed on the surface of the capsid dimer was identified from DV2 hyperimmune mouse sera and DV2-infected patient sera using pin-bound peptides [1]. Based on the structural determination of DV C protein by NMR, the first 20 amino

Fig. 8 Reactivity of anti-DV Pab by Western blot analysis. **a** DV2-infected C6/36 cell lysates were reacted with various Mabs to DV proteins; anti-E (3H5), anti-NS1 (1F11), anti-prM (1H10), anti-NS3 (1F), anti-C (D2-C1) (*lane 1–5*, respectively), or anti-DV Pab (at dilution 1:10,000). DV proteins are indicated on the *right*. **b** The purified MBP-C and MBP-C(Δ1-20) (*lane 1* and *3*, respectively) or factor Xa-digested MBP-C and MBP-C(Δ1-20) (*lane 2* and *4*, respectively) were analyzed by (*i*) SDS-PAGE followed by (*ii*) Western blot analysis with anti-DV Pab (at dilution 1:10,000). MBP-fusion proteins and C protein cleavage products are shown on the *right*

acid residues of C are unstructured or conformationally labile [3, 9]. We speculate that this region might be cryptic and inaccessible in the context of the viral nucleocapsid packed in the virion. Therefore, when mice were immunized with whole virions, a low titer of antibody to C protein was observed compared to the E, prM and NS1 proteins. Once the C protein was purified or produced as a recombinant protein, the first 20 residues could then be fully exposed and thus induce a high titer of antibody to C protein in mice after immunization. However, the actual relationship between the labile structure of the N terminus and the antigenicity of the DV2 C protein still requires further investigation.

In conclusion, two anti-C Mabs and Pab were generated by using a recombinant DV2 C protein produced in *E. coli* as an immunogen. The generated antibodies can be used to detect native DV2 C protein by various immunological assays. These anti-C Mabs could be further applied to explore in more detail the capsid protein function related to apoptosis in DV infection. Epitope mapping of these generated antibodies to the first 20 amino acids region on the N terminus of the DV2 C protein suggested that this region is predominantly immunogenic in mice.

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