INDUCTION OF NEUTRALIZING ANTIBODIES SPECIFIC TO DENGUE VIRUS SEROTYPES 2 AND 4 BY A BIVALENT ANTIGEN COMPOSED OF LINKED ENVELOPE DOMAINS III OF THESE TWO SEROTYPES

SAIMA KHANAM, BEHZAD ETEMAD, NAVIN KHANNA, AND SATHYAMANGALAM SWAMINATHAN*

Abstract. There is no vaccine to prevent dengue fever, a mosquito-borne viral disease, caused by four serotypes of dengue viruses. In this study, which has been prompted by the emergence of dengue virus envelope domain III as a promising sub-unit vaccine candidate, we have examined the possibility of developing a chimeric bivalent antigen with the potential to elicit neutralizing antibodies against two serotypes simultaneously. We created a chimeric dengue antigen by splicing envelope domain IIIIs of serotypes 2 and 4. It was expressed in Escherichia coli and purified to near homogeneity. This protein retains the antigenic identities of both its precursors. It elicited antibodies that could efficiently block host cell binding of both serotypes 2 and 4 of dengue virus and neutralize their infectivity (neutralizing antibody titers approximately 1:40 and ~1:80 for dengue virus serotypes 2 and 4, respectively). This work could be a forerunner to the development of a single envelope domain III-based tetravalent antigen.

INTRODUCTION

Dengue fever, a mosquito-borne viral disease, has become a major worldwide public health problem, with a dramatic expansion in recent decades. Globally, approximately a hundred million cases of dengue infections are believed to occur each year, with approximately half a million of these resulting in potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Currently, the number of people estimated to be at risk of dengue infections is approximately 2.5 billion. This is predicted to register almost a 100% increase in the coming 2–3 decades. There are four antigenically closely related, but distinct, serotypes of dengue (DEN) viruses designated DEN-1, -2, -3, and -4, which are members of the family Flaviviridae. Laboratory and epidemiologic data suggest that prior infection with one serotype, although conferring lifelong homologous immunity to that serotype, can sensitize an individual to DHF/DSS during a secondary infection with any of the remaining three serotypes through an antibody-dependent enhancement (ADE) mechanism. This, in conjunction with other host- as well virus-related factors, appears to contribute to disease pathogenesis.

A dengue vaccine has remained elusive despite decades of effort. An ideal dengue vaccine must be tetravalent, capable of affording protection against infection by all four serotypes. This consideration, together with the lack of a suitable animal model of the disease, has made the development of dengue vaccines a challenging task. Although early efforts to develop dengue vaccines have focused on conventional approaches, recent initiatives focus predominantly on recombinant strategies. Several recombinant strategies, based on infectious clone technology, DEN antigen-encoding viral and plasmid vectors, and recombinant DEN antigens, produced using Escherichia coli, yeast, and insect cell-based heterologous expression systems, are being explored.

The major envelope (E) protein of DEN viruses is a complex molecule organized into three distinct domains, a central domain (I), a dimerization domain (II), and an immunoglobulin-like domain (III). Several lines of evidence accumulated in recent years have identified domain III of the E protein (referred to here as EDIII) as the critical region from the perspective of vaccine development. EDIII-spanning amino acid residues 300–400 of the E protein is a highly stable, independently folding domain that lies exposed and accessible on the virion surface. Multiple type- and subtype-specific neutralizing epitopes of the E protein have been mapped to EDIII. A variety of studies have implicated this domain in host receptor-binding. Immunization of animals with either EDIII-encoding plasmids or chimeric proteins containing EDIII fused to maltose-binding protein (MBP) has been shown to elicit neutralizing antibodies to DEN viruses.

We recently demonstrated that the EDIII of DEN-2 could be successfully expressed in high yields in E. coli without the aid of an MBP carrier. Furthermore, we also showed that the in vitro-refolded protein was biologically functional on the basis of its ability to block the binding of DEN-2 virus to baby hamster kidney (BHK) cells. The EDIII-MBP protein studies referred to are based on generating a tetravalent dengue vaccine by physically mixing four monovalent (i.e., single serotype specific) EDIII-MBPs. The current work is based on the premise that the development of a single tetravalent
EDIII-based chimeric protein, rather than four monovalent EDIIIs, may offer a simpler and inexpensive alternative. As a first step in examining the feasibility of developing such a chimeric protein, we have spliced EDIIIs of two DEN serotypes (DEN-2 and DEN-4) by means of a flexible peptide linker, to create a novel bivalent antigen, rEDIII-4/2. We used this bivalent protein to address the following questions. 1) Would it retain the antigenic identity of its monovalent precursors? 2) Would it elicit antibodies specific to each of its constituent serotypes? 3) Would these antibodies be effective in recognizing and neutralizing the infectivity of DEN-2 and DEN-4 viruses? In this report, we describe the creation of the bivalent antigen, its expression in E. coli, the purification of the expressed bivalent protein, and preliminary proof-of-concept data in support of its potential to function as a bivalent dengue antigen.

MATERIALS AND METHODS

Materials. Escherichia coli host strain DH5α for routine recombinant plasmid manipulations was obtained from Invitrogen (Carlsbad, CA). The E. coli host strain SGI3009 for recombinant protein expression was obtained from Qiagen (Hilden, Germany). The expression plasmid pQE60 (amp+), Ni-NTA superflow resin, HisSorb enzyme-linked immunosorbent assay (ELISA) microtiter well strips, and anti-His monoclonal antibody (Mab) (catalog no. 34660) were also obtained from Qiagen. Plasmid pGEMT-Easy for TA cloning was obtained from Promega (Madison, WI). The Mab specific for DEN-2 virus (3H5) was obtained from the American Type Culture Collection (Manassas, VA). The DEN-4 virus-specific Mab (catalog no. MAB8704) was obtained from Chemicon International, Inc., Temecula, CA). The secondary antibody-enzyme conjugates (anti-mouse IgG-alkaline phosphatase [AP], anti-mouse IgG-horseradish peroxidase [HRPO]), and anti-mouse IgG-fluorescein isothiocyanate [FITC] conjugate) and the AP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were obtained from Calbiochem (San Diego, CA). The HRPO substrate 3, 3', 5', 5'-tetramethylbenzidine (TMB) was obtained from Kirkegaard and Perry Laboratories, Gaithersburg, MD).

The DEN-2 (NGC strain) and DEN-4 (H241 Dominican strain) viruses were kindly provided by Dr. A. Falconar (University of Oxford, Oxford, United Kingdom). BHK 21 cells, monkey cell line LLCMK2, mosquito cell line, C6/36, and the 3H5 Mab-producing hybridoma cell line HB46 were obtained from the American Type Culture Collection. All of these cell lines, with the exception of C6/36, were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in a humidified 10% CO2 incubator at 37°C. The C6/36 cells were maintained in Leibovitz L-15 medium supplemented with tryptose phosphate broth (2% v/v) and 10% (v/v) ΔFCS in a CO2-free incubator at 28°C. The QIAmp viral RNA mini kit was obtained from Qiagen and the reverse transcription–polymerase chain reaction (RT-PCR) Titan One Tube RT-PCR system was from obtained from Roche Diagnostics (Mannheim, Germany).

Isolation of EDIII-encoding cDNAs. Tissue culture supernatants obtained from C6/36 cells that were infected separately with either DEN-2 or DEN-4 virus (at low multiplicity) served as the starting material for the isolation of viral genome RNAs, using the commercially available QIAmp RNA mini kit. The viral RNAs were incorporated as templates in the RT-PCR to obtain the recombinant EDIII-encoding cDNAs designated as EDIII-2 and EDIII-4 genes, which corresponded to DEN serotypes 2 and 4, respectively. The EDIII-specific primers used in the RT-PCRs are shown in Table 1. Both of these genes are approximately 0.38 kilobases, and each is predicted to encode 121 amino acid residues corresponding to amino acids 296–416 (encompassing EDIII and spanning amino acids 300–400) of the E molecule. Since each of these two genes encode recombinant EDIII of a single DEN serotype (either DEN-2 or DEN-4), they are referred to as monovalent. These genes were engineered to have a Bgl II site at the 5’ end and a Bam HI site at the 3’ end to facilitate head-to-tail fusion with each other, as well as cloning into the E. coli expression plasmid pQE60. The RT-PCR-generated monovalent genes were purified, A-tailed using Taq polymerase, and ligated into the TA cloning site of the plasmid pGEMT-Easy to create the monovalent constructs pT-2 (harboring the monovalent gene EDIII-2) and pT-4 (harboring the monovalent gene EDIII-4). The ligated products were transformed into E. coli DH5α cells. The resultant transformants were selected on ampicillin plates and subjected to preliminary PCR screening using insert-specific primers. The PCR-positive clones were subjected to restriction analysis. Selected clones were further screened by in vitro coupled transcription and translation (TnT) using T7 RNA polymerase as previously described46 to identify those with intact open reading frames. One clone each of pT-2 and pT-4 was sequenced. No errors were seen in the EDIII-4 cDNA. However, the EDIII-2 cDNA had two point mutations, resulting in Ile to Phe (amino acid 402) and Thr to Ala (amino acid 404) substitutions. Since both of these were conservative changes, and outside domain III, they were ignored.

Creation of the bivalent gene EDIII-4/2. The monovalent genes EDIII-2 and EDIII-4 (carried by plasmids pT-2 and pT-4, respectively), were verified to be expressed in E. coli and then used as precursors to create the chimeric bivalent gene EDIII-4/2. To create this chimeric gene, which was composed of EDIIIs of two DEN serotypes (DEN-2 and DEN-4 in this case), the EDIII-2 gene was isolated from pT-2 by Bgl II/Bam HI double digestion and ligated into the Bam HI site of pT-4 to generate the construct pT-4/2. Once again, DH5α transformants were screened by PCR, restriction, TnT, and sequence analyses to choose the right bivalent clone. In the fusion gene EDIII-4/2 present in the pT-4/2 construct, the two monovalent genes are joined in-frame through ligation of

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>D4-F</td>
<td>ggagagctcGGAAAGTCATACAGTAGTGTCAGGA AAG</td>
</tr>
<tr>
<td>D4-R</td>
<td>gcggcttcACCTAGAATGGCCATCTGTTTATGGCAGGA</td>
</tr>
<tr>
<td>D2-F</td>
<td>ggagagctcGGAAAGTCATACAGTAGTGTCAGGA AAG</td>
</tr>
<tr>
<td>D2-R</td>
<td>gcggcttcACCTAAAATGGGACATTCCGTCATTCGCTC</td>
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* D2 and D4 represent primers specific to EDIIIs of dengue 2 (DEN-2) and DEN-4 viruses, respectively. F and R indicate forward and reverse, respectively.
† Sequences homologous to the template are shown in uppercase; in primers 1–4, the 5' lowercase sequences are non-homologous sequences designed to incorporate restriction sites (shown in italics).
Bam HI site (at the 3’ end of EDIII-4 gene) to Bgl II site (at the 5’ end of the EDIII-2 gene) with concomitant loss of both sites. As a result, the bivalent gene is also flanked by Bgl II site on the 5’ side and Bam HI site at the 3’ end, similar to the monovalent genes.

Construction of bivalent E. coli expression plasmid vector. The bivalent EDIII-4/2 vector was cloned into the bacterial expression vector pQE-60 under the control of the isopropyl-

thiogalactoside (IPTG)–inducible PTS/lac O promoter. To this end, the recombinant bivalent gene was isolated from plasmid pT-4/2 as a Bam HI/Bam HI fragment and ligated to pQE60 that had been digested with these same enzymes. In the resulting construct, pQ-EDIII-4/2, the inserted gene is fused in-frame with the ATG codon (at the 5’ end) and 6× His tag-encoding sequence (at the 3’ end) provided by the pQE-60 vector. This in-frame fusion would add 11 vector-derived amino acid residues to the bivalent protein (five and six residues at the N- and C-terminal ends, respectively). Insertion of the bivalent gene in the sense orientation was designed to abolish both Bam HI and Bgl II sites in the resultant construct. Recombinant clones were selected on ampicillin plates and subjected to direct colony PCR screening using insert-specific primers, followed by restriction analysis to identify recombinants harboring the inserted gene in the sense orientation.

Expression screening of clones harboring the bivalent fusion gene. Minipreps of plasmid pQ-EDIII-4/2 isolated from the DH5α recombinant were used to transform the E. coli expression host strain SG13009 containing the pREP4 plasmid. The pREP4 plasmid encodes the lacI repressor (required for regulated recombinant gene expression) and the kanamycin resistance marker. Double recombinants harboring both the pQ-EDIII-4/2 and pREP4 plasmids were selected in the presence of ampicillin and kanamycin. Several of these clones were inoculated into 3-mL test tube cultures and allowed to grow at 37°C in a shaker at 200 revolutions per minute (rpm). When the cultures were in logarithmic growth phase (corresponding to an optical density [OD] of approximately 0.5–0.6 at 600 nm), they were induced with 0.5 mM IPTG for approximately four hours. After induction, equivalent number of cells from the different cultures (normalized on the basis of OD₅₆₀ values) were lysed in sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Uninduced controls were analyzed in parallel. One clone that expressed maximal levels of the recombinant protein was chosen for further study.

Localization of recombinant rEDIII-4/2 bivalent protein in induced cells. To determine the solubility of the recombinant bivalent protein, test tube cultures were set up and induced with 0.5 mM IPTG at log phase for four hours, and processed essentially as previously described. Aliquots of the induced culture (corresponding to approximately 1.0 OD₅₆₀) were lysed separately using either native lysis buffer (50 mM potassium phosphate, pH 8, 0.3 M NaCl, lysozyme [1 mg/mL]) or urea lysis buffer (50 mM potassium phosphate, pH 8, 0.3 M NaCl, 8 M urea) and separated into supernatant (S) and pellet (P) fractions by centrifugation at 15,000 × g for 10 minutes. Equivalent aliquots of all four resultant fractions were analyzed by SDS-PAGE.

Purification of the recombinant monovalent and bivalent proteins. A pre-culture was set up by inoculating 20 mL of Luria Bertani (LB) medium containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) with 25 μL of glycerol stock (SG13009 cells transformed with the bivalent expression vector pQ-EDIII-4/2). The culture was grown overnight in a shaker at 37°C at 200 rpm and inoculated into 0.5 liters of LB medium containing both ampicillin (100 μg/mL) and kanamycin (25 μg/mL) in a four-liter Haffkine flask, which was placed in the shaker and incubated at 37°C for approximately 2–3 hours at 125 rpm. When the OD₅₆₀ of the culture reached approximately 0.5–0.6 (a small aliquot of the uninduced culture was set aside for subsequent analysis by SDS-PAGE), it was induced with IPTG to a final concentration of 0.5 mM. Induction was allowed to proceed for four hours before harvesting the cells. Aliquots of the induced and uninduced cell cultures were analyzed by SDS-PAGE prior to initiating purification.

Purification of the induced protein under denaturing conditions using Ni-NTA affinity chromatography was essentially as previously described with minor modifications. The induced culture was centrifuged in a Sorvall (Asheville, NC) GS3 rotor at 6,000 rpm for 15 minutes at 4°C. The cell pellet (approximately 1.5 grams wet weight) was lysed by resuspending in 50 mL of ice-cold lysis buffer (8 M urea dissolved in 100 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 10 mM imidazole, 1 mM freshly added phenylmethylsulfonyl fluoride) and sonication (model 550 sonicator; Fisher Scientific, Pittsburgh, PA) for 10 minutes. We used urea rather than guanidine hydrochloride for lysis under denaturing conditions, to permit direct SDS-PAGE analysis of the samples. The lysate was stirred for two hours at 4°C and clarified by centrifugation using a Sorvall SS34 rotor (17,000 rpm for one hour at 4°C). The resultant supernatant was mixed with 4 mL of Ni-NTA Superflow resin that had been pre-equilibrated with the lysis buffer. This suspension was gently rocked overnight at 4°C and then packed into a column. After collecting the flow through, the column was washed extensively with buffers 1 (8 M urea, 0.5 M NaCl, 50 mM sodium phosphate, pH 6.3) and 2 (8 M urea, 50 mM sodium phosphate, pH 5.9) and eluted with buffer 3 (8 M urea, 50 mM sodium phosphate, pH 4.5). Fractions of 3 mL were collected for analysis. All fractions obtained during the purification were analyzed by SDS-PAGE. Relevant fractions were pooled and dialyzed against 1× phosphate-buffered saline (PBS) containing 50 mM each of arginine and glutamic acid. The dialyzed protein was mixed with gentamicin to a final concentration of 50 μg/mL, flash-frozen in liquid nitrogen, and stored at −80°C until use. Additionally, to help characterize the rEDIII-4/2 protein and the antibodies it can induce in terms of their serotype specificity, we also expressed and purified all four monovalent rEDIII proteins (rEDIII-1, rEDIII-2, rEDIII-3, and rEDIII-4), essentially as described for the bivalent protein.

Western blot analysis. Western blot analysis of the Ni-NTA affinity-purified bivalent recombinant protein was performed as previously described. Briefly, the recombinant protein was subjected to electrophoresis under denaturing conditions (SDS-PAGE) along with relevant controls and pre-stained protein markers. Separated proteins were electro transferred (at 100V for 1 hour at 4°C) onto a nitrocellulose membrane using the Mini Trans-blot apparatus (Bio-Rad Laboratories, Hercules, CA). The pre-stained markers allowed visualization of successful protein transfer. After overnight incubation in blocking buffer (1% polyvinylpyrrolidone [PVP], 2% bovine
serum albumin, 1× PBS, pH 7.2), 0.1% Tween 20), the membrane was washed three times with 1× PBS, 0.1% Tween 20 (1× PBS-T) and incubated separately with either one of two primary antibodies for one hour at room temperature. The primary antibodies used were either the penta-His MAb (at a dilution of 1:7,500) or murine anti-rEDIII-4/2 polyclonal serum (at a dilution of 1:2,500). The blots were washed again with 1× PBS-T (three washes, 10 minutes/wash), and incubated with anti-mouse IgG-AP secondary antibody conjugate (at a dilution of 1:10,000) for 45 minutes at room temperature. The blots were washed again as above and protein bands were visualized after a 15-minute incubation at room temperature in BCIP/NBT substrate solution.

**ELISA for the bivalent r-EDIII-4/2 protein and EDIII-specific antibodies.** Recombinant protein in samples before and after affinity purification on Ni-NTA matrix was analyzed using a sandwich ELISA approach designed to detect only the recombinant protein. In this assay, appropriate dilutions of the 6× His-tagged recombinant protein—containing samples were coated onto commercially available Ni-NTA HisSorb microtiter wells (Qiagen). After a two-hour incubation at room temperature to allow binding, the wells were washed four times with 1× PBS-T and blocked for an additional two hours using a 5% solution of skimmed milk powder prepared in 1× PBS-T (blocking buffer). The wells were washed as before and incubated with 200 μL of 3H5 MAb (1 mg/mL diluted 1:500 in blocking buffer), for one hour at room temperature. After washing as before, the wells were incubated with anti-mouse IgG-HRPO conjugate (diluted 1:10,000 in blocking buffer). After incubation for 45 minutes at room temperature, the wells were washed as before and incubated with TMB substrate for 15 minutes at 37°C. The blots were washed and mounted in fluoroguard antifade reagent (Bio-Rad Laboratories) to limit subsequent photo bleaching. Fluorescent cells were visualized after a 15-minute incubation at room temperature, followed by ice-cold methanol (pre-chilled to −20°C) for 20 minutes. Fixed cells were rinsed with 1× PBS and blocked with 1% PVP in 1× PBS-T for two hours at 37°C. The cells were then washed three times with 1× PBS and incubated with 200 μL of primary antibody for one hour at room temperature. Two different primary antibodies were used. One was the DEN-2 virus-specific 3H5 MAb (1:300 dilution) and the other was the DEN-4 virus-specific MAb (catalog no. MAB8704 at a 1:400 dilution; Chemicon International, Inc.). Cells were then washed as before and incubated for 45 minutes with fluorescein-conjugated anti-mouse IgG (at a 1:50 dilution) at room temperature. Cells were washed and mounted in fluoroguard antifade reagent (Bio-Rad Laboratories) to limit subsequent photo bleaching. Fluorescent cells were visualized under a Nikon (Tokyo, Japan) microscope equipped for incident illumination with a narrow band filter combination selective for FITC.

**Plaque reduction neutralization test (PRNT).** LLCMK2 cells were seeded at a concentration of 0.5 × 10^6/well in 24-well plates approximately 24 hours prior to infection. Approximately 20–25 PFU each of DEN-1, DEN-2, and DEN-3, and DEN-4 viruses (50 μL) were separately pre-incubated with an equal volume of serial two-fold dilutions (up to a dilution of 1:160) of heat-inactivated pooled serum collected from rEDIII-4/2-immunized mice. After overnight pre-incubation at 4°C, the virus-serum mixture was diluted to a final volume of 200 μL with DME plus 2% FCS and used to infect a single well of 24-well plate. Each serum dilution was assayed in four replicate wells and the virus/serum pre-incubation mixture was prepared as a master mixture sufficient for four wells. After adsorption for two hours, the inoculum was aspirated off and the cells were overlaid with 1.25% methylcellulose in DME plus 6% ΔFCS (1 mL/well). Appropriate controls were set up in parallel with negative control (mock-infected) wells receiving 200 μL of DME plus 2% ΔFCS and positive control wells receiving DEN-1, DEN-2, DEN-3, or DEN-4 viruses separately, pre-incubated with DME plus 2% ΔFCS, instead of murine serum. The plates were incubated at 37°C in a humidified 5% CO_2 incubator. On day 6 post-infection, the overlay was gently decanted and the cells were fixed with 1 mL of 4% formaldehyde solution.
at room temperature for one hour. Wells were washed with tap water and then stained with a 1:40 diluted stock of 2% crystal violet solution in 20% ethanol for 30 minutes. Plaques revealed after staining were counted and the antiserum dilution resulting in a 50% reduction in plaque count (with reference to the number of plaques generated by the virus in the absence of antiserum), was expressed as the PRNT\textsubscript{50} titer.

RESULTS

Creation of EDIII-based monovalent precursors genes. Monovalent genes \textit{EDIII-2} and \textit{EDIII-4} encoding DEN-2 and DEN-4 virus-specific EDIIIs, respectively, were obtained as shown in Figure 1. Before proceeding to generate the bivalent gene, we sought to investigate if these two monovalent genes can be expressed successfully in \textit{E. coli} to produce the corresponding monovalent antigen proteins, which are predicted to be approximately 14 kD each. Accordingly, these genes were isolated as \textit{Bgl II/Bam} \textit{HI} fragments from the pT-2 and pT-4 plasmids and cloned into the \textit{E. coli} expression plasmid pQE60 under the control of an inducible \textit{PT5/lac} \textit{O} promoter. The IPTG-mediated induction of the resultant vectors is shown in Figure 2A. A comparison of the polypeptide profiles before and after induction showed that both the \textit{EDIII-2} (lanes 2 and 3) and \textit{EDIII-4} (lanes 4 and 5) genes express a new protein of approximately 14 kD, which is consistent with the predicted size of the monovalent \textit{rEDIII-2} and \textit{rEDIII-4} proteins. The \textit{rEDIII-2} protein was expressed at relatively higher levels than the \textit{rEDIII-4} protein. This was consistently observed in multiple experiments; however, the reason is not clear. We observed two conservative amino acid changes outside domain III in the gene encoding \textit{rEDIII-2}, as mentioned in the Materials and Methods. Since both of these proteins are engineered to carry a carboxy-terminal 6× His tag, we next performed a Western blot analysis using the commercially available penta-His MAb, as shown in Figure 2B. Both monovalent proteins were specifically recognized by the penta-His MAb, leading to the conclusion that both the recombinant monovalent genes can be successfully expressed in \textit{E. coli}.

Design and expression of a bivalent domain III-based chimeric protein. We created the chimeric gene \textit{EDIII-4/2} by in-frame fusion of the two monovalent genes (Figure 1). Since the chimeric gene incorporates sequences corresponding to two monovalent genes, we refer to it as the bivalent gene. Correspondingly, the recombinant protein it encodes, the \textit{rEDIII-4/2} protein, is referred to as the bivalent protein. Its amino-terminal half corresponds to the \textit{rEDIII-4} protein and the carboxy-terminal half corresponds to the \textit{rEDIII-2} protein. A tetrapeptide linker, Gly-Gly-Ser-Gly, joins the two halves. The chimeric \textit{EDIII-4/2} gene was inserted in-frame with the initiator codon and a carboxy-terminal 6× histidine tag-encoding sequence of the IPTG-inducible expression vector pQE60 described above. The resultant expression plasmid pQ-EDIII-4/2 is shown in Figure 3A. The induction profile of a typical \textit{E. coli} clone harboring this construct is shown in Figure 3B. Total lysates (prepared by directly boiling cell pellets in SDS-PAGE sample buffer) were examined in this

F\textsc{igure} 1. Strategy to create dengue (DEN) EDIII-based bivalent antigen.\textit{ cDNAs corresponding to the EDIII-encoding regions of the DEN-2 and DEN-4 genomes (indicated by the solid black and gray lines, respectively) were obtained by reverse transcription-polymerase chain reaction (steps A and C), using specific primers (see Table 1). The resultant cDNA products (indicated by the boxes labeled 2 and 4) were designed to have \textit{Bgl II} and \textit{Bam} \textit{HI} at the 5′ and 3′ ends, respectively. The DEN-2 and DEN-4 EDIII cDNAs (2 and 4), carrying \textit{Taq} polymerase-added 3′ adenylic acid residues (indicated by the As), were inserted into the TA cloning vector pGEMT-Easy (steps B and D), to generate the constructs pT-2 and pT-4, respectively. The DEN-2 EDIII cDNA was retrieved from pT-2 as a \textit{Bgl II/Bam} \textit{HI} fragment and inserted into the \textit{Bam} \textit{HI} site of pT-4 (step E). In the resultant construct, pT4-2, the DEN-2 EDIII cDNA is fused in-frame to the 3′ end of the DEN-4 EDIII cDNA. B/B denotes the \textit{Bgl II/Bam} \textit{HI} fusion site GGATCT, encoding Gly-Ser.
experiment. It is evident from a comparison of the polypeptide profiles obtained in the absence (lane 2) and presence (lane 3) of IPTG that induction of EDIII-4/2 gene expression results in the appearance of a new approximately 28-kDa band, which is consistent with the predicted size of the rEDIII-4/2 protein. To examine the relative distribution of the expressed recombinant protein in the soluble and insoluble fractions, induced cell lysates were prepared under native (lysozyme-mediated) and denaturing (urea-mediated) conditions, separated into the soluble (supernatant) and insoluble (pellet) fractions, and analyzed by SDS-PAGE. When the cells were lysed under native conditions, virtually all the expressed recombinant protein was associated with the pellet fraction (lane 5). The native lysate supernatant, which represents the soluble fraction, had no discernible recombinant protein (lane 4). The major band in this lane was the lysozyme used for the lysis. Analysis of the urea lysate confirmed the presence of the major proportion of the approximately 28-kD protein band in the urea supernatant (lane 6), with small amounts in the urea-insoluble pellet (lane 7). These data demonstrated that the rEDIII-4/2 protein expressed in E. coli is predominantly insoluble.

**Purification of the bivalent rEDIII-4/2 protein.** Since the rEDIII-4/2 protein was insoluble, we performed Ni-NTA affinity chromatography under denaturing conditions to purify the protein. Fractions collected during different steps of the purification were analyzed by SDS-PAGE as shown in Figure 4A. Approximately 80–90% of the induced protein bound to the column, as evident from a comparison of the polypeptide profile of the initial lysate loaded on the affinity matrix (lane 2) with that of the flow-through material (lane 3). Based on a comparison of the host band intensities in these two lanes, it

![Figure 2](image2.png)

**Figure 2.** Expression of the monovalent proteins rEDIII-2 and rEDIII-4 in Escherichia coli. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) polypeptide profiles of total lysates of E. coli harboring expression plasmids containing the dengue (DEN)-2 (D2, lanes 2 and 3) and DEN-4 (D4, lanes 4 and 5) EDIII cDNAs prepared before (lanes 2 and 4) and after (lanes 3 and 5) induction with isopropylthiogalactoside. U = un-induced; I = induced. Molecular weight marker proteins (M) were run in lane 1. Their sizes (in kD) are shown on the left. B, Immunoblot analysis of the rEDIII-2 and rEDIII-4 proteins. Aliquots of the induced lysates shown in A were subjected to SDS-PAGE, electro-transferred onto a nitrocellulose membrane, and subjected to Western analysis using monoclonal antibody to His. Lysates containing rEDIII-2 (D2) and rEDIII-4 (D4) proteins were analyzed in lanes 2 and 3, respectively. Pre-stained protein molecular weight markers (P) were run in lane 1. Their sizes (in kD) are indicated on the left of the blot. The arrows to the right indicate the positions of the recombinant rEDIII-2 and rEDIII-4 proteins.

![Figure 3](image3.png)

**Figure 3.** Expression of the bivalent rEDIII-4/2 protein in Escherichia coli. A, Map of the expression plasmid, pQ-EDIII-4/2. The chimeric EDIII-4/2 gene was inserted into the Bgl II and Bam HI sites of plasmid pQE60 in-frame with the vector-provided initiator codon and 6× His tag-encoding sequence. pt5/lacO = phage T5 lac operator hybrid promoter; t₁ and t₂ = lambda transcriptional terminators; Ori and Amp' = plasmid replication origin and ampicillin resistance marker, respectively. B, Coomassie-stained denaturing gel showing the induction and localization of the induced protein in E. coli harboring the plasmid shown in A. Polypeptide profiles of total E. coli lysates before (U = uninduced) and after (I = induced) induction with isopropylthiogalactoside are shown in lanes 2 and 3, respectively. An aliquot of induced culture was lysed under native conditions (N) and the resultant soluble (S, lane 4) and pellet (P, lane 5) fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie staining. In parallel, another aliquot was lysed under denaturing conditions (D) and the resulting soluble (S, lane 6) and pellet (P, lane 7) fractions were analyzed similarly. Protein molecular weight markers (M) were run in lane 1; their sizes (in kD) are shown on the left. The arrow at the right indicates the position of the rEDIII-4/2 protein. The arrowhead denotes the position of lysozyme used in the native lysis buffer.
also appears that there was no significant non-specific binding to the affinity matrix. This is consistent with virtually no protein appearing in the wash fractions (lanes 4 and 5). Elution with a pH 4.5 buffer resulted in the emergence of highly purified recombinant protein from the column (lanes 6–9). From a comparison of the protein profiles of the eluted material (for example, lane 6) and the crude lysate (lane 2), it is clearly evident that > 95% purity has been obtained.

We tested the purified chimeric protein in a Western blot assay using different antibodies to confirm its identity. To this end, we prepared a blot containing the affinity-purified bivalent protein and its monovalent precursors. We also included in this blot a previously described DEN-2 EDIII-based recombinant protein variant as a positive control. This variant also includes upstream amino acid sequences in addition to a 6× His tag. Consequently, it is larger (approximately 19 kD) and is referred to as \( \text{rEDIII-2}^{*} \) in this report to distinguish it from the approximately 14-kD rEDIII-2 generated in the present study. This blot was probed with a commercially available murine penta His MAb that was specific for the engineered 6× His Tag on the recombinant protein. The results showed that the penta His MAb, which can specifically recognize the r-EDIII-2* control protein, also recognized the approximately 28-kD bivalent protein, which is consistent with its ability to bind to the Ni-NTA matrix. In this immunoblot, the penta His MAb also recognized the monovalent protein precursors because they also carried the 6× His tag. We repeated the Western blotting experiment with murine polyclonal antibodies elicited by the rEDIII-4/2 protein. The results of this experiment are shown in Figure 4B as expected the murine antibodies recognized the rEDIII-4/2 protein (lane 4). The murine antibodies to rEDIII-4/2 also recognized the rEDIII-2 (lane 2) and rEDIII-4 (lane 3) proteins. This observation is consistent with the design of the bivalent protein, which is derived from these two monovalent precursor proteins. Once again, the previously characterized rEDIII-2* protein served as a corroborating control (lane 5).

Figure 4. Purification and characterization of the bivalent rEDIII-4/2 protein. A. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of Ni-NTA affinity column fractions obtained during the purification of the bivalent rEDIII-4/2 protein. Samples analyzed in the gel are load (L, lane 2), flow through (FT, lane 3), wash (W, lanes 4 and 5), and eluates (E, lanes 6–9). Low molecular weight protein markers (M) were run in lane 1; their sizes (in kD) are shown on the left. The arrow at the right indicates the position of the rEDIII-4/2 protein. B. Western blot analysis of the purified rEDIII-4/2 protein using murine anti-rEDII polyclonal antiserum. An aliquot of the purified recombinant bivalent protein (indicated as B) was run in lane 4. For comparison, the monovalent rEDIII-2 (D, lane 2) and rEDIII-4 (D, lane 3) and a previously described variant of rEDIII-2 (D, lane 5) were analyzed on the same gel. Pre-stained protein molecular weight markers (P) were run in lane 1; their sizes (in kD) are shown on the left of each panel. The arrow at the right indicates the positions of the bivalent rEDIII-4/2 (hatched arrow), the monovalent rEDIII-2 and rEDIII-4 generated in this study (dashed arrow), and the previously described rEDIII-2* variant (solid arrow). Starting with approximately 1.5 grams of induced cell pellet (equivalent to 500 mL of E. coli culture), we obtained approximately 14 mg of purified recombinant protein (pooled fractions in lanes 6–9). We recently described the use of a sandwich ELISA protocol designed for the specific detection of the 6× His-tagged rEDIII-2* protein mentioned in conjunction with the DEN-2 envelope EDIII-specific MAb 3H5. We had shown that in this assay an unrelated 6× His-tagged recombinant protein or an untagged E protein (provided by DEN-2 virus) did not elicit any significant antibody response. We adapted this assay for the detection and quantitation of the rEDIII-4/2 bivalent protein. In this assay, the 6× His-tagged bivalent protein in total lysates or in column fractions was captured in Ni-NTA HisSorb microtiter wells and then allowed to react with DEN-2 EDIII-specific 3H5 MAb. The resultant Ni-NTA-immobilized bivalent antigen/3H5 MAb complex was detected using anti-mouse IgG-HRPO conjugate. Using this sandwich assay, we estimate that our purification resulted in approximately 27% recovery. This assay clearly demonstrates the presence of the DEN-2 EDIII component in the bivalent protein. Ideally, this sandwich ELISA should also have been done using a DEN-4 EDIII-specific MAb instead of the 3H5 MAb. This would have independently demonstrated the presence of the DEN-4 EDIII component in the bivalent protein. However, the lack of such a DEN-4 EDIII-specific MAb precluded this assay. We therefore raised murine polyclonal antibodies to the monovalent precursor proteins rEDIII-2 and rEDIII-4 to address this issue.

Retention of the antigenic identities of its precursors by rEDIII-4/2 protein and formation of antibodies with preferential specificity for its constituent serotypes. A major objective of this study was to explore if a chimeric EDIII-based dengue virus antigen would retain the antigenic identities of its precursors. In other words, would the rEDIII-4/2 protein manifest the antigenic characteristics of its monovalent EDIII precursors? To address this question, we examined the reac-
tivity of the rEDIII-4/2 protein towards antisera raised against the monovalent rEDIII-2 and rEDIII-4 proteins. For comparison, we also generated antisera to rEDIII-1 and rEDIII-3 as well (all four monovalent proteins were expressed and purified using protocols described for the rEDIII-4/2 protein as described in the Materials and Methods). In the experiment shown in Figure 5A, serial dilutions of murine antisera raised against each of the four monovalent recombinant EDIII proteins were titrated against the rEDIII-4/2 protein as the capture antigen. We also tested in this experiment the reactivity of the rEDIII-4/2 protein towards anti-rEDIII-2/4 antisera and serum from mock-immunized mice as positive and negative controls, respectively. The data show that the rEDIII-4/2 protein displayed high levels of ELISA reactivity towards anti-rEDIII-2 (Figure 5A, curve c) and anti-rEDIII-4 (Figure 5A, curve d) antisera. The magnitude of the ELISA reactivity towards these two antisera was equal to that displayed by the rEDIII-4/2 protein towards antisera raised against itself (Figure 5A, curve f). In striking contrast, the rEDIII-4/2 protein manifested significantly lower reactivity towards anti-rEDIII-1 (Figure 5A, curves a and b) and anti-rEDIII-3 antisera (Figure 5A, curve c). In comparison with the anti-rEDIII-2 and anti-rEDIII-4 antisera, the anti-rEDIII-1 and anti-rEDIII-3 antisera displayed consistently lower (approximately three-fold) ELISA titers in the linear range of the dose-response curves (coinciding with serum dilutions in the range 1:4,000–1:8,000). These data suggest that the bivalent rEDIII-4/2 protein retains the antigenic properties of its monovalent precursors. This leads to the prediction that antibodies induced by the bivalent protein would be capable of specifically recognizing and binding to both rEDIII-2 and rEDIII-4 proteins.

We next did the converse experiment to test the above prediction. In this experiment, we used rEDIII-2 and rEDIII-4 as capture antigens and performed an ELISA using serial dilutions of anti-rEDIII-4/2 antiserum. We also used rEDIII-1 and rEDIII-3 proteins as coating antigens for comparison. As before, rEDIII-4/2 protein-coated wells were used as a positive control. As a negative control, mock-immunized serum was tested using a mixture of the four monovalent rEDIIIs as the capture antigen. The data are shown in Figure 5B. The data demonstrate that the reactivities of anti-rEDIII-4/2 antiserum towards rEDIII-2 (Figure 5B, curve d) and rEDIII-4 (Figure 5B, curve e) proteins are similar to each other over the entire range of dilutions tested. Conversely, ELISA titers observed using either rEDIII-1 (Figure 5B, curve b) or rEDIII-3 (Figure 5B, curve c), which were comparable to each other, were significantly lower than those seen for the rEDIII-2 and rEDIII-4 proteins. Again, these ELISA titers were consistently lower (approximately three-fold) when compared with those for the rEDIII-2 and rEDIII-4 proteins. These data suggest that the rEDIII-4/2 protein has the potential to evoke a bivalent immune response resulting in the induction of antibodies specific to EDIIIs of both DEN-2 and DEN-4 viruses.

Specific blocking of the binding of DEN-2 and DEN-4 viruses to host cells and neutralization of their infectivity by antibodies elicited by the bivalent protein. The ELISA data above showed that the rEDIII-4/2 protein has the capacity to induce a bivalent response targeting rEDIII proteins of DEN-2 and DEN-4 preferentially. However, this does not provide any information regarding the capacity of these antibodies to recognize and bind to infectious virus. We addressed this using an immunofluorescence assay. In this assay, we mixed DEN-2 and DEN-4 viruses separately with anti-rEDIII-4/2 antiserum (and two different control sera) and

**Figure 5.** Immunoreactivities of the rEDIII-4/2 protein and its antibodies. Antibody titers were determined using an HisSorb enzyme-linked immunosorbent assay (ELISA). HisSorb microtiter wells coated with the appropriate 6×-his tagged rEDIII protein were incubated with serial two-fold dilutions of murine serum (in duplicate). Captured antibodies were detected using anti-mouse IgG-horseradish peroxidase conjugate/3, 3′, 5′-tetramethylbenzidine substrate. Data points represent the average of two separate ELISAs. A. Sera from mice that were either mock-immunized (curve a) or immunized with the recombinant proteins, rEDIII-1 (curve b), rEDIII-2 (curve c), rEDIII-3 (curve d) and rEDIII-4 (curve e), or rEDIII-2/4 (curve f), were titrated against rEDIII-4/2 protein as the capture antigen. B. Anti-rEDIII-4/2 antiserum was titrated against rEDIII-1 (curve b), rEDIII-2 (curve d), rEDIII-3 (curve c), or rEDIII-4 (curve e) as capture antigens. As in A, serum from mock-immunized mice was used as a control. However, it was titrated against a mixture of all four rEDIII monovalent proteins as capture antigen (curve a).
then incubated the virus/serum mixture with BHK cells in culture for one hour to allow adsorption. We then monitored cell-bound viruses by immunofluorescence using serotype-specific murine MAbs in conjunction with anti-mouse IgG-FITC conjugate. The rationale for this experiment was that if the antibodies present in the anti-rEDIII-4/2 antiserum bound specifically and strongly to the virus (presumably to the surface-exposed EDIII region), it might interfere with the ability of the virus to interact with the host cell-surface receptors. The results of this experiment are shown in Figure 6. Pre-incubation of either DEN-2 or DEN-4 virus separately with sera drawn from mice either before immunization (Figure 6, a and d) or after mock-immunization (Figure 6, c and f) resulted in the detection of cell surface-associated virus. Pre-incubation of DEN-1 and DEN-3 viruses with anti-rEDIII-4/2 antiserum did not interfere with the detection of these viruses. In striking contrast, pre-incubation of these two viruses with the anti-rEDIII-4/2 antiserum resulted in barely discernible immunofluorescence (Figure 6, b and e). Presumably, the antibodies elicited by the recombinant bivalent protein had specifically bound to DEN-2 and DEN-4 viruses, thereby effectively preventing them from binding to the host cell surface.

The question we addressed next was will such binding neutralize virus infectivity? To this end, we performed PRNT assays, by plaquing the antibody-incubated DEN viruses on LLCMK2 cells. Figure 7 shows the percent reduction in virus infectivity as a function of anti-rEDIII-4/2 antiserum dilution for the four DEN virus serotypes. It is evident that the antiserum contains significant levels of virus-neutralizing antibodies specific to only DEN-2 and DEN-4 viruses. For example, a 1:10 dilution of the antiserum that could neutralize the infectivity of DEN-2 and DEN-4 viruses by approximately 80% could reduce the infectivity of DEN-1 and DEN-3 viruses by only approximately 25%. The PRNT_{50} titers of the anti-rEDIII-4/2 antiserum were approximately 1:40 and 1:80 against DEN-2 and DEN-4 viruses, respectively. In contrast, the titers were ≤1:5 for DEN-1 and DEN-3 viruses. This suggested that the bivalent protein is capable of eliciting neutralizing antibodies against both DEN-2 and DEN-4 viruses.

**DISCUSSION**

Developing a dengue vaccine has been a challenging task because of the need to provide solid and long-lasting immunity to four serotypes without eliciting the potentially fatal ADE response. Since conventional approaches based on live attenuated dengue viruses have not resulted in a licensed vaccine, increasing attention is being focused on recombinant strategies. These approaches, which are geared towards developing monovalent vaccines targeting single DEN serotypes, envisage the creation of a tetravalent vaccine by physically mixing the four monovalent components into a single formulation. To be of practical utility in the resource-poor regions of the world where the disease is endemic, a dengue vaccine has to be cost-effective in addition to being safe and efficacious. In this regard, the development of a vaccine based on a single tetravalent component, be it virus-, plasmid-, or protein-based, would definitely offer a significant cost advantage. Thus, the concept of a single tetravalent dengue vaccine offers an option worth investigating.

The present work is based on the emergence of EDIII as a potential lead candidate for the development of a sub-unit dengue vaccine. We believe that EDIII would be a valuable precursor in developing a tetravalent antigen representing all four serotypes in a single molecule. In a first step towards this objective, we created a chimeric molecule by splicing together the EDIII genes of two different DEN serotypes using a flex-

**FIGURE 6**. Prevention of binding of dengue (DEN)-2 and DEN-4 viruses to host cells by antibodies to rEDIII-4/2. Baby hamster kidney cells in culture were infected with either DEN-2 (a–c) or DEN-4 (d–f) virus pre-incubated with different murine sera. The murine sera were obtained from mice prior to immunization (a and d), after mock immunization with phosphate-buffered saline (PBS) (e and f), or immunization with purified rEDIII-4/2 protein (b and c). One hour later, cells were rinsed, fixed and virus-binding to the cell surface was visualized by indirect immunofluorescence using serotype-specific monoclonal antibodies in conjunction with fluorescein isothiocyanate–labeled anti-mouse antibody conjugate.
Accordingly, we used a BamHI site at the 5' end of one monovalent EDIII and a HindIII site at the 3' end of the other (Figure 5A). Commensurate with the high degree of sequence homology (approximately 65%) among the four rEDIII proteins, the rEDIII-4/2 protein cross-reacted with anti-rEDIII-1 and anti-rEDIII-3 antisera, but at significantly lower levels. Clearly, the rEDIII-4/2 protein displayed a marked preference for antibodies raised against its monovalent precursors. This suggests that the rEDIII-4/2 protein had largely preserved the unique antigenic integrity of its precursors. The prediction that stems from this, that the rEDIII-4/2 protein should potentially be able to elicit antibodies with specificities towards both DEN-2 and DEN-4 rEDIIIs, was borne out in the next experiment. In this experiment, we tested the capacity of the antibodies to rEDIII-4/2 to react with each of the four monovalent rEDIIIs. The results, which mirrored the earlier findings, showed that rEDIII-4/2-induced antibodies reacted preferentially with rEDIII-2 and rEDIII-4 (Figure 5B); these antibodies cross-reacted with rEDIII-1 and rEDIII-3, but again at low levels. Cross-reactive titers in both sets of experiments were consistently lower (approximately 3–4-fold). In this regard, it is worth mentioning that similar levels of cross-reactivity were reported using a mixture of the four recombinant EDIIIs (as MBP fusions) to immunize mice. The mutually complementary results in these two experiments reinforced the notion that the rEDIII-4/2 protein can function as a bivalent immunogen eliciting antibodies with specificities for EDIII of DEN-2 and DEN-4.

The question we addressed at this point was would these antibodies also bind DEN-2 and DEN-4 virus? If so, would such binding block virus adsorption to host cells and neutralize virus infectivity? Using an immunofluorescence approach to visualize cell surface-bound virus, we observed that the anti-rEDIII-4/2 antiserum could prevent the binding of both DEN-2 and DEN-4 viruses to the host cell surface (Figure 6). It is likely that because EDIII is involved in host receptor recognition, antibodies elicited by the bivalent protein specifically bind to EDIII on the DEN-2 and DEN-4 virion surface and thereby preclude its binding to host cell surface. This was borne out by PRNT data, which showed that the infectivity of both DEN-2 and DEN-4 viruses could be effectively neutralized by the anti-rEDIII-4/2 antiserum. The PRNT_{50} titer, was approximately 1:40 for DEN-2 and 1:80 for DEN-4, whereas it was < 1:5 for DEN-1 and DEN-3 (Figure 7). The PRNT_{50} titers reported recently using EDIII-based plasmids were approximately 1:10. In contrast, the EDIII-MBP proteins were reported to elicit much higher levels of neutralizing antibodies. The observed differences are very likely a reflection of the nature of antigen, route of immunization, and most importantly differences in the experimental parameters of the PRNT assay. The PRNT_{50} titers ≥ 1:10 are considered indicative of protective immunity. In the absence of a good animal model for dengue, neutralizing antibody titers
are widely accepted as surrogate markers of protective immunity. Our data clearly demonstrate the potential of the rEDIII-4/2 protein to elicit neutralizing, and therefore, presumably protective antibodies against both the DEN-2 and DEN-4 viral serotypes. However, additional studies are needed to assess if the reduced levels of cross-reactivity (to DEN-1 and DEN-3 viruses) observed in our experiments would be significant from the viewpoint of ADE.

In conclusion, we have developed a novel bivalent chimeric protein by fusing the EDIIIIs of DEN-2 and DEN-4 viruses using a flexible linker. The recombinant protein was expressed in E. coli and purified to near homogeneity. Data from an ELISA and imunofluorescence and PRNT assays strongly suggest that it retains the antigenic identity of its precursors and elicits neutralizing, and therefore, presumably protective antibodies against the DEN virus serotypes represented in the chimeric molecule. This warrants the exploration of the possibility of creating a single tetravalent antigen incorporating the EDIIIIs of all four DEN serotypes. The design of the bivalent protein based on a head-to-tail condensation strategy that relies on Bam HI/Bgl II fusion is compatible with insertion of additional EDIIIIs to create a single tetravalent molecule. Coupled with the high expression capacity of the E. coli system and easy one-step affinity purification, this strategy has the potential to lead to the development of a cost-effective tetravalent dengue vaccine.

Received June 1, 2005. Accepted for publication September 13, 2005.

Acknowledgments: We thank Dr. Andrew Falconar for providing all four serotypes of dengue virus.

Financial support: The work was supported by International Centre for Genetic Engineering and Biotechnology core funds. Saima Khanam is a senior research fellow supported by the Council of Scientific and Industrial Research, Government of India, India. Behzad Etemad is supported by an International Centre for Genetic Engineering and Biotechnology pre-doctoral fellowship.

Authors' address: Saima Khanam, Behzad Etemad, Navin Khanna, and Sathyamangalam Swaminathan, Recombinant Gene Products and Biotechnology pre-doctoral fellowship.

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