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Expression and Purification of Dengue Virus Type 2 Envelope Protein as a Fusion with Hepatitis B Surface Antigen in *Pichia pastoris*

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Received February 26, 2001, and in revised form May 7, 2001; published online August 20, 2001

The methyotrophic yeast, *Pichia pastoris*, has been used as a host to express the envelope protein (Den2E) of dengue type 2 virus (NGC strain) as a chimera with hepatitis B surface antigen (HBsAg); a protein known to self assemble into virus-like particles (VLPs) and to be efficiently expressed in *P. pastoris*. The Den2E gene used in this study is a truncated version encoding the first 395 amino acid (aa) residues of the mature Den2E protein; the HBsAg gene encodes the full length 226 aa HBsAg protein. Two in-frame gene fusions were constructed for intracellular expression in *P. pastoris*. The first one contains the HBsAg gene as the 5' partner and the Den2E gene as the 3' partner (HBsAg-Den2E). In the second one, the relative positions of the two partners of the gene fusion were reversed to create the hybrid Den2E-HBsAg gene. These fusion genes were integrated into the genome of *P. pastoris* under the control of the methanol-inducible alcohol oxidase (AOX1) promoter. Of the two fusions, the Den2E-HBsAg gene was expressed at higher levels in *P. pastoris* based on Northern analysis. The hybrid protein (~68 kDa) expressed by this clone was purified to near homogeneity using a combination of acid precipitation, hydrophobic interaction, and immunoaffinity chromatographic steps. Final purification achieved was ~1400-fold with a yield of ~26%. The chimeric protein was found to possess the ability to assemble into high molecular weight aggregates (akin to HBsAg particles). The recombinant fusion protein eluted close to the void volume of a Sepharose CL-4B column indicating its macromolecular nature. On a CsCl density gradient the recombinant fusion protein sedimented to a position very similar to that of HBsAg VLPs. The hybrid protein is recognized by the two neutralizing monoclonals against the two components of the chimeric protein.

Key Words: dengue envelope protein; hepatitis B surface antigen; *Pichia pastoris*.

Dengue is an acute flaviviral disease prevalent in over a hundred countries, mostly in tropical and subtropical areas of the world. Infection with dengue viruses, of which there are four closely related serotypes, is responsible for 50–100 million cases a year (1). Infection with dengue virus of one serotype does not offer cross-protection against other serotypes. A first infection sensitizes a person so that approximately 2% of second infections (with a different serotype) can develop into potentially fatal dengue hemorrhagic fever. The relative risk of experiencing the most severe form of the disease is 100-fold higher after a secondary than after a primary infection (2). A useful dengue vaccine, therefore, should ideally protect against all four serotypes of dengue viruses. The availability of the complete nucleotide sequences of all four dengue virus serotypes in recent years (3) has greatly stimulated efforts to develop candidate subunit vaccines by recombinant DNA technology as an alternate to live attenuated dengue virus vaccine (4).

Available data in the literature show that both structural and nonstructural proteins of dengue virus can serve as potential vaccine candidates (4, 5). Among the
several dengue viral proteins, the envelope (E) protein appears to be very promising vaccine candidate for several reasons. It is the most important antigen from the viewpoint of virus biology and humoral immunity. It is a multifunctional protein involved in virion assembly, host cell surface receptor binding, and membrane fusion.

It is the major component of the virion envelope and is responsible for eliciting the first and longest lasting, protective antibody response to dengue infection. Anti-E monoclonal antibodies (9) and purified E protein (10, 11) are capable of conferring protection against lethal dengue virus challenge, by passive immunization and induction of neutralizing antibodies, respectively.

The E protein has been expressed in several heterologous systems including Escherichia coli (12, 13), yeast (12, 14), insect (11, 15), and mammalian cells (16, 17). Potent flaviviral E protein antigens must be properly folded in a conformation that maintains the integrity of its neutralizing epitopes (18-20). In view of this constraint, the bacterial host system with a reducing intracellular environment is not very conducive to protein folding. The other expression systems have the potential to generate properly folded E protein. However, the production of recombinant protein, using the insect and mammalian cell based tissue culture systems, can be very expensive compared to the yeast based expression systems.

Yeast based expression system is unique in that it combines the advantages of both prokaryotic (high expression levels, easy scale-up, inexpensive growth media) and eukaryotic (capacity to carry out most of the posttranslational modifications characteristic of higher eukaryotes) expression systems. In recent years, the methylotrophic yeast, Pichia pastoris, has emerged as a powerful and inexpensive heterologous expression system for the production of high levels of functionally active recombinant proteins (21). From an expression perspective, the existence of well-established fermentation methods, that can generate very high cell densities using purely defined media (22) and the strong, tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter (23), make P. pastoris a very valuable host for heterologous protein expression.

In this study, we have sought to evaluate P. pastoris as a host to express the E proteins of dengue viruses. Our goal is to develop a P. pastoris vector capable of simultaneously expressing the E proteins of all four dengue virus serotypes. Our experience shows that it should be possible to express all the four E proteins in a single recombinant P. pastoris expression vector. Large inserts of up to 16 kb (containing up to 8 copies of the HBsAg gene) have been successfully cloned and expressed in this system (24). As a first step toward realizing this objective, we have focused on the E protein of dengue virus type 2. Since work reported in the literature suggests that dengue E protein may be susceptible to proteolysis in P. pastoris (12), we chose to express it as a hybrid protein with a suitable fusion partner in an attempt to minimize this potential problem. We chose the surface antigen of Hepatitis B virus (HBsAg) as the fusion partner for the following reasons. First, it can be expressed efficiently in P. pastoris using the AOX1 promoter (24, 25). Second, HBsAg expressed in P. pastoris is very stable as it spontaneously assembles into ~20 nm particles (24, 25). Such virus-like particles (VLPs) are highly immunogenic. Third, this inherent capacity of HBsAg to generate particulate structures is likely be retained in its fusion derivatives. Last, HBsAg has been used successfully as a carrier in the development of a circumsporozoite protein based malarial vaccine candidate (26). The specific objectives of this study were to (i) express a hybrid protein containing the E protein of dengue virus type 2 and HBsAg in P. pastoris, (ii) purify the hybrid protein, (iii) determine if the hybrid molecule assembles into particles, and (iv) determine if the hybrid protein is recognized by antibodies specific to the Den2E and HBsAg precursors.

**MATERIALS AND METHODS**

**Host Strain and Plasmid Vectors**

The P. pastoris host strain used in this study was the histidine requiring auxotroph, GS115 (his4). In vitro protein expression studies were performed using constructs created by inserting the recombinant gene downstream of the T7 promoter of the pVAX1 plasmid vector. For intracellular expression in P. pastoris, the plasmid pAO815 was employed. This plasmid contains the P. pastoris AOX1 promoter and transcription termination sequences separated by a unique EcoRI restriction site for insertion of the foreign gene of interest. It also contains a wild-type copy of the histidinol dehydrogenase (HIS4) gene necessary for selection of P. pastoris transformants. Both GS115(his4) host strain as well as the plasmids (pVAX1 and pAO815) were purchased from Invitrogen.

**PCR Primers and Templates**

The plasmid pAO815 + HBsAg described previously was used as the template for the amplification of the HBsAg gene (0.7 kb). This plasmid contains one copy of the HBsAg gene inserted into the unique EcoRI site of pAO815 described above. Dengue type 2 envelope (Den2E) gene (~1.3 kb) was amplified from a plasmid

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2 Abbreviations used: AOX1, Alcohol Oxidase 1; BMGY, buffered minimal glycerol medium with yeast nitrogen base; BMMY, buffered minimal methanol medium with yeast nitrogen base; Den2E, Dengue type 2 envelope protein; E, envelope protein; HBsAg, hepatitis B surface antigen; VLP, virus-like particle.
containing a DNA copy of the first ~2.2 kb of the RNA genome of dengue type 2 virus (NGC strain) (27), a kind gift from Professor R. Padmanabhan (University of Kansas Medical Center, KS). Nucleotide sequences of the primers for PCR amplification of these two genes are shown below.

1. GCGGAATTCGTACCATTGGAGAATCTACG
   EcoRI
2. AACGATTCGCGCGCAATGTATAACG
   NotI
3. GGTATACATGGCGCCGCAATGCCTTGCTACA
   GGAATAC
   NotI
4. CCGGAATTCCTCGAGTCCATATCTCCTCTTTTA
   EcoRI, XhoI
5. CGCGAATTCCTACGTACCATGCCTGATAG
   EcoRI, XhoI
6. GTTCCTAGCCGGCGCCTCCTTTCTTAAC
   NotI
7. TAAAGAAAGGAGCGCGCGCAATGGAGAACAT
   CACATC
   NotI
8. CCGGAATTCCTCGAGTCACTAAATGTATACC
   GGAATAC
   EcoRI, XhoI

Generation of Fusion Constructs

Two gene fusions, shown in Fig. 1, were constructed for this study. The Den2E gene used in our experiments was a truncated version encoding the first 395 amino acid (aa) residues of the Den2E protein; the HBsAg gene encoded the full-length 226 aa HBsAg protein. The first one contained the HBsAg gene as the 5' partner and Den2E gene as the 3' partner (referred to as HBsAg–Den2E in this paper). In the second one, the relative positions of the two partners of the gene fusion were reversed to create the hybrid Den2E–HBsAg gene. In both cases, the two partners were fused in-frame via a NotI site. The components of each gene fusion were amplified separately by PCR, restricted with NotI and then ligated together to generate the full-length hybrid genes. To construct HBsAg–Den2E, the HBsAg (amplified with primers 1 and 2) and Den2E (primers 3 and 4) PCR products were digested with NotI and ligated together to create the full-length chimeric gene. The ligated product was then digested with EcoRI and XhoI for insertion into the EcoRI and XhoI sites of pVAX1 (Fig. 2A) or with EcoRI alone for insertion into the unique EcoRI site of pAO815 (Fig. 3A). The reverse fusion construct, Den2E–HBsAg, was created using a similar strategy. The Den2E and HBsAg PCR products, amplified with primers 5 and 6 and 7 and 8, respectively, were cut with NotI and then ligated together. The full-length hybrid gene was then cut with EcoRI and XhoI as before to cloning into pVAX1 (Fig. 2A) or with EcoRI alone for cloning into pAO815 (Fig. 3A). Each of the two hybrid proteins encoded by the fusion genes described above is designed to carry the first 395 aa residues of Den2E and all 226 aa residues of HBsAg, with three in-frame alanyl residues linking the two fusion partners. Thus, both fusion genes are expected to encode hybrid proteins of 624 aa each, corresponding to a molecular weight of ~68 kDa.

In Vitro Transcription and Translation

The TNT quick coupled transcription/translation system from Promega was used for the in vitro expression of the recombinant constructs as per the manufacturer’s instructions. In brief, 1 µl (1.0 µg) of supercoiled plasmid carrying the recombinant gene of interest under the T7 promoter was used in a 25 µl TNT reaction containing 20 µl TNT Quick Master Mix, 2 µl Redivue L-[35S]methionine (1000 Ci/mmol at 10 mCi/ml from Amersham), 0.5 µl cold, 1 mM methionine, and 1.5 µl RNase-free water. The reaction was incubated at 30°C for 90 min and the products analyzed on a 12.5% denaturing polyacrylamide gel, followed by fluorography.

Immunoprecipitation

The in vitro-synthesized proteins were analyzed by immunoprecipitation as follows. About 20 µl of protein G–Sepharose was incubated with 20 µg anti-HBsAg mAb in 100 µl phosphate-buffered saline (PBS) for 15 min at room temperature (RT) and washed three times with 1 ml of PBS. Next, 5 µl TNT reaction was added to the anti-HBsAg mAb/Protein G complex in 100 µl and allowed to incubate 30 min at RT. The resultant immune complexes were washed with 1X radioimmunoprecipitation assay buffer, boiled in 25 µl Laemmli sample buffer and analyzed on SDS–12.5% PAGE followed by fluorography.

P. pastoris Transformation

Electrocompetent P. pastoris GS115 cells (80 µl) prepared from a culture growing in log phase were mixed with 5–20 µg of BgIII linearized expression vector in a 0.2-cm electroporation cuvette. The cells were then pulsed for ~10 ms with a field strength of ~7500 V/cm using a Bio-Rad Gene Pulser. Transformants harboring the plasmid-borne HIS4 marker were selected on minimal plates lacking histidine and screened by direct PCR analysis as described previously (28). Briefly, single colonies were digested with zymolase (7.5 ng/µl) for 1 h at 30°C in 35 µl of 10 mM Tris–HCl (pH 8) 10 mM
DENGUE ENVELOPE PROTEIN EXPRESSION IN Pichia pastoris

**FIG. 1.** Strategy for the creation of HBsAg-Den2E (A) and Den2E-HBsAg (B) fusion constructs. The HBsAg (hatched box) and Den2E (empty box) genes were PCR amplified using appropriate primers (indicated by the numbered arrows) from plasmids (templates) carrying these genes. The resultant PCR products were fused through a NotI site that was engineered into the relevant primers. The resultant fusion products were then restricted with appropriate enzymes to facilitate cloning into either pVAX1 or pAO815 vectors. Restriction enzyme recognition sites are indicated by capital alphabets (N, NotI; R, EcoRI; X, XhoI).

DTT, boiled for 10 min and used in 50 μl PCR reactions containing specific primers. Primer pairs used were 1 + 4 and 5 + 8, respectively, for identifying the HBsAg–Den2E and Den2E–HBsAg fusion genes. Selected clones were analyzed for expression of the recombinant fusion proteins by ELISA, as described below.

**Shake-Flask Cultures**

For most routine experiments, small-scale cultures of 100 ml were grown in 500 ml baffled flasks in BMGY medium (100 mM potassium phosphate buffer (pH 6)/1.347% (w/v) yeast nitrogen base/1% yeast extract/2% peptone/4 × 10^{-5} % biotin/1% (v/v) glycerol) at 30°C with shaking (250 rpm) for 16–18 h to logarithmic phase. For routine protein expression experiments, cultures were induced after 16–18 h with 1% methanol. At the time of induction, the optical density of the cells at 600 nm (OD_{600}) was in the range of 2–6. To initiate induction, cells growing in logarithmic phase, in BMGY, were pelleted down (5000 rpm, 10 min) in sterile centrifuge bottles and resuspended in 10 ml BMMY, transferred to the culture flask, and returned to the shaking incubator. The composition of BMMY is similar to BMGY except that 1% methanol replaces 1% glycerol. Induction was typically carried out for 72 h. During the course of the induction phase, methanol was added every 24 h to a final concentration of 1% (v/v).

**Preparation of Soluble Extracts for ELISA**

Aliquots of cells corresponding to 100 OD_{600} units were transferred into borosilicate tubes, pelleted by low-speed centrifugation (6000 rpm, 10 min) and washed twice with 10 ml lysis buffer (10 mM phosphate buffer (pH 7.2)/5 mM EDTA/0.1% (v/v) TritonX-100/1 mM PMSF). Washed cells were resuspended in 0.35 ml lysis buffer containing 0.6-g zirconia glass beads (0.45 μm) and lysed by 10 1-min bursts of vortexing at maximum speed, with chilling on ice for 1 min between bursts. The lysate was spun down and the supernatant collected. The levels of recombinant Den2E–HBsAg hybrid protein in this clarified lysate (soluble extract) were determined by using a commercially available ELISA kit (see below).

**ELISA for Soluble Recombinant Den2E-HBsAg Hybrid Protein**

Recombinant Den2E–HBsAg hybrid protein in soluble cell extracts, column fractions and CsCl-gradient
fractions were analyzed using the Hepanostika micro-ELISA system (specific for HBsAg 22-nm particles) purchased from Organon Teknika (The Netherlands). Sample aliquots ranging from 10–100 μl (after appropriate dilution when necessary) were assayed as per the manufacturer’s instructions. Den2E–HBsAg hybrid protein in fractions eluted from immunoaffinity column was detected using a sandwich ELISA. In this sandwich assay, the hybrid protein was captured on the ELISA plate using anti-Den2E-monomoclonal antibody, 3H5 mAb (neutralizing mAb, purchased from ATCC, Catalog No. HB46), and revealed by a particle-specific horse-radish peroxidase (HRPO) conjugated anti-HBsAg polyclonal antibody.

**Northern Analysis**

Total RNA was prepared from *P. pastoris* transformants harboring the two gene fusions using the RNeasy midi kit purchased from Qiagen. Twenty micrograms of each RNA sample was loaded on a 1.2% agarose gel containing 6% formaldehyde, transferred on to a nylon membrane, and probed separately with α-[^32P]-labeled H1S4 or Den2E DNA. The blots were visualized by autoradiography and scanned using Kodak Digital Science software.

**Purification of the Den2E-HBsAg Hybrid Protein**

A 2-liter culture of logarithmically growing *P. pastoris* transformant harboring the Den2E–HBsAg fusion gene was spun down and induction initiated by resuspending the cell pellet in 200 ml BMMY. Cells were harvested 72 h postinduction. The pellet (∼50 g wet weight) was washed twice with 200 ml cold distilled water, once with 100 ml incomplete lysis buffer [10 mM phosphate buffer (pH 7.2)/5 mM EDTA/0.1% Triton X 100], and finally resuspended in 100 ml complete lysis buffer (in addition to the ingredients listed for the incomplete buffer, this also contains 0.1% Chaps and 1 mM PMSF). This suspension was mixed with ∼300 g of glass beads (0.46 μm) and the cells disrupted using a bead beater (BioSpec) in 15 1-min pulses with 1 min off-time between pulses. The lysate was separated from the beads using a sintered glass funnel. The beads were washed twice with ∼50 ml cold complete lysis buffer and pooled with the initial filtrate. The volume of the lysate was made up to 250 ml with cold complete lysis buffer. The lysate was then clarified by centrifugation in a Sorvall GSA rotor at 10,000 rpm for 30 min at 4°C. The supernatant was further clarified by acid precipitation as described. The pH of the lysate was adjusted to 5 using ~2–3 ml 1 N phosphoric acid and stirred for 30 min at room temperature. This was centrifuged at 10,000 rpm for 50 min at 4°C. The resultant supernatant was adjusted to pH 6.5 with 1 N NaOH and allowed to bind to 10 grams of aerosil [50% (v/v) suspension in water] at room temperature for 10–30 min with stirring. The unbound proteins were removed by centrifugation at 10,000 rpm for 15 min at 4°C. The bound proteins were eluted from aerosil by gently shaking with 75 ml 0.5 M ammonia for 45 min at room temperature followed by centrifugation at 10,000 rpm for 30 min at 4°C in a GSA rotor. The ammonia elution was repeated a second time and the pooled aerosil eluate (150 ml) was dialyzed against 10 mM phosphate buffer, pH 7.2, and allowed to bind to anti-HBsAg antibody coupled to Sepharose (in-house immunoaffinity matrix) for 20–30 min at 37°C. After binding, the immunoaffinity matrix was packed into a column (3 × 4.5 cm). The column was washed with 10 bed volumes of 10 mM phosphate buffer (pH 7.2) and eluted with 100 mM phosphate buffer, pH 11.5, at a flow-rate of 0.5 ml/minute. Ten fractions of 2 ml each were collected and analyzed by a sandwich-ELISA procedure as described above.

**Gradient Analysis**

Gradient analyses were performed essentially as described before (24). An aliquot (50–100 μl) of the partially purified Den2E–HBsAg hybrid protein was layered on a 20% CsCl gradient (w/v) in 50mM Tris–HCl (pH 7.5) in an SW41 polyallomer tube and centrifuged at 35, 000 rpm for 48 h at 16–18°C in a Beckman ultracentrifuge. After the spin, the centrifuge tube was punctured at the bottom and 0.5 ml fractions of the gradient were collected and analyzed using the particle-specific ELISA kit described above.

**Electrophoresis**

Proteins were separated on 12.5% polyacrylamide gels under denaturing conditions (SDS–PAGE) as per the standard protocol (29). Nonradioactive proteins were visualized using Coomassie blue stain. ^35S-Labeled proteins were visualized by fluorography of dried gels.

**Protein Estimation**

Routine protein estimation was done according to Bradford (30), with bovine serum albumin (BSA) as the standard.
RESULTS

In Vitro Analysis of the Chimeric Gene Constructs

In order to verify if the two fusion genes did indeed code for the predicted protein chimeras, the pVAX1 based fusion constructs were transcribed with T7 RNA polymerase and translated in the presence of L-[35S]methionine in vitro using a commercial coupled TNT kit. The radioactive proteins generated in the in vitro reaction were analyzed on a 12.5% denaturing gel. The results are shown in Fig. 2B. The TNT reactions were analyzed directly in lanes 1 and 2. In both these lanes a major ~68-kDa band can be easily discerned, consistent with the predicted sizes of HBsAg-Den2E and Den2E-HBsAg hybrid proteins. In the case of the latter, a doublet was seen at the predicted position. A second translational initiation event can be ruled out, as the fusion gene does not have a second in-frame ATG codon at its 5' end. It is unlikely that the doublet is the result of proteolytic degradation. If this were to be the case, a similar doublet would likely have been evident in the HBsAg-Den2E lane (compare lanes 1 and 2). The slightly faster migrating band presumably represents the product of incomplete translation. While the TNT analysis demonstrated that proteins of the predicted sizes were made from the two gene fusions, it does not provide information regarding the identity of the in vitro-synthesized products. Therefore, the TNT products were analyzed by immunoprecipitation using an anti-HBsAg mAb. Examination of lanes 3 and 4 (Fig. 2B) shows that the anti-HBsAg mAb was able to recognize proteins of ~68 kDa. Further, it can be seen that the band pattern in lanes 3 and 4 exactly mirrors that seen in lanes 1 and 2, respectively. Thus, this analysis suggested that the two fusion genes did indeed encode the intended chimeric proteins.

Intracellular Expression of the Gene Fusions in P. pastoris

After confirming that the two fusion genes were capable of generating the predicted hybrid proteins in vitro, we next proceeded to express these genes in P. pastoris. To this end, we constructed yeast expression vectors by cloning the HBsAg-Den2E and Den2E-HBsAg fusion genes as EcoRI inserts into the unique EcoRI site of pA0815 (Fig. 3A) as described above. These vectors were then transformed into the histidine requiring auxotrophic P. pastoris host strain GS115. The transformation strategy adopted was designed to target integration of the hybrid protein-expression cassette plus the HIS4 marker into the AOX1 locus of the P. pastoris genome. Transformants were selected on minimal plates lacking histidine and screened for the presence of the hybrid gene by PCR using appropriate primer.

FIG. 2. In vitro expression analysis of the fusion constructs. (A) Plasmid pVAX1-based T7 expression vector. The fusion genes (described in the legend of Fig. 1) were digested with EcoRI and XhoI and inserted into pVAX1, immediately downstream of the phage T7 promoter to permit in vitro transcription and translation. (B) SDS-PAGE analysis of in vitro-synthesized, 35S-radiolabeled HBsAg-Den2E (lanes 1 and 3) and Den2E-HBsAg (lanes 2 and 4) fusion proteins before (lanes 1 and 2) and after (lanes 3 and 4) immunoprecipitation with anti-HBsAg mAb. 14C-labeled protein molecular weight markers were loaded in lane M. The molecular weights (in kDa) are shown on the left of the panel. The arrow to the right indicates the position of the fusion proteins.
pairs as described under Materials and Methods. Test-tube cultures of selected *P. pastoris* transformants were grown overnight and induced with 1% methanol to check for expression of the recombinant fusion proteins by ELISA (using Hepanostika kit). We consistently noticed relatively greater levels of recombinant protein expressed by *P. pastoris* clones harboring the Den2E-HBsAg fusion gene as compared to the clones harboring the reverse fusion gene, HBsAg-Den2E (data not shown). This prompted us to examine the steady-state levels of the corresponding mRNAs. To this end, total RNA was isolated from two representative *P. pastoris* clones (carrying the two different fusion genes) after methanol induction and subjected to Northern analysis using a Den2E-specific probe. HIS4 mRNA was analyzed in the same blot as an endogenous control in order to normalize the RNA levels. The results are shown in Fig. 3B. It is evident that the steady-state level of Den2E-HBsAg mRNA is two-to-three-fold greater than that of HBsAg-Den2E mRNA. Since these data showed that Den2E-HBsAg could be expressed comparatively more efficiently than HBsAg-Den2E, subsequent studies focused on the former hybrid protein.

**Optimization of Den2E-HBsAg Expression in Shake-Flask Cultures**

We next proceeded to investigate the conditions necessary to achieve optimal induction of Den2E-HBsAg expression in *P. pastoris*. First, the expression of Den2E-HBsAg was measured at varying inducer concentrations ranging from 0.5–3% methanol, keeping the duration of induction constant at 48 h (Fig. 4A). At 0.5% (v/v) methanol, the level of induction was quite modest. Increasing the methanol concentration to 1% (v/v) was accompanied by a significant enhancement (~4.5-fold) in the magnitude of induction. Increasing the methanol concentration thereafter resulted in diminished induction. At methanol concentrations between 1.5–3%, the level of induction was diminished by ~20–45% with respect to induction obtained at 1% (v/v) methanol. At concentrations exceeding 1% (v/v), the decrease in recombinant protein accumulation could perhaps be attributed to the toxic effects of increased formaldehyde production from methanol as well as decreased cell growth. It has been shown that growth of *P. pastoris* is markedly inhibited at high methanol concentrations (31).

Next, the expression of the recombinant protein was monitored as a function of duration of induction (Fig. 4B). In this experiment, a logarithmically growing *P. pastoris* culture was induced with 1% (v/v) methanol. Induction was maintained for a total period of five days by the addition of methanol to 1% (v/v) final concentration at 24-h intervals. Aliquots of the culture were withdrawn at daily intervals and analyzed by ELISA (Hepanostika kit). From the data in Fig. 4B, it can be seen that maximal expression was attained 72 h postinduction. Beyond three days, it is likely that toxic metabolites...
accumulate in the culture medium to growth-inhibitory levels and this could in turn adversely affect recombinant protein expression levels. Another possibility is that dissolved oxygen, which can be a limiting factor in shake-flask cultures, could be responsible for decreased cell growth and the resultant decline in the levels of recombinant protein production. The data in Figs. 4A and 4B indicate that induction times beyond three days and methanol concentrations higher than 1% (v/v) can result in a reduction in recombinant protein accumulation. Accordingly, in subsequent experiments, induction was carried out in the presence of 1% (v/v) methanol for 72 h.

**Purification of Recombinant Den2-HBsAg Hybrid Protein**

Based on the results above, ~50 g cells (wet weight, from a 2-liter culture of the appropriate *P. pastoris* clone) were induced with 1% (v/v) methanol for 3 days and used as the starting material for the purification of recombinant Den2E–HBsAg hybrid protein. The major purification steps are summarized in Table 1. Essentially, the purification strategy involved clarification of the initial lysate by acid precipitation followed by hydrophobic interaction chromatography on aerosil. Final purification was achieved by immunoaffinity chromatography on immobilized anti-HBsAg antibody column. Column fractions from this immunoaffinity step were assayed using a sandwich ELISA procedure designed to specifically detect the hybrid alone. This assay utilized two neutralizing antibodies, one specific for the Den2E component (3H5 mAb) and the other specific for the HBsAg component of the hybrid protein. In this sandwich assay, neither the component proteins (separate Den2E and HBsAg proteins) nor extracts prepared from untransformed *P. pastoris* GS115 cells elicit any significant positive signals. The immunoaffinity elution profile of Den2E–HBsAg obtained using this sandwich assay is depicted in Fig. 5A. The peak fractions from the immunoaffinity column were pooled together and dialyzed against 10 mM phosphate buffer, pH 7.2. An aliquot of this immunopurified preparation was analyzed on a denaturing polyacrylamide gel and visualized by Coomassie stain as shown in Fig. 5B. In this gel the purified Den2E–HBsAg fusion protein was run alongside bovine serum albumin (BSA) as a reference in addition to the regular low molecular weight protein markers. One major protein species migrating at ~67–

![FIG. 4. Optimization of methanol induction of Den2E-HBsAg expression in *Pichia pastoris*. (A) Effect of methanol concentration on the expression of recombinant fusion protein. Small-scale cultures, growing in logarithmic phase, were induced in parallel with varying concentrations of methanol (ranging from 0.5 to 3%) for 48 h. (B) Effect of duration of methanol induction on the expression of recombinant fusion protein. Aliquots were withdrawn from a culture at the indicated time points after initiating induction with 1% (v/v) methanol. In both experiments, the levels of recombinant protein in the samples were determined using the Hepanostika ELISA kit.](image-url)

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*Total protein was estimated with Bradford reagent using BSA as standard.

ELISA ODs in appropriately diluted samples were measured at 450 nm using Hepanostika Sandwich assay with developing time of 30 min.

Yields reported are based on ELISA ODs.

Fold purification is based on total protein recovered.
FIG. 5. Immunoaffinity purification and SDS-PAGE analysis of the recombinant hybrid protein. (A) Immunoaffinity chromatographic elution profile of recombinant Den2E-HBsAg hybrid protein. Column fractions were analyzed by a customized sandwich ELISA procedure, designed to score for both components of the hybrid protein (inset). (B) SDS-PAGE analysis of the immunoaffinity purified recombinant hybrid protein (lane R) run along with BSA (lane B) for comparison. Low molecular weight protein markers were loaded in lane M (their sizes, in kDa, are shown on the left of the panel).

68 kDa was present in the purified preparation. This electrophoretic mobility is consistent with the predicted molecular weight of the Den2E–HBsAg fusion protein (624 aa protein with a predicted molecular weight of ~68 kDa). Taken together, the data presented in Figs. 5A and 5B demonstrate that indeed the P. pastoris system is able to generate the intended hybrid protein consisting of Den2E and HBsAg components (based on sandwich ELISA data) of the predicted molecular weight (based on SDS–PAGE analysis). Further, the data presented in Table 1 taken in conjunction with SDS–PAGE analysis (Fig. 5B) show that the strategy we adopted to purify the recombinant hybrid protein resulted in approximately 1400-fold purification with a recovery of 26% (corresponding to ~1 mg pure protein) with respect to the initial crude lysate. The purity of the final preparation was judged to be ~95%.

Recombinant Den2E–HBsAg Assembles into High Molecular Weight Aggregates

One of the reasons for using HBsAg as the fusion partner for Den2E expression was the expectation that the HBsAg component would confer on the hybrid protein its inherent capacity to self-aggregate and give rise to high molecular weight VLPs. The ability of HBsAg expressed in P. pastoris to assemble into virus-like particles is well documented (24, 25). We therefore investigated whether the Den2E-HBsAg fusion protein also possesses this ability to generate VLPs. Figure 6A displays the elution profile of a partially purified preparation of Den2E–HBsAg on a Sepharose CL-4B gel filtration column (4.8 x 60 cm). The column fractions were analyzed using a HBsAg particle-specific ELISA (Hepanostika). The recombinant fusion protein eluted close to the void volume of the column indicating its multimeric nature. The sedimentation behavior of the recombinant fusion protein, centrifuged through a 20% CsCl density gradient, was investigated. The sedimentation analysis was performed with two different samples of the recombinant fusion protein. One was the crude lysate and the other was a partially purified preparation. This was done to estimate the relative proportion of monomers in the recombinant fusion protein preparation. Fractions were collected from the bottom of the gradient and analyzed by ELISA using the same particle-specific anti-HBsAg antibody described above. The resultant data profiled in Fig. 6B show a major peak sedimenting in the bottom half of the gradient for both samples of the recombinant fusion protein (fraction 8). In a parallel gradient, HBsAg VLPs displayed a very similar sedimentation profile (data not shown). The peak fraction containing Den2E–HBsAg particles (fraction 8) and the top fraction (fraction 20) expected to contain monomeric forms if any, from both samples, were analyzed using a linear epitope specific anti-HBsAg mAb (capable of recognizing HBsAg irrespective of its conformation).
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**FIG. 6.** Characterization of the particle-like behavior of recombinant hybrid protein. (A) Gel exclusion chromatographic elution profile of recombinant Den2E-HBsAg hybrid protein on a Sepharose CL-4B column (4.6 × 60 cm). The arrow above the peak indicates the void volume peak. (B) Sedimentation profile of the recombinant hybrid protein, through a 20% CsCl density gradient. The arrow above the peak represents the position of sedimentation of HBsAg particles run in a parallel gradient. Both Sepharose CL-4B as well as density gradient fractions were analyzed using a particle-specific anti-HBsAg antibody (Hepanostika). The inset in B shows a Western blot in dot-dot blot format (performed with a linear epitope-specific anti-HBsAg mAb) of the peak fraction (Fraction 8) and the top fraction (fraction 20) obtained from crude (dashed curve) and partially pure (solid curve) recombinant fusion protein. C represents a control dot-dot blot developed in parallel using an aliquot of 20% CsCl.

The results from this experiment, shown in Fig. 6B, inset, did not reveal the presence of any detectable monomeric form of the recombinant fusion protein both in crude as well as partially purified samples. The above data indicate that the recombinant Den2E-HBsAg fusion protein indeed assembles into high molecular weight aggregates akin to HBsAg VLPs when expressed in *P. pastoris*. The failure to detect monomers of the recombinant protein even in the crude lysate may be attributable to the slow methanol feed rate during an induction phase spanning three days. Under such conditions, it is likely that there is adequate opportunity for practically all of the recombinant protein monomers expressed in *P. pastoris* to assemble into particles. We have observed similar HBsAg particle assembly using a recombinant *P. pastoris* clone harboring as many as eight copies of the HBsAg gene (24).

**DISCUSSION**

We have explored the possibility of utilizing *P. pastoris* as a host system for the expression of the envelope proteins of dengue viruses. To begin with, we have focused on the E protein of dengue virus type 2 (Den2E). Several lines of evidence indicate that dengue E protein is a potential vaccine candidate. In experimental animals, passive immunization with anti-E-monoclonal antibodies has been shown to protect against dengue infection (9). Purified E protein can induce the production of neutralizing antibodies and confer protection against lethal dengue virus challenge (10, 11). Further, the E protein is responsible for eliciting the first and longest lasting antibody response (8). As a result, considerable effort has been focused on the expression of recombinant E protein in an attempt to create a subunit vaccine. The E protein has been expressed using both prokaryotic (12, 13) as well as eukaryotic systems (11, 12, 14–17). Although, the *E. coli*-based system has been used successfully to express defined domains of the E protein for the purpose of antigenic structure analysis (32), this system with its reducing intracellular environment does not appear to be appropriate for the expression of properly folded E protein. Therefore, an eukaryotic expression system is preferable as it can ensure proper folding of the recombinant E protein molecule. Of the eukaryotic systems available for the heterologous expression of the dengue E protein, the tissue culture based baculovirus- (11, 15) and vaccinia virus-expression (16, 17) systems can be very expensive. From the perspective of cost-effective large-scale expression of heterologous proteins, the methylotrophic yeast, *P. pastoris*, is an ideal host. However, information available in the literature regarding the use of *P. pastoris* for the expression of dengue viral antigens is very limited. Sugrue et al. (12) found that expression of full-length
E protein of dengue virus type 1 as a glutathione S-transferase fusion, in P. pastoris, was accompanied by extensive proteolysis of the recombinant protein. Deleting the carboxy terminal hydrophobic domain of the E protein could minimize this problem (12). Accordingly, we decided to express a similarly truncated version of Den2E. In order to minimize the potential for proteolysis even further, we chose to use as fusion partner the HBsAg protein, known to be quite stable in the host. Further, HBsAg has been used as a carrier to express antigens of poliovirus (33), human immunodeficiency virus 1 (34), and Plasmodium falciparum (26), because it is a good immunogen by virtue of its ability to generate high molecular weight polymers.

HBsAg has been successfully used as a fusion partner to augment the immunogenic potential of the circumsporozoite protein of P. falciparum to develop a malarial vaccine candidate, called RTS, S, which spontaneously assembles into particles (26). Thus, the choice of HBsAg has two obvious advantages. First, it may confer protection against proteolytic cleavage of the resultant fusion protein. Second, the inherent ability of HBsAg to spontaneously assemble into highly immunogenic virus-like particles (VLPs) may be retained in the fusion protein.

We successfully expressed the first 395 aa residues of the envelope protein of dengue virus type 2 virus (Den2E) fused in frame to the 226 aa major surface antigen protein of hepatitis B virus (HBsAg). The fusion protein, Den2E–HBsAg, was purified using a simple strategy that involved a preliminary acid precipitation step, followed by hydrophobic interaction chromatography on an aerosil (silica) matrix, and finally affinity chromatography on an anti-HBsAg antibody column. Fold purification obtained at the acid precipitation step (Table 1) is relatively insignificant. However, this step is essential as it greatly facilitates in clarifying the initial lysate, by getting rid of lipids that can interfere in the subsequent aerosil-binding step (35). The aerosil step, which resulted in ~9-fold enrichment was incorporated in the purification strategy to take advantage of the relatively strong hydrophobic property of the HBsAg component of the recombinant fusion protein. The final immunoaffinity step resulted in a further ~160-fold purification. In our experience, it was necessary to include the prior acid precipitation and aerosil binding steps to achieve maximal purification in the final immunoaffinity step. The purification strategy per se has worked satisfactorily considering that we have been able to achieve an overall purification of nearly 1400-fold with a recovery of 26%. However, in terms of absolute quantities, the yield is quite low. Starting from ~50 g (wet weight) of P. pastoris cells, ~1 mg purified recombinant protein could be obtained (Table 1). Proteolytic degradation contributing to low yields can be ruled out based on SDS–PAGE analysis of the purified hybrid protein, which revealed a single major band of the predicted electrophoretic mobility. No other bands, that may be indicative of the presence of degradation products, are evident. Inefficient and or improper particle assembly (in conjunction with the elimination of unassembled/improperly assembled Den2E-HBsAg during purification) contributing to the recovery of low levels of particulate Den2E-HBsAg can be ruled out based on the immunoblot data obtained with CsCl gradient fractions of crude and purified recombinant fusion protein. Apparently, low expression levels are responsible for the low yield of recombinant protein obtained. One obvious reason for low expression is that only one copy of the fusion gene has been integrated into the genome of P. pastoris. It should be possible to enhance expression levels using higher gene dosage as reported for HBsAg by Vassileva et al. recently (24). This was done by an in vitro sequential multimerization approach. The strategy relies on inserting additional copies of the recombinant gene in the form of 5′ BglII–BamHI 3′ fragments into the BamHI site of the P. pastoris integrative vector (see Fig. 3A). Using this strategy, we have cloned DNA inserts as large as 16 kb into genome of P. pastoris (24). The presence of an internal BamHI site in the Den2E gene is not compatible with this in vitro multimerization strategy. In order to circumvent this potential problem, we are currently deleting the internal BamHI site of the Den2E gene without altering the amino acid sequence of the encoded protein. We believe that increasing the copy number can contribute significantly to the enhancement of overall expression levels. Another factor that could be responsible for the low recombinant protein yield may pertain to the efficiency of induction. In shake flask cultures, we maintain the induction phase by periodic methanol addition to a final concentration of 1% (v/v). It is quite conceivable that induction is maximal immediately following the methanol “pulse,” and tapers off with time as the methanol is utilized. Thus, lack of maintenance of uniform and sustained induction phase could be partly responsible for low recombinant protein yields. It is, therefore, very likely that scale-up of the shake-flask to fermentor cultures can greatly improve recombinant protein yields, since critical parameters such as dissolved oxygen and methanol feeding strategy that influence cell growth and induction can be very precisely controlled. Extraction of soluble protein from methanol induced P. pastoris using glass beads and vortex mixer is inefficient and highly variable. More efficient and reproducible extraction methods have to be devised to improve overall yields of recombinant protein from P. pastoris cells.

We designed a sandwich ELISA protocol to specifically detect the hybrid protein. In this assay, the hybrid protein was first captured on the ELISA plate using
A significant finding is that this hybrid protein, Den2E-HBsAg molecule to self-associate and generates high molecular weight VLPs. Further, neutralizing antibodies specific to both components recognize it. This has important implications for multivalent vaccine development.

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ACKNOWLEDGMENTS

We are grateful to Professor R. Padmanabhan, University of Kansas Medical Center, KS, for his kind gift of the Den2E gene containing plasmid. H.B. is supported by a junior research fellowship awarded by the Council of Scientific and Industrial Research, India.

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Recombinant dengue virus type 2 envelope/hepatitis B surface antigen hybrid protein expressed in Pichia pastoris can function as a bivalent immunogen

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Abstract

A truncated version of the dengue virus type 2 envelope protein (Den2E) encoding the first 395 amino acid (aa) residues, and Den2E fused in-frame with the full-length 226-aa hepatitis B surface antigen (Den2E-HBsAg) protein were expressed in the methylotrophic yeast, Pichia pastoris. Both the recombinant proteins showed evidence of the capacity to form high molecular weight aggregates. Electron microscopic analysis of the purified proteins showed that while Den2E displayed an amorphous morphology, Den2E-HBsAg existed as well-structured virus-like particles (VLPs). Using immuno-gold electron microscopy, these VLPs were demonstrated to contain both components of the Den2E-HBsAg hybrid protein. Seroanalysis showed that the hybrid VLPs could function in vivo as bivalent immunogens, which could elicit immune responses directed against both components of the hybrid protein, as evidenced by ELISA, immunoprecipitation and immunofluorescence data.

Key words: Dengue envelope protein, Hepatitis B surface antigen, Pichia pastoris
Abbreviations: Den2E, Dengue type 2 envelope protein; E, envelope protein; HBsAg, Hepatitis B surface antigen; VLP, Virus-like particle.

Introduction

Dengue is an acute flaviviral disease prevalent in over a hundred countries, mostly in tropical and subtropical areas of the world (Gubler, 1997). Dengue viruses, of which there are four antigenically distinct serotypes, produce a broad spectrum of disease, ranging from mild febrile illness to a severe hemorrhagic, sometimes fatal, disease (George and Lum, 1997; Gubler, 1997). Epidemiological and laboratory data suggest that subneutralizing levels of antibodies, from a primary infection, may mediate enhancement of disease severity during a secondary infection (Bielefeldt-Ohmann, 1997; Morens, 1994) leading to the widely held view that a useful dengue vaccine should ideally protect against all four serotypes of dengue viruses. Among the several dengue viral proteins, the envelope (E) protein appears to be an effective vaccine candidate for several reasons. It is the most important antigen from the viewpoint of virus biology and humoral immunity. It is a multifunctional protein involved in virion assembly, host cell surface receptor binding, and membrane fusion (Chambers et al, 1990; Henchal and Putnak, 1990). It is the major component of the virion envelope and is responsible for eliciting the first and longest lasting, protective antibody response to dengue infection (Churdboonchart et al, 1991). Anti-E-monoclonal antibodies (Kaufman et al, 1987) and purified E protein (Feighny et al, 1992; Staropoli et al, 1997) are capable of conferring protection against lethal dengue virus challenge, by passive immunization...
and induction of neutralizing antibodies, respectively. Consequently, intense effort is being directed at heterologous expression of the dengue E protein, in order to develop it as a possible sub-unit vaccine candidate (Hermida et al., 2002; Kelly et al., 2000; Men et al., 1991, 2000; Pupo-Antúnez et al., 2001; Simmons et al., 1998; Staropoli et al., 1997). For the E protein to serve as a potent antigen, it is preferable to express it in a eukaryotic system, as it must be properly folded in a conformation that maintains the integrity of its neutralizing epitopes (Guinakhoo et al., 1992; Roehrig et al., 1989, 1990). Our laboratory has been interested in developing the methylotrophic yeast, Pichia pastoris, as a host to express the E proteins of dengue viruses. The existence of well-established fermentation methods, that can generate very high cell densities using purely defined media (Wegner, 1983) and the strong, tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter (Tschopp et al., 1987), make P. pastoris a very valuable host for heterologous protein expression.

We recently reported the expression and purification of a chimeric protein, Den2E-HBsAg, in which we had fused the first 395 amino acid (aa) residues of Den2E with the full-length (226 aa) hepatitis B surface antigen (HBsAg) (Bisht et al., 2001). Based on preliminary results of gel-exclusion chromatography and CsCl sedimentation analysis, it could be demonstrated that this hybrid protein had the potential to aggregate into high molecular weight entities, similar to virus-like particles (VLPs) (Bisht et al., 2001). We have focused on two major aspects in the current study. Firstly, we have examined if the Den2E-HBsAg hybrid protein aggregates, expressed in P. pastoris, do indeed assemble into VLPs, by direct electron microscopic visualization; we have also examined the effect of eliminating the HBsAg fusion partner on VLP formation. Secondly, we investigated if this hybrid molecule can elicit immune responses, specific to both its constituent components. We believe that, if it did, it could serve as a model to develop potential bivalent, and possibly multivalent immunogens.

MATERIALS AND METHODS

Host strain and plasmid vectors

The P. pastoris host strain used in this study was the histidine-requiring auxotroph, GS115 (his4). It has a mutant histidinol dehydrogenase gene (his4) to allow for selection of expression vectors containing the wild type allele (HIS4) upon transformation (Cregg et al., 1985). In vitro protein expression studies were performed using constructs created by inserting the recombinant gene downstream of the T7 promoter of the pVAX1 plasmid vector. For intracellular expression in P. pastoris, the plasmid pAO815 was employed. This plasmid contains the P. pastoris AOX1 promoter and transcription termination sequences separated by a unique EcoRI restriction site for insertion of the foreign gene of interest. It also contains a wild-type copy of the HIS4 gene necessary for selection of P. pastoris transformants. Both GS115 (his4) host strain as well as the plasmids (pVAX1 and pAO815) were purchased from Invitrogen.

Generation of constructs

The construction of the fusion gene, Den2E-HBsAg, has been described before (Bisht et al., 2001). This fusion gene encodes a hybrid protein containing the first 395 aa residues of dengue virus type-2 envelope protein followed by all 226 aa residues of the HBsAg protein. The dengue type-2 envelope (Den2E) gene (~1.2 Kb, encoding aa 1-395) was amplified from this fusion construct using the primers shown in Figure 1. For in vitro expression, the PCR amplified product was inserted into the vector pVAX1 using EcoRI and Xhol sites. For in vivo expression in P. pastoris, the ~1.2Kb Den2E gene was retrieved from the pVAX1 vector as an EcoRI fragment and cloned into the unique EcoRI site of the pichia integrative vector, pAO815.

Forward primer

5'-cgggaatttcatactactagcaatcgtgcatgcgttcataggaatatac-3'

EcoRI 6XHis-Tag

Reverse primer

5'-caggtttcgagcattccctttcctttaaaccagtttagtc-3'

EcoRI Xhol

Figure 1. PCR primers used for the amplification of Den2E gene. In-frame initiation and termination codons (indicated in bold) were incorporated into the primers. The boxed sequence in the forward primer represents a Kozak consensus sequence (Kozak, 1987) to facilitate efficient translation in P. pastoris. In addition, a sequence encoding a 6X-His tag (underlined) was inserted into the forward primer for affinity purification (Petty, 1996) of the expressed protein. EcoRI restriction sites were incorporated at the 5' ends of both primers to facilitate cloning into the plasmid; addition, the reverse primer had an Xhol site, just adjacent to EcoRI site for cloning into pVAX1. Restriction enzyme sites are shown in italics. The sequences homologous to dengue viral RNA are shown in capitals; non homologous sequences are in small case.

Expression of Den2E proteins in P. pastoris

Expression of the hybrid Den2E-HBsAg protein, in P. pastoris, has been described before (Bisht et al., 2001). Essentially the same strategy was utilized to express the Den2E protein. The recombinant gene encoding the his-tagged Den2E protein obtained after PCR amplification was first inserted into pVAX1, downstream of the phage T7 promoter so as to facilitate in vitro transcription and translation. After
verifying that the recombinant gene did indeed encode a protein of the predicted molecular size (data not shown), the Den2E gene was inserted into pAO815. The resultant vector was transformed into the histidine-requiring auxotrophic P. pastoris host strain, GS115, using a transplacement strategy designed to target integration of the transgene expression cassette into the AOX1 locus of the P. pastoris genome (Bisht et al., 2001; Vassileva et al., 2001). Transformants were selected on minimal plates lacking histidine and screened for the presence of the Den2E gene, by direct colony PCR (Arora et al., 1998), using the Den2E forward and reverse primers (Figure 1). Test tube cultures of selected P. pastoris transformants were grown overnight, and induced with 1% (vol/vol) methanol, to check for the expression of the recombinant Den2E protein by ELISA.

**Purification of recombinant Den2E protein**

The purification of the Den2E-HBsAg hybrid protein, from P. pastoris lysates, taking advantage of the HBsAg fusion partner has been described in our previous study (Bisht et al., 2001). We adopted a different strategy for the purification of the Den2E protein, which lacks the HBsAg fusion partner. We inserted a 6X-His tag at the N-terminus of Den2E to facilitate affinity purification using nickel-nitrilotriacetic acid (Ni-NTA) chromatography (Petty, 1996). A 1-liter culture of P. pastoris transformant, harboring the 6X-His-tagged Den2E gene growing at logarithmic phase, was spun down and induction initiated by resuspending the cell pellet in 1% (vol/vol) methanol containing medium as described before (Bisht et al., 2001; Vassileva et al., 2001). Cells were harvested 72 hours post-induction. The pellet (~25 grams wet weight) was washed twice with 200ml cold distilled water, once with 100ml lysis buffer [50mM phosphate buffer (pH 8.5)/150mM NaCl/10mM imidazole/0.1% Triton X-100/1mM phenyl methyl sulfonyl fluoride (PMSF)] and then resuspended in 30ml lysis buffer. This suspension was mixed with ~120g of glass beads (0.45-0.7mm) and the cells disrupted using a bead mill in three 1-minute pulses, with 1-minute ‘off’ time between pulses. The lysate was separated from the beads using a sintered glass funnel. The beads were washed twice with ~35ml cold complete lysis buffer and pooled with the initial filtrate. The volume of the lysate was made up to 100ml with cold lysis buffer, which was then clarified by centrifugation in a Sorvall GSA rotor at 10,000rpm for 30 minutes at 4°C, followed by centrifugation in a Sorvall SS34 rotor at 15,000 rpm for 60 minutes, at 4°C. This clarified supernatant was allowed to bind to 2ml of Ni-NTA agarose [50% (wt/vol) slurry, supplied by Qiagen] for 60 minutes at 4°C. Lysate/Ni-NTA mixture was loaded into a column and the flow-through was collected. The column was washed with 10 bed-volumes of wash buffer (composition was the same as lysis buffer, except that it had 20mM imidazole) and, the wash fractions were collected. The column was eluted twice with 1.5ml elution buffer (composition was the same as lysis buffer, except that it had 500mM imidazole). The pooled eluate (3ml), containing the purified recombinant his-tagged protein, was dialyzed against 50mM phosphate buffer pH 8.5/150mM NaCl.

**Immunoblot assay**

The purified 6X-His-tagged Den2E protein was detected by anti-Den2E (3H5, from ATCC) and anti-His (catalog number 34660, from Qiagen) monoclonal antibodies (mAbs). After appropriate dilution with 1X SDS sample disruption buffer, the samples were loaded onto a SDS-12.5% polyacrylamide gel. Separated proteins were electrotransferred using Mini Trans-blot Electrophoretic Cell (Bio-Rad) onto a nitrocellulose membrane (Hybond-C, Amersham, England), in the presence of 200 mM glycine/24 mM Tris/20% (vol/vol) methanol at 100V for 1.5 hours, at 4°C. Transfer of proteins onto the membrane was visualized by kaleidoscopic markers. The membrane was rinsed briefly in 1X PBS-T [10 mM phosphate buffer (pH 7.2)/150 mM NaCl (pH 7.5)/0.1% (vol/vol) Tween-20] and then incubated in blocking solution [1% (wt/vol) polyvinyl pyrrolidone (PVP, average Mr 40,000) /1% (vol/vol) horse serum in 1X PBS] for at least 2 hours with gentle shaking at room temperature. The blocking solution was replaced with the above mentioned mAb solutions (1:250 dilution of anti-Den2E mAb, 1:2000 dilution of anti-his mAb) and incubation was continued for a further hour with gentle shaking. Thereafter, the blots were washed 5 times with 1X PBS-T for 10 minutes each. After washing, blots were transferred into alkaline-phosphatase conjugated secondary antibody solution (1:5000 dilution in blocking solution) and further incubated for 1 hour. The blots were washed again as described above. The protein-antibody complex was developed in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5mM MgCl2 containing 150 μg ml-1 of Nitro Blue Tetrazolium and 75 μg ml-1 5-Bromo-4-chloro-3-indolyl phosphate. The reaction was stopped after ~30 minutes of incubation in a light-proof dish, by rinsing the blot in 10mM EDTA (pH 8.0).

**CsCl Gradient analysis**

Gradient analyses were performed essentially as described before (Bisht et al., 2001; Vassileva et al., 2001). An aliquot (50-100 μl) of the purified, recombinant Den2E protein was layered on a 20% (wt/vol) CsCl gradient in 50mM Tris-HCl (pH 7.5) and centrifuged in an SW60 rotor at 35,000 rpm for 48 hours at 16-18°C in a Beckman ultracentrifuge. After the spin, the centrifuge tube was punctured at the bottom and ~0.5ml fractions of the gradient were collected and analyzed by ELISA and dot blot, using the Den2E-specific monoclonal, 3H5 mAb.
ELISA for recombinant 6X-His-tagged Den2E protein

Recombinant 6X-His-tagged Den2E protein levels in soluble cell extracts and Ni-NTA column eluates were analyzed using Ni-NTA His-Sorb plates purchased from Qiagen. Sample aliquots (~100-200 μl) were added to each well and incubated overnight at 4°C. Wells were washed 4 times with 1X PBS-T. Next, 200 μl of anti-Den2E monoclonal antibody 3H5 (1:250 dilution in 1X PBS) was added and incubated overnight at 4°C. The wells were again washed with 1X PBS-T and incubated with 200 μl of horseradish peroxidase-conjugated secondary antibody for 60 minutes at 37°C. Color development was initiated by the addition of 100 μl 3,3',5,5'-Tetramethyl Benzidine (TMB) substrate. The reaction was stopped after 30 minutes, by the addition of 100 μl 1M H2SO4.

Electron microscopy

Direct visualization of purified recombinant Den2E proteins, expressed in P. pastoris, was carried out after negatively-staining the preparation with uranyl acetate as described before (Vassilev et al., 2001). Immunogold electron microscopy of Den2E-HBsAg was done as follows. Purified recombinant Den2E-HBsAg protein (in 1X PBS) was adsorbed onto carbon coated nickel grids. The grids (sample side down) were floated upon a drop of 3% (wt/vol) casein in 1X PBS-T for 30 minutes, for blocking. After blocking, the grids were washed by floating them on drops of 1X PBS-T (3 changes of 5 minutes each). Next, the grids were incubated with the first antibody (HBsAg-specific 5S mAb, Den2E-specific 3H5 mAb or a control antibody; all mAbs at ~10 μg ml-1) at room temperature for 30 minutes. Grids were washed as before, contrasted with 2% aqueous uranyl acetate, blotted on Whatman filter paper, dried under an infrared lamp and visualized using a JEOL 1200 EXII transmission electron microscope.

Immunization of mice

Four-to-six week-old Balb/c mice were immunized with intraperitoneal injections on days 0 and 30 with 0.2ml inoculum containing ~1 μg of purified recombinant hybrid protein supplemented with Freund’s complete adjuvant (FA) for the first and with Freund’s incomplete adjuvant (IFA) for the second injections. An additional group of Balb/c mice were inoculated with 0.2ml PBS alone (with FA for the first and with IFA for the second injections), as negative controls. Immunized mice were bled 3 weeks after the booster dose, by retro-orbital puncture.

Detection of anti-Den2E and anti-HBsAg antibodies by ELISA

Antibodies specific for Den2E and HBsAg in the sera of immunized animals were detected as follows. For the detection of anti-Den2E-specific antibodies, 96-well ELISA plates were coated with dengue type-2 virus preparation (at ~10^7 PFU/well, from Biodesign). Virus-coated ELISA plates were blocked with 1X PBS containing 1% PVP and 1% horse serum (blocking buffer) and incubated with pre-immune and immune sera samples (diluted 1:10 in blocking buffer) for 1 hour at 37°C. The wells were washed 5-6 times with 1X PBS containing 0.5% Tween-20 and re-incubated for a further 60 minutes with HRPO-conjugated antimouse IgG. After washing with 1X PBS-T, TMB substrate was added and optical density measured at 450nm.

Anti-HBsAg antibody was detected using anti-HBsAg Hepanostika microELISA system (specific for anti-HBsAg antibody) purchased from Organon Teknika, The Netherlands. ELISA was performed on the immunized sera samples as per the manufacturer’s instructions. Anti-HBsAg titers were calculated as International units (IU) litre-1, using International sera standards provided in the Organon Teknika kit.

Preparation of infected cell extracts and Immunoprecipitation

Monolayers of COS1 cells at 70-80% confluency (split ~24 hours before), in 60mm plates, were infected with dengue type-2 virus. After virus adsorption for 2 hours, the infected cells were fed with fresh medium and allowed to incubate at 37°C in a humidified, 10% CO2 incubator. Three days after infection, the cells were incubated for 3 hours in methionine- and cysteine-free DMEM containing 30 μg ml-1 actinomycin D. Cells were then metabolically labeled with [35S]-methionine (100 μCi ml-1) for 3 hours, harvested and lysed in 400 μl 1X radioimmunoprecipitation buffer (RIPA) buffer [50mM phosphate (pH 7.4)/150mM NaCl/1% (vol/vol) Triton X-100/2mM EDTA/2mM PMSF/0.5% (vol/vol) soybean trypsin inhibitor]. Prior to immunoprecipitation, lysates were pre-cleared by incubation with protein-G Sepharose for 30 minutes on ice, followed by centrifugation at 13,000 rpm in the microfuge at 4°C. About 30 μl of protein G-Sepharose was incubated separately with 50 μl each of pre-immune serum, immune serum and 3H5 mAb in 100 μl 1X PBS for 30 minutes on ice and washed 3 times with 1ml of 1X PBS. Next, 100 μl pre-cleared lysate was added and allowed to incubate for 1-2 hours on ice. The resultant immune complexes were washed twice with either L or H buffer. These two buffers are similar to the RIPA buffer described above except that salt and detergent concentrations are different [100mM NaCl and 0.1% (vol/vol) Triton X-100 in L buffer, 500mM NaCl and 0.5% (vol/vol) Triton X-100 in H buffer]. Washed
immunocomplexes were boiled in 25% Laemmli sample buffer and analyzed on SDS-12.5% PAGE followed by fluorography.

**Immunofluorescence assay**

Baby hamster kidney cells (BHK 21) were seeded on coverslips ~24 hours prior to infection. Cells were infected with dengue type-2 virus, when they were 80% confluent. At 24 hours post-infection, the growth medium was removed and the cells on cover slips were rinsed three times with 1X PBS and fixed for 20 minutes in cold acetone at ~20°C. Acetone was removed and the fixed cells were rinsed once with 1X PBS and blocked for 2 hours at 37°C with 1X PBS containing 2% (vol/vol) horse serum. Cells were then incubated, for 1 hour at 37°C, with one of the following primary antibodies (diluted in blocking buffer containing 0.1% Tween-20): pre-immune serum (1:50), immune serum (1:50), anti-Den2E 3H5 mAb (1:100) or an unrelated mAb (1:100; anti-HBsAg). Cells were washed extensively and incubated further for an hour with fluorescein-conjugated anti-mouse IgG from Calbiochem [1:80 dilution in blocking buffer containing 0.1% (vol/vol) Tween-20]. After rinsing in 1X PBS, the cells were mounted in polyvinyl alcohol supplemented with 40% (vol/vol) glycerol and saturated with n-propylgallate to limit subsequent photobleeding. Stained cells were visualized under a Nikon microscope equipped for incident illumination with a narrow band filter combination selective for fluorescein isothiocyanate.

**Plaque Reduction Neutralization Test (PRNT)**

Post-immunization sera were heat-inactivated at 56°C for 30 minutes. Thereafter, the sera samples were carried through a serial two-fold dilution [in DMEM supplemented with 1% (wt/vol) bovine serum albumin] up to a maximum dilution of 1:256, in a 96-well plate. Serially diluted sera (50μl each) were then incubated with an equal volume of dengue type-2 virus (30 plaque-forming units), for 1 hour at 37°C. Aliquots (100μl) of serum-virus mixture were inoculated onto 80% confluent BHK cells in a 6-well tissue culture plate. After adsorption for 1 hour at 37°C, infected cells were overlaid with 4ml of DMEM containing 4% heat-inactivated fetal calf serum, 1% methylocellulose and antibiotics (penicillin-streptomycin and gentamicin). The cells were incubated at 37°C in a humidified 10% CO2 incubator. On day 4, cells were overlaid again and incubated for a further 3-4 days. At 7-8 days post-infection, the overlay was gently decanted and the cells were fixed with 10% formalin at room temperature for an hour. Virus plaques were counted after staining with 2% (wt/vol) crystal violet in 20% ethanol for an hour. The percent reduction in virus plaques (50% end-point) was determined with reference to a control experiment, in which the virus used for infection was mixed with pre-immune mouse serum.

**RESULTS**

**Purification and preliminary characterization of 6X-His-Den2E expressed in P. pastoris**

A recombinant clone of P. pastoris harboring the gene encoding 6X-His-tagged Den2E protein in its AOX1 locus was created by a transplacement strategy as described under Materials and Methods. This approach placed the 6X-His-Den2E gene under the transcriptional control of the strong, tightly regulated methanol-inducible AOX1 promoter. Expression of the recombinant protein was achieved by methanol induction of a logarithmically growing shake-flask culture of the recombinant P. pastoris clone. Recombinant protein was recovered from the methanol-induced cell lysate by affinity chromatography on a Ni-NTA column. Figure 2A displays the SDS-PAGE profile of the affinity purification. A single major protein band of ~43kDa was obtained after Ni-NTA purification (Figure 2A, lane 5). The absence of any discernible low molecular weight bands indicates that there is no evidence of proteolytic degradation. This finding is consistent with that of Sugrue et al. (1997a) who reported that a truncated version of the dengue type-1 E protein, lacking the C-terminal 94 aa residues (expressed as a glutathione-S-transferase fusion), is relatively stable in P. pastoris. The purity of the Ni-NTA purified recombinant protein was judged to be ~90-95%. The calculated molecular mass of this recombinant protein (401 aa residues) is 44.6kDa. A slight discrepancy (less than 4%) in the apparent electrophoretic mobility (43 versus 44.6kDa) of the recombinant Den2E protein is evident from the gel shown in Figure 2A (lane 5). However, the Western blot data in Figure 2B demonstrate the authenticity of this recombinant protein. Both 3H5 mAb (specific for Den2E; Figure 2B, lane 1) and anti-His-mAb (specific for the 6X-His tag; Figure 2B, lane 2) recognized the purified recombinant protein attesting to its identity. An unrelated mAb (anti-HBsAg mAb) failed to recognize the recombinant Den2E protein (Figure 2B, lane 3), consistent with expectation. Apart from the ~43kDa protein, the presence of additional minor, high-molecular weight bands could be discerned in lanes 1 and 2 of Figure 2B. The apparent mobility of these additional bands suggests that they could perhaps represent dimeric or trimeric forms of the recombinant protein. This observation hinted at the possibility that the recombinant Den2E protein may have the capacity to associate into multimers. This possibility was investigated by subjecting the Ni-NTA purified preparation to sedimentation through a CsCl gradient. For comparison, dengue type-2 virus was co-sedimented in a parallel gradient.
Figure 2. Purification and characterization of recombinant Den2E protein expressed in Pichia pastoris.

(A) Affinity chromatographic purification of 6X-His-tagged Den2E protein from P. pastoris lysates. Samples (indicated above lanes 2 to 5) were analyzed by denaturing polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. Low molecular weight protein markers were loaded in lane 1. Their masses (in kDa) are shown on the left of the panel.

(B) Western blot analysis of recombinant 6X-His-tagged Den2E protein, prepared in A. Aliquots of the purified recombinant Den2E protein were run on SDS-12.5% PAGE, transferred to nitrocellulose membrane and probed with the monoclonal antibodies (mAbs), indicated on top of the lanes. The position of the protein markers are indicated by dots to the right of the blot; their masses (in kDa) are indicated alongside. The arrows on the right of the panels indicate the position of Den2E protein.

Figure 3. CsCl gradient analysis of recombinant Den2E expressed in P. pastoris.
Purified recombinant Den2E protein (solid curve) and dengue type-2 virus (dotted curve) were centrifuged through a 20% (w/v) CsCl density gradient. Fractions collected sequentially from the bottom of gradient were subjected to ELISA using dengue type-2 virus-specific 3H5 mAb as described in Materials and methods. The vertical arrows indicate the bottom (fraction 1) and top (fraction 7) of the gradient. The inset shows a dot-blot assay of the gradient fractions on nitrocellulose paper.

The sedimentation profiles of the recombinant Den2E protein and dengue type-2 virus, monitored by 3H5 mAb-based ELISA are shown in Figure 3. The data show that the Ni-NTA purified recombinant Den2E preparation did indeed sediment to the bottom of the CsCl gradient (fraction 1). Dengue type-2 virus migrated to a position close to the bottom of a parallel gradient (fraction 2). Results from an analysis of the gradient fractions by a dot-blot assay (Figure 3, inset) were essentially consistent with the ELISA profiles.

Den2E-HBsAg hybrid protein, but not 6X-His-Den2E protein, assembles into virus-like particles (VLPs)

To be effective immunogens, viral proteins must be capable of forming VLPs (Murphy and Chanock, 1996). The Den2E protein has the potential to generate higher order structures. This is borne out by the observation that P. pastoris expressed Den2E protein sediments to the bottom in a CsCl gradient (Figure 3 above). We have made similar observations regarding the sedimentation behaviour of hybrid Den2E-HBsAg protein as well (Bisht et al, 2001). However, such a sedimentation analysis alone is not sufficient to ascertain if the recombinant E proteins do assemble into characteristic VLPs. To address this issue, both the recombinant Den2E (this study) as well as the Den2E-HBsAg (Bisht et al, 2001) proteins purified from P. pastoris lysates were directly visualized by electron microscopy. This investigation revealed that the recombinant Den2E protein displayed an amorphous morphology; no particle-like structures could be discerned (data not shown). On the other hand, Den2E-HBsAg was found to exist as discrete particles (Figure 4A), reminiscent of the VLPs formed by HBsAg (Vassileva et al, 2001). Further characterization of these hybrid particles by immunoelectron microscopy showed that they were recognized by mAbs against both HBsAg (Figure 4B,
main panel) and Den2E (Figure 4B, inset) based on their visualization using gold-labeled anti-mouse IgG.

Figure 4. Recombinant Den2E-HBsAg hybrid protein assembles into virus-like particles (VLPs)

(A) Electron micrograph of purified recombinant Den2E-HBsAg expressed in P. pastoris, after direct negative staining with uranyl acetate. (B) Immuno-gold electron micrograph of recombinant Den2E-HBsAg. The VLPs shown in panel A were incubated sequentially, either with anti-HBsAg mAb (main panel) or 3H5 mAb (inset), followed by gold-labeled anti-mouse IgG, contrasted with uranyl acetate and then visualized by electron microscopy.

These data demonstrate that the VLPs formed in P. pastoris by the recombinant hybrid protein contain both Den2E and HBsAg.

The hybrid VLPs elicit immune responses against both components

Having demonstrated that the hybrid Den2E-HBsAg protein assembles into VLPs, we proceeded to examine if it had the potential to elicit immune responses specific to both its components. To this end, Balb/c mice were immunized intraperitoneally with the purified hybrid particles. Sera were collected from the immunized animals and analyzed for the presence of antibodies specific to Den2E and HBsAg, using an ELISA approach. For the detection of anti-Den2E antibodies, the wells in the ELISA plate were coated with a commercially available preparation of dengue type-2 virus (from Biodesign). Bound antigen was then used to capture antibodies in the immune sera. Resultant antigen/antibody complexes were revealed using HRPO-conjugated anti-mouse IgG. The results are depicted in Figure 5. Anti-Den2E-Ab titers in the sera (1:10 diluted) of immunized animals were 2-6 fold greater than the values seen for the control animals (Figure 5, inset).

![Figure 5. Determination of anti-dengue type-2 virus antibody levels in sera of Balb/c mice immunized with recombinant Den2E-HBsAg hybrid protein by ELISA.](image-url)

The presence of antibodies against dengue type-2 virus in serum samples was detected by using a commercially available dengue virus type-2 preparation (from Biodesign) as the capture antigen, and HRPO-conjugated anti-mouse IgG as the revealing antigen. The inset shows ELISA data obtained using pre-immune (Ctrl) and immune (1-3) sera, all at 1:10 dilution. The main panel shows antibody dilution curves obtained using pre-immune (dashed curve) and immune sera (solid curve). The immune sera used for constructing the antibody dilution curve was a pool of the three immune sera used in the ELISA shown in the inset.

The three samples of immune sera were pooled and the experiment repeated at several different dilutions. The antibody dilution curve (Figure 5, main panel) shows that the anti-Den2E antibody titres elicited by the hybrid antigen are not very high. Anti-HBsAg-Ab (‘a’ epitope-specific) titers were determined using the commercially available anti-HBs kit from Hepanostika. This kit is customarily used to monitor seroconversion following immunization with HBsAg antigen. Using this kit, a titre equivalent to 10 IU anti-HBs litre-1 is generally accepted to be the minimum level required to indicate protective immunity against Hepatitis B virus (Gamelkoorn, 1991). Anti-HBsAg titres in the three sera samples tested were quite high, ranging from ~1800-2000 IU litre-1 (data not shown). From these data it is evident that the Den2E-HBsAg protein can elicit antibody responses, in laboratory animals, against both its constituent components. Further, the anti-HBsAg response is protective in nature as evaluated by the Hepanostika anti-HBs kit (Gamelkoorn, 1991).
Antibodies in sera of immunized mice recognize Dengue type-2 virus

The ELISA data above demonstrated that the sera of immunized mice contained Den2E-specific antibodies. We were interested to examine if these antibodies were capable of interacting with the replicating dengue type-2 virus. To this end, an indirect immunofluorescence assay was performed wherein dengue type-2 virus-infected BHK cells were probed with antisera obtained from mice immunized with Den2E-HBsAg particles. The data are shown in Figure 6. Panels C and D represent positive and negative controls, probed with the dengue type-2-specific 3H5 mAb and an unrelated mAb (anti-HBsAg mAb), respectively.

Figure 6. Immunofluorescence-based detection of dengue type-2 virus in infected baby hamster kidney (BHK) cells using anti-Den2E-HBsAg antiserum

BHK cells, infected with the dengue type-2 virus for 24 hours, were fixed in cold acetone, and incubated with the appropriate mAb (panel A: immune serum; panel B: pre-immune serum; panel C: 3H5 mAb, dengue type-2 specific mAb, positive control; panel D: anti-HBsAg mAb, negative control), followed by fluorescence-labeled anti-mouse antibody to visualize Den2E expression.

The immune serum did specifically recognize and interact with dengue type-2 in the infected cells as is evident from a comparison of panels A (immune serum) and B (pre-immune serum). We next tested the ability of the antisera to immunoprecipitate the Den2E protein from dengue type-2 virus-infected cells. The results are shown in Figure 7. In this experiment, dengue type-2 virus-infected COS cells were metabolically labeled with [35S]-methionine, lysed and immunoprecipitated with antiserum, obtained from immunized mice. In parallel, immunoprecipitations were also set up with pre-immune serum (negative control) and 3H5 mAb (positive control). We found that when the protein-G-bound immune complexes, generated using anti-serum from immunized mice, were washed in the presence of high salt and detergent (500 mM NaCl and 0.5% Triton X-100), the E protein could not be detected (lane 6). Under these conditions the 3H5 mAb-containing immune complexes were stable, resulting in the appearance of the expected ~60-65kDa, full-length E protein during subsequent SDS-PAGE analysis (lane 7). In addition to the E protein, a protein band of ~20kDa was also seen (lanes 2, 3 and 7). This most likely represents the prM protein, which is associated with the E protein. It has been shown that prM tends to co-immunoprecipitate with E (Wang et al, 1999). The mobility of prM seen in this experiment is consistent with that reported in literature (Wang et al, 1999). In contrast to the situation described above (lane 6), when the washing buffer contained low salt and detergent, (150 mM NaCl and 0.1% Triton X-100), the presence of the E and the prM proteins was readily detectable (lane 2). Not surprisingly both these protein bands were seen in 3H5 mAb immunoprecipitates under the milder washing conditions (lane 3). The E protein in Lanes 2, 3 and 7 appears as a doublet. We believe that the two forms represent differentially glycosylated forms, with the faster band representing the un(der)glycosylated form and the slower band representing the glycosylated form. This notion, to be experimentally verified, is in agreement with the marginal size difference reported in the literature between these two forms of the E protein (Bray et al, 1989; Delenda et al, 1994; Men et al, 1991).

Figure 7. Radioimmunoprecipitation of viral proteins from dengue virus type-2 infected COS1 cells using anti-Den2E-HBsAg antiserum

Dengue virus type-2-infected COS1 monkey kidney cells were metabolically labeled with [35S]-methionine, lysed and immunoprecipitated using pre-immune (lanes 1 and 5) or immune serum (lanes 2 and 6). In parallel, control immunoprecipitations were performed using dengue type-2-specific 3H5 mAb (lanes 3 and 7). Immunoprecipitates were washed either with buffer H (high salt, high detergent; lanes 5-7) or buffer L (low salt, low detergent; lanes 2-4) and run on SDS-12.5% PAGE. Immunoprecipitated proteins were visualized by fluorography. The positions of protein markers and their masses (in kDa) are shown to the right of the panel. The arrows, on the left, indicate the positions of E and prM.

Lanes 1 and 5 represent immunoprecipitations performed with pre-immune serum. Pre-immune serum failed to precipitate the E and prM proteins (the faint bands discernible in lane 1 is the result of spill-over from lane 2). The seroanalysis data by radioimmunoprecipitation suggests that the Den2E-
HBsAg hybrid elicits antibodies that can specifically recognize and interact with Den2E protein in infected cell lysates. However, the binding affinity of these antibodies is apparently low as the immunoprecipitated viral proteins can be visualized only under low salt and low detergent conditions (compare lane 2 with lane 6). These data suggest that the Den2E-specific antibodies may not be capable of efficiently neutralizing virus infectivity. This, indeed, turned out to be the case when the antisera samples were analyzed by plaque reduction neutralization test (PRNT) assay. Although the antisera from mice immunized with Den2E-HBsAg could specifically recognize the E protein of dengue virus type-2 (Figures 5 and 6), analysis by PRNT assay did not show any significant neutralization activity against dengue type-2 virus (data not shown).

DISCUSSION

Several lines of evidence indicate that dengue E protein is a potential vaccine candidate (Churboonchart et al, 1991; Feighn et al, 1992; Kaufman et al, 1987; Staropoli et al, 1997). As a result, considerable effort has been focused on the expression of recombinant E protein in an attempt to create a subunit vaccine candidate (Kelly et al, 2000; Men et al, 1991, 2000; Simmons et al, 1998; Staropoli et al, 1997). In this study, we have examined two versions of the E protein of type-2 dengue virus. Both were expressed using the methylotrophic yeast, P. pastoris, as the heterologous host. One version, reported recently by us (Bisht et al, 2001), was a hybrid (Den2E-HBsAg protein) generated by fusing the Den2E gene in-frame with the HBsAg gene. The purification of this hybrid protein using the HBsAg component as an affinity handle has also been described earlier (Bisht et al, 2001). The second version (Den2E protein, this study) lacked the HBsAg fusion partner. To facilitate purification of this protein from crude P. pastoris lysates we inserted a 6X-His tag at the N-terminus of Den2E.

Carboxy-terminally truncated versions of the E proteins of dengue type-1 (Sugrue et al, 1997a & b) and type-4 (Hermida et al, 2002; Pupo-Antúnez et al, 2001) viruses have been expressed in P. pastoris. Sugrue et al (1997a) found that expression of full length E protein of dengue virus type 1 (as a glutathione S-transferase fusion), in P. pastoris, was accompanied by extensive proteolysis of the recombinant protein. Deleting the carboxy-terminal hydrophobic domain of the E protein could minimize this problem (Sugrue et al, 1997a). Accordingly, we decided to use a similarly truncated version of the Den2E gene, encoding the first 395 a residues of the mature E protein, in designing our constructs. This truncated version lacks the prM-recognizing C-terminal domain implicated in particle assembly (Wang et al, 1999). We compared the ability of both these P. pastoris-expressed recombinant envelope proteins (Den2E-HBsAg and Den2E) to assemble into VLPs. Multiple lines of evidence (based on size exclusion chromatography, sedimentation analysis and ELISA using HBsAg particle-specific mAb) indicated that the Den2E-HBsAg hybrid protein has the ability to assemble into high molecular weight aggregates (Bisht et al, 2001). In this study, we observed that the Den2E protein by itself also appeared to exist as high molecular weight aggregates, based on electrophoretic mobility as well as CsCl sedimentation behavior. These data, however, do not prove decisively that the recombinant proteins can indeed form VLPs. In order to address this issue conclusively, both the Den2E-HBsAg as well as Den2E proteins were directly examined by electron microscopy. This investigation revealed that the apparently high molecular weight aggregates of Den2E protein lacked any ordered structure, indicating that the truncated version of the E protein by itself does not have the capacity to assemble into VLPs. Presumably, the property of the E protein to form VLPs is dependent upon its C-terminal region (deleted in our constructs) as well as the presence of prM, as suggested by the work of Sugrue et al (1997b) and Wang et al (1999). Thus, we speculate that the high molecular weight aggregates of Den2E observed in our experiments (Figures 2B and 3) most likely are the result of random, intermolecular disulfide bonding that presumably occur due to air oxidation during extraction. In contrast to the amorphous morphology of the Den2E aggregates, the Den2E-HBsAg protein existed as discrete particles (Figure 4), reminiscent of the HBsAg VLPs (Vassileva et al, 2001). Further, these particles were demonstrated to be composed of the recombinant hybrid protein using gold-labeled anti-mouse IgG as secondary antibody for electron microscopic visualization. The immuno-gold electron microscopic data are consistent with our earlier finding that the hybrid protein is recognized by neutralizing mAbs, specific to each of the two components of the chimeric protein (Bisht et al, 2001). The ability of HBsAg to assemble into ~20nm immunogenic particles is well documented (Cregg et al, 1987; Vassileva et al, 2001). In the light of this fact, the electron microscopic data imply that the well-ordered particulate structure of the Den2E-HBsAg hybrid protein is very likely due to retention of the inherent particle-forming potential of the HBsAg component.

Particulate antigens are recognized to be more efficient immunogens (Murphy and Chanock, 1996). Since only the Den2E-HBsAg hybrid protein displayed the potential to assemble into VLPs, it was likely to be a better immunogen (by analogy with the ~20nm HBsAg particles) than the Den2E protein, which displayed an amorphous morphology. Therefore, we restricted our studies to investigating the immune responses elicited by the hybrid protein particles. We were interested to find out if the hybrid protein could act as a bivalent immunogen, i.e., elicit immune responses specific to
both its components. The data showed that indeed the sera from immunized mice contained antibodies directed against both Den2E as well as HBsAg. We utilized a commercial kit (Hepanostika anti-HBs kit from Organon Teknika, The Netherlands), which is customarily employed to assess sero-conversion in HBsAg-immunized individuals. Anti-HBsAg antibody titres of 10 IU litre-1 and above, obtained using this kit are regarded as adequate for protective immunity against Hepatitis B virus infections (Gamelkoorn, 1991). The magnitude of anti-HBsAg response (~1800-2000 IU litre-1) elicited by the recombinant Den2E-HBsAg protein was protective in nature, as judged using this kit. The antibody titers against Den2E appeared to be quite modest in comparison to the titers against HBsAg. However, the anti-Den2E antibodies could recognize and bind to dengue type-2 virus in infected cells in tissue culture; they could also immunoprecipitate the Den2E protein (along with the prM protein) from infected cell lysates. The binding affinity of these anti-Den2E antibodies appeared to be quite low, as the E and prM proteins could be detected in the immunoprecipitates, only if the Ag/Ab complexes captured on protein G-sepharose were washed under mild conditions. This was also reflected in the inability of these antibodies to effectively neutralize dengue type-2 virus in PRNT assays. It is possible that, the weak immune response against dengue type-2 virus could be due to un-optimized amount of the recombinant protein used, formulation (adjuvants), and route of administration during immunization. A major factor that has precluded a careful optimization of all these parameters has been the low yields of the recombinant hybrid protein. Efforts are on towards maximization of expression and recovery of the purified recombinant protein.

In conclusion, this work demonstrates that the truncated version of the Den2E protein (lacking the C-terminal 100 aa residues) which, by itself, is not capable of assembling into discrete particles can be incorporated into VLPs, by utilizing HBsAg protein as a carrier. A significant finding is that these Den2E-containing VLPs are generated in the absence other dengue viral structural proteins, known to be essential for virion morphogenesis in infected mammalian cells (Wang et al, 1999). Finally, these VLPs possess the unique capacity to elicit antibody responses specific to both components of the hybrid.

Acknowledgements

HB is supported by a senior research fellowship awarded by the Council of Scientific and Industrial Research, Government of India. We thank Dr. Ana Vassileva for her help during the initial stages of this work.

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