Adenovirus Fiber Shaft Contains a Trimerization Element That Supports Peptide Fusion for Targeted Gene Delivery

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Received 24 June 2006/Accepted 26 September 2006

Adenoviral (Ad) vectors have been widely used in human gene therapy clinical trials. However, their application has frequently been restricted by the unfavorable expression of cell surface receptors critical for Ad infection. Infections by Ad2 and Ad5 are largely regulated by the elongated fiber protein that mediates its attachment to a cell surface receptor, coxsackie and adenovirus receptor (CAR). The fiber protein is a homotrimer consisting of an N-terminal tail, a long shaft, and a C-terminal knob region that is responsible for high-affinity receptor binding and Ad tropism. Consequently, the modification of the knob region, including peptide insertion and C-terminal fusion of ligands for cell surface receptors, has become a major research focus for targeting gene delivery. Such manipulation tends to disrupt fiber assembly since the knob region contains a stabilization element for fiber trimerization. We report here the identification of a novel trimerization element in the Ad fiber shaft. We demonstrate that fiber fragments containing the N-terminal tail and shaft repeats formed stable trimers that assembled onto Ad virions independently of the knob region. This fiber shaft trimerization element (FSTE) exhibited a capacity to support peptide fusion. We showed that Ad, modified with a chimeric protein by direct fusion of the FSTE with a growth factor ligand or a single-chain antibody, delivered a reporter gene selectively. Together, these results indicate that the shaft region of Ad fiber protein contains a trimerization element that allows ligand fusion, which potentially broadens the basis for Ad vector development.

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The mechanisms that regulate Ad cell entry and Ad-mediated gene delivery involve receptor-mediated endocytosis. Thus, Ad2 and Ad5 use their fiber protein to attach with high affinity to a cell surface receptor, coxsackie and adenovirus receptor (CAR) (5, 7, 45). Cell surface-bound Ad is then internalized by receptor-mediated endocytosis initiated by the interaction of the Ad penton base protein with αv integrins (32, 52). Cells or tissues that lack CAR expression are highly resistant to Ad infection, while those that lack αv integrin expression suffer from a reduced rate of virus cell entry and poor efficiency of gene delivery (15, 52). Accordingly, strategies to genetically alter Ad tropism have focused mainly on the modification of viral capsid proteins, particularly on the fiber protein due to its role as the tropism determinant (49). However, such modification has been limited due to the trimeric nature of the fiber protein (28).

The Ad5 fiber protein is encoded by a single gene that expresses a polypeptide of 581 amino acid (aa) residues and exists as a homotrimer with an apparent molecular mass of 200 kDa (10). The monomeric fiber protein is composed of an N-terminal tail of approximately 47 aa residues that interact with the penton base protein of the capsid, a long, thin shaft comprising 21 pseudorepeats of 15 aa and an ~180-aa C-terminal globular head (also known as knob) that is responsible for cellular attachment. The formation of trimeric fiber is essential for its function and its assembly onto virus particles. It was found that the fiber knob at the C-terminus, but not the N-terminal half (1 to 260 aa), was required for fiber trimer formation (23) since fiber proteins containing the knob and two or more shaft repeats form functional trimers. Those find-
ings have since been used to guide fiber modification for improved gene delivery, including peptide insertion at the knob region or peptide fusion at the fiber C terminus (11, 19, 21, 27, 28, 35). In most cases, the modified viruses retain the native tropism while regaining some specificity associated with the inserted oligopeptides. More specific modifications require the use of trimerization elements, such as the bacteriophage T4 fibritin or the neck region of human lung surfactant protein D (14, 19, 21, 22, 24, 26, 33, 35, 40, 47).

Studies on fiber crystal structure shed light on the trimeric nature of the Ad fiber protein (48). The basic framework of the structure is maintained by the hydrophobic core along the shaft and the conserved inter- and intrachain hydrogen bonds, supplemented by nonconserved inter- and intrachain hydrogen bonds and salt bridges involving side chains. This structure is a highly cross-linked one that, together with a high proportion of buried surface, accounts for the high rigidity and stability of the shaft. Furthermore, the formation of a complete 360° spiral turn by seven repeats coincides with the fact that both Ad2 and Ad5 contain 21 repeats, underlining the importance of the seven repeats as a potential folding element for the formation and stabilization of the fiber trimer. We hypothesize that, because of these interactions, the fiber shaft region contains an independent trimerization element(s).

To test this hypothesis, we prepared a series of shortened fiber proteins containing the N-terminal tail and shaft repeats and investigated whether these fragments formed trimers. We show here that these knobless fiber fragments indeed formed stable trimers. Furthermore, we demonstrated that these fragments supported peptide fusion of protein motifs as large as a single-chain antibody (scFv). Modified Ad with the shaft fragment lost gene delivery capacity, while those targeting cell surface receptors regained ligand-mediated specificity. The identification of the fiber shaft trimerization element (FSTE) and the demonstration of its ability to support ligand fusion for targeting gene delivery may provide a platform for the generation of tissue-specific Ad.

MATERIALS AND METHODS

Cell lines and reagents. Cell lines were obtained from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, t-glutamic acid, and nonessential amino acids (Invitrogen). Anti-human insulin-like growth factor 1 (IGF-1) antibody was purchased from Sigma (St. Louis, MO). The monoclonal 4D2 antibody, specific against the N-terminal tail of Ad fiber, was purchased from Lab Vision (Fremont, CA), and the rabbit polyclonal anti-Ad5 fiber protein antibody was raised against recombinant Ad5 fiber protein and recognizes both monomeric and trimeric fiber proteins. The horseradish peroxidase-conjugated secondary antibody was from Sigma. The pAdEasy-1 and pAdTrack plasmids for the generation of recombinant Ad (18) were kindly provided by Bert Vogelstein (Johns Hopkins University). The cDNA of a single-chain antibody (F11-39) to human carcinoembryonic antigen (CEA) was cloned into pCR2.1 (Invitrogen). The plasmids were used as templates for the expression of fiber shaft proteins.

Constructions for fiber shaft protein expression. Fragments corresponding to the N-terminal tail and the shaft region of Ad5 fiber were generated by PCR amplification using wild-type (wt) Ad5 fiber gene as a template. The sequences of the oligonucleotides used to generate these constructs are as follows. The forward primer for all constructs (BamHI; the restriction site is underlined) was 5′-GATATCGATGGATGAGGCAGGCAAGCAGGTC. Reverse primers were (XbaI) 5′-TCTAGAGCCCGTGTTGCCTGATGTTGCC (F397); 5′-TCTAGACGTTAAT-GGCACGTGTGC (21 repeats [21R]); 5′-TCTAGAGTATACAGCGCTCTGTTT (14R); 5′-TCTAGACGTCGAGGCAGAGGATGAG (9R); 5′-TCTAGACCGTGCGGCTAGGAG (7R); and 5′-TCTAGACCGTGCGGCTAGGAG (6.5R). All oligonucleotides contain a stop codon (TAG) created by a frame shift with an inserted G or C prior to the XbaI site. The PCR products were ligated into pCR2.1 for automated sequence analysis and subcloning. The PCR products in pCR2.1 were then digested with AgeI (an internal site of fiber gene, 61 bp from the initiation site of Ad5 fiber gene) and XbaI. The Ad5/XbaI fragments were used to replace the existing Ad5/XbaI segment of the wild-type Ad5 fiber gene in pDV60, a plasmid previously prepared for fiber protein expression. To control the expression of a chimeric fiber-modified Ad2 cell was transduced with a plasmid that contained the 9R FSTE (18). The plasmid used for the expression of fiber shaft proteins was transiently transfected into HEK292T cells by using calcium phosphate precipitation or FuGENE 6 reagent (Roche) for 40 h. The cells were lysed with a buffer containing 1% NP-40. For protein separation under reducing conditions, solubilized proteins were heated at 95°C for 10 min in Laemmli sample buffer containing a reducing reagent. For the separation of proteins under nonreducing conditions, the protein samples were mixed with Laemmli sample buffer without reducing reagents and loaded onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Novex Tris-glycine gradient (Invitrogen) gels for separation. Protein expression and trimer formation were detected by immunoblotting against the fiber proteins.

Construction of 9R FSTE-modified adenoviral vectors. To replace the wild-type fiber gene of the Ad5 genome with a 9R FSTE, two fragments from the SpeI site (nucleotide [nt] 27241 of pAdEasy-1; sequence information was obtained from www.lazyDNA.org) to the end of the 9th shaft repeat (nt 29100) were amplified and subcloned into a shuttle vector derived from pCR2.1. The plasmid was then used to infect HEK293 cells. A SpeI/KpnI fragment from a resulting plasmid, which was designated pCR2.1-9R, was used to rescue the Ad5 genome by homologous recombination with SrfI-linearized pAdEasy-1 in Escherichia coli strain B5183 (18). The rescued plasmid contained the 9R FSTE in the position of the wild-type fiber gene and was therefore named pAd(9R). pAd(9R)/EGFP, a plasmid that has 9R FSTE and the enhanced green fluorescent protein (EGFP) reporter gene, was prepared by homologous recombination of pAdTrack and pAd(9R) (18).

To test protein expression and trimer formation, constructs for the expression of fiber shaft proteins were transiently transfected into HEK293T cells by using calcium phosphate precipitation or FuGENE 6 reagent (Roche) for 40 h. The cells were lysed with a buffer containing 1% NP-40. For protein separation under reducing conditions, solubilized proteins were heated at 95°C for 10 min in Laemmli sample buffer containing a reducing reagent. For the separation of proteins under nonreducing conditions, the protein samples were mixed with Laemmli sample buffer without reducing reagents and loaded onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Novex Tris-glycine gradient (Invitrogen) gels for separation. Protein expression and trimer formation were detected by immunoblotting against the fiber proteins.

Adenovirus preparation. To prepare adenoviruses that have modified fiber protein (9R FSTE or 9R-chimeric fiber proteins), Pacl-digested pAd(9R)/EGFP, pAd(9R-IGF)/EGFP, pAd(9R-IGF)/EGFP, or pAd(9R-CEA)/EGFP was transfected into HEK293 cells. Due to the reduced infectivity of the 9R or the chimeric fiber-modified Ad in 929 cells, a 293 cell line that constantly expresses wild-type Ad5 fiber protein instead of the parental 293 cells was transfected. The Ad recovered from these cells thus contains both wild-type and modified fiber protein. The stock virus was then used to infect HEK293 cells for the preparation of fiber-modified Ad. The viruses were purified on CsCl and dialyzed against Tris-buffered saline (20 mM Tris-HCl, pH 8.1) as previously described (19). The titers of the purified viruses were determined by measuring protein content using bovine serum albumin as a standard. The conversion equation of 1 μg protein equals 4 × 10^17 virus particles (vp) was used.

The identity of the modified Ad was characterized by PCR with primers that anneal to the 5′- and 3′-end flanking sequences of the Ad5 fiber gene (50) and by immunoblotting analysis for the presence of fiber proteins under both reducing and nonreducing conditions. For the separation of purified Ad under nonreducing conditions, the virus was incubated at a low pH (by the addition of 2 M acetic acid to a final concentration of 100 mM) at room temperature for 5 min. At a low pH, the fiber protein disassembles from Ad (19). The samples were then mixed with Laemmli sample buffer without reducing reagents and loaded onto a Novex Tris-glycine gel for separation. All modified viruses contained the modified fiber but not the wild-type fiber.
Assay for adenovirus-mediated gene delivery. Fiber-modified Ad [Ad(9R)/EGFP, Ad(9R-IGF)/EGFP, and Ad(9R-CEA)/EGFP] or an Ad5 with wild-type fiber protein (Ad/EGFP) (32) was incubated with detached cells on ice for 60 min. For the blocking assay, cells were preincubated with a blocking reagent (recombinant Ad5 fiber protein, His-tagged anti-CEA scFv protein, or anti-IGF-1 at 20 \( \mu \)g/ml) prior to the addition of Ad. After the removal of unbound virus by washing with ice-cold phosphate-buffered saline, the infected cells were cultured at 37°C. Infectivity was detected microscopically by direct immunofluorescence or by flow cytometry analysis for EGFP expression. The experiments were performed in duplicate, and the data were expressed as averages ± errors.

RESULTS

The Ad5 fiber shaft forms stable trimer independently of the C-terminal knob. We hypothesized that the Ad2/5 fiber shaft region contained the stabilization elements for fiber trimer formation due to the existence of hydrogen bonds and hydrophobic interactions. In addition, a unit composed of approximately seven shaft repeats may have the ability to form stable trimers. We thus generated a series of constructs corresponding to the N-terminal tail and the first 6.5, 7, 7.5, and 9 shaft repeats, respectively. The cDNA was cloned into a pcDNA3-based vector for protein expression in 293T mammalian cells. The expressed proteins were designated 6.5R, 7R, 7.5R, and 9R, respectively, based on the numbers of their shaft repeats. Protein expression and trimer formation were analyzed by immunoblotting analysis against fiber protein (Fig. 1A). Under reducing conditions, monomeric proteins were detected from the lysates of transfected cells. Under nondenaturing conditions, both monomeric and oligomeric proteins were detected in these samples. The major bands ranged from 50 kDa for the 6.5R fragment to 65 kDa for the 9R fragment, respectively, indicating that the N terminus of Ad fiber protein supported trimer formation. In further studies, we found that proteins corresponding to the first five or six shaft repeats did not form stable trimer (data not shown), suggesting a requirement for a minimal number of shaft repeats for protein oligomerization.

To investigate the contribution of the 360° turn to fiber shaft trimerization, we then generated constructs for the expression of both 14R and 21R fiber shaft proteins. These, comprising two and three turns of 360°, respectively, also formed trimer (Fig. 1B). The spacer region is known to destabilize fiber trimerization (23, 39), and consistent with this observation, no trimeric proteins were detected from cells that were transfected for fiber 1-397 (F397) protein expression (Fig. 1B).

Together, these data demonstrated that the N terminus of the fiber protein formed stable trimers independently of the C-terminal knob. Furthermore, these findings also indicated the importance of seven shaft repeats as a unit in fiber shaft trimerization. We thus designated these N-terminal fiber fragments fiber shaft trimerization elements.

The fiber shaft trimerization element assembles onto Ad particles. We next prepared an Ad that contained a FSTE instead of wild-type fiber and investigated whether a shortened fiber (9R FSTE) could assemble onto Ad virions. To this end, the fiber gene in pAdEasy-1 was replaced with 9R FSTE by homologous recombination (Fig. 2A). Although 6.5 to 9R FSTEs also formed stable trimers, we chose the 9R FSTE since we reasoned that the extra repeats in the 9R fiber might serve as a spacer for peptide presentation. The resulting plasmid, pAd(9R), which contained the 9R FSTE in the position of the wild-type fiber gene, was used for the generation of a reporter...
gene containing the plasmid pAd(9R)/EGFP by homologous recombination with pAdTrack (18) (Fig. 2B). pAd(9R)/EGFP was subsequently transfected to 293 cells to rescue the recombinant adenovirus.

The presence of the 9R FSTE gene and the protein of the purified virus were verified by PCR amplification using specific primers (data not shown) and immunoblotting analysis. As seen in Fig. 2C, the fiber protein of the Ad(9R)/EGFP virus migrated as a 20-kDa protein under reducing conditions; the same antibody detected a protein band of 65 kDa under non-denaturing conditions, indicating a trimeric 9R FSTE protein assembled onto the Ad virions. In addition, the relative intensities of the antifiber and anti-penton base signals of Ad(9R)/EGFP and the unmodified Ad (Ad5/EGFP) were measured and the ratios of these two signals of different viruses were compared to assess the degree of fiber assembly. The relative ratio of antifiber to anti-penton base signals of Ad(9R)/EGFP was 0.24, whereas that of the unmodified Ad was 0.30, suggesting that the 9R FSTE efficiently assembled onto Ad. To our knowledge, this represents the first study demonstrating that the shaft region of Ad fiber protein contains an independent trimerization element.

Ad modified with FSTE loses infectivity in Ad5-susceptible cells. Ad5 tropism is determined mainly by the interaction of the C-terminal fiber knob with the cell surface receptor CAR. Thus, Ad(9R) was anticipated to have a reduced infectivity due to the deletion of the knob region. This assumption was validated using an infection assay. We found that, compared to Ad5/EGFP, which delivered EGFP to over 90% of the A549 cells at a multiplicity of infection (MOI) of 10 viral particles/cell, Ad(9R)/EGFP failed to transduce A549 cells. Dotted lines, uninfected control. Solid lines, wild-type Ad(9R) virus.
cell (Fig. 2D), Ad(9R)/EGFP failed to transduce A549 cells even at an MOI of 3,000 particles/cell. These findings were consistent with the role of the fiber knob in mediating Ad infection. In addition, these results also demonstrated that the modification of Ad with FSTE ablated Ad gene delivery capacity.

Previous studies have mapped the trimerization domain of Ad fiber protein to its C-terminal knob region (19), a region which also mediates the high-affinity binding of Ad to its cell surface receptor. Based on the principle derived from these studies, modifications of fiber protein to improve gene delivery efficiency have been performed mainly within the content of the fiber knob. However, we found that the N-terminal shaft region of Ad fiber contained a novel trimerization element which is independent of the C-terminal knob. In contrast to the knob region, the C terminus of the FSTE imposes less steric hindrance due to the absence of the bulky knob region. This feature might facilitate the fusion of peptides or protein motifs of greater complexity.

The fiber shaft trimerization element accommodates C-terminal fusion of protein motifs. To explore the possibility that the newly identified FSTE could support peptide fusion, we chose human IGF-1, a peptide of 70 amino acid residues, and a single-chain antibody (25) as fusion partners (Fig. 3A). The fiber protein of Ad5 has been previously replaced with the counterpart of different Ad serotypes or a scFv with the use of the fibrinogen trimerization domain (19); however, the sequences of IGF-1 and anti-CEA scFv represent the largest peptides or protein motifs thus far to be directly fused onto Ad fiber protein or any Ad proteins.

The modified Ads, designated Ad(9R-IGF) and Ad(9R-CEA), were generated using standard protocols (18) and those described in Materials and Methods for the preparation of Ad(9R)/EGFP. Chimeric 9R-IGF-1 was detected as a protein of 90 kDa with an antifiber antibody relative to that of a 200-kDa band from the unmodified Ad under nondenaturating conditions (Fig. 3B). The same protein of Ad(9R-IGF)/EGFP migrated as a 30-kDa protein band after separation under reducing conditions. The chimeric nature of the 9R-IGF-1 protein was verified by immunoblotting with an anti-IGF-1 antibody (B, middle panel). MW, molecular weight in thousands; PB, anti-penton base protein as loading controls.
Ad5 infection due to the lack of appropriate receptor expression (45), express IGF-1 receptor which recognizes human IGF-1 (4, 37). NIH 3T3 cells were infected with Ad(9R-IGF)/EGFP or Ad5/EGFP, and the ability of these viruses to deliver the EGFP gene was analyzed by measuring GFP expression (Fig. 4A). As a positive control for infection, we also included A549 cells. In contrast to wild-type (wt) Ad5, which failed to deliver EGFP to NIH 3T3 cells at an MOI as high as 1,000 vp/cell, Ad(9R-IGF)/EGFP could transduce 13% NIH 3T3 cells at 100 vp/cell. In addition, Ad(9R-IGF)-mediated gene delivery was found to be dependent on IGF-1 protein fusion since pretreatment of this virus with a function-blocking anti-IGF-1 antibody blocked Ad-mediated gene delivery to NIH 3T3 cells. (B) Gene delivery to colorectal carcinoma cells with an Ad equipped with an anti-CEA antibody. Monolayers of Caco-2 cells in duplicate were infected with wild-type Ad or 9R-CEA-modified Ad at 100 vp/cell. Ad-mediated gene delivery was detected by fluorescence-activated cell sorter analysis. The specificity of the anti-CEA scFv in mediating gene delivery was verified with a recombinant protein of anti-CEA scFv (rscFv). Data are presented as averages ± errors and are representative of two independent experiments.

**DISCUSSION**

In this study, we identified a novel trimerization element within the Ad fiber protein. In addition, we demonstrated that a series of shortened fibers consisting of 6.5 to 9 shaft repeats, along with the N-terminal tail, assembled into stable trimers that also incorporated into mature Ad particles. The N-terminal trimerization element, designated FSTE, could serve as a versatile platform for the generation of tissue-specific Ad vectors. In this regard, we demonstrated that peptides or protein motifs could be fused to the N terminus of the trimerization element. Notably, trimeric fiber formed without the use of extrinsic trimerization motifs as reported recently (19, 21, 28, 33, 47).

Recent X-ray structure studies as well as biochemical analyses of fiber protein folding suggested that the shaft region contains stabilization elements for the fiber protein (36, 48). It was observed that a complete 360° rotation within the shaft could provide a stable conformation for hydrogen bond formation and hydrophobic interactions (48). Consistent with this observation, we found that proteins containing approximately 7, 14, and 21 shaft repeats all formed stable trimers (Fig. 1). In addition, a complete turn of 360° may be irrespective of whether the turn is supplied by the proximal repeats for fiber shaft trimerization since truncated fiber proteins containing deletions of the middle 6 or 12 repeats, but not 4 repeats, also formed stable trimers (23, 41).

Among the 50 or more serotypes of human Ad, the length of the shaft ranges from 5 repeats for Ad3, Ad7 and Ad11 to 7 repeats for Ad8 and Ad16 and up to 21 repeats for Ad2 and Ad5 and to 22 complete repeats for Ad12 (10, 48). The fibers of Ad5 and Ad16 or Ad12 likely share folding geometry similar to that of Ad2 and -5, while Ad3 fiber may have adopted a different configuration since the average length per shaft repeat of Ad3 is longer than that of Ad2 or Ad5 (10). A search of the Ad3 fiber shaft region against the protein database revealed that the amino acid sequence shares a higher homol-
ogy with the coiled-coil T(ii) region of the reovirus cell attachment σ1 protein (8, 13, 38) than with the β-spiral shaft of the Ad2 fiber (48). We speculate that some of the 11 repeats in Ad40 and Ad41 fibers may serve as a long spacer for the fiber knob since a flexible region in the shaft is critical for efficient fiber-receptor interactions (53). We indeed found that the 9R fiber, which may use the eighth and the ninth repeats as a spacer for the 7R fiber element, formed a stable trimer and supported peptide fusion.

The homotrimeric fiber protein attaches to Ad virion particles noncovalently with its N terminus, whereas the C terminus is essential for mediating Ad cell attachment and Ad infection. The trimerization domain has previously been mapped to the C-terminal knob region (23, 39). Therefore, efforts to improve Ad-mediated gene delivery have been focused primarily on the modification of the fiber knob region, including peptide insertion and fusion. Such modifications have yielded viruses of tissue specificity. However, both peptide insertion or fusion at the C terminus are limited by their tendencies to impair fiber trimerization (11, 28). The incorporation of exogenous trimerization elements, such as the foldon domain from the bacteriophage T4 fibrin and the neck region of surfactant D, has resulted in modified Ad with targeting ability. The identification of a novel trimerization element within the shaft region and the demonstration of its ability to support direct fusion of tissue-specific ligands broaden the basis for vector modifications.

It is worth noting that the assembly of 9R-CEA protein was less efficient than that of 9R or 9R-IGF protein on the corresponding virions. We found that the level of 9R-CEA protein on Ad(9R-CEA) virus was about 20% of that of the wild-type Ad, Ad(9R), or Ad(9R-IGF) virus (Fig. 3), suggesting that the assembly of a large protein motif like an scFv might affect chimeric fiber assembly. We are currently investigating whether the use of a flexible linker will improve the degree of chimeric fiber assembly. Nonetheless, our data obtained thus far demonstrate that the FSTE can support peptide fusion.

Ad5 vectors are particularly efficient at inducing cellular and humoral immune responses in a number of model systems, which is advantageous for the induction of mucosal immunity against sexually transmitted infections (3, 30). Several vaccine regimens involving Ad have been shown to afford partial protection in rhesus monkey against multiple AIDS virus isolates (31, 44). Although the prospect of such human immunodeficiency virus vaccine regimens remains to be determined, preliminary data of early-phase clinical studies suggest that Ad vaccines can elicit robust cellular immune responses in humans (43). However, the high prevalence of preexisting immunity to Ad5 in human populations may potentially limit the application of Ad5 vectors. The importance of the vector system described here is underlined by its usefulness in generating Ad of altered tropism, which, in turn, may avoid the neutralizing antibody response while retaining the immunogenicity.

ACKNOWLEDGMENTS

We thank Glen R. Nemerow for scientific discussion and support, Daniel von Seggern for providing the pDV60 plasmid, Swati Brown and Elaine Fukuda for technical assistance, and Patricia Rutledge and Joan Gausepohl for administrative assistance. This work was supported by an NIH grant (CA98160).


