Vault Nanoparticles Containing an Adenovirus-Derived Membrane Lytic Protein Facilitate Toxin and Gene Transfer

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ABSTRACT Nonviral methods of gene delivery possess several advantages over that of viral-based vectors, including having increased safety. However, the ability to achieve effective transport of therapeutic molecules across host cell membranes *via* nonviral methods remains a significant goal. Cell-derived nanoparticles known as vaults have been proposed as novel candidate transfer vehicles for various foreign molecules. Recombinant vault particles enter cells *via* macropinocytosis or phagocytosis but lack demonstrable membrane penetrating activity. To explore the feasibility of improving vault penetration into target cells, we incorporated the membrane lytic domain of adenovirus protein VI (pVI) into the interior of recombinant vault particles *via* fusion to the vault poly(ADP-ribose) polymerase (VPARP) interaction domain. The membrane lytic activity of the pVI domain was retained upon incorporation into vault particles. Moreover, internalization of vault-pVI complexes into murine macrophages promoted co-delivery of a soluble ribotoxin or a cDNA plasmid encoding GFP. These findings indicate that vault particles can be modified to enhance cell transfer of selected biomolecules.

KEYWORDS: adenovirus · protein VI · vault · nanoparticles · gene delivery · membrane penetration · ribotoxin

Substantial progress has been made on the use of different viral vectors for gene transfer *in vivo*. Nonetheless, significant safety concerns have yet to be fully overcome with any of the viral agents currently under investigation. In addition, pre-existing immunity to a particular viral vector can reduce efficacy by eliminating transduced cells or by preventing repeat administration. Thus, various nonviral delivery methods, including those involving synthetic polymer nanoparticles,[1,2] are under investigation to alleviate some of the safety and efficacy issues inherent in the use of replication-defective or conditionally replicating viral vectors.[3,4] However, nonviral methods of gene transfer, representing approximately 25% of all clinical gene transfer studies overall, remain relatively inefficient compared to viral-mediated gene transfer. Recent studies suggested that naturally occurring, barrel-shaped particles known as vaults deserve consideration as potential vectors for the delivery of therapeutic cargo.[5]

Vaults are evolutionarily conserved ribonucleoprotein particles that are present in large numbers in the cytoplasm of most eukaryotic cells.[6] Although their role in normal cell physiology has yet to be fully elucidated, previous studies raised the possibility that vaults can participate in multidrug resistance.[7] More recent findings suggest that vaults may also play a role in the innate immune response to microbial pathogens.[8] These host cell particles have a molecular mass of 12.9 ± 1 MDa and are composed of a thin (20 Å) protein shell surrounding an internal cavity volume of 5×10^7 Å^3.[9,10] Vaults are composed of three proteins, the major vault protein (MVP), the vault poly(ADP-ribose) polymerase (VPARP), the telomerase-associated protein 1 (TEP1), and a small RNA (86−141 bp) termed vRNA.[11] Cryoelectron microscopy and image reconstruction studies[10] revealed the structure of vaults at ~22 Å resolution, and a recent 8.8 Å structural model has been proposed based on a medium resolution X-ray crystallographic study.[12] These analyses demonstrated that vaults are hollow, barrel-like particles with two protruding caps and an invaginated waist with overall dimensions of 420 × 420 × 750 Å, larger in mass and size than some icosahedral viruses. MVP is thought to be present in 96 copies per vault, based on the observed symmetry of the particle and the fact that MVP accounts for 75% of the total protein mass of the particle. Expression of recombinant MVP alone in insect cells allows the formation of vault-

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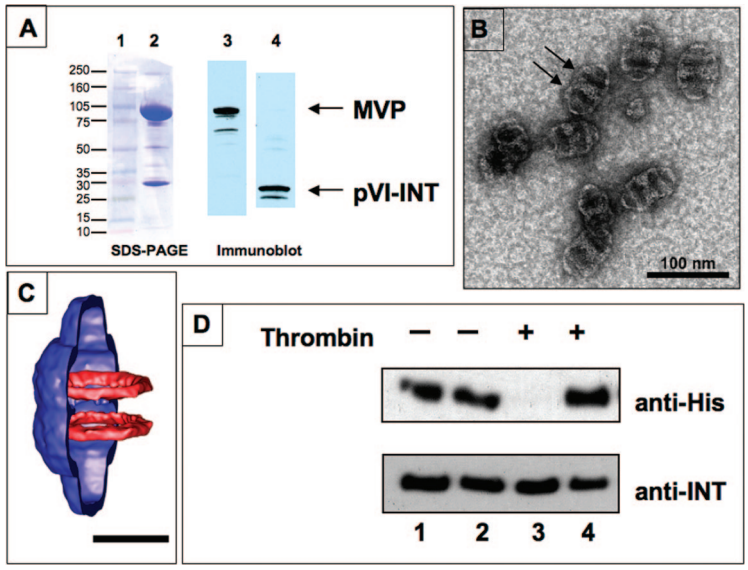

Figure 1. Incorporation of adenovirus pVI into recombinant vault particles. (A) Recombinant vaults were incubated with Ad pVI(34−114)-INT, purified, and fractionated on SDS-PAGE followed by staining with Coomassie or immunoblotting. Molecular weight standard (lane 1), purified vaults (lane 2). The immunoblots of purified vaults were probed with either anti-MVP monoclonal antibody 1023 (lane 3) or with rabbit polyclonal antisera directed against Ad pVI (lane 4). These results confirm the in-frame fusion of Ad pVI with the INT domain and co-purification with recombinant vaults. (B) Negative stain TEM image of vaults containing pVI-INT. Note the presence of additional protein density (lighter staining) near the waist of the vault barrel, which based on earlier structural studies is the expected location of pVI-INT. This extra density causes an adjacent depression in the particle where stain collects in two bands (indicated by arrows). (C) Cropped MVP vault cryoEM reconstruction showing the superimposed density of luciferase-INT in two rings near the waist. Scale bar is 250 Å. Courtesy of Dr. Phoebe Stewart at Vanderbilt University. (D) Immunoblot of thrombin cleaved proteins. The N-terminus of pVI-INT encodes a 6-His tag and thrombin cleavage site derived from the pET28 expression vector. Purified pVI-INT protein (lanes 1 and 3) or purified recombinant vaults containing pVI-INT (lanes 2 and 4) were either treated with thrombin (lanes 3 and 4) or not treated (lanes 1 and 2). Following digestion, the samples were separated on SDS-PAGE and probed by immunoblotting with either anti-His tag antibody (upper panel) or anti-INT antibody (lower panel). The pVI-INT protein is protected from thrombin digestion in the recombinant vaults but not when treated alone (compare lanes 3 and 4). Note that only 17 amino acids are cleaved off of pVI-INT and thus no change in pVI-INT mobility is observed.

like particles with a structure similar to wild-type particles.[10,13] Exogenous proteins fused to the interaction domain (INT) derived from VPARP can also be incorporated into the interior of recombinant vaults, and these proteins are functional.[5] Importantly, the dynamic nature of vault particles allows exposure of their interior (and presumably their cargo) to the cellular milieu.[14] The "breathing" of vault particles appears to be facilitated by exposure to low pH,[15] a process that would be expected to occur at greater frequency in the interior of cell endosomes.

Recombinant vault particles can be taken up by HeLa cells in culture, presumably into endosomes,[5] and specific targeting of the particle is possible by expression of targeting domains on the outside of the particle at the caps.[16] Despite the potential utility of vaults as gene transfer carriers, these nanoparticles have not been reported to display cell membrane penetrating activity, thus potentially limiting their cell transducing capacity. In an effort to overcome this deficiency, we sought to incorporate the membrane lytic domain of adenovirus protein VI into recombinant vault particles. Adenovirus internalization *via* endocytosis and the reduced pH of the endocytic compartment facilitates partial disassembly of the viral capsid concomitant with the release of protein VI.[17] The N-terminal region of pVI contains a putative amphipathic α-helical domain (amino acid residues 34−54) that exhibits potent membrane lytic activity as measured by the disruption of artificial lipid membranes (liposomes).[17] Thus, the studies reported herein were performed to examine whether the incorporation of the N-terminal domain of pVI into vault particles could increase membrane penetration as well as the co-delivery of reporter molecules.

RESULTS AND DISCUSSION

The delivery of therapeutic molecules to specific host tissues by viral or nonviral gene methods presents a significant challenge in terms of maintaining safety and achieving efficacy. Currently, nonviral methods have focused on the use of cationic liposomes as carriers for *in vivo* gene/drug transfer.[1,18−22] Although this approach appears to be well suited for local delivery, systemic administration has proved more difficult due to liposome aggregation and/or a failure of these particles to penetrate deep into target tissues. Thus, the application of other approaches may be required to achieve success in this arena.

Previous biochemical studies demonstrated that exogenous proteins could be targeted into the vault particle when fused to an MVP interaction domain (INT, amino acid residues 1563−1724 derived from the vault-associated VPARP protein[5,14]). Such fusion proteins bind with high affinity to an MVP binding site at the interior of the particle.[5] We reasoned that fusion of the INT domain to the membrane lytic domain of adenovirus protein VI (amino acid residues 34−114)[17] could allow this fusion protein to be targeted to the interior of recombinant vault particles. Incubation of recombinant MVP and pVI-INT proteins allowed the formation of a macromolecular vault complex that could be isolated by density gradient ultracentrifugation. The purified vaults contained both MVP as well as pVI-INT as indicated by immunoblot analyses (Figure 1A). On the basis of densitometric analysis of Coomassie stained SDS-PAGE, we estimate that, on average, each vault particle contains 20−30 molecules of the pVI-INT protein. This number is consistent with packaging of multiple copies of other INT fusion proteins into recombinant vaults.[14,5] Gas-phase electrophoretic mobility molecular analysis (GEMMA) indicates that INT fusion proteins are packaged into all

vaults (as the entire vault population shifts to a higher molecular mass; ref 14 and unpublished data). With an estimate of 20−30 pVI-INT proteins per vault, it is likely that this is at or near a saturating level for this size protein, suggesting that it is unlikely that there are any empty vaults in the population.

The pVI-vault complex also exhibited a very similar sedimentation profile on sucrose gradients as vault particles containing the INT domain fused to luciferase,[5] suggesting that incorporation of pVI-INT did not impact the normal structure of recombinant vault particles. To verify this, we examined purified vault-pVI complexes by negative stain transmission electron microscopy (Figure 1B). Vault-pVI complexes exhibited the characteristic barrel shaped morphology of vaults with additional protein density (seen as a lighter band where stain was excluded) near the "waist" of the vault barrel. This additional density results in two darker stained bands at areas of depression above and below the particle waist (Figure 1B arrows). This staining pattern is consistent with the previously established morphology of vaults containing recombinant-INT fusion proteins. This is illustrated in the cryoelectron microscopic reconstruction shown in Figure 1C, which was derived from recombinant vaults containing a luciferase-INT fusion protein that was localized by difference mapping (red bands).

We next confirmed that the pVI-INT protein was indeed located inside the vault particles using a protease protection assay. Vault-pVI particles (Figure 1D, lanes 2 and 4) or the purified pVI-INT protein (Figure 1D, lanes 1 and 3) were subjected to digestion with thrombin to determine if specific sites within the pVI-INT fusion protein were accessible to this protease. When the protease-treated samples were analyzed by immunoblotting with an anti-His tag mAb, the vault-pVI-INT complexes were resistant to thrombin digestion (Figure 1D, lane 4). In contrast, soluble recombinant pVI-INT alone, which had not been incorporated into vaults, was susceptible to thrombin cleavage (Figure 1D, lane 3). Control samples treated with thrombin and analyzed with an anti-INT polyclonal antibody did not reveal protease susceptibility, consistent with the retention of the C-terminal INT domain. Taken together, these findings indicate that pVI-INT could be incorporated into the interior of recombinant vaults without compromising vault assembly or gross morphology.

However, it remained important to determine whether the membrane lytic activity of this Ad-derived protein was retained upon fusion to the relatively large INT domain. We therefore measured the membrane lytic activity of pVI-INT fusion proteins alone or pVI-INT fusion proteins incorporated into vault particles using artificial membranes (liposomes) containing an entrapped fluorophore (sulforhodamine B) (Figure 2). The fluorescence emission of this dye is quenched at the high local concentrations of the liposome, but upon

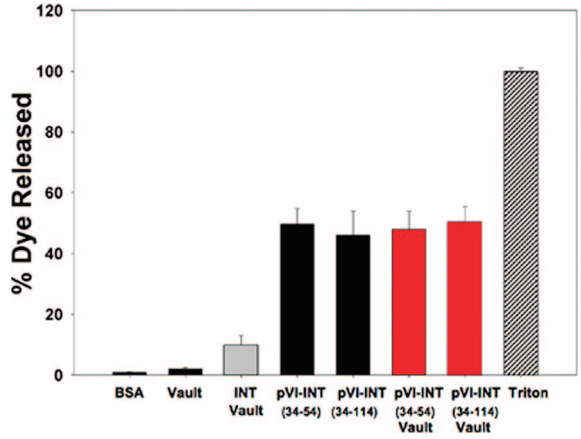

Figure 2. Disruption of liposomes by recombinant pVI-INT or pVI-INT incorporated into vaults. Unilamellar liposomes containing sulforhodamine B were used to measure fluorescence (dye release) at 585 nm in a cuvette with constant stirring. Background fluorescence was recorded for 60 s, and then the liposomes were incubated at 37 °C with 1 μg of different recombinant pVI-INT proteins (black bars) or 137 μg of vault-INT (gray bar) or 137 μg of different vault-pVI complexes (red bars) was recorded for an interval of 7 min. All measurements were performed in triplicate. The membrane lytic activity mediated by the proteins or complexes was expressed as the percentage of dye release relative to that achieved with Triton X-100. Disruption by bovine serum albumin (BSA) or empty vault particles alone were included as a negative control. The data shown are representative of three different experiments.

membrane disruption and subsequent dilution, it strongly emits fluorescence at 585 nm. The INT domain fused to the N-terminal 34−114 or just the N-terminal 34−54 residues of pVI exhibited substantial membrane lytic activity over a time interval of 7 min relative to complete release mediated by Triton X-100. The lytic activity of these pVI-INT fusion proteins was also similar to a purified synthetic peptide derived from pVI (pVI-syn, residues 34−54) that lacked the INT domain as well as the 34−114 residue pVI molecule (Figure S1 in the Supporting Information). These findings are consistent with previous pVI mutagenesis studies that show the predicted amphipathic α-helical domain (residues 34−54) of pVI is largely responsible for membrane insertion/disruption.[17] The current findings indicate that the membrane lytic domain of pVI retains membrane lytic activity even after fusion to the INT domain. We also sought to determine whether pVI-INT fusion proteins incorporated into recombinant vault particles were capable of mediating liposome disruption (Figure 2). Vault particles alone showed negligible membrane lytic activity, while vaults containing the INT domain exhibited a low level of membrane lysis that was not observed in every experiment. In contrast, vaults containing both the smaller pVI-INT (pVI residues 34−54) and the larger pVI (residues 34−114) fusion proteins caused substantial and consistent membrane disruption. These findings indicate that association of the membrane lytic domain of pVI with vault particles can facilitate membrane disruption.

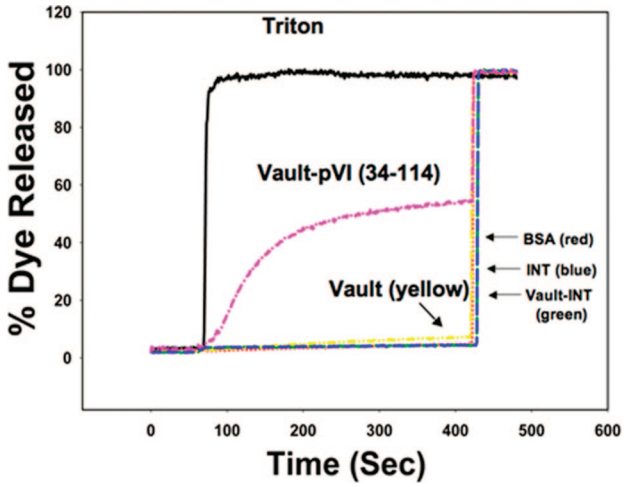

Figure 3. Time course of liposome disruption by pVI-vaults. Liposomes were incubated for varying lengths of time at 37 °C with 137 μg of empty vault particles (yellow line), vaults containing INT alone (green), pVI-INT (34−114) (magenta), or Triton X-100 to disrupt liposomes at 37 °C, and the fluorescence emission was recorded as described above. To assess maximum dye release, Triton X-100 was added to each sample at 420 s. Disruption by BSA (red) or INT (blue) alone was included as negative controls.

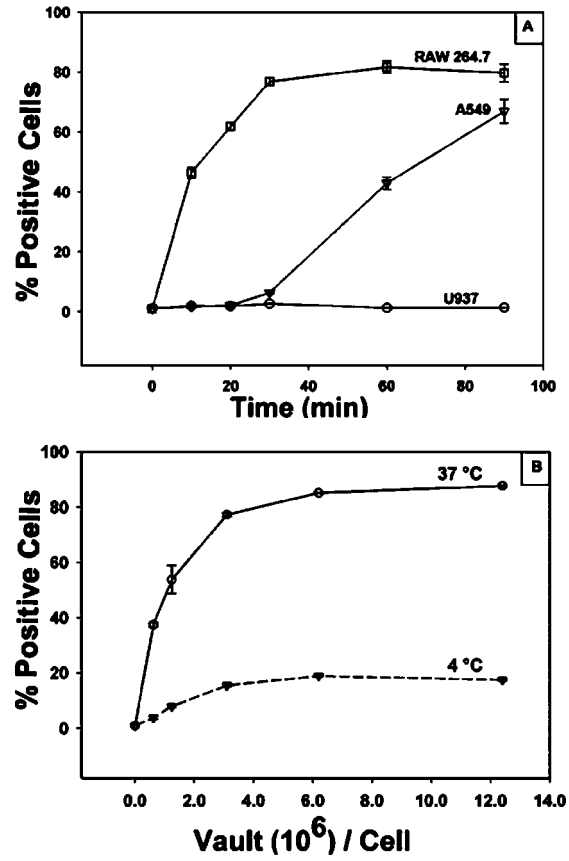

Figure 4. Analysis of vault internalization into different mammalian cells. (A) Internalization of Cy3.5-labeled fluorescent vaults into different cell types at 37 °C was measured by flow cytometry. The cells were incubated with labeled vaults for various times at 37 °C prior to measuring uptake by flow cytometry in the presence of trypan blue dye to quench the fluorescence of uninternalized particles. (B) Internalization of Cy3.5-labeled vaults into RAW 264.7 cells was measured at 4 or 37 °C.

Somewhat unexpectedly, however, we found that the smaller version of pVI (residues 34−54) was not as efficiently incorporated into vaults (data not shown) as the larger pVI domain (34−114). This result was possibly due to a folding of the smaller pVI-INT fusion protein into a conformation that interfered with the binding of INT to the vault interior. As the larger pVI domain was efficiently packaged, we employed this construct for all additional kinetic and functional analyses. Vaults containing pVI (34−114) caused a rapid and progressive disruption of liposomes as a function of time with 50% maximum disruption occurring at ~2 min (Figure 3). In contrast, empty vaults, the purified INT protein alone, vault-INT, or BSA caused little dye release over this time interval. These data suggest that vaults are dynamic and are breathing in solution thus exposing pVI-INT to the liposomes or that there is some sort of equilibrium of pVI-INT dissociation and reassociation with the vaults that allows it to disrupt the liposomes in solution.

We next examined whether different cell types could support entry of Cy3-labeled vault (empty) particles (Figure 4A). These studies used trypan blue dye to quench the fluorescence of externally bound vault particles while allowing detection of internalized vault particles. Murine macrophage RAW 264.7 cells exhibited rapid and substantial internalization of labeled vaults, whereas human U937 monocytic cells did not readily support vault internalization. Human A549 epithelial cells also supported significant vault uptake; however, this occurred with slower kinetics than that of RAW 264.7 cells. Vault association with RAW 264.7 cells was also substantially greater at 37 °C than at 4 °C (Figure 4B), indicating that the labeled vaults were undergoing internalization into cells rather than simply being bound to the cell surface. We previously demonstrated that vaults could be taken up by HeLa cells in culture,[5] presumably into the endosomal compartment. These data indicate that nonspecific vault uptake may vary depending on the cell type. Murine macrophage RAW 264.7 cells are likely to internalize the recombinant vaults *via* active phagoctosis, while uptake into other cell types appears saturable and thus likely to be *via* a receptor-mediated process, although specific receptors have not been identified.

We next asked whether pVI-containing vault particles were capable of facilitating entry of selected biomolecules into RAW 264.7 macrophages (Figure 5A). For these studies, we assessed vault-mediated delivery of a soluble ribotoxin (saporin) that blocks host cell protein synthesis and therefore decreases cell viability upon entry into the cytoplasm. Saporin alone in the absence of membrane penetrating agents had little effect on cell viability, consistent with its inability to cross cell membranes. Cell viability was also unaffected by vault particles or vault-pVI complexes in the absence of saporin. In contrast, vault-pVI (residues 34−114) in

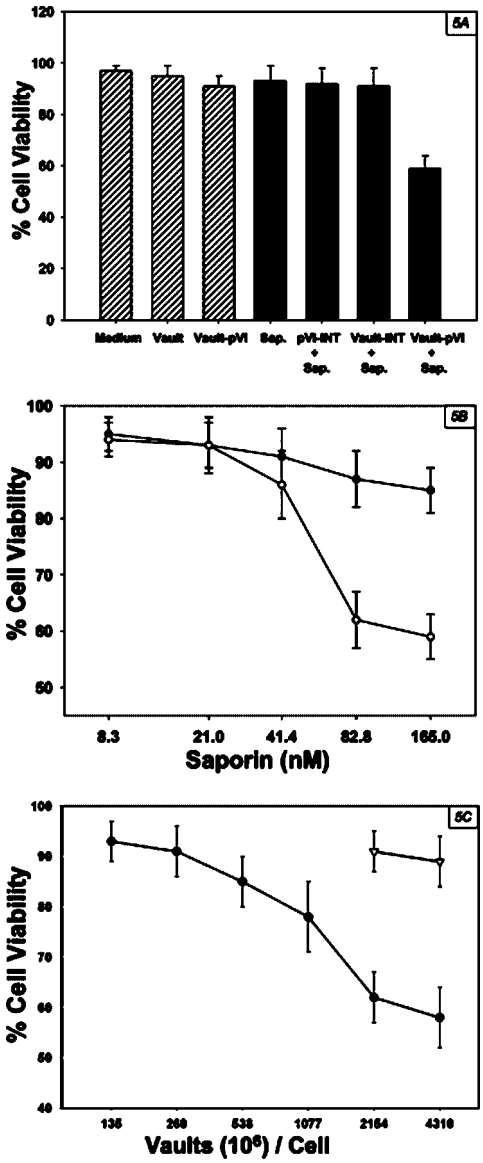

Figure 5. Co-delivery of a ribotoxin (saporin) into cells *via* pVI-vault particles. (A) RAW 264.7 cells were incubated with 1 μg of the indicated amounts of pVI-INT proteins or 137 μg of pVI-vault particles (4.310 × 109/cell) in the presence (black bars) or absence (stripped bars) of 1.65×10^{-7} M saporin, and cell viability was measured 48 h later using the XTT assay. All values were normalized to untreated cells. (B) Dose response of cell killing by pVI-vaults (4.310 × 109/cell) in the presence of saporin (open symbols) or with saporin only (closed symbols). Cytoxicity of RAW 264.7 cells was determined by XTT assay at 48 h following addition of these reagents. (C) Dose response induction of cytotoxicity in RAW 264.7 cells. Cells were incubated with varying amounts of pVI-vaults (solid circles) or INT-vaults (open triangles) in the presence of 1.65×10^{-7} M saporin for 4 h prior to measuring viability by XTT.

the presence of saporin caused a ~40% decrease in cell viability, consistent with significant endosomal membrane disruption by internalized particles. The decrease in cell viability mediated by vault-pVI particles was also directly proportional to the amount of saporin, with a threshold of 21 nM required to induce RAW 264.7 cell toxicity (Figure 5B). Cytotoxicity was also directly proportional to the amount of vault-pVI particles added per cell (Figure 5C).

In further studies, we examined whether pVI-vaults were capable of enhancing gene transfer to RAW 264.7 cells. For these studies, we assessed co-delivery of calcium phosphate precipitated cDNA plasmids encoding EGFP in the presence or absence of vaults (Figure 6). Briefly, cells were transfected in tissue culture with a EGFP-plasmid using calcium phosphate in the presence or absence of vault-pVI particles. The expression of EGFP in the transfected cells, as a measure of cell delivery, was assessed 24 h later using flow cytometry. Vault particles containing pVI enhanced delivery of EGFP plasmid DNA, achieving a ~7-fold higher level of delivery of EGFP plasmid at the highest dose than that observed with calcium phosphate precipitated DNA alone (Figure 6A). Transfection of calcium phosphate precipitated DNA was not significantly altered by the presence of 10 μg of purified pVI-INT or the INT protein alone (Figure 6A, inset). Vaults containing INT alone also conferred relatively low levels (~2−3-fold increase over CaPi alone) of gene transfer, and this was independent of input dose (Figure 6B). These findings indicate that vault-pVI particles are capable of stimulating the transfer of calcium phosphate precipitated DNA into target cells. Although not reflected in our studies assessing the *in vitro* membrane lytic activity of vault particles (Figure 3) or studies examining the ability of vaults to permeabilize cell membranes for ribotoxin entry (Figure 6A), recruitment of endogenous vault particles to lipid rafts during bacterial infection[8] leaves open the possibility that MVP may directly interact with lipid membranes to facilitate transient translocation of plasmid DNA. Vaults are also dynamic structures, and their interior surface may become exposed to cell membranes at low pH, thus facilitating disruption of intracellular vesicles.

We also observed that relatively large numbers of vault particles were needed to mediate toxin and gene delivery. It should be noted that relatively high amounts of adenovirus particles (*i.e.*, 20 000 per cell) are also needed to efficiently deliver ribotoxin.[17] The even higher amounts needed for vault-pVI delivery likely arises from the fact that these nanoparticles lack a cell targeting ligand unlike adenovirus. Moreover, pVI-vault complexes may not arrive in the same cell vesicular compartment as ribotoxin/DNA molecules, thus reducing the efficiency of gene transfer. To remedy this situation, it might be feasible to modify vaults to directly incorporate therapeutic nucleic acid sequences.

A potential advantage of vault particles for gene transfer is that because they are cell-derived they are unlikely to be targeted by host immune responses, a situation that could improve both safety and efficacy. However, the incorporation of viral proteins such as pVI into vault particles could present a target to host immune responses. This particular situation might be miti-

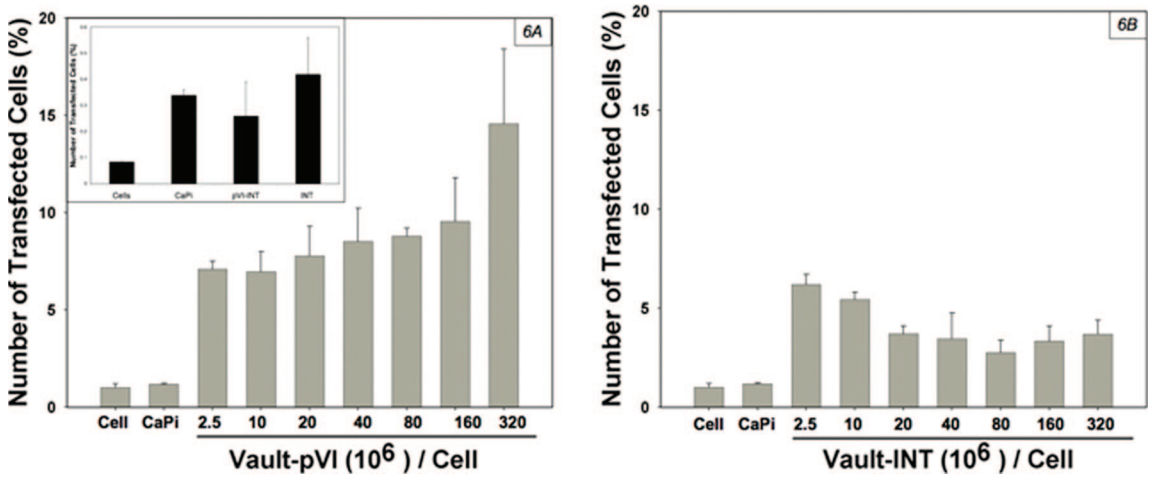

Figure 6. Enhancement of DNA delivery *via* pVI-vault particles. Raw 264.7 cells were transfected with calcium phosphate precipitated cDNA encoding GFP and 10 μg of purified pVI-INT or INT proteins alone (inset) or with increasing numbers of pVI-vaults (A) or INT-vault-particles (B) for 24 h prior to measuring GFP expression by flow cytometry. For comparison, untransfected cells or cells incubated with calcium phosphate precipitated DNA (CaPi) alone were analyzed in parallel. The data shown are the average of triplicate samples and are representative of at least three experiments.

gated by the fact that the majority of neutralizing human antibodies to adenovirus are directed to the hexon and to a lesser extent, the fiber and penton base.[23,24]

Thus, the studies presented herein offer new avenues for adapting naturally occurring cellular structures for cell delivery of therapeutic molecules. They also help to confirm the role of pVI in membrane penetration by adenovirus and could serve as convenient vehicles with which to study cell membrane disruption by truncated and mutant forms of pVI.

MATERIALS AND METHODS

cDNA Constructs for the Expression of Recombinant pVI Proteins. The cDNA plasmid constructs encoding the mature, full length protein VI (pVI), designated *p6*, or the N-terminal region of protein VI (NT) corresponding to residues ala-34 to glu-114, designated *nt*, were cloned into the *Bam*HI and *Eco*RI sites of the *Escherichia coli* expression vector pET28a (Novagen, Madison, WI). The constructs also included an N-terminal 6-His tag followed by a thrombin cleavage site. The 5′ and 3′ primers containing a *Bam*HI restriction site (underlined) in the 5′ primers and a TTA stop codon site (italics) and an *Eco*RI restriction site (underlined) in the 3′ primers were used as indicted: for P6 5′-CGGGATCCG-CCTTCAGCTGGGGCTCGCTCTGGAGC-3′ and 5′-CGGGAATTC-*TTA*CAGACCCACGATGCTGTTCAG-3′ for NT 5′-CGGGATCCGCCTT-CAGCTGGGGCTCGCTCTGGAGC-3′ and 5′-CGGGAATTC*TTA*CTCT CGGGAGGGCGGGGATC-3′and for TR, 5′-AAAGGATCCTATGG-CAGCAAGGC-3′ and 5′-AAAGAATTC*TTA*CAGACCCACGATG-CTGTT-3′. For vault incorporation experiments, we also expressed pVI (amino acid residues 34−54) in *E. coli* using the 5 end -CGGGATCCGCCTTCAGCTGGGGCTCGCTGTG-GAGCGGCATTAAAAATTTCGGTTCCACCGTTAAGAACT-ATGAATTCCGG- and 3′ end 5′-CCGGAATTCATAGTTCTTAA-CGGTGGAACCGAAATT TTTAATGCCGCTCCACAGCGAG-CCCCAGCTGAAGGCGGATCCCG-3′ primers. After endonuclease restriction digests with *Bam*HI and *Eco*RI, the resulting 60 bp cDNA fragment was inserted into pET 28a vector containing a His tag at the N-terminus. All plasmid constructs were confirmed by sequencing.

Recombinant pVI proteins were produced in the BL21 CodonPlus (Stratagene) strain of *E. coli*. Cells were grown in 1 L of LB media to an A_{600} of 0.6−0.8 prior to induction with isopropyl-ss-D-thiogalactopyranoside (IPTG, 0.5 mM). After 4 h, the cells were collected (7 g wet weight) and washed with buffer (200 mL of 50 mM Tris, 300 mM NaCl, pH 8) once. The cell pellets were then resuspended in 20 mL of Bugbuster (Novagen) protein extraction reagent supplemented with Benzonase (20 U/mL, Novagen), 1.0 mg/mL of lysozyme (Sigma), and one tablet of EDTA-free protease inhibitor cocktail (Roche). After 30 min rocking at 4 °C, the cell debris was removed by centrifugation at 10 000*g* for 30 min at 4 °C. Recombinant proteins were purified on nitrilotriacetic acid-Ni^{2+} agarose columns (QIAGEN). After extensive washing, the recombinant proteins were eluted with 50 mM Tris, 300 mM NaCl, 100 mM imidazole, pH 8, and then immediately dialyzed at 4 °C into 50 mM Tris, 300 mM NaCl, pH 8.

Construction of Vault-pVI Protein Complexes. The cDNA constructs encoding the INT and MVP were previously described.[5,14,15,25] The INT domain corresponds to the 162 aa C-terminal region of VPARP (amino acids 1563−1724) and is the smallest region identified for interaction with MVP. The INT domain coding region was PCR cloned into the *Bam*H1 and *Xho*1 sites of the *E. coli* expression vector pET28a using the sense primer, 5′-CGGGA-TTCGGCGGC*GAATTC*GATTACGATATCCCAACGACCGAA-3′, with *Bam*HI site (underlined) and *Eco*RI site (underlined italics). The sense primer contains a dipeptide flexible linker, Gly−Gly, and an added *Eco*R1 site designed for further insertion of recombinant pVI-NT. The antisense primer, 5′-CCCCTCGAG*TTA*GCCTT-GACTGTAATGGAGGACTCTATG-3′ contains an *Xho*I site (underlined) and a stop codon (italics).

The interaction domain chosen for pVI constructs encompassed amino acids 1563−1724 of VPARP. These pVI-INT fusion molecules were expressed in bacteria and purified as described above. Recombinant vaults based on expression of the MVP protein alone were produced in insect cells as previously described.[13,14] Purified vaults were also analyzed by immunoblot using polyclonal antibodies to MVP, INT, or pVI. The identity of CP-MVP-Z monomer present in vault particles was also confirmed by MALDI-TOF-MS, which is within the error range of expected molecular masses of CP-MCP-Z monomers.[25]

Generation of Recombinant Vault-pVI Particles. Expression of recombinant MVP in insect cells allows self-assembly of recombinant empty vault particles. In order to incorporate different pVI molecules into the interior of the vaults, ~2 mg of purified pVI-INT proteins in 10 mL of buffer A (50 mM Tris, pH 7.4, 75 mM NaCl, 0.5 mM $MgCl_2$) was added to 10 mL of recombinant vault containing Sf9 cell lysates, and the mixture was incubated on ice for 30 min before performing vault purification as previously

described.[13,14] Alternatively, we mixed an extract containing empty vaults (from a 50 mL culture of CP-MVP infected Sf9 cells) with an extract from 100 mL of pVI-INT induced bacteria in a buffer containing 150 mm NaCl, the mixture was incubated on ice for 30 min prior to purifying vaults as previously described.[13,14]

The 40 and 45% sucrose density gradient fractions containing the assembled vaults were pelleted separately and resuspended in 20 mM MES buffer (pH 6.5) for further analysis. To confirm the successful loading of pVI-INT into the vaults, immunoblotting of the isolated vault particles was performed using monoclonal antibody to MVP (1032, Santa Cruz). The presence of pVI in the vaults was simultaneously detected with an affinity purified pVI polyclonal antibody.

Protease Sensitivity and Transmission Electron Microscopy. To examine the protease sensitivity of purified vault particles, 30 μL of purified pVI-vaults (150 μg/mL) was incubated with 6 μL of 10X thrombin cleavage buffer, 3 μL of thrombin (1 U/μL, Novagen, Madison, WI), and 21 μL of water to a total volume of 60 μL. The reaction mixture was incubated at 25 °C. Aliquots (10 μL) of this mixture were collected after 24 h and analyzed by immunoblot as described above. Purified vault particle morphology was assessed by negative stain transmission electron microscopy as previously described.[14] EM grids were examined on a JEOL 1200 EX electron microscope, and micrographs were captured with a BIOScan 600W digital camera (Gatan Inc., Pleasanton, CA).

Liposome Disruption Assay. To assess the ability of pVI or pVI-vaults to mediate membrane disruption, we used model membranes (liposomes) containing an entrapped fluorescent dye. Unilamellar liposomes having an average diameter of 500 μm were prepared using bovine liver phosphatidylcholine (PC), bovine brain phosphatidylserine (PS) (Avanti Polar Lipids), and sulforhodamine B (SulfoB) (Molecular Probes, Invitrogen) as previously described[26] with slight modifications. Lipid vesicles were prepared by mixing lipids in a molar ratio of PC/PS of 4:1 in a total amount of 5.0 μmol in 1 mL of chloroform. The solution was then evaporated under argon to generate a lipid film which was vacuum-dried for 12 h to remove residual chloroform. The dried lipid film was then hydrated for 1 h in a 1 mL solution sulforhodamine B (100 mM) in HBS buffer (20 mM HEPES/NaOH buffer, 100 mM NaCl, 0.02% sodium azide pH 7.5). Small unilamellar vesicles (SUVs) were prepared by vortexing the reaction tube vigorously to completely resuspend the lipid mixture followed by sonication for 1 h in a bath sonicator (Laboratory Supplies Inc.). This final solution was then gel-filtered on a Sephadex G-25 column and eluted with HBS buffer. The liposomes, which eluted as a pink band, were collected and used within 24 h. The final lipid concentration was determined by using an inorganic phosphorus assay[27,28] and then adjusted to 0.15 mM for the vault and pVI-INT assays as described below.

Time-dependent fluorescence was used to analyze liposome disruption using an Aminco Bowman Series luminescence spectrometer equipped with 535/20 nm excitation and 585/20 nm emission filters, respectively. Briefly, 12.5 μL of liposome solution was added to HBS buffer (1 mL) in a fluorescence cuvette equipped with a stir bar, and fluorescence measurements were taken at 1 s intervals under stirring conditions for a total time of approximately 7 min. After 60 s to record background fluorescence, 10 μL of the pVI-INT proteins (1 μg total) or vault-pVI complexes at 13.7 mg/mL (137 μg total) in 50 mM Tris, 300 mM NaCl, pH 8, was added to the liposome solutions. After reaching a plateau in the fluorescence signal, 25 μL of a 10% aqueous solution of Triton X-100 was added and the percentage of SulfoB release was calculated using the following formula: % SulfoB released $= 100 \times [(F - F_0)/(F_t - F_0)]$, where F_0 and F are the fluorescence before and after the addition of protein, respectively, and F_t is the total fluorescence intensity in the presence of Triton X-100.

Preparation of Fluorescent Vault Nanoparticles. Recombinant vaults were fluorescently labeled using the NHS-ester Cy3.5 bisreactive dye (Amersham Biosciences). Briefly, 1.0 mg of the free bisfunctional NHS-ester Cy3.5 dye was dissolved in 1 mL of 0.1 M carbonate buffer, pH 8.5, and then mixed with 0.65 mL of 13.7 mg/mL purified vaults, resulting in the conjugation at a molar ratio of 1.1 dye molecules per 1 MVP molecules. The conjugation mixture was incubated at 4 °C for 40 min with occasional rocking, and the remaining nonconjugated dye was removed by filtration on a PD-10 column (Amersham Biosciences) pre-equilibrated with buffer A (see above) that was also used for elution of the conjugation product. The colored (pink) fraction containing the dye-conjugated vault nanoparticles was collected and loaded onto a discontinuous 20−60% sucrose density gradient and ultracentrifuged as described above. The pink band corresponding to the 45% fraction was pelleted by centrifugation using a Beckman Ti70 rotor (39 000 rpm for 2 h at 4 °C) and resuspended in 20 mM MES buffer pH 6.5. The yield of labeled vault particles was estimated by linear regression analyses of the UV absorbance of the labeled vaults assuming Cy3.5 dye λ_{ex}= 581 nm and λ_{ex} of proteins at 280 nm, resulting in a ratio of 1 Cy3.5 dye molecules per MVP protein subunit.

Vault Interactions with Mammalian Cells. RAW 264.7 mouse macrophages and A549 human epithelial cells were maintained in Dulbecco's complete modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, 100 U of penicillin G/mL, 0.3 mg of gentamicin/mL, and 10% fetal bovine serum (D-10). U937 human monocytic cells, maintained in RPMI-1640 modified medium, were supplemented with 10 mM HEPES, 2 mM glutamine, and 10% fetal bovine serum.

To measure vault−host cell interactions, cells were cultured in 6-well tissue culture plates at a density of 2×10^5 cells/well 24 h prior to measuring vault internalization. The cells were then incubated with varying amounts of Cy3.5-labeled vault nanoparticles in MES buffer for varying times at 37 or 4 °C. Internalized vaults were then quantified by flow cytometry following the detachment of cells by trypsinization and resuspension in 1 mL of cold Ca^{2+} and Mg^{2+} free PBS buffer, pH 7.0, containing 1 mM EDTA, 25 mM HEPES, and 1% heat inactivated fetal bovine serum. Trypan blue dye was then added to each cell sample at a final concentration of 200 μg/mL in cold FACS sort buffer in order to quench the fluorescence of noninternalized Cy3.5-labeled vault particles as previously described.[29] The cell suspensions were analyzed by flow cytometry with a Becton-Dickinson FACSCan cytometer using a 488 nm laser for red emitting fluorochrome excitation. Cy3.5 fluorescence was detected using PL2 channel in conjunction with a 585 nm band-pass filter. An electronic gate was set around cells based on the forward and side scatter properties of the population, and a minimum of 10 000 gated events per sample were collected. Data analysis was performed with CellQuest software (BD Bioscience, San Jose, CA).

Cell Membrane Penetration Assay. To examine vault-mediated endosome penetration, we used an assay that measures the co-delivery of a ribotoxin (saporin) into the cytosol of host cells *via* a membrane lytic virus.[17] RAW 264.7 cells were seeded at a density of 3000 cells per well and allowed to attach for 4 h in a 96-well tissue culture plate. One microgram of vaults alone, or vault-PVI complexes, or pVI protein was incubated with cells in the presence or absence of the ribotoxin saporin 29[30] in D-10 medium for 4 h. The saporin concentration was varied from 1.65×10^{-7} to 8.25×10^{-9} M in two-fold dilutions. The cultured cells were then washed two times with PBS buffer and media and then cultured in medium for 48 h before measuring cell metabolic activity using the colorimetric XTT assay[31,32] (Promega, Madison, WI). The absorbance was measured at 485 nm on a Molecular Devices SpectraMAX 250 microplate reader. All experiments were performed in triplicate.

Vault-Mediated Delivery of CaPi:DNA Complexes. The murine macrophage RAW 264.7 cells were cultured in 60 mm dishes in D-10 medium (DMEM + 10% FCS medium and antibiotics) and seeded onto 96-well plates (6×10^3 cells per well, in 0.2 mL growth medium) 24 h prior to performing transfection experiments. A plasmid encoding a red-shifted variant of GFP (pEGFP-N1, Clontech, Palo Alto, CA) was amplified in the *E. coli* (DH5α) and purified according to the manufacturer's protocol (Qiagen, USA). The isolated DNA was resuspended in Tris-EDTA (pH 8.0) at a concentration of 0.5 μg/μL and used for the preparation of calcium phosphate precipitates for transfection experiments as previously described.[33−36] Briefly, 1.5 μL of DNA plasmid encoding GFP (0.75 μg) along with calculated volumes of 10 μg of purified pVI-INT or INT or pVI-Vault solution (5.39×10^{-7} M, 5.68 ×

10^6 particles /cell) and 1.00 μL of a 2 M $CaCl_2$ (Profection, calcium phosphate mammalian transfection system, Promega, Madison, WI) aqueous solution were mixed with EMEM (11090-81, GIBCO) in a final volume of 50 μL. The mixtures were allowed to incubate for 30 min at 4 °C. The cell culture medium was replaced by 150 μL of fresh EMEM medium, and 50 μL of the complex was then applied to cells with gentle agitation. After incubation for 1 h at 37 °C, the transfection mixture was removed by washing the cells with 0.15 M NaCl and cultured with D-10 medium. pEGFP gene expression was measured by FACS after 1 day post-transfection.

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Supporting Information Available: Description of liposome disruption by recombinant protein VI peptides in a kinetic analysis. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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