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Award Number: DAMD17-98-1-8321

TITLE: Viral Vectors Selective for Metastatic Breast Cancer Tumor Cells

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REPORT DATE: November 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20030805 075

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2002	Final (1 Oct 98 - 1 Oct 02)

4. TITLE AND SUBTITLE
Viral Vectors Selective for Metastatic Breast Cancer Tumor Cells

5. FUNDING NUMBERS
DAMD17-98-1-8321

6. AUTHOR(S) :
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The relative ease with which polyomavirus VP1 quasi-equivalent assemblies (VLPs) can be expressed and purified has prompted us to develop them as vectors for gene therapy. We have introduced sequences into the surface-exposed loops of VP1 that will target VLPs to receptors frequently expressed on breast cancer cells. We have introduced into VP1 loops BC, DE, EF and HI, sequences capable of binding to the urokinase plasminogen activator (uPA) receptor or the ErbB2 receptor. Introduction of these sequences renders most of the modified VP1 proteins insoluble when expressed at high levels in insect cells, although some modified proteins such as VP1/EF-uPA(10-34), -uPA(1-60) and -ErbB26.1 are partly soluble. Conditions have been developed to improve yield and solubility. The efficiency of self-assembly of these modified VP1 proteins appears to be reduced as compared to VP1/wt protein, however co-expression with VP1/HI-FLAG improves yields. That the VLPs contain the co-expressed VP1 proteins has been established by demonstrating VP1/EF-uPA(1-60) proteins migrating at the position of native VLPs in sucrose velocity sedimentation and co-precipitation with VP1/EF-FLAG proteins captured by anti-FLAG antibody. Specificity for receptors present on cancer cell surfaces has been defined.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
breast cancer, IDEA	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction: The most life-threatening aspect of cancer is its capacity to invade normal tissue and to establish new foci of tumor cells at distant sites. While there has been great progress in understanding the genetic and cellular mechanisms involved in the conversion of normal cells to metastatic tumor cells, less progress has been made in utilizing what has been learned to reduce cancer morbidity and mortality. The objective of this work has been to develop novel gene therapy vectors selective for metastatic cells. Selectivity relies upon metastatic cells expressing urokinase plasminogen activator (uPA) to which the vectors can bind.

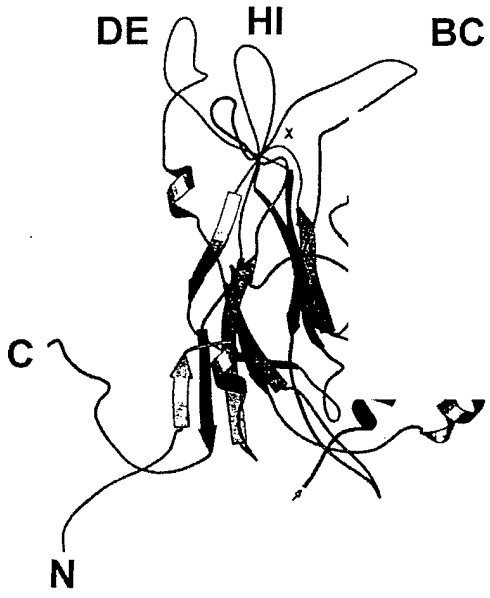
Research Accomplished:

1. Construction of modified polyomavirus (Py) virion protein 1 (VP1) genes

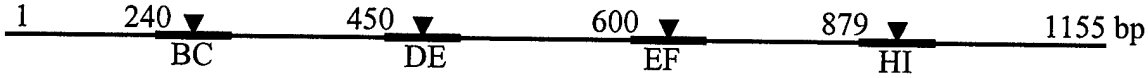
The Py major capsid protein VP1 will self-assemble to make virus-like particles (VLP) without involvement of other capsid proteins (Salunke DM. *et al.* 1986; An K *et al.*, 1999). The relative simplicity of self-assembly makes these VLPs attractive as gene therapy vectors.

VP1 is composed of 384 amino acids and its 3-dimensional structure has been reported (Soeda E *et al.* 1980). Four loops, BC, DE, EF and HI are exposed on the outside surface in capsomere structure and hence may not be involved in forming virus-like particles. We selected those four loops as target sites to insert foreign peptides.

Structure of Py VP1 protein and sites of foreign peptide insertion



Amino acid sequence 13-32 of uPA is responsible for its binding to urokinase plasminogen activator receptor (uPAR; Appella *et al.* 1987). Consequently, we have chosen to insert residues 10-34 of uPA into the VP1 protein so that it might interact with the uPAR receptor. Also, we have inserted the entire amino terminal fragment (ATF) into this site. Goodson RJ *et al.* (1994) selected 15-mer peptides with high affinity for uPAR using random bacteriophage display. The selected peptides have two short conserved sequences, neither of which is found in uPA protein. One such peptide, AEPMPHSLNFSQYLWYT, showed a higher affinity for the uPA receptor than original uPA peptide. We also inserted this peptide into the loops of VP1. To help in purification and analysis, a FLAG epitope was inserted in the HI loop of VP1.

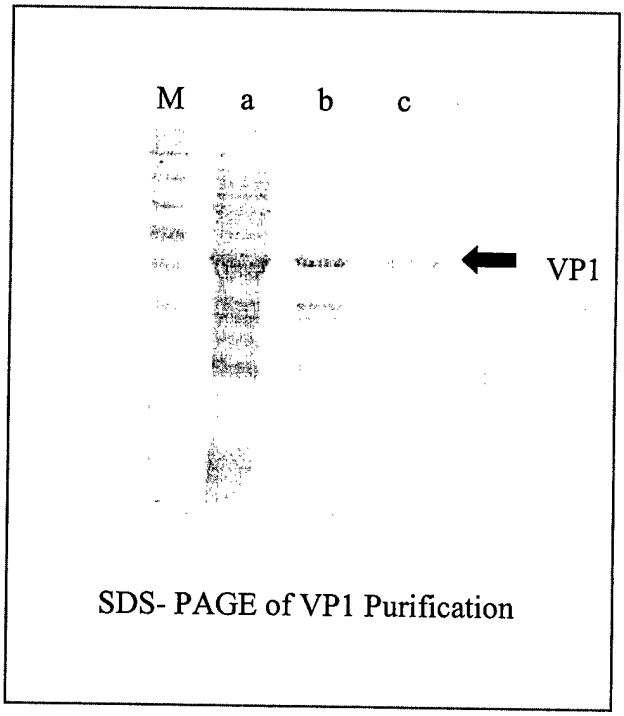


VP1 protein: (▼ : Site of foreign peptide insertion)

2. Cloning of wild type and modified VP1 gene(s) into baculovirus and VP1 protein expression:

Wild type and modified VP1 gene(s) were cloned into the pFastBac expression plasmid and transformed into DH10Bac competent cells containing Bacmid plasmid DNA. Recombinant bacmids containing wild type or modified VP1 gene(s) were purified and transfected into Hi-5 insect cells to obtain recombinant baculovirus. These were amplified to obtain high titer virus stocks. To produce VP1, confluent monolayers of Hi-5 cells were infected with recombinant baculoviruses and incubated for 4 days. Protein production was evaluated by western blot analysis.

1. Infection of Hi-5 cells with baculovirus (1 x 10e7 cells/T150 flask, m.o.i = 10)
2. Incubation for 3.5 days(2 % FCS)
3. harvest and sonication sedimentatio (40sec x 3, take supernatant: **a**)
4. Sucrose sedimentation (sucrose 20%, SW 28 rotor, 27K, 3 hr: **b**)
5. Sucrose sedimentation velocity (Sucrose 10-50%, SW40 rotor, 35k, 2hrs: **c**)

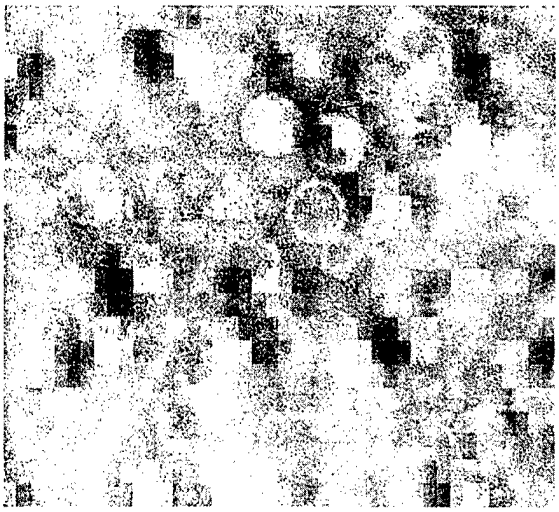


SDS- PAGE of VP1 Purification

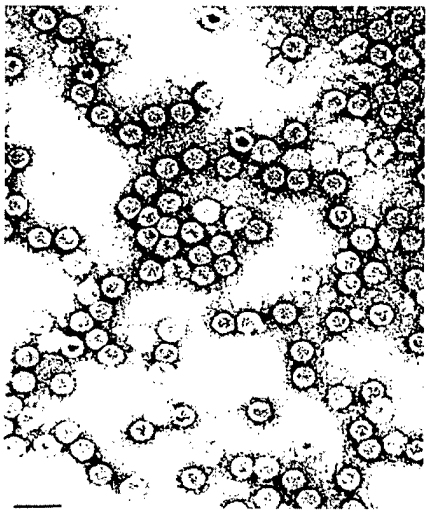
3. Purification of VLP and transmission electron microscopy

After harvesting the infected Hi-5 cells by low speed centrifugation, they were disrupted by brief sonication and the lysate was centrifuged at 10.000g for 30minutes, with the supernatant being saved. The pellet was re-extracted by sonication and centrifuged same as above. The supernatant was saved and combined with the original supernatant. VLPs were concentrated through 2ml of a 20% sucrose shelf and centrifuged at 35,000rpm for 120 minutes in a Beckman SW40 rotor. The pellet containing the partially purified VLPs was resuspended in small volume of buffer and layered on top of a preset cesium chloride gradient (1.35g/ml-1.23g/ml) and centrifuged at 33,000rpm for 15 hours in a SW40 rotor and then fractions were collected. The fractions containing VLP's were dialyzed and used for transmission electron microscopy.

Electron microscopy images of VLPs



VLPs made from insect cell expressed VP1

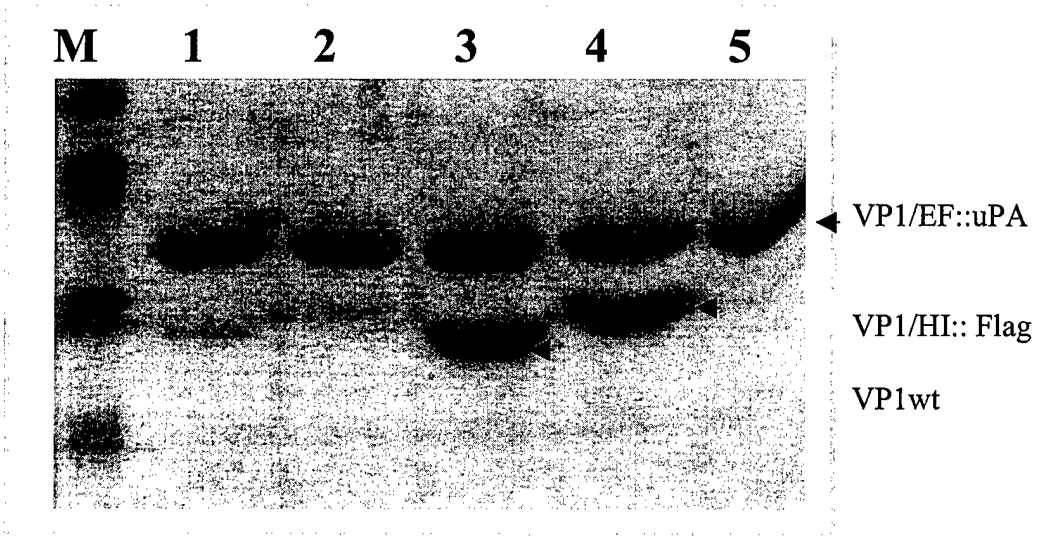


Native polyoma virus

4. The solubility of modified VP1 proteins was examined by measuring the distribution between supernatant and pellet. Hi-5 cells were infected with baculoviruses which express each modified VP1. Cells were harvested at 84 hrs pi and sonicated, followed by high speed centrifugation (15,000g for 30 min). SDS PAGE and western blots were performed to evaluate the distribution of modified proteins. All of the uPA and ERB-B2 inserts into the EF loop of VP1 yielded soluble proteins, whereas these inserts into the BC, DE and HI loops generally produced insoluble proteins. Insertion of the FLAG peptide into the EF loop produced soluble proteins.

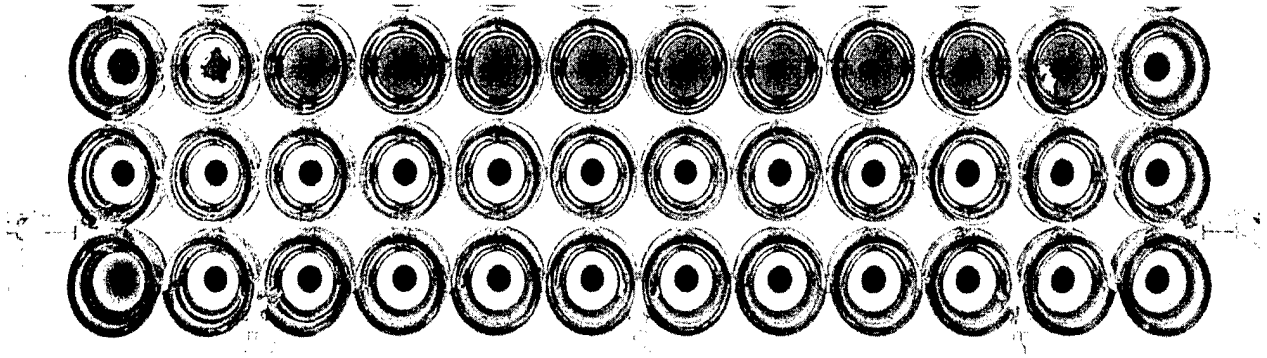
5. The reactivity of the modified proteins against anti-uPA antibodies was determined. American Diagnostica Antibody #3921 reacted strongly against uPA sequences introduced into the EF loop, indicating the native structure of uPA was retained.

6. To produce heterotypic VLPs, (those containing different forms of VP1), Hi-5 cells were co-infected with baculoviruses expressing VP1/wt and VP1/EF::uPA1-60. VP1 proteins were purified by pelleting through sucrose and were observed to co-sediment. To obtain firm evidence that the VLPs contained both species of VP1, heterotypic VLPs containing FLAG ligand and uPA 1-60 were prepared by co-infection of Hi-5 cells and immunoaffinity purified with anti-FLAG antibody. These VLPs were demonstrated to contain the uPA 1-60 insert by subsequent immunoprecipitation with anti-uPA antibody #3921.



Cells Infected with:
1. VP1/wt
2. VP1/HI:: FLAG
3. VP1/wt + VP1/EF::uPA 1-60
4. VP1/HI:: FLAG + VP1/EF::uPA 1-60
5. control

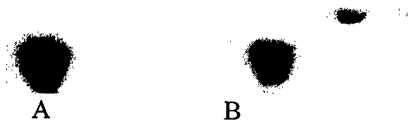
7. To determine whether the insertion of the ligands into VP1 affected adsorption of the virus to sialic acid receptors, hemagglutination assays were performed with the VLPs. The assay indicated that the modified VLPs (rows 2&3) did not hemagglutinate.



8. Binding of the modified VLPs to DU145 cells expressing uPAR indicates that VLPs containing the uPA ligand bind preferentially to PMA treated cells, in which expression of the uPAR is stimulated. Wild type VLPs bind even more strongly (because of their inherent affinity for sialic acid). The binding to the U-937 cells was specifically blocked by ATF.

SDS-PAGE of proteins eluted from U-937 cells after adsorption of VLPs and washing to remove unbound proteins.

A. Binding of VLPs to unstimulated U-937 cells.
B. Binding of VLPs to PMA stimulated U-937 cells.



(VLPs composed of: lane 1- VP1/wt, lane 2 - VP1/HI-FLAG; lane 3- VP1/EF-uPA(1-60); lane 4 - VP1/EF-uPA(1-60) with ATF competitor.

Key research Accomplishments to date:

1. We have expressed and purified polyomavirus VP1 proteins containing inserts derived from sequences that bind uPAR.

2. We have assembled virus like particles (VLPs) from these modified proteins.

3. We have shown the inserts are expressed on the surface of the VLPs in a form that can be detected by antibodies.

4. We have expressed VLPs containing inserts that bind to both uPAR.

5. We have demonstrated that the modified VLPs lose the capacity to bind to normal cells.

6. We have demonstrated affinity of the modified VLPs for cells expressing uPAR.

Reportable Outcomes:

1.) Development of expression clones for chimeric VP1 proteins.

2.) Development of procedures for the purification and self-assembly of modified VP1 proteins containing inserts into the EF and HI loops.

3.) Development of VLPs with modified VP1 proteins that preferentially bind to uPAR.

4. These results have been reported at two international conferences, the 2001 ICRF/UCSF DNA Tumor Virus Meeting and the 2002 Era of Hope DOD Breast Cancer Meeting. A manuscript describing these results is being prepared for submission (draft is attached).

Conclusions: This work demonstrates the feasibility of preparing virion like particles (VLPs) composed of modified polyomavirus capsid proteins into which ligands for the urokinase plasminogen activator have been introduced. Selective binding by the modified VLPs for the urokinase plasminogen activator receptor on cell surfaces has been demonstrated. We have attempted to introduce DNAs into the VLPs, but more work will be required to establish the conditions for this to be efficient.

Personnel receiving support: Ms Sarah Scanlon, Ms Olga Kenzior, Mr. Young Cheol Shin, Mr. Anyong Xie and several undergraduate research assistants.

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Development of polyomavirus VLPs that bind to the urokinase plasminogen activator (uPA) receptor

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Abstract

Virus-like particles (VLPs) derived by self-assembly of polyomavirus VP1 can deliver DNA into mammalian cells, and hence might be useful for gene therapy. To achieve cell-type specific targeting, the native tropism of polyoma VLPs for sialic acid on the surface of cells must be replaced by new binding specificities. Introduction of the FLAG epitope into the HI loop of VP1 caused polyoma VLPs to lose their capacity for sialic acid binding. To target polyoma VLPs to the urokinase plasminogen activator receptor (uPAR), several sequences related to or derived from the urokinase plasminogen activator (uPA) amino terminal region responsible for binding to its receptor were introduced into surface-exposed loops of VP1. Analysis of the modified VP1 proteins revealed that the EF loop of VP1 is able to accommodate these sequences better than the BC, DE and HI loops, and one of modified VP1 proteins containing uPA (1-60) sequence in the EF loop, together with the modified VP1 containing the FLAG epitope formed heterotypic VLPs that specifically bound to uPAR expressed on the surface of U937 cells.

Introduction

Polyomavirus capsids are formed by three structural proteins VP1, VP2 and VP3(1). The major capsid protein, VP1, self-assembles into virus-like particles (VLPs) resembling the native virion, composed of 72 pentameric subunits arranged in an icosahedral lattice with an approximate diameter of 50nm(2-8). The structural framework of VP1 is antiparallel β–sheet with jelly-roll topology from which emanate four major loops(9-11). The BC, DE and HI loops closely interact and are located at the outward facing end of the β-sheet.

The EF loop, originating from the inward facing end, is located on the side of the β-sheet (11, 12). Even though these loops may have little impact on the structure of VP1, they are involved in the recognition of receptors on host cells. A shallow groove formed between the BC1 and HI loops binds oligosaccharides terminating in A-5 acetylneuraminic acid (sialic acid)(10, 12, 13) expressed on many animal cells(11). A motif for integrin binding (LDV) also is found in the DE loop of the VP1 protein (personal communication, Dr. P. Amati). These surface-exposed loops exhibit significant sequence variability among different strains of polyomavirus (9), perhaps because of the influence of the host immune system (14). Furthermore, they do not contribute to intramolecular interactions, which makes them attractive sites for incorporation of foreign sequences. However, it is not known if the loops differ in their capacity to accept foreign sequences and once modified, affect VLP formation.

Polyomavirus VLPs might be useful for gene delivery vehicles(15) (16), however, the native tropism of polyomavirus VLPs must be replaced by new binding specificities to achieve cell-type specific binding. Various peptide and protein sequences have been employed to decorate the surface of VP1, including the *E.coli* dihydrofolate reductase (DHFR)(17), protein Z(18), a WW domain(19) and a polyanionic adapter sequence(20). However, VLPs formed from these modified VP1 proteins do not directly target specific cell types because they lack affinity ligands for cellular receptors. In this study, we attempted to target polyoma VLPs to the urokinase plasminogen activator receptor (uPAR) that is expressed on the surface of many cell types including leukocytes(21, 22), endothelial cells(23, 24) and a variety of neoplastic cells(25, 26-28). The interaction of

uPA and its receptor plays a key role in cell migration(29, 30) and proliferation(31, 32) and in metastasis of cancer cells (26, 28, 29, 33-39).

To abolish the native cell tropism of polyoma VLPs, a putative integrin-binding motif was disrupted by site-directed mutagenesis, and the native sialic acid-binding capacity of VP1 was destroyed by the insertion of the FLAG epitope into the HI loop of VP1. To establish an uPAR-specific binding, modified VP1 genes were constructed by introducing a variety of uPA sequences into each loop region of VP1 and expressed them in insect cells. The peptides sequences tested in this study were a phage display (clone-20) peptide, and uPA sequences uPA (10-34), uPA (1-60) and uPA (1-135). The clone-20 peptide is composed of 17 amino acids, and no sequence homology with uPA, but it has high affinity for uPAR (IC_{50} = 0.01 uM)(40). The uPA (10-34) sequence was chosen because it includes principal binding determinant for uPAR(41). The uPA (1-135) sequence, also called ATF, has as high affinity for its receptor (IC_{50} = 0.00012 uM) as the whole uPA protein(41, 42) and has been widely used in the studies of uPA-uPAR interaction(42-45). The uPA (1-60) sequence, which comprises roughly half of ATF, was chosen because it is more hydrophilic than uPA (1-135) (ExPASy ProtScale, http://us.expasy.org/).

Construction and biochemical analysis of each modified VP1 protein indicates that the EF loop is more capable of accommodating these uPA sequences than the other loops. One modified VP1 protein containing uPA (1-60) in the EF loop could self-assemble to make heterotypic VLP, and when mixed with VP1/wt or VP1/HI-FLAG formed heterotypic VLPs that bind specifically to uPAR present on the surface of U-937 cells.

Materials and Methods

Modification of VP1 genes

The VP1/wt gene of polyoma virus (A3 strain) cloned in pFastBac1 plasmid (GIBCO-BRL) was used as a backbone for the construction of modified VP1 genes. A putative LDV integrin binding sequence in VP1 (P. Amati, personal communication) was changed to LAA by site-directed mutagenesis using the GeneEditor™ kit (Promega). Insertions into the BC (80), DE (150), EF (200) and HI (293) loops of VP1 (numbers indicate the residue of VP1 protein prior to the insertion) were made as exemplified by the insertion of the FLAG epitope into the HI loop: two DNA fragments of VP1 containing the FLAG sequence were synthesized by PCR using primer pairs, FBac-F / HI-FLAG-R and FBac-R / HI-FLAG-F. The PCR products were purified by agarose gel-electrophoresis and isolated with the Compass DNA purification kit (American Bioanalytical, Natick, MA) then annealed to make the intact VP1/HI-FLAG gene. This DNA was amplified by PCR using FBac-F and FBac-R primers, purified and then cloned into the pFastBac-1 plasmid at the XbaI and XhoI sites. Essentially the same method was employed to insert uPA (10-34) and clone-20 peptide sequence(40) into the HI, BC, DE and EF loops. The following primers were used to insert these sequences (residues complementary to VP1 are underlined):

FBac-F: gattattcataccgtcccaccatcg

FBac-R: ctgattatgatcctctagtacttct

HI-FLAG-F: aaggacgacgatgacaagcccgggaactatgatgtccatcactg

HI-FLAG-R: gtcatcgtcgtccttgtagtcgaattcttgtaactctcctgccca

BC-uPA (10-34)-F: caacaagtacttctccaacattcactggtgcaactgcccaaatttggctacatcagatac

BC-uPA (10-34)-R: gagaagtacttgttggacacacatgttcctccatttagacagtcacagttaatccctctgctccaaccat

DE-uPA (10-34)-F: caacaagtacttctccaacattcactggtgcaactgcccaacaaaagtaatttccactcc

DE-uPA (10-34)-R: gagaagtacttgttggacacacatgttcctccatttagacagtcacagttgtttactgtatctgtgggtt

EF-uPA (10-34)-F: caacaagtacttctccaacattcactggtgcaactgcccagacatggtcaacaaagacca

EF-uPA (10-34)-R: gagaagtacttgttggacacacatgttcctccatttagacagtcacagttcttctttgtgattgttttga

HI-uPA (10-34)-F: caacaagtacttctccaacattcactggtgcaactgcccaaactatgatgtccatcactg

HI-uPA (10-34)-R: gagaagtacttgttggacacacatgttcctccatttagacagtcacagtttcttgtaactctcctgccca

BC-clone-20-F: tctctgaacttctctcagtacctgtggtacaccgaggattccccagaaaataat

BC-clone-20-R: agagaagttcagagagtgcggcatcggttccgctgtatctgatgtagccaaatt

DE-clone-20-F: tctctgaacttctctcagtacctgtggtacaccacaaaagtaatttccactcca

DE-clone-20-R: agagaagttcagagagtgcggcatcggttccgcgtttactgtatctgtgggttt

EF-clone-20-F: tctctgaacttctctcagtacctgtggtacaccgacatggtcaacaaagaccaa

EF-clone-20-R: agagaagttcagagagtgcggcatcggttccgccttctttgtgattgttttgat

HI-clone-20-F: tctctgaacttctctcagtacctgtggtaccaccaactatgatgtccatcactgg

HI-clone-20-R: agagaagttcagagagtgcggcatcggttccgctcttgtaactctcctgcccat

For the insertion of longer sequences such as uPA (1-60) or uPA (1-135) into VP1, restriction enzyme sites for NheI and XmaI were introduced into each VP1 loop by the same procedure employed to make the FLAG insertion using the following primers (sequences complementary to VP1 are underlined and sequences of restriction enzymes are indicated in bold letters):

BC-F: **gctagc**agccgt**cccggg**gaggattccccagaaaataat

BC-R: **cccggg**acggct**gctagc**tgtatctgatgtagccaaatt

DE-F: **gctagc**agccgt**cccggg**acaaaagtaatttccactcca

DE-R: **cccggg**acggct**gctagc**gtttactgtatctgtgggttt

EF-F: **gctagc**agccgt**cccggg**gacatggtcaacaaagaccaa

EF-R: **cccggg**acggct**gctagc**cttctttgtgattgttttgat

HI-F: **gctagc**agccgt**cccggg**aactatgatgtccatcactgg

HI-R: **cccggg**acggct**gctagc**tcttgtaactctcctgcccat

A cDNA of human uPA was synthesized by reverse transcription using uPA (1-135)-R primer and amplified with uPA (1-60)-F / uPA (1-60)-R and uPA (1-135)-F / uPA (1-135)-R primer pairs to produce the uPA (1-60) and uPA (1-135) sequences respectively. The PCR products were inserted into each loop of VP1 using NheI and XmaI restriction enzymes. The sequences of primers used for amplification of uPA sequences are as follows (sequences of uPA are underlined, sequences of restriction enzymes are indicated in bold letters):

uPA (1-60)-F: agct**gctagc**agcaatgaacttcatcaagtt

uPA (1-60)-R: taat**cccggg**tcctcggtaaaagtgaccatt

uPA (1-135)-F: ccg**gaattc**agcaatgaacttcatcaagtt

uPA (1-135)-R: tccc**cccggg**ttttccatctgcgcagtcatg

Construction of VP1/EF-uPA (1-60) / HI-FLAG was performed by substituting part of the VP1 sequence of pFastBac-VP1/HI-FLAG with that of pFastBac-VP1/EF-uPA (1-60) using XbaI and ApaI restriction enzymes.

All of the constructs were confirmed by DNA sequencing.

Cell Culture and Baculovirus Infections

Hi-5 insect cells were grown with EX-CELL TM 405 medium (JRH biosciences, Lenexa, KS) supplemented with 5% fetal calf serum (FCS). Baculoviruses expressing wild type and modified VP1 proteins were prepared according to procedures described in the Bac-to-Bac instruction manual (GIBCO-BRL) and titered by plaque assay. Cells (5X10E(6)) in T-150 flasks were infected at an MOI of 10 and harvested 84 hrs after infection. For the production of heterotypic VLPs, Hi-5 cells were infected as above by baculoviruses expressing VP1/wt or VP1/HI-FLAG together with baculoviruses expressing VP1/EF-uPA (1-60)/HI-FLAG with an MOI of 2 to 20.

Preparation of Purification of VLPs

Purification of VLPs was performed as described by Gillock *et al*(46) with slight modifications. Baculovirus-infected cells in T-150 flasks were harvested by low-speed centrifugation and suspended in 3ml of buffer A (10mM Tris / 1 M NaCl / 0.01mM $CaCl_2$ / 0.01 % Triton X-100) and sonicated for 20 sec three times. Proteinase inhibitors (Complete™, Roche Diagnostics, Mannheim, Germany) were added to the lysate, which was centrifuged at 12,000 rpm for 30 min in a Beckman JA21 rotor, and the supernatant saved. The pellet was resuspended with 2ml of buffer A and sonicated, then centrifuged as above. The two supernatants were combined and placed on top of 3ml of 20% sucrose solution in buffer A and centrifuged at 28,000 rpm for 3 hr in a Beckman SW28 rotor. The resulting pellet was resuspended in 1ml of buffer A and sonicated for 20 sec to disrupt aggregates. When specified, the VLPs were further purified by sedimentation through 10-50 % sucrose gradients at 35,000 rpm for 2 hrs in a Beckman SW40 rotor. For the analysis of heterotypic VLPs, 10-40 % sucrose gradients were employed with centrifugation for 1.5 hrs.

To immunopurify VLPs, the supernatant after sonication was loaded on a M2-FLAG agarose column (Sigma) and washed with 5 bed volumes of buffer A. Proteins bound to M2-FLAG agarose were eluted with glycine/HCl buffer (100 mM, pH 3.0), which were immediately neutralized with Tris/HCl (1.5M, pH 8.8).

Immunoprecipitation of VLPs

VLPs (0.4 ml) were mixed with anti-uPA monoclonal antibody (0.1ml, #3921 American Diagnostica) and incubated for 1hr at 4 degrees with rotary agitation. Protein A - Sepharose CL-4B (50%, v/v) was added (80ul) and incubated for another 1 hr. The mixture was centrifuged and the pellet washed with 1 ml of buffer A, then bound protein was eluted with 100 mM glycine/HCl (pH 3.0) and immediately neutralized with Tris/HCl (1.5M, pH 8.8) before further analysis.

Electron microscopy of VLPs

A preparation of purified VLPs (0.5-1mg/ml) was placed on a carbon-coated copper grid for 5min and negatively stained with 1% phosphotungstic acid (pH 7.0) for 5min. Transmission electron microscopy was performed with a JEOL 1200EX transmission electron microscope operating at 100 kV and photographs were taken with a magnification factor of 100K.

Hemagglutination of VLPs

Purified VLPs (0.5mg/ml, 100 ul) were serially diluted with saline in a u-bottomed 96-well plate. Equal volumes of guinea pig red blood cells (2 %, v/v) were added to each well and incubated for 3 hrs at room temperature

VLP binding to U-937 cells

Human U-937 cells (ATCC, CRL 1593) growing in RPMI-1640 medium supplemented with 10% FCS and gentamycin (20mg/L) were harvested and resuspended at a concentration of 5X10E(6) cells/ml. Induction of uPAR was performed as described by Stoppelli et al (44) with slight modifications. Phorbol 12-myristate 13-acetate (PMA) was added at a concentration of 50nM and the cells were cultured for 24 hrs in T-150 flasks. Most of cells were attached by this time. The medium was changed and the cells incubated for another 2 days, at which time the cells were detached with PBS-EDTA and incubated in 50mM glycine/100mM NaCl (pH 3.0) for 3 min to remove bound endogenous uPA, washed with phosphate buffered saline containing 0.1 mg/ml of bovine serum albumin (PBSA) and resuspended at 5X10E(7) cells/ml in PBSA.

Expression of uPAR was verified by Western blot analysis using anti-uPAR antibody (#3936, American Diagnostica). Purified VLPs (20 ug) with or without competitor were mixed with 0.1ml of cell suspension from above and incubated for 40 min on ice. As a competitor, 30 ug of high molecular weight uPA (American Diagnostica) was added in each reaction. At the end of incubation, unbound VLPs were removed by washing twice with PBSA and cells were lysed with 100ul of SDS-PAGE sample buffer. SDS-PAGE and Western blot analysis were performed to evaluate the amount of bound VLPs.

Results

Expression and preliminary analysis of modified VP1 proteins

SDS-PAGE analysis of the lysates from cells infected with baculoviruses expressing polyomavirus VP1 revealed that all the modified VP1 proteins were expressed as well as unmodified VP1/wt protein. The lysates were subjected to centrifugation to separate soluble from insoluble protein. The distribution of VP1 between the two fractions was determined by Western blot analysis using anti-VP1 antibody (Table 1). The VP1/HI-FLAG occurred mainly in the supernatant fraction, like VP1/wt, and approximately two

thirds of VP1/EF-Clone-20, VP1/EF-uPA (10-34) and VP1/EF-uPA (1-60) proteins were present in the supernatant (notably, all of these have peptide insertions at EF loop of VP1). They also were predominantly presented in the supernatant when co-expressed with VP1/wt or VP1/HI-FLAG. However, all other modified VP1 proteins were found predominantly in the pellet whether or not they were co-expressed with VP1/wt or VP1/HI-FLAG.

We ascertained if uPA sequences introduced into VP1 could be recognized by antibodies such as #3921 and #389 (American Diagnostica) directed against uPA (monoclonal and polyclonal antibodies, respectively). It was observed that uPA (1-60) and uPA (1-135) in all of the VP1 loops reacted with these antibodies, but uPA (10-34) did not (data not shown). The uPA (1-60) introduced into the EF loop showed markedly stronger signals in Western blots than uPA (1-60) introduced into BC, DE and HI loops of VP1 (Fig. 1). This might mean that the native structure of uPA (1-60) is retained better when introduced into the EF loop. Consequently, we focused subsequent studies on VP1/EF-uPA (1-60), thinking that it might be more capable of binding to its receptor.

Formation of VLPs by modified VP1 proteins

The supernatants of cell lysates containing VP1/wt, VP1/HI-FLAG, VP1/EF-uPA (1-60) and VP1/EF-uPA (1-60) + VP1/HI-FLAG (co-expression of both proteins) were pelleted through 20% sucrose to determine whether they formed VLPs. As shown in Fig.2 B, VP1/HI-FLAG sedimented like VP1/wt, suggesting it self-assembles into VLPs. However, VP1/EF-uPA (1-60) did not sediment like VP1/wt, indicating it might not self-assemble into VLPs or that it makes smaller VLPs that cannot be sedimented by this procedure. When VP1/EF-uPA (1-60) and VP1/HI-FLAG were co-expressed, both

sedimented like VP1/wt (B lane 4), suggesting these two proteins might interact to form heterotypic VLPs. We further analyzed the partially purified VP1 proteins by sedimentation through 10-40 % sucrose. All fractions were analyzed by SDS-PAGE and Western blotting. VP1/EF-uPA (1-60) + VP1/HI-FLAG were found to sediment at the same rate as VP1/wt, but VP1/EF-uPA (1-60) expressed alone sedimented much more slowly (data not shown). This supports the notion that VP1/HI-FLAG and VP1/EF-uPA (1-60) might form heterotypic VLPs when co-expressed.

Purified VP1/wt, VP1/HI-FLAG, VP1/EF-uPA (1-60) and VP1/EF-uPA (1-60) + VP1/HI-FLAG were examined by transmission electron microscopy (Fig. 3). Both VP1/wt and VP1/HI-FLAG preparations contained typical 50nm VLPs, but only smaller particles of approximately 20nm diameter were observed with VP1/EF-uPA (1-60), suggesting they might be 24-ICOSA structures(3). The VLPs preparation from cells co-infected by VP1/HI-FLAG and VP1/EF-uPA (1-60) contained particles approximately 50nm, suggesting VP1/EF-uPA (1-60) was incorporated together with VP1/HI-FLAG into the larger VLPs. To demonstrate the formation of heterotypic VLPs, VP1/HI-FLAG and VP1/EF-uPA (1-60) were co-expressed and purified as above. The proteins were immunoprecipitated with anti-uPA antibody (#3921) and analyzed by Western blotting (Fig. 4). VP1/HI-FLAG was precipitated by this antibody as well as VP1-EF-uPA (1-60). In a similar experiment using anti-FLAG antibody, VP1-EF-uPA (1-60) was co-precipitated as well as VP1/HI-FLAG. Electron microscopy of these precipitates could not be distinguished from those that had been purified by sucrose sedimentation. This evidence suggests that VLPs formed by co-infection are composed of two kinds of modified proteins.

Specific binding of heterotypic VLPs to uPAR

It has been observed that peptide insertion into HI loop results in disruption of sialic acid-binding capacity of VP1(17, 18, 20). The insertion of FLAG epitope into the HI loop showed a similar effect, as hemagglutination of red blood cells by this modified protein was blocked, indicating sialic acid binding was destroyed. Since it is necessary to disrupt the sialic acid binding of VLPs to test the affinity between uPAR and uPA (1-60) introduced into VP1, we introduced the FLAG epitope into VP1/EF-uPA (1-60); A doubly modified protein, VP1/EF-uPA (1-60)/HI-FLAG, formed heterotypic VLPs along with VP1/HI-FLAG or VP1/wt, as shown when immunoprecipitation was performed using #3921 antibody (data not shown). To assess the affinity of heterotypic VLPs for uPAR, U-937 cells were chosen as a source of uPAR. They express high levels of uPAR by stimulation with PMA, while they hardly expressed any uPAR without PMA stimulation(42, 42, 44).

Since VP1/wt binds to sialic acids that are abundant on eukaryotic cells, it is expected to bind to U-937 cells regardless of stimulation with PMA (Fig. 5). On the other hand, VP1/HI-FLAG, which lost sialic acid binding capacity, did not bind to U-937 cells. Heterotypic VLPs composed of VP1/EF-uPA (1-60)/HI-FLAG and VP1/HI-FLAG could bind to PMA stimulated U-937 cells only but not to unstimulated U-937 cells. This binding was inhibited by uPA protein added as competitor indicating it is due to the specific interaction between uPA (1-60) introduced into VP1 and uPAR of U-937 cells.

Discussion

The BC, DE, EF and HI loops of the polyomavirus are surface-exposed and thus, provide potential sites for the insertion of affinity ligands. The HI loop has been

extensively exploited as an insertion site of foreign peptide sequences such as the DHFR of *E.coli,* protein Z sequence, or a polyanionic adaptor sequence. Modification of the HI loop is attractive in that sialic acid binding can be disrupted at the same time with the insertion of foreign peptide sequences. However, the introduction of certain peptides into the HI loop affected self-assembly of VP1, often leading to aberrant VLP structure. For example, introduction of the WW domain into the HI loop destroyed the correct folding of the WW domain and additionally the VP1/HI-WW protein failed to form any VLPs(19). Also, VP1/HI-DHFR showed decreased self-assembly, with about 20 % failing to make VLPs, and most were smaller than typical polyoma 50nm VLPs (17).

Each peptide sequence might have its own ideal site into which it can be incorporated with minimal interference to the structure of VP1. It is known that BC loop of poliovirus and HI loop of adenovirus and C-terminus of papillomavirus L1 protein are quite tolerant to genetic modification(14, 47, 48). To determine the general acceptor site of polyomavirus VP1, we have performed comparative study by inserting four different kinds of sequences, clone-20 peptide, uPA (10-34), uPA (1-60) and uPA (1-135), into four different loops of the VP1 protein. The modified VP1 proteins containing these foreign peptide sequences in each loop were expressed in insect cells. Even though their expression levels were similar to that of VP1/wt, their solubilities were quite different. Most of modified VP1 proteins were insoluble and found in pellet after cell lysis. But some modified VP1 proteins exhibited moderate solubilities, all of which had peptide insertions in the EF loop, suggesting that insertion of foreign peptides into the EF loop has less effect on the native structure of VP1 than into the other three loops.

We also tried to detect uPA sequences introduced into VP1 by using #389 and #3921 antibodies. They could detect peptide sequences uPA (1-60) and uPA (1-135) regardless of insertion site but not uPA (10-34). It was possible that uPA (10-34) lost its structural feature that could be recognized by these antibodies when inserted into VP1. However, the FLAG epitope introduced into HI loop seemed to retain its native structure, because we could immunoprecipitate VP1/HI-FLAG or VP1/EF-uPA (1-60)/HI-FLAG with anti-FLAG antibody. From the study with #3921 Ab, we observed an interesting fact that, even though #3921 could detect uPA (1-60) inserted into any loop of VP1, the extent of detection was quite different depending on the site of insertion. With the same amount of protein, VP1/EF-uPA (1-60) showed stronger signal than VP1/BC-uPA (1-60) or VP1/DE-uPA (1-60) or VP1/HI-uPA (1-60) in Western blot analysis. This suggests structural feature of uPA (1-60) might be more preserved when it was inserted into EF loop than any other loops of VP1. The solubilites and antibody responses of modified VP1 proteins, combined together, suggest the EF loop is better site for ligand insertion than the other loops of VP1. It is conceivable that EF loop, fairly isolated at the side of pentamer might be more flexible and accommodating than BC, DE or HI loop, which are interlocked to form the top surface of pentamer(9) and hence may be less flexible than the EF loop.

There are several factors that appear to be critical for successful VP1 engineering. First of all, site of ligand insertion seems to be critical. As mentioned above, insertion of same ligand into different loops of VP1 gave totally different results. In the case of WW domain, DE loop was more compatible than HI loop(19), even though BC and EF loop were not tested. Our results indicate EF loop is more favorable for uPA-related sequences

than the other loops of polyomavirus VP1. Of course, more research has to be done to generalize this idea.

Second, size of ligand might be a limiting factor. Since polyoma VLP is composed of 360 copies of VP1 monomers, introduction of a certain ligands into VP1 results in 360 copies of them at the surface of VLP, which might cause serious space problem. The molecular weight of *E.coli* DHFR and uPA (1-135) are ~18 KD and ~15KD respectively. It seemed to be difficult to display 360 of these fairly large peptides on the surface of VLP. On the other hand, relatively small peptides such as protein Z (~6.8 KD) and WW domain (~4 KD) could be introduced without disrupting the self-assembly of VP1. The size constraint of incoming ligand was also reported in the papillomavirus L1 protein, which could accommodate ~60 amino acids(49). One of the ways to overcome size constraint would be formation of smaller particles, which would have larger curvatures. The size shift observed from VP1/HI-DHFR might be explained by the fact that smaller particles could have more space on the surfaces to accommodate foreign peptide(3). Another way to overcome size constraint would be formation of heterotypic VLPs. The VP1/wt or VP1/HI-FLAG has no or very small peptide insert and hence, may relieve some steric hinderence exerted by incoming ligands. In fact, the VP1/EF-uPA (1-60) in our study could form heterotypic VLPs along with VP1/wt or VP1/HI-FLAG even thought it could not form 50nm VLPs by alone. When the relative ratio of VP1/EF-uPA (1-60) to VP1/HI-FLAG is varied, by adjusting the m.o.i., during the formation of heterotypic VLPs, the size of resulting VLPs also changed. It is observed that the more VP1/EF-uPA (1-60) protein is present in VLPs, the smaller the particles become, shifting the size equilibrium to the 20nm as a main population (data not shown).

Another factor to be considered in VP1 engineering might be the hydrophilicity/hydrophobicity of ligand and it maybe directly related to the correct folding of ligands itself. The ligand introduced into VP1 would be displayed at the surface of VLPs and hence, it should be hydrophilic in nature. It is plausible to display a short anionic sequence on the surface of VLP without interfering with the ionic feature of VLP surface that is mainly negatively charged in neutral and alkaline solutions(50). The FLAG epitope, composed of 8 amino acids (DYKDDDDK), has 5 negatively charged aspartates, which make FLAG epitope an ideal candidate to be displayed on the surface of VLP. In fact, FLAG epitope was successfully inserted into the HI loop of adenovirus without any loss of fiber knob function(48). The polyanionic adaptor (E8C) is also hydrophilic in nature, and has 8 negatively charged glutamic acids(20) and it should be relatively easy to display them on the surface of VLPs. In our study, VP1/EF-uPA (1-60) was moderately soluble unlike VP1/EF-uPA (1-135). It might be due to the fact that uPA (1-60) is overall more hydrophilic than uPA (1-135) as well as it has smaller size. In fact, the uPA (1-135) itself was expressed in inclusion body in *E.coli*(51) and expressed as insoluble protein in insect cells (our own study, data not shown), which suggest uPA (1-135) is difficult to fold correctly in cytoplasm of *E.coli* and insect cells. It might be even more difficult for uPA (1-135) to fold correctly when it is introduced into VP1 protein.

Apart from the potential for delivery of DNA, the formation of heterotypic VLPs presents some interesting prospect in the use of VP1 as polyvalent targeting vector i.e., multiple ligands can be displayed at the same time in one particle and hence, targeting specificity to a certain cell type might be increased by using more than one ligand. There are many peptide sequences that can be used to target specific cell-types. One of

examples is EGF-like domain of heregulin. It is composed of 60 amino acids and known to sufficient for the binding to epidermal growth factor (EGF) receptor(52) that is expressed on many breast cancer cells(53). Also, C-terminal 21 amino acids of gastrin-releasing protein (GRP) has proved its potential to target to its receptor(54), which is over-expressed in a variety of carcinoma and melanoma(55-57). It seems feasible to incorporate these ligands, due to their compactness, into heterotypic VLPs in combination with other ligand such as uPA (1-60) to increase specificity of targeting to a certain cell-type. These enhanced specificity by polyvalent VLPs maybe also valuable in imaging of a certain cancer cells that express multiple tumor-associated antigens.

Another possible use of heterotypic VLPs would be as carriers of multiple antigenic epitopes. It is known that VLP elicits similar immune response to a virus with inherent adjuvant activity and thus, might be applied to the preparation of vaccine. In fact, VLPs of HIV gag protein(58), bovine papillomavirus L1 protein(59) and hepatitis surface antigen(60) have proven as efficient antigen delivery tools. Especially in the work of Liu *et al*(47), multiple CTL epitopes were introduced at the C-terminus of L1 protein to induce immune response against HIV and HPV at the same time. But its use as polyvalent carrier is limited by the fact that only upto 60 amino acids can be introduced without affecting VLP self-assembly. Even though polyomavirus VLPs also showed similar size limitation to incoming peptide in our study, the formation of heterotypic VLP might overcome such limitation because it may be formed by several kinds of VP1 proteins that have different peptide inserts in each of them.

Acknowledgments

We thank Dr. Mark Martin, University of Missouri, Columbia, for providing pET-15b-VP1/wt and also thank Dr. Richard A. Consigli, Kansas State University, Manhattan, for his kind gift of anti-VP1 antibody. All electron microscopic pictures were taken by Molecular Biology Electron Microscopy core of University of Missouri-Columbia. This research was supported by U.S. Army Medical research and material command under DAMD17-98-1-8321.

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