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Enhanced Baculovirus-Mediated Transduction of Human Cancer Cells by Tumor-Homing Peptides

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Tumor cells and vasculature offer specific targets for the selective delivery of therapeutic genes. To achieve tumor-specific gene transfer, baculovirus tropism was manipulated by viral envelope modification using baculovirus display technology. LyP-1, F3, and CGKRK tumor-homing peptides, originally identified by in vivo screening of phage display libraries, were fused to the transmembrane anchor of vesicular stomatitis virus G protein and displayed on the baculoviral surface. The fusion proteins were successfully incorporated into budded virions, which showed two- to fivefold-improved binding to human breast carcinoma (MDA-MB-435) and hepatocarcinoma (HepG2) cells. The LyP-1 peptide inhibited viral binding to MDA-MB-435 cells with a greater magnitude and specificity than the CGKRK and F3 peptides. Maximal 7- and 24-fold increases in transduction, determined by transgene expression level, were achieved for the MDA-MB-435 and HepG2 cells, respectively. The internalization of each virus was inhibited by ammonium chloride treatment, suggesting the use of a similar endocytic entry route. The LyP-1 and F3 peptides showed an apparent inhibitory effect in transduction of HepG2 cells with the corresponding display viruses. Together, these results imply that the efficiency of baculovirus-mediated gene delivery can be significantly enhanced in vitro when tumor-targeting ligands are used and therefore highlight the potential of baculovirus vectors in cancer gene therapy.

Lately, tumor vasculature has received increased attention as a target for potential anticancer therapies. The vascular endothelium is implicitly important in cardiovascular diseases and cancer but is relatively poorly transduced by most present vector systems (4, 6). Selective targeting to tumors has been hampered by the unavailability of targeted vectors. Peptides that home to specific sites in the tumor vasculature are therefore attractive as carriers of therapeutic genes as well as diagnostic agents. Peptides and antibodies that recognize tumor-specific vascular signatures have been identified by novel methods such as in vivo screening of phage libraries, revealing extensive heterogeneity in tumor blood vessels and lymphatics (3, 46, 51). These homing molecules can consequently be exploited in the targeted delivery of therapeutic agents to inhibit tumor growth. Specifically, the LyP-1 (37), F3 (48), and CGKRK (9) peptides are known to home to either tumor lymphatics (LyP-1) or the tumor neovasculature (F3 and CGKRK) of experimental tumors. These peptides are capable of being internalized by certain tumor cells and tumor endothelial cells and delivering therapeutic-agent-like payloads such as T7-phage, fluorescein, and quantum dots into the target cells in vivo (2, 22, 36, 37, 48). However, their ability to target a bulky enveloped virus, such as baculovirus, has not been previously evaluated.

For the achievement of efficient and safe gene delivery vehicles, increasing interest has been directed toward the development of viral vectors for targeting specific cells and tissues. The insect virus *Autographa californica* multiple nucleopolyhedrovirus (Ac*M*NPV), a prototype of the *Baculoviridae* family, is a fresh and attractive candidate for gene therapy applications. Baculovirus is able to mediate transient and sustained gene delivery to a variety of primary and established cell lines (7, 10, 17–20, 25, 32, 40, 55) as well as to some tissues (21, 26, 38, 39, 53). Transgene expression has been demonstrated in vivo with an efficiency comparable to that of adenovirus (1). Several display strategies have been developed to engineer the surface characteristics of baculovirus (44). Pseudotyped viruses can be efficient tools for gene delivery; for example, the vesicular stomatitis virus G protein (VSVG) has been shown to enhance baculovirus-mediated transduction both in vitro and in vivo (5, 16, 29, 31, 45, 47, 56). However, this strategy lacks the possibility of targeting the virus to desired cells or tissues. Although targeting of baculovirus has, in most cases, resulted in enhanced binding, this interaction has not led to improved transduction (42, 43). To date, only avidin-displaying baculovirus has been shown to mediate both targeted and enhanced transduction to biotinylated mammalian cells (49). Generation of envelope-modified baculovirus vectors therefore enables new applications of the system, including targeted in vivo and ex vivo gene delivery (31, 33, 44).

Baculovirus is regarded as a safe gene delivery vehicle due to its high species specificity and lack of replication in mammalian cells. Ac*M*NPV can be readily manipulated, is easily produced at high titers, and has a large capacity for insertion of foreign DNA (18, 32, 33, 44). Moreover, the cytotoxic effects mediated by this insect virus in mammalian cells appear to be comparatively small (19, 53). Complement sensitivity of baculovirus particles has been overcome by using recombinant soluble complement receptor type 1 or by displaying decay-accelerat-

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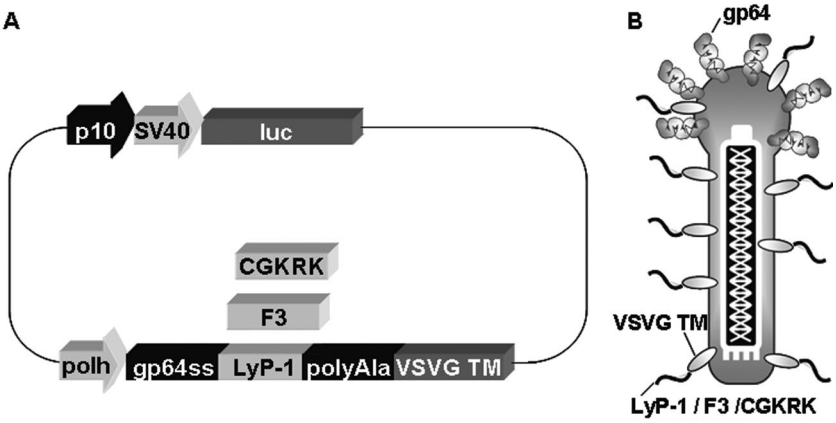

FIG. 1. (A) Schematic representation of the recombinant baculovirus constructs. (B) A schematic illustration of a recombinant baculovirus displaying the LyP-1-VSVG, F3-VSVG, or CGKRK-VSVG fusion proteins on the viral envelope. Abbreviations: gp64ss, the signal sequence of Ac*M*NPV gp64; LyP-1, F3, and CGKRK, targeting peptides; polyAla, a linker sequence encoding 20 alanine residues; VSVG TM, transmembrane/cytoplasmic domains of vesicular stomatitis virus G protein; p10, polh, and SV40, promoters of the p10, polyhedrin, and simian virus 40 genes, respectively.

ing factor on the viral envelope (23, 27). Overall, baculovirus is a promising and safe alternative for potential gene-therapeutic applications compared to pathogenic viral vectors.

We have endeavored to genetically modify baculovirus to improve transduction of human cancer cells in an efficient and selective manner by employing cyclic LyP-1 or linear F3 and CGKRK tumor-homing peptides originally identified by phage display (22, 37, 48). Here, we report the construction and evaluation of these novel baculovirus vectors and demonstrate improved binding and transgene delivery to both human breast carcinoma and hepatocarcinoma cells.

MATERIALS AND METHODS

Cell culture. *Spodoptera frugiperda* (Sf9; ATCC CRL-1711) insect cells were maintained in monolayer and/or suspension cultures at 28°C using serum-free Insect-XPRESS culture medium (Cambrex, Walkersville, MD) without antibiotics. The MDA-MB-435 human breast carcinoma cell line (Pirjo Laakkonen, University of Helsinki, Finland) was grown in a monolayer culture in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). The HepG2 human hepatocarcinoma cell line (ATCC HB-8065) was grown in a monolayer culture using minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (all from Invitrogen, Carlsbad, CA), and 10% FBS.

Generation of the recombinant baculoviruses; pLyP-1-VSVG/luc, pF3-VSVG/luc, and pCGKRK-VSVG/luc plasmid constructs. The 2,394-bp transcription unit of firefly luciferase composed of the simian virus 40 (SV40) promoter, *luc* gene, and the SV40 late poly(A) signal, in addition to the SV40 enhancer sequence, was amplified by PCR from the pGL3-Control Vector (Promega, Madison, WI). The 5′-terminal XhoI and 3′-terminal KpnI restriction sites (underlined) were concurrently introduced into the PCR product using 5′-AA AAA CTC GAG TGC ATC TCA ATT AGT CAG CAA CC-3′ forward and 5′-AA AAA GGT ACC GCT GTG GAA TGT GTG TCA GTT AG-3′ reverse primers (Oligomer, Helsinki, Finland), respectively. The PCR product was XhoI/KpnI double digested and subcloned into pFastBac Dual downstream of the p10 promoter, resulting in intermediate plasmid p10SV40-luc. The synthetic gene encoding the LyP-1-VSVG fusion protein (MVSAIVLYVL LAAAAHSAFA CGNKRTRGCA AAAAAAAAAA AAAAAAAAAS SIASFIIGLI IGLFLV LRVG IHLCIKLKHT KKRQIYTDIE MNRLGK) was purchased from GeneArt (Regensburg, Germany) and designed to contain the N-terminal signal sequence (gp64ss, residues 1 to 20) derived from the baculovirus major envelope glycoprotein, gp64, the sequence of the LyP-1 tumor-homing peptide (37), a linker region encoding 20 alanine residues (polyAla), and the reading frames of the transmembrane (TM) (residues 463 to 482) and cytoplasmic (CT) (residues 483 to 511) domains of VSVG. The codon usage of LyP-1-VSVG, F3-VSVG, and CGKRK-VSVG fusion genes (see below) was adapted to the codon bias of *S. frugiperda* genes. To complete the structure of the pLyP-1-VSVG/luc expression vector, the synthetic DNA sequence encoding the LyP-1-VSVG fusion gene was isolated by EcoRI/PstI double digestion from a commercial pPCR-Script vector (GeneArt, Regensburg, Germany), and subsequently inserted into pFastBac Dual under the transcriptional control of the polyhedrin promoter.

The vector intermediate, p10SV40-luc, was utilized as a backbone for the construction of the pF3-VSVG/luc and pCGKRK-VSVG/luc expression vectors. Similar cloning procedures were performed for the establishment of these plasmids as for pLyP-1-VSVG/luc. The synthetic genes encoding F3-VSVG (MVS AIVLYVL LAAAAHSAFA KDEPQRRSAR LSAKPAPPKP EPKPKKAPAK KAAAAAAAAA AAAAAAAAAA ASSIASFFFI IGLIIGLFLV LRVGIH LCIK LKHTKKRQIY TDIEMNRLGK) or CGKRK-VSVG (MVSAIVLYVL LAAAAHSAFA CGKRKAAAAA AAAAAAAAAA AAAAASSIAS FFFIIG LIIG FLVLRVGIHL CIKLKHTKKR QIYTDIEMNR LGK) fusion proteins were isolated by EcoRI/PstI double digestion from commercial pPCR-Script vectors (GeneArt, Regensburg, Germany) and transferred into the p10SV40-luc cloning intermediate under the polyhedrin promoter. The nucleotide sequences of the inserts were verified by sequencing.

Recombinant viruses were generated using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). Viral titers were determined by end point dilution assays from nonconcentrated baculovirus stocks using standard protocols. The resultant luciferase-expressing display viruses were named *Ac*LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc. Possessing a wild-type surface phenotype, the *Ac*-luc construct served as a control virus in all experiments.

Western blot analysis. Equal PFU counts of each virus were concentrated and solubilized in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing β-mercaptoethanol (5%). Samples were heat denatured (100°C, 5 min) and separated by reducing 15% SDS-PAGE. Molecular-weight marker was purchased from MBI Fermentas (Vilnius, Lithuania). Separated proteins were transferred from the gel onto nitrocellulose membranes (PROTRAN; Schleicher & Schuell Bioscience, Germany) by electroblotting, and the blots were blocked (5% dried milk powder in Tris-buffered saline–Tween) and probed with rabbit anti-VSVG tag antibody (1:4,000; Sigma-Aldrich, St. Louis, MO) or mouse anti-vp39 monoclonal antibody (MAb) (1:1,000) (60). Detection was performed using alkaline phosphatase-conjugated secondary antibodies (Promega, Madison, WI), Nitro Blue Tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' instructions.

Confocal microscopy. Sf9 cells, grown in suspension culture (2×10^6 cells/ml), were infected with an MOI (multiplicity of infection) of 3 for each recombinant virus. At 38 h postinfection (p.i.), cells were harvested, washed with phosphate-buffered saline (PBS), and labeled (1 h, 4°C) with rabbit anti-VSVG-tag antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:400 in 3% bovine serum albumin in PBS (BSA-PBS). Subsequent to washes with BSA-PBS, cells were incubated in

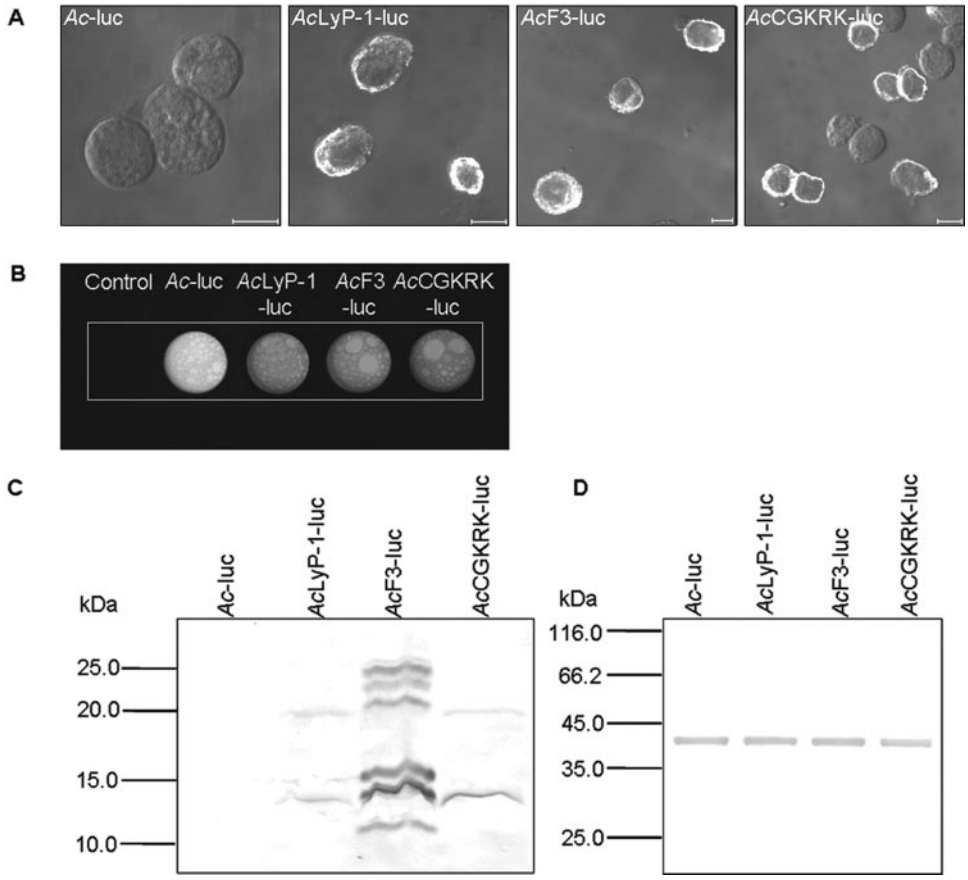

FIG. 2. (A) Expression of the LyP-1-VSVG, F3-VSVG, and CGKRK-VSVG fusion proteins on the surface of infected Sf9 cells at 38 h p.i. Fusion proteins were detected with rabbit anti-VSVG-tag antibody and analyzed by confocal microscopy. Images are single confocal optical midsections of cells, approximately 0.8 μm in thickness. Differential interference contrast and fluorescence images are merged. Scale bar, 10 μm. (B) Bioluminescence of luciferase-expressing infected Sf9 cells in microtiter plate wells at 38 h p.i. The images were captured with a digital camera (Nikon Coolpix 4100). (C) Immunoblot analysis of the recombinant baculoviruses (10^8 PFU) displaying LyP-1-VSVG, F3-VSVG, and CGKRK-VSVG fusion proteins on the viral envelope. *Ac*-luc served as a negative control. Viral proteins were probed under reducing conditions with rabbit anti-VSVG-tag antibody. (D) Determination of the ratio of total particle number versus the count of infectious virus (2.5×10^7 PFU) by immunoblot analysis with Ac*M*NPV anti-vp39 MAb.

the dark (30 min, 4°C) with goat anti-rabbit Alexa Fluor 555 immunoglobulin G (Molecular Probes, Eugene, OR) diluted in BSA-PBS, followed by washes with BSA-PBS. Finally, cells were resuspended in Mowiol (Calbiochem, Darmstadt, Germany) supplemented with DABCO (25 mg/ml; Sigma-Aldrich, St. Louis, MO) and mounted on microscope slides under glass coverslips. Samples were inspected under a confocal laser scanning microscope (LSM510, Zeiss Axiovert 100 M; Jena, Germany) using appropriate excitation and emission settings.

Flow cytometry. Binding of each virus to MDA-MB-435 and HepG2 cells was investigated by incubating 1×10^6 cells with each virus (100 PFU/cell) for 1.5 h at 4°C. All the steps throughout the experiment were performed at 4°C. Unbound virus particles were removed by washes with 1.5% BSA-PBS, and bound virus was detected by incubation with Ac*M*NPV gp64-specific MAb B12D5 (30) for 1 h followed by washes with 1.5% BSA-PBS and incubation with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 30 min. Fluorescence of cells was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software.

Binding inhibition experiments were performed using the same protocol with a few exceptions: 0.2×10^6 cells were incubated (15 min, 4°C) with synthetic LyP-1 (37), F3 (48), or CGKRK (22) peptides (50, 200, 500, and/or 1,000 μM) prior to virus exposure, and 0.2% FBS-PBS was used for washes throughout the experiment.

Transduction experiments. For all transduction experiments, 0.5×10^6 to 2×10^6 MDA-MB-435 or HepG2 cells were plated and allowed to attach overnight. Virus dilutions of desired counts of PFU/cell were prepared in appropriate culture medium (Dulbecco's modified Eagle medium or minimum essential medium) containing 1% FBS. Transduction was performed for 1.5 h at 37°C followed by addition of growth medium supplemented with 10% FBS. At 24 h or 48 h posttransduction (p.t.), cells were detached by scraping, concentrated by centrifugation (3 min, $500 \times g$), and resuspended in PBS. Samples were transferred onto a white pigmented 96-well plate (CellStar; Greiner-Bio One, Frickenhausen, Germany) followed by addition of 1 mM D-luciferin (Sigma-Aldrich, St. Louis, MO) diluted in 0.1 M Na citrate buffer (pH 5). Immediately after, the enzymatic activity of luciferase was measured with a Wallac 1420 Victor multilabel counter (Wallac Oy, Turku, Finland). The luminescence mode and 1-second counting time were applied using the software (version 2.00).

Transduction experiments were also performed in the presence of cyclic LyP-1 (37) or linear F3 (48), CGKRK (22), or RKK (28) peptides (250, 500, and/or 1,000 μM). Cells were preincubated in the presence of the peptides for 30 min at 4°C followed by addition of virus (100 PFU/cell) and further incubation for 60 min at 4°C. Cells were then washed with cold culture medium to remove unbound virus, followed by virus adsorption and transduction for 24 h at 37°C in complete growth medium. Cells were monitored for luciferase activity as described above.

Blocking of endocytosis was performed using different concentrations of ammonium chloride. Virus preparations (100 PFU/cell) were diluted with ice-cold medium and allowed to adsorb to HepG2 cells for 1 h at 4°C prior to addition of ammonium chloride-containing medium to ascertain that ammonium chloride did not adversely affect virion structure and stability or interfere with viral binding. The virus-containing medium was replaced with warm (37°C) medium, followed by addition of ammonium chloride (1, 2, 4, 6, 8, and 10 mM). Trans-

duction was performed for 24 h at 37°C and the luciferase activity was measured as described above. To exclude the possibility that ammonium chloride quenched the luciferase signal, 10 mM ammonium chloride was added to the transduced control cells 1 h (37°C) prior to harvesting.

RESULTS

Construction and characterization of tumor-homing, peptide-displaying baculovirus vectors. We developed three surface-modified baculovirus vectors displaying tumor-targeting peptides (Fig. 1). The coding regions of the LyP-1, F3, or CGKRK targeting peptides were fused to the transmembrane cytoplasmic domains of VSVG through a polyalanine linker and coupled with the strong polyhedrin promoter of Ac*M*NPV. All vectors were further equipped with an expression cassette encoding firefly luciferase, enabling sensitive luminescent detection of transgene expression in both insect and mammalian cells.

The LyP-1-VSVG, F3-VSVG, and CGKRK-VSVG fusion proteins were directed to the surface of the recombinant baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells by the signal sequence of the major Ac*M*NPV envelope glycoprotein gp64. Expression of the fusion proteins was studied by confocal microscopy using an anti-VSVG-tag antibody that recognizes 11 C-terminal amino acids of the VSVG cytoplasmic tail. As shown in Fig. 2A, the LyP-1-VSVG, F3-VSVG, and CGKRK-VSVG fusion proteins were clearly detectable on the plasma membrane of the infected insect cells at 38 h p.i., indicating successful expression and transport to the surface of Sf9 cells. These proteins appeared to be uniformly distributed on the cell surface. Minor labeling was also detected in the cytoplasm, representing fusion proteins undergoing synthesis and translocation towards the cell membrane. Neither mock- nor *Ac*-luc control virus-infected cells showed reactivity with this antibody. In addition, strong expression of luciferase was demonstrated in Sf9 cells infected with the corresponding viruses at 38 h p.i. (Fig. 2B).

Samples of the recombinant viruses were exposed to immunoblot analysis to investigate incorporation of the LyP-1-VSVG (calculated molecular mass of a monomer, 8 kDa), F3-VSVG (10.5 kDa), or CGKRK-VSVG (7.6 kDa) fusion proteins into virus particles. Bands positive for the anti-VSVG-tag antibody, representing mainly dimeric and trimeric forms of the fusion proteins (50) (Fig. 2C), were identified, confirming incorporation of these proteins into the budded virions. In addition, bands most likely representing posttranslationally modified forms of the F3-VSVG were detectable. The control virus *Ac*-luc, possessing the wild-type surface phenotype, showed no reactivity with the anti-VSVG-tag antibody.

The ratio of the total particle number (TP) versus the number of infectious virus particles (IP) was determined by immunoblotting with anti-vp39 antibody, revealing a similar TP/IP ratio between the control virus and the surface-modified viruses (Fig. 2D).

Improved binding of the tumor-homing, peptide-displaying viruses to MDA-MB-435 and HepG2 cells. The LyP-1, F3, and CGKRK peptides all bind to and accumulate within certain tumor cells and tumor endothelial cells in vivo (22, 37, 48). Based on these studies, MDA-MB-435 cells were chosen for binding and transduction studies performed in vitro. In addition, the HepG2 cell line, known to be susceptible to baculovirus transduction, was used (24, 25). Both MDA-MB-435 and HepG2 cells are known to express nucleolin (9, 12), the cell surface receptor responsible for F3 internalization (9), and common cell surface components such as heparan sulfate proteoglycan, the putative cellular attachment and/or internalization molecule for F3 and CGKRK (9, 22). Viral binding was quantitatively measured by flow cytometry (Fig. 3) using Ac*M*NPV-specific anti-gp64 antibody. The results showed that the tumor-homing, peptide-displaying viruses exhibit two- to fivefold-greater binding, determined in terms of mean fluorescence intensity, for both MDA-MB-435 and HepG2 cells compared to that of the control virus *Ac*-luc, suggesting that the displayed peptides improve viral binding to target cells.

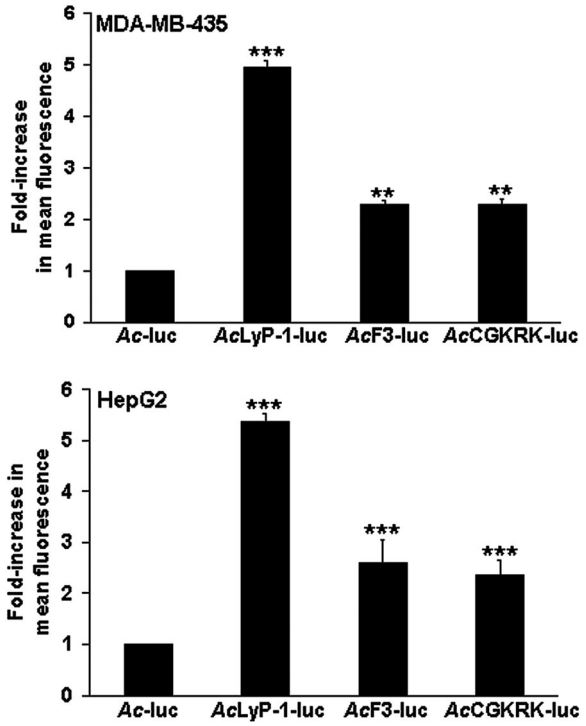

FIG. 3. Flow cytometric analysis of the binding of *Ac*LyP-1-luc, *Ac*F3-luc, *Ac*CGKRK-luc, and the control virus, *Ac*-luc (100 PFU/cell), to the surface of MDA-MB-435 and HepG2 cells. Viral binding was detected with mouse anti-gp64 MAb. Normalized mean fluorescence values (*Ac*-luc = 1) for each virus ± standard deviations of triplicate samples are indicated. The data were compared using the unpaired Student *t* test with a two-tailed *P* value, and statistical significance was determined. ***, $P < 0.001$; **, $P < 0.002$.

Enhanced transduction of MDA-MB-435 and HepG2 cells by the tumor-homing, peptide-displaying viruses. Luciferase molecules produce light in direct proportion to their numbers in mammalian cells. Therefore, the luciferase-expression cassette was exploited in order to scrutinize ligand-directed marker gene delivery by the recombinant viruses. The transduction efficiency, in terms of transgene expression level, was assessed by measuring the luciferase activity (Fig. 4A). Up to 7- and 24-fold increases in luciferase activity were achieved by the surface-modified viruses compared to the control virus in MDA-MB-435 and HepG2 cells, respectively. As expected, the increase in transduction efficiency was most prominent at low

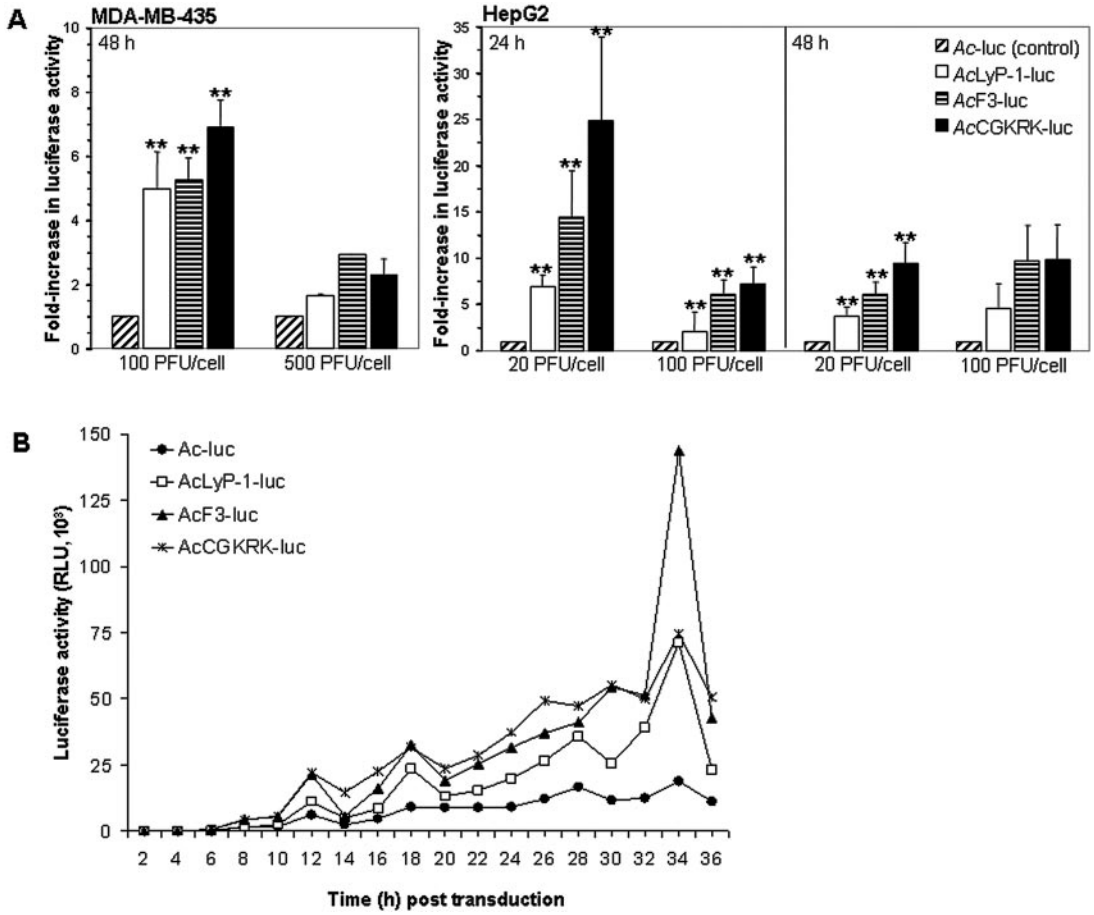

FIG. 4. (A) Viral transduction efficiency of MDA-MB-435 and HepG2 cells measured by luciferase activity. The cells were transduced with *Ac*-luc (control), *Ac*-LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc viruses, followed by monitoring the luciferase activity at 24 and/or 48 h p.t. Mock-infected cells were used as the background control. The PFU counts used are indicated. The results are shown as means of relative luciferase activity (*Ac*-luc = 1) ± standard deviations of three individual experiments, each performed in triplicate per variable. The data were compared using the unpaired Student *t* test with a two-tailed *P* value, and statistical significance was determined; **, $P < 0.05$. (B) Expression profile of *Ac*-luc, *Ac*-LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc viruses (100 PFU/cell) in HepG2 cells at 0 to 36 h p.t. The luciferase activity (relative light units [RLU]) was monitored every 2 h.

counts of PFU/cell, and the luciferase activity increased in a virus titer-dependent manner. With each virus, the luciferase expression climaxed at approximately 48 h p.t. in MDA-MB-435 cells (data not shown) and at 34 h p.t. in HepG2 cells (Fig. 4B). The MDA-MB-435 cell line was found to be rather nonpermissive for baculoviral transduction; luciferase activity was only weakly detectable earlier than 48 h p.t. even with high counts of PFU/cell, whereas in HepG2 cells, luciferase expression was detectable already at 4 h p.t. Use of sodium butyrate, a histone deacetylase inhibitor (11), had no effect on the transgene expression level in MDA-MB-435 cells.

To study whether the display viruses use the common entry route of wild-type Ac*M*NPV into HepG2 cells (41), different concentrations of ammonium chloride were used to prevent endosomal acidification. The transduction efficiency, determined by luciferase activity at 24 h p.t., progressively decreased for each virus with increasing concentrations of ammonium chloride (Fig. 5). The transgene delivery by each virus was completely abolished at 6 mM ammonium chloride, indicating

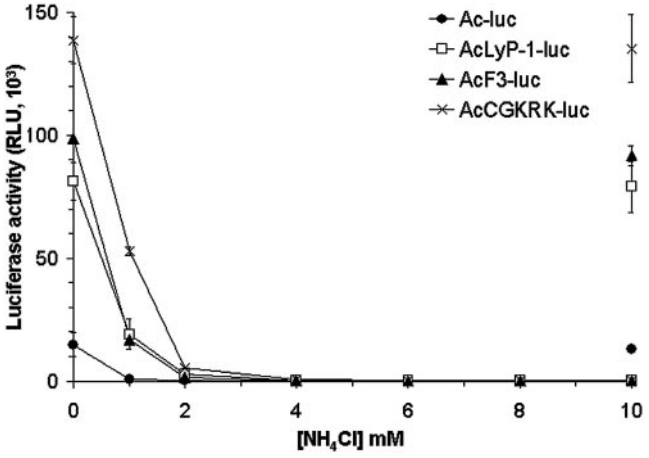

FIG. 5. Effect of ammonium chloride on gene transduction in HepG2 cells at 24 h p.t. Transduction efficiency of the *Ac*-LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc display viruses and the control virus, *Ac*-luc, was monitored by measuring luciferase activity. Influence of ammonium chloride per se on the luminescence signal was determined by addition of 10 mM ammonium chloride to transduced control cells 1 h prior to harvesting (single values on the right).

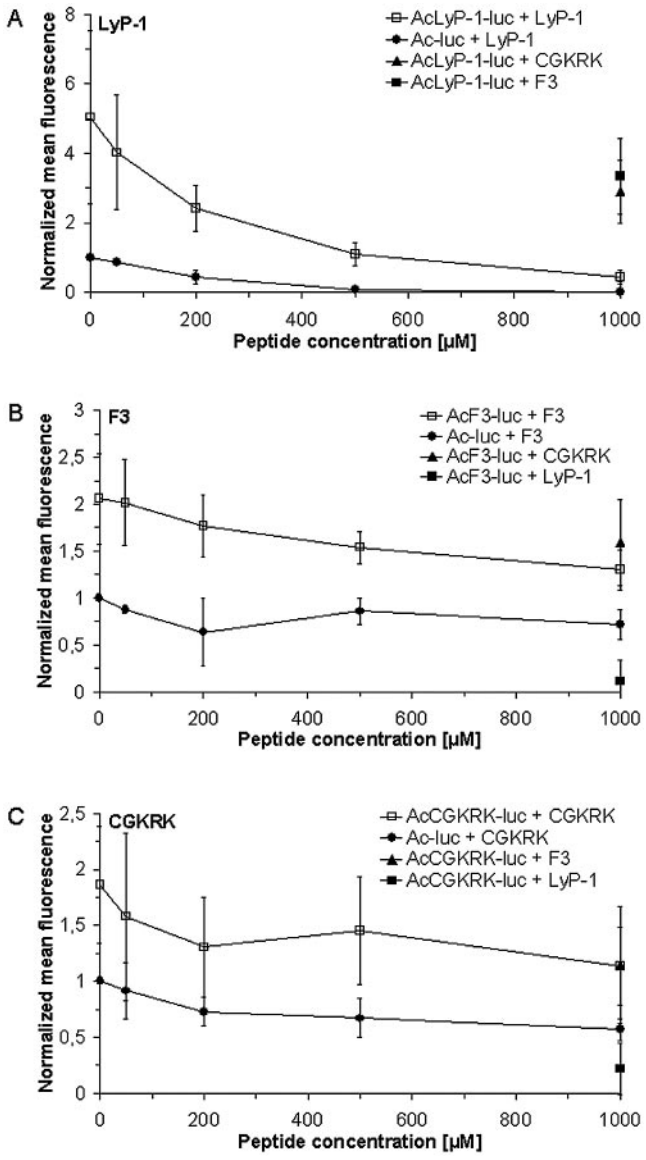

FIG. 6. Cross-inhibitory effect of the synthetic LyP-1 (A), F3 (B), and CGKRK (C) peptides on the binding of recombinant viruses *Ac*Lyp-1-luc (A), *Ac*F3-luc (B), and *Ac*CGKRK-luc (C) and control virus *Ac*-luc (A to C) to MDA-MB-435 cells. Cells were incubated with the corresponding soluble peptides (indicated in the figure) prior to virus attachment at 4°C followed by detection of the bound viruses by flow cytometry using anti-gp64 MAb. The data represent the means ± standard deviations of three independent experiments, each performed in duplicate.

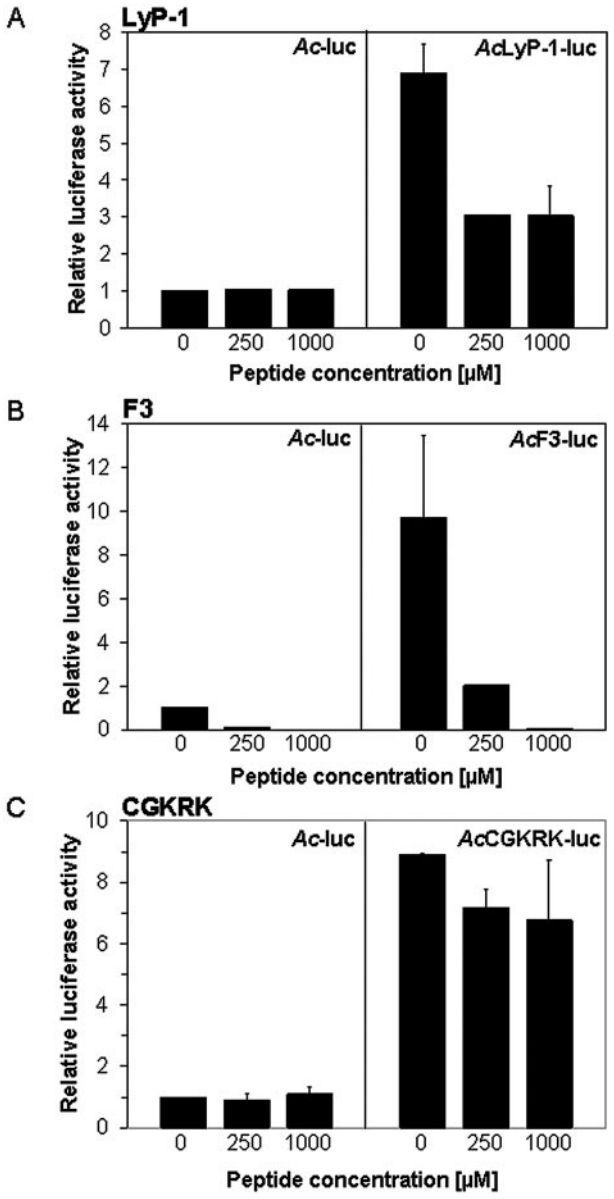

FIG. 7. Inhibitory effect of the synthetic LyP-1 (A), F3 (B), and CGKRK (C) peptides on the transduction efficiency of *Ac*LyP-1-luc, *Ac*F3-luc, and *Ac*CGKRK-luc, respectively. HepG2 cells were preincubated with the corresponding peptides at 4°C prior to addition of the virus (100 PFU/cell). An irrelevant RKK peptide was used as a control (not shown). The luciferase activity was monitored at 24 h p.t. The peptide concentrations and viral constructs are indicated.

the use of a similar entry mechanism by the control and surface-modified viruses.

Inhibition of virus binding and transduction by LyP-1, F3, and CGKRK peptides. Flow cytometry was used in a binding competition assay to measure the inhibitory effects of LyP-1, F3, and CGKRK tumor-homing peptides on binding of *Ac*LyP-1-luc, *Ac*F3-luc, *Ac*CGKRK-luc, and *Ac*-luc viruses to MDA-MB-435 cells. Cells were preincubated with different concentrations of soluble tumor-homing peptides, followed by virus binding and staining for the presence of the bound virus on the cell surface. The LyP-1 peptide was the strongest inhibitor of binding of all virus constructs, both in terms of magnitude of binding inhibition and apparent affinity (Fig. 6). The LyP-1 peptide bound to cells with an apparent dissociation constant in the hundreds micromolar range, CGKRK and F3 peptides being less specific. All three peptides self- and cross-inhibited the binding of each peptide-displaying virus and the control virus, with the F3 and CGKRK peptides causing less inhibition than the LyP-1 peptide in all cases (Fig. 6). Again, the enhancement of virus binding to MDA-MB-435 cells conferred by the presence of the tumor-homing peptides on the viral

surfaces is clear, the LyP-1 peptide causing the greatest, approximately fivefold, increase in virus binding to cells in the absence of peptide inhibitors. Together, the LyP-1 peptide inhibits binding of the tumor-homing, peptide-displaying baculoviruses and the *Ac*-luc control virus to MDA-MB-435 cells with a greater magnitude and specificity than CGKRK and F3 peptides.

The inhibitory effect of the synthetic LyP-1, F3, and CGKRK tumor-homing peptides on the transduction efficiencies of the corresponding viruses was studied by a luciferase activity assay using HepG2 cells. Preincubation of cells with 250 μM or higher LyP-1 peptide reduced the transduction of *Ac*LyP-1-luc virus by half but had no effect on the transduction efficiency of the control virus *Ac*-luc (Fig. 7A). Preincubation with 500 μM F3 peptide resulted in almost full inhibition of transduction by both *Ac*F3-luc and *Ac*-luc (Fig. 7B). Preincubation with 1,000 μM or lower CGKRK peptide reduced the transduction efficiency of the *Ac*CGKRK-luc to a lesser extent but did not affect the transduction of the control virus *Ac*-luc (Fig. 7C). A nonrelevant cationic peptide, RKK (28), had no effect on the transduction efficiencies (data not shown).

DISCUSSION

Limitations of current virus-based gene therapy vehicles for malignant tumors include a lack of cancer-specific targeting and inadequate gene delivery. Here, cancer-selective tropism of baculovirus was pursued by introducing tumor-targeting ligands into the envelope of Ac*M*NPV. We showed that display of the LyP-1, F3, and CGKRK tumor-homing peptides on the viral surface results in enhanced binding and transduction of two human cancer cell lines.

We developed three recombinant baculovirus vectors, each displaying a fusion protein composed of a tumor-homing peptide, LyP-1, F3, or CGKRK, coupled with the transmembrane anchor of VSVG through a polyalanine linker. A truncated VSVG composed of a 21-amino-acid ectodomain together with the TM and CT domains is known to enhance display and enable uniform distribution of the fusion proteins on the baculoviral surface (8, 42). This contrasts with the gp64-based fusion strategy, where the displayed polypeptides are mainly located at the pole of the virion (42, 44, 49). In addition, display by a fusion to VSVG-TM/CT leaves gp64 intact to facilitate viral infection and transduction. To improve specificity, we further modified the VSVG membrane anchor by excluding the 21-amino-acid truncated VSVG ectodomain, known to mediate unselective viral binding and transgene delivery to target cells (29, 42). The polyalanine linker, possessing inert, nonpolar side chains, was anticipated to provide distance and flexibility for the amino-terminal targeting peptides to be displayed correctly without compromising binding or transduction specificity. The VSVG TM and CT domains alone were demonstrated to be sufficient for promoting incorporation of these fusion proteins to the plasma membrane of infected insect cells in high quantities, and subsequently into budded virions. Native VSVG is known to form spikelike, noncovalently associated trimers, for the formation of which the cytoplasmic domains are sufficient (13, 14, 34). Here, the fusion proteins were most probably expressed as trimers (50), as trimerization is known to be necessary for the transport of VSVG from the endoplasmic reticulum to the Golgi apparatus and subsequently to the cell membrane (34). The presence of multimeric forms, despite the reducing conditions, was demonstrated by immunoblot analysis of the purified viruses. The fusion proteins may possess partial resistance to denaturation by SDS treatment, since SDS-resistant forms, conferred by the cytoplasmic tail of VSVG, have previously been reported (59). Thus, the subunits may stay bound together by noncovalent interactions during SDS-PAGE.

The virus binding experiments showed that the recombinant viruses, displaying either LyP-1, F3, or CGKRK peptides, each bound to the cellular membrane of both MDA-MB-435 and HepG2 cells at significantly higher levels than the control virus. Improved binding of the F3-displaying baculovirus vectors to both MDA-MB-435 and HepG2 cells was expected, as nucleolin, the cell surface receptor of F3 (9), is known to be present on the surfaces of both of these cell lines (9, 12). The positively charged F3 peptide is known to bind to nucleolin and is internalized in an energy-dependent manner involving the N-terminal acidic domain of nucleolin. The peptide also binds to cell surface heparan sulfate, although these negatively charged glycosaminoglycans are not required for internalization (9). In contrast, the cell surface receptor molecules for LyP-1 and CGKRK are unidentified (22, 36, 37). Binding of CGKRK to these cell lines in vitro has not been documented; nevertheless, this peptide has been demonstrated to home to MDA-MB-435 tumor xenografts in vivo and is known to possess rather broad tropism among cultured tumor cells (22). Also, CGKRK may recognize heparan sulfates and phosphatidylserine, and internalization may be mediated by these cell surface molecules (22). The receptor molecules for Ac*M*NPV attachment and entry have been suggested to include common constituents of the cell membrane including phospholipids or heparan sulfate proteoglycans (15, 57). The presentation of F3, LyP-1, and CGKRK on the viral surface may therefore augment the natural attachment and entry process of baculovirus to mammalian cells, facilitating enhanced binding and uptake of the virus by both specific and nonspecific interactions.

Results of the binding inhibition assay suggest that the LyP-1 peptide is superior to F3 and CGKRK peptides in terms of mediating the degree and probably also the specificity of viral binding to MDA-MB-435 cells. Interestingly, all three peptides cross-inhibited the binding of both the *Ac*-luc control virus and peptide-displaying viruses, suggesting that the peptides bind to similar or adjacent sites on the cell surface. This could be related to their grossly similar positively charged structures and potential interactions with cell surface molecules such as heparan sulfate and phosphatidyl serine. The LyP-1 peptide strongly inhibits binding of both *Ac*-luc and, consequently, all the peptide-displaying viruses. The reason for this is unclear, since the receptor for LyP-1 is presently unknown. However, it is possible that baculovirus naturally binds to the receptor(s) of LyP-1 in MDA-MB-435 cells and that this binding is augmented by the presence of the LyP-1 peptide on the viral surface.

Being positively charged and largely composed of basic residues, LyP-1, F3, and CGKRK have the striking property of being internalized into their target cells following binding. In this regard, LyP-1 and CGKRK resemble certain cationic cell-penetrating peptides, which are able to cause adsorptive en-

docytosis and subsequent energy-independent internalization (54, 61–63). However, internalization of F3 into cells is known to be energy dependent (48). Nevertheless, the most striking advantage of these peptides over other cationic cell-penetrating peptides is their specific targeting preference to certain malignant cell types in vivo (2, 22, 36, 37, 48).

LyP-1, F3, and CGKRK are able to carry diverse payloads, such as T4 phage, fluorescein, and quantum dots, into target cells in vivo (2, 22, 36, 37, 48). As baculovirus has been shown to enter mammalian cells, including HepG2 cells, by endocytosis (35, 41, 52, 58), we wanted to investigate whether the tumor-homing, peptide-displaying baculoviruses enter cells by the same mechanism. Our endocytosis inhibition results with ammonium chloride suggest that the entry of the display viruses is predominantly endocytosis dependent in HepG2 cells, as has been reported for wild-type virus (35, 41, 52). Thus, the displayed tumor-homing peptides likely improve baculoviral binding and consequently enhance virus internalization via the natural entry pathway rather than an alternative entry route. It is unlikely that such a bulky load as Ac*M*NPV would be capable of internalizing in an energy-independent manner.

The increase in transduction, due to the displayed peptides on the viral envelope, was considerable in both MDA-MB-435 and HepG2 cells. Although the baculovirus-mediated transduction of MDA-MB-435 cells turned out to be challenging, the enhancement in transgene expression was significant. Since the use of sodium butyrate, a selective histone deacetylase inhibitor (11), had no effect on the transduction efficiency, the barrier in the permissiveness of the MDA-MB-435 cells is a matter of speculation. Several steps including internalization, cytoplasmic trafficking, and/or nuclear import may be involved (35, 52, 58).

We also investigated the inhibitory effect of the synthetic LyP-1, F3, and CGKRK tumor-homing peptides on the transduction efficiency of the corresponding display viruses. The LyP-1 and F3 peptides significantly inhibited transduction of HepG2 cells by the corresponding recombinant viruses. This implies that the increased transgene expression by these modified viruses is at least partly a result of improved viral binding to LyP-1 and F3 receptors and/or enhanced uptake mediated by the peptides. The F3 peptide also inhibited transduction of the control virus, suggesting that wild-type Ac*M*NPV may use protein moieties similar to F3 in virus attachment and/or entry into HepG2 cells. In addition, being positively charged, F3 could block transduction of both *Ac*F3-luc and *Ac*-luc by binding to cell surface heparan sulfate (9, 15). The CGKRK peptide appeared to have only a modest inhibitory effect on the transduction of *Ac*CGKRK-luc. Since a nonrelevant cationic RKK peptide (28) had no inhibitory effect on viral transduction, the enhancement of transduction by the tumor-homing peptides displayed on the baculoviral surfaces might be mediated by more specific interactions than provided solely by the cationic nature of the peptides. Together, the complexity of the binding and transduction inhibition results likely reflects the involvement of more than one receptor in both the binding and the internalization of the virus. In addition, evidence of sequence-specific as opposed to charge-mediated enhancement of viral binding and internalization was clearest for the LyP-1 peptide.

To summarize, we have shown that introduction of tumor-targeting peptides into the viral envelope of baculovirus has facilitated modification of the tropism of this insect virus. This single-component system resulted in enhanced binding and transduction of certain human carcinoma cell lines, highlighting the potential of baculoviral vectors in cancer therapy. Introduction of tissue-specific promoters, suicide genes, and complement resistance (27) into these vectors may assist in more effective in vivo use of baculovirus for gene therapy applications in cancer treatment.

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