Immovobilized Cobalt Affinity Chromatography Provides a Novel, Efficient Method for Herpes Simplex Virus Type 1 Gene Vector Purification

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Herpes simplex virus type 1 (HSV-1) is a promising vector for gene therapy applications, particularly at peripheral nerves, the natural site of virus latency. Many gene vectors require large particle numbers for even early-phase clinical trials, emphasizing the need for high-yield, scalable manufacturing processes that result in virus preparations that are nearly free of cellular DNA and protein contaminants. HSV-1 is an enveloped virus that requires the development of gentle purification methods. Ideally, such methods should avoid centrifugation and may employ selective purification processes that rely on the recognition of a unique envelope surface chemistry. Here we describe a novel method that fulfills these criteria. An immobilized metal affinity chromatography (IMAC) method was developed for the selective purification of vectors engineered to display a high-affinity binding peptide. Feasibility studies involving various transition metal ions (Cu2+, Zn2+, Ni2+, and Co2+) showed that cobalt had the most desirable features, which include a low level of interaction with either the normal virus envelope or contaminating DNA and proteins. The introduction of a cobalt-specific recognition element into the virus envelope may provide a suitable target for cobalt-dependent purification. To test this possibility, we engineered a peptide with affinity for immobilized cobalt in frame in the heparan sulfate binding domain of HSV-1 glycoprotein B, which is known to be exposed on the surface of the virion particle and recombined into the viral genome. By optimizing the IMAC loading conditions and reducing cobalt ion leakage, we recovered 78% of the tagged HSV-1 recombinant virus, with a >96% reduction in contaminating proteins and DNA.

Human herpes simplex virus type 1 (HSV-1) is a neurotropic DNA virus that has been engineered for gene transfer applications, including human gene therapy (6, 18, 31). HSV-1-based vectors exhibit the advantages of a broad host cell range, a large transgene packaging capacity, and potentially lifelong transgene expression in neurons mediated by components of the natural virus latency promoter system (19, 20). Recent reports describe extensive efforts to improve the quality of replication-defective, genome-based HSV-1 gene vectors, including reducing vector cytotoxicity (32, 33, 61, 62, 77), exploiting viral persistence in neurons for long-term gene therapy (19, 20, 33, 76), and targeting vector tropism by glycoprotein modification (1, 7, 35). This class of HSV-1 vectors has been exploited for treatment in animal models of pain (5, 23, 24, 27, 29), peripheral neuropathy (12, 13, 21, 22), Parkinson’s disease (16, 69, 79), multiple sclerosis (17, 41, 42), cystitis (80), and cancer (3, 15, 26, 39, 40, 44, 46, 47, 58). In addition, characteristics of the optimum conditions for replication-defective vector growth enabled the efficient production of clinically relevant quantities of this vector (51, 52, 74, 75). However, efficient, validated methods for the purification of vectors that are nearly free of contaminating proteins and nucleic acids are in development and are required for patient applications.

Currently, HSV-1 vector preparations are harvested by clarifying the virus from cell debris by low-speed centrifugation followed by a high-speed centrifugation step to pellet the virus away from soluble protein and DNA molecules (37, 63). However, cell organelles, proteins, and DNA aggregates cosediment along with the viral particles during the second centrifugation step. The removal of those impurities from the virus preparation conventionally relies on density gradient centrifugation (63, 70, 71, 73), which is both time- and labor-intensive and is not easily amenable to scaling up for manufacturing processes. Moreover, gradient centrifugation may not remove particular contaminants that have size and density properties very close to those of the viral particles. Replacing the density gradient centrifugation step of HSV-1 vector purification with a more efficient and scalable purification method such as column chromatography will provide more efficient and cost-effective vector purification.

Column chromatography, such as ion-exchange or size exclusion chromatography, has been used for the purification of retroviruses (4, 34), adenoviruses (25), and adeno-associated virus (AAV) (25, 30, 50, 64, 83), which are all gene therapy vectors, as well as for the purification of HSV vaccines (49). Although these procedures can be effective, they may prove to be less efficient than ligand affinity chromatography (68). Im-
mobilized metal affinity chromatography (IMAC) is a powerful and inexpensive ligand affinity purification method that has been used for the purification of a variety of proteins and peptides (2, 55, 56, 72). IMAC utilizes the affinity interaction between the immobilized transition metal ions, such as Cu²⁺, Zn²⁺, Ni²⁺, and Co²⁺, and protein surface electron-donating groups, such as histidine, cysteine, and tryptophan residues. In comparison to the biomolecules such as antibodies or receptors that are used for affinity separation, the chromatography substrate used for IMAC is more stable and inexpensive and provides the same high selectivity (54). Although IMAC was introduced two decades ago, it has been mainly applicable to protein and peptide purification (55). Recently, this technique was reported for the purification of AAV (82) and baculovirus (81).

Here we report the use of IMAC for the purification of recombinant HSV-1 preparations that have been clarified and concentrated by centrifugation or other methods, such as large-scale batch filtration. In order to select the proper IMAC column, we first evaluated elution profiles for both the HSV-1 vector and contaminants (protein and DNA) on iminodiacetate (IDA)-chelating columns charged with different transition metals (Cu²⁺, Zn²⁺, Ni²⁺, and Co²⁺). Chromatographic analysis showed that neither the virus nor the contaminants bound IDA-Co²⁺, suggesting that this column could be useful for HSV-1 vector purification if the vector could be endowed with the ability to bind to immobilized cobalt. Accordingly, we constructed an HSV-1 recombinant with a cobalt affinity tag replacing the heparan sulfate (HS) binding domain of virion envelope glycoprotein B (gB). The binding and elution properties of the recombinant virus on IDA-Co²⁺ columns confirmed that efficient purification was possible. This method may also prove to be broadly applicable to the purification of gene transfer vectors derived from different virus backgrounds.

**MATERIALS AND METHODS**

**Viruses and cells.** All viruses used for this study were derived from the KOS strain of HSV-1. Vector OQZHG (14), derived from d106 (61, 62), was used as the untagged virus in the initial IMAC study. The d106 vector (kindly provided by Neal DeLuca, University of Pittsburgh) has deletions of the essential immediate early (IE) genes ICP4 and ICP27. Two other IE genes, ICP22 and ICP47, have mutations in their promoters such that they are expressed as early genes.

Southern blot hybridization. Southern blot analysis was performed to confirm that the KgBHAT genome carried the HAT coding sequence. Viral DNAs of KgBHAT and the control virus KgBpK were digested with BamH1 or HpaI, separated in an agarose gel, and transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.) for analysis. A biotin-labeled gB probe was synthesized from a 1.3-kb XhoI- and SalI-digested pTZ18UgBpK and used to detect the gB coding region in recombinant virus preparations by using the Northern blotting method (10). The hybridization and detection procedures were performed according to the instructions of a North2South chemiluminescent nucleic acid hybridization and detection kit (Pierce Biotechnology, Rockford, Ill.). The hybridization and detection procedures were performed according to the instructions of a North2South chemiluminescent nucleic acid hybridization and detection kit (Pierce Biotechnology, Rockford, Ill.).

Western blot analysis. Western blot analysis was performed to verify the presence of the HAT protein in the KgBHAT particles. The KgBHAT and KgBpK samples were submitted to Northern blotting and hybridized with a rabbit polyclonal antibody (Cointech, Palo Alto, Calif.) against the HAT peptide or with an HSV gB-specific monoclonal antibody (Viruses, Sykesville, Md.) overnight at 4°C in PBS containing 2% dry milk. The membrane was washed with PBS containing 0.05% Tween 20 (Sigma, St. Louis, Mo.) and incubated with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Sigma) in 2% milk-PBS for 1 h at 25°C. Additional washes were performed before detection was performed on a ChemiDoc XRS system (Bio-Rad, Hercules, Calif.). The X-ray film was exposed to X-ray film for 5 min. The X-ray film was then exposed to X-ray film for 5 min.

**Preparation of clared virus sample for chromatography.** The OQZHG virus was pelleted on 7B cells and virus was concentrated in a roller bottle containing 100 mL of DMEM supplemented with 10% fetal bovine serum (FBS), while the KgBHAT virus and its counterpart, the KgBpK virus, were propagated on Vero cells in Ultra-
MDCK serum-free medium (Cambrex Bioscience Inc., Baltimore, Md.) by the same procedure. Confluent cells in roller bottles were infected with virus at a multiplicity of infection (MOI) of 0.01. At 72 h postinfection, the cultures were harvested and centrifuged (314 × g) to remove cell debris. The supernatants were then centrifuged (40,000 × g) to pellet the virus. The virus pellets were subsequently resuspended in PBS (Roche Diagnostics, Indianapolis, Ind.), divided into aliquots, and stored at −80°C.

IMAC. Chromatography was performed with a 1-ml HiTrap IDA metal-chelating column (Amersham Pharmacia Biotech) using a Pharmacia LKB P-1 pump to load samples and buffers. The new column wasflushed with 5 ml volumes of 50 mM cuSO4, ZnCl2, NiSO4, and CoCl2 in water. The column was then subsequently washed with 7 CV of distilled water and then 7 CV of PBS–0.5 M NaCl (pH 4.0) buffer to wash out any nonspecifically bound metal ions. The column was then equilibrated with 7 CV of loading buffer (PBS–0.5 M NaCl, pH 7.0, unless otherwise stated). QOZHG or KgBHAT virus stocks were thawed and resuspended in 10 ml of loading buffer. The virus suspension was then passed through a 1.2-μm-pore-size syringe filter and loaded onto the column. After the column was washed with 5 CV of loading buffer, bound virus was eluted with 5 CV of PBS–0.5 M NaCl buffer, using step changes in the pH. Finally, an EDTA solution (50 mM EDTA, PBS, 0.5 M NaCl, pH 7.0) was used to strip the metal and any residual virus from the column. The loading virus suspension and all of the elution fractions were assayed for virus, total protein, and DNA concentrations. The flow rate was used 0.5 ml/min.

Plaque assay. The quantification of infectious viral particles was accomplished by a standard plaque assay. Serial dilutions of virus were added to 8 × 104 cells (Vero cells for the KgBHAT, KOS, or KgBpK virus and 7B cells for the QOZHG virus) in a 1.7-ml Eppendorf tube and rocked on a Nutator rocker (Becton Dickinson, San Diego, Calif.) at 37°C. After a 1-h adsorption period, the cells were plated in six-well plates. After 12 h, the inoculum was replaced with complete medium containing 0.5% (wt/vol) methylcellulose (Aldrich, Milwaukee, Wis.) and incubated for an additional 2 days for the KgBHAT virus and 3 days for the QOZHG virus. The plates were then stained with 1% (wt/vol) crystal violet in 50% methanol–50% H2O (vol/vol), and the numbers of plaques were counted. Titers were calculated as PFU per milliliter of virus suspension.

Protein and DNA assays. Total protein concentrations in loading samples and elution fractions from KgBHAT virus chromatography assays were measured with a Bio-Rad (Hercules, Calif.) protein assay kit. Protein concentrations in loading samples and elution fractions from KgBHAT virus chromatography assays were measured with a CBQCA protein assay kit (Molecular Probes, Eugene, Oreg.) due to the low protein concentrations in these samples. Bovine serum albumin was used as the standard protein for calibration for both protein assays. A Picogreen double-stranded DNA quantitation kit (Molecular Probes) was used to measure the DNA concentrations in all of the samples, with lambda DNA used as a calibration control. All assays were performed in triplicate under the conditions specified by the manufacturers.

Virus adsorption assay. Monolayers of confluent Vero cells in six-well plates were incubated at 4°C with 250 PFU of KOS, KgBpK-, or KgBHAT virus per well and suspended in 1 ml of serum-containing medium (DMEM containing 10% FBS) per well for 10 to 110 min, after which the unbound virus was removed and the cell monolayers were washed three times with cold serum-containing medium. The cell monolayers were subsequently overlaid with 2 ml of DMEM–10% FBS containing 0.5% methylcellulose, shifted to 37°C, and incubated for 2 days to allow plaques to form. In control wells, the cells were incubated with the virus for 2 h at 4°C, the plates were shifted to 37°C for an additional 1-h incubation, and the previous medium was replaced with 2 ml of methylcellulose medium. The plaques formed in each well were stained, counted, and normalized to control wells. The amounts of increase in normalized plaque number with increases in adsorption time were taken as a measure of the amount of virus adsorption rate.

Virus penetration assay. The penetration rates of the gB recombinants were assessed by determining the rates at which penetrated viruses became resistant to inactivation by the treatment of infected monolayers with a low-pH glycine buffer. Confluent Vero cells in six-well plates were incubated with 250 PFU of KOS, KgBpK-, or KgBHAT per well in 1 ml of serum-containing medium per well for 2 h at 4°C. After adsorption, the cells were rinsed three times, overlaid with serum-containing medium, and shifted to 37°C to allow virus penetration. At selected times after the temperature shift, the cells were treated with 1 ml of 0.1 M glycine (pH 3.0) per well for 1 min to inactivate viruses that had not penetrated the cells. The infected control monolayer was treated with 1 ml of PBS for 1 min. The cells were washed three times with complete medium, overlaid with methylcellulose medium, and incubated at 37°C for 2 days. Plaques were visualized and counted as described above.

RESULTS

IMAC of untagged HSV-1. The binding selectivity of IMAC can be tailored through the choice of metal ions or buffer conditions or by surface modification of the target product (a protein, cell, or virus). The most desirable scenario would be that IMAC using one of the commonly employed transition metal ions (Cu2+, Zn2+, Ni2+, or Co2+) could be used to substantially purify HSV-1-based vectors. Therefore, we first examined the feasibility of using metal ion columns for the purification of untagged HSV-1.

The HSV-1-based vector QOZHG (14) was used to test the binding and elution properties of untagged HSV-1 in IMAC. QOZHG is a replication-defective virus with a deletion of the essential IE genes and contains expression cassettes for both LacZ and green fluorescent protein. The vector was grown on 7B cells, which complement the missing essential viral genes in trans. QOZHG was harvested from virus-infected cells, clarified by low-speed centrifugation, and concentrated by high-speed centrifugation (see Materials and Methods for details). Aliquots of virus were resuspended in loading buffer and introduced onto IDA columns that were charged with different metal ions. The columns were washed with loading buffer to remove any nonspecifically bound material, eluted with a pH step gradient, and finally stripped with an EDTA-containing buffer. The eluent samples were collected and assayed for total protein and DNA contents as well as for numbers of infectious viral particles. The resulting elution profile showed that the binding strength of the virus particles to the IDA columns followed the order Cu2+ > Zn2+ > Ni2+ > Co2+ (Fig. 1). The IDA-Cu2+ column captured most of the loaded untagged virus, with only a very small percentage of virus present in the flowthrough (0.67%) and the pH 7.0 wash fraction (0.29%). Protein and DNA were also efficiently bound to Cu2+ and were only removed by stripping of the column with EDTA. Only a small percentage of infectious virus was recovered by this method, suggesting that exposure to the copper column resulted in virus inactivation. The Zn2+, Ni2+, and Co2+ columns displayed decreasing protein, DNA, and virus binding, respectively. The protein, DNA, and virus elution profiles for each of these four columns did not provide a means of separating the virus from contaminants. However, it was interesting that the Co2+ column bound minimal amounts of protein, DNA, and virus in certain fractions (pHs 5.5 and 5.0); negligible amounts of contaminant proteins and DNA were eluted from the column, thus creating a low background region. These results suggested that IMAC might be useful for separating virus from contaminants if the virus particles could be tagged with a cobalt-specific ligand to enhance virus binding to cobalt under loading conditions and subsequently eluted in the low background region identified.

Construction of cobalt affinity peptide-tagged HSV-1 virus KgBHAT. Based on the above elution profiles, we postulated that the fusion of a short metal binding peptide to a virion surface protein might endow the virus with sufficient affinity for immobilized cobalt. This enhanced binding under loading conditions could also direct its elution to the low background
For the peptide to be effective, it must be exposed on the virus envelope and accessible for binding to the immobilized cobalt on the column. Moreover, the peptide-bearing virus should only be minimally adversely affected in its ability to efficiently bind to the normal HSV-1 cell surface receptors and enter into target host cells.

A 19-mer polyhistidine affinity tag (HAT), KDHLIHNVIHK...
EEHAHAIK, naturally exists in the N terminus of chicken lactate dehydrogenase, has a high affinity for cobalt ions, and can be eluted under conditions that are milder than those for the His, tag (11). Therefore, we selected HAT as a cobalt affinity peptide for incorporation into the HSV-1 virion.

A replication-competent HSV-1 viral recombinant carrying the HAT peptide in the HS binding site of gB was constructed to evaluate the binding and elution properties of tagged HSV-1 on the IDA-Co$^{2+}$ column. A plasmid (pgBHAT) encoding HAT-tagged gB (Fig. 2B) was constructed with annealed oligonucleotides encoding the HAT peptide inserted in place of the deleted HS binding site in pTZ18UgBpK$^{-}$ (Fig. 2A), which encodes an HS-binding-deficient form of gB (36). The HAT-tagged gB construct was recombined into the HSV-1 genome by marker rescue of a gB nonsense mutant virus, K082 (10) (Fig. 2C). Recombinant viruses were selected on noncomplementing Vero cells, which cannot support the replication of the gB-deficient K082 parent virus.

Southern blot analysis was performed to confirm the presence of the HAT coding sequence in the recombinant virus genome. Viral DNAs from K082 and two recombinant isolates were extracted from virus-infected cells, digested with BamHI and HpaI, subjected to electrophoresis, transferred to a Nytran membrane, and then hybridized with a biotin-labeled gB-specific probe (Fig. 2C). The gB probe hybridized to two fragments (3.1 and 5.1 kb) of the parental K082 virus genome (Fig. 2E, lane 1) because of the existence of the HpaI site in the gB locus (10). This HpaI site incorporates the stop codon in the parental virus and is a marker for the gB null phenotype. The gB probe hybridized to a single 8.2-kb fragment in the putative HAT-bearing isolate KgBHAT (Fig. 2E, lanes 2 and 3), demonstrating that this region in the K082 genome was replaced with the sequences from pgBHAT by homologous recombination or that the isolates were revertants that had rescued the gB nonsense mutation during passage on the gB-complementing cell line. For verification of the insertion of the HAT sequence within the gB locus of the recombinant viruses, candidate recombinant viruses were subjected to PCR with two primers flanking the locus of HAT insertion. The ~250-bp PCR products were analyzed by DNA sequencing, and the results verified that the HAT-encoding sequences were inserted into the recombinant viral genomes in frame in the proper orientation (data not shown).

To verify the expression of the HAT epitope in the gB proteins of the recombinant viruses, proteins were extracted from control KgBpK$^{-}$ and KgBHAT virus-infected Vero cells and subjected to Western blot analysis with an anti-gB monoclonal antibody or an anti-HAT polyclonal antibody for detection. As shown in Fig. 3, the gB antibody detected the HS-binding-deficient form of gB in KgBpK$^{-}$-infected cells (Fig. 3, lane 1) and the HAT-tagged gB protein in KgBHAT-infected Vero cell samples (Fig. 3, lane 2). The HAT-tagged gB protein is 28 amino acid residues longer than its HS-binding-deficient counterpart, resulting in a 3.3-kDa molecular mass difference. This size difference is readily seen in Fig. 3, as the HAT-tagged gB protein migrated slightly slower through a NuPAGE Tris-acetate gel. The HAT antibody detected a protein with a molecular weight equivalent to that of HAT-tagged gB in KgBHAT-infected Vero cell samples (Fig. 3, lane 4), confirming that the HAT peptide was successfully incorporated into gB. As expected, no HAT epitope was detected in the untagged KgBpK$^{-}$-infected control sample (Fig. 3, lane 3). These experiments verify the insertion of the HAT sequences within the HS binding region of gB and the expression of the HAT peptide during productive infection.

**Chromatography of KgBHAT with IDA-Co$^{2+}$ column.** The binding and elution properties of KgBHAT for an IDA-Co$^{2+}$ column were investigated by using a virus stock harvested from a KgBHAT-infected Vero cell supernatant. The infections were carried out in serum-free medium to reduce the level of contaminating cellular proteins to aid in the purification process. Initially, the same chromatography procedures were employed for the tagged virus as those for untagged virus (Fig. 1). The results demonstrated that by tagging gB with the HAT peptide, the elution of the KgBHAT virus was directed to the pH 5.5 elution step (Fig. 4A), a region shown to have insignificant amounts of contaminating proteins and DNA (Fig. 1D). The amounts of KgBHAT virus in the pH 7.0 wash step and in the pH 6.5 elution fraction were negligible, demonstrating that the tagged virus efficiently binds cobalt ions above pH 6.5. Small amounts of bound virus were eluted at pH 6.0, and there was a major elution peak at pH 5.5, suggesting that the pH 5.5 buffer was sufficient to elute the majority of the bound virus from the immobilized cobalt column. In contrast, the control untagged virus, KgBpK$^{-}$, did not efficiently bind to the IDA-Co$^{2+}$ column (Fig. 4B), as expected. Although the elution of the tagged virus from the column was directed to the low background area (Fig. 4A), the recovery of virus as measured by the percentage of recovered infectivity was relatively low (approximately 20%) for this experiment. Pilot experiments suggested that loading the virus onto the column at different pHs modified the strength of virus binding to the column. Therefore, we attempted to increase virus recovery by manipulating the virus loading conditions.

**IDA-Co$^{2+}$ chromatography loading conditions.** The effect of altering the loading pH on the amount of recoverable virus was examined for a range of pHs (Fig. 5). For these assays, the KgBHAT virus was resuspended in buffer at pH 7.0, 6.5, 6.0, or 5.5 and then loaded onto an IDA-Co$^{2+}$ column. The column was washed with loading buffer and eluted at pH 5.5, after which each column was stripped with EDTA to remove any remaining virus. The treatment of virus preparations with EDTA did not affect virus infectivity (data not shown). The summation of the viable KgBHAT virus percentages from all fractions increased as the loading pH was decreased (Fig. 5), although initial virus binding to the column was also reduced as the loading pH was reduced. Viruses loaded at pH 7.0 showed the highest level of column binding, as demonstrated by the negligible amount of virus found in the flowthrough. However, the strength of this binding appeared to inactivate viruses on the column, resulting in a low total virus recovery. Lowering the loading pH to 6.5 also enabled high-affinity binding to the column, as confirmed by the minimal amount of virus collected from the flowthrough fraction, and resulted in increased virus recovery at the pH 5.5 elution step. A reduction in the loading pH to 6.0 resulted in reduced virus binding to the column and increased the amount of virus in the flowthrough fraction. This reduction in virus binding resulted in a reduced relative recovery in the pH 5.5 elution fraction compared to that for the pH 6.5 loading condition. When the samples were loaded at pH...
FIG. 2. Construction of recombinant HSV vector expressing HAT tag in gB. HAT-encoding oligonucleotides containing BglII sticky ends were introduced into the BamHI site of the plasmid pTZ18UgBpK (A), in which the sequence encoding the HS binding domain of gB was replaced by a BamHI site, to create the plasmid pgBHAT (B). The pgBHAT plasmid was used to rescue the gB-defective mutant virus K082 (C) to create the recombinant virus KgBHAT (D). (E) Southern blot analysis of two rescued virus isolates (lanes 2 and 3) digested with BamHI and HpaI and probed with the SphI-SalI fragment from BamHI-F of HSV KOS genomic DNA compared to the parental K082 (lane 1) virus. U₁, long unique segment; Uₛ, short unique segment; TMD, transmembrane domain.
5.5, at which most viruses do not bind, the vast majority of the loaded virus was collected in the flowthrough, with a small amount recovered from the pH 5.5 elution step, thereby suggesting that at lower pHs, virus binding was inefficient and not suitable for purification.

These results suggest that loading the virus at a higher pH (7.0) results in avid column binding and a lower recovery for the pH 5.5 elution step, whereas loading at a lower pH (6.0) weakens the binding of the virus to the column, resulting in overall virus loss due to flowthrough. The optimal balance of binding and recovery was seen for loading at pH 6.5 and eluting at pH 5.5, which was deemed optimal for purification of a HAT-tagged virus on an immobilized cobalt column.

**Two-column IDA-Co$^{2+}$ chromatography.** Experiments were conducted to test whether there was cobalt leakage from the column because this could be an important consideration for the acceptance of this method for the purification of vectors for clinical purposes. An atomic absorption analysis indicated that 4 to 5 ppm of cobalt was present in the eluted samples (data not shown). To address the problem of residual cobalt, we placed an IDA column which was not charged with any metal ions downstream of the immobilized cobalt column. The aim was to capture any cobalt ions that may have leaked from the

**FIG. 3.** Western blot analysis of HAT-tagged gB. Protein lysates from Vero cells infected with KgBHAT (lanes 2 and 4) and the control virus KgBpK$^-$ (lanes 1 and 3) were separated by using the NuPAGE system and were transferred to a polyvinylidene difluoride membrane. The membrane was then cut in half, and each part was probed separately either with a monoclonal antibody against HSV gB (lanes 1 and 2) or with a polyclonal antibody against the HAT peptide (lanes 3 and 4), followed by detection with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody, respectively. M, molecular size marker; Ab, antibody.

**FIG. 4.** Elution profile of HAT-tagged and control virus on IDA-Co$^{2+}$ column. Approximately $4.0 \times 10^7$ PFU of clarified KgBHAT virus (A) or KgBpK$^-$ control virus (B) was resuspended in 10 ml of loading buffer (PBS–0.5 M NaCl, pH 7.0) and applied to an IDA-Co$^{2+}$ column. After being washed with 5 CV of loading buffer, the column was eluted with a pH step gradient using 5 CV per step and with 5 CV of an EDTA solution (50 mM EDTA, PBS, 0.5 M NaCl, pH 7.0). The flow rate of the chromatography procedure was 0.5 ml/min. The percentages of recovered virus in wash and step gradient elution fractions were added, and summations are depicted on the right side of each plot.
FIG. 5. Effect of loading conditions on recovery of KgBHAT virus from IDA-Co²⁺ column. Clarified KgBHAT virus (4.0 × 10⁷ PFU) was resuspended in 10 ml of different loading buffers (PBS–0.5 M NaCl at different pHs). (A) pH 7.0; (B) pH 6.5; (C) pH 6.0; (D) pH 5.5. After virus loading (flow rate = 0.5 ml/min), the column was washed with 5 CV of the same loading buffer. The column was eluted with 5 CV of PBS–0.5 M NaCl, pH 5.5, if it was loaded with pH 7.0, pH 6.5, or pH 6.0. All columns, regardless of loading conditions, were finally stripped with 5 CV of 50 mM EDTA–PBS–0.5 M NaCl, pH 7.0. The percentages of recovered virus in wash and step gradient elution fractions were added, and summations are depicted on the right side of each plot.
purification column. This simple modification to the protocol indeed reduced the cobalt contamination in the final viral product to undetectable levels, as measured by atomic absorption. In addition, we hypothesized that residual cobalt associated with the purified virus could reduce infectivity, particularly since we were unable to account for the total input virus once it was avidly bound to a column. A second uncharged column should remove cobalt from the virus stock and thereby potentially increase virus infectivity and yield. As expected, the addition of an uncharged IDA column downstream of the cobalt-charged column increased the total virus recovery to 78% of the total loaded virus (Fig. 6) compared to about 40% recovery observed with a single cobalt-charged column (Fig. 5B). The final protein concentration in the purified virus stock was 54 ng/ml, a reduction of >96.8% from that in the loaded virus stock. The final DNA contaminant concentration was 3.5 ng/ml, for an overall reduction of >96.7%. Overall, the second column served the dual purpose of increasing the infectious particle yield and resulting in far less DNA or protein contamination.

**Infectivity of KgBHAT.** gB is believed to be involved in both the adsorption and penetration steps of HSV-1 infection. Replacement of the HS binding region with the cobalt affinity HAT peptide within the HS binding domain of this glycoprotein may alter its function and affect the infectivity of the recombinant virus. The infectivity of the KgBHAT virus was compared to that of KOS and KgBpK<sup>−</sup> by measuring adsorption and penetration kinetics on Vero cells. The adsorption studies were performed at 4°C to avoid the complication of viral entry. As shown in Fig. 7A, the adsorption rates of the three viruses on Vero cells were essentially the same within a 2-h incubation period. The rate of virus entry into host cells was determined as the rate at which penetrated viruses became resistant to a low-pH glycine buffer treatment relative to an untreated virus control. The results showed that the KgBpK<sup>−</sup> virus displayed an approximately 20% reduction in penetration rate compared to the wild-type virus KOS (Fig. 7B), which is consistent with published results (36), while the penetration rate of the HAT-tagged virus KgBHAT was slightly lower than that of the KgBpK<sup>−</sup> recombinant. Together, the data indicated that although virus binding was not affected by the insertion of the HAT tag into the HS binding site of gB, virus entry with this insertion was less efficient than that of the wild-type virus but not substantially different from that of the gB mutant lacking the HS binding element.

**DISCUSSION**

HSV-1 vectors provide a promising platform gene delivery technology, especially for applications involving gene delivery to sensory nerves, the natural site of virus latency. Near-term clinical applications include the treatment of certain solid tumors and sensory nerve conditions, such as chronic pain and peripheral nerve degeneration. For patient applications, a scalable and efficient purification process is an essential step in providing clinical-grade HSV-1 vectors for early-phase human trials to assess vector safety and eventual efficacy. The objective of this research was to test the feasibility of utilizing IMAC column purification and a surface glycoprotein tagged virus to replace the current methods for purification of recombinant HSV-1 virus vectors.

Initial experiments were aimed at determining whether one of the commonly used immobilized metal ion columns was able to separate untagged HSV-1 virus from protein and DNA contaminants. The relative affinity of untagged HSV-1 virus for different immobilized metal ions (Fig. 1) followed the order Cu<sup>2+</sup> > Zn<sup>2+</sup> > Ni<sup>2+</sup> > Co<sup>2+</sup>. The binding of untagged HSV-1 to transition metal ions infers the existence of metal ion binding sites on the native virion surface. The outermost layer of the HSV-1 virion is a lipid membrane envelope from which at least 10 HSV-encoded glycoproteins (gB to gE, gG to gJ, gL, and gM) project beyond the lipid bilayer (59, 65). Every gly-
actions among the envelope components may cooperate to fortuitously form potent histidine-rich intermolecular metal ion binding sites. These histidines may explain the high affinity of the virus for immobilized zinc and the reduced binding of native viral glycoproteins to nickel and cobalt columns.

The affinities of protein and DNA contaminants to each of the above immobilized metal ions followed the same trend as the untagged HSV-1 virus (Fig. 1). No purification of untagged HSV-1 was achieved by solely selecting one of the above immobilized metal ion columns. However, the selectivity of IMAC can also be tailored by surface modification of one or more of the viral glycoproteins. Since the majority of virus or contaminants did not bind to the immobilized cobalt ion (Fig. 1D), augmentation of the affinity of HSV-1 virus to cobalt could enhance binding and direct the elution of the virus into a fraction that is low in nonviral contaminants. We tested this possibility by engineering a cobalt affinity peptide into a virus surface glycoprotein.

For the construction of an effective tagged HSV-1 recombinant, the peptide tag and the locus of tag insertion should be appropriately selected so that the tag is accessible and able to bind to the cobalt ion. Moreover, the tag insertion must have minimal impairment on virus infectivity. It has been established that HAT is an efficient tag for cobalt ions (11). gB was selected as the target for tag insertion because gB has the longest amino acid ectodomain among the HSV-1 glycoproteins (57) and because the clustering of gB spikes in protrusions of the virion envelope (66) implies that regions of gB are surface exposed and thus accessible for binding to the immobilized cobalt on the column. It is well known that gB participates in mediating the adsorption of HSV-1 virions to susceptible cells by binding to the HS moieties of cell surface proteins (28, 36, 78) and subsequently by contributing to virus penetration into cells through envelope-membrane fusion (9, 45). The HS binding domain (amino acids 68 to 76 [KPKKNKKPK]) of gB was selected to be replaced by the HAT peptide for the following reasons. First, the existence of an HS binding domain (36) and the presence of a neutralizing epitope localized to this region of the molecule (43, 53) suggest that this domain of gB is exposed on the virion surface and can be easily recognized by one of its natural receptors (HS). Thus, replacement of the HS binding domain with the HAT tag increased the likelihood that the tag may also be available for binding cobalt. Second, most of the HS binding function of HSV-1 is contributed by gC, the tag may also be available for binding cobalt. Second, most of the HS binding function of HSV-1 is contributed by gC, the tag insertion must have minimal impairment on virus infectivity on Vero cells (36). Third, it has been predicted by neural network computational analysis that insertional mutagenesis in the secondary loop structure region of the gB protein can minimize the outcome of a misfolded mutant (48), with the HS domain of gB predicted to be in a loop conformation (48). The rationale for the placement of the HAT tag into gB was supported by the experimental findings with the recombinant virus. These studies demonstrated that the tagged virus was bound to the immobilized cobalt column, and the elution profiles indicated that the virus particles could be directed to a fraction that was nearly devoid of contaminants.

The recovery of viable virus from IMAC varies with different loading conditions and different metal ions. It appeared that...
the stronger the virus bound to metal ions, the larger was the extent of metal ion-induced virus inactivation. For example, the immobilized copper ion appeared to strongly bind to untagged virus, and thus the recovery of infectious virus from copper IMAC was significantly lower than that for the other columns tested. Sagripanti et al. (60) found that HSV-1 can be inactivated in a Cu²⁺ solution by free radicals generated from a series of Cu²⁺-catalyzed redox reactions, referred to as Fenton reactions (67). The occurrence of Fenton reactions has been described for copper ion IMAC systems (8), which could account for the inactivation of untagged virus in the IDA-Cu²⁺ column (Fig. 1A). Elution from a single cobalt-charged column resulted in the loss of infectious virus and the concomitant presence of leached cobalt in the eluate. Thus, cobalt bound to gB on the viral envelope may interfere with the essential function of gB, similar to the inhibition seen by adding Co²⁺ directly to an aliquot of KgBHAT stock (data not shown). We found that Co²⁺ leakage was substantially mitigated simply by connecting a metal-free IDA column downstream to capture the free and/or virus-bound Co²⁺. By loading the virus in pH 6.5 buffer and using a second uncharged column downstream (Fig. 6), we found that the overall recovery of HAT-tagged HSV-1 from IDA-Co²⁺ was increased to approximately 80%, while protein and DNA contamination was reduced by >96.5%, to 54 and 3.5 ng/ml, respectively. Together, these results suggest that the introduction of a HAT tag into HSV-1 gB enabled the rapid purification of the recombinant virus through IMAC Co²⁺ columns.

The effect on virus infectivity of the replacement of the HS binding domain with the HAT peptide was assessed. In previous work (36), it was found that compared with the wild-type virus, a virus mutant with an HS-binding-deficient form of gB (KgBpK⁻) showed a 20% reduction in binding capacity on mouse L cells after a 5-h incubation. High numbers of radio-labeled virus particles (>1,000 viruses/cell) were used in the adsorption assay for that study in order to see the capacity differences of different virus mutants. In HSV-1 vector gene transfer applications, the MOI applied is normally far from the virus binding capacity on target cells. We were more interested in the rate of virus adsorption. In this work, we found that when a low MOI (250 PFU/10⁶ cells) was used, KgBpK⁻ or KgBHAT showed essentially the same adsorption rate as KOS on Vero cells (Fig. 7A). The rate of penetration of KgBHAT on Vero cells was found to be slightly lower than that of its KgBpK⁻ counterpart (Fig. 7B). This minimal loss in tagged virus infectivity is compensated for by gains in vector purity and more economical manufacturing. We are currently investigating the use of this system to purify an HSV-1 vector under scaled-up manufacturing conditions. The efficacy of IMAC-purified tagged HSV-1 vectors will be evaluated in animal models and compared to that of an untagged vector purified by conventional methods such as gradient centrifugation.

Several recent reports described IMAC purification of hexahistidine (His₆)-tagged AAV (82) and baculovirus (81). The addition of a hexahistidine (His₆) tag to the C terminus of AAV VP3 did not affect AAV tropism and productivity while providing the capability of purifying tagged AAV on a nitrolotriacetic acid-Ni²⁺ column (82). The fusion of His₆ to gp64 of baculovirus allowed purification of the tagged baculovirus with nitrolotriacetic acid-Ni²⁺ to 87% purity, but there was only a poor (2 to 3%) recovery (81). These studies demonstrated that IMAC could be adapted for virus purification; however, recovery appeared to be low in one case and was not reported in the other. Therefore, the transition metal and corresponding binding peptide must be carefully selected to ensure separation from contaminating DNA and protein and the recovery must be high in order for the method to be amenable to scalable vector manufacturing. These parameters in turn may be specific to each virus structure and production technology. Our experience with HSV indicates that cobalt binds fewer contaminants from HSV-1 preparations than nickel; however, it remains to be determined whether cobalt will be similarly useful for other vector systems.

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