The L1 Major Capsid Protein of Human Papillomavirus Type 11 Recombinant Virus-like Particles Interacts with Heparin and Cell-surface Glycosaminoglycans on Human Keratinocytes

(Received for publication, April 17, 1998, and in revised form, October 21, 1998)

Joseph G. Joyce‡§, Jwu-Sheng Tung‡, Craig T. Przysiecki‡, James C. Cook‡, E. Dale Lehman‡, Jeffrey A. Sands‡, Kathrin U. Jansen‡, and Paul M. Keller‡

From the ‡Department of Virus and Cell Biology, Merck Research Laboratories, West Point, Pennsylvania 18486, the ¶Department of Bioprocess and Bioanalytical Research, Merck Research Laboratories, Rahway, New Jersey 07065, and the ‡Department of Biological Sciences, Lehigh University, Bethlehem, Pennsylvania 18015

The L1 major capsid protein of human papillomavirus (HPV) type 11, a 55-kDa polypeptide, forms particulate structures resembling native virus with an average particle diameter of 50–60 nm when expressed in the yeast Saccharomyces cerevisiae. We show in this report that these virus-like particles (VLPs) interact with heparin and with cell-surface glycosaminoglycans (GAGs) resembling heparin on keratinocytes and Chinese hamster ovary cells. The binding of VLPs to heparin is shown to exhibit an affinity comparable to that of other identified heparin-binding proteins. Immobilized heparin chromatography and surface plasmon resonance were used to show that this interaction can be specifically inhibited by free heparin and dextran sulfate and that the effectiveness of the inhibitor is related to its molecular weight and charge density. Sequence comparison of nine human L1 types revealed a conserved region of the carboxyl terminus containing clustered basic amino acids that bear resemblance to proposed heparin-binding motifs in unrelated proteins. Specific enzymatic cleavage of this region eliminated binding to both immobilized heparin and human keratinocyte (HaCaT) cells. Removal of heparan sulfate GAGs on keratinocytes by treatment with heparinase or heparitinase resulted in an 80–90% reduction of VLP binding, whereas treatment of cells with laminin, a substrate for α5 integrin receptors, provided minimal inhibition. Cells treated with chlorate or substituted β-D-xylolides, resulting in undersulfation or secretion of GAG chains, also showed a reduced affinity for VLPs. Similarly, binding of VLPs to a Chinese hamster ovary cell mutant deficient in GAG synthesis was shown to be only 10% that observed for wild type cells. This report establishes for the first time that the carboxyl-terminal portion of HPV L1 interacts with heparin, and that this region appears to be crucial for interaction with the cell surface.

Papillomaviruses are non-enveloped, double-stranded DNA viruses containing a circular genome of approximately 8,000 base pairs. The viral capsid is composed of a major and minor capsid protein, both products of late gene expression and termed L1 and L2, respectively (1). The L1 protein has a molecular mass of 55–60 kDa by polyacrylamide gel electrophoresis and is well conserved across types. In the virion it accounts for 80–90% of total viral protein (2, 3). The viral capsid is built up from pentameric capsomers of L1, with 72 such structures arranged in a T = 7 icosahedral array (4, 5). The localization of L2 in this ordered structure is not known at present nor is the mechanism of assembly, although recent reports suggest disulfide bonding may play a role (6, 7). Over 80 types of human papillomavirus are currently identified, and many have been shown to be associated with various forms of warts occurring on the surface epithelia of skin and mucous membranes. HPV3 types can be broadly grouped into those that cause benign lesions such as condyloma acuminata and others that show the potential for malignant transformation. HPV types 11 and 6 are representative of the former and are the etiological agents responsible for approximately 90% of all benign warts associated with the anogenital tract and respiratory mucosa (8). Members of the second group include HPV types 16 and 18, which cause cervical intraepithelial neoplasia (9–11) that may progress to squamous cell carcinoma (12).

Progress has recently been made producing either L1 alone or L1 and L2 proteins recombinantly in a number of expression systems (13–16). It has been shown that newly synthesized L1 is translocated to the nucleus where it self-associates to form capsid-like structures that resemble the native virus but lack viral nucleic acid (14, 17, 18). These virus-like particles (VLPs) are capable of eliciting neutralizing antibodies and can competitively block infection by live virus (18, 19). Because native virus has proved exceedingly difficult to propagate in vitro, the ability to produce large amounts of recombinant VLPs has greatly aided cell-based binding studies aimed at identifying candidate HPV receptors.

Although papillomaviruses can bind to a wide variety of cell types (20, 21), their productive tropism is generally limited to epithelial keratinocytes and fibroblasts (20). The fact that most HPV’s can bind to cells of epithelial origin in vitro but cannot be propagated suggests that intracellular events following uptake of virus are crucial for establishment of successful infection and that host specificity is not linked to the presence of a given cell-surface receptor (20). This is particularly true in keratinocytes where establishment of infection has been linked to cel-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Virus and Cell Biology, Merck Research Laboratories, Sumneytown Pike, West Point, PA 19486. Tel.: 215-652-5617; Fax: 215-652-2142.
lular differentiation (22). The ability of HPV to bind to a wide range of nonpermissive tissue- and species-specific cell lines argues for a receptor that is either highly conserved or of low specificity. Recently, Evander et al. (23) have proposed the αβ₄ integrin as a candidate receptor based on the ability of HPV 6b VLPs to immunoprecipitate proteins with molecular weights corresponding to the integrin subunits and the ability of laminin to block VLP binding.

Alternatively, HPVs may exhibit broad binding ranges as a result of a multiple receptor mechanism. In this model a receptor of relatively low specificity causes interaction of virus with the cell followed by binding to a specific protein component resulting in internalization and infection. One group of molecules able to serve as putative receptors is cell-surface glycosaminoglycans. This mode of binding has been established for herpes simplex virus 1 (HSV-1) (24) and human herpesvirus 7 (25) in which binding of viral glycoproteins to cell-surface heparan sulfate molecules provides initial contact. Recent reports have suggested that GAGs may play a similar role in binding of varicella-zoster virus to human embryonic lung fibroblasts (26). Similarly, infection of cells by human immunodeficiency virus 1 can be blocked by polyanions such as dextran sulfate (27) although proteoglycans are not implicated as viral receptors.

Our laboratory has been involved in the purification and characterization of various HPV VLP types (13, 28). In this report we have made a sequence comparison based on the method of Pearson and Lipman (29) to identify a conserved domain in the L1 carboxyl terminus containing amino acid sequences of the general type XBBBXXB where B is either Arg or Lys. These are similar to the XBBXBX and XBBXBBX sequences identified by Cardin and Weintraub (30) as putative heparin-binding motifs. We therefore investigated the ability of L1 VLPs to bind to heparin and characterized the nature of this interaction with regard to charge and polymer size. Finally, we examined the role of cell-surface glycosaminoglycans in binding VLPs. The HaCaT human keratinocyte cell line was chosen for binding studies because it represents a cell type that serves as an in vivo infection target, and its ability to internalize HPV VLPs has been established (31). We show in this study that the carboxyl-terminal portion of HPV 11 L1 interacts with heparin and that this interaction displays a certain degree of specificity. Furthermore, this region is shown to be crucial for interaction with the cell surface.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

HaCaT cells were maintained as monolayer cultures at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM: Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.). For sulfate-free medium, the DMEM formulation was the same except that magnesium sulfate was omitted and dialyzed FCS (Sigma) was used. CHO-K1 and XYXYX cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were grown as monolayer cultures at 37 °C, with a 5% CO2 atmosphere in F-12K medium (ATCC) supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were routinely subcultured every 14–21 days.

**Expression and Purification of VLPs**

Recombinant HPV type 11 L1 VLPs were expressed in *Saccharomyces cerevisiae* using the pGal110-11 vector as described previously (32). VLPs were purified from clarified yeast cell lysate by ion exchange chromatography using a modification of previously described methodology (13). The presence of VLPs was confirmed by electron microscopy, and purity was determined by denaturing polyacrylamide gel electrophoresis (PAGE) and immunoblotting. VLPs were quantitated using a commercial bicinchoninic protein assay (Pierce) and a specific radiomunnoassay described elsewhere (28).

**Polyacrylamide Gel Electrophoresis and Immunoblotting**

Aliquots (100 µg) of purified VLP preparations were concentrated by precipitation with 10% trichloroacetic acid at 0 °C for 1–2 h. The pellets were resuspended at a concentration of 1.0 µg/ml in 2× Laemmli buffer containing 200 mM dithiothreitol and heated at 100 °C for 15 min. Sufficient volume to give 2.5–20 µg of total protein was loaded on pre-cast 4–20% polyacrylamide gels (NOVEX, San Diego, CA) utilizing a Tris glycerine buffer system. The gels were electrophoresed at a constant current of 30 mA/gel for 1 h. Protein bands were fixed with 12% trichloroacetic acid and visualized with a commercial colloidal Coomasie Blue staining kit (Sigma). Immobilon Western membranes (Millipore, Bedford, MA) according to manufacturer’s recommendations. Quantitation of bands was performed using a Molecular Dynamics Personal Densitometer model SI (Sunnyvale, CA). For immunoblotting, proteins were electrophoretically transferred to polyvinylidene difluoride membranes at a constant voltage of 25 V for 1 h. Type 11 L1 was detected using a polyclonal goat antibody and alkaline phosphatase-conjugated rabbit anti-goat antiserum (Pierce) as secondary antibody. Visualization was by nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine reagent (Pierce).

**Electron Microscopy**

Electron microscopy was performed by Advanced Biotechnologies Inc. (Columbia, MD). Briefly, an aliquot of diluted VLPs was placed on a 300-mesh carbon-coated copper grid and air-dried. A drop (20 µl) of 2% glutaraldehyde, pH 7.0, was placed on the grid for 30 s. The grid was allowed to air-dry prior to transmission electron microscopy examination. All microscopy was performed using a Hitachi-HU-12A transmission electron microscope with micrographs taken of random sections at various magnifications.

**Heparin Affinity Chromatography**

Chromatography was performed on a Waters 2690XE Alliance system (Milford, MA) at ambient temperature using 1- or 5-mL cartridges of Hi-Trap® heparin-Sepharose (Amersham Pharmacia Biotech) equilibrated with 50 mM MOPS, pH 7.0, and varying concentrations of NaCl. Flow rates were 1 or 4 mL/min for the small and large columns. Purified VLPs were diluted to the appropriate NaCl concentration in buffer and applied to the column. For most studies, the column was washed with 5 volumes of equilibration buffer, and proteins were eluted with a 10-volume linear salt gradient to 2.0 M NaCl. Absorbance was monitored using either Waters 990 or 996 photodiode array detector. For inhibition studies, VLP preparations were diluted to 100 µg/ml in MOPS buffer containing 0.4 M NaCl. Compounds tested as inhibitors were prepared in 50 mM stock solutions in MOPS buffer and added to diluted VLPs. The samples were held at 4 °C for 16–20 h and brought to ambient temperature prior to chromatography.

**Enzymatic Degradation of L1**

VLPs (300 µg) were treated with bovine factor Xa (bFXa) (Hematological Technologies, Essex Junction, VT) to remove the carboxyl terminus of the L1 molecule as described elsewhere. Briefly, digests were performed by blowing air saturated with water over the surface of a 1.0-mL VLP sample in a sealed vial for 45–60 min in a 25 °C circulating water bath. The appropriate volume of bFXa or buffer (control) was added to give an enzyme/substrate weight ratio of 1/20. The sample was mixed and incubated at 25 °C for 16 h. To remove bFXa, soybean trypsin inhibitor-agarose resin (50 µl) was added, and the sample was mixed on a rotor shaker for 1 h at room temperature. Resin was pelleted by centrifugation at 2,000 × g for 15 s, and the supernatant was recovered. Denaturing PAGE was performed to determine the extent of digestion. Reversed phase identification of released peptide, intact L1, and truncated L1 was performed using a high pressure liquid chromatography assay described elsewhere.

**Surface Plasmon Resonance**

The BIAcore system, CM5 sensor chip, amine coupling kit, ethanolamine, and surfactant P20 were obtained from Pharmacia Biotech (Amersham Pharmacia Biotech). Running buffer (HBS) was 10 mM HEPES, pH 7.4, 0.15 M NaCl. Heparin was oxidized and immobilized to
the CM5 chip according to BIApplications handbook (Pharmacia Biosensor). Heparin was dissolved in 100 mM sodium acetate buffer, pH 5.5, at 1 mg/ml. A 1/50 volume of freshly prepared sodium metaperiodate solution was added to give a final concentration of 1 mM. The sample was incubated on ice for 20 min, and the reaction was stopped by dialysis against 10 mM sodium acetate buffer, pH 4, at 4 °C. The sensor chip surface was prepared by equilibration with HBS, 0.05% (v/v) surfactant P20 at 5 µl per min, followed by successive injections of (i) 35 µl of a mixture of equal volumes of 0.1 M N-hydroxysuccinimide, 0.1 M N-ethyl-(dimethylamino)propyl)carbodiimide; (ii) 35 µl of 5 M carbodiimide; (iii) 35 µl of 1 M ethanolamine hydrochloride, pH 8.5; and (iv) 35 µl of oxidized heparin at 1 mg/ml. The flow rate was lowered to 2 µl/min, and 40 µl of 0.1 M sodium cyanoborohydride in 0.1 M sodium acetate buffer, pH 4, was injected. The immobilized heparin was treated with two 5-µl pulses of 20 mM HCl to remove noncovalently attached ligand. VLP preparations were diluted to the appropriate concentration of NaCl in HBS and injected (20-µl volume) onto the heparin sensorchip at a constant eluent flow rate of 5 µl/min. The chip was regenerated by a 7-µl injection of 20 mM HCl. Data points corresponding to the initial binding rates were routinely collected manually except during quantitative free heparin inhibition analysis where collection was automatic. For inhibition studies, samples of VLPs were prepared as above at constant protein concentration, and inhibitors were added from concentrated stocks in 50 mM MOPS buffer, pH 7. Samples were allowed sufficient time to reach equilibrium prior to analysis.

**Cell Binding Assays**

**VLP Binding Enzyme-linked Immunosorbent Assays**—The standard assay employed to study VLP binding to cells and the effects of various inhibitors on binding was a sandwich enzyme-linked immunosorbent assay. Cells were seeded on 96-well plates at a density of 5 x 10^4 (HaCaT) or 1 x 10^4 (CHO-K1 and pGUS-745) cells/well. Plates were maintained in standard media for 48–72 h until ≥90% confluence was reached. All subsequent steps were done at ambient temperature. Cells were washed once manually with phosphate-buffered saline (PBS) and fixed with 4% aqueous paraformaldehyde for 15–30 min at ambient temperature. All subsequent washes were done in Tris-buffered saline containing 0.1% Tween 20® (TTBS) using a Denley WellWash 5000 plate washer. Blocking buffer for VLP and antibody incubations was PBS containing 1% non-fat milk. VLPs at the proper dilutions were added for 1–2 h. Bound VLPs were detected with the conformation-specific monoclonal antibody 8740 (Chemicon, Tercula, CA). Mouse IgG was detected with goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce) utilizing a 3,3',5,5'-tetramethylbenzidine in hydrogen peroxide buffer detection system (Pierce). Absorbance at 450 nm was read with a Bio-Tek EL312 microplate reader. A modified form of the assay was used for certain studies. In this protocol, confluent cells were washed with PBS but not fixed. Antibody and VLP dilutions were made in PBS containing 1% bovine serum albumin, and all washes were done manually in PBS. Incubation and wash steps were performed on ice at 4 °C to minimize VLP internalization.

**Inhibition Assays**—Compounds previously determined to inhibit VLP binding to immobilized heparin were added from concentrated stock solutions to a fixed concentration of VLPs, and the samples were held for 16–20 h at 4 °C. Treated or control VLPs were diluted to the appropriate concentrations and added to fixed cells as described above.

**Glycosaminoglycan Modification Assays**—HaCaT cells were treated with a variety of reagents known to modify or inhibit synthesis of cell-surface glycosaminoglycans. For most experiments, cells were grown to ≥90% confluence on 96-well plates as described. Growth medium was removed, and fresh or sulfate-free medium containing appropriate concentrations of reagent was added. Cells were maintained for a given time at 37 °C after which they were washed and used for assay. See individual figure legends for details specific to each reagent.

**Heparinase Treatment**—HaCaT cells were grown to confluence on 96-well plates. Culture medium was removed, and cells were washed once with PBS. Heparinase (forms I and II) or heparitinase (heparinase III) was diluted in digestion buffer (20 mM Tris-HCl, 50 mM NaCl, 4 mM CaCl₂, pH 7.5, containing 0.01% BSA) and added to cells at a final concentration of 1 or 3 units/well. Control wells consisted of buffer without enzyme. Duplicate plates were prepared and incubated at room temperature or 37 °C for 1 h. Plates were washed once with PBS. Cells were fixed and used for assay as described above. All enzymes were purchased from Sigma.

**Characterization of Purified VLPs**—Recombinant HPV 11 L1 VLPs were isolated from yeast cells as described under “Experimental Procedures.” Fig. 1A shows the results of SDS-PAGE analysis on 20 µg of purified VLPs. The major protein band migrating at 55,000 Da (p55) is confirmed to be HPV L1 by immunoblotting and accounts for 84% of all L1-reactive material. In addition to p55, approximately 6% of L1-reactive bands appear as high molecular weight multimers, probably representing non-denaturable aggregates, whereas the remaining 10% migrate at molecular weights below p55 and represent proteolytic degradation products of L1. Such degradates have been previously observed in this expression system (15). Purity of the VLP preparation was determined to be 96% by densitometric analysis. Fig. 1B shows an electron micrograph of a purified preparation. VLPs routinely exhibited an average diameter of 50–60 nm and were present as individual, well-defined particles with minimal aggregation.

**Interaction of VLPs with Immobilized Heparin**—The carboxy-terminal sequence (residues 447–501) of HPV 11 L1 is shown in comparison with other HPV L1 types in Fig. 2. This alignment was generated using the “FastA” and “Pileup” programs contained in Intelligenetics version 8.0 software (Genetics Computing Group Inc., Madison, WI). “FastA” was used to identify sequences similar to the complete HPV 11 L1 query sequence. Out of more than 50 hits, human sequences containing at least 64% amino acid identity are shown. The carboxyl terminus is fairly well conserved among HPV types and is characterized by a cluster of 6–8 basic amino acids within the final 15 residues and a shorter 3–4 basic amino acid cluster upstream from this. Eight of nine types contain the putative heparin-binding motif BXXBXX where B is a basic residue (Lys or Arg). To explore the ability of HPV 11 L1 to interact with heparin, VLPs were applied to a column of immobilized heparin-Sepharose. Preliminary experiments showed that binding could be achieved at salt concentrations up to 0.5 M NaCl. The VLPs bound quantitatively as judged by the lack of a significant breakthrough peak. VLPs were eluted with porcine mucosal high molecular weight (HMW) heparin of average molecular mass (M̄) 17,500 using a series of step gradients of increasing concentration. Pools collected during elution were analyzed by denaturing PAGE (Fig. 3A). Approximately 93% of bound...
VLPs could be eluted with free heparin, with 60% eluting at 1.0 mg/ml heparin and an additional 33% being recovered at concentrations up to 10 mg/ml. The remaining 7% of applied protein was recovered in a 2M NaCl strip of the resin and most likely represents aggregated species. VLPs could also be eluted from immobilized heparin using a linear NaCl gradient in which they eluted at approximately 0.8M NaCl (Fig. 3B). The broadness and asymmetry of the chromatographic peak further indicates microheterogeneity with regard to affinity for the resin. All subsequent studies of inhibition of VLP binding were performed using NaCl gradient elution.

**Inhibition of VLP Binding**—A variety of compounds with structural similarities to heparin were tested as inhibitors of VLP binding by heparin-Sepharose chromatography. For these experiments, the concentration of VLPs was held constant while inhibitor concentration was varied. Mixtures of VLPs and inhibitor were held at 4 °C for various times (1–24 h) prior to analysis (data not shown). It was found that inhibition was relatively rapid, occurring within 1 h, but in order to ensure that equilibrium had been obtained, samples were typically incubated 16–20 h prior to chromatography. The total amount of p55 was calculated as the sum of retained and breakthrough peak areas, and recoveries were typically 90–95% that of control VLPs. This was done for each sample in order to account for slight variations in injection amount and recovery. The percent p55 bound was then calculated from the area of the retained peak with the same retention time as untreated controls (average of triplicate injections). The effect of various inhibitors is shown in Fig. 4. Binding data were fit to two sigmoidal dose-response models and a one-site competition model. The best fits were achieved using a sigmoidal dose-response model with variable slope, and this was used to calculate IC50 values. Binding was inhibited by HMW heparin with a calculated IC50 of 14.9 μM. By contrast, a commercial low molecular weight (LMW) preparation (Mr 6,000) gave 50% inhibition at a concentration of 762 μM. Furthermore, whereas VLP binding was completely eliminated by HMW heparin at concentrations above 60 μM, approximately 15–20% residual binding was observed.
were incubated in MOPS-buffered 0.5 M NaCl with increasing concentrations of inhibitor for 16–20 h prior to chromatography on heparin-Sepharose as described in Fig. 3. Compounds tested for their ability to inhibit binding were HMW heparin, $M_r$ 17,500 (●); LMW heparin, $M_r$ 6,000 (○); N-desulfated heparin, $M_r$ 17,500 (×); and HMW dextran sulfate, $M_r$ 500,000 (■). The abscissa shows the percent of VLPs which bound the column relative to control VLPs incubated under same conditions in absence of inhibitor.

served for the LMW species at concentrations up to 1.7 mM. Since chemical depolymerization may be accompanied by some degree of desulfation, enzymatically depolymerized heparin was used for these experiments. Independent charge analysis and size exclusion experiments (data not shown) confirmed that the depolymerized heparin populations retained charge densities similar to HMW heparin. HMW sulfated dextran ($M_r$ 500,000) exhibited a dose-dependent inhibitory effect similar to heparin, although its IC$_{50}$ (11.9 nM) was dramatically lower.

The importance of sulfation was evident in that chemical N-desulfation of glucosamine residues in heparin led to a complete loss of the inhibitory effect at all concentrations tested. Furthermore, inhibition by dextran sulfate was dependent on the anionic nature of the polysaccharide as non-sulfated dextrans of low or high $M_r$ (40,000 and 2,000,000, respectively) had no effect on binding (data not shown).

Effect of Polymer Molecular Weight on Inhibition—The difference in IC$_{50}$ values exhibited by HMW and LMW heparins suggested a dependence of inhibition strength on molecular weight. This was tested using a series of sized dextran sulfate preparations with $M_r$ ranging from 5,000 to 500,000. The molecular weights used were those estimated from low angle laser light scattering by the manufacturer. Independent assessment of these preparations was made by high performance size exclusion chromatography (data not shown). Although some polydispersity was evident in all preparations, the gel filtration peaks were fairly symmetrical and eluted in the predicted order. Fig. 5A shows that HMW polyelectrolytes are much better at inhibiting VLP binding to heparin than LMW ones, but the surprising conclusion was how abrupt the difference was between dextran sulfate of $M_r$ 5,000 (○); 8,000 (○); 10,000 (×); 50,000 (■); and 500,000 (■). B, inhibition by the glycosaminoglycans chondroitin sulfate A, $M_r$ 20,000 (●); and chondroitin sulfate C, $M_r$ 50,000 (○).

The type of glycan residues present in a given structure play a role. Sulfated monosaccharides such as glucose 6-sulfate and galactose 6-sulfate were tested as inhibitors at concentrations up to 27 mM. No reduction in binding was provided by these low molecular weight molecules (data not shown).

Dependence of VLP Binding on an Intact L1 Carboxyl Terminator—Treatment of HPV 11L1 VLPs with bFXa releases a 28-residue carboxyl-terminal peptide, generating a truncated L1 with a molecular mass of approximately 53,000 Da (p53) as determined by denaturing PAGE analysis. Following removal of enzyme by soybean trypsin inhibitor-agarose treatment, the reaction products were analyzed by heparin-Sepharose chromatography. Fig. 6A shows the profiles of control (VLP without enzyme) and digested VLPs. A characteristic VLP peak (I) accounting for 92% of $A_{220\text{ nm}}$ area is present in the control sample. Approximately 94% of retained peak area is lost and recovered in the column breakthrough upon treatment with enzyme. The small amount of peak I remaining after bFXa treatment (6%) correlates well with the 5% intact p53 observable by gel electrophoresis of the digest. Reverse phase analysis (data not shown) was used to identify the species present in each peak from the heparin-Sepharose column. The carboxy-terminal peptide was recovered in peak III and truncated L1 with a molecular mass of approximately 53,000 Da (p53) as determined by denaturing PAGE analysis. Following removal of enzyme by soybean trypsin inhibitor-agarose treatment, the reaction products were analyzed by heparin-Sepharose chromatography. Fig. 6A shows the profiles of control (VLP without enzyme) and digested VLPs. A characteristic VLP peak (I) accounting for 92% of $A_{220\text{ nm}}$ area is present in the control sample. Approximately 94% of retained peak area is lost and recovered in the column breakthrough upon treatment with enzyme. The small amount of peak I remaining after bFXa treatment (6%) correlates well with the 5% intact p53 observable by gel electrophoresis of the digest. Reverse phase analysis (data not shown) was used to identify the species present in each peak from the heparin-Sepharose column. The carboxy-terminal peptide was recovered in peak III and truncated L1 in peak II. Peak II was re-injected in order to determine if p53 did not bind to heparin as a result of inhibition by the highly basic free peptide. An identical elution profile was obtained, confirming that competition by free carboxy-terminal peptide did not inhibit p53 from binding. To study further the effect of free
peptide, a synthetic 15-mer carboxyl-terminal peptide was analyzed by heparin-Sepharose chromatography. The peptide showed lower affinity for the resin compared with intact VLPs. When peptide or VLPs were injected on a column equilibrated in 0.36 M NaCl, the 15-mer was retained but eluted at a lower ionic strength than VLPs (data not shown). When the initial concentration of NaCl was raised to 0.5 M, the peptide interacted weakly, eluting isocratically in the wash portion of the run (Fig. 6B, top). Co-injection of peptide and protein confirmed this difference in affinity and revealed that the 15-mer was unable to inhibit VLP binding even at a 67-fold molar excess (Fig. 6B, bottom). The study was repeated with a 28-mer peptide which corresponded more closely to the region removed by bFXa. Although this peptide eluted at higher ionic strength than the 15-mer, it was also less retained than VLPs and did not inhibit VLP binding (data not shown). Given the weak affinity of the synthetic peptides, the failure of the enzymatically generated carboxyl-terminal fragment to bind the heparin-Sepharose column (Fig. 6A) may have been a result of chromatographic displacement by p53.

Analysis of Heparin Binding by Surface Plasmon Resonance—Surface plasmon resonance performed on a BIAcore system was used to characterize further the nature of VLP binding to heparin. This technique was chosen since binding has been shown to be directly proportional to the change in reflection units (RU) (33) and because it eliminates some of the uncertainty associated with column chromatography, notably nonspecific interactions with the resin matrix. Heparin was immobilized to a carboxymethylated dextran surface following the manufacturer’s suggested protocol, and this biosensor chip was used for subsequent studies. For inhibition studies, percent residual binding in the presence of inhibitor was calculated from RU values measured during the dissociation phase. The RU measured in the absence of inhibitor was taken as 100% bound, and inhibition samples were calculated relative to this value. A typical sensorgram of VLPs in the absence of inhibitor is presented in Fig. 7A. The initial vertical rise in RU was due to introduction of sample containing higher concentrations of salt than the running buffer. Antigen bound well to the heparin surface at 0.5 M NaCl, characterized by a further RU increase occurring from 800 to 1020 s.

Equilibrium binding was not achieved at the protein concentrations (4.3 μM) used in this experiment. At 1020 s, the wash step was initiated, resulting in an initial sharp decrease of RU, a refractive index artifact caused by the change in NaCl concentration between sample and wash buffer. A flat dissociation phase was then observed, indicating that under these conditions VLPs bind with a very high affinity, and no Kd constant could be measured. At 1180 s, a pulse of 20 mM HCl was used to strip VLPs and regenerate the surface.

Fig. 7A also shows that VLPs did not bind at 0.7 M NaCl, as indicated by the flat binding phase (130–380 s) and immediate return of RU to base line as wash buffer is introduced. The higher absolute value of RU initially attained was reflective of the higher salt concentration of the sample.

Inhibition by HMW heparin was assessed using three different experimental conditions (Fig. 7B). In the first trace, inhibitor was injected immediately following injection of VLPs (340 s). The sensorgram shows a pronounced decrease in RU during the wash phase (580–990 s) corresponding to 72% inhibition. In the second case inhibitor was added after VLPs had bound and been washed with running buffer (1590 s). Here, a slower dissociation was observed, reaching an equilibrium value of approximately 40% inhibition. When VLPs were preincubated with inhibitor as in column experiments (2500–3000 s), inhibition approached 98%. The preincubation method was used for subsequent studies to generate inhibition curves for heparin and HMW dextran sulfate in an analogous manner to heparin-Sepharose studies. The observed IC50 of 4.91 mM for dextran sulfate was in good agreement with that obtained by chromatography; however, HMW heparin gave a much lower value of 75.9 nM. This discrepancy may be explained in part to differences in the way VLPs are able to interact with the immobilized ligand in a given system. Analysis of bFXa-treated VLPs is shown in Fig. 7C. Injection of reaction control (VLPs without enzyme) (0–840 s) showed typical high avidity binding of uncleaved p55 VLPs. Digested VLPs (840–1450 s) in which >90% of the L1 had been converted to p53 failed to interact with heparin as indicated by the flat association curve from 900 to
1200 s. Analysis of a reaction blank (enzyme without VLPs) (1450–2040 s) showed no measurable interaction of enzyme with the heparin surface at the concentration used. The ability of the 15-mer carboxyl-terminal peptide to compete with VLPs was examined. Even at 1000-fold molar excess of peptide, less than 30% inhibition was observed, suggesting that a structural feature or the multiply charged nature of intact VLP contributes to high affinity binding (data not shown).

Interaction of VLPs with HaCaT Cells—For initial binding studies VLPs were added to fixed cells in the standard assay and allowed to bind for 1–3 h at ambient temperature. Fig. 8A shows that when VLP titrations were performed using low or high ionic strength buffers, saturation was achieved at approximately 80–160 ng of L1 protein, which was similar to previously reported results describing HPV 33 L1 VLP binding to HeLa cells (21). The interaction exhibited some dependence on ionic strength in the linear region of the curve since the absorbance values at a given concentration were lower for VLPs diluted in 0.5 M NaCl as opposed to 0.15 M NaCl. At low VLP concentrations, approximately 65% reduction in binding was observed when the ionic strength was raised. The effect of bFXa treatment is shown in Fig. 8B. Denaturing gel electrophoresis and densitometry of reaction products confirmed that 89% of p55 was converted to p53. Replicates of reaction control and digest samples were assayed for their ability to bind HaCaT cells. The A$_{450}$ nm values for control replicates were averaged, and this value was taken as 100% bound. Individual replicates were calculated relative to the average. In all cases, binding of treated VLPs was nearly quantitatively reduced with an average decrease of 93%. As was observed for heparin-Sepharose chromatography and SPR, reduction in binding correlated well with the percent degradation measured by densitometry of Coomassie-stained gels.

Binding of VLPs to HaCaT Cells Is Inhibited by Sulfated Polysaccharides—The ability of HMW heparin and high or low molecular weight dextran sulfate to inhibit VLP binding to HaCaT cells was assessed by preincubating a constant concentration of VLPs with increasing amounts of inhibitor prior to assay. Controls in which (i) untreated VLPs were added to cells or (ii) high concentrations of inhibitor were added during the primary antibody incubation step were run on the same plate. These showed that the polysaccharides did not affect binding of the monoclonal detector antibody to VLPs. Inhibition curves for the three polysaccharides are shown in Fig. 9. All exhibited a dose-dependent effect similar to that observed with immobilized heparin-Sepharose and SPR. Significant dissociation of VLP-inhibitor complexes did not occur upon dilution in binding buffer as judged by good agreement of IC$_{50}$ values with those derived from non-cell-based assays. The IC$_{50}$ for HMW heparin (293 nM) agreed closely with the value determined by SPR but was lower than that observed with column chromatography. Similarly, the 5,000 M$_{r}$ dextran sulfate preparation gave an IC$_{50}$ of 21.7 μM, which was comparable with that determined for the 8,000 M$_{r}$ preparation during dextran sizing experiments on heparin-Sepharose. The best agreement between all meth-
Human Papillomavirus L1 Protein Interacts with Heparin

5817

FIG. 8. Interaction of VLPs with keratinocytes. A, the effect of ionic strength on binding of VLPs to HaCaT cells was determined by diluting increasing concentrations of VLPs in 0.15 M (○) or 0.5 M (●) NaCl buffer and performing the standard binding assay described under “Experimental Procedures.” For all assays, the reported absorbance (450 nm) values have been corrected for background absorbance of cells in the absence of VLPs. B, treatment of VLPs with bFXa was performed as in Fig. 6. Control and digested VLPs were assayed for their ability to bind HaCaT cells and by denaturing PAGE on 8% Tris glycine gels with Coomassie staining. The average absorbance for five control replicates was taken as 100% VLPs bound, and the individual control and digest absorbances were expressed relative to this value.

ods was obtained with HMW dextran sulfate, most likely because the extended, highly charged polymer offers more flexibility and less heterogeneity than smaller sized dextran sulfates or heparins.

Effect of Laminin on VLP Binding—A previous study (23) proposing the α6β1 integrin as a candidate receptor for papillomavirus showed that HPV 6b VLP binding to HaCaT cells was completely inhibited by laminin when added at 500 ng/well in a 96-well assay format. VLP binding in this study was performed on cells that had been trypsinized, washed, and held in suspension. Since keratinocytes in vivo are normally polarized and associated with complex basement membranes, we examined the effect of laminin on VLP binding to HaCaT cells maintained as adherent monolayers. For these experiments live cells were used in order to eliminate any artifacts caused by fixation, and all manipulations were performed on ice to minimize VLP internalization. Both mouse and human laminin were tested over a wide concentration range (250–10,000 ng/well) and in different binding buffers. Initial experiments examined pretreatment of cells, with the expectation that laminin would bind to the integrin and prevent binding of VLPs. Fig. 10A shows that VLP binding to cells treated with 500 ng/well human laminin was 95–99% that of control cells. Concentrations of laminin 10–20-fold higher showed a variable 30–50% decrease in VLP binding when added in PBS. When human laminin was added to cells in growth medium no effect on binding was seen, most likely due to desorption of the protein by components of bovine serum present in the medium. Mouse laminin failed to inhibit VLP binding when added in either PBS or growth medium. It would seem unlikely for the inhibitory effect to be narrowly species-specific, so the reason for this result is unclear. A second experiment (Fig. 10B) examined whether inhibition of binding was dependent on the time of laminin addition. A fixed amount of laminin (5000 ng/well) was preincubated with cells as in the initial study or added at the time of VLP addition. VLP titrations were performed, and the concentrations chosen for analysis were those giving the highest signal above background. Results of preincubation were similar to the initial experiment. Human laminin co-added with VLPs inhibited binding by 20% compared with approximately 35% when preincubated. A surprising 30–40% enhancement of binding was observed when mouse laminin was co-added with VLPs, although the mechanism for this effect is not known. The similar effectiveness of pretreatment and co-addition observed with human laminin stands in contrast to inhibition by heparin as measured by SPR where co-addition was much less effective at preventing binding.

Binding of VLPs to HaCaT Cells Is Reduced by Heparinase and Chlorate—In order to probe more directly the role of cell-surface anionic polysaccharides in VLP binding, cells were treated with a variety of agents designed to reduce or alter glycosaminoglycan content. For all analyses untreated controls were included on every plate, and binding of VLPs to treated cells was expressed relative to the amount bound to control cells. In all experiments VLPs were added at various concentrations, and quantitative data were calculated using those concentrations that gave the best signal above background.

Treatment of GAGs with heparinases (Fig. 11A) produces oligosaccharides of varying length containing an unsaturated uronic acid residue. Heparin lyases (heparinase I and II) cleave the polysaccharide chain at GlcNSO3-L-iduronic (2-O-sulfate) linkages whereas heparitinase (heparinase III) cleaves less sulfated regions containing l-glucuronic-hexosaminidic linkages. These enzymes are specific for heparin and heparan sulfate, respectively (34). Confluent cells were treated with 1 or 3 units/well of enzyme at 25 or 37 °C for 1 h and subsequently fixed. Control cells incubated at either temperature showed no
difference in VLP binding, confirming that the digestion buffer had no effect on cell-surface properties. No significant differences in the data were observed between cells treated with 1 or 3 units of enzyme; for clarity, data results were averaged. At 25 °C, all enzymatic digestions led to a decrease in VLP binding. The heparin lyases provided 70–85% inhibition, which was slightly more efficient than the 60–75% decrease in VLPs bound observed using heparitinase. At elevated temperatures, a more significant difference was noted. The activity of heparin lyases was slightly enhanced to give 80–90% inhibition, whereas heparitinase treatment reduced it by only 20–30%. This result was not due to temperature-induced inhibition of enzyme since the optimum for heparitinase is 43 °C but may reflect differences in the type and accessibility of surface proteoglycans. Treatment of cells with sodium chlorate (Fig. 11B) was used to produce undersulfated GAG chains. Confluent cells maintained in standard medium were washed and cultured for 48 h in sulfate-free medium containing sodium chlorate. At the highest concentrations used for this experiment cell morphology was somewhat altered, and at concentrations above 100 mM acute toxicity was observed. Cells treated with increasing concentrations of reagent stained less intensely with Alcian blue, indicative of a loss of anionic cell-surface components (data not shown). The effect of undersulfation was comparable to removal of GAG chains enzymatically. At 20 and 50 mM sodium chlorate, less than 25% of VLPs bound relative to controls. Interestingly, cells treated with 100 mM chlorate bound approximately 50% as many VLPs as controls. At this concentration of chlorate some morphological changes were noted, particularly in loss of cells from the surface. If a significant portion of fixed
cells had been lysed, VLP binding might be enhanced due to nonspecific binding to intracellular components.

Inhibition of GAG Synthesis Reduces VLP Binding—Substituted β-D-xylosides serve as artificial acceptors of nascent GAG chains, resulting in a large increase in the percentage of glycosaminoglycans which are secreted into the medium. Although not covalently linked to core proteins, the highly charged structure of the GAGs can cause them to remain non-covalently associated with the cell through ionic interactions. Commercially available ρ-nitrophenyl (ρNP) and 4-methylumbelliferyl (4MU) xylosides were employed for this study (Fig. 12, A–C). Reagents were added to cells in sulfate-free medium in order to directly compare results with those observed using chloride treatment. Medium was replenished every 2–4 days throughout the course of the experiment to avoid build-up of potentially toxic hydrolysis products of the xylosides. VLP binding was most effectively reduced by treatment with ρNP-Xyl (Fig. 12A) which showed a typical dose-dependent effect at all concentrations tested. This effect was enhanced when cells were washed with PBS containing 0.6 M NaCl instead of PBS alone (data not shown), and the high salt wash was used for all subsequent assays. It is reasonable to assume that high ionic strength can remove non-covalently associated GAGs which would be expected to retain their ability to bind VLPs. The 4MU-Xyl analog provided a somewhat lower degree of inhibition in the concentration range of 0.2 to 1 mM. When higher concentrations of each reagent were tested an effect similar to that which occurred at high chloride concentrations was observed (Fig. 12B) for 4MU-Xyl. VLPs bound approximately 40% better to treated cells than to controls. This effect was not noted for ρNP-Xyl, which provided greater than 70% inhibition at elevated concentrations. No adverse morphological changes were evident in cells treated with either reagent, suggesting that the 4MU-Xyl effect may be specific to the form of β-xyloside used and not to cellular damage. The effect of xylosides on actively growing cultures as opposed to confluent, contact-inhibited cells was examined in a second experiment (Fig. 12, C). The xyloside concentration selected was one which provided significant inhibition in initial studies. Reagents were added to cells that were 30–40% confluent, 16 h after seeding. Total growth time in the presence of xyloside was 6 days, after which the cells were washed with high salt, fixed, and assessed for VLP binding. Treated cells were found to bind approximately 80% as many VLPs as untreated controls, and no difference was noted between ρNP or 4MU substrates. The degree of inhibition provided by xylosides under these conditions was approximately 50% less than when confluent cells were treated. Light microscopy indicated that cells reached confluency much slower in medium containing xylosides suggesting that intact proteoglycan structure may be a requirement for proper spreading and growth of keratinocytes.

In order to determine if N-linked glycosylation played a significant role in VLP binding cells were cultured in the presence of tunicamycin for 20 h (Fig. 12D). No true dose-dependent
effect was seen, although at higher concentrations approximately 10–20% inhibition was observed at some VLP concentrations. No changes in cellular morphology or growth rate were observed.

Binding of VLPs to CHO-K1 and pgsA-745 Cells—Several well characterized mutants of CHO cells have been identified in which GAG production is hampered to various degrees. The cell line pgsA-745 is deficient in the enzyme UDP-D-xylose:serine-1,3-D-xylosyltransferase and does not produce glycosaminoglycans (35). Cultures of CHO-K1 and pgsA-745 cells were grown under standard conditions and used for VLP binding studies (Fig. 13). Both lines grew at a comparable rate under the conditions employed, although wild type cells presented a more typically striated morphology and tended to shed less cells into the medium than did the mutant. Initial experiments (Fig. 13A) were performed using conditions developed for HaCaT binding assays, and these indicated that wild type CHO-K1 cells bound fewer VLPs than did HaCaT cells as evidenced by the higher concentrations of protein required to obtain signals above background. For HaCaT cells, saturation was reached at VLP concentrations of 1600 ng/ml whereas linear response was still evident when 12,800 ng/ml VLPs was added to CHO-K1 cells (Fig. 13B). Under these conditions the linear range of the assay was approximately 10–20% inhibition was observed, although at higher concentrations approximately 10–20% inhibition was observed at some VLP concentrations. No changes in cellular morphology or growth rate were observed.

DISCUSSION

In this report we have identified a heparin-binding region on the carboxyl-terminal portion of human papillomavirus L1 protein, and we have shown that this region plays an important role in binding of VLPs to cell surfaces. An overwhelming majority of “heparin-binding” proteins share at least one motif in common, that being one or more regions of clustered basic amino acids which provide the site of interaction with negatively charged polysaccharides. However, more stringent efforts designed to identify definitive sequence requirements have met with only marginal success (36). Sequence comparison of nine HPV types identified a conserved region in the final 15 amino acid residues of the L1 protein of the general type XBBBXXB where B is Lys, Arg, or His. This pattern is similar to the XBBXBX and XBBBXBX consensus sequences identified through molecular modeling of known heparin-binding proteins (30). Although tight groupings of basic residues are somewhat atypical, the heparin-binding region of extracellular superoxide dismutase (37, 38) contains the sequence XBBBXYBBX, which is similar to that proposed for L1. Consensus sequence searching can be misleading when investigating interactions between proteins and anionic polysaccharides. The high affinity interaction of heparin and antithrombin III has been well characterized, leading to the identification of a unique polysaccharide sequence required for binding (39); however, for other proteins such as those of the fibroblast growth factor family (40), binding to heparin and related glycosaminoglycans appears to require less stringency. Still, for most heparin-binding proteins the specificity of interaction generally surpasses that expected from simple cation exchange chromatography.

HPV 11 L1 VLPs could be eluted from heparin-Sepharose columns using free heparin or NaCl. The ionic strength required for elution (0.8 M) was similar to that seen for HSV glycoprotein C (24) as well as non-viral proteins such as lactoferrin (41), thrombin (42), and the insulin-like growth factor-binding proteins (43). By contrast, high affinity heparin-binding proteins such as basic fibroblast growth factor (44) and antithrombin III (45) typically require 1 M NaCl or greater. Enzymatic removal of the HPV L1 carboxyl terminus completely abolished binding to immobilized heparin. The high affinity interaction displayed by HPV L1 is most likely due to the presence of multiple exposed carboxyl termini on the surface of the particle. This is suggested by synthetic 15-mer peptide binding studies in which the free peptide elutes at lower ionic strength and does not effectively compete with intact VLPs for binding sites. An alternative explanation is that a secondary structural motif present in the intact particle may confer higher affinity on the VLP in a manner analogous to basic fibroblast growth factor (44). This possibility was explored using a longer peptide of 28 residues with the assumption that this might more easily adopt a secondary structure in solution. Although some enhancement of binding was observed, most likely due to the contribution of a second short grouping of basic residues (Arg<sup>477</sup>–Arg<sup>485</sup>), the degree of interaction was less than that exhibited by VLPs.

Inhibition of VLP binding by polyanions revealed a dependence on both mass and charge density of the polymer. The heparinbinding region on the carboxyl-terminal portion of human papillomavirus L1 protein, and we have shown that this region plays an important role in binding of VLPs to cell surfaces. An overwhelming majority of “heparin-binding” proteins share at least one motif in common, that being one or more regions of clustered basic amino acids which provide the site of interaction with negatively charged polysaccharides. However, more stringent efforts designed to identify definitive sequence requirements have met with only marginal success (36). Sequence comparison of nine HPV types identified a conserved region in the final 15 amino acid residues of the L1 protein of the general type XBBBXXB where B is Lys, Arg, or His. This pattern is similar to the XBBXBX and XBBBXBX consensus sequences identified through molecular modeling of known heparin-binding proteins (30). Although tight groupings of basic residues are somewhat atypical, the heparin-binding region of extracellular superoxide dismutase (37, 38) contains the sequence XBBBXYBBX, which is similar to that proposed for L1. Consensus sequence searching can be misleading when investigating interactions between proteins and anionic polysaccharides. The high affinity interaction of heparin and antithrombin III has been well characterized, leading to the identification of a unique polysaccharide sequence required for binding (39); however, for other proteins such as those of the fibroblast growth factor family (40), binding to heparin and related glycosaminoglycans appears to require less stringency. Still, for most heparin-binding proteins the specificity of interaction generally surpasses that expected from simple cation exchange chromatography.

HPV 11 L1 VLPs could be eluted from heparin-Sepharose columns using free heparin or NaCl. The ionic strength required for elution (0.8 M) was similar to that seen for HSV glycoprotein C (24) as well as non-viral proteins such as lactoferrin (41), thrombin (42), and the insulin-like growth factor-binding proteins (43). By contrast, high affinity heparin-binding proteins such as basic fibroblast growth factor (44) and antithrombin III (45) typically require 1 M NaCl or greater. Enzymatic removal of the HPV L1 carboxyl terminus completely abolished binding to immobilized heparin. The high affinity interaction displayed by HPV L1 is most likely due to the presence of multiple exposed carboxyl termini on the surface of the particle. This is suggested by synthetic 15-mer peptide binding studies in which the free peptide elutes at lower ionic strength and does not effectively compete with intact VLPs for binding sites. An alternative explanation is that a secondary structural motif present in the intact particle may confer higher affinity on the VLP in a manner analogous to basic fibroblast growth factor (44). This possibility was explored using a longer peptide of 28 residues with the assumption that this might more easily adopt a secondary structure in solution. Although some enhancement of binding was observed, most likely due to the contribution of a second short grouping of basic residues (Arg<sup>477</sup>–Arg<sup>485</sup>), the degree of interaction was less than that exhibited by VLPs.

Inhibition of VLP binding by polyanions revealed a dependence on both mass and charge density of the polymer. The heparin-binding region on the carboxyl-terminal portion of human papillomavirus L1 protein, and we have shown that this region plays an important role in binding of VLPs to cell surfaces. An overwhelming majority of “heparin-binding” proteins share at least one motif in common, that being one or more regions of clustered basic amino acids which provide the site of interaction with negatively charged polysaccharides. However, more stringent efforts designed to identify definitive sequence requirements have met with only marginal success (36). Sequence comparison of nine HPV types identified a conserved region in the final 15 amino acid residues of the L1 protein of the general type XBBBXXB where B is Lys, Arg, or His. This pattern is similar to the XBBXBX and XBBBXBX consensus sequences identified through molecular modeling of known heparin-binding proteins (30). Although tight groupings of basic residues are somewhat atypical, the heparin-binding region of extracellular superoxide dismutase (37, 38) contains the sequence XBBBXYBBX, which is similar to that proposed for L1. Consensus sequence searching can be misleading when investigating interactions between proteins and anionic polysaccharides. The high affinity interaction of heparin and antithrombin III has been well characterized, leading to the identification of a unique polysaccharide sequence required for binding (39); however, for other proteins such as those of the fibroblast growth factor family (40), binding to heparin and related glycosaminoglycans appears to require less stringency. Still, for most heparin-binding proteins the specificity of interaction generally surpasses that expected from simple cation exchange chromatography.

HPV 11 L1 VLPs could be eluted from heparin-Sepharose columns using free heparin or NaCl. The ionic strength required for elution (0.8 M) was similar to that seen for HSV glycoprotein C (24) as well as non-viral proteins such as lactoferrin (41), thrombin (42), and the insulin-like growth factor-binding proteins (43). By contrast, high affinity heparin-binding proteins such as basic fibroblast growth factor (44) and antithrombin III (45) typically require 1 M NaCl or greater. Enzymatic removal of the HPV L1 carboxyl terminus completely abolished binding to immobilized heparin. The high affinity interaction displayed by HPV L1 is most likely due to the presence of multiple exposed carboxyl termini on the surface of the particle. This is suggested by synthetic 15-mer peptide binding studies in which the free peptide elutes at lower ionic strength and does not effectively compete with intact VLPs for binding sites. An alternative explanation is that a secondary structural motif present in the intact particle may confer higher affinity on the VLP in a manner analogous to basic fibroblast growth factor (44). This possibility was explored using a longer peptide of 28 residues with the assumption that this might more easily adopt a secondary structure in solution. Although some enhancement of binding was observed, most likely due to the contribution of a second short grouping of basic residues (Arg<sup>477</sup>–Arg<sup>485</sup>), the degree of interaction was less than that exhibited by VLPs.
arin and dextran sulfate preparations used in these studies contained an average sulfate content of 2.5 to 2.7 mol/mol disaccharide monomer unit and effectively competed for binding to immobilized heparin. The strength of inhibition was dependent on average polymer size as evidenced by the 50-fold higher IC50 value of LMW heparin. Carlson et al. (46) showed a similar effect of polymer weight on the activation of a "very high" affinity isoform of antithrombin III. The molecular mass effect for VLPs was more definitively shown by analysis of a series of sized dextran sulfates. The dramatic decrease in IC50 observed between polymers of M<sub>b</sub> 8,000 and 10,000 indicates that a critical size requirement was attained in this range. It is generally accepted that highly charged hydrophilic polymers exist in solution as extended, flexible rod-like structures, and it is reasonable to assume that these would tend to bind particles carrying multiple positively charged protein chains in a cooperative manner. Interestingly, Abbott et al. (47) showed that a critical dependence of the partitioning coefficient existed for the two-phase aqueous poly(ethylene oxide)-dextran system at poly(ethylene oxide) M<sub>b</sub> below 10,000. Models were proposed for different degrees of interaction between protein and polymer chains, and it was shown that when the attractive forces were strong (i.e., ionic binding) the polymer tended to form an encapsulated "shell" surrounding the globular protein. In the case of very large, rigidly spherical particles such as a VLP, higher molecular weight chains would be able to effectively cover more surface area at lower concentrations. Such strong, multiple site attachments can be viewed in a sense as being irreversible and offer a likely explanation why dissociation constants could not be measured under the binding conditions used for SPR measurements. This mechanism also can account for the SPR results showing different degrees of inhibition exhibited by HMW heparin depending on its time of addition. Preincubation of VLPs and free ligand would result in a uniformly coated particle carrying a negative surface charge and unable to bind to immobilized heparin. Alternatively, free VLPs bound to the biosensor chip strongly with no measurable dissociation and could not be quantitatively displaced using the same IC50 concentration of heparin calculated from preincubation experiments.

Inhibition studies using chondroitin sulfates illustrate that, in addition to molecular weight, sulfate content and possibly saccharide composition are factors in binding. Chondroitin sulfate A and C, both of which were less effective inhibitors compared with HMW heparin, contain D-glucuronic acid (β1–3) N-acetylgalactosamine (β1–4) core disaccharides instead of the L-iduronic acid (α1–4) N-acetylgalactosamine (α1–4) unit found in heparin. The presence of iduronic acid in heparin is postulated to offer a greater degree of flexibility to the GAG chain, allowing more possible conformations for binding to proteins (36). Chondroitin sulfates are monosulfated on either C<sub>a</sub> or C<sub>b</sub> of the GalNAc residue and thus possess a lower sulfate to carboxyl ratio than heparin. Fromm et al. (48) showed that synthetic peptides of the form BB(X)<sub>c</sub>BB and BBB(X)<sub>c</sub>B bound more effectively to heparin when n = 0 or 1 and more effectively to heparan sulfate when n = 1–3. Furthermore, chemically desulfated heparin and non-sulfated dextrans were completely unable to inhibit VLP binding, even when very high molecular weight preparations were used. These data demonstrate that VLP binding to heparin was electrosstatic in nature but not of sequence-defined high specificity, which explains why dextran sulfates of equal or higher M<sub>b</sub> than heparin were more effective inhibitors.

The binding of VLPs to keratinocytes closely coincided with most of the results observed with immobilized heparin, suggesting an interaction with cell-surface GAGs. Preincubation of VLPs with heparin or dextran sulfate resulted in a dose-dependent inhibition of binding that was dependent on the molecular mass of the polymer preparation. The IC<sub>50</sub> for HMW heparin (293 nM) was lower than that determined by heparin-Sepharose chromatography (14.9 μM) but comparable to the SPR results (76 nM). This value corresponded to a concentration of 5.1 μg/ml that was very comparable with values of 2.5 and 1.2 μg/ml measured for heparin binding of PrV gIII (56) and HSV gC (24) viral glycoproteins, respectively. The elevated value calculated from chromatographic studies could be the result of secondary nonspecific interactions between VLPs and the resin matrix, i.e., mixed modal binding mediated by regions distinct from the carboxyl terminus. For HMW dextran sulfate, good agreement was noted among all three methods. Given the relationship between polymer mass and charge with regard to inhibitory effectiveness, it is not surprising that this compound was the most effective identified in this study.

Of course, binding of inhibitor might have masked an unrelated site which was the true receptor target. If so, the bFXa studies indicate that this region is also localized to the last 28 amino acids since removal of these residues eliminated binding. Treatment of VLPs with a combination of reducing and chelating agents had only a marginal effect on reducing binding. This indicates that either the treatment was ineffective for type 11 L1 VLPs under the experimental conditions employed or that capsomers also bind to cells and retain the conformational epitope recognized by the monoclonal antibody. Based on similar gel filtration profiles exhibited by treated and control VLPs, the former explanation is more likely and suggests possible stability differences among various VLP types.

Specific enzymatic removal of heparin-like GAGs was very effective at reducing VLP binding as was reduction of sulfate content by chlorate treatment. Previous studies have shown that heparan sulfate GAGs account for 60–70% of proteoglycan on HaCaT cells (49). Although heparan sulfate was not tested specifically as an inhibitor, the question was addressed through the use of specific heparin lyases. Interestingly, heparinase was a more effective enzyme than heparitinase, especially at 37 °C, suggesting that the heparan sulfate chains of HaCaT cells may contain increased regions of tri- and tetrasulfated di- and tetrasaccharides and thus more closely resemble heparin. This has been demonstrated for BALB/c 3T3 fibroblasts (50), and recent studies have indicated that keratinocytes produce more highly sulfated glycosaminoglycans in confluent, high density cultures than under proliferative conditions (51). Similarly, treatment of cells with pNP-Xyl reduced VLP binding at all concentrations tested, whereas the 4MU-Xyl analog caused a 40% enhancement in binding at high concentrations. The efficiency of GAG priming by substituted xylosides has been shown to depend on the nature of the aglycone used (52); this is further defined by the cell type chosen for analysis. In most cases chondroitin sulfate is primarily transferred to the xyloside, but at high enough concentrations heparan sulfate synthesis is also affected. Because GAG chains can be associated with membranes in the absence of protein cores and because this interaction does not appear to be ionic (50), some cell-surface GAG may still be present under the conditions used for this assay. Furthermore, the degree of sulfation can be increased in the presence of xylosides resulting in chains containing higher contents of trisulfated disaccharides (53). It can be postulated that 4MU-Xyl is more efficient at producing structures of these types in keratinocytes. Both of these studies suggest that heparan sulfate may serve as a less effective inhibitor than heparin.

The evidence for VLP interaction with cellular GAGs was further supported using the CHO cell pgsA-745 mutant. This cell line lacks the xylosyltransferase enzyme that mediates the
initial step in glycosaminoglycan synthesis and as a result produces no cell-associated GAGs (35). VLPs were found to bind to CHO cells with lower affinity than they bound to HaCaT cells, which is consistent with previous reports (21). This suggests that CHO cells may carry less of the specific receptor molecule for HPV. Alternatively, if GAGs are involved in binding, the lower affinity may be related to the proportion and composition of heparan sulfate chains on the CHO cell surface. Binding to pgsA-T45 cells was significantly reduced relative to wild type, demonstrating that cells that do not produce GAGs show a markedly reduced capacity to bind HPV VLPs.

The search for a putative VLP receptor has focused on a protein constituent based in part on the observation that trypsinization of cells eliminates binding. This observation is not inconsistent with a proposed role for GAGs since the majority of these are present covalently bound to core proteins and are also removed by mild trypsinization (50). Although Evander et al. (23) reported that laminin was effective in preventing binding of type 6b L1 VLPs to the α6 integrin, this result was not observed in the current study except at very high laminin concentrations. This discrepancy may be in part due to differences in assay methods. In the former, cell monolayers were disrupted with trypsin and suspended prior to binding assays, whereas in this report all assays were performed on intact cell monolayers. In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.

In vivo, the α6 integrin is localized to hemidesmosomes on HaCaT cells and since cultured keratinocytes produce rudimentary, poorly formed structures of this type (54), the amount of integrin available for binding may be reduced. Inhibition by laminin may also reflect the fact that the long arm of the laminin A chain can bind to heparan sulfate (55). Finally, VLP binding to the CHO cell mutant was reduced by the same amount as that observed for heparinase and chlorella.