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Heparan sulfate proteoglycan mediates the selective attachment and internalization of serotype 3 human adenovirus dodecahedron

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Abstract

During adenovirus type 3 (Ad3) infection cycle, the penton (Pt) of the viral capsid, a noncovalent complex of fiber and penton base proteins, is produced in large excess and self-assembles to form a highly organized dodecahedral structure, termed dodecahedron (Dd). The physiological role of these particles is poorly understood, but we have recently reported that they can penetrate cells with high efficiency and thus may constitute an attractive tool for gene or protein delivery approaches. Surprisingly, Dd displayed the ability to enter cells non-permissive to Ad3, suggesting the existence of additional internalization modes. In this study, we show that Ad3 Dd binds to cell surface heparan sulfate (HS) through high affinity interaction with the penton base. Furthermore, binding to HS was found to be the prerequisite for a novel and Dd specific entry pathway that could not be used by Ad3. Overall, these data provide new insights in the possible role of Dd during viral infection and potential therapeutic applications.

Keywords: Heparan sulfate; Adenovirus dodecahedron; Receptor; Cell attachment; Internalization; Vector

Introduction

Heparan sulfate proteoglycans (HSPGs) are glycoproteins ubiquitously expressed at the surface of mammalian cells and in extracellular matrices. During the past 15 years, progress in the study of these molecules has highlighted their central role in most biological processes including cell proliferation, cell adhesion, chemoattraction, inflammation, wound healing, coagulation, matrix assembly, embryo development … (Delehedde et al., 2002; Esko and Selleck, 2002; Gallagher, 2001; Iozzo and San Antonio, 2001). HSPG properties are mostly mediated by the O-linked heparan sulfate (HS) polysaccharide chains present on the protein core that bind and regulate a wide range of proteins through motifs of specific saccharide sequence (Lindahl et al., 1998). Binding of proteins to HS serves a range of functional purposes, from simple immobilization and localization to specific regulation of signal transduction, as initially demonstrated for basic fibroblast growth factor (bFGF) signaling (Lundin et al., 2000; Pye et al., 1998). Besides its physiological functions, it is widely recognized that HS binding properties can also be exploited by a great number of pathogen microorganisms, particularly viruses, for attachment to the host cell surface (Sawitzky, 1996; Spillmann, 2001). Binding to HS is generally believed to promote infectivity by increasing local concentration of pathogens at the cell surface and thus favoring access to specific entry receptors. Hence, HS can play the role of a viral attachment receptor, as described for adenoviruses type 2 and 5 (Dechecchi et al., 2000, 2001), adeno-associated parvovirus-2 (AAV-2) (Summerford and Samulski, 1998), several members of the herpes virus family (Shukla and Spear, 2001), Flavivirus including hepatitis C (Germi et al., 2002) and Dengue (Chen et al., 1997) virus, Sindbis virus (Byrnes and Griffin, 1998) or human T cell leukemia virus type-1 (Q. Sattentau, personal communication). However, several studies have recently highlighted a deeper role of HS in infection mechanisms, for which a direct participation in viral entry was demonstrated. This has been described at least for herpes simplex virus-1 (HSV-1), for which viral fusion with the cell membrane is triggered by the binding of

Abbreviations: Ad, adenovirus; Bs-Dd, base-dodecahedron; CS-A, chondroitin 4-*O*-sulfate; CS-C, chondroitin 6-*O*-sulfate; CAR, coxsackievirus and adenovirus receptor; DS, dermatan sulfate; Dd, dodecahedron; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; Pt-Dd, penton dodecahedron.

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the viral glycoprotein D to rather rare HS motifs, in cooperation with other viral envelope proteins (Shukla et al., 1999; Spear et al., 2000). Finally, HS expressed on nonpermissive cells can capture, protect, and transmit viruses such as HIV, with increased infectivity, to cells permissive for the replication. This kind of novel class of in-trans receptor may have a deep impact on the tropism and dissemination of the virus in vivo (Bobardt et al., 2003).

Human adenoviruses (Ads) are a large family of non-enveloped viruses responsible for respiratory, ocular, and enteric infections. They share a common structural organization, in which the 36 kb dsDNA genome is concealed within a 90-nm icosahedral capsid composed of three major proteins: the trimeric hexon, the pentameric base, and the trimeric fiber. The last two form a noncovalent complex called the penton (Pt), which protrudes from each of the 12 vertices of the capsid (Ginsberg et al., 1966; Valentine and Pereira, 1965).

Ad penton is the major structural element responsible for viral attachment and entry into host cells. It has been demonstrated that purified penton alone was able to enter human epithelial cells (Fender et al., 1997; Hong et al., 1999). The infection process involves a first interaction of the fiber with a high affinity primary receptor that will facilitate subsequent binding of the penton base to cellular integrins and eventually trigger endocytosis (Mathias et al., 1994; Wickham et al., 1993). For most Ads, a 42-kDa protein of tight junctions, termed coxsackievirus and adenovirus receptor (CAR), is the primary receptor for infection (Bergelson et al., 1997). Members of Ad subgroup B, including adenovirus type 3 (Ad3), do not use CAR (Roelvink et al., 1998; Segerman et al., 2003). Recently, CD46 has been identified as the primary receptor used by several subgroup B Ads, but not Ad3 (Gaggar et al., 2003). However, this latter observation concerning Ad3 appears to be challenged by a recent work from another group (Greber and Hemmi, personal communication, manuscript submitted).

During Ad infectious cycle, free pentons are produced in excess and released with the virus progeny. The physiological relevance of this remains unclear, but a recent study suggested that free penton proteins enhanced infectivity by disturbing tight junctions and thus favored virus spreading (Walters et al., 2002). In contrast with most Ads, expression of Ad3 penton resulted in the formation of a highly organized dodecahedral structure (49 nm in diameter), in which the 12 penton base proteins interact with each other (Fig. 1) (Schoehn et al., 1996). Although its physiological role is not known, this complex, termed penton-dodecahedron (Pt-Dd), displayed high efficiency ability to bind and enter human epithelial cells, thus indicating that no other Ad protein was required during early endocytosis.

We have recently demonstrated that Ad3 Pt-Dd could constitute a good tool for gene or protein delivery (Fender et al., 1997, 2003). However, potential applications require the clarification of the mechanisms involved in cell attachment and entry. Intriguingly, we found that the expression of Ad3

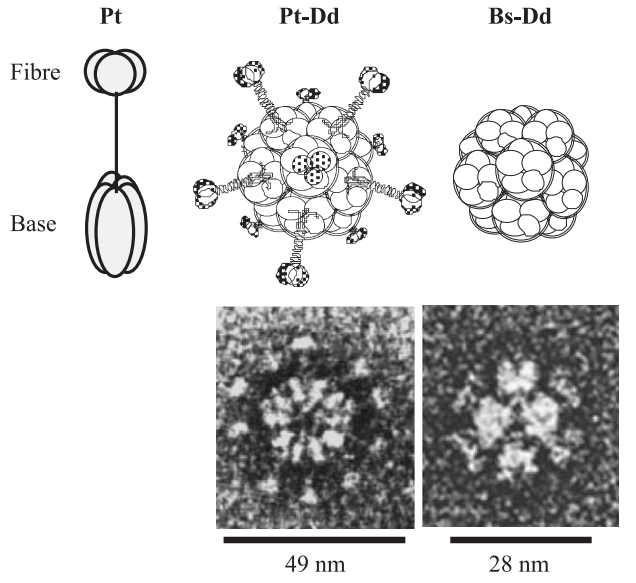

Fig. 1. Structure of Ad3 penton and Ad3 Dds. Schematic representation of Ad3 penton (Pt), which comprises the pentameric penton base (Bs) and the trimeric fiber. Self-association of Pt or Bs results in the formation of highly organized dodecahedral complexes, termed, respectively, Pt-Dd and Bs-Dd. The structure of Pt-Dd and Bs-Dd is represented schematically, along with the respective electron microscopy pictures.

penton base alone also yielded dodecahedral structures, termed base-dodecahedron (Bs-Dd, see Fig. 1). These smaller particles (28 nm in diameter) could also get into human cells, though with lower efficiency than Pt-Dd, indicating that the fiber plays an important role for cell attachment or entry (Fender et al., 2003), but that the existence of alternative internalization pathways had to be considered. In this study, we provide evidence that HS is a receptor involved in binding and entry of Dd into cells.

Results

Ad3 Dds use cell surface GAGs for attachment to CHO cells

To study novel Dd internalization pathways, we first investigated the ability of Ad3 Dd to bind to the surface of Ad3 nonpermissive cells, that is, cells that would not express Ad3 primary receptor. CHO-K1 cells were incubated with Pt-Dd at 4 °C and in presence of NaN_3 to prevent Dd internalization. After washing, bound dodecahedron was detected by FACS analysis, using a set of anti-Dd primary and FITC-conjugated secondary antibodies. No background was observed for control assays performed in absence of primary antibody (data not shown). Results showed that Pt-Dd efficiently bound to CHO-K1 cell surface, as indicated by a shift of the cell-associated fluorescence peak (Fig. 2A). However, no Dd binding could be detected on CHO-2241 cells, a mutant clone defective in glycosaminoglycan (GAG) synthesis, at both concentrations tested (Fig. 2B). These data suggested that Dd interaction with the CHO cell surface

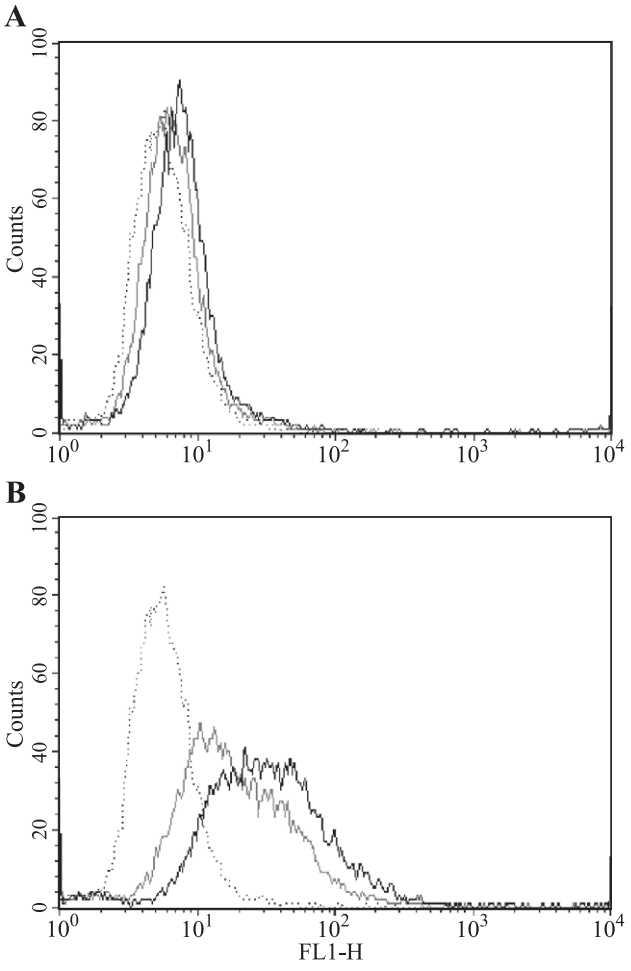

Fig. 2. FACScan analysis of Pt-Dd binding to CHO-K1 and CHO-2241 cells. CHO-K1 (A) and CHO-2241 (B) cells were incubated at 4 °C with 0, 25, or 120 nM of Pt-Dd (dotted, gray, and black lines, respectively), followed by successive incubations with anti-Pt-Dd primary antibody and FITC-conjugated secondary antibody. FL1-H: fluorescence intensity.

involved GAGs, and that no other appropriate attachment receptors were present on these cells. To confirm such hypothesis, CHO-K1 and CHO-2241 cells were incubated with FITC-labeled Dd and observed by laser confocal microscopy (Fig. 3). On CHO-K1 cells, a green halo resulting from bound Dd could be clearly seen at the cell surface (Fig. 3A). In contrast, no cell-associated green fluorescence could be distinguished on CHO-2241 cells, indicating absence of Dd attachment. Interestingly, similar observations were made for experiments performed with FITC-labeled Bs-Dd. This indicated that Dd binding to CHO cells was fiber independent. Such a mechanism is distinct from the putative Ad3 primary attachment process, which by analogy with subgroup C Ads, should require the fiber. Finally, the involvement of GAGs suggested by the above data was confirmed by competition assays with exogenous heparin. Preincubation of Dd with the polysaccharide before cell exposure indeed abrogated Dd attachment, with a dramatic reduction observed at heparin concentration as low as 0.01 μg/ml (Fig. 3B).

Ad3 Dd binding to GAGs is restricted to HS and heparin

The nature of cell surface GAGs actually involved in Dd binding was then determined, using filter binding analysis. Pt-Dd was incubated with biotinylated heparin (Hpb), after which the reaction mixture was drawn through a nitrocellulose membrane. Free Hpb was washed away, while Pt-Dd-bound Hpb remained trapped on the membrane and was detected by exposing the blots to peroxydase conjugated extravidin, followed by ECL detection (Fig. 4). The absence of signal for the negative control (Fig. 4, top lane, row 1) confirmed that free heparin was not retained by the nitrocellulose membrane. In contrast, binding of Hpb to Pt-Dd was clearly shown by a spot of strong density (Fig. 4, top lane, row 3). The Pt-Dd/Hpb interaction could be inhibited with an excess of non-biotinylated heparin or HS resulting, respectively, in total or severe signal loss (Fig. 4, top lane, rows 4 and 5, respectively). However, chondroitin 4-*O*-sulfate (CS-A), Dermatan sulfate (DS), and chondroitin 6-*O*-sulfate (CS-C) failed to compete with Hpb (Fig. 4, top lane, rows 6–8), indicating that Dd GAG-binding properties were restricted to heparin and HS. Experiments performed with Bs-Dd yielded a similar binding pattern (Fig. 4, bottom lane), with spots of equivalent intensity, thus further supporting a location of the heparin/HS binding site on the base protein of Ad3 Dd.

Ad3 Dds bind heparin with high affinity and form very stable complexes

Ad3 Dd interaction with heparin was then studied, using surface plasmon resonance (spr) analysis. For each sample, a set of sensorgrams was obtained by injection of a range of Pt-Dd or Bs-Dd concentrations (from 0 to 60 μg/ml) over both heparin-immobilized and negative control surfaces, and on-line subtraction of the recorded signals (Figs. 5A and 5B, respectively). Sensorgrams indicated potent interaction of both Pt-Dd and Bs-Dd to heparin, with significant signal being observed even at the lowest concentration tested (2.5 μg/ml, i.e., 0.52 and 0.69 nM, for Pt-Dd and Bs-Dd, respectively).

Preliminary kinetic analysis of the sensorgrams revealed a complex binding process, which could not be fitted to any obvious model of ligand–receptor interaction (data not shown). This is in agreement with the complexity of the Dd structure, in which the 12 penton bases, each composed of 5 identical monomers, display 60 potential heparin binding sites and thus could bind heparin in many different ways. Such multivalency is likely to cause strong avidity. Kinetic analysis of the sensorgram dissociation phases, using the BIAevaluation 3.1 software, yielded apparent dissociation rate constants (k_{off}) of 5.6 10^{-5} and 9.3 10^{-5} s^{-1} for Pt-Dd and Bs-Dd, respectively, indicating that Ad3 Dd formed highly stable complexes with heparin. To analyze the Dd–heparin interaction in more detail, we generated data in which the association phase was allowed to

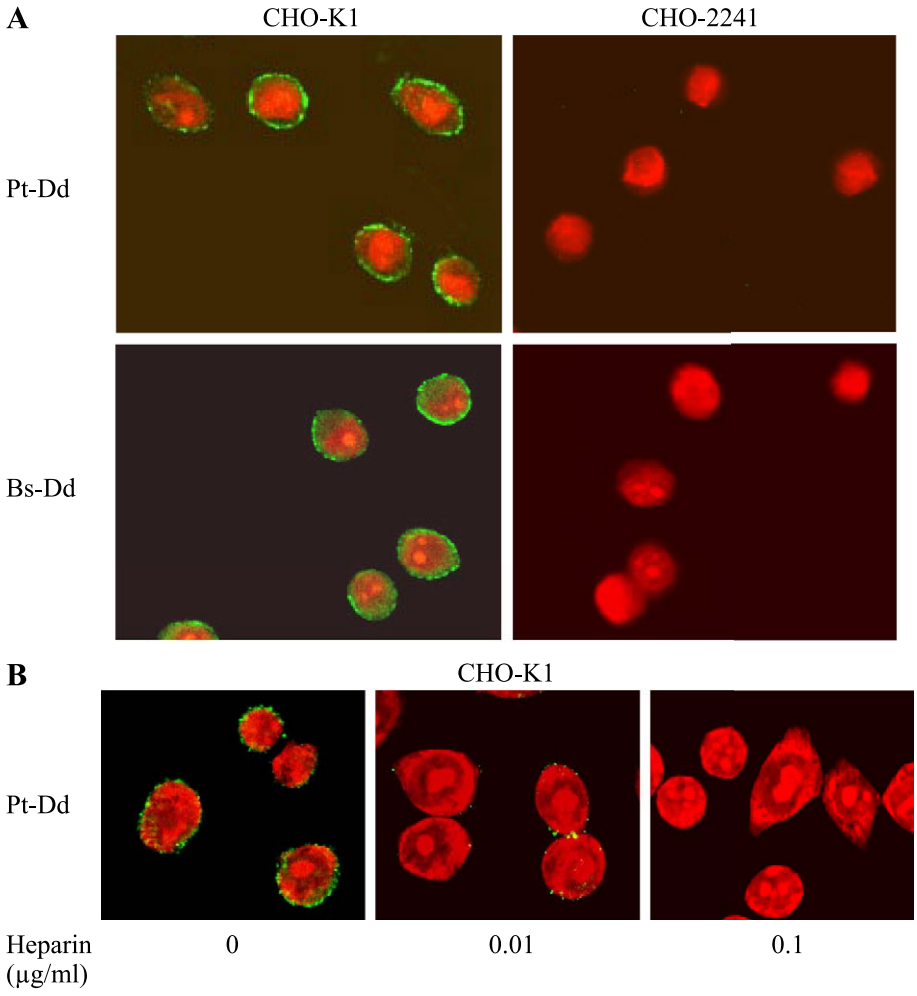

Fig. 3. Analysis of Dd binding to CHO-K1 and CHO-2241 cells by confocal microscopy. (A) CHO-K1 and CHO-2241 cells were incubated for 2 h at 4 °C with 1 nM of FITC-labeled Pt-Dd or Bs-Dd (green signal). After permeabilization, cell nuclei were counter-stained with propidium iodide (red signal). (B) Before CHO-K1 cell incubation, FITC-labeled Pt-Dd (1 nM) was pre-incubated with heparin (0, 0.01, or 0.1 μg/ml) for 30 min at room temperature.

proceed to equilibrium (20 min injections, Figs. 5A and 5B). Equilibrium data were extracted from sensorgrams that displayed a clear binding plateau, that is, for concentrations of 10 μg/ml and above, and these were plotted according to the Scatchard representation (Figs. 5C and 5D) to provide an affinity value independently of the kinetic aspect of the binding. This confirmed the high affinity of these interactions, with calculated Kd values in the nanomolar range: 0.7 and 1.4 nM for Pt-Dd and Bs-Dd, respectively.

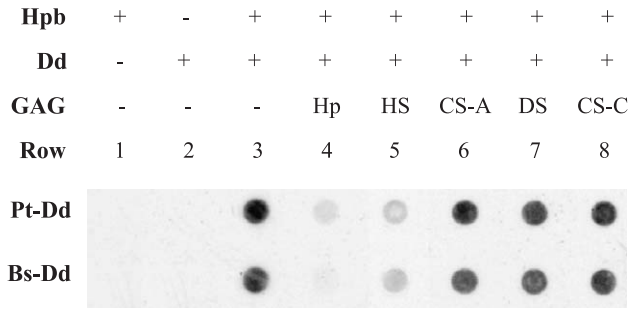

Fig. 4. GAG specificity of Dd interaction. Filter binding analysis of Dd/GAG interaction. Pt-Dd/Bs-Dd were incubated with (rows 3–8) or without (row 2) biotinylated heparin (Hpb), in absence (row 3) or presence of a 10-fold excess of non-biotinylated competing GAG, including Hp, HS, CS-A, DS, and CS-C (rows 4–8, respectively). Negative control (Hpb in absence of Dd) is shown on the top lane, row 1.

Cell surface HS is required for Ad3 Dd entry into CHO cells

Having highlighted the critical role of HS in Dd cell attachment, we then investigated a possible involvement in the cell internalization process. Here, incubation of CHO-K1 cells with FITC-Dd was performed in PBS for 1 h at 37 °C to allow internalization. Cell observation by confocal microscopy showed a very bright and punctuated intracellular signal, indicating that both Pt-Dd and Bs-Dd could efficiently enter CHO cells (Figs. 6A and 6B, respectively), despite absence of Ad3 primary receptor. Furthermore, Pt-Dd and Bs-Dd showed no difference in the endocytosis efficiency, thus supporting that this process

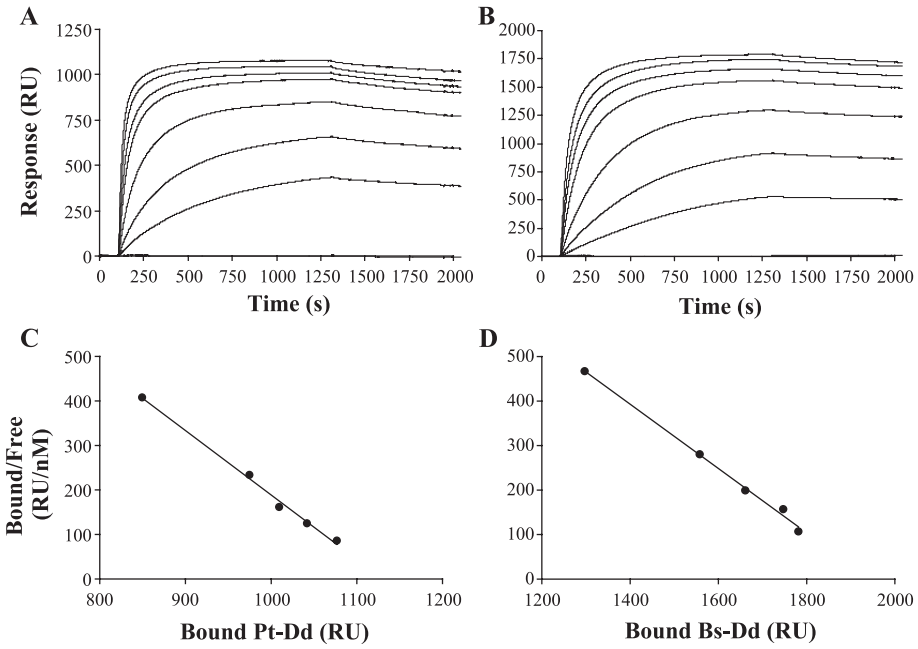

Fig. 5. Analysis of Pt-Dd and Bs-Dd binding to heparin by spr. Sensorgrams were obtained by injections of Pt-Dd (A) and Bs-Dd (B) at 0, 2.5, 5, 10, 20, 30, 40 and 60 μg/ml onto a heparin immobilized sensorchip. Data for which equilibrium was achieved were used for Scatchard analysis of the interaction (C and D, respectively).

was independent of the presence of the fiber. Addition of heparin (10 μg/ml) or cell treatment with chlorate that blocks GAG sulfation totally abolished cell internalization of both Pt-Dd (Figs. 6C and 6D, respectively) and Bs-Dd (data not shown). These results demonstrate that Dd binding to HS is a prerequisite for endocytosis of these particles into CHO cells.

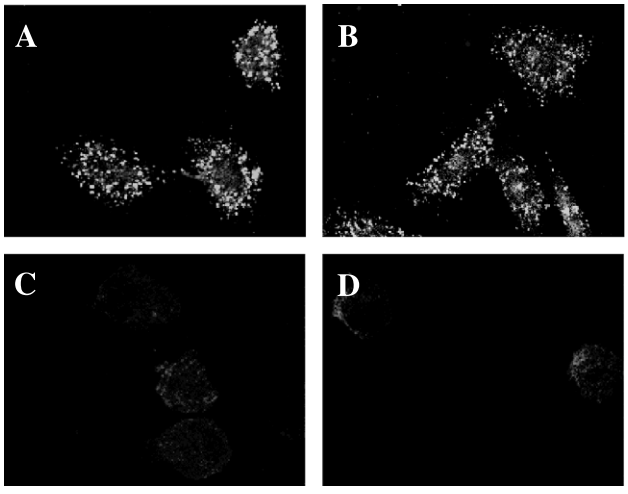

Fig. 6. HS-dependent Dd entry into Ad3 nonpermissive CHO-K1 cells. FITC-labeled Pt-Dd (A) and Bs-Dd (B) were incubated with HeLa cells for 1 h at 37 °C. After washing and fixation with 2% PFA, cells were observed by laser confocal microscopy. Pt-Dd internalization was inhibited by either pre-incubating Dd with 10 μg/ml of heparin (C) or treating cells with chlorate (D).

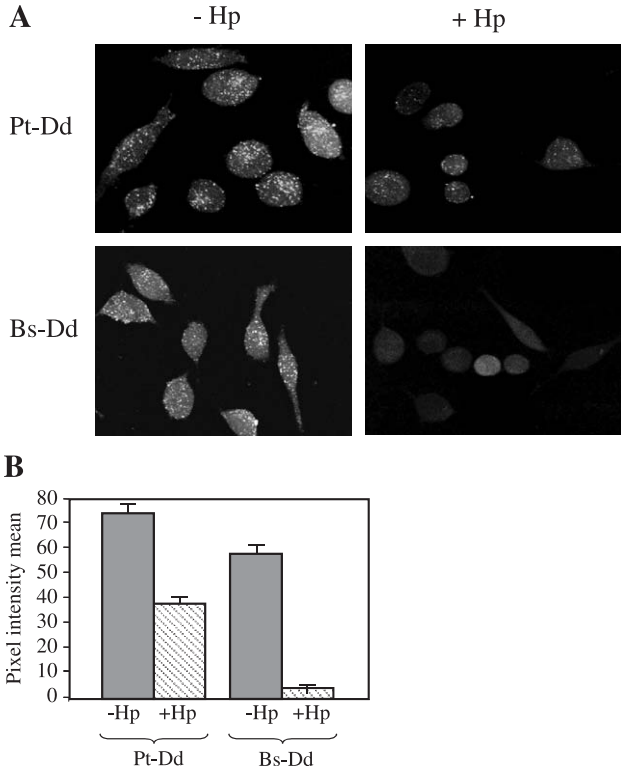

Fig. 7. Role of HS for Dd entry into Ad3 permissive HeLa cells. FITC-labeled Dd (1 nM) was pre-incubated or not with 10 μg/ml of heparin (Hp), then incubated with HeLa cells at 37 °C. After washing and fixation with 2% PFA, cells were observed by laser confocal microscopy (A). Cell fluorescence was quantified by photo-counting. The means of pixel intensity were determined from 12 individual cells (B).

HS constitutes an alternative pathway for Dd entry into Ad3 permissive HeLa cells

The role of HS in Dd cell attachment and internalization was defined on CHO-K1 cells, which are not the natural target of Ad3. To study this process in a more physiological context, we repeated our direct fluorescence assays on Ad3 permissive HeLa cells. FITC-Dd were incubated with HeLa cells for 1 h at 37 °C, before cell washing and fixation. In agreement with our previous study (Fender et al., 2003), results indicated that both Pt-Dd and Bs-Dd could enter HeLa cells, with slightly lower yields for the latter (Fig. 7). Addition of heparin resulted in a twofold reduction of Pt-Dd endocytosis, and a complete abrogation of Bs-Dd entry, as shown by cell fluorescence quantification (Fig. 7B). These data support the existence on HeLa cells of a fiber-independent, HS-mediated Dd internalization pathway, which is exclusively used by Bs-Dd and accounts for about half of Pt-Dd endocytosis.

Discussion

Due to its high ability to enter cells, Ad3-Dd has been thoroughly studied as a putative vector for gene and protein delivery. The advantage of such a structure is that it only comprises two distinct proteins, the base and the fiber, and is devoid of foreign nucleic acid. However, the mechanism of Dd cell attachment and entry remains poorly understood. By analogy with subgroup C adenoviruses, it is thought that Ad3 fiber head binds to a cell surface primary receptor, which will in turn facilitate interaction of Ad penton base RGD motif with αvβ3 and αvβ5 integrins and trigger subsequent endocytosis (Wickham et al., 1993). However, our previous data had already suggested the existence of a Ad3 primary receptor independent internalization pathway, as fiber devoid Bs-Dd was still able to bind to and enter cells, though to a lesser extent than Pt-Dd (Fender et al., 2003). Here, we provide evidence of a novel Dd cell attachment pathway, in which HS promotes binding and is required for cell entry.

The present study first showed that both Bs-Dd and Pt-Dd could bind to non-permissive CHO-K1 cells (Figs. 2 and 3). Interestingly, binding occurred with a similar efficiency for both Dd forms, suggesting that the fiber was not involved in this interaction. GAGs were identified as the docking site, because Dd attachment to CHO-K1 cells could be efficiently inhibited by competition with free heparin (Fig. 3B) and that both Bs-Dd and Pt-Dd failed to bind to GAG-deficient CHO-2241 cells (Fig. 3A). GAG-specific requirements for Ad3 Dd cell attachment were then investigated by filter binding analysis (Fig. 4). Our results indicated that both Bs-Dd and Pt-Dd bound to the biotinylated heparin with similar efficiency, thus further supporting the view that the base only participates to the binding. Competition performed with non-biotinylated GAGs showed that only heparin and HS could significantly inhibit the interaction, although addition of CS-A, DS, or CS-C had no effect. These results demonstrated that the base protein of Dd specifically bound to HS amongst cell surface GAGs.

The Dd/heparin interaction was further characterized by spr analysis. Injections of Pt-Dd and Bs-Dd on a heparin-immobilized surface yielded sensorgrams with very similar features, that is, a strong association with the polysaccharide followed by a very slow dissociation phase (Fig. 5). Due to the complexity of the binding reaction, we did not attempt to fully describe the kinetic aspects of the interaction. However, the very slow dissociation phase observed indicated that the complexes formed were very stable. Kd values could be deduced from Scatchard analysis of the data obtained at the binding equilibrium. Pt-Dd and Bs-Dd bound heparin with similar affinity (0.7 and 1.4 nM, respectively). These particularly high affinities may result from an avidity mechanism caused by the multiplicity of HS binding sites present on the Dd. We recently reported that the trimerization of the Ad2 fiber protein elicited a 25-fold affinity increase for the CAR receptor, this being due to an avidity effect (Lortat-Jacob et al., 2001). Such a phenomenon has also been observed when comparing the binding to heparin of HIV virions and isolated gp120 (unpublished observation). Moreover, the high affinity values measured are consistent with the low heparin concentration (0.01 μg/ml) needed to inhibit Dd cell attachment (Fig. 3B). Interestingly, surface maximum occupancy of Pt-Dd was half that of Bs-Dd (42 and 18 heparin chains per Pt-Dd and Bs-Dd, respectively), this reflecting the radius differences of Dd particles (49 nm versus 28 nm, see Fig. 1) that may limit the maximum density that could be bound onto the heparin surface.

In addition to cell attachment, our data provided evidence that Dd binding to HS was the prerequisite of a novel cell internalization pathway. Such process involved interaction of the polysaccharide with the penton base, as both Pt-Dd and Bs-Dd failed to enter GAG-deficient CHO-2241 cells, but could penetrate CHO-K1 cells with similar efficiency (Fig. 6). HS requirement was further demonstrated by blockage of Dd cell entry, using exogenous heparin, or cell treatment with chlorate that prevents endogenous GAG sulfation (Cardin and Weintraub, 1989). This HS-dependent internalization pathway could be clearly distinguished from the conventionally assumed Ad3 cell entry process that required the binding of the fiber to the Ad3 primary receptor, as shown by our results on Dd entry into Ad3 permissive HeLa cells (Fig. 7). On these cells, preincubation of Dd with exogenous heparin completely abolished attachment and entry of fiber devoid Bs-Dd, indicating an exclusive use of the HS-dependent pathway, and significantly reduced the internalization of Pt-Dd, which nevertheless retained a partial ability to enter cells, most likely through the Ad3 primary receptor pathway. The mechanisms by which HS initiates Dd cell entry remain unclear. A first hypothesis is that HS could facilitate the binding of Dd with

entry receptors, such as integrins. The structural basis of HS interaction with the penton base has not been investigated yet. However, analysis of the protein sequence revealed several positively charged arginine and lysine residues gathered in clusters typical of HS binding sites. Interestingly, one of this cluster, $K_{337}QKR_{340}$, is found near the $R_{329}GD_{331}$ sequence believed to interact with integrins. The close proximity of these two putative binding sites could suggest that HS and integrins may act in synergy to trigger cell internalization. This would be in agreement with our data showing that αv integrins, which are expressed at the surface of CHO-2241 cells, could not mediate Dd attachment or entry on their own. Another hypothesis is that HSPGs themselves could mediate internalization of Dd, as observed for several ligands, including growth factors and lipoproteins (Reiland and Rapraeger, 1993; Williams and Fuki, 1997). Interestingly, work from Fuki et al. (1997) reported that this endocytosis process could be triggered by the clustering of HSPG transmembrane and cytoplasmic domains. For Dd, HSPG clustering is likely to be favored by the multiplicity of HS binding sites at their surface and thus could permit efficient endocytosis of these particles.

In contrast to the high affinity binding of Ad3 Dd to HS, the Ad3 virus does not interact with either heparin or HS (Dechecchi et al., 2000 and unpublished data). Such a discrepancy raises several questions from a structural and a functional point of view. Pt-Dd and Ad3 viral capsid both comprise 12 pentons protruding in a similar manner from the particle. However, in the former, pentons directly interact with each other, while they are separated by hexons on the virus. It is possible that the HS binding site on the penton base is located near the penton–hexon interface, and thus the hexon on the virus partially or fully overlaps HS binding residues. Alternatively, the HS binding site could be created by the penton–penton interface that occurs on the Dd. Such situation has been already described for several dimerized chemokines, for which the HS binding site was formed at the protein–protein interface (Lortat-Jacob et al., 2002). Another hypothesis is that the negatively charged hexon, which is the major component of the viral capsid, but is not found on the Dd, may significantly influence the surface electrostatic potential and thus prevent possible interaction of the base with polyanionic HS. This is supported by data reporting that Ad endocytosis is sensitive to the global charge of the capsid (Arcasoy et al., 1997; Shayakhmetov and Lieber, 2000). Other Ads do use HS for cell attachment. In particular, HS has been recently described as an alternative receptor to CAR for wild-type Ad2/Ad5 (Dechecchi et al., 2000, 2001). In this study, a BBxB consensus motif (Humphries and Silbert, 1988) within the Ad2 fiber shaft sequence was presented as a putative HS binding site. This basic cluster is absent in the Ad3 fiber, suggesting that Ad2 penton and Ad3 Dd interact with HS through distinct binding sites. Pt-Dd is naturally found during the Ad3 replication cycle and in vivo, large amounts of Pt-Dd are released at the cell lysis (Norrby, 1966). The physiological role of Dd during viral infection is still unknown, but its ability to bind to HS, a molecule involved in a huge array of biological functions, is likely to be critical. It has been recently shown that the excess of synthesized Ad2 fiber contributed to virus spreading by disrupting the junctional integrity mediated by CAR (Walters et al., 2002). In addition, the base protein is also known to trigger cell detachment (Boudin et al., 1979), supporting further the ability of penton components to interfere with cell adhesion processes. As HS participates to cell cohesion, its interaction with Ad3 Dd may also be involved in the weakening of cell–cell contacts and contribute to the viral dissemination.

In this study, we have identified HS as a major attachment receptor for Ad3 Dd to the cell surface, and as a trigger for endocytosis through a novel, fiber-independent pathway. Our data highlighted a major divergence between Ad3 and Ad3 Dd cell entry mechanisms that may have an important physiological significance. Clarifying the structural and functional features involved in Ad3 Dd/HS interaction will constitute a major challenge for the understanding of the role played by these particles during viral infection, and for a potential use, as a gene/protein delivery vector.

Material and methods

BIAcore 3000, B1 sensorchip, amine coupling kit, and HBS-EP buffer were purchased from BIAcore AB. FACScan is from Becton Dickinson. EMEM and Ham's F12 medium were from Gibco. Heparin of 9 kDa was a generous gift from Maurice Petitou (Sanofi-Synthélabo). Heparin of 6 kDa and chondroitins sulfate were supplied by Sigma. MRC-600 laser scanning confocal microscope was purchased from BioRad. Tissue culture reagents were from Gibco Lifesciences.

Cell culture

Chinese Hamster Ovary (CHO) cell lines K1 (GAG positive, American Type Culture Collection [ATCC] code CCL-61) and 2241 (GAG negative, pgsB-618, ATCC code CRL-2241) were obtained from the European Collection of Cultured Cells. Cells were cultured at 37 °C, under 5% CO_2 atmosphere, in Ham's F12 (for CHO cells) or EMEM (for HeLa cells), supplemented with 10% fetal calf serum (FCS), penicillin (50 IU/ml), and streptomycin (50 μg/ml). Chlorate treatment was performed by incubation of trypsinized cells for 36 h in EMEM, 2% FCS, 30 mM sodium chlorate (Cardin and Weintraub, 1989).

Production, purification, and labeling of Ad3 Dd

Both Pt-Bs and Bs-Dd were expressed using the baculovirus system. Particles were purified by sucrose density gradient, as previously described (Fender et al., 1997). Dd

(300 nM) were fluorescently labeled by incubation with 1 mM FITC, in 20 mM HEPES, pH 8, for 3 h at 4 °C. Free FITC was removed by dialysis against 20 mM HEPES, 150 mM NaCl, pH 8.

Flow cytometry analysis

Cells (2×10^6/ml) in FACS buffer (PBS, 1% BSA, 0.02% azide, 1 mM EDTA) were incubated with Pt-Dd (25 and 120 nM) for 45 min at 4 °C. After washing with FACS buffer, cells were incubated with primary anti-Dd Ab (1/1000, 1 h at 4 °C in FACS buffer, followed by washing), then with FITC-conjugated secondary antibody (1/200, 1 h at 4 °C in FACS buffer, followed by washing), and analyzed by flow cytometry.

Filter binding assay

The interaction of Ad3 Dd with heparin was analyzed by filter binding assay (Sadir et al., 2001). Ad3 Pt-Dd and Bs-Dd (100 ng) were co-incubated with biotinylated heparin (0.05 μg/ml) in 200 μl of Tris-buffered saline (TBS) for 45 min at room temperature. In competition assays, a 10-fold excess of non-biotinylated GAGs (0.5 μg/ml) was also added. Samples were then drawn through a nitrocellulose membrane using a vacuum-assisted dot-blot apparatus. The membrane was washed twice with 200 μl of TBS and blocked with 5% dry milk in TBS containing 0.05% Tween 20. Protein-bound biotinylated heparin was revealed by incubation of the membrane with extravidin peroxydase (0.5 μg/ml) and enhanced chemiluminescence (ECL) detection reagents, followed by autoradiography.

Heparin/Dd interaction analysis by surface plasmon resonance

Heparin (9 kDa) was biotinylated and immobilized on a B1 BIAcore sensorchip, as described before (Vives et al., 2002). Briefly, two flowcells were prepared by sequential injections of EDC/NHS, streptavidin, and ethanolamine. One of these flowcells served as negative control, while biotinylated heparin was injected on the other one, to get an immobilization level of 80–90 response units (RU). All spr experiments were performed, using HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4), at a flow rate of 10 μl/min. Interaction assays involved 20 min injections of different dodecahedron concentrations (ranging from 0 to 60 μg/ml) over the heparin and negative control surfaces, followed by a 10-min washing step with HBS-EP buffer to allow dissociation of the complexes formed. At the end of each cycle, the heparin surface was regenerated by sequential injections of 0.05% SDS (1 min) and 2 M NaCl (2.5 min). Sensorgrams shown correspond to on-line subtraction of the negative control to the heparin surface signal. Data from sensorgrams that reached binding equilibrium were used for Scatchard analysis.

Confocal microscopy

Cells grown overnight on coverslips (about 10^5 cells/slide) and washed in PBS were incubated with either FITC-labeled Pt-Dd or Bs-Dd (1 nM) for 2 h at 4 °C. For heparin competition assays, Dd were first incubated for 30 min at RT with 0.01 or 0.1 μg/ml of 6 kDa heparin. Cells were then permeabilized with cold methanol for 10 min at −20 °C and cell nuclei counter-stained with propidium iodide (5 μg/ml). For internalization assays, cells were incubated with either Pt-Dd or Bs-Dd (1 nM) in PBS for 1 h at 37 °C. After PBS washes, cells were fixed with 2% paraformaldehyde. Coverslips were then mounted for observation on a microscope slide with 1,4-diazabicyclo-octane.

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