Rapid Communication

Synthesis, cellular localization, and quantification of penton-dodecahedron in serotype 3 adenovirus-infected cells

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Abstract

Adenovirus penton is a non-covalent complex composed of the penton base and fiber proteins, localized at the twelve vertices of the icosahedral virus capsid. In cells infected by adenovirus serotype 3 (Ad3), penton is found not only in the virus capsid but also self-assembled in dodecahedra formed through interactions between the twelve penton bases. In this study, the intracellular trafficking of penton proteins from the cytoplasm to the nucleus has been followed, and the nuclear re-arrangement induced by viral infection has been observed by electron microscopy of ultrathin sections. The amount of dodecahedra has been assessed in relation to the number of Ad3 infectious virions produced during the Ad3 replication cycle. It appears that dodecahedra are produced in a large excess over viral infectious particles and that they are located intranuclearly along the nuclear membrane of Ad3-infected cells at late times of infection.

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Introduction

Human adenoviruses (Ads) are non-enveloped viruses causing respiratory, ocular, and enteric infections. Their icosahedral capsid, containing the 36 kpb dsDNA genome, is composed of three major proteins, the hexon, the penton base, and the fiber. At the twelve vertices of the capsid, the protruding fiber together with the penton base embedded in the capsid form a complex called penton (Pt). Ad penton is the major structural element responsible for viral attachment and entry into host cells. Interestingly, in some Ad serotypes, penton proteins (penton base and fiber) are over-expressed during replicative cycle and can be purified from Ad-infected cell either separately or in form of penton complex (Boulanger and Puvion, 1973). Pentons in the complete virion are separated by hexon proteins but amazingly, in some serotypes, pentons can self-assemble into dodecameric particles called adenovirus dodecahedra (Ad-Dd). This has been reported for the first time for Ad3-infected cells (Norrby, 1966) and later the presence of Ad-Dd has been observed for other Ad serotypes. Ad-Dd can be also obtained by the penton expression in the baculovirus system thus demonstrating that other adenoviral components are not required for dodecahedra formation (Fender et al., 1997). The structure of these particles has been solved by cryo-electromicroscopy (Schoehn et al., 1996) and it has been reported that Ad-Dd enters human cells with a high efficiency, likely through interaction of the fiber with the Ad3 primary receptor (CD46) (Gaggar et al., 2003; Sirena et al., 2004), but also through direct interaction of the penton base with heparan sulphate (Vives et al., 2004).

Despite all these functional and structural data, the formation, the localization, and the quantification of Ad3-Dd produced during the adenoviral replicative cycle are not well established. Here, we report the kinetics and the site of penton synthesis in Ad3-infected cells. In addition, we present the data concerning the amount of Ad-Dd produced in comparison with the infectious virus and we provide
information on the respective nuclear localization of Ad3 and Ad-Dd.

Results

Penton synthesis during the Ad3 replicative cycle

For the determination of the kinetics of penton synthesis during the Ad3 replicative cycle, HeLa cells were infected with Ad3 at MOI 1 for different periods of time. Infected cells were lysed and analyzed by Western blot using an anti-Pt-Dd serum that recognized SDS-denatured penton base and fiber proteins. Although the anti-Pt-Dd serum is able to detect less that 1 ng of protein, neither fiber nor penton base protein could be detected before 12 h of infection (Fig. 1A). As expected for late viral proteins, the penton synthesis started at 12 h p.i. as attested by the detection of the monomeric forms of the penton base at 60 kDa and a faint signal for the fiber at 35 kDa. Penton protein expression increased rapidly between 12 and 24 h and seemed to stabilize after 24 h.

Cellular localization of pentons during the Ad3 replication cycle

The expression and localization of penton proteins in Ad3-infected cells were studied by confocal microscopy using anti-Pt-Dd serum and anti-rabbit FITC-labeled secondary antibody while nuclei were counterstained in red by propidium iodide (Fig. 1B). At 12 h p.i., penton proteins were observed solely in the cytoplasm of Ad3-infected cells indicating entry into the late stage of the Ad replication cycle. Interestingly, at this stage, no dodecahedra were found in the heavy fractions of sucrose gradient density suggesting that dodecamerization of penton does not take place in the cytoplasm (data not shown). Four hours later (16 h p.i.), although penton synthesis was still detectable in the cytoplasm, the majority of the signal was observed in the nucleus where the diffuse yellow signal reflected the co-localization of penton proteins with the cellular DNA. At 24 h p.i., no detectable penton signal was seen in the cytoplasm, indicating that Ad late proteins are not sufficiently expressed to be detectable under these experimental conditions. Interestingly, the diffuse green penton signal

Fig. 1. Kinetics and localization of penton proteins in Ad3-infected HeLa cells. (A) Expression of penton base and fiber proteins monitored by Western blot with anti-Pt-Dd serum recognizing both monomeric penton base and monomeric fiber. Time post-infection is shown on the figure. NI—lysate from non-infected cells; Pt-Dd—purified penton dodecahedron expressed in the baculovirus system. (B) Localization of penton proteins, monitored by immunofluorescence. Ad3-infected HeLa cells were permeabilized at indicated times of infection and incubated with anti-Pt-Dd. Penton proteins are seen in green with a FITC-conjugated antibody while the nuclei are counterstained in red with propidium iodide. Picture acquisitions were performed at the same exposure settings on MRC600 (Bio-Rad) LASER confocal microscopy.
seen at 16 h in the nucleus was now punctated and predominantly located at the nuclear membrane of the infected cells (Fig. 1B, last panel). Taken together, these results show that penton proteins are expressed in the cytoplasm and transported to the nucleus where they tend to localize close to the nuclear membrane later in infection.

**Specific location of encapsidated and non-encapsidated pentons in infected cell**

At 24 h p.i., a complete cycle of adenovirus replication has occurred yielding progeny virions in infected cells. Penton can then be found either incorporated in the progeny virions (encapsidated pentons) or can remain free in form of dodecahedron (non-encapsidated pentons). To determine whether the penton signal seen at the nuclear membrane is due to the dodecahedron or to the virus, an immunofluorescence study was performed using either anti-Pt-Dd or anti-hexon antibodies. While the anti-Pt-Dd serum will interact with both virions and dodecahedra, the latter antibody will recognize virions but not the Dd. Interestingly, the signal raised with anti-hexon was also punctated but arranged all over the infected cell nucleus (Fig. 2A, left panel). Of note, a majority of hexon synthesized in the Ad3-infected cells is used for the formation of 240 hexon capsomers of virions since infection by Ad3 contrary to that by Ad2 does not produce a large excess of free hexon (White et al., 1969, Boulanger and Puvion, 1973). It seems plausible that the weak intranuclear signal observed with anti-Pt-Dd antibody (Fig. 2A, middle panel) is due to encapsidated pentons whereas the bright perinuclear signal raised by this antibody is likely emitted by non-encapsidated pentons. To confirm this hypothesis, ultrathin sections of Ad3-infected HeLa cell have been observed by electron microscopy (Fig. 2B). As previously described for other Ad serotypes (Henry et al., 1971), the Ad3 progeny viruses are seen concentrated in form of nuclear inclusions, possibly explaining the large punctate signal observed with the anti-hexon antibody in the immunofluorescence study. It is relevant that individual Dd with about 3-fold smaller diameter than that of virus cannot be directly detected by this technique. Nevertheless, the punctate signals observed
on Fig. 2A with anti-Pt-Dd (middle panel) can be correlated with the dark punctate structures seen at the perinuclear membrane space of infected cells nuclei on ultrathin sections (Fig. 2B, right panel). It can be thus concluded that a large excess of penton is synthesized during the Ad3 replicative cycle and that this non-encapsidated pentons tends to accumulate in the nucleus lining the external side of the nuclear membrane at late stage of infection.

**Estimation of dodecahedra and infectious virus in Ad3-infected cell**

We have elaborated a strategy enabling both the quantification of Dd and the titration of Ad3 to be performed on the same batch of infected cells (Fig. 3). Lysate recovered from HeLa cell infected for 24 h has been either subjected to sucrose gradient ultracentrifugation to separate Dds from the virus particles or applied to cesium chloride gradient to purify Ad3 virions. As expected, Dd was localized in the 28–38% part of the sucrose gradient density with very low contamination by virus particles as attested by electron microscopy (below 0.5%, data not shown). These fractions were then pooled and different volumes were run on SDS-PAGE in the presence of known amounts of purified Dd, and subjected to Western blot with anti-Pt-Dd serum. By comparing the band intensity of our sample to that of the purified Dd range, we found a total amount of 45 µg of Dds in the lysate of 10^7 cells meaning that, on average, 7.5 \times 10^5

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**Fig. 3. Titration of infectious virus and quantification of Pt-Dd and total viral particles. (A) Principle: Ad-infected HeLa cells were lysed after 24 h of infection. A part of the lysate was centrifuged on a 15–40% sucrose gradient to recover Pt-Dd. Quantification of Dd was performed by Western blot using anti Pt-Dd serum. For this, aliquots of Pt-Dd produced during the Ad3 cycle were run on SDS-PAGE and compared to known amounts of Pt-Dd expressed in the baculovirus system and purified. For the infectious virus titration, Ad3 was purified on a cesium chloride gradient and titrated by FFU determination. (B) Results obtained in three independent experiments. Dodecahedron particle molecular weight of 3.6 \times 10^6 Da was used in calculations.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Dodecahedron amount (ng)</th>
<th>Dodecahedron particles</th>
<th>Infectious virus FFU total</th>
<th>Ratio (Dds/Virus)</th>
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<tr>
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<td>42</td>
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<td>1.3 \times 10^{8}</td>
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<tr>
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<td>7.3 \times 10^{12}</td>
<td>1.2 \times 10^{8}</td>
<td>6.1 \times 10^{6}</td>
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<tr>
<td><strong>Average</strong></td>
<td><strong>45</strong></td>
<td><strong>7.5 \times 10^{12}</strong></td>
<td><strong>1.4 \times 10^{8}</strong></td>
<td><strong>5.5 \times 10^{6}</strong></td>
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particles were found per cell. This amount is likely to be underevaluated as some cells were not infected.

Infectious Ad3 particles were titrated by fluorescent forming unit (FFU) determination. For this, adenoviruses were purified from infected cell lysate by cesium chloride gradient centrifugation in order to eliminate Dds and other contaminants that could interfere with Ad3 infection. Results of three independent experiments demonstrated that a total of $1.4 \times 10^6$ FFU were present in the lysate. These results show that there is a large excess of Dd over infectious Ad3 particles in infected cells with an average of $5.5 \times 10^6$ Dds per one infectious virus. Total viral particles have also been assessed by Western blot by comparing the penton base band displayed by the virus to that of a range of known amounts of purified dodecahedron, as described in Fig. 3. With a ratio of 26,000 non-infectious viral particles per infectious virus, it has been calculated that about 200-fold more Dds than total viral particles are found in Ad3-infected cell, at 24 h p.i.

Discussion

Adenovirus penton base is a major player not only in adenovirus entry but also during the adenovirus replication cycle. Indeed, even if it has been known for long time that penton base has a critical role in viral endocytosis (Wickham et al., 1993), another role for this protein has been reported more recently. Due to their strictly conserved PPxY sequence in their N-terminus, human adenovirus penton bases interact with WW domain-containing proteins such as the ubiquitin ligases family (Galinier et al., 2002, Chroboczek et al., 2003). In our study, we showed that neo-synthesized penton as a typical late product appears in the cytoplasm of infected cell 12 h p.i and its synthesis increases steadily until 24 h, as reflected by the kinetic study (Fig. 1). During this period, the penton is susceptible to interact with several cytoplasmic protein partners including WW-containing proteins before being transferred to the nucleus where the virus assembly takes place. Interestingly, the diffuse penton base signal seen inside the nucleus at 16 h p.i. seems to become arranged along the nuclear membrane 8 h later. At 24 h p.i., one cycle of the virus replication has been accomplished and thus virion progeny is already present in the nucleus of infected cell (White et al., 1969; Boudin et al., 1979).

It has been reported that a highly symmetric particle made of twelve penton bases is produced in Ad3-infected cells (Norrby, 1966). This particle, called adenovirus dodecahedron (Dd), is well characterized from the structural point of view (Schoenl et al., 1996; Zubieta et al., 2005) as well as functionally (Fender et al., 1997; Vives et al., 2004) but little is known about its formation. In this study, we bring new elements showing that this particle is likely assembled in the nucleus as no Dd can be recovered from cell infected for 12 h when penton base is expressed in the cell cytoplasm but not yet found in the nucleus (Fig. 1B). Nuclear assembly of virus-like particles has already been described for other viruses such AAV or polyomavirus (Hoque et al., 1999; Johne and Muller, 2004).

Another point investigated in this work is the total number of Ad-Dd produced per infected cell and how it correlates with the virus titer. Surprisingly, Ad3 infection in cell culture yielded very low titer of infectious virus. This low number is somewhat in contradiction with the number and the size of adenoviruses para-crystals (nuclear inclusion) seen in the nucleus of Ad3-infected cells (Fig. 2B, right panel). A ratio of about 26,000 non-infectious viral particles per infectious particle has been determined. Such a low yield of infectious Ad3 might suggest that the virion maturation requires an additional step taking place during normal cell lysis and which does not occur in freeze–thaw cycles. However, whatever the number of defective and infectious viruses, a massive amount of Dd is found in the nucleus of Ad3-infected cell and we have investigated their distribution. Remarkably, these complexes that at 16 h p.i. are randomly spread all over the nucleus, are arranged inside the nucleus along the nuclear membrane at 24 h p.i. This distribution contrasts with the virion nuclear inclusions that are found in different area of the nucleus as seen by immunofluorescence with anti-hexon antibody and confirmed on infected cell ultrathin sections (Fig. 2). The mechanism leading to such localization of Dd is not known and unfortunately the size of the particle does not permit obtaining precise details of their organization on ultrathin sections.

The amount of dodecameric particles produced in infected cell nuclei has been estimated. On average, $7.5 \times 10^5$ particles are found per infected cell representing a ratio of $5.5 \times 10^6$ Dds per infectious virus. Such an excess of pentons synthesized during the Ad3 replication cycle strongly suggests that Dd has a role in virus strategy. It is relevant that the Ad2 penton base protein is known to triggers cell detachment (Boudin et al., 1979) and we have reported that Ad3-Dd interacts with high affinity with heparan sulfate that participates in cell cohesion (Vives et al., 2004). It can be hypothesized that during cell lysis, the release of massive amounts of Dd can affect cell cohesion and/or that competition with virus for its receptor during secondary infection contributes to virus escape and spreading as reported for the Ad2 fiber (Walters et al., 2002). With regards to our results, it can also be hypothesized that Dd plays a role in the nucleus of infected cells. It could be envisaged that Dd localizes with nuclear pore complex and regulates nuclear import and export. Taken together, our results show that only a low proportion of Ad3 penton proteins migrating from cytoplasm to the nucleus is used for virion formation while the major part self-assembles in form of Dd and accumulates along the nuclear membrane reinforcing the idea of the crucial role of this particle in the Ad3 life cycle.
Materials and methods

Cells and virus

HeLa cells were cultured at 37 °C, under 5% CO₂ atmosphere, in EMEM (BioWhittaker), supplemented with 10% fetal calf serum (FCS), penicillin (50 IU/ml), and streptomycin (50 μg/ml). Ad3 stock was purified from a lysate of HeLa cells infected at MOI 1 for 48 h, by double banding on CsCl gradient according to Kanegae et al. (1994). This stock was dialyzed against Tris 20 mM pH 7.4–150 mM NaCl and stored in presence of 20% glycerol at −20 °C.

Pt-Dd expression and detection

Pt-Dd was expressed in the baculovirus system and purified on sucrose density gradient, as previously described (Fender et al., 1997). Pt-Dds were detected by Western blot using anti-Pt-Dd serum (diluted at 1/40,000 in PBS-0.1% Tween-20) and a subsequent incubation with anti-rabbit HRP-conjugated antibody at 1/5000 (Jackson).

Virus titration and Dd quantification

HeLa cells grown in a 25-cm² dish (about 10⁷ cells) were infected with Ad3 at MOI 1. At the indicated time after infection, cells were washed with PBS and lysed by three freeze/thaw cycles in 1 ml hypotonic buffer (Tris 20 mM pH 7.5–50 mM NaCl). The cell lysate was recovered after 5 min centrifugation at 400 × g and one part was used for titration of infectious virus while the other part was used for Dd quantification (summarized in Fig. 3).

Ad3 titration

Titration of Ad3 infectious particles was performed by immunofluorescence. Serial virus dilutions prepared in 250 μl of EMEM without serum were incubated for 1 h with HeLa cells in a 24-multiwell plate (10⁵ cells/well). Then, 750 μl of EMEM containing 10% SVF was added. After different periods post-infection (p.i.), the inoculum was removed, cells were washed, and 750 μl EMEM containing 10% FCS was added. After different periods post-infection (p.i.), the medium was removed, cells were rinsed with PBS and then permeabilized with cold methanol for 10 min. Immunofluorescence detection of penton was performed by incubation of coverslips with anti-Pt-Dd rabbit polyclonal serum at 1/1000 in PBS–0.1% Tween-20 and a subsequent incubation with FITC-labeled goat anti-rabbit antibody (Jackson), diluted 1/250 in the same buffer. Cell nuclei were counterstained with propidium iodide (5 μg/ml). A similar experiment was performed with mouse monoclonal anti-Ad3 hexon antibody (Chemicon) at 1/100 and FITC-labeled anti-mouse antibody. Localization of Dd and adenoviruses in the same cell was performed by using an anti-Bs-Dd rabbit polyclonal serum (recognizing only the penton base) at 1/1000 and an anti-Ad3 hexon antibody at 1/100. Penton base signal was revealed in red by rhodamine-labeled anti-rabbit antibody and hexon signal in green by FITC-labeled antibody. Laser scanning confocal microscopy was performed on MRC600 (Bio-Rad).

Epon inclusion and thin sections of infected cells

HeLa cells grown in a Labtek chamber slide (Nunc) were infected with Ad3 at MOI 1 for 24 h. Cells were fixed with 2.5% glutaraldehyde in 100 mM HEPES buffer pH 7.4, post-fixed with 1% osmium tetroxide and dehydrated with ethanol. Ultrathin sections (80 nm) of infected cells embedded in Epon were performed and collected on carbon-coated grids. Grids were stained with saturated uranyl acetate in 50% ethanol and then with 1 M lead citrate. Observations were made with Philips CM10 microscope at 80 kV.
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References


