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The 100K-Chaperone Protein from Adenovirus Serotype 2 (Subgroup C) Assists in Trimerization and Nuclear Localization of Hexons from Subgroups C and B Adenoviruses

Saw See Hong^{1*†}, Ewa Szolajska^{2,3†}, Guy Schoehn^{4†}
Laure Franqueville¹, Susanna Myhre^{5,6}, Leif Lindholm^{5,6}
Rob W. H. Ruigrok⁴, Pierre Boulanger¹ and Jadwiga Chroboczek^{3*}

¹Laboratoire de Virologie et Pathogénèse Virale, CNRS UMR 5537, Faculté de Médecine RTH Laennec Institut Fédératif de Recherche IFR-62, 69372 Lyon, France

²Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02196 Warsaw, Poland

³Institut de Biologie Structurale 41 rue Jules Horowitz, 38027 Grenoble Cedex 1, France

⁴Laboratoire de Virologie Moléculaire et Structurale FRE 2854 CNRS/UJF, c/o EMBL BP 181, 38042 Grenoble Cedex 9, France

⁵Department of Medical Microbiology and Immunology University of Göteborg, P.O. Box 435, SE 40530 Göteborg Sweden

⁶Got-A-Gene AB, Stena Center 1B, SE 41292 Göteborg, Sweden

*Corresponding authors

Recombinant hexons from subgroup C adenoviruses (Ad2 and Ad5) and from a member of subgroup B (Ad3) adenoviruses have been expressed in insect cells. When expressed alone, all three hexons were found to be insoluble and accumulated as inclusion bodies in the cytoplasm. However, co-expression of recombinant Ad2, Ad5 or Ad3 hexon with Ad2 L4-100K protein resulted in the formation of soluble trimeric hexons. EM analysis of hexons revealed that they were indistinguishable from native hexon capsomers isolated from Ad2-infected human cells, or released from partially disrupted adenovirions. This suggests that 100K acts as a chaperone for hexon folding and self-assembly into capsomer in insect cells. Since 100K protein assists in the trimerization of subgroup C hexon, and of subgroup B hexon protein, it implies that it functions in a manner that is both homo- and heterotypic. During the course of recombinant protein expression, the 100K protein was found in association with hexon monomers and trimers within the cytoplasm. In the nucleus, however, 100K was found in complexes with hexon trimers exclusively. EM observation of purified 100K protein samples showed a dumb-bell-shaped molecule compatible with a monomeric protein. EM analysis of hexon-100K protein complexes showed that interaction of hexon with the 100K protein occurred *via* one of the globular domains of the 100K protein molecule. Our data confirm the role of the 100K protein as a scaffold protein for hexon, and provide evidence suggesting its function in hexon nuclear import in insect cells.

Keywords: adenovirus; 100K protein; hexon; trimerization; nuclear import

Introduction

Adenoviruses (Ad) are medium size, non-

enveloped DNA viruses with an icosahedral capsid. The capsid is made up of a dozen different structural proteins, among which hexon, penton base and fibre represent the most accessible and thus the most antigenic components. The most abundant capsid protein is the hexon, with 240 copies forming the 20 facets of the icosahedron, followed by pentons, complexes of fibre and penton base, located at each of the 12 vertices. Each hexon capsomer is trimeric and the crystallographic structure of Ad2 hexon at 2.9 Å resolution showed the protein to possess a pseudo-hexagonal base and a triangular top.¹ This was confirmed recently by

† S.S.H., E.S. & G.S. contributed equally to the work.

Abbreviations used: Ad, adenovirus; EM, electron microscopy; HF cells, High Five (*Trichoplusia ni*) cells; IF, immunofluorescence; MOI, multiplicity of infection; PCR, polymerase chain reaction; PEI, polyethylenimine; pi, post infection; Sf cells, *Spodoptera frugiperda* cells; TBST, Tris-buffered saline containing 0.05% Tween 20.

E-mailed addresses of the corresponding authors: sawsee.hong@sante.univ-lyon1.fr; wisia@ibs.fr

the determination of the Ad5 hexon structure at 2.5 Å resolution, where the refined structure showed that the monomers are composed of two eight-stranded β -barrels and four extended loops.² These loops, which intertwine in the hexon trimer and are exposed on the virus surface, contain most of the type-specific epitopes,^{3,4} plus a recently identified post-entry neutralizing epitope.⁵

The capsid proteins of viruses quite often have additional functions besides their structural role, and the hexon protein of Ad is no exception. We have shown recently that other than its major role in capsid formation, the hexon also plays a role in Ad entry. Ad virions were able to attach to cellular phospholipids *via* the hexons, thus permitting Ad cell entry independent of primary and secondary receptors.⁶ Furthermore, hexon is the only major structural protein of incoming Ad that docks at the nuclear pore complex and can be, in part, imported into the nucleus, possibly guiding the injected viral DNA.^{7,8} Accordingly, it was shown that the hexon protein enhanced by tenfold the nuclear delivery of polyethylenimine (PEI)/DNA complexes *via* the nuclear pore complex.⁹

The hexon trimer (330–360 kDa) contains three identical polypeptides, the length of which varies slightly for each serotype, 967 amino acid residues for Ad2, 951 residues for Ad5 and 943 residues for Ad3. The assembly of hexon trimers is dependent upon the presence of a viral scaffold protein, the late phase L4-100K non-structural protein,^{10–12} containing 805 residues for both Ad2 and Ad5. It was suggested that the 100K protein is required in stoichiometric amounts to assist in the proper folding and trimerization of hexon, as well as its transfer to the nucleus.^{12,13} A direct association between hexon monomers and the 100K protein was demonstrated by immunoprecipitation with 100K protein-specific antibodies,¹² and 600–800 kDa protein complexes were isolated by gel-filtration chromatography.^{12,14} In addition, a scaffolding role of 100K protein in the intranuclear assembly pathway of Ad virions has been suggested, on the basis of phenotypic analysis of temperature-sensitive mutants of 100K protein.¹⁵

Another property of the 100K protein consists of its interaction with viral mRNA,¹⁶ allowing for the efficient translation of late viral mRNA species.^{17,18} More recently, it has been determined that 100K protein blocks cellular protein synthesis *via* its binding to eIF4G and its inhibition of eIF4G–Mnk1 interaction.^{19,20} In addition, it has been shown that the 100K protein is a substrate for granzyme B, a cellular protease present in granules of cytotoxic lymphocytes.²¹ At the same time, 100K protein inhibits granzyme B proteolytic activity strongly, preventing apoptosis of infected cells targeted by cytotoxic lymphocytes. The considerable half-life of the 100K protein/granzyme B complex (about three hours), and the vast excess of the 100K protein compared to granzyme B in Ad-infected cells undergoing granule-induced cytotoxicity,²¹ ensured the survival of the Ad-infected cells so

that the virus could complete its life-cycle. All these data point to the multiple roles that the 100K protein plays in various phases of the Ad life-cycle. This is confirmed by the phenotype of 100K protein-deleted Ad5 vectors in non-complementing cells, showing a severely decreased capacity to express several late viral genes (including the hexon monomer) and to encapsidate viral genomes.²²

Here, we describe the expression of recombinant Ad2 100K protein, and hexon proteins from Ad2, Ad5, both from subgroup C, and Ad3, a member of subgroup B. We found that the 100K protein acted as a scaffold protein for hexon capsomers in insect cells, and assisted in the trimerization of subgroup C hexon proteins and of hexon of subgroup B. The 100K protein therefore functioned in a manner that was both homotypic and heterotypic. The recombinant hexons produced in insect cells in the presence of 100K were trimeric, and were indistinguishable from hexon capsomers isolated from Ad2-infected human cells. We provided evidence that trimeric hexon was transported to the cell nucleus in the presence of the 100K protein, and independently of any other Ad proteins. Our biochemical data suggested that the 100K protein interacted with both hexon monomers and trimers within the cytoplasm, whereas it interacted predominantly with hexon trimers in the nucleus. The Ad2 100K protein could be observed for the first time under the electron microscope (EM), and was seen as a dumbbell-shaped molecule, with two globular domains linked by a rod-like structure. The size and the volume of the L4-100K protein is compatible with a monomeric form.

Results

Expression and oligomeric status of recombinant hexon proteins from different Ad serotypes in insect cells

The two components of the Ad penton have been produced in insect cells in proper oligomeric forms; namely, trimeric fibres for serotypes 2, 5 and 3,^{23–25} and pentameric penton base for serotypes 2 and 3.^{25,26} In addition, it has been shown that the fibre and penton base were capable of assembly within the insect cell to form either complete pentons or symmetric complexes of 12 pentons called dodecahedra.^{25,26} In our aim to study the assembly of Ad capsids, we cloned and expressed the hexon genes of Ad serotypes 2, 5 and 3 in the baculovirus-insect cells system, alone or in co-expression with the scaffold protein 100K. The sequence of the Ad3 100K protein gene is unknown, but available sequences of various non-structural proteins of Ad2 and Ad3 exhibit a high level of identity. However, when PCR was performed using primers derived from the Ad2 100K protein gene and Ad3 DNA as template, no PCR product could be obtained under the different reaction conditions

tested. Ad2 was therefore the only 100K serotype analyzed in the present study.

We assayed for the solubility of 100K protein and hexon proteins, either individually in single expression, or in co-expression of 100K protein with the hexons. When expressed alone, Ad2 100K accumulated in a soluble form, as most of the recombinant protein was recovered in the supernatant of cell lysis (Figure 1(a)). As expected, anti-100K protein antibody revealed a protein migrating at 100 kDa (major species), and a minor band at approximately 98 kDa in SDS-PAGE and Western blot analysis (Figure 1(a)). This protein band pattern has been observed in Ad2-infected human cells,¹⁵ and likely resulted from some proteolytic cleavage of the primary gene product. By contrast, hexon proteins were found to be completely insoluble in single expressing cells (data not shown). However, the hexon proteins from each of the three Ad serotypes were recovered as soluble proteins from insect cells co-expressing the 100K protein (Figure 1(b)). In SDS-PAGE, heat-denatured protein samples showed bands migrating with the molecular mass expected for Ad2, Ad3 and Ad5 hexon monomers, i.e. 110–120 kDa (Figure 1(b), lanes 100 °C). In non-boiled samples, Ad2, Ad3 and Ad5 hexon bands migrated with apparent molecular mass of >250 kDa, suggesting the

occurrence of a major oligomeric form for all three proteins, consistent with the trimeric nature of native hexon capsomers (Figure 1(b), lanes 4 °C). These results demonstrated that the presence of 100K was indispensable for the proper oligomerization of hexon proteins. This suggested also that the 100K protein could not be substituted for by chaperone protein(s) provided *in trans* either by the recombinant baculovirus vector or the insect cell.

Interestingly, our data showed that the 100K protein from Ad2 (subgroup C) was able to assist in the proper folding of hexons from serotypes belonging to the same subgroup (e.g. Ad5), or to a different one, subgroup B Ad3. Thus, the chaperone function of the 100K protein was apparently not type-specific or subgroup-specific, at least between subgroups C and B. This implied that, even though the nucleotide sequence was not similar enough between Ad2 and Ad3 serotypes to allow for amplification of the Ad3 100K protein gene by PCR using Ad2-derived primers, the 100K protein regions involved in hexon conformation are functionally conserved between the 100K proteins from subgroups B and C Ad.

The oligomeric nature of the recombinant Ad 100K and hexon proteins was also analysed using centrifugation in sucrose-density gradients. The 100K protein sedimented between the 4.1S and 7.6S markers, with its peak slightly above the 6 S marker (Figure 2A), a value for the S coefficient consistent with the value of 6.2 S reported for the 100K protein isolated from Ad2-infected human cells.^{27,28} This suggested a monomeric status for recombinant Ad2 100K protein isolated from insect cells. When lysates from cells co-expressing Ad2 100K protein with Ad2, Ad5 or Ad3 hexon were analysed by sucrose-gradient centrifugation, all three hexon proteins peaked in fractions corresponding to a sedimentation coefficient of 12 S (Figure 2(b)–(d)), a value previously reported for hexon trimers.^{27,29}

Morphological analysis of recombinant hexons and 100K protein

Recombinant hexon was produced in the presence of 100K protein. Hexon from these samples was purified and examined by EM. In most preparations, the hexons showed end-on views with either the top or the bottom of the molecule in contact with the carbon support film and, thus, only the top or the bottom of the molecules outlined by the stain (Figure 3(a)–(c)). The height of the hexons could be measured only on the Ad2 molecules, since only this preparation showed a few side-views (Figure 3(a)). The size of the hexons ($96(\pm 3)$ Å in diameter, $105(\pm 3.5)$ Å in height) was similar to that obtained from the Ad5 hexon X-ray structure.² We made averages of the end-on views of the three hexon preparations and compared these with similar views of the atomic structure of the Ad5 hexon,² filtered to 20 Å

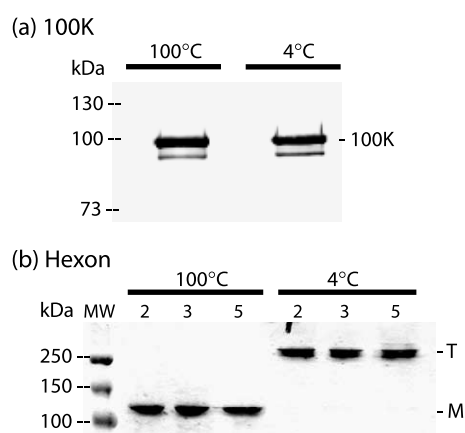


Figure 1. Purified recombinant 100K and hexon proteins. Proteins were analysed by 10% polyacrylamide gel electrophoresis, after heat-denaturation (100 °C, SDS-PAGE) or without heat-denaturation (4 °C, NDS-PAGE). (a) The 100K protein was expressed in insect cells infected with Bac100K2 and purified as described in Materials and Methods. A Western blot was performed with anti-100K rabbit polyclonal antibody. Note the minor band of a 98K product. (b) Coomassie brilliant blue-stained gel of the hexon proteins, which were expressed in insect cells infected with BacHex2 and Bac100K2 (lane 2), or BacHex3/100K2 (lane 3), or BacHex5 and Bac100K2 (lane 5), and purified as described in Materials and Methods. Purified recombinant hexons of Ad2, Ad3 and Ad5 were run in lanes 2, 3 and 5, respectively. Hexon monomers (M) migrated with an apparent molecular mass of 110–120 kDa in heat-denatured samples, and hexon trimers (T) migrated above the 250 kDa marker in the non-boiled samples.

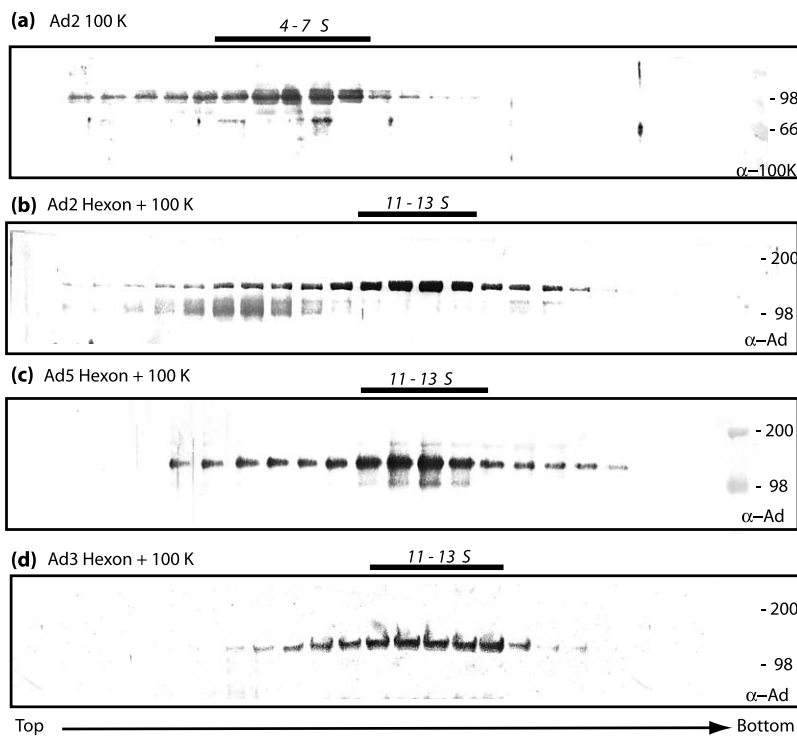


Figure 2. Sucrose gradient ultracentrifugation analysis of 100K and hexon proteins isolated from lysates of single or double recombinant baculovirus-infected cells. (a) Ad2 100K; (b) Ad2 hexon +100K; (c) Ad5 hexon +100K; (d) Ad3 hexon +100K. Gradient fractions were analysed by SDS-PAGE, and immunoblots with anti-100K polyclonal antibody (α -100K) in (a), or with anti-Ad virion antibody in B-D, followed by phosphatase-labelled anti-rabbit IgG conjugate. The position and S range for 100K and hexon proteins are shown on top of each panel. Top and bottom refer to the centrifugation tube, and indicate the direction of sedimentation.

(Figure 3(a)–(c), top insets averaged views, lower insets, similar views of the filtered atomic structure). The Ad2 and Ad3 hexons had associated mainly with their tops to the carbon film (triangular top-views), whereas the Ad5 hexon showed mainly the hexagonal imprints of the bottom half of the molecule. Figure 3(a) also shows the comparison of a single side-view of a hexon with the side-view of the filtered image. All these results clearly show that the recombinant hexons of Ad2, Ad5 and Ad3, synthesized in the presence of 100K protein in insect cells, are trimeric and have the same structural characteristics as the hexons released from spontaneously disrupted virions (Figure 3(d)).

EM observation of 100K protein samples showed a dumbbell-shaped molecule, consisting of two globular domains, about 23 Å in radius, linked by a rod-like structure of 30 Å in length and 30 Å in thickness (Figure 4(a) and (b)). The total length of the molecule was about 120 Å. The volume for each sphere ($51,000 \text{ \AA}^3$) and for the intermediate cylinder ($17,600 \text{ \AA}^3$) gave an estimated volume of $120,000 \text{ \AA}^3$ for the whole structure. Considering the value of 0.84 Da/\AA^3 as the average density for a protein, this volume corresponded to a molecular mass of 103 kDa, compatible with a monomeric status for the native 100K protein. EM analysis of hexon–100K protein complexes present in insect cell extracts from hexon plus 100K protein co-expressing cells (Figure 4(c)) showed that interaction of trimeric hexon with 100K protein occurred *via* one of the globular domains of the 100K protein. These observations are confirmed by the EM analysis performed on proteins purified from the Ad5-infected 293 cells (Figure 4(d) and (e)).

It is worth commenting on technical difficulties concerning EM visualization of the 100K protein. The protein is quite unstable which forced us to use freshly purified preparations each time. In addition, this flat elongated molecule was easily embedded in the EM net and we could finally see it due to the presence of the hexons, which retained the dye very well (meniscus effect).

Time-course of Ad 100K protein and hexon expression

The process of hexon synthesis and trimerization was followed using insect cells infected by recombinant baculovirus after synchronization by cold shock. Cells were harvested at the appropriate times, lysed, fractionated and the cell lysates centrifuged through a sucrose-density gradient. The collected gradient fractions were then analyzed by PAGE under denaturing and non-denaturing conditions, followed by Western blotting (Figure 5). At 15 hours post infection (pi), only a small amount of 100K protein was detected in the top fractions of the gradient (Figure 5(a), panel 100 °C, fractions 2 and 3). At this time-point, the production of hexon (indicated with an asterisk) was low and consisted predominantly of monomeric protein, as indicated by the band pattern under the non-denaturing conditions (Figure 5(b), panel 4 °C, fractions 3 and 4). At 22 hours pi, the 100K protein was visible in all gradient fractions with a significant proportion colocalizing with hexon trimers (compare Figure 5(c) with (d), panel 4 °C). Hexon synthesis was increased significantly and the predominant hexon form was trimeric at this time-point

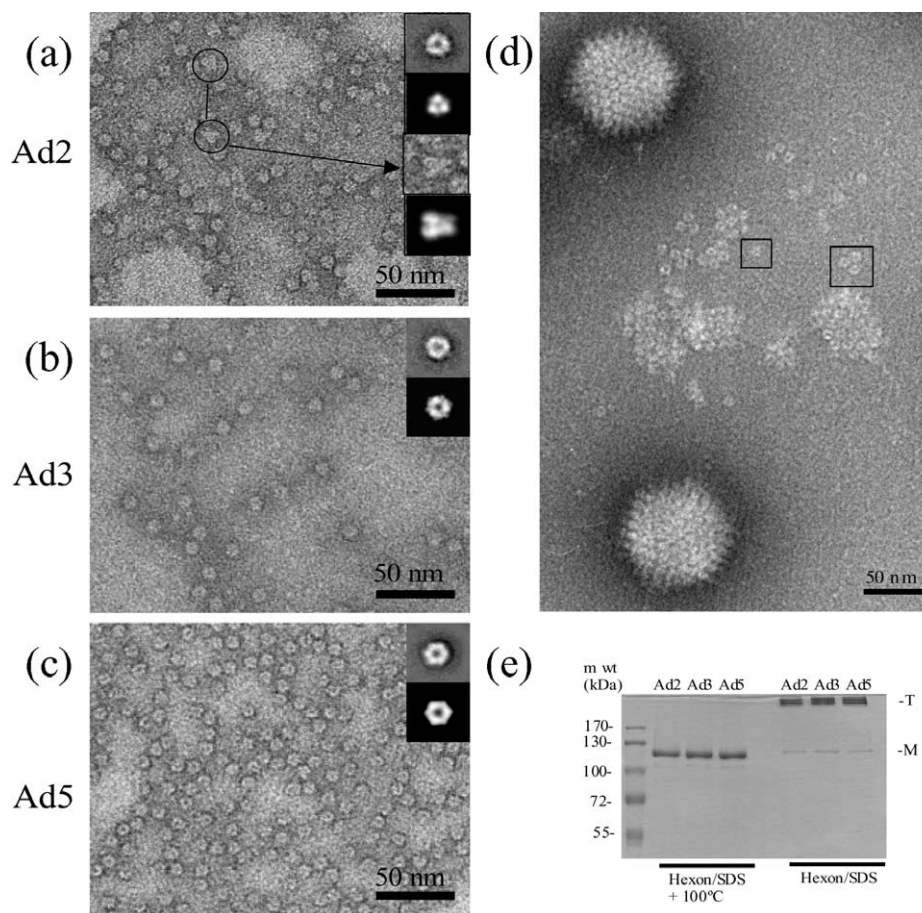


Figure 3. EM analysis of recombinant hexons. (a)–(c) Electron micrographs of Ad2, Ad3 and Ad5 hexon trimers. The proteins bound preferentially with their tops or their bottoms to the carbon support film, but hexons some side-views could be observed for Ad2 (circles in (a)). In each field, as shown on the right, there was an average of 20 EM views of the hexon (upper insets). The averages were also compared with the re-projection of the atomic structure of the Ad5 hexon filtered to 20 Å (lower insets). In (a) the averaged image looks trimeric, implying that the hexon bound preferentially with its apical region to the carbon atom. In order to confirm this, only the apical half of the hexon X-ray structure filtered to 20 Å was re-projected. The re-projection and the average are similar. A comparison between a side-view of the hexon in EM and the re-projected side view is presented. In (b), the averaged image is probably a mixture between top and bottom views. It is similar to the total re-projection of the hexon. In (c), the averaged image looks hexagonal, indicating that most of the 20 averaged hexons were associated with their bottom part to the support film. To confirm this, only the bottom half of the X-ray hexon is re-projected. All the inset squares (average and re-projection) are 22 nm × 22 nm. In (d) EM of Ad2 virions; the isolated and the group of hexons seen in the background (in rectangles) are derived from disrupted virions. The scale bars represent 50 nm. In (e) Coomassie brilliant blue-stained gel of purified hexon proteins used for EM analysis. Hexon monomers (M) are obtained in heat-denatured samples, and hexon trimers (T) in non-boiled samples.

(Figure 5(d), panel 4 °C, fractions 2–4). It is noteworthy that our anti-100K protein antibody did not recognize hexon (compare control hexon lanes Hx in Figure 5(a) and (b) versus (c) and (d)), and our anti-Ad3 antibody used for hexon identification reacted much more strongly with trimeric hexons. So, the bands at the top of the gel in Figure 5(c), panel 4 °C, fractions 2–4, likely represented 100K protein–hexon complexes. Late after infection (48 hours pi), 100K protein was detected in nearly all gradient fractions, visible as a doublet band reminiscent of the pattern observed during protein purification and the large portion of hexon protein was found at the top of the gel, in a position

corresponding to the trimeric form (Figure 5(e) and (f)).

These results suggest that (i) at early times pi hexon molecules were in the monomeric form, but not all were associated with 100K protein, since the hexon signal was predominant in fractions 3 and 4, whereas 100K protein was predominant in fraction 2 (Figure 5(a) and (b)); (ii) midway through the infection, a sizable portion of hexon was in a trimeric form, and both monomeric and trimeric forms seemed to co-localize with 100K protein, suggesting interaction of both forms with 100K; (iii) at late times pi, most hexon protein was apparently trimeric and likely in association with 100K protein.

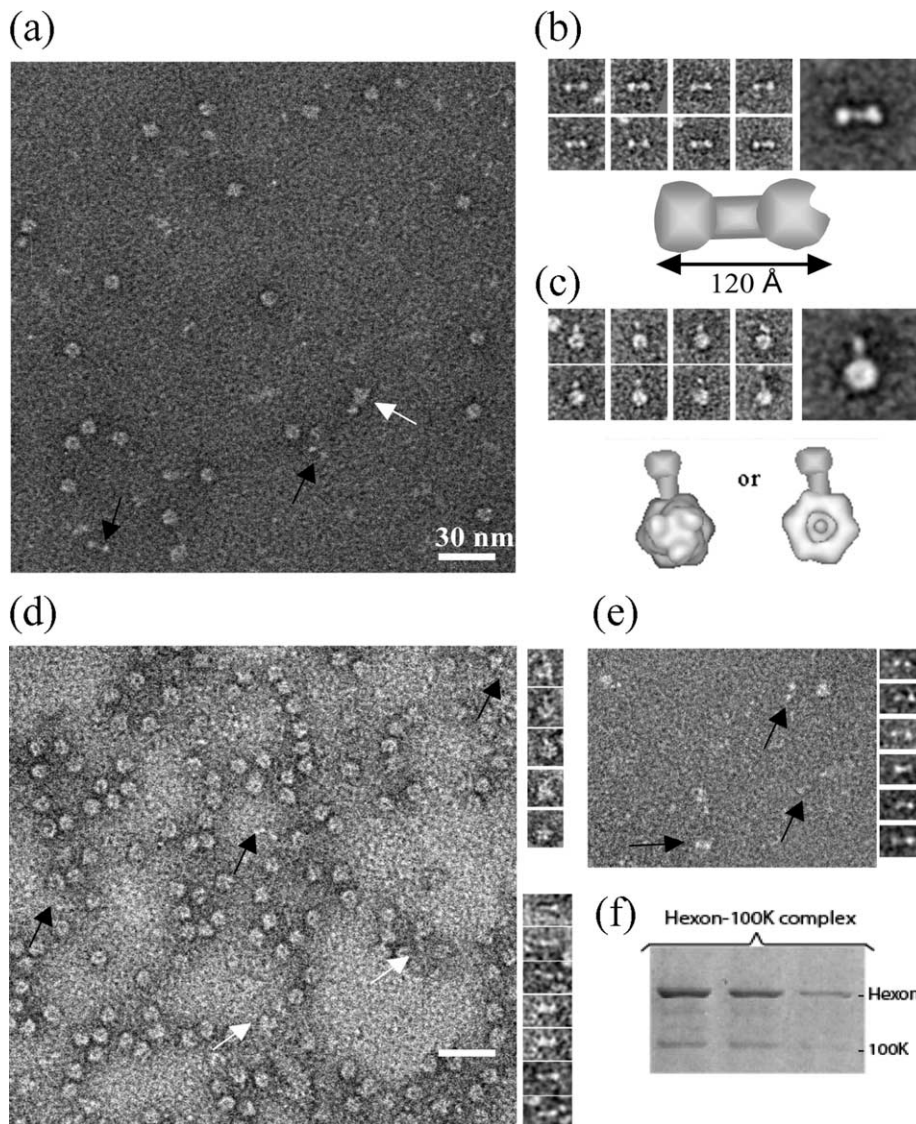


Figure 4. EM analysis of recombinant Ad3 hexon and Ad2 100K. (a) Part of an electron micrograph of a fraction containing both proteins. Most of the visible structures are hexons (about 20 particles that are not marked), but there are also two 100K protein molecules (black arrows) and one hexon-100K protein complex (white arrow). The scale bar represents 30 nm. (b) Gallery of eight images of the 100K protein, the averaged image, and the structural model of the 100K protein (from left to right). Each single image is $310 \text{ \AA} \times 310 \text{ \AA}$ in size. The averaged image shows the 100K molecule composed of two globular domains connected in the middle with a rod. (c) Gallery, the averaged image and the model of the hexon-100K protein complex with the hexon in end-on view (from left to right). The model shows an interpretation of the averaged image with one globular domain of the 100K protein attached either to the distal domain or to the apical domain of the 3D structure of the hexon filtered to 20 \AA . (d) and (e) EM analysis of purified hexon and 100K protein from Ad5-infected 293 cells. The scale bar represents 30 nm. (d) Electron micrograph of a fraction containing the 100K protein plus hexon as seen in the gel in (f). Most of the visible structures are hexons. Some 100K protein molecules and hexon-100K protein complexes are marked with black arrows and white arrows, respectively. The column on the right represents five images of hexon-100K protein complexes and five images of 100K protein molecules (upper and lower columns, respectively). (e) Electron micrograph of fraction containing mainly the 100K protein (black arrows). The column on the right represents six images of the 100K protein, very similar to the recombinant Ad2 100K protein shown in (b). (f) SDS/PAGE gel of salt-gradient fractions of chromatography-purified hexon and 100K proteins from Ad5-infected 293 cells. The densitometry of bands containing denatured proteins suggests the stoichiometry of hexon to 100K as 3:1.

Cytological analysis of hexon and 100K proteins localization in insect cells

The insect cell protein expression system allowed us to study the cellular behaviour of the 100K and

hexon proteins individually and independently of other Ad gene products. Moreover, since hexon and 100K are partner proteins, we used a single baculovirus recombinant, which expressed Ad3 hexon and Ad2 100K simultaneously. Their

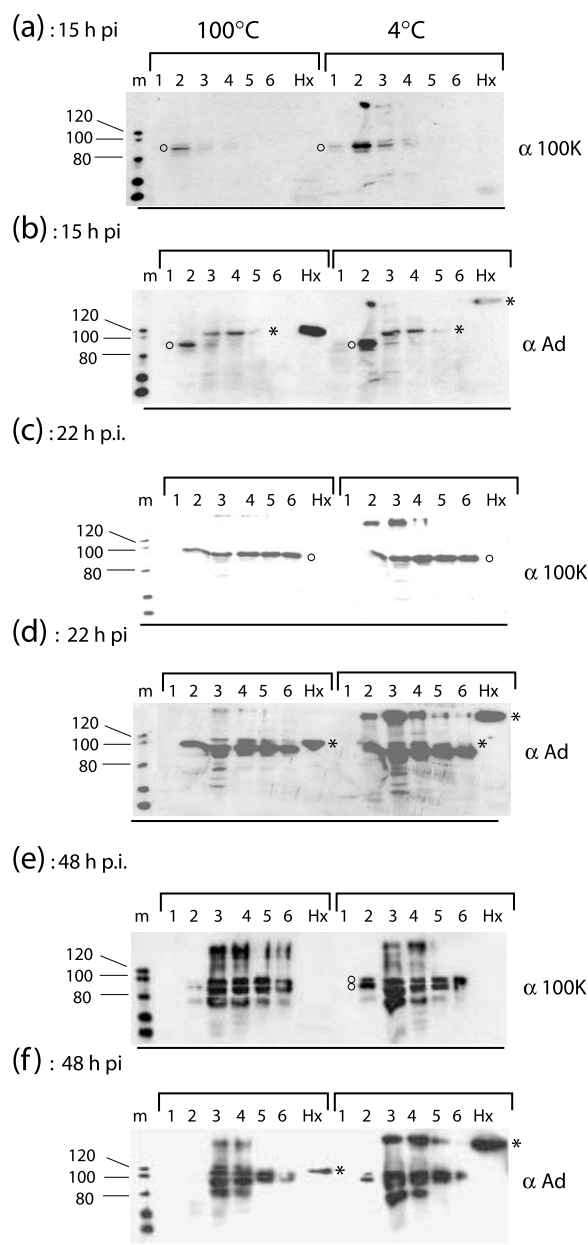


Figure 5. Time-course analysis of Ad3 hexon and Ad2 100K protein co-expression. Synchronized HF cells were infected with double-expressing baculovirus vector BacHex3/100K2 virus at MOI 5, and cells were harvested at different times pi as indicated at the top left of the panels. Cell lysates were fractionated in 2.2 ml sucrose-density gradients, as described in Materials and Methods. Aliquots (20 μ l) of boiled (left-half panels, 100 $^{\circ}$ C) or non-boiled (right-half panels, 4 $^{\circ}$ C) gradient fractions were electrophoresed in SDS/10% polyacrylamide gels and analyzed by Western blot using antibodies specific for the 100K protein (α 100K; (a), (c) and (e)) and for major capsid proteins including hexon (α Ad; (b), (d) and (f)), respectively. Lanes 1 (top of the gradient) to 6 (bottom) show the protein pattern of each gradient fraction. Control lanes show FPLC-purified Ad3 hexon (lane Hx), and molecular mass markers (lane m). The position of the 100K protein is indicated with an open dot, and that of hexon monomers and trimers with asterisks.

expression kinetics and cellular localization were studied by immunofluorescence (IF), using insect cells synchronized by cold shock before infection. It is relevant to note that the recombinant protein-expressing insect cells have quite large nuclei but the cytoplasmic region was discernible owing to the 4',6-diamidino-2-phenylindole nuclear counterstain. The hexon signal (in green) was already detected at 22 hours pi in both cytoplasm and in the nucleus, with increasing nuclear accumulation observed from 36 hours pi (Figure 6(a) and (b)). The cellular localization pattern of the 100K protein was somewhat different from that of hexon. Although 100K protein was localized both in the cytoplasm and in the nucleus throughout the course of infection, it was concentrated in the perinuclear region from the beginning of the observation (Figure 6(a), a, e and i). Co-localization of 100K protein and hexon was observed in the cytoplasm during early and late times of infection, and a significant perinuclear overlap was visible at late times (Figure 6(a), d, h and l). However, when insect cells were infected with only hexon-expressing baculovirus, no detectable hexon signal within the nucleus could be seen, and hexon fluorescence observed in the cytoplasm was in the form of bright speckles, indicative of insoluble protein inclusions (Figure 6(c)). These cells had shapes that were irregular and distorted due to the accumulation of the inclusion bodies.

Time-course analysis of recombinant protein cellular distribution

In order to confirm the IF observations, cell fractionation followed by protein analysis was performed on cold-synchronized cells expressing both hexon and 100K protein, and harvested at different times pi. Hexon monomers were detected early after infection (15 hours pi), but this signal was almost entirely restricted to the cytoplasmic compartment. Only trace amounts of monomeric hexon were found to be associated with the nuclear pellet, which apparently contained mainly hexon trimers (compare Figure 7(a) and (b), rightmost panels 4 $^{\circ}$ C). A faint band of hexon trimers was visible in both the cytoplasm and nuclear fractions from the 15 hours pi samples, and the intensity of this band increased with the time of infection in the two compartments. As the pool of nuclear hexon trimers increased during the course of infection, a higher molecular mass band was apparent at 48 hours pi (Figure 7(b), rightmost panel 4 $^{\circ}$ C), which could represent higher oligomeric structures of hexons.

A significantly different pattern was shown by the 100K protein. Small amounts of 100K protein were observed in both the cytoplasm and the nucleus at 15 hours pi (Figure 7(a) and (b)), and the levels of 100K protein increased in the two compartments until it plateaued at 22 hours pi. There was no significant accumulation of the 100K protein gene primary product at late times pi, as a

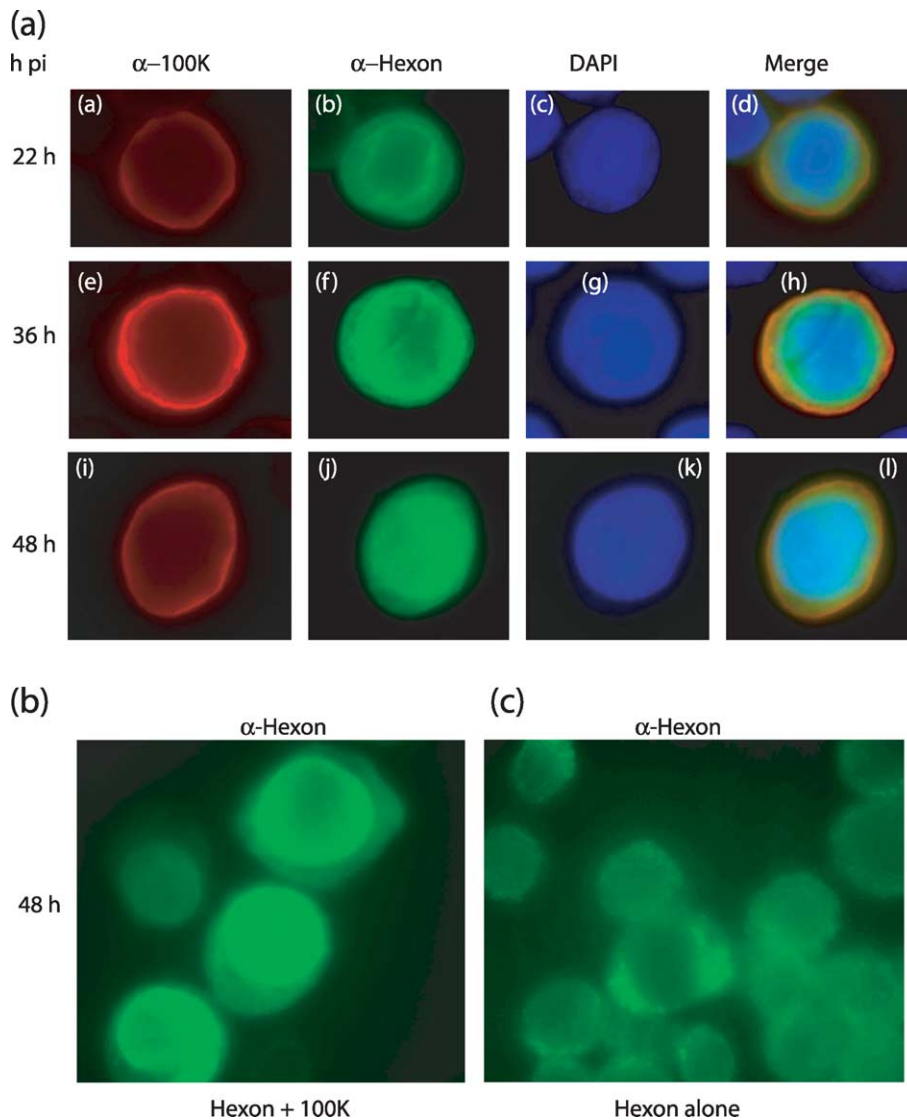


Figure 6. Immunofluorescence microscopy of recombinant hexon- and 100K-expressing cells. (a) Sf9 cells were infected with double-expressing baculovirus vector BacHex3/100K2 virus at MOI 5. The cells were harvested at the indicated times pi (left side of the panels) and processed for IF as described in Materials and Methods. The 100K protein is shown by the red signal (leftmost column a, e and I), the hexon protein by the green signal (b, f and j), and the nuclei are stained in blue by 4',6-diamidino-2-phenylindole (c, g and k). Merging of green and red signals is shown in the rightmost column (d, h and l). (b) Field of hexon-expressing insect cells after 36 hours infection with BacHex3/100K2. (c) Sf9 cells were infected with single-expressing baculovirus vector BacHex2 at MOI 5, then processed as above for IF detection of hexon protein.

major proteolytic processing of the 100K protein occurred in both compartments (Figure 7(a) and (b) leftmost panels 100 °C). Interestingly, some 100K protein was found to be associated with the nucleus as early as 15 hours pi (Figure 7(b), leftmost panel 100 °C, and middle panel 4 °C), and in non-denatured samples this nuclear 100K species appeared to co-migrate with the trimeric form of hexon (Figure 7(b), middle panel 4 °C, band T). In non-denatured cytoplasmic fractions, however, two discrete anti-100K protein antibody-reacting bands were found to co-migrate with the hexon monomers (Figure 7(a), middle panel 4 °C, band M) and trimers (Figure 7(a), middle panel 4 °C, band T). Since there was no evidence for oligomeric 100K

protein species (refer to Figures 1, 2 and 4), this co-migration pattern confirmed the interaction of 100K protein with monomeric and trimeric hexons, as shown in Figure 5.

Taken together, our cytological and biochemical results showed that the L4-100K behaved both as a cytoplasmic and nuclear protein. Moreover, the cellular localization of hexon apparently depended upon its oligomeric state; within the cytoplasm, both hexon monomers and trimers were present, whereas only hexon trimers were found in the nucleus. Our data also implied that the interaction of 100K protein with hexon occurred in both cytoplasmic and nuclear compartments, and that both hexon monomers and trimers were associated

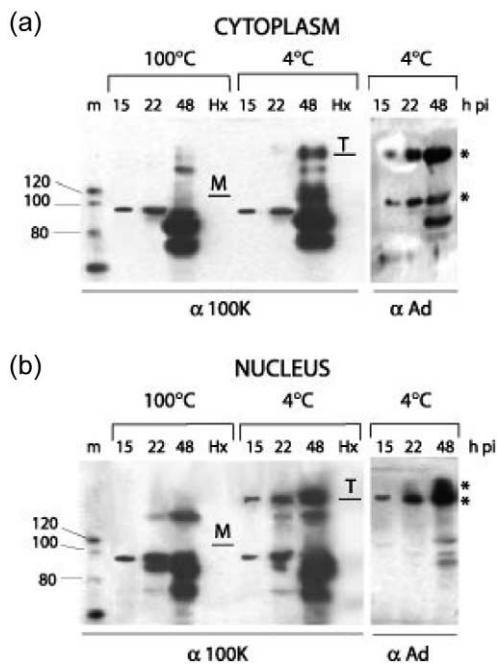


Figure 7. Interaction of hexon with 100K protein in the cytoplasmic and nuclear compartments of baculovirus-infected cells. Synchronized cells (aliquots of 10^7 HF cells) were infected with BacHexon3/100K2 and harvested at 15 hours, 22 hours and 48 hours pi, as indicated on top of each panel. After cell fractionation into (a) cytoplasm and (b) nucleus, proteins were analyzed by SDS-PAGE (100 °C) or NDS-PAGE (4 °C) in 10% polyacrylamide gels followed by Western blot using anti-100K (α 100K) or anti-Ad3 antibody (α Ad). Control lanes: Hx, FPLC-purified Ad3 hexon; m, molecular mass markers. The positions of hexon monomers and trimers in lanes Hx of blots with α -100K are indicated by (M) and (T), respectively. The positions of hexon (monomer or trimer) in blots with anti-Ad3 are indicated with asterisks.

with the 100K protein (Figures 6 and 7). It is noteworthy that hexon synthesis and trimerization were accompanied by a massive proteolysis of the 100K protein (Figure 7).

Discussion

In the present study, Ad2 100K protein and the hexons from subgroup B (Ad3) and C (Ad2, Ad5) were expressed in insect cells using recombinant baculoviruses. When expressed individually, 100K protein accumulated in a soluble, monomeric form, whereas all hexon species were found to be insoluble and to accumulate as cytoplasmic inclusion bodies. Fully soluble, trimeric hexon proteins were recovered from insect cells co-expressing the Ad2 100K protein, demonstrating that a proper folding of hexon proteins into trimers was possible only in the presence of the viral chaperone 100K protein, confirming earlier data on the role of 100K in hexon biogenesis in mammalian

cells.^{10–12,14} Our results obtained with recombinant 100K and hexon proteins expressed in a heterologous eukaryotic system also implied that no early or late adenoviral proteins other than 100K were required to achieve hexon trimerization. Thus, the presence of 100K protein represents the necessary and sufficient condition for hexon trimer formation. EM analysis of negatively stained samples showed that all three recombinant hexons had the morphological characteristics of native hexon capsomers.^{28,30–32}

In addition, our data showed that 100K protein-hexon interaction was not serotype-specific, since Ad2 100K protein could assist in the trimerization of subgroup C hexon proteins and of hexons of subgroup B, acting in a manner that was both homotypic and heterotypic. This suggests that the regions of the 100K protein involved in hexon trimerization are highly conserved between the different serotypes of subgroups B and C. This could have important implications in terms of Ad vector development, in particular in strategies where hexon switching between Ad serotypes is required to overcome pre-existing humoral immunity against the vector. However, hexon switching did not always result in viable chimeric Ad, and this failure was attributed to the lack of compatibility between hexon and 100K proteins of different serotypes.³³ On the basis of our results, it seems that non-viable Ad would more likely result from multiple parameters, including (i) incompatibility between hexon and 100K protein, (ii) illegitimate interactions between hexon and other capsid proteins such as penton base, IIIa or pIX, or (iii) fault in proper folding and solubility of fibre and its assembly with penton base.³⁴ In this context, the baculovirus expression system could be used advantageously as a biological tool for testing the compatibility between the 100K protein of one serotype and hexon proteins from different serotypes, before embarking on the tedious task of the construction of chimeric adenoviruses.

Stable complexes of 100K protein with hexon monomers have been shown to occur in Ad-infected cells.^{12,35,36} However, although the role of 100K protein in hexon trimerization was hypothesized and confirmed by the phenotype of Ad2 carrying temperature-sensitive (*ts*) mutations in the 100K protein gene,¹⁴ the occurrence of trimeric hexon in complex with the 100K protein was not demonstrated previously. Previous data obtained with 100K protein *ts*-mutants maintained at the restrictive temperature showed that hexon accumulated in the cytoplasm, as it failed to be transported to the nucleus.^{37,38} A role of 100K protein in hexon nuclear transport was therefore postulated. This, combined with the inability to detect hexon trimers in the cytoplasm, led to the hypothesis that hexon protein trimerized in the nucleus. In the light of our results, this constitutes a rather unlikely scenario.

Kinetic analysis of protein expression and cellular localization of the two partners, 100K protein and

hexon, demonstrates that 100K protein interacts with hexon monomers during hexon trimerization, and with hexon trimers. Interaction of 100K protein with hexon monomers and trimers occurred in the cytoplasm, whereas in the nucleus this interaction involved only hexon trimers (Figure 7). It might be inferred, therefore, that within the cytoplasm, hexon monomers are assisted by the 100K chaperone protein during their folding into trimers and, subsequently, the 100K protein-hexon trimer complexes are transferred to the nucleus. At a certain step of the virus assembly pathway, the 100K protein, which is apparently not a component of the mature virions,¹⁵ has to dissociate from the virus particles. The trigger for hexon-100K protein complex dissociation is unknown but might involve the proteolytic cleavage of the 100K protein, a phenomenon that we observed at late times pi.

It has been proposed recently that in COS-7 cells the nuclear import of hexons is mediated by the Ad late protein L3-pVI (39). This protein has an important structural role, as in mature virions polypeptide VI apparently interacts with both the inner face of peripentonal hexons,³⁹ and the virion core, thus connecting the apical region with the virion interior.⁴⁰ In the study by Wodrich *et al.*, the nuclear transport of hexon was achieved through co-transfection of pVI and hexon genes, or by means of microinjection of hexon protein in pVI-transfected cells.⁴¹ When hexon was expressed in the presence of a 100K protein, no hexon was observed in the nucleus and hexon localized exclusively within the cytoplasm. Partial nuclear localization of hexon could be observed only when trimeric hexon was injected into the cytoplasm of pVI-transfected cells. These data are in apparent contradiction to previous results.^{7,9} In addition, our data show unambiguously that the expression of 100K protein alone is sufficient to cause both trimerization and nuclear transport of hexon. Some plausible explanations could be put forward to explain the results observed with pVI. First, considering the multiple and versatile strategies used by viruses, it is conceivable that the hexon nuclear import could be mediated by two different partners, 100K protein and pVI, at different stages of the Ad life-cycle. Consistent with this, the synthesis of 100K protein in Ad-infected cells precedes that of pVI by at least four hours.¹⁷ Nevertheless, even at 18 hours pi, at the peak level of the Ad structural proteins synthesis, there seems to be much more 100K protein in the infected cells than pVI (op. cit). Second, COS-7 cells derived from monkey kidney cells might be somehow deficient in the nuclear transport of adenoviral products, as they are deficient in the splicing of adenovirus mRNAs⁴² Finally, at this stage we cannot exclude the possibility that insect cells might transport adenoviral proteins differently from either monkey (COS-7) or human (e.g. HeLa) cells, or that a baculovirus protein might functionally substitute for L3-pVI.

Trotman *et al.* have shown that during the import

of Ad2 DNA at the early step of Ad infection, the viral capsid docks to the nuclear pore complex (NPC) filament protein CAN/Nup241.⁴³ Such NPC-bound Ad2 particles can then bind nuclear mobile histone H1 through Ad2 hexons. This binding probably triggers Ad2 capsid disassembly and permits importin beta and importin 7 (nuclear import factors of H1) to facilitate the translocation of H1-hexon complexes into the nucleus. The interaction of H1 is thought to occur through the lysine-rich tails of H1 and Ad2 hexon surface loops containing 16 acidic residues. However, these residues are absent from the Ad3 hexon and, indeed, Trotman *et al.* show that histone H1 does not bind the Ad3 capsids. It seems unlikely, therefore, that the nuclear translocation of both Ad2/Ad5 (subgroup B), and Ad3 (subgroup C) hexons could be mediated by a similar, histone H1-based mechanism. And so, this mechanism cannot account for the nuclear translocation of all three hexons observed in this study.

For the first time since the discovery of Ad and the morphological identification of its major capsid components about 40 years ago,^{31,32,44,45} the Ad2 viral chaperone 100K protein could be observed under the EM and characterized morphologically. The 100K protein is a nearly symmetrical, dumb-bell-shaped molecule consisting of two globular domains, joined by a rod-like structure (Figure 4). The overall volume of the structure allowed calculation of an estimated molecular mass compatible with that of a monomeric 100K protein, confirming its electrophoretic behaviour under denaturing and non-denaturing conditions (Figure 1). EM observation of 100K protein-hexon trimer complexes suggested that the 100K protein-hexon interaction occurred *via* one of the globular domains of the 100K protein molecule. Further biochemical and morphological analyses of 100K protein deletion mutants will hopefully allow us to map the hexon binding site(s) on the 100K protein.

Materials and Methods

Genes, plasmids, baculoviruses and cells

Hexon genes of Ad2 (2907 bp), Ad3 (2829 bp) and Ad5 (2856 bp) were generated from the appropriate viral genomic DNAs by PCR using the following forward and reverse primers: 5' CCG CCG TGC GCC AGA TCT CCA AGA TGG CTA CC 3' with a BglIII restriction site and 5' GGC AGC TGT TCT AGA TGT TTA TTG CTT CTT 3' carrying an XbaI restriction site for Ad2; 5' CCG GAA TCC ATG GCT ACC CCT TCG 3' with Ec I restriction site and 5' GCG TCT AGA TTA TGT GGT GGC GTT 3' with XbaI restriction site for Ad3; and 5' ACA GCG GCC GCA TGG CTA CCC CTT CGA 3' (NotI) and 5' ACA GGT ACC TTA TGT TGT GGC GTT GCC G 3' (KpnI) for Ad5. The restriction sites are in italics.

The Ad2 hexon gene was inserted into the transfer vector pGmAc115T,²⁶ and the recombinant baculovirus, BacHex2, was obtained by *in vivo* recombination in insect cells between the transfer vector and Bsu36I-digested

DNA from BacPAK6 virus (Clontech). For the Ad5 hexon, the PCR product was cloned directly in TA-cloning vector (Invitrogen, excised with *Ec* RI and inserted into the vector pBacPAK9. The baculovirus BacHex5 was obtained after recombination using BacVector 1000 DNA (Novagen). Both Ad2 and Ad5 hexons were expressed under the control of the polyhedrin promoter. Ad3 hexon was expressed from the double-expression baculovirus obtained as follows. First the Ad2 100K protein gene (2418 bp) was synthesized by PCR using Ad2 genomic DNA as template with the forward primer 5' TCC CCC GGG ATG GAG TCA GTC GAG 3' containing *Sma*I site and reverse primer 5' CCA ATG CAT CTA CGG TTG GGT CGG 3' with an *Nsi*I site. Total untreated PCR products containing A3 hexon and Ad2 100K protein genes were used separately for the intermediate cloning into the pCR-Script SK(+) (Stratagene). The DNA carrying these genes were recovered with QIAquick gel extraction kit (Qiagen) and the compatible ends were created with appropriate restriction enzymes. Then the gene for Ad3 hexon was inserted under the control of the polyhedrin promoter, between *Eco*RI and *Xb* I restriction sites of pFastBacDual vector (Gibco), and the gene for Ad2 100K protein was subsequently inserted under control of the p10 gene promoter, between *Sma*I and *Nsi*I sites. The recombinant baculovirus that expressed simultaneously Ad3 hexon and Ad2 100K protein genes, BacHex3/100K2, was generated using the Bac-to-Bac Baculovirus Expression System (Life Technology). All constructs were verified by DNA sequence analysis. An additional construct of Ad2 100K protein was made with the 100K protein gene inserted in pGmAc115T under the polyhedrin promoter, which produced the baculovirus Bac100K2. The baculoviruses were propagated in Sf9, Sf21 or in HF (High Five or BTI-TN-5B1-4) cells (Invitrogen) maintained in TC-100 medium supplemented with 5% (v/v) fetal calf serum. HF cells in suspension were cultured in Express Five SFM medium (both from Invitrogen). Virus stocks were prepared by infection of Sf21 monolayers at MOI 0.1 with the supernatants obtained after cell transfection with recombinant baculovirus DNA in the presence of CellFectin™ (Gibco). The total cell extracts were analysed for the presence of hexon and 100K proteins by Western blot performed using polyclonal rabbit sera against hexon and 100K protein, used at the respective dilutions of 1:1000 and 1:20,000.

Antibodies, immunological and biochemical analyses

Native hexon protein purified from Ad2-infected HeLa cells was used for production of polyclonal rabbit antibody. Anti-Ad3 and anti-Ad5 rabbit polyclonal antibodies were raised against a mixture of complete and sonication-disrupted Ad3 and Ad5 virus, respectively. Rabbit polyclonal antibody to the 100K protein raised against a full-length recombinant His-tagged protein expressed in *Escherichia coli* was a kind gift from Dr F. Andrade, Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, Mexico). The anti-hexon monoclonal antibody (4C3) against Ad5 was obtained from Professor Willie Russell (University of St Andrews, Scotland). Secondary rhodamine-labelled anti-rabbit and fluorescein isothiocyanate (FITC)-labelled anti-mouse antibodies were from Sigma. Polyacrylamide gel electrophoresis under denaturing (PAGE) and semi-denaturing (NDS-PAGE) conditions and immunoblot analyses have been described in detail.^{23,25,26,34,46} Sedimentation velocity gradient analysis was performed in linear sucrose gradients (5%–27% (w/v) sucrose) as

described.^{27,46} Sedimentation markers, purchased from Sigma, were human carbonic anhydrase (3.3 S), bovine serum albumin (4.1 S), yeast alcohol dehydrogenase (7.6 S) and guinea pig apoferritin (17.7 S).

Production and purification of recombinant proteins

Ad2 and Ad5 hexons

For the production of Ad2 and Ad5 recombinant hexons, Sf9 or Sf21 adherent cells were co-infected with baculoviruses BacHex2 and Bac100K, or BacHex5 and Bac100K, respectively, at a multiplicity of infection (MOI) of 5. At 48 hours pi, the cells were harvested and resuspended in ten volumes of hypotonic TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) containing protease inhibitors, then subjected to five strokes in a Dounce homogenizer. The cell lysate was then clarified by centrifugation at table top Eppendorf centrifuge at 10,000 rpm for ten minutes, and the supernatant was dialysed against buffer A (50 mM phosphate buffer (pH 6.8), 5 mM DTT). The hexons were purified as described.⁴⁷ Briefly, hexons were first eluted at 550 mM NaCl from a DEAE-Sepharose column developed with a linear gradient of NaCl in buffer A. Pooled fractions containing hexon were dialysed against buffer A and applied to a hydroxyapatite column equilibrated with buffer A. The proteins were fractionated with the phosphate gradient made in buffer A and hexon was recovered at 180 mM phosphate. The proteins were then analysed by SDS-PAGE and Western blotting.

Ad3 hexon

HF cells were infected with BacHex3/100K2 baculovirus at MOI 5 and cultured in suspension. Three days later cells were collected and suspended in 20 mM Tris (pH 6.5), 1 mM EDTA containing protease inhibitors, and lysed by three cycles of freezing and thawing. The soluble fraction recovered after 30 minutes of centrifugation at 13,000 rpm was subjected to ion-exchange chromatography on S2-Sepharose equilibrated in 40 mM Mes buffer (pH 6.5). Under these conditions, Ad3 hexon did not bind to the resin and was recovered in the flow-through fractions. It was further purified on Q-Sepharose column using a gradient of NaCl in 40 mM Mes (pH 6.5). Fractions containing hexon were pooled and dialysed against 20 mM Tris (pH 7.5), 2 mM EDTA. The hexon-containing pool was applied to a Q-Sepharose column equilibrated in the dialysis buffer, and proteins eluted using a gradient of NaCl. Hexon was recovered at 400 mM NaCl. The co-purifying *Spodoptera frugiperda* chitinase (identified by N-terminal amino acid analysis) was removed on a chitin affinity column using the same buffer. Protein fractions after ion-exchange chromatography were analysed in SDS/10% or 12% polyacrylamide gels, followed by Western blotting, or stained with Coomassie brilliant blue R250.

Ad2 100K protein

HF cells were infected with BacHex3/100K2 baculovirus at MOI 5 and cultured in suspension. Cells were collected at 48 hours pi, resuspended in 20 mM Mes (pH 6.5), 20 mM NaCl, 1 mM EDTA containing protease inhibitors and lysed by three cycles of freezing and thawing. The soluble fraction recovered after 30 minutes of centrifugation at 13,000 rpm was subjected to ion-exchange

chromatography on S2-Sepharose equilibrated in 40 mM Mes buffer (pH 6.5). Under these conditions, Ad3 hexon and Ad2 100K did not bind to the resin and were recovered in the flow-through fraction. This fraction was dialysed against 20 mM Tris (pH 7.5), 20 mM NaCl, 2 mM EDTA and applied to a Q-Sepharose column equilibrated in the same buffer and developed with a gradient of NaCl. Fractions containing hexon trimers, 100K protein-hexon trimer complexes and 100K protein (200 mM–450 mM NaCl) were pooled, precipitated with ammonium sulphate at 45% saturation for two hours at 4 °C, and centrifuged at 13,000 rpm for 15 minutes. The resulting pellet was dissolved in 50 mM sodium phosphate buffer (pH 7), 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, and applied to a Superdex 200 column (Amersham Pharmacia, 1 cm×30 cm) equilibrated with the same buffer. Peak fractions of eluted proteins were pooled and analysed under the EM.

Electron microscopy analysis of recombinant hexon and 100K

Ad3 hexon and Ad2 100K proteins were isolated from insect cells infected with BacHex3/100K2. Samples were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 1% (w/v) sodium silicotungstate (pH 7.5) or 1% (w/v) ammonium molybdate (pH 7.5) (Ad2). A grid was placed on top of the carbon film, which was subsequently air-dried. Micrographs were taken under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV and a calibrated magnification of 39,750× (based on the helical pitch of tobacco mosaic virus). Selected negatives were then digitized on a Zeiss scanner (Photoscan TD) with a pixel size of 14 µm (3.5 Å at the sample scale). The atomic structure of Ad5 hexon² was filtered to 20 Å resolution and re-projected in a top view and a side view using SPIDER,⁴⁸ which was used also to create averaged top or bottom views of single hexons, 100K protein molecules and of 100K protein-hexon complexes.

Immunofluorescence (IF) microscopy

Insect cells infected with recombinant baculovirus expressing Ad3 hexons and Ad2 100K protein were fixed with 2% (v/v) paraformaldehyde in PBS, and allowed to attach to glass coverslips. The cells were then permeabilized with 0.1% (v/v) Triton X-100 in PBS, blocked with 1% (w/v) bovine serum albumin in PBS for 30 minutes at room temperature and incubated for one hour with either anti-100K protein rabbit polyclonal or anti-hexon monoclonal (mAb 4C1) antibodies at dilutions of 1:15,000 and 1:1000 in PBS, respectively. Subsequently, the coverslips were incubated with the secondary, rhodamine-labelled anti-rabbit antibody (Sigma) or FITC-labelled anti-mouse antibody (Sigma) for one hour at room temperature. The coverslips were then mounted on glass slides for observation under an inverted UV microscope (Zeiss Axiophot).

Kinetics of recombinant protein expression in insect cells

HF cells were infected with BacHexon3/100K2 at MOI 5. Infection was synchronized by incubation of cells with the virus inoculum for 90 minutes at 4 °C. The cells were then cultured at 27 °C in suspension and collected at different times pi. Collected cells were suspended in 20 mM Tris

(pH 8), 1 mM EDTA containing protease inhibitors and lysed by three cycles of freezing and thawing. For each time-point, the lysate derived from 3×10⁶ baculovirus-infected cells was applied onto a 15–40% (w/v) sucrose-density gradient (2.2 ml) made in 10 mM Tris (pH 7.5), 10% glycerol; 150 mM NaCl; 2 mM EDTA, and centrifuged for four hours in a Beckman TLS 55 rotor at 55,000 rpm. Fractions of 320 µl were collected from the top and aliquots of fractions were analysed by Western blot with anti-100K protein serum followed by anti-Ad3 serum.

Cell fractionation

HF cells were infected with BacHexon3/100K2, cultured as described above and collected at different times pi by centrifugation at 2000 rpm for ten minutes. Cell pellets were washed twice with PBS, and cell fractionation performed as described.⁴⁹ Nuclei were separated from the soluble fraction by centrifugation through a 1 ml sucrose cushion (30% (w/v) sucrose in hypotonic buffer), and lysed in 80 µl of hypotonic buffer.⁴⁹

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References

1. Athappilly, F. K., Murali, R., Rux, J. J., Cai, Z. & Burnett, R. M. (1994). The refined crystal structure of hexon, the major coat protein of adenovirus type 2, at 2.9 Å resolution. *J. Mol. Biol.* **242**, 430–455.
2. Rux, J. J. & Burnett, R. M. (2000). Type-specific epitope locations revealed by X-ray crystallographic study of adenovirus type 5 hexon. *Mol. Ther.* **1**, 18–30.
3. Toogood, C. I., Murali, R., Burnett, R. M. & Hay, R. T. (1989). The adenovirus type 40 hexon: sequence, predicted structure and relationship to other adenovirus hexons. *J. Gen. Virol.* **70**, 3203–3214.
4. Toogood, C. I., Crompton, J. & Hay, R. T. (1992). Antipeptide antisera define neutralizing epitopes on the adenovirus hexon. *J. Gen. Virol.* **73**, 1429–1435.
5. Varghese, R., Mikyas, Y., Stewart, P. L. & Ralston, R. (2004). Postentry neutralization of adenovirus type 5 by an antihexon antibody. *J. Virol.* **78**, 12320–12332.
6. Balakireva, L., Schoehn, G., Thouvenin, E. &

- Chroboczek, J. (2003). Binding of adenovirus capsid to dipalmitoyl phosphatidylcholine provides a novel pathway for virus entry. *J. Virol.* **77**, 4858–4866.
7. Saphire, A. C., Guan, T., Schirmer, E. C., Nemerow, G. R. & Gerace, L. (2000). Nuclear import of adenovirus DNA *in vitro* involves the nuclear protein import pathway and hsc70. *J. Biol. Chem.* **275**, 4298–4304.
 8. Greber, U. F., Willetts, M., Webster, P. & Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, **75**, 477–486.
 9. Carlisle, R. C., Bettinger, T., Ogris, M., Hale, S., Mautner, V. & Seymour, L. W. (2001). Adenovirus hexon protein enhances nuclear delivery and increases transgene expression of polyethylenimine/plasmid DNA vectors. *Mol. Ther.* **4**, 473–483.
 10. Leibowitz, J. & Horwitz, M. S. (1975). Synthesis and assembly of adenovirus peptide III, reversible inhibition of hexon assembly in adenovirus type 5 temperature-sensitive mutants. *Virology*, **66**, 10–24.
 11. Oosterom-Dragon, E. A. & Ginsberg, H. S. (1980). Purification and preliminary immunological characterization of the type 5 adenovirus, nonstructural 100,000-dalton protein. *J. Virol.* **33**, 1203–1207.
 12. Cepko, C. L. & Sharp, P. A. (1982). Assembly of adenovirus major capsid protein is mediated by a nonvirion protein. *Cell*, **31**, 407–415.
 13. Philipson, L. (1984). Structure and assembly of adenoviruses. *Curr. Top. Microbiol. Immunol.* **109**, 1–52.
 14. Morin, N. & Boulanger, P. (1984). Morphogenesis of human adenovirus type 2, sequence of entry of proteins into previral and viral particles. *Virology*, **136**, 153–167.
 15. Morin, N. & Boulanger, P. (1986). Hexon trimerization occurring in an assembly-defective, 100K temperature-sensitive mutant of adenovirus 2. *Virology*, **152**, 11–31.
 16. Tasserou-De Jong, J. G., Brouwer, J., Rietveld, K., Zoetemelk, E. M. & Bosch, L. (1979). Messenger ribonucleoprotein complexes in human KB cells infected with adenovirus type 5 contain tightly bound viral-coded 100K proteins. *Eur. J. Biochem.* **100**, 271–283.
 17. Hayes, B. W., Telling, G. C., Myat, M. M., Williams, J. F. & Flint, S. J. (1990). The adenovirus L4 100-kilodalton protein is necessary for efficient translation of viral late mRNA species. *J. Virol.* **64**, 2732–2734.
 18. Riley, D. & Flint, S. J. (1993). RNA-binding properties of a translational activator, the adenovirus L4 100-kilodalton protein. *J. Virol.* **67**, 3586–3595.
 19. Cuesta, R., Xi, Q. & Schneider, R. J. (2000). Adenovirus specific translation by selective disassembly of cap-initiation complex. *EMBO J.* **19**, 3465–3474.
 20. Cuesta, R., Xi, Q. & Schneider, R. J. (2004). Structural basis for competitive inhibition of eIF4G-Mnk1 interaction by the adenovirus 100-Kilodalton protein. *J. Virol.* **78**, 7707–7716.
 21. Andrade, F., Bull, H. G., Thornberry, N. A., Ketner, G. W., Casciola-Rosen, L. A. & Rosen, A. (2001). Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. *Immunity*, **14**, 751–761.
 22. Hodges, B. L., Evans, H. K., Everett, R. S., Ding, E. Y., Serra, D. & Amalfitano, A. (2001). Adenovirus vectors with the 100K gene deleted and their potential for multiple gene therapy applications. *J. Virol.* **75**, 5913–5920.
 23. Novelli, A. & Boulanger, P. (1991). Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fibre. *Virology*, **185**, 365–376.
 24. Santis, G., Legrand, V., Hong, S. S., Davison, E., Kirby, I., Imler, J.-L. *et al.* (1999). Molecular determinants of adenovirus serotype 5 fibre binding to its cellular receptor CAR. *J. Gen. Virol.* **80**, 1519–1527.
 25. Fender, P., Ruigrok, R. W., Gout, E., Buffet, S. & Chroboczek, J. (1997). Adenovirus dodecahedron, a new vector for human gene transfer. *Nature Biotechnol.* **15**, 52–56.
 26. Karayan, L., Gay, B., Gerfaux, J. & Boulanger, P. A. (1994). Oligomerisation of recombinant penton base of adenovirus type 2 and its assembly with fibre in baculovirus-infected cells. *Virology*, **202**, 782–795.
 27. Lemay, P. & Boulanger, P. (1980). Physicochemical characteristics of structural and non-structural proteins of human adenovirus 2. *Ann. Virol. (Inst. Past.)*, **131E**, 259–275.
 28. Pettersson, U. (1984). Structural and nonstructural adenovirus proteins. In *Adenoviruses* (Ginsberg, H. S., ed.), pp. 205–270, Plenum Press, New York and London.
 29. Velicer, L. F. & Ginsberg, H. S. (1970). Synthesis, transport, and morphogenesis of type 5 adenovirus capsid proteins. *J. Virol.* **5**, 338–352.
 30. Pettersson, U., Philipson, L. & Höglund, S. (1967). Structural proteins of adenoviruses I. Purification and characterization of adenovirus type 2 hexon antigen. *Virology*, **33**, 575–590.
 31. Valentine, R. C. & Pereira, H. G. (1965). Antigens and the structure of the adenovirus. *J. Mol. Biol.* **13**, 13–20.
 32. Wilcox, W. C. & Ginsberg, H. S. (1963). The structure of type 5 adenovirus. I. Antigenic relationship of virus structural proteins to virus specific soluble antigens from infected cells. *J. Expt. Med.* **118**, 295–306.
 33. Wu, H., Dmitriev, I., Kashentseva, E., Seki, T., Wang, M. & Curiel, D. T. (2002). Construction and characterization of adenovirus serotype 5 packaged by serotype 3 hexon. *J. Virol.* **76**, 12775–12782.
 34. Magnusson, M. K., Hong, S. S., Henning, P., Boulanger, P. & Lindholm, L. (2002). Genetic retargeting of adenovirus vectors, functionality of targeting ligands and their influence on virus viability. *J. Gene Med.* **4**, 356–370.
 35. Cepko, C. L. & Sharp, P. A. (1983). Analysis of Ad5 hexon and 100K ts mutants using conformation specific monoclonal antibodies. *Virology*, **129**, 137–154.
 36. Gambke, C. & Deppert, W. (1983). Specific complex of the late nonstructural 100,000-dalton protein with newly synthesized hexon in adenovirus type 2-infected cells. *Virology*, **124**, 1–12.
 37. Russel, W. C., Newman, C. & Williams, J. F. (1972). Characterization of temperature-sensitive mutants of adenovirus type 5-Serology. *J. Gen. Virol.* **17**, 265–279.
 38. Stinski, M. F. & Ginsberg, H. S. (1974). Antibody to the type 5 adenovirus hexon polypeptide, detection of nascent polypeptides in the cytoplasm of infected KB cells. *Intervirology*, **4**, 226–236.
 39. Stewart, P. L., Fuller, S. D. & Burnett, R. M. (1993). Difference imaging of adenovirus, bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.* **12**, 2589–2599.
 40. Russell, W. C. (1982). Precious B Nucleic acid-binding properties of adenovirus structural polypeptides. *J. Gen. Virol.* **63**, 69–79.
 41. Wodrich, H., Guan, T., Cingolani, G., Von Seggern, D.,

- Nemerow, G. & Gerace, L. (2003). Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *EMBO J.* **22**, 6245–6255.
42. Klessig, D. F. & Chow, L. T. (1980). Incomplete splicing and deficient accumulation of the fibre messenger RNA in monkey cells infected by human adenovirus type 2. *J. Mol. Biol.* **139**, 221–242.
43. Trotman, L. C., Mosberger, N., Fornerod, M., Stidwill, R. P. & Greber, U. F. (2001). Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nature Cell Biol.* **3**, 1092–1100.
44. Ginsberg, H. S., Pereira, H. G., Valentine, R. C. & Wilcox, W. C. (1966). A proposed terminology for the adenovirus antigens and virion morphological subunits. *Virology*, **28**, 782–783.
45. Norrby, E. (1969). The structure and functional diversity of adenovirus capsid components. *J. Gen. Virol.* **5**, 221–236.
46. Karayan, L., Hong, S. S., Gay, B., Tournier, J., d'Angeac, A. D. & Boulanger, P. (1997). Structural and functional determinants in adenovirus type 2 penton base recombinant protein. *J. Virol.* **71**, 8678–8689.
47. Boulanger, P., Yoshinaga, S. & Berk, A. J. (1987). DNA-binding properties and characterization of human transcription factor TFIIC2. *J. Biol. Chem.* **262**, 15098–15105.
48. Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, M. & Leith, A. (1996). SPIDER and WEB, processing and visualisation of images in 3D electron microscopy and related fields. *J. Struct. Biol.* **116**, 190–199.
49. Xu, B., Yoo, S. & Guarino, L. A. (1995). Differential transcription of baculovirus late and very late promoters: fractionation of nuclear extracts by phosphocellulose chromatography. *J. Virol.* **69**, 2912–2917.

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