Adenovirus (AdV) is thought to follow a sequential assembly pathway similar to that observed in dsDNA bacteriophages and herpesviruses. First, empty capsids are assembled, and then the genome is packaged through a ring-like structure, referred to as a portal, located at a unique vertex. In human AdV serotype 5 (HAdV5), the IVa2 protein initiates specific recognition of viral genome by associating with the viral packaging domain located between nucleotides 220 and 400 of the genome. IVa2 is located at a unique vertex on mature capsids and plays an essential role during genome packaging, most likely by acting as a DNA packaging ATPase. In this study, we demonstrated interactions among IVa2, 33K and DNA-binding protein (DBP) in virus-infected cells by in vivo cross-linking of HAdV5-infected cells followed by Western blot, and co-immunoprecipitation of IVa2, 33K and DBP from nuclear extracts of HAdV5-infected cells. Confocal microscopy demonstrated co-localization of IVa2, 33K and DBP in virus-infected cells and also in cells transfected with IVa2, 33K and DBP genes. Immunogold electron microscopy of purified HAdV5 showed the presence of IVa2, 33K or DBP at a single site on the virus particles. Our results provide indirect evidence that IVa2, 33K and DBP may form a complex at a unique vertex on viral capsids and cooperate in genome packaging.
mutant results in the accumulation of empty capsids, indicating that the genome packaging was blocked (Ostapchuk et al., 2011); and (5) IVa2 is located at a unique vertex of mature virions (Christensen et al., 2008).

Since IVa2 is considered the putative DNA packaging ATPase, it is anticipated to interact with other unknown components of the DNA packaging complex and proteins involved in capsid assembly. Here, we describe the interactions between IVa2, L4 33K and E2 DNA-binding protein (DBP), and show that these proteins are located at a single site on viral capsids. Our results suggest that IVa2, 33K and DBP form a complex at the unique vertex during the genome packaging.

RESULTS

IVa2 associates with viral and cellular proteins

In order to identify the viral and cellular proteins that interact with IVa2, we employed in vivo cross-linking of protein complexes followed by Western blot. Mock or HAdV5-infected 293-IVa2 cells were harvested at 24 h post-infection and treated with paraformaldehyde to stabilize protein–protein interactions. The 293-IVa2 cells were used in order to differentiate between the viral and cellular interacting partners of IVa2. The cross-linked (or non-cross-linked) cell lysates were processed for Western blot using anti-IVa2 antibody. A single IVa2 band was detected in the non-cross-linked cell lysates (Fig. 1). Supershifted bands were detected in the lysate from cross-linked HAdV5-infected cells indicating an association of IVa2 with viral or virus-induced cellular proteins. The supershifted bands disappeared when the cross-links were reversed prior to processing the sample for Western blot (data not shown). Three distinct bands (Band 1, Band 2 and Band 3) of higher molecular mass than that of IVa2 were consistently detected in repeated experiments. A smear-like pattern was detected above 150 kDa. Mass spectrometric analyses followed by peptide sequencing of Band 1, Band 2 and Band 3 revealed the presence of both the cellular and viral proteins (Table 1). Cross-linking of uninfected 293-IVa2 cells did not result in any supershifted bands; interactions of IVa2 with cellular proteins or the non-stabilization of those interactions in our experimental conditions may have been below detection levels.

Proteins that were detected in the supershifted bands could be classified into two groups: (1) those with a molecular mass similar to that of the corresponding band and (2) those with a molecular mass smaller than that of the corresponding band. Predictably, the proteins in the second group were more likely to be associated with IVa2. Mass spectrometry followed by peptide sequencing of Bands 1, 2 and 3 indicated the presence of IVa2, 33K and DBP, IVa2 and DBP, and IVa2 and 33K, respectively, implying that these three proteins interact with each other and may coexist as a single complex. Two cellular proteins, beta actin-like 2 (42 kDa) and histone cluster 1 (14 kDa) were also found in Band 1. The focus of this article will be on the interactions of IVa2 with the AdV proteins, 33K and DBP.

IVa2, 33K and DBP co-immunoprecipitate with an anti-IVa2, anti-33K or anti-DBP antibody

Results of in vivo cross-linking indicated that both 33K and DBP interact with IVa2. Since these interactions were detected in cross-linked cell lysates, it cannot be ruled out

<table>
<thead>
<tr>
<th>Band</th>
<th>Constituent proteins</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>IVa2, 33K, DBP, 100K</td>
<td>Viral</td>
</tr>
<tr>
<td></td>
<td>Beta actin-like 2, Histone cluster 1, Elongation factor 2</td>
<td>Cellular</td>
</tr>
<tr>
<td></td>
<td>HSP90, Proteasome 26S subunit</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IVa2, DBP, 100K</td>
<td>Viral</td>
</tr>
<tr>
<td></td>
<td>Human IMP-1, Human nucleolin</td>
<td>Cellular</td>
</tr>
<tr>
<td>3</td>
<td>IVa2, 33K</td>
<td>Viral</td>
</tr>
<tr>
<td></td>
<td>HSP70, Transketolase, Human IMP-1</td>
<td>Cellular</td>
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that the proteins identified in Band 1, Band 2 and Band 3 may have migrated to their respective positions by virtue of their association with some other viral and/or cellular protein(s), rather than IVa2. To rule out this possibility, nuclear extracts of HAdV5-infected 293 cells were immunoprecipitated with anti-IVa2, anti-33K, anti-DBP, normal rabbit IgG or normal mice IgG. The immunoprecipitated complexes and untreated nuclear extracts were analysed by Western blots using anti-IVa2 (Fig. 2a), anti-33K (Fig. 2b), anti-DBP (Fig. 2c) and anti-fibre (Fig. 2d) antibodies. Western blots with anti-IVa2, anti-33K or anti-DBP antibody detected IVa2, 33K or DBP, respectively, in samples immunoprecipitated with anti-IVa2, anti-33K or anti-DBP antibody. Since anti-IVa2, anti-33K and anti-DBP antibodies do not cross-react (data not shown) and none of the three proteins were immunoprecipitated using normal rabbit or mice IgG, co-immunoprecipitation of IVa2, 33K and DBP with either an anti-IVa2, anti-33K or anti-DBP antibody strongly suggests that IVa2, 33K and DBP interact with each other in virus-infected cells. Additionally, the fibre protein, which is expressed at high levels in virus-infected cells and is not known to interact with IVa2, 33K or DBP, could not be detected in immunoprecipitated samples (Fig. 2d) implying specificity of the co-immunoprecipitation.

**In vivo co-localization of IVa2, 33K and DBP**

The in vivo cross-linking and immunoprecipitation experiments were performed using virus-infected cells; and therefore, do not rule out the role of a virus-specific factor in the interactions among IVa2, 33K and DBP. In fact, the IVa2 is known to associate with the viral packaging domain (Ostapchuk et al., 2005; Perez-Romero et al., 2005), and DBP is known to bind to both single and double-stranded viral DNA during virus replication (Stuiver & van der Vliet, 1990). To determine whether interactions of IVa2, 33K and DBP could occur in the absence of other AdV protein(s) or the viral genome, co-localization of IVa2, 33K and DBP expressed as IVa2-EGFP, 33K-EBFP and DBP-mCherry fusion proteins was studied by confocal microscopy.

For studying co-localization of IVa2, 33K and DBP, 293 cells were transfected with plasmids pIVa2-EGFP, p33K-EBFP and pDBP-mCherry in various combinations, and the transfected cells were analysed by confocal microscopy. Cells expressing IVa2-EGFP and 33K-EBFP (Fig. 3a–d) showed a similar pattern of distribution of intranuclear green and blue fluorescence, respectively. The fluorescence changed to cyan with the merging of the images from the green and blue channels. Similarly, cells expressing IVa2-EGFP and DBP-mCherry (Fig. 3e–h), or 33K-EBFP and DBP-mCherry (Fig. 3i–l) also showed a comparable pattern of intranuclear distribution. Merging of the images from the green and blue channels resulted in yellow fluorescence and the merging of the images from the red and blue channels resulted in purple fluorescence. Images of the cells expressing all three proteins (Fig. 3m–p) revealed granular structures of green, red and blue fluorescence with identical intranuclear distribution. The granular structures exhibited white fluorescence upon merging of the images from the green, red and blue channels. These results suggest that IVa2, 33K and DBP co-localize even in the absence of the viral genome or viral packaging domain. However, transient expression of the proteins as fusions in the absence of other viral factors may not mimic the context in which their interactions occur. In
order to determine whether the three proteins co-localize in virus-infected cells, HAdV5 infected 293 cells were stained with anti-IVa2, anti-33K and/or anti-DBP antibody at 18 DBP forms large intranuclear inclusion bodies that are known to be viral DNA replication and transcription centres (Bosher et al., 1992; Puvion-Dutilleul & Puvion, 1990). A similar distribution of DBP in virus-infected cells was observed (Fig. 4). Immunostaining with various combinations of two or all three antibodies demonstrated co-localization of IVa2, 33K and/or DBP as granular structures. Co-localization of DBP and 33K was found to be extensive. However, anti-IVa2 resulted in more diffused staining even though the staining was more intense in the regions stained by anti-33K and anti-DBP. Earlier studies have also reported diffused distribution of IVa2 (Lutz et al., 1996; Winter & D’Halluin, 1991). In addition, IVa2 formed speckled granular nuclear structures in the background of more diffused nucleoplasmic staining during infection (Lutz et al., 1996).

**Presence of IVa2, 33K or DBP at a single site on the AdV virion**

IVa2, the putative packaging ATPase, has been shown to be located at a single site on the virion (Christensen et al., 2008). Studies in bacteriophages and herpesviruses have shown that the DNA packaging ATPase associates with the portal and small terminase proteins at the portal vertex (Rao & Feiss, 2008; Sun et al., 2010). To investigate

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**Fig. 3.** Co-localization of IVa2-EGFP, 33K-EBFP and DBP-mCherry fusion proteins in 293 cells. 293 cells were transfected either with pIVa2-EGFP + p33K-EBFP (a–d), pIVa2-EGFP + pDBP-mCherry (e–h), p33K-EBFP + pDBP-mCherry (i–l) or pIVa2-EGFP + p33K-EBFP + pDBP-mCherry (m–p). The set of four panels shows expression of EGFP, EBFP, mCherry and merged image of same field of cells. The images were taken at 60× and zoomed to ~5×.

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**Fig. 4.** Co-localization of IVa2, 33K and DBP in HAdV5-infected 293 cells. HAdV5 infected 293 cells were immunostained for IVa2 and 33K (a), IVa2 and DBP (b), DBP and 33K (c) or IVa2, DBP and 33K (d) at 18 h post-infection. Merged images obtained by combining images captured in green, blue or red channel are shown on the right of each panel. The images were taken at 60× and zoomed to ~5×.
whether IVa2, 33K and DBP are located on the surface of AdV capsids, purified AdV particles were subjected to immunogold electron microscopy using anti-IVa2, anti-33K and anti-DBP. Immunogold staining with anti-IVa2 (Fig. 5c, d) or anti-33K (Fig. 5a, b) resulted in labelling of approximately 22% and 11% of the capsids, respectively, with either a single gold particle or two to three gold particles located closely together. Immunogold staining with an anti-DBP antibody (Fig. 5e, f) yielded approximately 15% of particles having one gold label particle at a single site. Immunogold labelling with an anti-HAdV5 hyperimmune serum resulted in the labelling of capsids at more than one location (data not shown). No signal was detected with an anti-mouse IgG (Fig. 5g, h) or a secondary antibody control (data not shown). These results indicate that individually, IVa2, 33K or DBP are located at a single site on the AdV virions.

**Identification of IVa2, 33K and DBP in the empty or mature HAdV5 particles**

Virus morphogenesis is mediated by several steps including the incorporation of the viral genome into empty capsids and the release of both viral scaffolding protein(s) and the proteins involved in genome packaging. In order to understand the role of IVa2, 33K or DBP in virion morphogenesis, purified preparations of the empty or mature HAdV5 capsids were analysed for the presence of IVa2, 33K or DBP. The empty or mature AdV capsids were purified by caesium chloride equilibrium gradient from lysates of ADLC8cluc-infected 293cre cells or HAdV5-infected 293 cells, respectively. The empty or mature particles banded at the expected densities of 1.29–1.30 g cm\(^{-3}\) or 1.34 g cm\(^{-3}\), respectively (Fig. 6a). The particle to plaque-forming unit (pfu) ratio of the empty or mature virions was found to be approximately 10\(^7\) or 10\(^3\), respectively. PCR analysis using 33K coding region-specific primers confirmed the absence of the viral genome in the empty particles and its presence in the mature particles (data not shown). The particle to pfu ratios and the absence or presence of the viral genome in the empty or mature particles confirmed their purity. Purified empty or mature particles were analysed by Western blot with anti-IVa2, anti-33K or anti-DBP antibody. The identification of an approximately 50 kDa band showed the presence of IVa2 in both the empty and mature particles (Fig. 6b). In addition to the 50 kDa band corresponding to the full-length IVa2 protein, a faster migrating band possibly representing the 40 kDa isoform of IVa2 (Pardo-Mateos & Young, 2004) was detected. The 33K protein band was detected in the empty particles, but not in the mature virions (Fig. 6c), whereas DBP was found in both empty and mature particles (Fig. 6d). The nature of the two bands with lower molecular mass than that of DBP in the empty particle sample is not clear. To rule out experimental error, three independent experiments were performed, which yielded similar results.

**DISCUSSION**

IVA2 plays an important role in viral genome recognition and in its selective encapsidation, possibly by acting as a packaging ATPase (Ostapchuk et al., 2005; Ostapchuk et al., 2011). Based on the studies with dsDNA-containing bacteriophages and herpesviruses, the packaging ATPase, portal and small terminase are the minimal components of a packaging complex that assembles at a unique vertex (Rao & Feiss, 2008; Sun et al., 2010). Our results indicate that IVa2, 33K and DBP interact in virus-infected cells and are located at a single site on viral capsids. Given the interactions of IVa2 and 33K with packaging domains (Ali et al., 2007) and based on our results, it seems likely that IVa2, 33K and DBP constitute a complex at a unique vertex that helps in genome translocation during genome packaging.

The most striking observation from the *in vivo* cross-linking experiments was the detection of IVa2 and 33K, IVa2 and DBP, and IVa2, 33K and DBP in Bands 3, 2 and 1, respectively (Fig. 1). From studies in dsDNA bacteriophages, packaging proteins are known to function as oligomers. The complete packaging machinery is a heteromeric complex consisting of multiple copies of each component. It is likely that *in vivo* cross-linking may not stabilize interactions between all subunits of the complex. Partial cross-linking of the subunits of different components of the complex may result in cross-linked complexes containing submaximal but variable number of subunits. Such variable sized multi-subunit complexes could have resulted in the appearance of the smear-like pattern observed above 150 kDa in the cross-linking experiment.
Interactions observed by in vivo cross-linking were confirmed by co-immunoprecipitation followed by Western blot analyses using anti-IVa2, anti-33K and anti-DBP antibodies, thus providing strong evidence that IVa2, 33K and DBP interact in virus-infected cells. In addition, the confocal microscopy experiments demonstrated co-localization of IVa2, 33K and DBP in transfection experiments and also in virus-infected cells. The co-localization of IVa2-EGFP, 33K-EBFP and DBP-mCherry fusion proteins in the absence of AdV genome suggests the formation of a single complex comprising all three proteins. IVa2 has been shown to interact with E1B 55-kDa protein (Harada et al., 2002), which in turn co-localizes with viral DNA replication centres containing the DBP in HAdV5-infected cells (Gonzalez & Flint, 2002). Our confocal data demonstrate the appearance of granular structures in the presence of IVa2 and DBP in 293 cells suggesting interplay between the proteins involved in DNA replication and virion morphogenesis. Localization of DNA replication and virion morphogenesis proteins in close vicinity should not be surprising as it may allow coupling of DNA replication and packaging. Similarly, the replication centre protein pUL112-113 of HCMV has been shown to co-localize with the putative portal protein pUL104 and also with the large terminase protein pUL56 in infected cells (Dittmer et al., 2005).

Proteins involved in capsid assembly and/or genome packaging are expected to be present in the viral particles. IVa2 is known to be present in all assembly intermediates and mature virions (Winter & D’Halluin, 1991). The 33K protein was detected in empty capsids, but not in mature virions, raising the possibility that 33K may be the scaffolding protein for capsid assembly. The characteristic features of scaffolding proteins are their presence in the empty or procapsids, their release from the virions following DNA packaging, their absence from the mature particles and their presence as a requirement for capsid assembly. However, localization of 33K at a single location on the capsids and its presence in lesser amounts in empty capsids than that expected of a scaffolding protein argues against its role as a scaffolding protein. Based on the presence of 33K in empty capsids, its interaction with the putative packaging ATPase IVa2, its presence at a unique vertex and its interaction with viral packaging domain (Ali et al., 2007), it is possible that 33K functions as a small terminase. Further experimentation is necessary to precisely dissect the role of 33K during AdV morphogenesis.

**Fig. 6.** Purification of HAdV5 empty and mature particles and analysis for the presence of IVa2, 33K and DBP. HAdV5 empty and mature particles were purified as described in Methods. (a) Tubes showing the relative position of the empty and mature particle bands following caesium chloride-gradient ultracentrifugation. Lysates of 2 or 10 µg of purified particles were separated on SDS–PAGE gels and processed for Western blot analysis with an anti-IVa2 (b), anti-33K (c) or anti-DBP (d) antibody. Bands corresponding to full-length IVa2, 33K and DBP are indicated. Bands of lower molecular mass are shown by stars (a and d).
Given the interactions of DBP with IVa2 and 33K, and its presence in capsids, it seems likely that DBP plays a role in capsid assembly or packaging. However, considering the vital roles that DBP plays in viral DNA replication and stability of viral mRNAs (Kitchingman, 1995), it will be important to demonstrate its separate role in capsid assembly or packaging. Nonetheless, a temperature-sensitive mutant of HAdV5 with point mutations in DBP coding region was found to be defective for capsid assembly (Nicolas et al., 1983).

Immunogold electron microscopy is a proven technique that has been used to locate components of the genome packaging machinery at the unique vertex of capsids of various phages and herpes simplex virus 1 (HSV-1) (Fu & Prevelige, 2009; Newcomb et al., 2001). As all capsids are expected to contain a unique vertex, immunogold labelling theoretically should result in the labelling of 100 % of the virions with gold particles. However, this has not been observed practically, possibly due to the non-availability of the unique vertex of all capsids when the capsids are applied to the EM grid surface resulting in steric hindrance. Immunogold labelling of proteins at a unique vertex generally results in labelling of 16–41 % of the capsids (Christensen et al., 2008). In this manuscript, immunogold labelling with an IVa2-, 33K- or DBP-specific antibody resulted in labelling of 22, 11 and 19 % of capsids at a single site, respectively. In some cases, more than one closely placed gold particle were observed on capsids labelled with an anti-IVa2 or anti-33K antibody. That was probably due to the polyclonal nature of these antibodies and/or to the presence of more than one copy of IVa2 or 33K at the unique sites. The presence of IVa2, 33K and DBP at a unique vertex is significant because those proteins also interact with each other. It is likely that a single heterotrimeric complex forms at the unique vertex of capsids; however, this would need further confirmation. The DNA packaging machinery of dsDNA bacteriophages consists of the portal, the packaging ATPase or large terminase, and the small terminase. The portal directly associates with the packaging ATPase, which in turn associates with a small terminase (Rao & Feiss, 2008). IVa2 is likely to be the packaging ATPase for AdV, however, the portal component is yet to be identified. Since the portal is invariably retained after the genome packaging in all viral systems studied to date, it seems that 33K is not the AdV portal. The presence of DBP in both empty and mature capsids and its location at a single site on the virion suggests that it may serve as the AdV portal. Determination of the copy number of DBP in capsids and the structural analysis of purified DBP will be necessary to ascertain if it exists as a dodecamer and forms a ring-like structure similar to other known portals.

**METHODS**

**Cell lines and viruses.** Cell lines used in this study were: (1) A549, a human lung carcinoma cell line (Giard et al., 1973); (2) 293, a human embryonic kidney cell line transformed with E1 region of HAdV5 (Graham et al., 1977); and (3) 293cre, a cell line derived from 293 cells and constitutively expresses Cre recombinase (Parks et al., 1996). These cell lines were maintained in minimum essential medium (MEM) with 10 % FetalClone III (Thermo Scientific, Rockford, IL) and gentamicin (50 ?g ml⁻¹). The 293-IVa2 cells were derived from 293 cells and constitutively express IVa2 of HAdV5. This cell line was generated by transfecting 293 cells with pcDNA3.1-IVa2 by Lipofectamine 2000 (Invitrogen, Green Island, NY)-mediated transfection following manufacturer’s instructions and as described previously (Bangari & Mittal, 2004; van Olphen et al., 2002).

Plasmid construction. To express IVa2, 33K or DBP fused with the enhanced green fluorescent protein (EGFP), enhanced blue fluorescent protein (EBFP) or mCherry fluorescent protein (mCherry) gene, respectively in mammalian cells, the IVa2, 33K or DBP gene was amplified by reverse transcriptase PCR (RT-PCR) using the total RNA extracted from HAdV5-infected cells and following primer sets: pEGFP-N1/IVa2For: ATTAGCTTATGGGAGAGGCGCAAGG and pEGFP-N1/IVa2Rev: ATTTGATCCGGATTTAGGGGTTTTGCGC and pEBFP-N1/33KFor: TTTAAGATCTATGGCACACAAAAAGAAGCTG and pEBFP-N1/33KRev: ATTTAATTCCTCTTGAAGTCCAGCGG and pmCherry-C1/DBPFor: TTATCCGGAATGCGCAGTTCGCGAGAGG and pmCherry-C1/DBPRev: TAACTGAGTATTTCTAAATAACAGGGGTTCGT.

The RT-PCR products of IVa2, 33K and DBP were inserted into pEGFP-N1, pEBFP-N1 and pmCherry-C1 (Clontech, Mountain View, CA) to generate pEGFP/IVa2, pEBFP/33K and pmCherry/DBP, respectively. The plasmid pcDNA3.1-IVa2, used for generating the 293-IVa2 cell line, was constructed by inserting the IVa2 gene into pcDNA3.1+. All constructs were confirmed by digestion with restriction enzymes and DNA sequencing.

Antibodies and Immunoblotting. For generating anti-IVa2 and anti-33K antibodies, 250 µg of IVa2 or 33K protein, expressed in bacteria as a His-Tag protein and purified on a nickel affinity chromatography column, was emulsified with an equal volume of Freund’s incomplete adjuvant and injected intramuscularly in 3–4 week old New Zealand white rabbits. Two (IVa2) or three (33K) booster inoculations were given 2 weeks apart starting at 3 weeks post-priming. Serum samples were collected at various intervals and evaluated for the presence of IVa2- or 33K-specific antibody by Western blot analyses. The IgG fraction from the hyperimmune serum was purified by protein-A Sepharose (GE Healthcare, Piscataway, NJ) affinity chromatography. The anti-DBP antibody clone B6 (Reich et al., 1983) was a kind gift from Dr Arnold Levine (Institute of Advanced Study, Princeton, NJ). Anti-fibre antibody (clone 4D2) was obtained from Abcam (Cambridge, MA). For immunoblotting, purified proteins, cell lysates or purified virus particles were separated on SDS–PAGE and processed for immunoblotting as described earlier (van Olphen et al., 2002).

In vivo cross-linking. The 293-IVa2 cells were mock infected or infected with HAdV5 at a multiplicity of infection (moi) of 20 pfu per cell. At 24 h post-infection, cells were washed once with PBS,
trypsinized and collected along with the medium. The cell suspension was centrifuged at 3000 g for 10 min at 4 °C. The cell pellet was washed once with PBS [PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2H2O and KH2PO4, pH 7.4)], centrifuged at 3000 g for 10 min at 4 °C, resuspended in 0.5% paraformaldehyde (PFA) in PBS for stabilizing protein–protein interactions, and incubated at 37 °C for 15 min. Free PFA was quenched by adding Tris, pH 7.5 to 125 mM final concentration, followed by incubation at room temperature for 5 min. Cross-linked cells were collected by centrifugation at 3000 g for 10 min at 4 °C, washed once with PBS, resuspended in 1 ml radioimmunoprecipitation buffer (30 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) containing protease inhibitors (5 μg/ml 1-aprotinin and 100 μM PMSEF) per 107 cells, and incubated on ice for 15 min. Cells were sonicated briefly and the lysate was centrifuged at 21,000 g for 30 min at 4 °C. Protein concentration of the clarified lysate was estimated by Coomassie protein assay (Thermo Scientific). The cross-linked lysates were analysed by Western blot with an anti-IVa2 antibody.

Protein identification by mass spectrometry and peptide sequencing. To identify protein constituents in supershifted bands detected by Western blot with an anti-IVa2 antibody in cross-linking experiments, protein samples were run in duplicate on SDS–PAGE. Protein bands from one-half of the gel were transferred to a nitrocellulose membrane for Western blot analysis using an anti-IVa2 antibody. X-ray film with resulting bands was aligned with the other half of the gel. Areas corresponding to major supershifted bands were excised and sent to ProtTech (Norristown, PA) for mass spectrometry analysis and peptide sequencing.

Co-Immunoprecipitation. 293 cells (1.5 x 106) in monolayer cultures were infected with HAdV5 at an moi of 20 pfu per cell. At 24 h post-infection, cells were processed for preparation of nuclear extract. Briefly, cells were washed once with PBS, scraped off and collected along with the medium and centrifuged at 2800 g for 10 min at 4 °C. The cell pellet was resuspended in 1 ml hypotonic lysis buffer (10 mM Tris/HCL, pH 7.5, 1.5 mM MgCl2, 0.25 M sucrose, 5 μg/ml 1-aprotinin and 100 μM PMSEF), incubated on ice for 15 min and centrifuged at 2800 g for 10 min at 4 °C. The pellet was resuspended in 5 ml hypotonic lysis buffer, homogenized with 15 strokes of a Dounce homogenizer and centrifuged at 2800 g for 10 min at 4 °C. Following this, the pellet (nuclei) was resuspended in 3 ml high salt lysis buffer (40 mM Tris/HCL, pH 7.5, 0.5 M NaCl, 0.2 mM EDTA, 5 μg ml−1 1-aprotinin and 100 μM PMSEF), incubated on a nutator for 30 min at 4 °C and centrifuged at 21,000 g for 30 min at 4 °C. The supernatant was dialysed against a dialysis buffer (20 mM Tris, pH 7.9, 5 mM MgCl2, 50 mM KCl). Nuclear extracts were pre-cleared by incubating with protein-A Sepharose beads (GE Healthcare) for 1 h at 4 °C on a rotator followed by treatment with an anti-IVa2, anti-33K, anti-DBP, normal rabbit IgG or normal mice IgG overnight at 4 °C. The complexes were purified by treatment with protein-A Sepharose beads for 1 h at 4 °C on a rotator. Beads were washed six times with dialysis buffer containing 0.1 M KCl. The purified complexes were analysed by Western blot with an anti-IVa2, anti-33K, anti-DBP or anti-fibre antibody. In order to avoid reaction with the antibody used during immunoprecipitation, Protein-A–HRP conjugate (Thermo Scientific), was used in-place of secondary antibody in Western blot analyses.

Confocal microscopy. 293 cells were grown on coverglasses (Corning, Corning, NY) pre-coated with poly-L-lysine. At about 60% confluency, cells were transfected with 3–5 μg of various combinations of plasmids pEGFP-IVa2, pEBFP-33K and pmCherry-DBP (pIVa2-EGFP, p33K-EBFP, pDBP-mCherry, pIVa2-EGFP + p33K-EBFP, pIVa2-EGFP + pDBP-mCherry, p33K-EBFP + pDBP-mCherry or pIVa2-EGFP + p33K-EBFP + pDBP-mCherry) using Lipofectamine 2000 following manufacturer’s instructions. At 36 h post-transfection, cells were washed once with PBS and fixed with 4% PFA in PBS for 15 min at room temperature. The coverglasses were mounted on glass slides using Fluoro-gel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Fluorescence imaging was performed using a Nikon C1+ confocal microscope (Nikon, Melville, NY) with 60× objective. Image analyses were performed using NIS Elements software (Nikon).

For immunofluorescence, 293 cells were grown to ~70% confluency on poly-l-lysine coated coverglasses and infected with HAdV5 at 5 pfu per cell. At 18 h post-infection, cells were washed twice with PBS and fixed with 4% PFA in PBS for 15 min at room temperature followed by blocking and permeabilization using permeabilization blocking buffer (PBB) (Biotium, Hayward, CA). Cells were incubated overnight at 4 °C with anti-DBP (1:100), CF488-tagged anti-IVA2 (1:100) or CF350-tagged anti-33K (1:100) antibody diluted in PBB. Anti-mouse CF568 (Biotium) at 1:1000 dilution in PBB was used as a secondary antibody to detect anti-DBP antibody. The washings after antibody incubations were done with PBB. Fluorescence imaging and image analysis were performed as described above.

Immunogold electron microscopy. Purified HAdV5 in dilution buffer (DB) [10 mM Tris, pH 8.0, 150 mM NaCl and 10 mM MgCl2] was applied to glow-discharged 300 mesh Formvar/carbon-coated nickel electron microscopy grids (Electron Microscopy Sciences) for 5 min at room temperature. Grids were fixed for 5 min with 10 μl of 1% glutaraldehyde in DB, followed by washings with DB twice for 1–2 min each. Blocking was done for 2 h at room temperature by floating the grids face down on drops of blocking solution (5% fetal bovine serum, 5% BSA in DB). Grids were incubated with a primary antibody (anti-IVA2, anti-33K, anti-DBP, anti-rabbit IgG and anti-HAdV5) at 1:50 dilutions overnight at 4 °C in blocking solution followed by five to six washings for 5 min each by floating the grids face down on drops of blocking solution. The incubation with a secondary antibody was carried out for 2 h at room temperature with either a goat anti-rabbit- or goat anti-mouse-conjugated to 6 nm gold particles (Electron Microscopy Sciences) at 1:100 dilution followed by 6 washings for 5 min each by floating the grids face down on drops of blocking solution. Negative staining was done with 10 μl of 3% phosphotungstic acid (PTA) for 1 min. Dried grids were examined on a JEM-1200EX transmission electron microscope (Joel, Peabody, MA).

Purification of empty and mature capsids. Empty and mature HAdV5 capsids were purified as described earlier (Ostapchuk et al., 2011), with some modifications.

ACKNOWLEDGEMENTS

This work was partially supported by a Purdue Research Foundation Grant and Hatch funds. We thank Jane Kovach for secretarial assistance and Shanker Thangamani for expertise in confocal microscopy.

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