Nuclear import of adenovirus DNA involves direct interaction of hexon with an N-terminal domain of the nucleoporin Nup214

Running title: Direct binding of hexon with Nup214 N-terminal domain

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Abstract

In this study, we characterized the molecular basis for binding of adenovirus (AdV) to the cytoplasmic face of the nuclear pore complex (NPC), a key step during delivery of the viral genome into the nucleus. We used RNAi to deplete cells of either Nup214 or Nup358, the two major FG repeat nucleoporins localized on the cytoplasmic side of the NPC, and evaluated the impact on hexon binding and AdV infection. The accumulation of purified hexon trimers or partially disassembled AdV at the nuclear envelope (NE) was observed in digitonin permeabilized cells in the absence of cytosolic factors. Both in vitro hexon binding and in vivo nuclear import of the AdV genome were strongly reduced in Nup214-depleted cells but still occurred in Nup358-depleted cells, suggesting that Nup214 is a major binding site of AdV during infection. The expression of an NPC-targeted N-terminal domain of Nup214 in Nup214-depleted cells restored the binding of hexon at the NE and the nuclear import of pVII, indicating that this region is sufficient to allow AdV binding. We further narrowed the binding site to a 137 amino acid segment in N-terminal domain of Nup214. Together, our results have identified a specific region within the N-terminus of Nup214 that acts as a direct NPC binding site for AdV.

Importance

AdV, which have the largest genome of non-enveloped DNA viruses, are being extensively explored for use in gene therapy, especially in alternative treatments for cancers that are refractory to traditional therapies. In this study, we characterized the molecular basis for binding of AdV to the cytoplasmic face of the NPC, a key step for
delivery of the viral genome into the nucleus. Our data indicate that a 137 amino acid region of the nucleoporin Nup214 is a binding site for the major AdV capsid protein hexon, and that this interaction is required for viral DNA import. These findings provide additional insight on how AdV exploits the nuclear transport machinery for infection. The results could promote the development of new strategies for gene transfer, and enhance understanding of the nuclear import of other viral DNA genomes, such as those of papillomavirus or hepatitis B virus that induce specific cancers.

INTRODUCTION

Adenoviruses (AdV) are non-enveloped DNA viruses consisting of an icosahedral capsid of ~90 nm diameter and an inner nucleoprotein core containing a linear double-stranded DNA genome of ~36 kbp (1–3). The major structural component of the capsid is the hexon trimer that is present in 240 copies. On the outer surface of the capsid at each of the twelve vertices, fiber proteins are anchored to the penton base. A number of minor capsid proteins on the outer and inner surface of the virus particle help to stabilize the capsid (4). The DNA is directly associated with the core proteins including protein X, the “terminal protein”, which is covalently linked to the 5' DNA termini, protein VII and protein V, which connects the core to the outer capsid.

AdV enter the cells by receptor-mediated endocytosis during which the virion becomes partially uncoated (3). Uncoating involves a series of events culminating with endosomal membrane lysis by protein VI, which allows access of the particle to the cytosol (5). The partially disassembled capsid is then translocated along microtubules to the nucleus using the dynein/dynactin motor complex (6, 7). AdV then interacts with the nuclear envelope (NE) at nuclear pore complexes (NPCs) (8) and the viral genome is
translocated into the nucleus by means of nuclear import receptors and/or histone H1
(8–10).

NPCs are evolutionarily conserved large protein complexes of ~100 MDa spanning
the NE that mediate trafficking into and out of the nucleus. Although small molecules
passively diffuse through the NPC, macromolecules larger than ~20-40 kDa are
transported in an active manner. The latter pathway is mediated by cellular transport
receptors, including the karyopherin beta family that facilitates the translocation of most
proteins and certain RNAs (11, 12). NPCs are formed by ~30 proteins, which are
thought to be present in multiples of 8 copies (13). A third of all nucleoporins (Nups)
contain intrinsically disordered regions enriched in Phe-Gly (FG) repeats. The FG
repeat domains directly bind karyopherins (12) and play an essential role in trafficking of
receptor-cargo complexes through the NPC (13). Many of the FG nucleoporins are
localized to central regions of the NPC, but some occur at the NPC periphery. The
peripheral FG nucleoporins include Nup214 and Nup358, which are located in fibrils that
emanate from the cytoplasmic face of the NPC, and Nup153, which is concentrated in
the “basket” that projects from the nuclear face of the NPC (14, 15).

Since the upper size limit for signal-mediated transport through the NPC is ~40 nm,
few viruses are sufficiently small to be imported into the nucleus without disassembly
(17,18). Accordingly, AdV genome import requires binding of the virus at the NPC and
full capsid disassembly (18–20). A priori, AdV binding to the NPC is likely to be
mediated by the hexon protein, which is the most abundant and outermost capsid
protein, and by the cytoplasmic NPC fibrils, which are the most exposed structural
components. AdV binding to the NPC was suggested to directly or indirectly involve the
nuclear export receptor CRM-1 (21) and the cytoplasmic FG nucleoporins Nup214 (8) and Nup358 (22), which are known to be involved in binding of karyopherins during nuclear import/export (23, 24). The specific region involved in the docking has not been precisely evaluated.

To promote understanding of the mechanism of AdV interaction with the NPC, we used cells where Nup214 and Nup358 were depleted by RNAi to study AdV and hexon binding to the NPC and import of the viral genome. Our studies revealed a specific role for Nup214 in hexon binding and genome import, and mapped hexon binding to a 137 residue potentially unstructured region in the N-terminus of Nup214.

MATERIALS AND METHODS

Cell lines and transfection. Normal rat kidney (NRK) cells, human HEK293-T cells and human HeLa cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. For transfection assays, 0.5 x 10^6 cells were seeded into each well of 6-well dishes the day prior to transfection. For one well, 2 µg of DNA with 2 µl of Plus Reagent and 5 µl of Lipofectamine in Opti-MEM were used (Invitrogen). The next day, the cells were plated onto 10-well slides (ICN Biomedical) at 15,000 cells/well. The binding and AdV infection experiments were performed 48 h post-transfection.

Plasmids. All the plasmids were amplified by PCR using pBluescript containing human Nup214 as a template. The N-terminal (1-1058) and central (586-1058) of Nup214 were inserted in the pcDNA TM3.1D/V5-His-TOPO vector (Invitrogen). The N-
terminal domains of Nup214 (1-450; 1-586, 450-586) were inserted in the pET101D/V5-His-TOPO vector (Invitrogen). These N-terminal domains and aa 587-723 were amplified by PCR using primers containing V5 (sequence found in the P and V proteins of the paramyxovirus, SV5) and His tags and inserted in pcDNA3.1 vector. The fragment Nup214 (450-586), which was amplified as a fusion with V5 and His tags by PCR, was inserted into pMalc2x vector (NEB) between HindIII and EcoR1 sites. The pcDNA™3.1D/LacZ-V5-His vector (Invitrogen) was used for a mock transfection control.

Expression of recombinant proteins. V5-His fusion proteins were expressed in the BL21(DE3) E. coli strain. Cells were lysed and purification was performed on cleared lysate using Ni-NTA agarose beads (Qiagen). The proteins were eluted in transport buffer TB (20 mM Heps pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM EGTA and protease inhibitors) with 200 mM imidazole. After imidazole removal, the proteins were concentrated, snap-frozen in liquid nitrogen. For the Nup214 (450-586) fragment, the MBP tag was removed by the addition of Factor Xa for 3 h at RT. The Nup214 (450-586) fragment was eluted from beads, the buffer was exchanged and the proteins were stored as described above.

Other recombinant protein fragments and proteins were expressed in E. coli and purified as described before: Nup358-1 and Nup358-4 fragments (25), C-terminal domain (aa 1861 to 2090) of Nup214 (26).

RNA interference. For gene silencing by RNAi, cells were transfected with the pSUPER vector (Oligoengine) expressing short hairpin RNAs (shRNAs) against Nup214.
and Nup358. The oligonucleotides used for silencing of Nups were cloned in the pSUPER vector, and had the following sequences: Nup214 (sh214-1: 5'-gatcccttgcccaaggaacgctcgattcaagagatcgcgtcccttgggcaattttta-3'; sh214-2: 5'-gatcccccctagccaaacatcgggaatctttaagagatcgcgtcccttgggcaattttta-3'; sh214-3: 5'-gatcccccgatagcacaatgcttgccacgaaattcaagagatttcgtggcaagcattgtgctatcgttttta-3') or Nup358 (sh358, 5'-gattcccccaggtcaatggcaactattcaagagatttcgtggcaagcattgtgctatcgttttta-3') (27). A peGFP-H1 vector without inserted shRNA-encoding sequence containing two expression cassettes (one for eGFP and one for shRNA) was used as a control and for the reconstitution experiments (kindly provided by Claire Waterman).

**Purification of virus and acid treated virus.** An AdV5 vector (AdV) containing an eGFP expression cassette in the E1 region was propagated in the HEK293-T cells. Virus was purified as previously described (28). AdV particles were partially disassembled following the protocol of Wiethoff et al (5). For this, the virus was dialyzed against the acetate buffer pH 5.0 and incubated at 37°C for 10 min before being loaded onto a Nycodenz step density gradient. The partially disassembled virus was dialyzed against 5 mM Hepes pH 7.4, 1 mM MgCl₂ and 10% glycerol at 4°C. The fraction was analyzed for protein content by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

**Purification of hexon.** Hexon was purified following a protocol modified from Wodrich et al (29). Infected cells were disrupted and the lysate was cleared by two steps of centrifugation. The hexon containing band was extracted and dialyzed against
10 mM Bis-Tris propane pH 7.0 using 100 kDa cut-off Float-A-Lyser (Spectrum Lab), then applied to a MonoQ FPLC column (Pharmacia). Highly enriched hexon fractions were pooled and concentrated. The buffer was exchanged to TB. The purified hexon corresponds to trimeric hexon, as observed by negative staining electron microscopy. The fraction was analyzed for protein content by SDS-PAGE after Coomassie staining and by immunoblot using an affinity purified rabbit anti-hexon antibody against peptides (230-243 and 430-445). No pVI protein was detected by immunoblot using specific protein VI antibody (30).

**Binding experiments in permeabilized cells.** The binding experiments in permeabilized cells were performed in NRK and HeLa cells. Cells were plated on 10-well slides the day before the experiment. Cells were permeabilized by treatment with 0.005% digitonin (Calbiochem) in TB for 5 min at RT. The cells were pre-incubated with TB alone for 15 min at 30°C. Binding reaction mix was incubated for 30 min at 30°C or 4°C. The binding reaction mix contained 0.75 μM purified hexon, cytosol with or without ATP-regenerating system, TB alone or TB with competitors (see below). Cytosol was isolated by digitonin lysis of HeLa cells as described by Kehlenbach et al (31) and used at a final concentration of 4 mg/ml. Cells were fixed and processed for hexon and Nup detection as described below. All experiments were repeated at least three times.

**Adenovirus infection following knockdown.** After initial transfection with pSUPER expressing different shRNAs and/or pcDNA3.1 expressing the central domain of Nup214 or the N-terminal domain of Nup214, HeLa cells were infected with AdV at
48h post transfection. Cells were infected using 10,000 virus particles/cell for FISH experiments or 1,000 virus particles/cell for pVII detection. For infection experiments, the virus was pre-bound to cells for 1 h on ice, followed by 2 h or 3 h incubation at 37°C for FISH or 3h at 37°C for pVII detection. Prior to detection, the cells were fixed with 3.7% formaldehyde as described above. The experiments were repeated at least three times with three independent transfections.

**Immunofluorescence (IF).** The fixed cells were permeabilized with 0.2% Triton for 5 min and detection of protein was performed by incubation with antibodies for 1 h. The primary antibodies used were: 8C4 mouse anti-hexon antibody (Fitzgerald Industries), rabbit anti-Nup214 antibody (Bethyl Laboratories), affinity purified guinea pig anti-Nup358 directed against the FG-rich domain (aa 996-1963) in the Nup358-1 clone provided by Nabeel Yaseen (32), rabbit anti-pVII antibody (kindly provided by Daniel A. Engel) were used. FITC, Texas Red (Jackson Laboratories) or Alexa labeled antibodies (Invitrogen) were used as secondary antibodies. The Nup214 expressed domains were detected with FITC-labeled mouse monoclonal anti-V5 antibody (Invitrogen). Nuclei were counter-stained with DAPI (Sigma) or with TOPRO-3 (Molecular Probes). The cells were examined using a Bio-Rad 1024 or a Leica TCS SP5 laser scanning confocal microscope with a 63x oil immersion objective (Zeiss Plan Apo 1.4 NA or Leica Plan Apo 1.4 NA). Images were collected with Bio-Rad Lasersharp 2000 software or with Leica LAS AF software. Images were analyzed using Simple PCI software (Compix) or Image J software (NIH). The nuclear quantification or rim staining quantification was based on the nuclear space defined by the DAPI or TOPRO-3 staining. In quantitative
analysis, error bars indicate the standard deviation in three different experiments except for representative analysis in which error bars indicate the standard deviation in three different fields. Statistical analysis was done using two-tailed Student’s t-test. For the localization of the AdV genome and protein VII, Leica SP8 laser scanning confocal microscope with a 63x oil immersion objective was used and a representation of the staining was obtained using Image J software (NIH). Maximal projection of image stacks in the nucleus was performed; a Top hat filter and StackReg plugin (33) were applied.

**Fluorescence in situ hybridization (FISH).** The detection of AdV DNA was performed following a modified protocol from Greber et al. (19). The probes were generated using random priming on purified AdV genome with the digoxigenin DNA labeling kit (Roche). The cells were stained and IF images were acquired before the cells were further processed for FISH. The probe and the slide were pre-denatured for 10 min at 95°C in hybridization buffer then mixture was additionally denatured for 5 min before hybridization overnight at 37°C. Mouse anti-digoxigenin antibody (Roche) and Alexa 488-labeled goat anti-mouse (Invitrogen) were used to detect AdV genome. The cells were examined as described above.

**Pull down and immunoblot.** Total cell lysates from $10^6$ cells were prepared and analyzed by Western blot using standard procedures. Mouse monoclonal anti-V5 antibody (Invitrogen), rabbit anti-hexon (34), mouse RL1 (35), anti-hexon antibody (36) and HRP secondary antibodies (Jackson Immunoresearch) were used for immunoblotting. The pull-down was performed using Dynabeads® His-Tag Isolation &
Pull-down following the manufacturer's protocol (Invitrogen). 4 µg of purified recombinant proteins or buffer alone and 2.5 µg of purified hexon were incubated together then 20 µl beads were added. The unbound fraction was removed and complexes were eluted with 300 mM imidazole. 1/10 of the input, 1/3 of the unbound fraction and ½ of bound fraction was loaded on SDS-PAGE for Western blotting.

RESULTS

Binding of partially disassembled AdV and purified hexon at the NE in digitonin permeabilized cells. We used digitonin permeabilized cells, which are functional for nuclear import (37), to analyze binding of AdV and purified hexon trimers to the NPC in vitro. Digitonin treatment selectively permeabilizes the plasma membrane while leaving the NE intact, thereby allowing virus entry into the cytosolic space. To mimic the exposure of virus to the low pH environment of the endosome (3), the virus was pretreated with pH 5.0 buffer followed by Nycodenz gradient purification. As described previously (5), this treatment induced dissociation of the penton base, fiber, protein IIIa and protein VI (Fig. 1A, left). Removal of these proteins has been previously shown to be required for infection (41, 42). The low pH treatment generates partially disassembly capsid and the loss of capsid integrity was confirmed by transmission electron microscopy (data not shown). The purity of trimeric hexon preparation was validated by SDS-PAGE (Fig. 1A, right) and by Western blotting using hexon and pVI specific antibody (data not shown).

Purified trimeric hexon or low pH-treated AdV were added to digitonin permeabilized NRK cells in the absence or presence of cytosol and their localization
was determined by IF using anti-hexon antibody. In the presence of cytosol, purified hexon strongly accumulated in cytoplasmic areas around the nucleus and weakly associated with the nuclear rim; however, it was not transported into the nucleus (data not shown). By contrast, in the absence of cytosol purified hexon was concentrated mainly in a perinuclear rim (Fig. 1B) and no staining can be observed in absence of hexon indicating any cross-reactivity of the antibody for the NE (antibody control). The hexon localization largely overlapped with that of Nups (Fig. 1C), indicating that hexon accumulates at the NPC with this assay. Nuclear rim accumulation also was seen for acidified AdV added to permeabilized cells in the absence of cytosol, although binding to cytoplasmic areas occurred as well. This pattern resembled the distribution of hexon in HeLa cells 2 h after AdV infection (Fig. 1B). Because the cytosol-independent binding of hexon trimers to the NPC in permeabilized cells was very robust, this assay is used in the experiments below to dissect this interaction.

**Loss of hexon binding at the NE in Nup214 depleted HeLa cells.** To determine whether Nup214 or Nup358 are required for binding of purified hexon to the NPC, we used RNAi to deplete HeLa cells of these Nups. Three separate shRNAs targeting different domains of the Nup214 RNA (Fig. 2A) were analyzed. In the majority of cells, most Nup214 was absent from the NE as seen by IF, which ever shRNA used (Fig. 2D and data not shown). Transfection with the sh214-2 plasmid reduced Nup214 expression by ~80% by Western blotting (Fig. 2B and C), and also led to a smaller decrease (~30%) in Nup358 expression. Transfection with the sh358 plasmid reduced
the expression of Nup358 by 80% compared to non-transfected cells but did not impact Nup214 expression (Fig. 2B and C).

Cells transfected with shRNA plasmids to deplete Nup214 or Nup358 were analyzed for the binding of purified hexon to the NE in absence of cytosol after digitonin permeabilization. Hexon hardly bound to the NE of cells lacking Nup214 expression (Fig. 3D). In Nup214 depleted cell populations, hexon rim staining on average was reduced by 70% (Fig. 3E), which correlates well with the efficiency of Nup214 silencing. In contrast, hexon rim staining remained unchanged in Nup358 depleted cells (Fig.3D, lower panels) compared to non-transfected cells. In summary these data show that binding of hexon at the NE required Nup214 but not Nup358, suggesting that Nup214 is the primary binding partner for hexon exposed on the outer face of the NE.

Reduction of viral DNA import in Nup214 depleted HeLa cells. The AdV genome is transported into the nucleus via the NPC in order to initiate virus replication and particle assembly. To assess the relationship of hexon association with Nup214 and the import of the genome, we infected HeLa cells with AdV 48h after Nup214/358 shRNA transfection. The infection was limited to 2 or 3 h incubation of AdV in order to prevent interference by newly synthesized viral proteins. The AdV genome was detected by fluorescence in situ hybridization (FISH) under denaturing conditions. Fluorescent dots representing the AdV genome were observed in the nucleus and in the cytoplasm in non-transfected and in Nup358-depleted cells (Fig. 3A). In contrast, the presence of AdV DNA was substantially reduced in the nucleus in Nup214-depleted...
cells (Fig. 3A). Detection was specific as no fluorescent dots were observed in non-infected cells (data not shown).

Quantification of the fluorescence images revealed a 50% reduction for the AdV genome signal in the nucleus of Nup214 depleted cells compared to non-transfected cells (Fig. 3B), while no significant reduction was observed for Nup358-depleted cells. To confirm the FISH results, we also analyzed the localization of the genome-associated protein VII by IF. Prior to nuclear import, protein VII is inaccessible to antibody staining since it is located inside the capsid (1–3). Similar to our observations of AdV DNA, pVII fluorescent dots accumulated in the nucleus of untreated and Nup358-depleted cells (Fig. 3C). In contrast, a > 75% reduction for the protein VII IF was observed when cells were depleted for Nup214 compared to non-transfected cells (Fig. 3D), while no major reduction was observed for Nup358-depleted cells.

To extend these results, we analyzed the localization of protein VII and the AdV genome in the same samples by a protocol involving immunostaining combined with FISH. With this method, protein VII was detectable in the nucleus as dots very similar to the dot-like signal of AdV genome, and partially overlapping with the latter (Fig. 3E). The AdV genome but not protein VII also was seen in the cytoplasm at the vicinity of the nucleus. Complete overlap of pVII and AdV genome in the nucleus was not apparent, probably due to the denaturation treatment required for the FISH. For subsequent analyses of nuclear delivery of AdV genome, pVII staining was used as a surrogate for the AdV genome.

Collectively, these data show that Nup214 but not Nup358 is required for efficient delivery of the AdV genome to the nucleus in infected cells, similar to the requirements
for binding of hexon to the NPC in permeabilized cells. Our observation that protein VII was detectable in the nucleus only in the presence of Nup214 suggests that Nup214 is a prerequisite for capsid disassembly leading to genome release.

Identification of hexon binding region in Nup214. We expressed fragments of human Nup214 in Nup214-depleted HeLa cells to identify regions that can reconstitute hexon binding to the NE in permeabilized cells. Nup214 contains 2090 aa and has an N-terminal domain (1-586) containing a β-propeller structure (38), a coiled coil-containing domain (586-1058) with two leucine zippers, and a C-terminal domain (1058-2090) enriched in FG repeats (see Fig 2A). Our initial analysis involved two amino terminal fragments of Nup214 that contained the coiled coil domain required to target the protein to the NPC(39): Nup214 (586-1058) and Nup214 (1-1058) (see Fig. 4B). Knockdown was achieved with the sh214-3 plasmid, which does not target these exogenous Nup214 fragments. The Nup214 constructs were expressed as fusion proteins with a V5-His tag at the C-terminus for discrimination from the endogenous Nup214. The antibody against endogenous Nup214 recognizes a domain of Nup214 (1250-1300) that is not present on the ectopically expressed N-terminal Nup214 proteins (see Fig. 2A and 4B). Western blotting verified expression of the recombinant proteins with some minor degradation products and two slower migrating bands, presumably reflecting post-translation modifications (Fig. 4A). IF staining validated the incorporation of these proteins into the NE (Fig. 4A, 3rd column).

In the permeabilized cell assay for hexon binding to the NE, no increase in nuclear rim staining of hexon was observed in cells expressing the coiled coil domain of
Nup214 (Fig. 4B, 3rd row and Fig. 4C), as compared to the sh214 control. In contrast, hexon staining was restored to the levels of the NT control when the larger N-terminal region of Nup214 was expressed (Fig. 4B, 4th row and Fig. 4C). This finding indicates that the coiled coil domain of Nup214 by itself is not sufficient for the binding of hexon to the NE, whereas hexon binding is fully supported by the entire N-terminal region (1-1058).

Reconstitution of the nuclear import of protein VII by complementation with recombinant Nup214 proteins. Since the N-terminal region of Nup214 could restore nuclear binding of hexon, we asked if this domain also could restore nuclear delivery of the AdV genome in Nup214-depleted HeLa cells. Cells first were co-transfected with plasmids encoding eGFP and sh214 on the one hand and exogenous fragments of human Nup214 on the other hand then were infected with AdV, and cells were analyzed for the localization of protein VII. The eGFP expression was used to track transfected cells. In Nup214-depleted HeLa cells, we observed a strong reduction of specific pVII staining in the nucleus. Reduced nuclear accumulation of pVII also was observed after expression of the coiled coil domain of Nup214 (Fig. 4D). In contrast, a strong nuclear staining with pVII was observed after complementation with the N-terminal region of Nup214 as observed for NT cells (Fig.4D). This suggests that hexon binding to the N-terminal region of Nup214 is required for nuclear import of pVII, and by inference, AdV DNA.
Competition of Nup214 proteins for hexon binding at the NE. The previous experiments showed that the coiled coil domain of Nup214 was not sufficient for the binding of hexon to the NE in permeabilized cells but that the first N-terminal 586 residues also were needed. To test if a specific domain of this N-terminal extension is involved for hexon binding, we expressed several soluble N-terminal Nup214 domains in bacteria and used the purified molecules in competition experiments. These included Nup214 (1-586), Nup214 (1-450), and Nup214 (450-586) (Fig. 5A and 5B). All proteins appeared as prominent bands at the predicted molecular weight by SDS PAGE and Coomassie blue staining, although Nup214 (1-586) also produced smaller additional bands around 25 kDa that contained the V5 epitope in addition to the full-length protein (66 kDa) (data not shown).

We analyzed the localization of exogenously added hexon in digitonin permeabilized HeLa cells when cells were incubated with various Nup214 fragments at excess molar concentrations. Competition with the longer Nup214 domain (1-586) strongly inhibited hexon staining around the nucleus whereas the shorter Nup214 domain (1-450) had little effect on hexon binding to the NE (Fig. 5C). The addition of the Nup214 domain (450-586) also strongly reduced hexon staining around the nucleus. Quantification of hexon binding in presence of the three domains indicated 80% reduction for Nup214 (1-586) and Nup214 (450-586) while only 20% reduction for Nup214 (1-450) in these competition studies. These data indicate that the most N-terminal part of Nup214 region did not compete for hexon binding while the region between aa 450-586 of Nup214 reduced the binding significantly, thus further delineating the hexon binding site within Nup214 to aa 450-586 (Fig. 5C). This domain
contains only two FG repeats, unlike the C-terminal domain of Nup214, which contains many repeats.

Although our data argue that the N terminal part of Nup214 is the only critical region for hexon binding, we sought to determine whether the FG repeat-rich C-terminal region of Nup214 (1861-2090) and other known FG repeat-rich regions of Nup358 (Nup358-1 and Nup358-4) also could compete for hexon binding (Fig. 5D). However, hexon staining was not diminished significantly after the addition of 2-fold molar excess of these regions. Since other FG repeats of different Nups did not interfere with hexon binding to the NE, we concluded that FG repeats do not play a role in the hexon binding to Nup214.

**Reduction of protein VII nuclear localization following overexpression of soluble Nup214 fragments.** To complement the *in vitro* experiments involving competition of hexon binding to the NPC by Nup214 fragments (Fig. 6), we overexpressed N-terminal Nup214 fragments in cells and infected the cells with AdV. We analyzed pVII subcellular localization by IF 3 h after infection. The Nup214 proteins were expressed at the expected molecular weights as shown by Western blotting (Fig. 6A) and were localized throughout the entire cell (Fig. 6B). Nuclear accumulation of pVII was observed after overexpression of Nup214 (1-450) as well as in mock-transfected cells, showing that this region of Nup214 does not prevent the pVII nuclear import. A small decrease of nuclear accumulation of pVII was observed after overexpression of Nup214 (1-586) and pVII accumulation in the nucleus was strongly reduced after overexpression of the Nup214 (450-586) fragment (Fig. 6B and C). Although the
Nup214 (1-586) fragment also contains aa 450-586, these results suggest that full accessibility of the domain may not occur when it is not anchored in the NPC. The absence of pVII staining in the cytoplasm after overexpression of the Nup214 (450-586) fragment indicates that the fragment was sufficient to prevent AdV binding to the NPC and the release of the genome but not sufficient to allow the capsid disassembly in the cytoplasm. The sequence of Nup214 (450-586) is rich in Proline/Alanine/Serine (18%/17%/24%). To test the specificity of this fragment for inhibition of pVII nuclear import, we also overexpressed a fragment of the same length enriched in similar amino acids just downstream of 586 position, Nup214 (587-723) (16%/12%/20%). This fragment does not contain coiled coil domain so it does not dimerize. Nuclear accumulation of pVII was observed after overexpression of Nup214 (587-723) fragments, showing that this fragment did not compete for binding and pVII nuclear import. These data further indicate that nuclear binding is required for nuclear transport of the genome and that a specific small region (450-586) in the N-terminal part of Nup214 binding is required for nuclear import of AdV DNA.

**Direct interaction of hexon with the N-terminal region (450-586) of Nup214.**

Having obtained evidence for a direct, transport receptor-independent binding of hexon to Nup214 and localized the binding site to the aa 450-586 of Nup214, we next confirmed the interaction biochemically. We incubated the soluble recombinant His tagged Nup214 N-terminal proteins with purified hexon: Nup214 (1-450) and Nup214 (450-586) and captured the formed complexes on Ni-agarose beads (Fig. 7). The complexes bound were eluted and analyzed on SDS-PAGE. Western blotting revealed...
that purified hexon showed substantial interaction with Nup214 (450-586) (Fig. 7, lane 3), but not Nup214 (1-450) (Fig. 7, lane 2). A weak unspecific interaction of purified hexon for the beads alone was observed. Our finding that purified hexon showed a direct interaction with 450-586 of Nup214 is consistent with the ability of this fragment to inhibit AdV genome nuclear import.

DISCUSSION

Because the partially uncoated AdV particle is suggested to bind to the NPC via hexon, we used a permeabilized cell assay to map the Nup binding site for hexon binding at the NPC. The information from this assay was then used to validate the relevance of this interaction for AdV genome import into the nucleus. For initial insight on the NPC binding site for hexon, we used a shRNA knock-down approach to deplete either Nup214 or Nup358, the two major Nups localized at the cytoplasmic side of the NPC previously suggested to be involved in AdV nuclear import (8, 22). The depletion of each Nup was achieved without markedly affecting the expression of the other, as reported previously (24). The in vitro binding of hexon to the NE was specifically lost in Nup214-depleted cells, and not in Nup358-depleted cells, consistent with work of Strunze et al (22). Correspondingly, we observed loss of AdV genome import in Nup214-depleted cells but not in Nup358-depleted cells. These results strongly support an essential role of Nup214 in AdV docking to the NPC and genome import. In other studies, it has been suggested that kinesin-1 is involved in an active process engaging Nup214 and Nup358 to disrupt the permeability of the NPC and to uncoat the AdV DNA (22). Although a requirement for Nup358 was not detected with our conditions, the
discrepancy between the two studies might be due to different MOI and infection time
after the Nup knock-down or to differences in silencing efficiency.

Here we have showed a direct interaction of hexon with an N terminal region of
Nup214 by biochemical assays and in the context of the NPC in digitonin-permeabilized
cells. We found that highly purified hexon as well as low pH treated AdV particles
efficiently bound to the NE of permeabilized cells in the absence of added cytosolic
factors, suggesting that this interaction does not involve nuclear transport receptors.
Consistent with the notion that this binding represents a docking step for AdV, we did
not observe hexon nuclear import when energy and cytosol were added to the binding
reaction. These results agree with the previous report that hexon import is mediated by
protein VI (29). In an effort to map the binding site on Nup214 for AdV, we performed
reconstitution with Nup214 N-terminal fragments that retained information for targeting
to the NPC and competition experiments. The expression of the N-terminus of Nup214
(1-1058) in Nup214-depleted cells, but not a fragment containing aa 586-1058 was
sufficient to fully restore the binding of hexon to the NE. Consistently, a recombinant N-
terminal fragment (Nup214, aa 1-586) strongly reduced the binding of hexon to the NE
in competition experiments. These results showed the coiled-coil domain (586-1058),
the minimal region for NPC binding of Nup214, was not involved in hexon binding,
excluding a potential role of Nup88 or other associated Nups. We also showed direct
binding in \textit{in vitro} binding assay using purified hexon and recombinant fragment
supporting that hexon binds to aa 450-586 of Nup214. A multitude of \textit{in vivo} competition
assays indicate that binding to this region is important for AdV DNA import- both
involving expression of NPC-targeted regions of Nup214, and competition with fragments of Nup214 that are not targeted to NPC.

Here we identified a new binding site at the N-terminus of Nup214 while a previous study reported that the C-terminus of Nup214 (1549-2090) is associated with AdV 

AdV in vitro using crosslinking approaches (8). Nevertheless the total restoration of binding and protein VII accumulation after expression of the N-terminal domain of Nup214 in Nup214-depleted cells does not exclude the role of other regions of Nup214 in AdV import. The N-terminal region of Nup214 contains a β propeller structure (40) and two FG repeats. The β propeller structure of Nup214 does not seem to be part of the binding site because the soluble N-terminal fragment (Nup214, aa 1-450) encompassing the β propeller structure does not compete for hexon binding. This restricts the putative binding site to a 137 amino acid fragment of highly disordered structure and encoding for two FG repeats. A role of the FG repeats in the binding is not supported by our experiments showing that several FG enriched fragments were not able to compete for the hexon binding in digitonin permeabilized cells. Several tools for protein disorder prediction are in agreement to identify this fragment as disordered structure: DisEMBL identified aa 375-582 disordered by loops, aa 487-517 and 527-543 disordered by hot loops and aa 454-584 was disordered by GlobPlot software (42, 43). Intrinsically disordered proteins are involved in cell signaling and can bind to multitude interacting proteins (44). A recent study used this disorder property to explain the specificity of interaction between Nup153 and transport receptors (45). Further analysis will be required to understand how hexon binds to this restricted highly disordered fragment.
Nups including Nup214 and Nup358 are likely involved in the binding and nuclear transport of several other nuclear replicating viruses, however each virus seems to have developed its own nuclear association strategy. The nuclear entry of the human immunodeficiency virus-1 (HIV-1) (46) was reduced in Nup358-depleted cells. A hypothetical model for HSV-1 binding and uncoating at the NPC involved the binding of the capsid to the cytoplasmic filaments mediated by Nup358 (47), then Nup214 resulting in capsid destabilization (48). Indeed the knockdown of Nup214 also delayed the onset of DNA replication in the nucleus of HSV-1 infected cells (49). In contrast to AdV, the NPC-binding of HSV-1 capsid was importin β-dependent (50). AdV could also bind in a non-specific manner to the FG-enriched domain of Nup214 or other FG-enriched Nup of cytoplasmic fibrils thereby allowing capsid dissociation from microtubules in the vicinity of a specific binding site in the N-terminal of Nup214. Further delineation of the complex steps involved in virus binding and nuclear penetration will require concerted efforts in both structural and molecular cell biology. The identification of the specific domain on hexon interacting to the Nup214 could promote the development of new strategies for gene transfer to target efficiently the nucleus.

ACKNOWLEDGMENTS

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REFERENCES


FIG1 Binding of acid treated AdV and purified hexon at the NE. (A) Intact AdV or low pH-treated AdV purified (1.5 µg) were analyzed by SDS-PAGE and silver staining (left). Purified hexon (2.5 µg) was analyzed by SDS-PAGE and Coomassie blue staining (right). The hexon trimer (left) is seen only with short heating in SDS sample buffer. (B) Digitonin permeabilized NRK cells were incubated in transport buffer with low pH-treated AdV or with purified hexon, washed and analyzed by IF. HeLa cells were infected for 2 h by AdV. The hexon (upper panels) and nuclear (DAPI/Topro-3) stainings are shown. (C) Permeabilized HeLa cells were incubated with purified hexon and analyzed as described in B. The nucleus stained with DAPI (in blue), hexon staining (in green), FG repeat nucleoporin staining (in red) and the merge of hexon and Nups staining are shown. Orthogonal views of NE contained in white boxes are shown below.

FIG2 Loss of hexon binding at the NE in Nup214-depleted HeLa cells. (A) Schematic representation of targeting sites of shRNA directed against Nup214 (short horizontal bars). Nup214 full-length (aa 1-2090) is represented as a large horizontal bar. Black hatched box indicates the β propeller structure; grey boxes, indicate leucine zipper domains; thick vertical bars, FG repeats. Short horizontal bar indicates the epitope (1250-1300) recognized by anti-Nup214 antibody. (B) Knock down in HeLa cells 48 h after transfection. Cells were transfected with shRNA expression plasmids against Nup214 (sh214-2) or against Nup358 (sh358) or were not transfected (NT). Total lysates of cells were analyzed by SDS-PAGE and Western blot using RL1 antibody. In each condition, the expression of Nup358, Nup214 and Nup62 (as internal control) is shown. (C) Quantification of the knock down in HeLa cells 48 h after transfection. Cells
were transfected as described in A and stained for Nup214 and Nup358. The mean fluorescence intensity of endogenous Nups staining was measured at the whole circumference of the nucleus (N=31-63 for each condition of each experiment). The histogram shows the mean fluorescence intensity of endogenous Nup214 (white bar) or Nup358 (grey bar) staining compared to NT cells fixed to 100 percent (*, p<0.05). (D) Representative images of hexon binding at the NE. Purified hexon was added 48 h after transfection to digitonin permeabilized HeLa cells of expression plasmids encoding shRNA against Nup214 (sh214-2) (upper panels) or against Nup358 (sh358) (lower panels) or no plasmid (NT). The cells were analyzed by IF staining to detect the hexon, Nup214 (upper panels) or Nup358 (lower panels). The nucleus was stained with DAPI. (E) Quantitative analysis of hexon binding at the NE. HeLa cells were treated as described in D. The mean fluorescence intensity of hexon staining at the whole circumference of the nucleus was measured (N=31-63 for each condition of each experiment). The histogram shows the mean fluorescence intensity of hexon staining in knock down cells and compared to NT cells fixed to 100 (*, p<0.05).

**FIG3** Reduction of viral DNA import in Nup214-depleted HeLa cells. HeLa cells were transfected with shRNA expression plasmids against Nup214 (sh214-2) or against Nup358 (sh358) or were not transfected (NT). The cells were infected with AdV adenovirus for 2 h (A, B) or 3 h (C, D, E). (A) Reduction of AdV genomes in the nucleus of infected Nup214-depleted HeLa cells. Cells were immunostained to detect Nup214 or Nup358 (as indicated) and incubated with DAPI to detect the nucleus. The AdV genome was specifically detected by FISH using anti-digoxigenin antibody. The white arrows
show the nucleus with decreased Nup214 and AdV genome staining and the nucleus with depleted Nup358 but with AdV genome staining present. (B) Quantification of AdV genomes in the nucleus of HeLa cells. The histogram shows the mean fluorescence intensity of AdV genome staining indicated as a percentage. The mean fluorescence intensity of AdV genome staining into the nucleus was measured (N=17-36 for each condition of each experiment) and compared to NT cells fixed to 100 (**, p<0.01). (C) Reduction of protein VII in the nucleus of infected Nup214-depleted HeLa cells. Cells were immunostained as described in A. The protein VII was detected using anti-pVII antibody. The white arrows showed the nucleus with a loss of Nup214 and protein VII staining and the nucleus with a loss of Nup358 but with protein VII staining present. (D) Quantification of protein VII in the nucleus of HeLa cells. The histogram shows the mean fluorescence intensity of pVII staining indicated as a percentage of stained cells. The mean fluorescence intensity of pVII staining into the nucleus was measured (N=64-114 for each condition of each experiment) and compared to NT cells fixed to 100 (**, significant p<0.01). (E) AdV genome and protein VII localization in the nucleus of HeLa cells. Cells were immunostained to detect protein VII using anti-pVII antibody (in red) and with DAPI to detect the nucleus (in blue). The AdV genome was detected by FISH using anti-digoxigenin antibody (in green). Representations of maximal projections of image stacks and merge pictures are shown. The white arrows highlight overlapping staining of pVII and AdV genome, seen as yellow dots.

**FIG4** Reconstitution of hexon binding by expression of the N-terminal domain of Nup214. (A) Expression of Nup214 domains in HeLa cells. HeLa cells were transfected
with expression constructs corresponding to the N-terminal domain Nup214 (1-1058) or coiled-coil domain of Nup214, Nup214 (586-1058) or were not transfected (NT). Total lysates were analyzed by immunoblot using V5-antibody. The predicted sizes of the domains are indicated by the arrows. (B) Representative images of hexon binding at the NE. HeLa cells were transfected with shRNA expression plasmid against Nup214 (sh214-3) alone or were co-transfected with the Nup214 coiled coil domain expression plasmid (sh214-3/Nup214 (586-1058)) or with the Nup214 N-terminal domain (sh214-3/Nup214 (1-1058)) or were not transfected (NT). Purified hexon was added to digitonin permeabilized HeLa cells. The cells were analyzed by IF staining using FITC anti-V5 antibody to detect the overexpressed Nup214 fragments, or with the anti-hexon antibody and anti-Nup214 antibody. The white arrows show hexon staining around the nucleus of Nup214-depleted cells after overexpression of N-terminal domain of Nup214 (1-1058). (C) Quantitative analysis of hexon binding at the NE. The histogram presents the mean fluorescence intensity of hexon staining indicated in percentage. The mean fluorescence intensity of hexon staining around the nucleus was measured (N=43-91 for each condition of each experiment) and compared to NT cells fixed to 100 (***, p<0.001). (D) Reconstitution of the nuclear import of protein VII by expression of the N-terminal domain of Nup214 in Nup214-depleted cells. HeLa cells were transfected with expression plasmid eGFP-shRNA against Nup214 (eGFP-sh214-3) alone or were co-transfected with the Nup214 coiled coil domain expression plasmid (eGFP-sh214-3/Nup214 (586-1058)) or with the Nup214 N-terminal domain (eGFP-sh214-3/Nup214 (1-1058)) or were not transfected (NT). Cells were infected with AdV for 3 h. The cells were analyzed by IF staining using anti-pVII antibody. The histogram presents the mean
fluorescence intensity of pVII staining in the nucleus indicated as a percentage. The mean fluorescence intensity of pVII staining around the nucleus was measured (N=40-114 for each condition of each experiment) and compared to NT cells fixed to 100 (*, p<0.05).

**FIG5** Competition of hexon binding at the NE. Permeabilized HeLa cells (A,B,C,D) or NRK cells (E) were incubated with purified hexon in transport buffer for 30 min in the presence or absence of soluble Nup fragments used as competitors and analyzed by IF staining using anti-hexon antibody. (A) Schematic representation of soluble Nup214 proteins used for hexon binding competition in HeLa cells. Legend described in Fig. 2A. (B) Expression of purified recombinant N-terminal proteins analyzed by SDS-PAGE and Coomassie staining. The expected size of each protein is indicated by the arrows. The migration and sizes of standard markers are shown on the left. (C) Quantitative analysis of hexon binding at the NE in HeLa cells in the presence of 2 µM soluble Nup214 proteins. The histogram shows the mean fluorescence intensity of hexon staining indicated as a percentage in presence of Nup214 (1-586), or Nup214 (1-450), or Nup214 (450-586). The mean fluorescence intensity of hexon staining around the nucleus was measured (N=36-57 for each condition of each experiment) and compared to no Nup214 proteins fixed to 100 (* p<0.05). (D) Representative analysis of hexon binding at the NE in HeLa cells in the presence of 2.5 µM soluble Nup proteins. The histogram shows the mean fluorescence intensity of hexon staining indicated as a percentage in presence of Nup358-1 (996-1963), or Nup358-4 (2500-3224), Nup214 (1861-2090). The mean fluorescence intensity of hexon staining around the nucleus
was measured (N=52-70 for each condition) and compared to no Nup proteins fixed to 100 (* p<0.05).

**FIG6** Reduction of nuclear localization of protein VII in HeLa cells overexpressing soluble Nup214 proteins. HeLa cells were transfected with expression plasmids encoding soluble Nup214 proteins, Nup214 (1-450), Nup214 (1-586), Nup214 (450-586), Nup214 (587-723) or with an empty plasmid expressing V5-His tag (Mock) or were not transfected (NT). Cells were infected with AdV for 3 h. The cells were analyzed by IF staining using anti-V5 to detect the Nup214 proteins and anti-pVII antibody. (A) Expression analysis of soluble Nup214 proteins 48 hours post transfection in HeLa cells. Cell lysates of NT cells or cells transfected cells with the different expression constructs were analyzed by Western blot using anti-V5 antibody. The expected sizes of the Nup214 domains are indicated with the arrows. The migration and size of standard markers is shown on the left side of each Western blot. (B) Representative images of protein VII localization in the nucleus of HeLa cells. Nuclei were stained with DAPI. Cells transfected by empty plasmid expressing V5-His tag is shown (Mock). (C) Quantitative analysis of pVII in the nucleus of HeLa cells transfected with soluble Nup214 proteins. The histogram shows the mean fluorescence intensity of pVII staining indicated as a percentage. The mean fluorescence intensity of pVII staining around the nucleus was measured (N=36-76 for each condition of each experiment) and compared to mock cells fixed to 100 (*, p<0.05; **, p<0.01).
**FIG7** Direct binding of hexon to Nup214 (450-586) fragment. Soluble recombinant N-terminal fragments of Nup214 containing His tags were expressed and purified and coupled to nickel beads. The beads were incubated with purified hexon alone, no Nup214 (lane 1), with purified hexon and Nup214 (1-450) (lane 2) or with purified hexon and Nup214 (450-586) (lane 3). The hexon was detected using anti-hexon antibody in the input (1/10) and unbound fractions (upper first and second images) or pull down fractions (middle images). The presence of Nup214 fragments on the beads was detected using anti-V5 antibody (lower images).
FIG 2

A

B

C

D

E

Nucleoporin knockdown

Hexon binding

Nup358
Nup214
DNA

NT
sh214-2
sh358

Hexon
Nup214
DNA

NT
sh214-2
sh358

Beta propeller | FG | LeuZipper

Nup358
Nup214
Nup62

Nucleoporin staining, %

Hexon staining, %

120
100
80
60
40
20
0

10 μm

10 μm
FIG 3

A

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B

Nuclear quantification of AdV genome

![Graph](image10)

C

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D

Nuclear quantification of protein VII

![Graph](image20)

E

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FIG 7

+ Hexon

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Input

Unbound

Pull down

IB: hexon

Nup214 (1-450)

IB: V5

Nup214 (450-586)