The EMBO Journal Vol.16 No.19 pp.5998–6007, 1997

The role of the nuclear pore complex in adenovirus DNA entry

Urs F.Greber[1], Maarit Suomalainen, Robert P.Stidwill, Karin Boucke, Melanie W.Ebersold[2] and Ari Helenius[2]

University of Zurich, Department of Zoology, Winterthurerstrasse 190, 8057 Zurich, Switzerland and [2]Yale University School of Medicine, Department of Cell Biology, 333 Cedar Street, New Haven, CT 06510, USA

[1]Corresponding author

Adenovirus targets its genome to the cell nucleus by a multistep process involving endocytosis, membrane penetration and cytoplasmic transport, and finally imports its DNA into the nucleus. Using an immunochemical and biochemical approach combined with inhibitors of nuclear import, we demonstrate that incoming viral DNA and DNA-associated protein VII enter the nucleus via nuclear pore complexes (NPCs). Depletion of calcium from nuclear envelope and endoplasmic reticulum cisternae by ionophores or thapsigargin blocked DNA and protein VII import into the nucleus, but had no effect on virus targeting to NPCs. Calcium-depleted cells were capable of disassembling incoming virus. In contrast, inhibitors of cytosolic *O*-linked glycoproteins of the NPC blocked virus attachment to the nuclear envelope, capsid disassembly and also nuclear import of protein VII. The data indicate that NPCs have multiple roles in adenovirus entry into cells: they contain a virus-binding and/or dissociation activity and provide a gateway for the incoming DNA genome into the nucleus.

Keywords: adenovirus/calcium/DNA/nuclear import/nuclear pore complex

Introduction

Viruses carry genetic information from one cell to the other. Viruses, which replicate in the nucleus, import their genome into the nucleus at early stages of infection. They either access the nuclear interior after mitotic breakdown of the nuclear envelope, as in the case of oncoretroviruses, or transport their genome across the nuclear envelope into the interphase nucleus (for review, see Fields *et al.*, 1996).

To reach the interphase nucleus, the viral genome must cross three barriers, the plasma membrane, the cytosol and the nuclear envelope. After passing the plasma membrane and the cytosol, nuclear RNA viruses, such as human immunodeficiency virus (HIV) or influenza virus, import a high molecular weight nucleoprotein complex into the nucleus. This process requires signals, cellular factors, functional nuclear pore complexes (NPCs) and a special configuration of the nucleoprotein complex (Gallay *et al.*, 1996; Stevenson, 1996; Whittaker *et al.*, 1996). The small DNA virus SV40 accesses the nucleus through the nuclear pores by some kind of a conformational change in its capsid and uncoats the DNA for transcription and replication within the nucleus (for review, see Greber and Kasamatsu, 1996). Larger DNA viruses, such as adenoviruses, herpesviruses, baculoviruses or hepadnaviruses, uncoat their genome to a variable extent before they reach the nuclear membrane (Miller, 1996; Roizman and Sears, 1996; Shenk, 1996; Kann *et al.*, 1997; Sodeik *et al.*, 1997). When they dock at the NPC, they are still enwrapped by a protein capsid, ready to undergo a final disassembly reaction and genome injection into the nucleoplasm.

To analyze the mechanisms of DNA import into the nucleus, we are using adenovirus type 2 in cultured human epitheloid cells. Adenoviruses naturally enter human airway cells and produce progeny virions within the nucleus of the infected cell (for review, see Horwitz, 1990). Adenovirus particles have a diameter of ~90 nm and contain at least 11 different structural polypeptides and a linear double-stranded DNA molecule. The DNA is condensed with proteins V, VII and μ, and covalently associated with the terminal protein and non-covalently with the cysteine protease L3/p23 (Mangel *et al.*, 1993; Stewart *et al.*, 1993). The DNA is connected to the inside wall of the capsid via protein VI. The icosahedral capsid consists of six or seven different proteins. More than 75% of the capsid mass is contributed by the hexon protein. Hexon is held together by the cementing protein IX, which accounts for ~4% of the capsid mass (van Oostrum and Burnett, 1985). Protein IIIa, another capsid-stabilizing component, links adjacent facets of the icosahedron. The vertices of the capsid are made up of pentameric penton base and protruding trimeric fiber proteins. Fibers make a primary contact with a cell surface receptor of the immunoglobulin gene family (Bergelson *et al.*, 1997; Hong *et al.*, 1997; Tomko *et al.*, 1997).

Adenovirus enters the cells by a stepwise uncoating program (Greber *et al.*, 1994). First, the fibers are released, followed by other coat proteins. Interactions between the viral penton base and a secondary cell surface receptor, αvβ5 integrins, mediate virus uptake into endosomes (Wickham *et al.*, 1993). Integrins and the penton base are thought to assist the acid-stimulated virus release from early endosomes, ~15 min after internalization into cold synchronized cells (Greber *et al.*, 1993; Wickham *et al.*, 1994; Prchla *et al.*, 1995). By lysing the endosome, virus passes to the cytosol and then to the nuclear membrane. Penton base–integrin interactions together with reducing agents in endosomes or the cytosol also reactivate the viral cysteine protease L3/p23 inside the capsid. L3/p23 then degrades the internal protein VI (Cotten and Weber, 1995; Greber *et al.*, 1996). This step thus weakens the capsid for final dissociation and DNA import into the nucleus.

The nucleus is separated from the cytoplasm by the nuclear envelope, a double membrane with an intermediate filament network, the lamina (for review, see Goldberg and Allen, 1995). The nuclear envelope membranes are contiguous with the endoplasmic reticulum (ER) membrane. Their cisternae are major intracellular calcium stores (for review, see Pozzan *et al.*, 1994). Embedded in both nuclear membranes are large macromolecular structures, the NPCs. NPCs control transport of proteins and nucleic acids in and out of the nucleus. They are anchored in the membranes via the pore membrane domain, a specialized subdomain of the nuclear envelope which lines the pore and links the outer and inner nuclear membranes (for reviews, see Davis, 1995; Panté and Aebi, 1996b). In higher eukaryotic cells, the pore membrane domain is an integral part of the scaffolding framework and contains transmembrane proteins, such as the major NPC glycoprotein gp210 or Pom121 (for review, see Gerace and Foisner, 1994). Small diffusional channels with a diameter of ~5–9 nm are situated in the scaffolding framework (Hinshaw *et al.*, 1992). These sites are thought to allow passive diffusion of small ions and molecules smaller than 30–50 kDa in and out of the nucleus (for review, see Davis, 1995).

Nuclear import of most cellular proteins depends on nuclear localization signals (NLSs) and signal decoding machineries (for different models, see Duverger *et al.*, 1995; Melchior and Gerace, 1995; Rexach and Blobel, 1995; Görlich and Mattaj, 1996; Panté and Aebi, 1996a; Pollard *et al.*, 1996). Nuclear import can be operationally separated into two steps, energy-independent attachment of the transport ligand to the cytoplasmic face of the NPC and energy-dependent translocation. Stable attachment of transport ligands to NPCs in higher eukaryotic cells involves cytosolic factors and *O*-linked *N*-acetylglucosamine-containing NPC glycoproteins (Finlay *et al.*, 1987; Adam and Adam, 1994). Translocation of NLS-bearing proteins occurs via the central transporter region of the NPC in cooperation with additional cytosolic factors (for review, see Davis, 1995; Panté and Aebi, 1996a).

In addition, lumenal factors of the nuclear envelope have a role in maintaining the functionality of the NPC. Binding of a monoclonal antibody to a lumenal epitope of the transmembrane glycoprotein gp210 reduces the nuclear envelope permeability to small macromolecules and signal-bearing proteins in living cells (Greber *et al.*, 1990; Greber and Gerace, 1992). Recent experiments have identified an additional lumenal factor, calcium ions, affecting NPC function. Depletion of calcium from the nuclear envelope by calcium ionophores or the calcium ATPase inhibitor thapsigargin blocks passive diffusion and signal-mediated transport across the nuclear envelope in somatic mammalian cells and frog oocytes (Greber and Gerace, 1995; Stehno-Bittel *et al.*, 1995; Sweitzer and Hanover, 1996). The inhibition is most likely due to a steric block of the central and peripheral NPC transport channels, as suggested by atomic force microscopy in *Xenopus* nuclear envelopes (Perez-Terzic *et al.*, 1996).

In this study, we addressed the mechanisms of adenovirus DNA import into the nucleus in living cells. Our results demonstrate that viral DNA is imported through the NPCs. Depletion of ER/nuclear envelope calcium inhibited DNA and protein VII translocation into the nucleus, but not virus targeting to the nuclear pores or virus disassembly. Wheat germ agglutinin (WGA) or antibodies against *O*-linked NPC glycoproteins blocked protein VII import, virus attachment to the nuclear envelope and virus disassembly. The data indicate that pore complexes have multiple roles in adenovirus entry into the nucleus. They provide a docking site for a cytoplasmic virus at the nuclear envelope, they contain a virus disassembly activity and they are a gateway for the incoming genome into the nucleus.

Results

Calcium requirements during adenovirus infection

To investigate the mechanisms by which incoming adenovirus DNA is imported into the cell nucleus, we tested whether calcium depletion from intracellular stores of HeLa cells by thapsigargin would affect the efficiency of adenovirus infection. Thapsigargin is an irreversible high affinity inhibitor of the ER-associated calcium ATPase (Thastrup *et al.*, 1990). It rapidly depletes calcium from internal ER and nuclear envelope cisternae in many permanent cell lines, including epidermal carcinoma cells (Chao *et al.*, 1992). Calcium depletion from the ER and nuclear envelope cisternae with thapsigargin or calcium ionophores previously has been shown to inhibit nuclear transport of classical NLS-bearing proteins and small dextrans in mammalian cells (Greber and Gerace, 1995; Stehno-Bittel *et al.*, 1995). However, low calcium concentrations (~100 nM free calcium, buffered with EGTA) in the extracellular medium for up to 1 h did not entirely deplete internal calcium stores and did not inhibit nuclear protein import.

Virus was allowed to bind at a multiplicity of infection (m.o.i.) of 5–10 to the surface of HeLa cells in the cold using a normal calcium-containing medium. Infection was started by adding medium at 37°C. At various times thereafter, extracellular and lumenal calcium stores were depleted by adding 37°C medium containing ~100 nM free calcium buffered with EGTA (low calcium medium), in the presence or absence of thapsigargin. The extracellular calcium concentration was kept low to prevent influx of calcium across the plasma membrane in response to depleting the internal stores (Fasolato *et al.*, 1994; Clapham, 1995). After 3 h, the inhibitors (EGTA and thapsigargin) were washed out and the calcium concentration returned to normal. Infection was scored by counting the number of cells positive for the immediate early transcription factors E1A at 14 h using indirect immunofluorescence microscopy.

About 90% of the cells, which were not depleted of calcium, were E1A positive (Figure 1A). This corresponded well with the expected efficiency of virus binding to cells in cold medium, which is ~10% of the input virus (Greber *et al.*, 1993). When thapsigargin/EGTA was added to the cells at 0, 10, 20 or 50 min after warming, the number of E1A-positive cells was reduced 5- to 10-fold as compared with control cells kept in regular calcium medium. Depleting calcium at 100 min or later post-warming still had a marginal effect on E1A expression, yielding ~60% positive cells. It is possible that translation of E1A was inhibited due to calcium store depletion. In separate experiments, we showed that store depletion

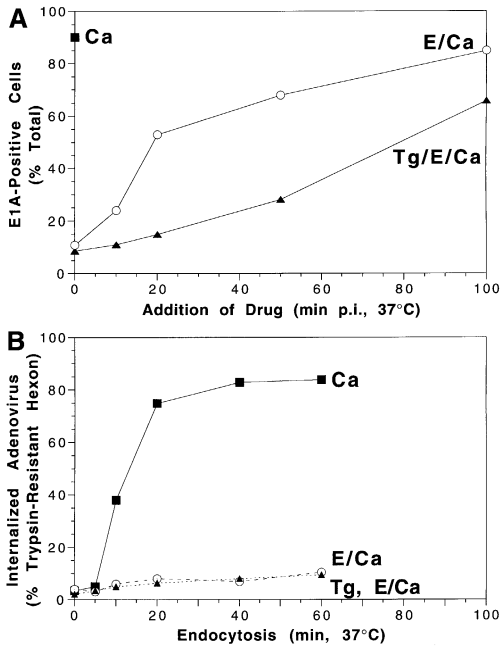

Fig. 1. Calcium depletion from the ER/nuclear envelope inhibits adenovirus infection. (**A**) Purified adenovirus (10 p.f.u./cell) was bound in the cold to HeLa cells grown on glass coverslips. Samples were warmed in either normal calcium-containing medium, EGTA/calcium (E/Ca) medium or E/Ca medium containing 0.5 μM thapsigargin (Tg). After 3 h, medium was replaced with DMEM containing fetal bovine serum. Infection was scored by immunostaining against E1A 14 h after warming. (**B**) Purified [^{35}S]methionine-labeled adenovirus (50 000 c.p.m. per dish) was bound to HeLa cells in the cold. Samples were warmed for 60 min as described in (A) and virus internalization measured by trypsin digestion of cell surface-bound hexon protein as described (Greber *et al.*, 1993).

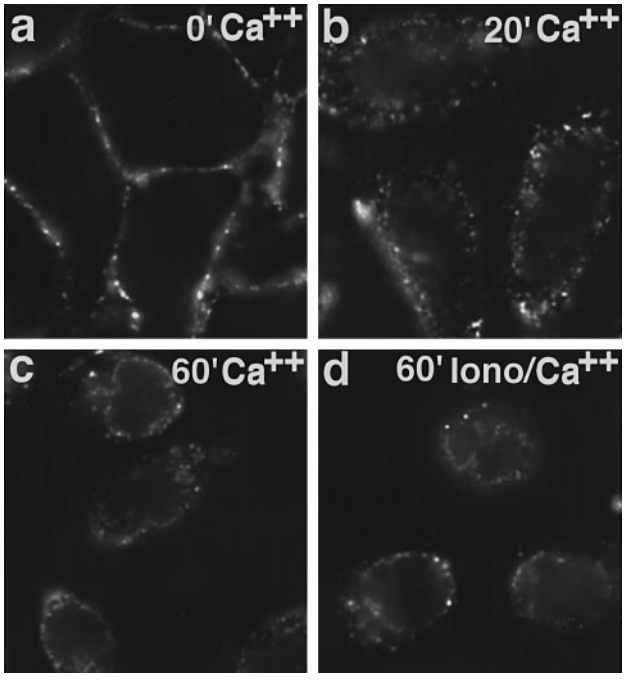

Fig. 2. Adenovirus transport to the nuclear envelope in calcium-depleted cells. Purified FITC-labeled adenovirus was bound to the cell surface in the cold at 20 μg/ml and internalized in warm calcium medium for 0, 20 or 60 min (**a**–**c**). Cells were also warmed for 20 min in calcium medium, followed by incubation in EGTA/calcium medium containing 5 μg/ml ionomycin up to 60 min post-warming (**d**). Samples were fixed in paraformaldehyde and analyzed on a Reichert-Jung Polyvar fluorescence microscope equipped with a CCD camera.

reduced the incorporation of [^{35}S]methionine into cellular proteins by 30–50% (data not shown; see also Brostrom and Brostrom, 1990). Adding low calcium medium without thapsigargin at 0, 10 or 20 min, but not at 50 or 100 min after warming, also delayed E1A expression. We concluded that internal calcium stores and perhaps extracellular calcium were important for one or several early step(s) in cell infection with adenovirus.

Earlier studies using anti-virus antibody accessibility measurements had suggested that virus uptake into cells requires divalent cations (Svensson and Persson, 1984). To test whether extracellular calcium was required for virus endocytosis, we determined the efficiency of virus uptake using a trypsin digestion assay and [^{35}S]methionine-labeled virus pre-bound in the cold (Figure 1B). Either with or without thapsigargin in low concentrations of extracellular calcium, no trypsin-resistant hexon was detected up to 60 min post-warming, indicating that virus was unable to enter these cells. In contrast, in the presence of normal amounts of extracellular calcium, viruses were internalized rapidly and efficiently (Figure 1B). Approximately 80% of the pre-bound viruses became trypsin resistant 20 min post-warming, in agreement with earlier results in unperturbed cells (Greber *et al.*, 1993).

Nuclear envelope/ER calcium is not required for virus transport to the nuclear envelope

To investigate the nature of the intracellular calcium-dependent step(s), extracellular calcium and internal calcium stores were depleted after the virus had been internalized into cells, but before it had reached the nucleus, i.e. 20–25 min post-warming. Depletion of calcium was performed with the reversible calcium ionophore ionomycin (Greber and Gerace, 1995). Thapsigargin and A23187 were also tested and gave essentially the same results as ionomycin.

We first analyzed whether virus transport to the nucleus was affected in ionomycin-treated cells using fluorescein isothiocyanate (FITC)-labeled adenovirus. Virus labeling was performed without inhibiting viral infectivity, as determined by plaque titration (data not shown). Approximately 58% of the FITC was incorporated into the major capsid protein hexon and ~27% into the minor hexon-associated protein IX. Penton base and fiber each contained ~7.5% of the total FITC. Entry of FITC-labeled adenovirus into HeLa cells was analyzed by fluorescence microscopy at different times after warming (Figure 2). Cell surface-bound viruses gave a typical plasma membrane staining pattern, with a prominent signal at cell borders (Figure 2a). After 20 min of warming in calcium-containing medium, a discretely punctate cytoplasmic pattern was observed, and at 60 min a predominant nuclear envelope rim staining was seen (Figure 2b and c). The majority of the punctate fluorescence signals were most likely individual virus particles, as concluded from serial confocal microscopy sections across whole cells (data not shown). In addition, we found no sign of virus aggregation in transmission electron micrographs (see also Figure 3). At 60 min after warming, a large fraction of viruses was distributed around the nuclear envelope. The same result was obtained with cells that were treated with ionomycin

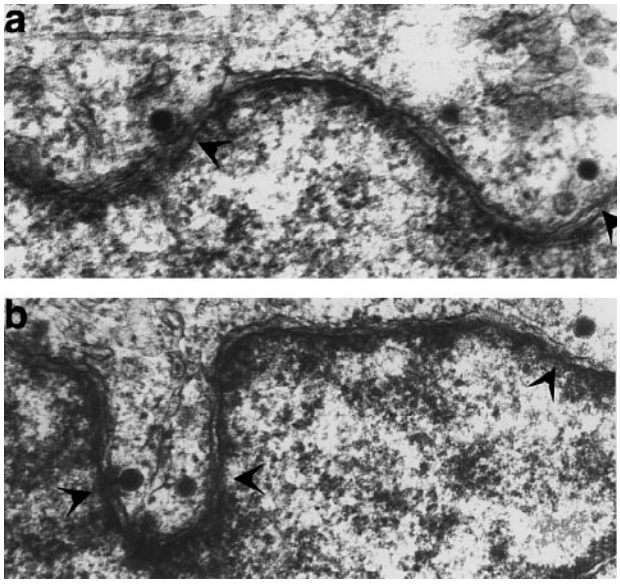

Fig. 3. Adenovirus docking to nuclear pore complexes in calcium-depleted cells. Purified adenovirus was bound to HeLa cells in the cold at 120 μg/ml and internalized into cells for 60 min in calcium medium (**a**) or EGTA/calcium medium (E/Ca) containing ionomycin (**b**) as described in Figure 2. Samples were fixed in 2% glutaraldehyde, embedded in Epon and processed for thin section electron microscopy as described in Materials and methods.

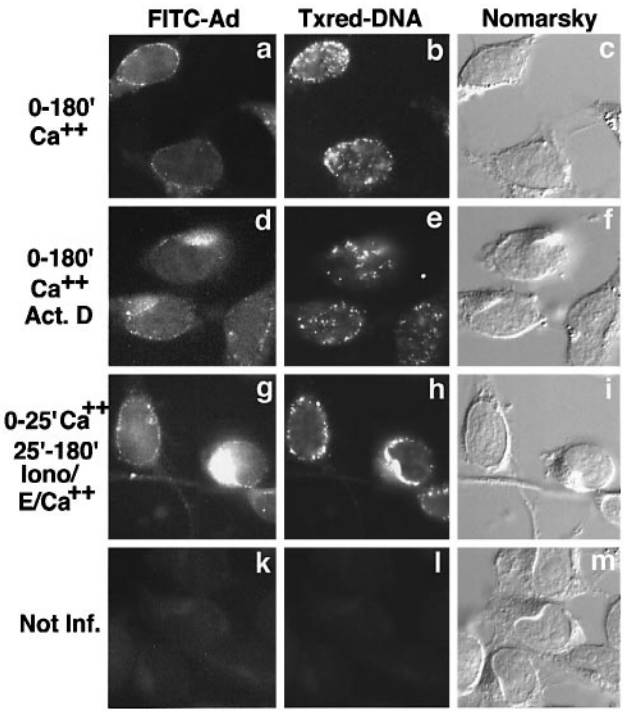

Fig. 4. Nuclear transport of viral capsid and DNA. FITC-labeled adenovirus bound to HeLa cells at 20 μg/ml was internalized in calcium medium (**a**–**c**), calcium medium containing 5 μM actinomycin D (**d**–**f**) for 180 min or in calcium medium for 25 min followed by EGTA/calcium medium (E/Ca) containing 5 μg/ml ionomycin for up to 180 min (**g**–**i**). Samples were fixed in methanol and paraformaldehyde, and processed for *in situ* hybridization using Texas Red-labeled adenovirus type 2 DNA as a probe. Non-infected cells were processed identically (**k**–**m**). Double fluorescence images and Nomarsky differential interference optics pictures were recorded with a CCD camera on a Reichert-Jung Polyvar microscope.

in EGTA/calcium medium at 20 min post-virus internalization (Figure 2d).

Transmission electron microscopy indicated that adenovirus particles were associated with NPCs in ionomycin-treated cells, indistinguishably from mock-treated cells (Figure 3). Identical results were obtained with FITC-labeled virus (data not shown). We thus concluded that depletion of lumenal calcium had no effect on adenovirus transport across the cytoplasm to the nuclear membrane and attachment to NPCs.

Nuclear envelope/ER calcium is needed for DNA transport into the nucleus

We next investigated whether nuclear import of incoming viral DNA and DNA-bound protein VII was disturbed in calcium-depleted cells. We determined the subcellular localization of viral DNA by a fluorescence *in situ* hybridization (FISH) assay at different times after warming cells containing filled or ionomycin-depleted internal calcium stores (Figures 4 and 5). DNA fragments of 200–500 bp in length from isolated adenovirus were labeled by nick translation in the presence of dUTP–Texas Red according to standard protocols (Sambrook *et al.*, 1989). Hybridization of probe DNA to target nucleic acids was performed by denaturation at 92°C and subsequent hybridization at 37°C as described (Ishov and Maul, 1996).

We first analyzed the DNA localization of FITC-labeled adenovirus in control cells under normal calcium conditions at 180 min post-warming, either in the presence or absence of 5 μg/ml actinomycin D (Figure 4a–f). At this concentration, actinomycin D is a potent inhibitor of DNA polymerase I and II transcription (Perry and Kelley, 1970). When images across the centers of the cells were recorded with a sensitive charge coupled device (CCD) camera, FITC-labeled viral capsid proteins could be seen at the nuclear envelope. The Texas Red fluorescent DNA probe was found throughout the nucleus and occasionally in the cytoplasm. The Texas Red signal represented incoming viral genomes, since first, the distribution was the same whether actinomycin D was present or absent. Second, neither FITC, nor Texas Red staining was found in uninfected cells (Figure 4k–m). Third, the Texas Red signal was quantitatively quenched by incubation of the infected cells with DNase, but not with RNase post-fixation in methanol (data not shown). When internal calcium stores were depleted of calcium with ionomycin from 25 to 180 min post-internalization, capsid proteins were still found at the nuclear envelope, but viral DNA was now concentrated around the nuclear envelope (Figure 4g–i). DNA was not seen across the nucleus. Taken together, these results indicate that in cells depleted of internal calcium stores, DNA remained in apparently close association with the capsid, excluded from the nucleus. Under normal calcium conditions, DNA effectively separated from the capsid and was apparently imported into the nucleus.

To confirm that viral DNA was indeed transported into the nucleus under normal calcium conditions, we analyzed the FISH assays by confocal microscopy. The nuclear envelope was visualized with anti-lamin A, B and C antibodies by indirect immunofluorescence (Figure 5A, green signal). After 25 min of warming cells containing unlabeled adenovirus, punctate signals of viral DNA (in red) were found almost exclusively in the cytoplasm

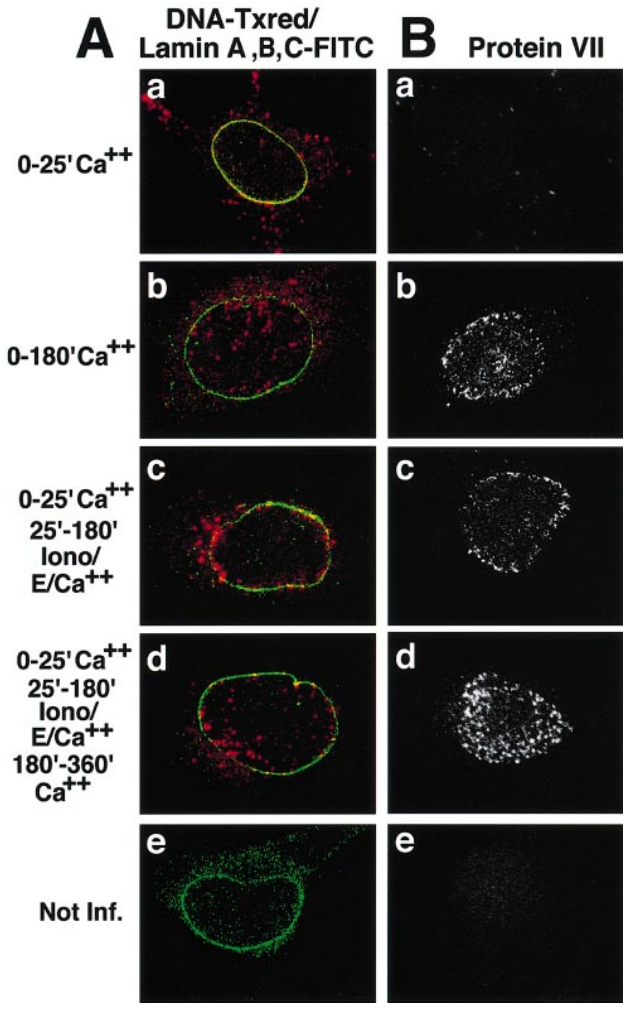

Fig. 5. Nuclear import of viral DNA and associated protein VII is blocked at the nuclear envelope in calcium-depleted cells. Purified adenovirus bound to HeLa cells at 20 μg/ml was internalized in calcium medium in the presence of 0.5 mM cycloheximide (CHX) for 25 min (**A** and **B**, a) or 180 min (A and B, b), respectively. Other cells were incubated in calcium medium for 25 min followed by EGTA/calcium medium (E/Ca) containing 5 μg/ml ionomycin for up to 180 min (A and B, c). Samples were either fixed directly or washed in warm calcium medium and incubated in regular medium for up to 360 min post-warming (A and B, d). Non-infected cells were processed identically (A and B, e). Samples were either processed for *in situ* hybridization with Texas Red-labeled virus DNA (A) or processed for immunostaining of protein VII (B). Confocal fluorescence micrographs were recorded across the centers of the cells using the FITC and Texas Red filter sets, respectively. Results are displayed as single optical slices with an estimated thickness of 0.2 μm each (A) or as a stack of four slices, each ~0.2 μm thick and 0.5 μm apart from the next slice (B).

(Figure 5A, a). Most probably, the majority of the fluorescent dots in these confocal sections represented single DNA molecules. A few particles appeared already at the nuclear envelope, as indicated by the yellow color of the merged lamin and DNA stainings. No DNA signal was detected in mock-infected cells (Figure 5A, e). After 180 min of warming in normal calcium medium, the majority of adenovirus DNA was found inside the nucleus, enclosed by the lamina rim (Figure 5A, b). Some DNA was located near the nuclear envelope, suggesting that this DNA was transported rather slowly into the nucleus. DNA staining could be detected inside the nucleus as early as 60 min post-warming (data not shown). In cells depleted of lumenal calcium by ionomycin from 25 to 180 min post-internalization, viral DNA was excluded from the nucleus and localized in the vicinity of the cytoplasmic face of the nuclear envelope (Figure 5A, c). When ionomycin/EGTA was removed and cells incubated in normal calcium-containing medium, DNA was imported into the nuclei, as shown at 360 min post-internalization (Figure 5A, d). Similar results were also obtained after 270 min (data not shown). These results confirmed that the cells were not poisoned irreversibly by the ionomycin/EGTA treatment. No significant inhibition of DNA import was observed when cells were treated with EGTA/calcium alone from 25 to 180 min post-internalization, suggesting that calcium was not depleted sufficiently from internal stores under these conditions (data not shown). When internal calcium was depleted by ionomycin/EGTA at 180 min post-warming, viral DNA was still found inside the nucleus, indicating that calcium depletion did not extract previously imported DNA from the nucleus (data not shown).

In a parallel experiment, the localization of incoming DNA-associated protein VII was analyzed by confocal microscopy and indirect immunofluorescence labeling in the presence of cycloheximide, which blocks viral and cellular protein synthesis (Figure 5B). Simultaneous staining of protein VII and viral DNA was unfortunately not possible under the conditions used for *in situ* hybridization (data not shown). As expected, protein VII was not detected after 25 min of warming, since viral DNA and associated proteins were still enwrapped by an essentially intact coat (Figure 5A and B, panels a, see also Greber *et al.*, 1993). No protein VII was detected in uninfected cells (Figure 5B, e). In the presence of calcium, protein VII was found inside the nucleus and also at the nuclear envelope after 180 min, similarly to the viral DNA (Figure 5B, b). In calcium store-depleted cells, protein VII was enriched at the nuclear envelope, but not detected inside the nucleus, suggesting that virus disassembly could occur, but not nuclear import (Figure 5B, c). When calcium was restored, protein VII was found inside the nucleus, in agreement with the *in situ* hybridization results (Figure 5B, d). The data demonstrated that depletion of calcium from internal cisternae had no effect on intracellular virus targeting to the nuclear envelope, but inhibited translocation of DNA and associated protein VII across the nuclear envelope.

Attachment to nuclear pore complexes is needed for capsid disassembly

It had been shown earlier by biochemical and genetic data that capsid disassembly was required for the transport of the DNA-associated protein VII into the nucleus (Miles *et al.*, 1980; Greber *et al.*, 1996). In order to test if the inhibition of DNA and protein VII import into the nucleus of calcium-depleted cells was due to reduced virus dissociation, we quantitated capsid disassembly using two different biochemical assays. Confocal microscopy together with the Bio-Rad area software was used to determine the abundance of hexon epitopes for a polyclonal anti-hexon antibody (Baum *et al.*, 1972; Greber *et al.*, 1995, 1996). We also determined the amount of hexon-associated DNA by co-immunoprecipitation experiments of [^{3}H]thymidine-

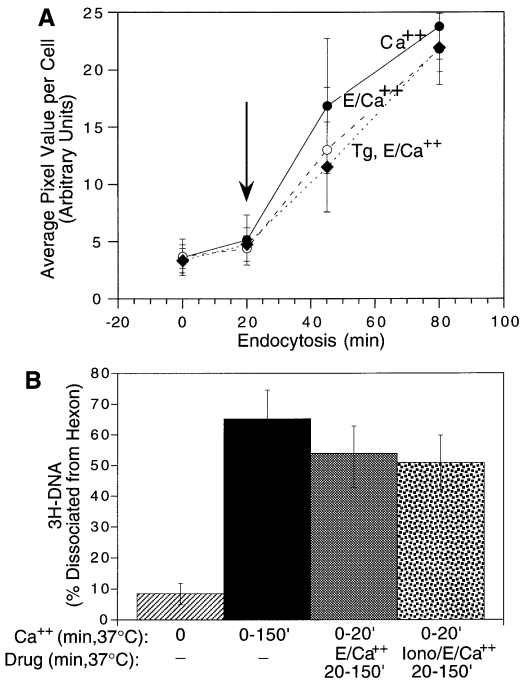

Fig. 6. Adenovirus disassembly in calcium-depleted cells. (**A**) Adenovirus (40 μg/ml) was bound to HeLa cells and internalized in calcium medium for 20 min. Infected cells were incubated further either in calcium medium, EGTA/calcium medium (E/Ca) or E/Ca medium containing 0.5 μM thapsigargin (Tg) for the indicated times (arrow). Samples were fixed and processed for indirect anti-hexon immunofluorescence microscopy. The amount of hexon fluorescence in single cells was quantitated in confocal micrographs across the cell centers and expressed as average pixel values including standard deviations as described previously (Greber *et al.*, 1995). (**B**) The amount of hexon-associated DNA was determined using anti-hexon immunoprecipitations of [^{3}H]thymidine-labeled adenovirus at different times of warming HeLa cells exactly as described (Greber *et al.*, 1996).

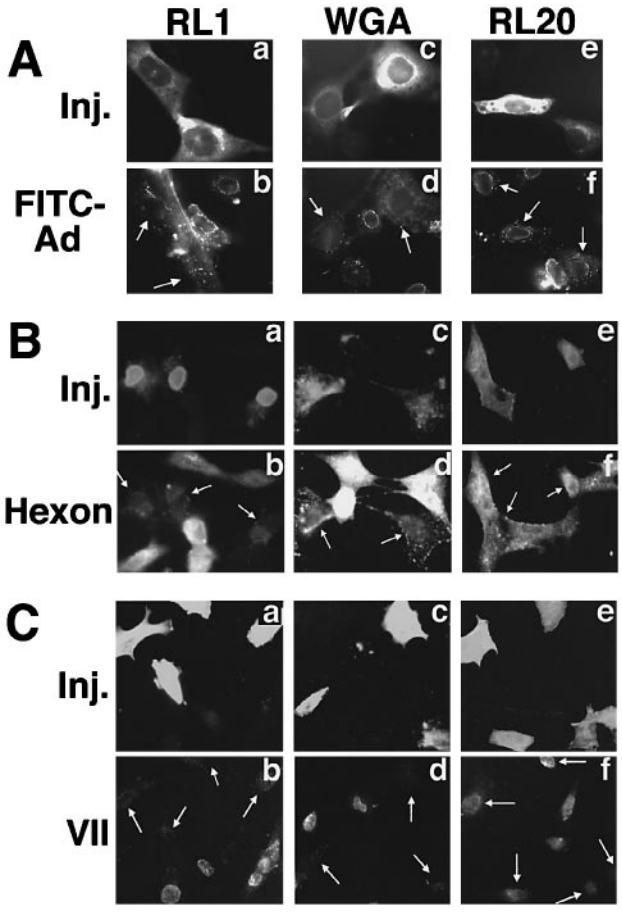

Fig. 7. Microinjections of nuclear pore complex inhibitors into cells containing internalized adenovirus. FITC-labeled adenovirus (**A**) or unlabeled virus (**B** and **C**) was bound at 20 μg/ml to HeLa cells in the cold and internalized in warm growth medium for 25 min. Cells were chilled in cold RPMI medium and injected with anti-NPC antibody RL1 (2 mg/ml IgM), WGA (1 mg/ml) or with anti-gp210 lumenal domain antibody RL20 (2 mg/ml IgG), in either the presence (A and C) or absence (B) of 0.2 mg/ml tracer substance, Texas Red-conjugated BSA (Inj, see also arrow). In (B), FITC-labeled WGA was microinjected and the RL1- and RL20-injected cells identified by immunofluorescence staining using FITC-conjugated goat anti-mouse IgG. After injections, cells were returned to regular growth medium for 35 min (A), or for 125 min, respectively (B and C). Samples were fixed in paraformaldehyde and processed for indirect immunofluorescence. Hexon was detected by the rabbit R70 antibody and goat anti-rabbit–Texas Red (B). Protein VII was detected by the rabbit R3 antibody and FITC-conjugated goat anti-rabbit IgG (C). Double fluorescence micrographs were recorded on a Zeiss Axiovert microscope equipped with FITC and Texas Red filter sets.

labeled virus DNA with an anti-hexon antibody exactly as described before (Greber *et al.*, 1996). The antigens of the major coat protein picked up by the first assay are of low abundance within the native particle, but are more abundant in stripped down or disassembled particles. The second assay measures the dissociation of virus DNA from hexon, provided that the degradation of the linker protein between the capsid and the viral chromosome, protein VI, has occurred. In both assays, we detected no significant differences in virus uncoating between calcium-depleted cells and control cells (Figure 6). Cells treated with thapsigargin in low calcium medium at 20 min of warming showed the typical increase in hexon fluorescence with time, reaching, at 80 min, nearly the same levels as the control cells or the cells treated with low calcium-containing medium alone (Figure 6A). Likewise, in the inhibitor-treated and the control cells, ~50 and 65%, respectively, of the viral DNA was dissociated from hexon after 150 min of internalization (Figure 6B). This was in good agreement with previous results (Greber *et al.*, 1996). The data suggested that depletion of lumenal calcium did not impair virus attachment to NPCs or virus disassembly.

To test whether NPCs were involved directly in virus disassembly, we microinjected WGA or the RL1 antibody into HeLa cells. WGA and RL1 bind to central domains of the NPC and block classical NLS-mediated nuclear import (Finlay *et al.*, 1987; Panté and Aebi, 1996a). Prior to injection, we internalized adenovirus into the cells for 25 min to ensure that virus was located in the cytoplasm, but not yet at the nuclear membrane. FITC-labeled adenovirus did not attach to the nuclear envelope in cells injected with WGA or RL1, i.e. 60 min post-internalization (Figure 7A, a–d, arrows). Cells injected with the control anti-gp210 lumenal domain antibody, RL20 (Greber *et al.*, 1990), showed clear enrichment of virus at the nuclear envelope after 60 min of internalization, similarly to uninjected cells (Figure 7A, e–f). RL1- or WGA-injected cells contained lower amounts of hexon fluorescence than either the control RL20-injected cells, or the uninjected cells at 150 min post-internalization (Figure 7B, a–f). RL1

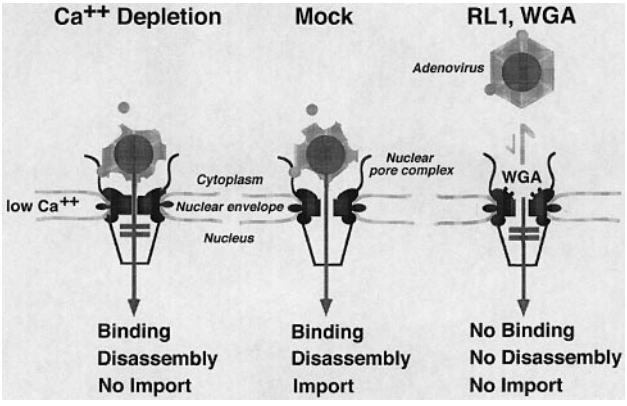

Fig. 8. The role of the nuclear pore complex in binding, disassembly and transport of adenovirus. In untreated cells, stripped down adenovirus particles attach to the cytosolic side of the NPC, disassemble and import DNA and associated protein VII into the nucleus. When calcium is removed from the nuclear envelope and the endoplasmic reticulum by ionophores or thapsigargin, incoming adenovirus attaches to NPCs and disassembles, but cannot import DNA and protein VII into the nucleoplasm. When either the RL1 antibody or WGA is bound to peripheral components of the NPC, adenovirus does not associate with the nuclear envelope, does not disassemble and is unable to import protein VII into the nucleus.

or WGA also blocked the appearance of protein VII in the nucleus, as observed 150 min post-internalization (Figure 7C, a–d, arrows). No protein VII staining in the cytoplasm was detected in these cells either, confirming that virus disassembly was blocked. As expected, protein VII was found across the nucleus, but not in the cytoplasm in the control cells, which were either injected with RL20 or were not injected (Figure 7C, e–f). These results demonstrated that peripheral NPC protein(s), alone or in conjunction with cytosolic factors, trigger virus dissociation, such that the DNA and associated proteins are liberated and accessible to the nuclear import machinery.

Discussion

This study demonstrated that the NPC has at least three distinct functions in adenovirus DNA delivery to the nucleus. It serves as a docking site for incoming virus particles, contains a particle dissociation activity and provides a gateway for nuclear import of the uncoated viral DNA (see also Figure 8).

When adenovirus enters cells, its capsid becomes weakened and partially stripped of stabilizing proteins (Greber *et al.*, 1996). The cytosolic capsid still contains ~80% of its original mass, including most of the hexon protein and a large fraction of penton base and protein IX (Greber *et al.*, 1993). It attaches to the cytoplasmic side of the NPC. We showed here that inhibitors of *O*-linked NPC glycoproteins, WGA and the RL1 antibody, prevented stable virus docking at the nuclear envelope and also disassembly of the capsids and nuclear import of the DNA-associated protein VII. WGA and RL1 are general inhibitors of NPC function. They block classical NLS-containing protein and ribonucleoprotein import into the nucleus (Finlay *et al.*, 1987; Martin and Helenius, 1991; Pollard *et al.*, 1996). Morphological evidence suggests that WGA acts by plugging the central transporter region of the NPC (Akey and Goldfarb, 1989; Panté and Aebi, 1996a). It can also block the association of NLS proteins with the nuclear envelope (Adam and Adam, 1994). *O*-linked NPC glycoproteins could thus directly, or together with cytosolic factors, interact with viral capsid proteins, such as hexon or penton base, and trigger capsid dissociation. WGA and RL1 could, however, also indirectly inhibit docking (and disassembly) of the capsid at cytoplasmic fibrils, perhaps by hindering the flexibility of these fibrils (Panté and Aebi, 1996a). In this case, the fibrils, perhaps in conjunction with cytosolic factors, might have a capsid dissociation function.

Although soluble or insoluble cytosolic factors might weaken the incoming capsid, it is unlikely that such factors trigger the capsid to disassemble and release the DNA within the cytoplasm. We know that adenovirus can still be recognized as virus particles at the nuclear envelope, specifically at the NPCs (Horwitz, 1990; Greber *et al.*, 1996). Furthermore, virus disassembly, as indicated by the appearance of hexon and protein VII epitopes, was blocked completely by the RL1 antibody, a highly specific probe for NPC-associated glycoproteins (Snow *et al.*, 1987; see also Figure 7B, a). In a normal infection, protein VII epitopes are never seen in the cytoplasm. They are only found at or within the nucleus, after dissociation of the capsid (Greber *et al.*, 1996; see also Figure 5). Protein VII could be seen outside the nucleus only if the nuclear pores were blocked by lumenal calcium depletion (Figure 5) or virus penetration across endosomes was inhibited (M.Suomalainen and U.F.Greber, unpublished results). Protein VII, together with FITC-labeled capsid proteins, localized to the vicinity of the nuclear envelope and lysosomes, respectively.

Following capsid dissociation at the nuclear membrane, viral DNA was transported into the nucleus. Our fluorescence micrographs suggested that the DNA was imported into the nucleus at the same time as the DNA-associated protein VII. Whether these proteins entered as a complex remains to be determined. The majority of fluorescent capsid proteins, including the heavily labeled hexon and protein IX, stayed at the nuclear envelope, indicating that the DNA physically separated from the capsid. Another DNA-associated protein, the covalently bound terminal protein, however, enters the nucleus together with the DNA. Inside the nucleus it serves to initiate viral DNA replication (for review, see Shenk, 1996). Since the terminal protein contains a classical NLS, it is possible that it also plays a role in threading the linear DNA molecule through the narrow opening of the NPC. At what level(s) DNA import requires the consumption of ATP (Chardonnet and Dales, 1972) remains to be determined.

Our results also showed that at least two calcium-dependent steps are involved in nuclear import of the viral DNA. The first step occurred before virus reached the cytosol. If extracellular calcium was depleted within the first 10 min of warming cells containing pre-bound virus, infectivity was reduced 5- to 10-fold. Since the half-time of virus internalization into cold-synchronized cells is ~10 min and virus penetration occurs 15–20 min post-warming, the first calcium-dependent step was either virus uptake or pentration of the endosomal membrane. Internalization and penetration both depend on interactions between the viral penton base and integrins (Wickham *et al.*, 1994). Extracellular divalent cations, particularly

calcium, are required for integrin binding to extracellular ligands (Hynes, 1992; Diamond and Springer, 1994). Integrin adhesiveness can, however, be modulated by intracellular calcium bursts originating from internal stores (Diamond and Springer, 1994). It is possible that, in our experiments, the reduction of extracellular calcium down to ~100 nm free calcium triggered calcium release from interal stores. Such an effect has been observed by complete depletion of extracellular calcium using chelators in the absence of any free calcium (Chao *et al.*, 1992). It cannot, therefore, be entirely excluded that, in our experiments, the functionality of integrins was also altered from inside out. The reduction of extracellular calcium to 100 nM in the absence of ionophores, however, generally leaves enough calcium within internal stores to allow fully efficient nuclear import of proteins (Greber and Gerace, 1995).

Nuclear import of the viral DNA was the second calcium-sensitive step (see also Figure 8). Cells depleted of internal calcium stores immediately after endosome penetration were unable to import viral DNA and the associated protein VII into the nucleus, as directly demonstrated by *in situ* hybridizations and confocal microscopy. This result was in agreement with infectivity measurements 14 h post-warming of cold-synchronized cells. Infectivity was reduced maximally when internal calcium stores were depleted until ~70 min post-warming. Depleting internal calcium stores at 100 min post-warming had little effect on infectivity, suggesting that the fastest viruses had already delivered their DNA into the nucleus. The inhibiton of DNA import was not due to a failure of virus disassembly, as demonstrated by immunoprecipitations and indirect immunofluorescence labeling experiments using antibodies against the internal capsid protein VII and the capsid protein hexon. Most probably, DNA import was blocked due to impermeable NPCs.

Calcium depletion from internal stores inhibits classical NLS-dependent protein import and passive diffusion into the nucleus in living cells (Greber and Gerace, 1995). Selective depletion of calcium from isolated nuclear envelope cisternae by inositol (1,4,5)-trisphosphate (IP3) decreases the permeability of the envelopes to small dextran molecules (Stehno-Bittel *et al.*, 1995). The permeability reduction is associated with the appearance of a central plug in the NPC (Perez-Terzic *et al.*, 1996). A conformational change in the NPC induced by low calcium in the nuclear envelope cisternae may sterically block the passage of macromolecules through the central pore and the diffusional channels. Possibly, one of the integral membrane proteins in the NPC scaffold is a calcium sensor that changes its conformation at low calcium levels.

In the living cell, the ER/nuclear envelope cisternae are, besides the mitochondria, a major reservoir for calcium. They can be used as a source and a sink to regulate calcium levels within the nucleus and the nearby cytosol (Lanini *et al.*, 1992; Humbert *et al.*, 1996). The extent of calcium depletion from the nuclear envelope upon hormone stimulation might regulate the permeability of the NPCs and could influence the duration and/or the extent of the nuclear calcium spike (Badminton *et al.*, 1996). Indeed, transient increases in nuclear calcium concentrations can contribute to the regulation of gene expression (Bachs *et al.*, 1994). However, hormone-stimulated cells may not reduce all trafficking of macromolecules across the nuclear envelope (Berridge, 1995). It is possible that cells containing low nuclear envelope calcium shut down certain pathways of nuclear import, such as GTP/importin-dependent nuclear transport involving classical NLSs (Sweitzer and Hanover, 1996). These cells could, in turn, activate a calcium/calmodulin-dependent import pathway, which operates through the NPCs independently of known cytosolic factors at the consumption of ATP (Pruschy *et al.*, 1994). It will be important to decipher the physiological control mechanisms of calcium contents in the nuclear envelope cisternae and how calcium affects molecular trafficking of proteins and nucleic acids across the NPC.

Materials and methods

Cells, virus and antibodies

Cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Switzerland) containing 7% Clone III serum or 7% fetal bovine serum (FBS) (Hyclone, Integra Biosciences AG, Switzerland), 1% non-essential amino acids, 1% glutamine and 100 U/ml streptomycin, 0.1 mg/ml penicillin (all from Gibco-BRL, Switzerland). Cell culture grade bovine serum albumin (BSA) was from Sigma (Chemie Brunschwig, Switzerland). HeLa cells (cervical epitheloid carcinoma) were used for adenovirus type 2 entry studies, KB cells (nasopharyngal carcinoma) for virus growth and A549 cells (human lung carcinoma) for virus plaque titration as described (Greber *et al.*, 1993). The mouse monoclonal antibody M73 against the early adenovirus proteins of the E1A transcription unit was obtained from E.Harlow (Charlestown, USA). Rabbit anti-hexon serum R70 was a gift from M.Horwitz (New York) and rabbit anti-protein VII was obtained from U.Pettersson (Uppsala, Sweden). Rabbit anti-nuclear lamin A, B and C peptide antibody 8188 and mouse monoclonal anti-NPC antibody RL1 were kindly supplied by L.Gerace (La Jolla, CA).

FITC and Texas Red labeling of virus and BSA

Adenovirus was isolated from Freon-extracted KB cells by double CsCl gradient centrifugation. Homogeneity was verified by negative staining with uranyl acetate and electron microscopy and SDS–PAGE as described (Horne, 1986; Greber *et al.*, 1993). Protein determination was by Micro BCA assay (Pierce, Socochim, Switzerland).

FITC-labeled adenovirus was prepared by dialyzing virus against 0.01 M sodium bicarbonate containing 0.1 M NaCl, 1 mM $MgCl_2$, pH 9 for 2 h, and the protein concentration was determined using Micro BCA assay (Pierce). The pH of the virus solution was adjusted to 9 if necessary by adding 0.5 M sodium bicarbonate, pH 9.0. Labeling was initiated by adding 18 µl of 0.5 mg/ml FITC solution [diluted immediately before use into 0.1 M sodium bicarbonate pH 9 from a 5 mg/ml stock solution in dimethylsulfoxide (DMSO)] to 125 µl of 1.8 mg/ml virus suspension. After 1 h in the dark, the reaction was terminated by adding 40 µl of freshly made 1.5 M hydroxylamine pH 8.5 for 1 h at room temperature. Virus was purified by CsCl centrifugation, dialyzed against 0.01 M Tris–HCl, 0.15 M NaCl, 1 mM $MgCl_2$ pH 8.1, assayed for protein concentration, frozen in 10% glycerol under liquid nitrogen and stored at –70°C for several months. Assuming that one per every three or four FITC molecules conjugated to virion proteins (according to Molecular Probes), approximately five FITC molecules are incorporated per hexon monomer. Densitometric analysis of FITC-labeled virus by SDS–PAGE by fluoroimaging (Molecular Dynamics) indicated that ~58% of the fluorochrome was incorporated into hexon, 8% was found in penton base, 7% in fiber/IIIa and 27% in protein IX.

For Texas Red labeling, purified virus was dialyzed against 0.1 M sodium bicarbonate, 0.05 M sodium chloride, 1 mM $MgCl_2$ pH 8.2 using 75 kDa cut off collodion membrane (Schleicher & Schuell, Germany). To 0.4 ml of 0.8 mg/ml adenovirus, 8 µl of 0.5 mg/ml Texas Red (diluted from 5 mg/ml stock solution in DMSO into dialysis buffer immediately before use) were added and incubated in the dark on a rocker at room temperature for 1 h. The reaction was terminated with hydroxylamine, and virus was repurified on a CsCl gradient as described above. Both Texas Red- and FITC-labeled virions had exactly the same

infectivity per protein as unmodified wild-type virus determined in plaque assays.

Virus entry and calcium depletion

HeLa cells were seeded 1–2 days before the experiment on alcian blue-coated glass coverslips placed in 24-well dishes (Costar, Integra Biosciences, Switzerland) containing FBS–DMEM or Clone III–DMEM in a humidified 37°C CO_2 incubator. On the day of infection, cells were 60–90% confluent. Adenovirus was bound to the cell surface for 90 min in 0.2 ml of cold RPMI medium (Gibco-BRL, Switzerland) containing 0.2% BSA, 15 mM HEPES–NaOH, pH 7.4. Virus was internalized in DMEM–0.2% BSA at 37°C in a CO_2 incubator for different times. Calcium was depleted from internal stores with 5 μg/ml ionomycin, A23187 (Sigma) or 0.5 μM thapsigargin (Gibco-BRL) for the indicated times in S-MEM medium containing 0.2% BSA, 1.1 mM EGTA, 0.1 mM $CaCl_2$ (E/Ca), L-glutamine and non-essential amino acids. Cells were either fixed in 3.3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min or the drugs were washed out with calcium-containing DMEM supplemented with 0.2% BSA, followed by fixation. Unreacted paraformaldehyde was quenched with 25 mM NH_4Cl in PBS for 5 min at room temperature, coverslips washed briefly with PBS and mounted on glass slides in either PBS containing 2% 1,4,-diazabicyclo[2,2,2]octane (DABCO) or *N*-propylgallate (10%), 70% glycerol, 0.03 M Tris–HCl, pH 9.5. Edges were sealed with nailpolish and coverslips observed immediately by fluorescence microscopy.

Fluorescence in situ hybidization (FISH)

Adenovirus DNA probe was prepared from twice-isolated CsCl-banded virions as described (Pettersson and Sambrook, 1973). DNA fragments of average size 200–500 bp were labeled with Texas Red–12-dUTP (ChromaTide™, Molecular Probes) by nick translation at 0.08 mg/ml according to standard protocols (Sambrook *et al.*, 1989). To detect incoming adenovirus genome, HeLa cells grown on glass coverslips in 24-well culture dishes were infected with adenovirus (4 μg in 0.2 ml cold RPMI–HEPES, pH 7.4 medium) and warmed for different times in DMEM–BSA medium with or without calcium. Samples were fixed in pre-chilled methanol at –20°C for 6 min, post-fixed in 1% paraformaldehyde in PBS for 30 min and quenched in 25 mM ammonium chloride in PBS at room temperature for 5 min. Samples were also treated with DNase I (50 μg/ml) or RNase A (50 μg/ml) in PBS containing 5 mM $MgCl_2$ pH 7, for 30 min at room temperature and fixed in paraformaldehyde. Coverslips were washed in PBS and inverted onto a glass slide over 5 μl of hybridization mix containing 0.9 ng/μl Texas Red-labeled adenovirus DNA probe and 15 ng/μl salmon sperm DNA in 50% formamide, 7.3% dextran sulfate and 0.74× SSC, as modified according to Ishov and Maul (1996). Coverslips were sealed with rubber cement and samples denatured for 3 min at 92°C on a dry block or at 90°C in a water bath. Slides were placed immediately into a moist chamber in a 37°C water bath and incubated for 2 h. Coverslips were washed in 50% deionized formamide–2× SSC in a 42°C water bath (3×5 min), followed by three 5 min washes in 0.1× SSC at 60°C and two quick washes in PBS at room temperature. Coverslips were mounted onto *N*-propylgallate (10%) in 70% glycerol, 0.03 M Tris–HCl, pH 9.5, sealed with nailpolish and analyzed by fluorescence microscopy. Cells treated with DNase I (Boehringer-Mannheim) for 30 min after methanol fixation gave no Texas Red signal. RNase A (Boehringer-Mannheim) treatment for 30 min, or DNase I for 1 min, however, gave discretely punctate cytoplasmic or nuclear Texas Red signals.

Fluorescence microscopy

Micrographs were aquired on a Reichert-Jung Polyvar microscope (Merck, Switzerland) equipped with Nomarski differential interference optics (DIC), a Texas Red filter set (excitation filter 530–585 nm, emission filter LP 615) and a fluorescein filter set (excitation filter 475–495 nm, emission filter BP 520–560) linked to a CCD video camera (Hamamatsu C5405, Hamamatsu Photonics, Germany). Images were obtained with the Argus-20 imaging acquisition software (Hamamatsu Photonics, Germany) and Photoshop Version 3.05 (Adobe) on a Macintosh computer on-line to the camera system.

Confocal microscopy

Samples were recorded using a Leica DM IRBE inverted microscope equipped with a Leica TCS 4D confocal laser-scanning device. All images were acquired with a 100× lens; the distances between neighboring sections was 0.5 μm. To localize the DNA by FISH assays, six successive sections in the middle of each cell nucleus were recorded simultaneously for both channels (FITC for nuclear lamins and Texas Red for viral DNA) at a pinhole setting of 100, corresponding to a diameter of 254.5 nm. Electronic zoom factors used were between 1.2 and 1.69. To analyze protein VII, the cells were fully scanned at a pinhole setting of 50 (corresponding to a diameter of 139.5 nm). Zoom factors used were between 1 and 1.87. The raw images were transferred to a Macintosh computer and processed using the software package NIH image (http://rsb.info.nih.gov/nih-image/).

Electron microscopy

Cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences), 0.1 M sodium cacodylate, pH 7.4 containing 0.2% tannic acid (Sigma) for at least 1 h at room temperature, washed in 0.1 M sodium cacodylate, pH 7.4 and post-fixed in 2% OsO_4 (Merck, Switzerland) for 10 min in 0.05 M Na cacodylate, pH 7.4. Samples were stained *en bloc* with 2% uranyl acetate (Merck, Switzerland) in 0.05 M Na maleate pH 5.2 (Sigma) for 60 min in the dark, dehydrated in acteon p.a. (Fluka Chemicals, Switzerland), embedded in Epon 812-ERL4206 (Fluka and Electron Microscopy Sciences) and ultrathin sectioned in an ultramicrotome (Reichert-Jung, Germany) onto pallodium film and carbon-coated electron microscopy grids (SYNAP TEK G-2793, Plano, Germany). Samples were analyzed in a transmission electron microscope (Zeiss EM 902A) at an acceleration voltage of 80 000.

Acknowledgements

We thank M.Nakano for analysis of FITC-labeled adenovirus. This work was supported by a grant from the Swiss National Science Foundation to U.F.G., by an EMBO long term fellowship to M.S. and the Kanton of Zurich as well as a grant from the NIH to A.H.

References

Adam,E.J.H. and Adam,S.A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.*, **125**, 547–555.

Akey,C.W. and Goldfarb,D.S. (1989) Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.*, **109**, 971–982.

Bachs,O., Agell,N. and Carafoli,E. (1994) Calmodulin and calmodulin-binding proteins in the nucleus. *Cell Calcium*, **16**, 289–296.

Badminton,M.N., Campbell,A.K. and Rembold,C.M. (1996) Differential regulation of nuclear and cytosolic calcium in HeLa cells. *J. Biol. Chem.*, **271**, 31210–31214.

Baum,S.G., Horwitz,M.S. and Maizel,J.V. (1972) Studies of the mechanism of enhancement of human adenovirus infection in monkey cells by simian virus 40. *J. Virol.*, **10**, 211–219.

Bergelson,J.M., Cunningham,J.A., Droguett,G., Kurt-Jones,E.A., Krithivas,A., Hong,J.S., Horwitz,M.S., Crowell,R.L. and Finberg,R.W. (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, **275**, 1320–1323.

Berridge,M.J. (1995) Calcium signalling and cell proliferation. *BioEssays*, **17**, 491–500.

Brostrom,C.O. and Brostrom,M.A. (1990) Calcium-dependent regulation of protein synthesis in intact mammalian cells. *Annu. Rev. Physiol.*, **52**, 577–590.

Chao,T.S., Byron,K.L., Lee,K.M., Villereal,M. and Rosner,M.R. (1992) Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor. *J. Biol. Chem.*, **267**, 19876–19883.

Chardonnet,Y. and Dales,S. (1972) Early events in the interaction of adenoviruses with HeLa cells: III. Relationship between an ATPase activity in nuclear envelopes and transfer of core material: a hypothesis. *Virology*, **48**, 342–359.

Clapham,D.E. (1995) Intracellular calcium: replenishing the stores. *Nature*, **375**, 634–635.

Cotten,M. and Weber,J.M. (1995) The adenovirus protease is required for virus entry into host cells. *Virology*, **213**, 494–502.

Davis,L.I. (1995) The nuclear pore complex. *Annu. Rev. Biochem.*, **64**, 865–896.

Diamond,M.S. and Springer,T.A. (1994) The dynamic regulation of integrin adhesiveness. *Curr. Biol.*, **4**, 506–517.

Duverger,E., Pellerinmendes,C., Mayer,R., Roche,A.C. and Monsigny,M. (1995) Nuclear import of glycoconjugates is distinct from the classical nls pathway. *J. Cell Sci.*, **108**, 1325–1332.

Fasolato,C., Innocenti,B. and Pozzan,T. (1994) Receptor activated calcium influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.*, **15**, 77–83.

Fields,B.N., Knipe,D.M. and Howley,P.M. (1996) *Fundamental Virology.* Lippincott-Raven, New York.
Finlay,D.R., Newmeyer,D.D., Price,T.M. and Forbes,D.J. (1987) Inhibition of *in vitro* nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.*, **104**, 189–200.
Gallay,P., Stitt,V., Mundy,C., Oettinger,M. and Trono,D. (1996) Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J. Virol.*, **70**, 1027–1032.
Gerace,L. and Foisner,R. (1994) Integral membrane proteins and dynamic organization of the nuclear envelope. *Trends Cell Biol.*, **4**, 127–131.
Goldberg,M.W. and Allen,T.D. (1995) Structural and functional organization of the nuclear envelope. *Curr. Opin. Cell Biol.*, **7**, 301–309.
Görlich,D. and Mattaj,.W. (1996) Protein kinesis—nucleocytoplasmic transport. *Science*, **271**, 1513–1518.
Greber,U.F. and Gerace,L. (1992) Nuclear protein import is inhibited by an antibody to a lumenal epitope of a nuclear pore complex glycoprotein. *J. Cell Biol.*, **116**, 15–30.
Greber,U.F. and Gerace,L. (1995) Depletion of calcium from the lumen of the endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus. *J. Cell Biol.*, **128**, 5–14.
Greber,U.F. and Kasamatsu,H. (1996) Nuclear targeting of adenovirus and simian virus SV40. *Trends Cell Biol.*, **6**, 189–195.
Greber,U.F., Senior,A. and Gerace,L. (1990) A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large lumenal domain and a small cytoplasmic tail. *EMBO J.*, **9**, 1495–1502.
Greber,U.F., Willetts,M., Webster,P. and Helenius,A. (1993) Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, **75**, 477–486.
Greber,U.F., Singh,I. and Helenius,A. (1994) Mechanisms of virus uncoating. *Trends Microbiol.*, **2**, 52–56.
Greber,U.F., Webster,P., Weber,J. and Helenius,A. (1996) The role of the adenovirus protease in virus entry into cells. *EMBO J.*, **15**, 1766–1777.
Hinshaw,J.E., Carragher,B.O. and Milligan,R.A. (1992) Architecture and design of the nuclear pore complex. *Cell*, **69**, 1133–1141.
Hong,S.S., Karayan,L., Tournier,J., Curiel,D.T. and Boulanger,P.A. (1997) Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J.*, **16**, 2294–2306.
Horne,R.W. (1986) Electron microscopy of crystalline arrays af adenoviruses and their components. In Harris,J.R. and Horne,R.W. (eds), *Electron Microscopy of Proteins: Viral Structure*. Academic Press, New York, Vol. 5, pp. 71–101.
Horwitz,M.S. (1990) Adenoviruses. In Fields,B.N. and Knipe,D.M. (eds), *Virology.* Raven Press, New York, Vol. 1, pp. 1723–1740.
Humbert,J.P., Matter,N., Artault,J.-C., Köppler,P. and Malviya,A.N. (1996) Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium signaling by inositol 1,4,5-trisphosphate. *J. Biol. Chem.*, **271**, 478–485.
Hynes,R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, **69**, 11–25.
Ishov,A.M. and Maul,G.G. (1996) The periphery of nuclear domain 10 (Nd10) as site of DNA virus deposition. *J. Cell Biol.*, **134**, 815–826.
Kann,M., Bischof,A. and Gerlich,W.H. (1997) *In vitro* model for the nuclear transport of the hepadnavirus genome. *J. Virol.*, **71**, 1310–1316.
Lanini,L., Bachs,O. and Carafoli,E. (1992) The calcium pump of the liver nuclear membrane is identical to that of endoplasmic reticulum. *J. Biol. Chem.*, **267**, 11548–11552.
Mangel,W.F., McGrath,W.J., Toledo,D.L. and Anderson,C.W. (1993) Viral DNA and a viral peptide can act as cofactors of adenovirus virion proteinase activity. *Nature*, **361**, 274–275.
Martin,K. and Helenius,A. (1991) Transport of incoming influenza virus nucleocapsids into the nucleus. *J. Virol.*, **65**, 232–244.
Melchior,F. and Gerace,L. (1995) Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.*, **7**, 310–318.
Miles,B.D., Luftig,R.B., Weatherbee,J.A., Weihing,R.R. and Weber,J. (1980) Quantitation of the interaction between adenovirus types 2 and 5 and microtubules inside infected cells. *Virology*, **105**, 265–269.
Miller,L. (1996) Insect viruses. In Fields,B.N., Knipe,D.M. and Howley,P.M. (eds), *Fundamental Virology.* Lippincott-Raven, New York, pp. 401–424.
Panté,N. and Aebi,U. (1996a) Sequential binding of import ligands to distinct nucleopore regions during their nuclear import. *Science*, **273**, 1729–1732.
Panté,N. and Aebi,U. (1996b) Toward the molecular dissection of protein import into nuclei. *Curr. Opin. Cell Biol.*, **8**, 397–406.
Perez-Terzic,C., Pyle,J., Jaconi,M., Stehno-Bittel,L. and Clapham,D.E. (1996) Conformational states of the nuclear pore complex induced by depletion of nuclear calcium stores. *Science*, **273**, 1875–1877.
Perry,R.P. and Kelley,D.E. (1970) Inhibition of RNA synthesis by actinomycin D: characteristic dose–response of different RNA species. *J. Cell Physiol.*, **76**, 127–139.
Pettersson,U. and Sambrook,J. (1973) Amount of viral DNA in the genome of cells transformed with adenovirus type 2. *J. Mol. Biol.*, **73**, 125–130.
Pollard,V.W., Michael,W.M., Nakielny,S., Siomi,M.C., Wang,F. and Dreyfuss,G. (1996) A novel receptor-mediated nuclear protein import pathway. *Cell*, **86**, 985–994.
Pozzan,T., Rizzuto,R., Volpe,P. and Meldolesi,J. (1994) Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.*, **74**, 595–636.
Prchla,E., Plank,C., Wagner,E., Blaas,D. and Fuchs,R. (1995) Virus-mediated release of endosomal content *in vitro*: different behavior of adenovirus and rhinovirus serotype 2. *J. Cell Biol.*, **131**, 111–123.
Pruschy,M., Ju,Y., Spitz,L., Carafoli,E. and Goldfarb,D.S. (1994) Facilitated nuclear transport of calmodulin in tissue culture cells. *J. Cell Biol.*, **127**, 1527–1536.
Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*, **83**, 683–692.
Roizman,B. and Sears,A.E. (1996) Herpes viruses and their replication. In Fields,B.N., Knipe,D.M. and Howley,P.M. (eds), *Fundamental Virology.* Lippincott-Raven, New York, pp. 1043–1107.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shenk,T. (1996) Adenoviridae: the viruses and their replication. In Fields,B.N., Knipe,D.M. and Howley,P.M. (eds), *Fundamental Virology.* Lippincott-Raven, New York, pp. 979–1016.
Sodeik,B., Ebersold,M.W. and Helenius,A. (1997) Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell Biol.*, **136**, 1007–1021.
Snow,C.M., Senior,A. and Gerace,L. (1987) Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.*, **104**, 1143–1156.
Stehno-Bittel,L., Perez-Terzic,C. and Clapham,D.E. (1995) Diffusion across the nuclear envelope inhibited by depletion of the nuclear envelope calcium store. *Science*, **270**, 1835–1838.
Stevenson,M. (1996) Portals of entry: uncovering HIV nuclear transport pathways. *Trends Cell Biol.*, **6**, 9–15.
Stewart,P.L., Fuller,S.D. and Burnett,R.M. (1993) Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J.*, **12**, 2589–2599.
Svensson,U. and Persson,R. (1984) Entry of adenovirus 2 into HeLa cells. *J. Virol.*, **51**, 687–694.
Sweitzer,T.D. and Hanover,J.A. (1996) Calmodulin activates nuclear protein import—a link between signal transduction and nuclear transport. *Proc. Natl Acad. Sci. USA*, **93**, 14574–14579.
Thastrup,O., Cullen,P.J., Drobak,B.K., Hanley,M.R. and Dawson,A.P. (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. *Proc. Natl Acad. Sci. USA*, **87**, 2466–2470.
Tomko,R.P., Xu,R. and Philipson,L. (1997) HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl Acad. Sci. USA*, **94**, 3352–3356.
van Oostrum,J. and Burnett,R.M. (1985) Molecular composition of the adenovirus type 2 virion. *J. Virol.*, **56**, 439–448.
Whittaker,G., Bui,M. and Helenius,A. (1996) The role of nuclear import and export in influenza virus infection. *Trends Cell Biol.*, **6**, 67–71.
Wickham,T.J., Mathias,P., Cheresh,D.A. and Nemerow,G.R. (1993) Integrin-alpha-v-beta-3 and integrin-alpha-v-beta-5 promote adenovirus internalization but not virus attachment. *Cell*, **73**, 309–319.
Wickham,T.J., Filardo,E.J., Cheresh,D.A. and Nemerow,G.R. (1994) Integrin αvβ5 selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.*, **127**, 257–264.

Received on May 21, 1997; revised on July 9, 1997