The role of the adenovirus protease in virus entry into cells

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Adenovirus uncoating is a stepwise process which culminates in the release of the viral DNA into the nucleus through the nuclear pore complexes and dissociation of the capsid. Using quantitative biochemical, immunochemical and morphological methods, we demonstrate that inhibitors of the cysteine protease, L3/p23, located inside the capsid block the degradation of the capsid-stabilizing protein VI, and prevent virus uncoating at the nuclear membrane. There was no effect on virus internalization, fiber shedding and virus binding to the nuclear envelope. The viral enzyme (dormant in the extracellular virus) was activated by two separate signals, neither of which was sufficient alone; virus interaction with the integrin receptor (inhibited with RGD peptides) and re-entry of the virus particle into a reducing environment in the endosome or the cytosol. Incorrectly assembled mutant viruses that lack the functional protease (ts1) failed at releasing fibers and penetrating into the cytosol. The results indicated that L3/p23 is needed not only to assemble an entry-competent virus but also to disassemble the incoming virus.
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Introduction

Entry of adenovirus into a host cell is a complex, stepwise process. First, the virus attaches with high affinity to an as yet unidentified cell surface receptor via the globular head domains of the protruding viral fibers (Philipson *et al.*, 1968; Louis *et al.*, 1994; Xia *et al.*, 1995). A secondary receptor, the fibronectin binding integrin, then associates with the penton base protein, and as a result the virus is endocytosed via coated pits and coated vesicles (Varga *et al.*, 1991; Bai *et al.*, 1993; Belin and Boulanger, 1993; Wickham *et al.*, 1993). Penetration of the viral particles from endosomes into the cytosol occurs by acid-triggered lysis of the endosomal membrane, a reaction most likely mediated by the integrins and the penton base protein (Pastan *et al.*, 1986; Wickham *et al.*, 1994). Having thus entered the cytosol, the viral particles are next transported through the cytoplasm to the nuclear mem-

brane, and attach to the cytosolic face of the nuclear pore complexes (Lonberg-Holm and Philipson, 1969; Chardonnet and Dales, 1970; Dales and Chardonnet, 1973; Greber *et al.*, 1993). The DNA is released through the pore, and the viral capsid dissociates (Horwitz, 1990).

At different stages during passage from the cell surface to the nuclear pore, several proteins are detached from the virion and some of them are even degraded (Greber *et al.*, 1993). The first to be lost are the fibers which dissociate already during endocytic uptake. Some of the penton base complexes are also removed during the early stages of entry, followed by proteins IIIa, VIII and IX, which detach from the surface of the virion in the cytosol (Greber *et al.*, 1994). The degradation of protein VI, a protein located inside the capsid, underneath the peripentonal hexons, starts already in endocytic vacuoles and is completed when the virus reaches the nuclear envelope (Greber *et al.*, 1993). The stepwise dismantling most likely results in the weakening of the capsid structure so that the DNA can be released efficiently at the nuclear pore, and the remaining empty capsid dissociated.

Since protein VI is thought to link the DNA to the inside of the capsid shell (Russell and Precious, 1982), we have speculated that its elimination allows the release of the viral DNA and is therefore a necessary part of the uncoating process. How the protein gets degraded is not known at present. However, since it is located inside the capsid and, thus, is presumably poorly accessible to cellular proteases, we have turned our attention to a viral protease, the cysteine protease L3/p23, located in the internal cavity at ~10 copies per virion (Anderson, 1990). This protease, encoded in the late cassette L3 as a 23 kDa polypeptide, is essential during the assembly of the virus in the nucleus of infected cells (Weber, 1995). It is responsible for cleaving six different capsid protein precursors during the assembly process, and is itself packaged with the DNA into viral particles.

L3/p23 has eight cysteine residues, of which at least one is needed for proteolytic activity (Weber, 1995). A thiol–disulfide exchange mechanism is thought to provide activation of the protease during virus assembly via a disulfide-linked peptide dimer corresponding to the C-terminal peptide of the protein VI precursor (Mangel *et al.*, 1993; Webster *et al.*, 1993). When the activating peptide is present, the activity of the enzyme is stimulated by small concentrations of dithiothreitol (DTT) (Webster *et al.*, 1994). Enzymatic activity is destroyed by alkylating agents such as *N*-ethylmaleimide (NEM), indicating that free cysteines are involved in the catalytic mechanism (Webster *et al.*, 1989, 1994). A point mutation in the protease gene, the ts1 mutation, causes the enzyme to misfold irreversibly and prevents its packaging into particles (Weber, 1976; Rancourt *et al.*, 1995). The resulting unprocessed particles do contain the DNA genome of the
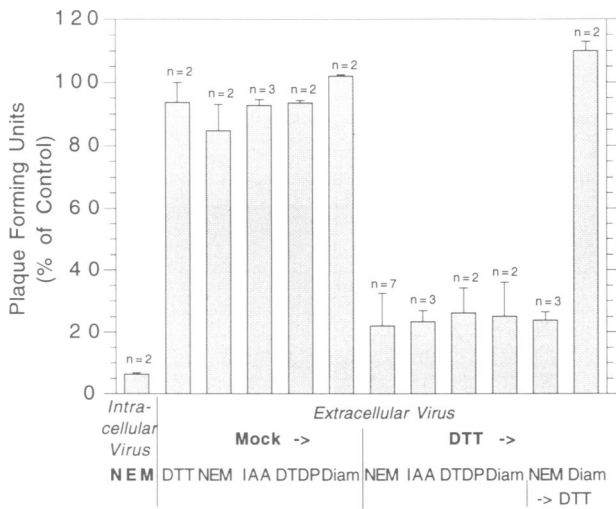
Fig. 1. Inhibition of cell infection with reduced/alkylated adenovirus. Intracellular adenovirus or isolated adenovirus were treated with reducing and alkylating agents as described in Materials and methods. Infectivity was determined by taking the mean plaque number from two 10-fold dilutions. 100% infectivity was obtained with untreated virus. n indicates the number of independent experiments and bars represent the standard deviations.

virus, but are unable to penetrate from endosomes, and are obviously non-infectious (Miles *et al.*, 1980; Hannan *et al.*, 1983; Cotten and Weber, 1995).

The experiments described here indicate that the L3/p23 protease is not only needed for virus assembly but also for uncoating during entry into the host cell. Active protease packaged into viral particles during assembly in the reducing cell nucleus is inactivated in the oxidizing extracellular environment after virus release from the infected cells. The protease is apparently reactivated during virus entry into a reducing environment and by integrin binding to penton base. As a result, the capsid-stabilizing protein VI is degraded, the capsid disassembles at the nuclear pore complex and the viral DNA can be released into the nucleus.

Results

Sulfhydryl groups are important in adenovirus infectivity

As a first test of whether L3/p23 might be needed during virus entry, we added the protease activator DTT to isolated virus and then inactivated the reduced protease by treating virus with different alkylating and oxidizing agents. These conditions have been reported to cause inactivation of the isolated protease (Webster *et al.*, 1989, 1994). As alkylating agents, we used NEM, iodoacetamide (IAA) and (4′,4′)-dithiodipyridine (DTDP). A strong sulfhydryl oxidant, diamide, was also used (Kosower and Kosower, 1987).

These treatments were all found to lower infectivity 4- to 5-fold, as measured by plaque assays 5 days after infection (Figure 1). When infectivity was measured by scoring cells that expressed the early protein E1A using indirect immunofluorescence (Whyte *et al.*, 1988), we found that the DTT/NEM-treated virus infected only 3% of the cells, while mock-treated control virus infected 60% (data not shown). The DTT/NEM-inactivated virus

could not be rescued by DTT reduction. In contrast, the DTT/diamide-treated virus regained full infectivity. Treatment of native virus with NEM, IAA, DTDP, diamide or DTT alone had no effect on infectivity (Figure 1).

These results indicated that free thiols, either in the viral protease, or in other structural proteins of the virus, were critical for infectivity. Since both reduction and alkylation were needed, these results suggested that the critical cysteines were in the oxidized form in the isolated viral particles, and had to be reduced to become a target for alkylation.

Since the nucleoplasm in which the viruses assemble provides a reducing environment, it seemed likely that the cysteines in intranuclear, newly synthesized viral particles were in the reduced form. When NEM was added to infected cells shortly before the end of the infectious cycle and the virus isolated, the infectivity was found to be 15- to 20-fold lower than in mock-treated control (Figure 1). In other words, no prior reduction with DTT was needed to make the virus sensitive to alkylation. Apparently, the critical cysteines become oxidized only when a virus is released from the cell into the extracellular space. However, the oxidation event was not required to render the virus infectious because adenovirus isolated under reducing conditions from cells and applied to uninfected cells in the continuing presence of 5 mM DTT was equally infectious as control viruses (data not shown).

The data suggested that release of intracellular virus from the reducing cellular milieu into the oxidizing extracellular medium resulted in the formation of one or more disulfide bonds in the viral proteins, which protected critical sulfhydryl residue(s) against alkylation. However, if these bonds were first artificially reduced, they could be alkylated or re-oxidized in such a way that infectivity was impaired.

The L3/p23 protease as a target of alkylation

To determine which viral proteins were alkylated, we incubated DTT-reduced and non-reduced viruses with [³H]NEM and then analyzed the proteins by SDS–PAGE and autoradiography. A number of bands were labeled in both preparations: the hexon, penton base, protein V and two unidentified proteins with apparent mol. wts of 60 kDa and ~22 kDa (Figure 2A, lanes 1 and 2). Protein VI, which is known to be cysteine-free and migrate near the 32.5 kDa marker protein (Akusjärvi and Persson, 1981; Greber *et al.*, 1993), was not labeled, confirming the specificity of labeling.

The most strongly labeled protein band was located between proteins VI and VII and had an apparent mol. wt of 28 kDa. It was labeled specifically in the reduced virus, but not in native or reduced and alkylated DTT/NEM virus. It migrated identically with recombinant L3/p23 protease and with protease isolated from purified adenovirus (Anderson, 1990). On Western blots, it could be labeled with anti-L3/p23 protease antiserum (Figure 2B). We concluded that the band corresponded to the viral protease, and that the protease was a major target for alkylation after DTT reduction.

Interestingly, when extracted from reduced virus, a large fraction of the protease migrated faster (Figure 2B, lane 7) than the protease from the non-reduced virus (Figure 2B, lane 6). Generally, reduced proteins migrate more
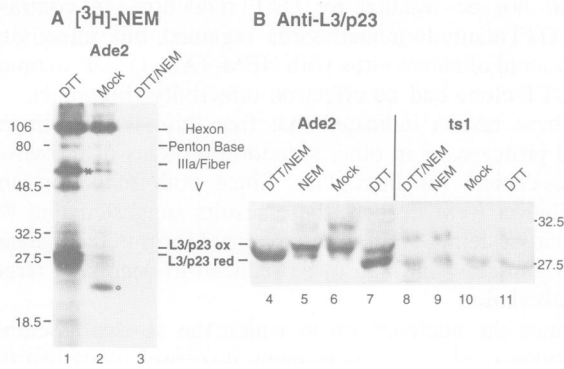
Fig. 2. [³H]NEM labeling of adenovirus-associated L3/p23 protease. Wild-type adenovirus was mock, DTT or DTT/NEM treated, dialyzed, labeled with [³H]NEM for 20 min, quenched with cold NEM (10 mM), TCA precipitated and equal quantities of protein were separated by SDS–PAGE and analyzed by fluorography (**A**). Star and ring symbols indicate unidentified proteins. In a parallel experiment, purified wild-type adenovirus or ts1 virus (grown at the restrictive temperature of 39°C) was treated or not treated with DTT, NEM or DTT/NEM, TCA precipitated, separated by SDS–PAGE, transferred to nitrocellulose and probed with an anti-protease antiserum. Bound IgG was detected by ECL with a goat anti-rabbit IgG coupled to horseradish peroxidase (**B**). The identity of bands migrating at 31 kDa (lane 6) and at 30 kDa (lanes 8 and 9) are unknown.

slowly than their oxidized counterparts. In fact, the expected slower migration after reduction has been described for the recombinant version of the L3/p23 protease in its chemically or air-oxidized versus reduced forms (Tihanyi *et al.*, 1993). The aberrant gel mobility of the viral protease in our gels was therefore not likely to be caused by intrachain disulfides. Instead, it was consistent with the previously suggested presence of an interchain disulfide that connects the protease to its activator peptide, the C-terminal peptide from the protein VI precursor (Mangel *et al.*, 1993; Webster *et al.*, 1993). That the viral protease was covalently associated with a peptide was supported by the observation that the defective ts1 protease, which cannot generate the protein VI precursor peptide (Webster *et al.*, 1993), did not show the aberrant gel mobility after reduction (Figure 2B).

Internalization of ts1 and DTT/NEM-treated viruses is normal

Next, we determined whether viruses that had been inactivated by DTT/NEM treatment and viruses lacking the protease (ts1 mutant virus) were affected in their early interactions with host cells. To monitor the kinetics and efficiency of binding and endocytic internalization, the [³⁵S]methionine-labeled viruses were allowed to bind to KB cells in the cold. Endocytosis was initiated by adding warm medium, whereafter surface viruses and endocytosed viruses were assayed by determining trypsin accessibility of the cell-associated viruses in the cold (Greber *et al.*, 1993). Degradation of the viral proteins was determined using trichloroacetic acid (TCA) precipitation.

No abnormalities in binding to cells in the cold were detected for either virus. For the DTT/NEM viruses, the kinetics and the efficiencies of internalization were normal and degradation was minimal (Figure 3). Similar results were obtained with viruses treated with NEM or DTT alone (data not shown). Internalization of all three virus
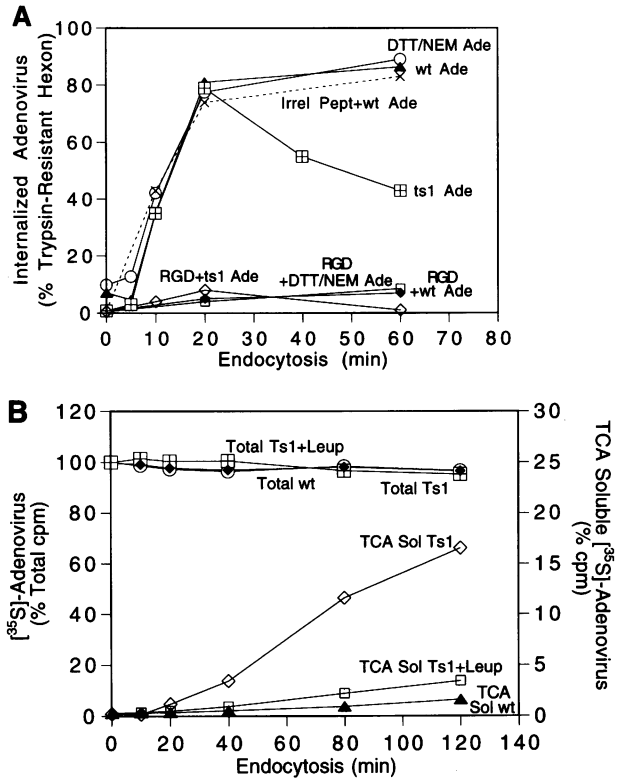

Fig. 3. Internalization and degradation of wild-type and ts1 adenovirus in the presence of inhibitors. (**A**) [³⁵S]Methionine-labeled wild-type or ts1 adenovirus was treated or not treated with DTT/NEM, dialyzed, bound to KB cells in the cold (10⁵ c.p.m. per dish), and endocytosed in the presence or absence of GRGDSP peptides or RGES peptides (irrelevant) for different times at 37°C. Cells were chilled in PBS and treated with cold trypsin. Virus was TCA precipitated, and cleaved (surface-bound) and uncleaved hexon (internalized) analyzed by SDS–PAGE and fluorography. Internalized adenovirus was scored as the percentage of trypsin-resistant hexon normalized to the 0 min time point determined by densitometric scanning. (**B**) [³⁵S]Methionine-labeled wild-type or ts1 adenovirus was bound in the cold to KB cells, which were pre-treated or not with 20 μg/ml leupeptin (Sigma, St Louis, MO) for 16 h, and endocytosed at 37°C for different times. TCA-soluble and -insoluble radioactivity was determined by liquid scintillation counting at non-quenching dilutions and data were normalized to the 0 min time point. 100% was in the range 40 000–50 000 c.p.m. for wild-type adenovirus (wt Ade) and 60 000–70 000 c.p.m. for ts1 virus (B).

preparations was inhibited with RGD peptides, indicating that it was integrin-mediated (Figure 3A) (Wickham *et al.*, 1993). Control peptides had no effect (Figure 3A).

When the entry kinetics of the ts1 virus were determined, an interesting abnormality was observed. Whereas the initial uptake looked normal (Figure 3A), the amount of cell-associated virus began to drop after 20 min, reaching a value half that of control virus after 60 min. Since this was not accompanied by a corresponding rise in virus degradation (only 12% was degraded after 40 min, Figure 3B), we concluded that a large fraction of the internalized viruses was recycled back to the plasma membrane. This was consistent with the observation that the ts1 virus is unable to penetrate from endosomes into the cytoplasm (Miles *et al.*, 1980; Cotten and Weber, 1995; see below). Despite recycling back to the plasma membrane, an increasingly large fraction of the ts1 virus was degraded, reaching >60% after 120 min (Figure 3B). Ts1 degradation
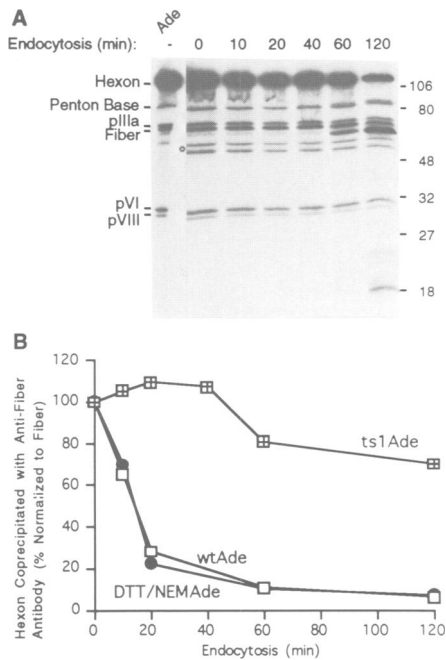
A

B

Fig. 4. Inhibition of fiber dissociation and protein VI precursor degradation in ts1 virus, but not DTT/NEM adenovirus. [^{35}S]Methionine-labeled ts1 or wild-type adenovirus treated or not with DTT/NEM was endocytosed into KB cells for different times and the polypeptide composition determined by anti-fiber immunoprecipitations and SDS–PAGE and autoradiography (**A**: ts1 virus). The amount of hexon co-precipitated with fiber was quantitated by densitometry and expressed as a percentage of co-precipitated hexon at time 0 (**B**).

included all the labeled capsid proteins (not shown), but was significantly reduced if cells were pre-treated with the lysosomal protease inhibitor leupeptin, a membrane-impermeable agent that enters endosomes and lysosomes by fluid phase endocytosis and serves as an effective inhibitor of proteases in these compartments (Seglen et al., 1979). The data supported the notion that ts1 was degraded in endosomes and/or lysosomes. In contrast, the wild-type and DTT/NEM viruses were saved from recycling and degradation perhaps by their efficient penetration into the cytoplasm. The integrin receptors that adenovirus uses are known to undergo endocytosis (Felding-Habermann and Cheresh, 1993; Panetti and McKeown-Longo, 1993; Shakibaei et al., 1993; Diamond and Springer, 1994).

Aside from this recycling phenomenon with ts1 virus, we thus found that the early interactions during entry were normal. The activity of the L3/p23 protease was apparently not essential for virus binding to cells nor endocytosis via integrin receptors.

Fiber shedding and penetration into the cytoplasm
We have shown previously that prior to virus penetration from endosomes into the cytoplasm the fibers are quantitatively released, and that this release does not require a low pH in the endosomal compartment (Greber et al., 1993). Using a co-precipitation assay, we found that the protease-free ts1 particles did not release their fibers quantitatively (Figure 4). After 120 min of warming, 80% of the non-degraded hexon was still precipitable with anti-fiber antibodies. In contrast, the DTT/NEM-inactivated viruses released their fibers as quickly and efficiently as control

virus (Figure 4B). This observation implied that protease activity as such was not required for fiber shedding. The poor shedding of fibers from ts1 virus was most likely due to the same cause as failure of the viruses to penetrate: the incomplete assembly status of capsids in which precursor proteins are uncleaved (Philipson, 1983; Weber, 1995).

To test whether the DTT/NEM-inactivated viral particles were able to penetrate into the cytoplasm, we performed electron microscopy after a 60 min incubation at 37°C with control virus and DTT/NEM-inactivated virus at high multiplicity (Figure 5). In both cases, most of the particles seen were in the cytoplasm, many near the nuclear envelope and close to the pore complexes. Quantitative analysis of >200 particles located closer than two virus diameters from the nuclear envelope in 30–40 different cells (50–60 micrographs) revealed that, for both viruses, an average of 4.3 viral particles per micrograph sample were associated with the nuclear envelope. Of the nuclear envelope-associated viruses, 29% of control and 21% of the DTT/NEM viruses were associated with pore complexes, which contained on average 1.3 mock-treated viruses and 1.1 DTT/NEM-treated viruses, respectively (Table I). Since the pore complexes cover only ~10% of the total nuclear envelope in these cells (Gerace and Burke, 1988), the association was clearly specific.

We concluded that DTT/NEM-inactivated viruses were able to penetrate into the cytoplasm efficiently, move to the nuclear envelope and bind to nuclear pore complexes.

Protein VI degradation is inhibited in protease-defective or -deficient viruses
As already mentioned, protein VI is located inside the capsid in contact with the DNA (Russell and Precious, 1982; Stewart et al., 1993; Matthews and Russell, 1994). It is thought to form a bridge between the peripentonal hexons and the DNA, thus stabilizing the capsid structure. Our previous studies have shown that, during virus entry, protein VI is degraded (Greber et al., 1993).

To test whether protein VI is degraded in the absence of L3/p23 protease activity, [^{35}S]methionine-labeled DTT/NEM virus and ts1 viruses were allowed to be endocytosed at 37°C for different times and were analyzed by SDS–PAGE and autoradiography (Figure 6). While ~80% of the protein VI in control virus was degraded within 2 h, the protein remained unchanged in ts1 virus (Figures 4A and 6B). In DTT/NEM-inactivated virus, only ~25% of protein VI was degraded. In reduced viruses that had been alkylated with IAA or DTDP, the protein VI behaved similarly to the DTT/NEM-treated virus, whereas in virus preparations treated with DTT or NEM alone protein VI was degraded with efficiencies similar to control virus (Figure 6B). Degradation was not inhibited by addition of a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), or inhibitors of trypsin and chymotrypsin-like enzymes, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) (data not shown). This suggested that endosomal or lysosomal proteases were not involved.

The time course of protein VI degradation for the control virus showed that half of protein VI was degraded already within 20 min of internalization, i.e. shortly after virus penetration into the cytosol, which occurs with a
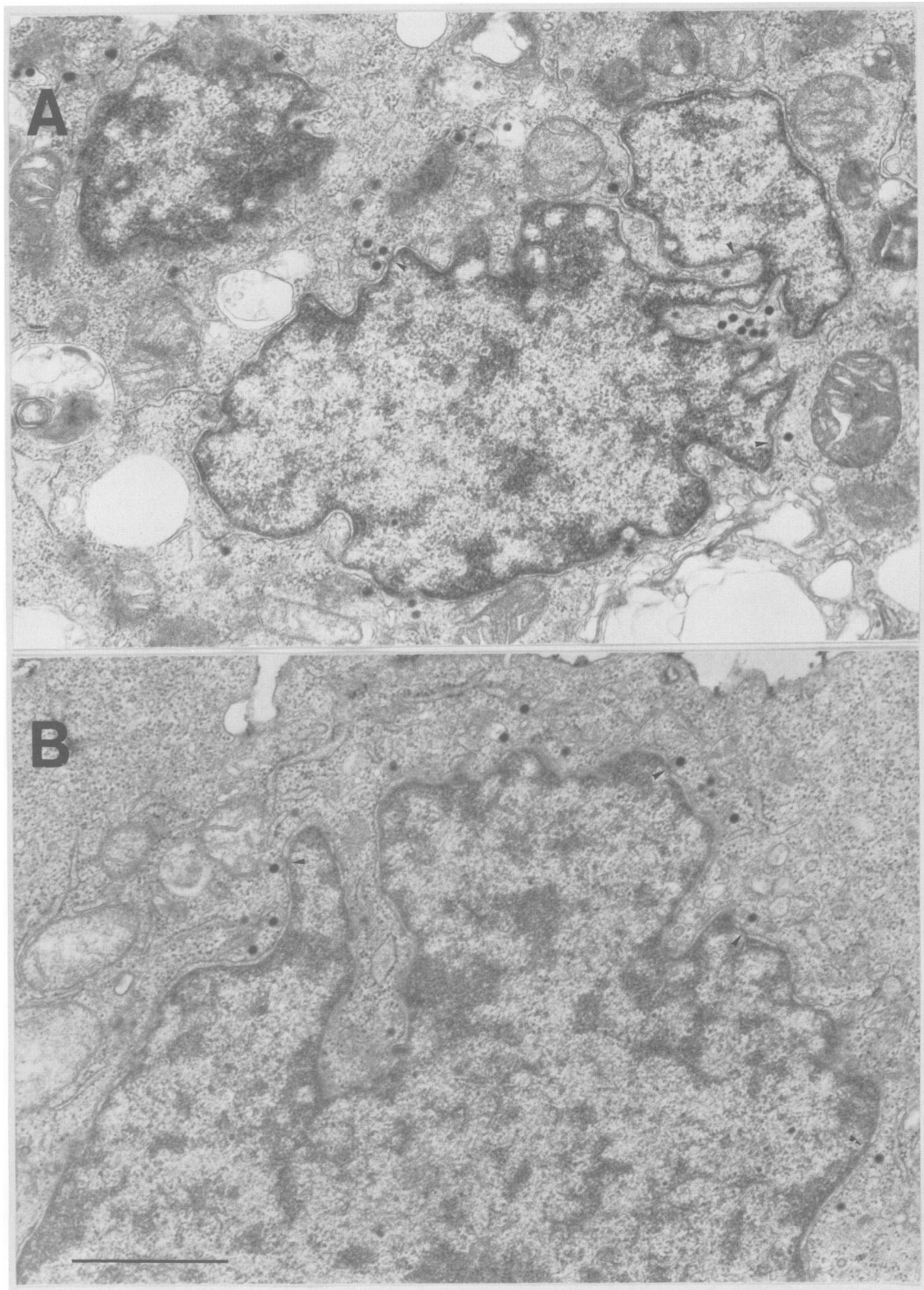
Fig. 5. Localization of incoming DTT/NEM adenovirus to the nuclear pore complex. Mock- or DTT/NEM-treated adenovirus was internalized into KB cells at 1000 p.f.u. per cell for 60 min at 37°C [(**A**) Mock, (**B**) DTT/NEM)]. Samples were fixed, embedded in Epon and processed for thin section electron microscopy. Pore complex-associated virus is labeled with an arrowhead. Bar = 1 μm.

half-time of 15 min (Greber *et al.*, 1993). The endosomal protease inhibitor leupeptin had no effect on the degradation. Monensin, a carboxylic ionophore that increases vacuolar pH and inhibits acid hydrolases, also had no effect (Figure 6). These findings suggested that the degradation of

protein VI was independent of low pH and of endosomal and lysosomal proteases. Also, since monensin blocks virus penetration into the cytosol (Seth *et al.*, 1984; Yoshimura, 1985), these results demonstrated that the degradation could occur while the virus was inside the

Table I. Pore complex association of incoming DTT/NEM- and mock-treated adenovirus

	Micrographs	Virus at NE	Virus per NE sample (mean)	Virus at NPC	Virus per NPC (mean)	Virus at NPC/NE (mean×100, ±SE)
Mock-Ade	54	240	4.3	67	1.3	28.9 ± 4.2%
DTT/NEM-Ade	61	267	4.3	68	1.1	21.4 ± 2.8%

Mock- or DTT/NEM-treated adenovirus was incubated with KB cells at 1000 p.f.u. per cells for 60 min at 37°C and samples were processed for thin section electron microscopy. Two hundred and forty mock- and 267 DTT/NEM-treated viral particles closer than two virus diameters at the nuclear envelope (NE) were counted blindly in 34 and 37 different cells, respectively. The mean number of mock and DTT/NEM-treated viruses per nuclear envelope sample was 4.3. Sixty-seven mock- and 68 DTT/NEM-treated viruses were unequivocally associated with nuclear pore complexes (NPCs). The average number of particles per pore complex was 1.3 mock- and 1.1 DTT/NEM-treated viruses. The mean percentage pore complex-associated virus per nuclear envelope was 28.9% mock- and 21.4 % DTT/NEM-treated viruses. Standard errors (SE) were derived from 54 mock- and 61 DTT/NEM-treated virus micrographs.
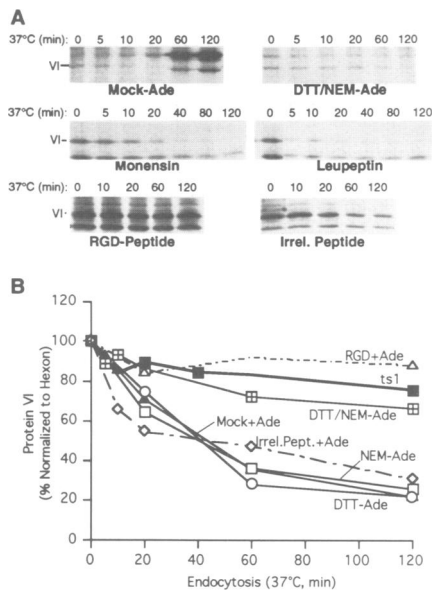

Fig. 6. Protein VI degradation in incoming DTT/NEM-treated adenovirus is inhibited. DTT/NEM-, DTT-, NEM- or mock-treated [^{35}S]methionine-labeled wild-type adenovirus or ts1 virus was bound and endocytosed into KB cells in the presence or absence of monensin, RGD or irrelevant peptides (RGES) for different times. TCA-precipitated cell extracts were analyzed by SDS–PAGE and autoradiography (**A**) and the amount of protein VI determined by densitometry normalized to the amount of hexon present in each sample (**B**).

endocytic vacuoles, i.e. the virus did not have to reach the cytosol for the degradation to occur.

However, the endosomal and lysosomal proteases were apparently, at least in part, responsible for another degradation step, the partial degradation of incoming hexon which normally begins much later, between 40 and 150 min after internalization (Figure 6) (see also Greber et al., 1993). Unlike protein VI, hexon degradation was slowed down markedly by both leupeptin and monensin. Hexon of the ts1 virus was degraded even though the virus does not reach the cytoplasm, suggesting that hexon degradation occurs in the endocytic pathway (Figure 4A). In contrast, the degradation of hexon in the DTT/NEM-treated virus was reduced compared with the untreated or NEM alone-treated virus, supporting the notion that the DTT/NEM-treated virus was not degraded by endosomal/lysosomal enzymes.

Binding to integrin receptors is needed to trigger degradation

To test whether receptor binding was a prerequisite for protein VI degradation, wild-type virus was bound to KB cells in the cold, whereafter the cells were warmed to 37°C in the presence of RGD peptides which prevent virus attachment to the integrin receptors and virus endocytosis (Wickham et al., 1993). Protein VI degradation was clearly inhibited by the RGD peptides, but not by control peptides (Figure 6). Thus, degradation of protein VI was dependent on changes either in the virus itself induced by the binding to integrins or by the subsequent receptor-mediated endocytosis into endosomes.

Since it was possible that endocytosis exposed the virus to a more reducing milieu inside the endosomes, we tried to bypass the RGD peptide block by addition of 5 mM DTT or 10 mM glutathione. This did not lead to protein VI degradation (data not shown), indicating that attachment of the virus to the cell via the fibers alone, even under reducing conditions, did not activate protein VI degradation. Attachment via the penton base proteins to the integrin receptor may therefore induce a necessary change in the capsid structure without which protein VI degradation cannot occur. It might be significant that protein VI is located just underneath the penton structure at the peripentonal hexons.

Disassembly and nuclear import are inhibited in protease-defective virus

Up to the point of virus binding to the nuclear pore complex, our results had indicated no difference between DTT/NEM and control virus entry except that in the DTT/NEM virus protein VI did not get digested.

To analyze whether the final disassembly process was also normal for this inactivated virus, we monitored capsid uncoating by immunofluorescence and confocal microscopy. We took advantage of the observation that anti-capsid or anti-core antibodies often bind more efficiently to disassembled capsid proteins than to intact capsids (Martin and Helenius, 1991; Singh and Helenius, 1992). The major increase in fluorescence intensity can therefore be used to monitor the uncoating process.

We used two different antibodies, a non-neutralizing anti-hexon antibody and an anti-protein VII-specific antibody (Prage and Pettersson, 1971; Baum et al., 1972). The hexon antibodies gave only faint labeling of surface-bound mock-, NEM- or DTT/NEM-treated viruses (images not shown, for quantitated data see Figure 7B). However,
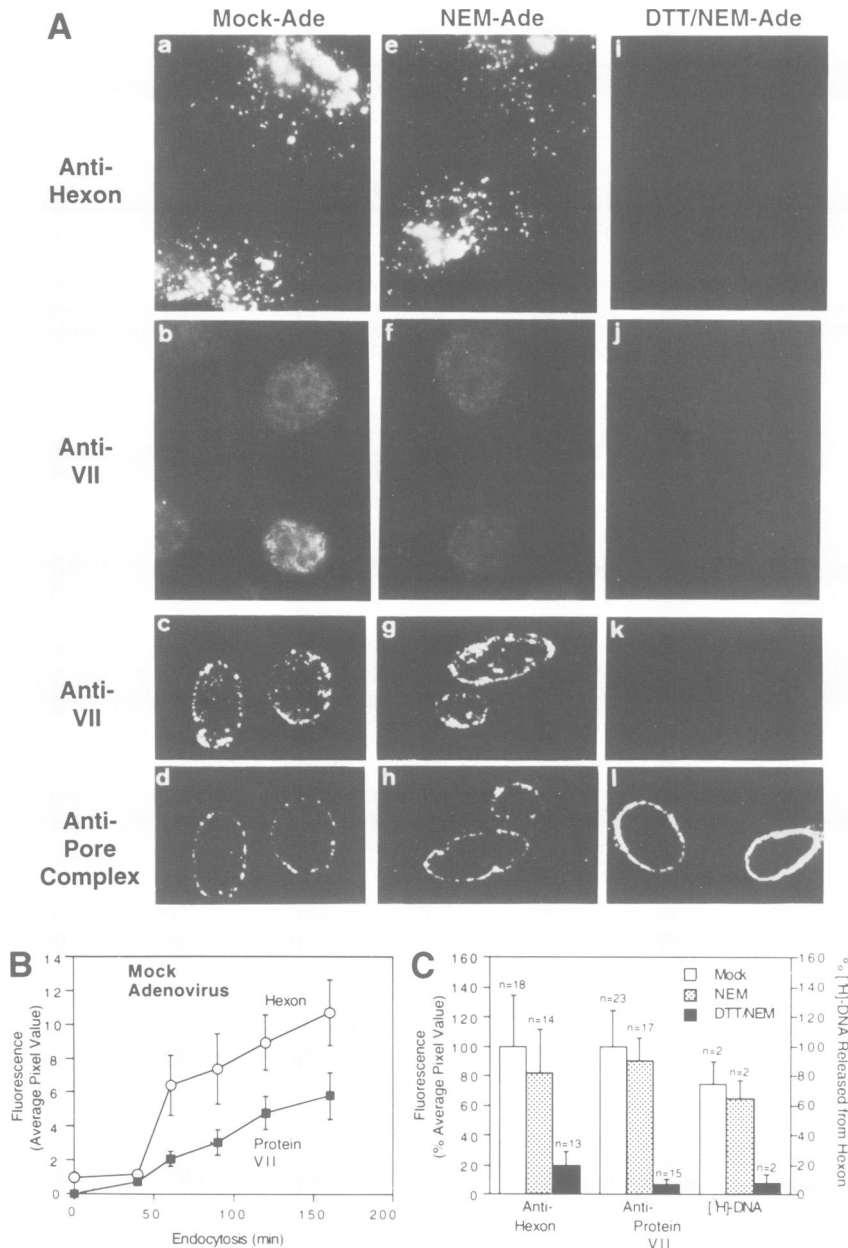
Fig. 7. DTT/NEM-treated adenovirus is uncoating-defective. (**A**) Mock- (a–d), NEM- (e–h) or DTT/NEM-treated adenovirus (i–l) was bound in the cold and endocytosed into HeLa cells for 150 min at 37°C. Cells were processed for double labeling indirect immunofluorescence microscopy using rabbit anti-hexon (a, e and i), rabbit anti-protein VII (b, f and j; c, g and k) and mouse anti-pore complex antibodies (d, h and l) followed by secondary goat anti-rabbit–Texas red and goat anti-mouse–fluorescein antibodies, respectively. Photographs were taken using a regular fluorescence microscope (a, e and i; b, f and j) and a confocal microscope (c, g and k; d, h and l), respectively. (**B**) Mock-treated adenovirus was bound and internalized into HeLa cells for the indicated times at 37°C. Cells were fixed and processed for indirect immunofluorescence microscopy using anti-hexon and anti-protein VII antibodies, respectively. The average pixel intensity was determined in optical sections across the cell centers and plotted as mean values together with the standard deviations derived from 20–37 different cells per time point. (**C**) Mock-, NEM- or DTT/NEM-treated adenovirus labeled or not labeled with [3H]thymidine was internalized into HeLa cells for 150 min at 37°C. Cells were either analyzed for fluorescence intensity as described above or hexon-associated viral DNA was determined by co-immunoprecipitation with anti-hexon antibodies as described (Greber *et al.*, 1993). Between 30 and 40% of the total cell-associated 3H-labeled DNA from wild-type or NEM-treated virus and ~20% of the DNA from DTT/NEM-treated virus was precipitated with the anti-hexon antibody. The data were normalized to the signal from mock-treated virus (100%). n represents the number of cells analyzed by fluorescence and the number of independent DNA-anti-hexon co-precipitation experiments, respectively.

after 150 min of infection with either mock- or NEM alone-treated virus, the anti-hexon antibody gave intense and predominantly cytoplasmic punctuate labeling (Figure 7A). A small amount of labeling was also observed inside the nucleus. Quantification of the hexon signal in the cytoplasm revealed a rapid increase starting after a lag of

~40 min (Figure 7B). This time course was consistent with biochemical analysis of DNA dissociation from hexons (Greber *et al.*, 1993), and with the disappearance of visible capsids by thin section electron microscopy. The data would suggest that ~50% of the particles disassembled within 50 min of infection.

In contrast to native virus, the DTT/NEM-inactivated virus did not show a dramatic increase in hexon fluorescence (Figure 7A and C). The amount of fluorescence after 150 min of warming was only 20% of that observed for native virus. The lack of an increase in signal was not due to diminished reactivity of the alkylated antigen with the antibodies, since the antibodies bound strongly to both alkylated and reduced/alkylated hexon on Western blots (data not shown).

The result thus indicated that the defect in the DTT/NEM-treated virus manifested itself at the level of final capsid disassembly. While the viruses were able to bind to the nuclear pore complexes, they did not disassemble so that the hexons would become more accessible to antibodies.

This conclusion was supported by the results obtained with the anti-protein VII antibody. Protein VII is one of the structural proteins associated directly with the viral DNA (Horwitz, 1990). No staining of cell surface-bound or of cytosolic virus was ever seen with this antibody, consistent with the antigen being inaccessible inside the intact viruses and cytosolic capsids. However, after a 40 min lag, the antibody showed relatively strong fluorescence in the nucleus (Figure 7A and B). The staining was present in a discrete punctate and sometimes also more diffuse pattern. Double labeling confocal fluorescence microscopy with the nuclear pore complex-specific antibody RL1 as a marker (Snow *et al.*, 1987) confirmed that the protein VII entered the nucleus, and localized in structures near the periphery of the nuclear envelope (Figure 7A). The apparent half-time of protein VII arrival in the nucleus was delayed compared with capsid disassembly, suggesting that full exposure of protein VII to the antibody occurred later.

When DTT/NEM virus was used, practically no protein VII staining was detected in the nucleus nor anywhere else in the cell (Figure 7). Apparently, protein VII was not released from the virus nor transported into the nucleus under these conditions.

We have described previously a biochemical assay for virus disassembly based on co-precipitation of viral DNA with hexons (Greber *et al.*, 1993). This assay showed that ~80% of incoming [^{3}H]thymidine-labeled adenovirus DNA dissociated from the hexons within 150 min of warming (Figure 7C). A similar result was obtained for viruses treated with NEM alone. When we used DTT/NEM-inactivated virus in this assay, only 10–15% of the DNA was released from the hexon after 150 min of infection (Figure 7C). In the presence of RGD peptides, no dissociation of DNA from hexon was detected (not shown).

Taken together, these results indicated that the DTT/NEM-treated virus, in which the viral protease was inactivated and protein VI was not digested, was unable to undergo the final disassembly step that normally occurs when the virus binds to the nuclear pore complex. Since the DNA did not dissociate from the hexons, and since protein VII, a DNA binding protein, did not get exposed, it was likely that the DNA remained associated with the capsids in the cytosol. The apparent reason for this effect was the failure of the viral protease to cleave protein VI and thereby liberate the DNA from its association with the capsid.

Discussion

When viruses enter a host cell, they receive signals that activate their uncoating program. These may involve interactions with surface receptors, exposure to low pH in endosomes, association with intracellular factors or other changes in the immediate environment of the virus (see Greber *et al.*, 1994). In the case of adenovirus, a series of different signals is needed for the transport of the incoming virus from the plasma membrane to the nucleus. The signals include the interaction of the penton base with the integrin receptor which leads to endocytosis and fiber dissociation, low pH-activated membrane lysis in the early endosome, and association with the nuclear pore complex. Our data now suggest that for capsid dissociation and DNA release the structural protein VI has to be degraded by the viral L3/p23 protease, dormant in the extracellular virus. Two separate signals are both needed to trigger this event, virus interaction with integrin receptors and re-entry into a reducing environment.

About 10 copies of the L3/p23 protease are present in each viral particle (Anderson, 1990). They are located in the central cavity and bound to the DNA (Everitt and Ingleman, 1984; Mangel *et al.*, 1993) and require specific sequence motifs in the substrate proteins (Webster *et al.*, 1994). Protease isolated from adenovirus has no measurable activity unless first treated by urea and reducing agents (Webster and Kemp, 1993; Rancourt *et al.*, 1995). It is clear that the enzyme requires free thiols for its activity.

While proteolytic degradation events have rarely been described during virus entry (Greber *et al.*, 1994), viral proteases are known to play a crucial role during the assembly of numerous viruses (see Hellen and Wimmer, 1992). For adenovirus, the role of a protease during virus assembly is illustrated by the ts1 mutant which lacks L3/p23 protease (Weber, 1976; Rancourt *et al.*, 1995). As a result, several of the structural proteins remain unprocessed, and the released viruses have an immature surface structure and remain non-infectious (Hannan *et al.*, 1983). We found that while the particles bound to cell surface receptors and were endocytosed, they failed to shed their fibers subsequently. They ended up in a futile recycling circuit between endosomes and the plasma membrane. Moreover, the ts1 particles did not respond properly to low pH and failed to penetrate from the endosomes into the cytosol (Cotten and Weber, 1995; Greber, unpublished data). Importantly, protein VI remained undegraded.

To uncouple the role of the L3/p23 protease during assembly from a possible role during disassembly, we treated isolated wild-type virus with the L3/p23 inhibitor NEM. While it is clear that NEM alkylates cysteines in other capsid proteins besides the protease (see also Figure 2A), our subsequent data argue that alkylation of the reduced virus had a selective effect on uncoating and nuclear import, but did not affect the function of capsid proteins needed for cell binding, endocytosis, penetration, transport to the nucleus and localization to the pore complex. Consistently, DTT reduction of the virus causes no conformational changes in capsid proteins, except in the protease (Anderson *et al.*, 1973; Figure 2B). NEM treatment of native, non-reduced virus, on the other hand, had no effect on infectivity, uncoating and nuclear import, and correlated with the absence of alkylation of the
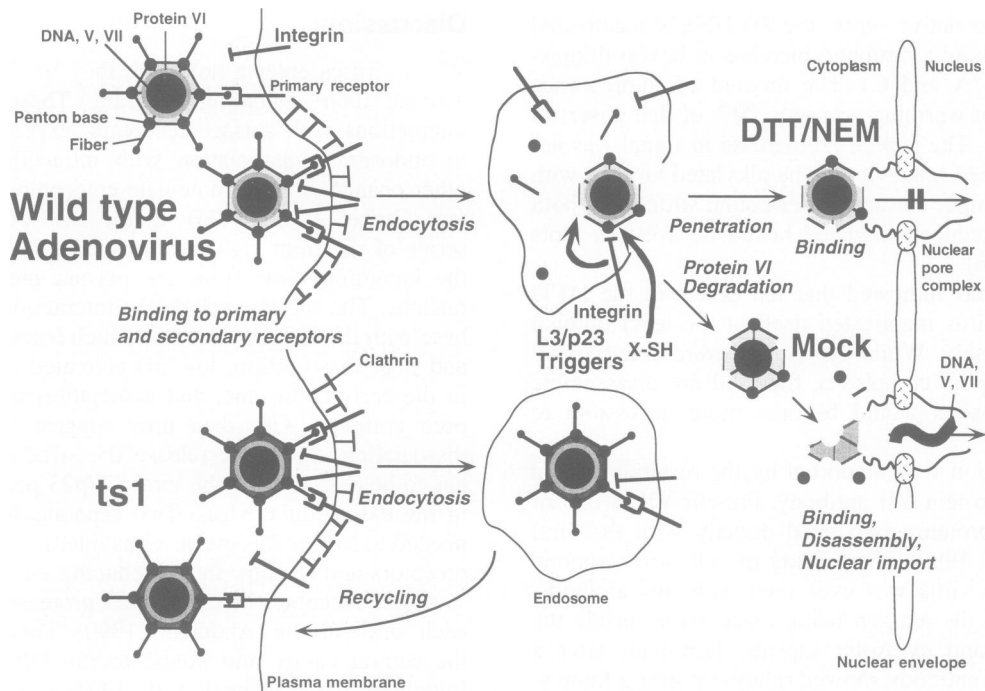
Fig. 8. Cell entry and uncoating of protease-defective adenoviruses. Protease-lacking, incompletely assembled ts1 and fully assembled DTT/NEM-protease inactivated adenovirus bind to cells and endocytose via integrins with efficiencies and kinetics similar to wild-type virus. In contrast to wild-type and protease-inactivated virus, ts1 virus does not shed fibers and penetrate from endosome into the cytoplasm, but is recycled to the plasma membrane. Binding of wild-type virus to integrins together with reducing agents in the cytoplasm or endosomes (X-SH) triggers the L3/p23 protease-dependent degradation of the internal capsid protein VI (curved arrows) and enables the capsid to disassemble completely at the nuclear membrane and import its DNA into the nucleus. Protease-inactivated virus, in contrast, does not degrade protein VI. It penetrates into the cytoplasm, is transported to the nuclear envelope and pore complexes like wild-type virus, but is unable to disassemble and import its DNA-associated protein VII into the nucleus.

L3/p23 protease. Interestingly, DTT reduction was not absolutely necessary to make the virus susceptible to NEM, since virus infectivity was reduced 2- to 3-fold more effectively if virus was alkylated in the reducing milieu of the infected cell before release into the oxidizing medium. It is possible that protease in the intracellular virus or the cysteine-containing activating peptide is more susceptible to NEM than protease or peptide of DTT-treated isolated virus. Protease from the latter virus was only partially reduced with DTT (see Figure 2B, lane 7). Lastly, another L3/p23 protease inhibitor, copper chloride, recently was reported effectively to block adenovirus-mediated delivery of carrier genes into the nucleus (Cotten and Weber, 1995). Where the block occurred in these experiments was, however, not investigated.

Our results suggest that the L3/p23 protease is responsible for the degradation of the capsid protein VI, a capsid-stabilizing factor. According to image reconstruction analysis of isolated virus, protein VI is located entirely inside the capsid, and is, therefore, poorly accessible to cellular proteases (Stewart *et al.*, 1993). A variety of inhibitors of proteases of the endocytic pathway, such as leupeptin and monensin, had no effect on degradation of protein VI, whereas they inhibited the partial degradation of hexon protein later in infection.

Moreover, our experiments with native, reduced and alkylated virus show that a viral activity was needed for the degradation of protein VI and for the subsequent disassembly of the capsids at the nuclear membrane. Since protein VI has no cysteines and was not labeled by

[³H]NEM, it seems clear that the effects of these sulfhydryl reagents were not on the substrate. L3/p23 was, in fact, strongly labeled by [³H]NEM after prior DTT reduction, correlating with the capacity of the alkylating agent to inhibit protein VI digestion.

That the cysteine protease L3/p23 is involved in the degradation of protein VI is supported by several indirect observations. First, under certain conditions, the L3/p23 protease can cleave degenerate consensus sites (unpublished observations). One such site in protein VI is PEGR-G, located between residues 94 and 95. Second, a major fraction of recombinant protein VI precursor is degraded completely when incubated with recombinant protease in the presence of the protease-activating C-terminal peptide from protein VI (pVIc-peptide) (Matthews and Russell, 1995). Third, reducing agents are required for protease activity *in vitro*, presumably for disulfide interchange with the disulfide-linked dimer of the activating pVIc-peptide (Webster *et al.*, 1994). Finally, the reduced but not the oxidized protease can be inactivated completely by alkylating agents *in vitro*, suggesting that the catalytically active cysteine is accessible for modification and catalysis (Bhatti and Weber, 1979; Webster *et al.*, 1989). Either the active site cysteine or another absolutely conserved cysteine residue at position 122 or 104, which is thought to form a disulfide bond with the activating pVIc-peptide, could be modified with the alkylating agent, destroying protease activity (Grierson *et al.*, 1994; Rancourt *et al.*, 1994).

It is interesting that re-entry into a reducing environment

can serve as a cue for virus uncoating. Although the block in uncoating caused by the failure of protein VI degradation in the DTT/NEM-treated virus is manifested only at the last step in the uncoating program, the degradation of the protein normally occurs much earlier. Degradation occurs already at the time of endocytosis and penetration from endosomes into the cytosol. The lack of monensin inhibition indicates that L3/p23 can be activated, and protein VI can be digested, within endocytic vacuoles, a compartment which is thought to contain a reducing milieu causing disulfide bond reduction (Collins et al., 1991; Pisoni et al., 1995). However, it is likely that during the normal infection most of the degradation occurs shortly after the virus reaches the cytosol (Greber et al., 1993). Protein VI degradation does not require low pH, which is consistent with the neutral pH optimum previously reported for L3/p23 (Bhatti and Weber, 1979; Webster et al., 1989).

Another factor needed for protease reactivation was the RGD-dependent interaction of the penton base with integrin receptors. Integrins mediate virus endocytosis and are a determinant for membrane penetration at low pH (Bai et al., 1993; Wickham et al., 1993, 1994; Goldman and Wilson, 1995). We think that the binding of penton base to integrins induces a conformational change in the vertex region of the capsid, exposing hidden protease cleavage sites in protein VI.

Taken together, the data suggest the following scenario (see also Figure 8). When newly assembled viruses are released from the nucleus of the lysing host cell, the L3/p23 proteases encounter the oxidizing extracellular milieu. The enzymes are inactivated by the formation of intrachain disulfides. When the viruses bind to a new host cell, the integrin receptors on the cell surface associate with the penton base proteins. This interaction leads not only to the release of the fibers but also to a conformational change in the penton complexes such that previously hidden protease L3/p23 cleavage sites in the penton-associated protein VI become exposed. When, moments later, the virus enters a more reducing environment in the endosome or the cytosol, the inactivating disulfide bond is reduced, and the protease is re-activated. This leads to degradation of protein VI. Since protein VI is bound to the DNA and to the peripentonal hexons (Russell and Precious, 1982; Stewart et al., 1993; Matthews and Russell, 1994), the cleavage results in the release of the anchored DNA from the inside wall of the capsid. Later, when the virus undergoes additional changes at the nuclear pore complex, the DNA is free to escape.

Adenoviruses have evolved a finely tuned disassembly program which allows them to deliver their genes into the nuclei of non-dividing cells. To develop efficient gene delivery and expression systems based on the virus paradigm, more detailed information about uncoating and intracellular transport of the incoming viral particles is needed (Mulligan, 1993; Greber et al., 1994; Michael and Curiel, 1994). The crucial points illustrated by our results are the close interplay between cellular and viral functions and the complexity of the disassembly program involved in targeting and liberating the genome for transcription and replication in the nucleus.

Materials and methods

Virus, cells and antibodies

Adenovirus type 2 (wild-type, from Dr E.White, The State University of New Jersey) and H2ts1 adenovirus (ts1) were grown in HeLa cells

and purified by CsCl density gradient centrifugation and plaque-assayed as described (Weber, 1976; Greber et al., 1993). Monolayers of A549, HeLa and KB cells (obtained from American Type Culture Collection, Rockville, MD) were maintained as described (Greber et al., 1993).

[35S]Methionine- and [3H]thymidine-labeled wild-type adenovirus was prepared as described (Greber et al., 1993). [35S]Methionine-labeled ts1 virus was prepared by infecting HeLa cells for 12 h at 39°C with ~5 p.f.u./cell of a p2 stock grown at 32°C. Endogenous methionine was depleted for 30 min in methionine-free MEM containing 5% fetal bovine serum, and viral proteins were labeled for 9 h at 39°C with 1 mCi of TRAN35S-Label (1000 Ci/mmol, ICN Biomedicals Inc., Irvine, CA) per 7 cm dish, followed by a chase with excess cold methionine for 48 h. Virus was released from cells by repeated freeze–thawing and freon extraction and purified by CsCl gradients as described (Greber et al., 1993).

Rabbit antisera against hexon (R70), fiber (R72) and protein VII were generous gifts of Dr M.Horwitz (Albert Einstein University, New York) and Dr U.Pettersson (University of Uppsala, Sweden) (Prage and Pettersson, 1971; Baum et al., 1972). IgG fractions were obtained by pH 3.0 elution from protein G–Sepharose as described (Greber et al., 1993).

Virus inactivation

Intracellular adenovirus (62 h post-infection) was treated with 10 mM NEM for 20 min on ice in phosphate-buffered saline (PBS), extracted by freon, purified by CsCl density gradient centrifugation, dialyzed and tested for infectivity by plaque assays as described (Greber et al., 1993).

Extracellular virus, purified from HeLa cells by freon extraction and CsCl density gradient centrifugation (0.5 mg/ml protein as determined by the BCA assay, Pierce, Rockford, IL), was dialyzed in a Collodion membrane bag (Schleicher and Schuell, Keene, NH) against 0.02 M Tris, 0.15 M NaCl, pH 7.5 and reduced with 10 mM DTT in the presence of 1 mM EDTA at 4°C for 60 min. DTT and EDTA were removed by dialysis against 0.02 M Tris, 0.15 M NaCl, pH 7.5 at 4°C for 30 min. Sulfhydryl groups were alkylated with 10 mM NEM, IAA or DTDP, or oxidized with diamide (20 mM) (all reagents from Sigma, St Louis, MO) or with 0.1 mCi [3H]NEM (44.1 Ci/mmol, 1 mCi/ml, NEN-Du Pont, Wilmington, DE) at 4°C for 20 min. The inhibitors were removed by dialysis against 0.01 M Tris, 0.15 M NaCl, pH 7.5 at 4°C for 2 h and treated with 10 mM DTT followed by dialysis, or virus was incubated with cells or analyzed by SDS–PAGE. Mock-treated virus was prepared in parallel by incubation of virus without inhibitors. Infectivity was tested by plaque assays on A549 cells.

Virus entry into cells

Mock- or inhibitor-treated virus was bound to cells and internalized as described (Greber et al., 1993). Internalization was measured by trypsin digestion of surface-bound adenovirus hexon protein in the cold in the presence or absence of GRGDSP peptides (3 mM, Telios Pharmaceuticals, San Diego, CA) or control peptides (e.g. CGYGPKNKRKVED, Yale, CT, Peptide Synthesis Facility, or RGES peptides, Sigma, St Louis, MO) (Greber et al., 1993). Monensin (10 μM, Sigma, St Louis, MO) was added to cells at 37°C, 30 min prior to infection in slightly alkaline growth medium. Overall degradation of incoming [35S]methionine-labeled virus was determined by TCA precipitation of cell lysates and liquid scintillation counting of TCA-soluble and -insoluble material.

Incoming [35S]methionine- or [3H]thymidine-labeled wild-type adenovirus or ts1 virus was immunoprecipitated with anti-fiber and anti-hexon antibodies, respectively, and analyzed by SDS–PAGE or liquid scintillation counting as described (Greber et al., 1993). In brief, cells were lysed in 0.5% Empigen BB (Calbiochem, San Diego, CA) in MNT buffer [20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 30 mM Tris, pH 7.5, 100 mM NaCl] containing 0.1 mM EDTA, 0.5 mM PMSF and benzamidine, and 10 μg/ml of chymostatin, leupeptin, antipain, pepstatin, TPCK and TLCK. Lysates were cleared by centrifugation and either immunoprecipitated with anti-fiber R72 antibodies and fixed Staphylococcus aureus bacteria (Zymed Laboratories Inc, San Francisco, CA) or precipitated with 15% TCA and analyzed by SDS–PAGE.

SDS–PAGE and Western blotting

Virus or virus/cell extracts were concentrated by TCA precipitation, washed in ice-cold acetone (90%, 10% 10 mM HCl), dried and dissolved in SDS sample buffer (Greber et al., 1993). Reduced (20 mM DTT) or unreduced samples were boiled at 95°C for 3 min and separated on 12.5% polyacrylamide gels in SDS (Laemmli, 1970). Fluorography was done by fixing the gels in 20% methanol–PBS for 30 min on an orbital

shaker, and incubation with Amplify™ (Amersham, Arlington Heights, IL) for 45 min.

Proteins from SDS gels were electrotransferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a semi-dry blotter (Millipore, Bedford, MA) and labeled with anti-protease antiserum (1:100) (Rancourt *et al.*, 1994). Bound IgG was identified by ECL (Amersham, Arlington Heights, IL) using goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma, St Louis, MO).

Indirect immunofluorescence and laser scanning confocal microscopy

Inhibitor- or mock-treated wild-type adenovirus (200 p.f.u. per cell) was bound to HeLa cells on glass coverslips at 4°C for 2 h. Unbound virus was washed off and warm MEM–BSA was added and the cells incubated for different times at 37°C. Cells were washed with cold PBS, fixed with 3% paraformaldehyde for 10 min, quenched with 25 mM ammonium chloride in PBS, pH 7.4, for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed in PBS and incubated with anti-hexon R70 IgGs (10 µg/ml) or anti-protein VII IgGs (10 µg/ml) for 1 h at 4°C in the presence or absence of the anti-pore complex antibodies RL1 [5 µg/ml mouse IgM (Snow *et al.*, 1987)]. Viral antibodies were pre-adsorbed against fixed and Triton X-100-extracted uninfected HeLa cells at 10 µg/ml IgG for 12 h at 4°C before use. Primary antibodies were visualized with goat anti-rabbit IgG conjugated to Texas red and goat anti-mouse IgM conjugated to fluorescein, respectively, at 10 µg/ml (Zymed Laboratories, San Francisco, CA). Coverslips were mounted in Mowiol containing 2% 1,4-diazobicyclo-[2,2,2]-octane (Harlow and Lane, 1988) and analyzed on a Bio-Rad MRC-600 confocal imaging system equipped with a Zeiss Axiovert 10 microscope and a 63× Planapochromat lens. Confocal settings were: 0.3 mW laser power, Kalman filters, 1 s/frame, 8 frames/image. The photomultiplier gain was set at maximum and the confocal aperture was adjusted to 5, yielding optical slices of ~0.7 µm thickness. Images of an area of $200 \times 280 \ \mu m^2$ and a raster size of 512×756 pixels were taken through the center of the cells, collected through the rhodamine and fluorescein channels in dual channel mode and photographed on Kodak TMAX 400 film (Eastman Kodak Co., Rochester, NY). For fluorescence quantification, the average fluorescence intensity (pixel units) across the cytoplasm and the nucleus of 13–40 randomly chosen cells was determined for hexon epitopes and protein VII epitopes, respectively, using the Bio-Rad area software. Where indicated, photographs were also taken on TMAX 400 film using an Axiophot microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a 63× Planapochromat lens.

Electron microscopy and stereology

Pellets of infected cells were fixed in 2.5% glutaraldehyde buffered in 100 mM sodium cacodylate, pH 7.4, post-fixed in 2% osmium tetroxide in the same buffer and stained *en bloc* with 1% uranyl acetate in 50 mM sodium maleate, pH 5.2 (Griffiths, 1993). The pellets were dehydrated in ethanol, embedded in Epon and sectioned. The thin sections were mounted on Formvar/carbon-coated specimen grids, contrasted with 3% aqueous uranyl acetate and lead citrate, and examined in a Phillips 410 transmission electron microscope operating at 80 kV. Micrographs were taken from randomly sampled cells, and the numbers of viruses localized to the nuclear envelope and nuclear pore complexes were counted. For association with the nuclear envelope, only viral particles that were closer than two virus diameters were scored. For association with the nuclear pore complex, only those events were counted where unequivocal identification of the pore complex was possible.

Acknowledgements

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