JOURNAL OF VIROLOGY, Aug. 1987, p. 2559–2566
0022-538X/87/082559-08$02.00/0

Vol. 61, No. 8

Processing of Vimentin Occurs during the Early Stages of Adenovirus Infection

MARIE-THÉRÈSE BELIN AND PIERRE BOULANGER†*

Unité de Virologie 233 de l'Institut National de la Santé et de la Recherche Médicale, 59045 Lille Cédex, France

Received 29 December 1986/Accepted 17 April 1987

Evidence is presented that a fraction of vimentin, a component of cytoskeleton recently found to be associated with intracytoplasmic, migrating adenovirus type 2 (Ad2), is processed into smaller polypeptides at early times after infection. The extent of vimentin cleavage appears to depend upon both the multiplicity of infection and the adenovirus serotype. Ad2, Ad5, Ad4, and Ad9 induced similar vimentin cleavage in infected cells, whereas Ad3, Ad7, and Ad12, for which most infecting particles are found sequestered within phagosomes, induced very little, if any, vimentin breakdown. This suggests that vimentin processing is in some way related to the number of virus particles migrating through the cytoplasm. Experiments performed in vitro and in vivo with adenovirus temperature-sensitive mutants H2 *ts*1 and H2 *ts*112 and UV-inactivated wild-type Ad2 indicated that vimentin processing is due to a nonvirion, cytoskeleton-associated, proteolytic enzyme activated by adenovirus and sharing characteristics with the protease described by Nelson and Traub (W. J. Nelson and P. Traub, J. Cell Sci. 57:25–49, 1982). The activity of this protease appears to be required for productive infection by adenovirus serotypes 2 and 5 (subgroup C), 4 (subgroup E), and 9 (subgroup D) but not by the oncogenic serotypes 3 and 7 (subgroup B) and 12 (subgroup A).

During the initial stages of infection, human adenovirus, as well as other animal viruses, interacts with the cytoskeletal elements of the host cell (for reviews, see references 18 and 25), particularly with microtubules (14, 26, 28). Thus, the cytoskeletal framework is believed to play a role in the vectorial transport of the virions from the penetration site at the cell surface to the nuclear membrane pores. Interactions of adenovirus particles with microtubules have also been observed in vitro (41). It is generally accepted that penetration and migration of a virus into the different cell compartments is controlled by both the virus and the host (13). Thus, an understanding at the molecular level of the process of transcytoplasmic migration of adenovirus requires at least three different routes of investigation: (i) biochemical analysis of the cytoskeletal elements during virus migration, (ii) identification of the viral components involved, and (iii) determination of the nature of the interactions between the viral and cellular components.

In a recent study (2), we demonstrated that at least two cytoskeletal proteins are in close association with adenovirus as early as 30 min after infection. These proteins were identified as alpha-tubulin (the major microtubular component) and vimentin (one of the intermediate filament [IF] constituents). Both are cross-linked to form an adenovirus-containing complex (2). The adenovirus particles present in this complex were found to be structurally altered, as suggested by significant modifications occurring in the neighbor relationships among virion proteins (2).

In the present study, in an attempt to further explore the mechanism of virus transport to the nucleus, we analyzed the modifications undergone by the proteins constituting the IFs in the early stages of an adenovirus infection. We found that vimentin (and desmin as well) was cleaved by a cellular protease, apparently activated by adenovirus particles soon after their penetration into the cell. This protease coextracts with the IFs and shares biological properties with the calcium-dependent, stress-induced protease (6, 31, 32).

It seems that the activity of this protease is required for a productive viral infection by adenovirus serotypes which mainly undergo a transcytoplasmic route of penetration (adenovirus type 2 [Ad2] and Ad5) but not by the serotypes whose major pathway is within the phagocytotic vesicles (Ad7 and Ad12).

MATERIALS AND METHODS

Cells. Hamster cell line BHK-21 and human cell lines HeLa, HEp-2, and HEK-293 were cultured as monolayers in Eagle minimal essential medium supplemented with 10% calf serum.

Virus. Human adenovirus serotypes 2, 3, 4, 5, 7, 9, and 12 were produced in HeLa cell monolayers or suspension-grown KB cells. The virus titers were determined by a fluorescent-focus assay, as previously described (17) and expressed as fluorescent-focus units (FFU). Adenovirus H2 *ts*112, a temperature-sensitive mutant altered in virion morphogenesis, was used to produce empty particles at 39.5°C devoid of DNA-containing cores (16, 17, 27). H2 *ts*1 was kindly provided by J. Weber and has been shown to be mutated in the virus-encoded 21-kilodalton (kDa) protease (5).

Analysis of IF proteins by one-dimensional and two-dimensional gel electrophoresis. Isolation of IFs was done basically as described previously (2). Cells were lysed in lysis buffer consisting of 10 mM Tris hydrochloride (pH 8.9), 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone for 30 min at 4°C with gentle rotatory shaking. This buffer releases soluble cytoskeletal proteins and leaves the cytoskeletal lattice and microfilaments in the insoluble bulk. The cell lysate was centrifuged at 8,000 × *g* and 4°C for 10 min. The residual pellet was washed with buffer consisting of 50 mM Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100, and 0.05 M KCl. The IFs were

* Corresponding author.
† Present address: Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90024.

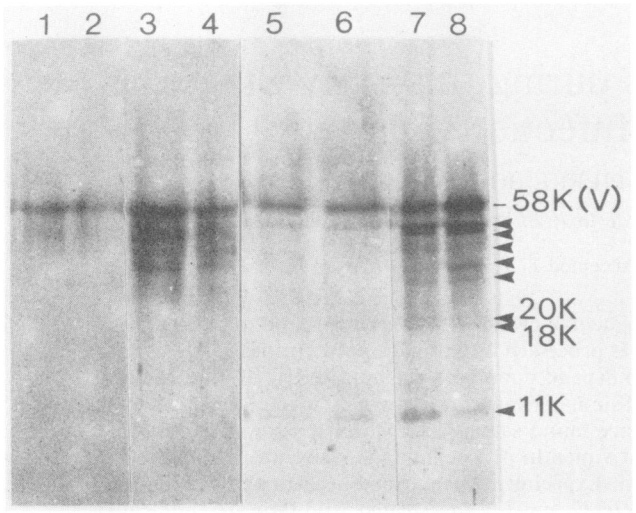

FIG. 1. Identification of immunoreactive vimentin-derived polypeptides in SDS-polyacrylamide gels of BHK-21 cell extracts. IFs of mock-infected cells (lanes 1, 2, 5, and 6) or Ad2-infected cells (lanes 3, 4, 7, and 8) harvested 30 min after infection were electrophoresed on a 5 to 15% polyacrylamide gradient gel, transferred to nitrocellulose filters, and reacted with anti-human vimentin rabbit serum (lanes 1 to 4) or anti-bovine vimentin serum (lanes 5 to 8). The antigen-antibody complexes were revealed by peroxidase-labeled anti-rabbit immunoglobulin. The arrowheads indicate the positions of antivimentin-reacting polypeptides. 58K(V), Intact vimentin.

isolated by centrifugation at 8,000 × *g* for 10 min and washed three times with the same buffer with alternate high (1.5 M) and low (60 mM) KCl concentrations to remove the maximum amount of actin (23).

The proteins of the IFs were solubilized in sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 4% SDS, 10% mercaptoethanol, 6 M urea, 0.005% bromophenol blue) and electrophoresed in an SDS-containing 6 to 15% polyacrylamide gradient slab gel in the discontinuous buffer system of Laemmli (22). Two-dimensional electrophoresis was performed by the method of O'Farrell (33). When IF proteins were labeled with ^{14}C-amino acids, the gels were dried and autoradiographed. For unlabeled samples, the IF proteins and their processing products were identified by immunoblotting.

Gel immunoblotting. Proteins electrophoresed in polyacrylamide gels were transferred electrically onto nitrocellulose sheets (9) and reacted with antivimentin, antitubulin, or antiactin rabbit serum. Antigen-antibody complexes were revealed by a second peroxidase-conjugated anti-rabbit immunoglobulin antibody (Institut Pasteur, Paris, France) or by ^{35}S-labeled protein A (Amersham). Antiserum to bovine vimentin was raised in rabbits by injection of calf lens vimentin (2). Rabbit sera against human vimentin, tubulin, and actin were purchased from Miles Laboratories. In some experiments, an antivimentin mouse monoclonal antibody (Amersham) was used followed by a peroxidase-labeled anti-mouse immunoglobulin antibody (Institut Pasteur).

Isotopes and labeling conditions. IF proteins were labeled with [^{14}C]valine or [^{14}C]arginine at 0.1 μCi/ml for 4 h in a medium containing 20% of the normal concentration of the corresponding unlabeled amino acid. [^{14}C]valine and [^{14}C]arginine were purchased from the Commissariat à l'Energie Atomique, Saclay, France. The specific activity for [^{14}C]valine- or [^{14}C]arginine-labeled IF proteins was usually 500 to 1,000 cpm/μg of protein.

Determination of the extent of cleavage of peptide bonds X-Arg in vimentin products from adenovirus-infected cells. [^{14}C]arginine-labeled proteins of cells lysed in lysis buffer were electrophoresed in polyacrylamide gels, electrically transferred to nylon membranes (Byodine; pore size, 0.2 μm; Pall Ultrafine Filtration Corp.), and autoradiographed. A lane was reacted with antivimentin rabbit serum, and each major [^{14}C]arginine-labeled peptide band which corresponded to a cleavage product of vimentin was identified. Pieces of the nylon blot corresponding to each major antivimentin-reacting peptide were suspended in a solution of 0.5 mg of aminopeptidase M (Boehringer GmbH, Mannheim, Federal Republic of Germany) per ml in 50 mM NaCl–50 mM Tris hydrochloride (pH 7.4) at 37°C. The kinetics of liberation of [^{14}C]arginine was determined by counting the radioactivity of the blots at intervals in a liquid scintillation spectrometer.

RESULTS

In vivo cleavage of vimentin in WT Ad2-infected cells at early times of infection. BHK-21 cells were infected with wild-type (WT) Ad2 at 300 FFU per cell at 37°C and harvested 60 min after infection. The cells were processed for isolation of IFs, and the IF pellet was suspended in SDS buffer and analyzed in an SDS-polyacrylamide gradient gel. Vimentin and vimentin-derived products were revealed by immunoblotting by using anti-human vimentin serum and anti-bovine vimentin serum (Fig. 1). Immunoreactive bands of lower molecular weights than the vimentin subunit (58 kDa) were visible in preparations from Ad2-infected cells (Fig. 1, lanes 3, 4, 7, and 8) but were not visible or scarcely visible in IF prepartions from mock-infected cells (Fig. 1, lanes 1, 2, 5, and 6). Major bands in the 55- to 50- and 45- to 35-kDa domains were revealed by both types of antisera. However, our anti-bovine vimentin serum appeared more reactive than did the commercial anti-human vimentin serum and elicited extra discrete bands, particularly a doublet band in the 18- to 20-kDa zone and a band at 11 kDa. Since no viral proteins were found to react with any of the antisera used, all of these bands were identified as cleavage products of vimentin. The breakdown product bands at 18 to 20 and 11 kDa, which appear to be good markers for vimentin degradation, occasionally appeared in the soluble IFs from mock-infected cells (Fig. 1, lanes 5 and 6) but never in IF preparations from untreated cells. In two-dimensional gels, these cleavage products, migrating with an apparent pI ranging from 5.3 to 4.9, appeared to be more acidic than vimentin (results not shown).

Kinetics of vimentin cleavage during the early stages of Ad2 infection. BHK-21 cells were infected at a constant multiplicity of 100 FFU per cell and harvested at different times after infection. Vimentin alterations were analyzed by immunoblotting on an SDS-polyacrylamide gradient gel by using our anti-bovine vimentin rabbit serum. Vimentin proteolysis seemed to increase with time of infection. A cleavage product migrating as a discrete band of 20 kDa and a doublet band at 58 to 56 kDa appeared as early as 5 min after infection. An 11-kDa species appeared at 10 min, and an 18-kDa species appeared between 45 and 60 min (Fig. 2A).

Influence of MOI on vimentin cleavage in BHK-21 cells infected with WT Ad2. Cultures of BHK-21 cells were infected with different multiplicities of infection (MOIs), ranging from 10 to 1,000 FFU per cell, and harvested 30 min

after infection. The degree of cleavage of vimentin as evidenced by the intensity of the 18- and 11-kDa products appeared to be related to the number of particles infecting each cell (Fig. 2B). Except for a doublet band at the vimentin position occurring with an MOI of 3,000 FFU, the extent of vimentin processing did not change significantly above 100 FFU per cell. These data and data shown in Fig. 2A indicate why an MOI of 300 to 500 FFU per cell was chosen for most of the subsequent experiments and why cells were harvested at 30 min after infection. WT Ad2 therefore induced proteolytic cleavage of vimentin during the early stages of infection, soon after the virus had penetrated into the cell, and likely during transcytoplasmic migration (10, 11, 24, 38).

Incubation of Ad2 particles with purified vimentin or IFs in vitro. A virus-encoded protease found in human adenovirus (1, 12, 21) seems to be responsible for the cleavage of the viral protein precursors PVI, PVII, and PVIII (29) and PIIIa (7). The protease is temperature sensitive in the Ad2 temperature-sensitive mutant H2 *ts*1 (1, 5, 21). The expression of the protease is a late event in the adenovirus cycle (4, 34). However, the protease is virion incorporated (12) and might be active in the early phase of the infection process. The following experiments were designed to determine whether the protease involved in vimentin processing was of viral or cellular origin.

Samples of [^{14}C]valine-labeled vimentin (1 μg; 1,000 cpm), purified as described previously (2), were incubated with increasing amounts of purified WT Ad2 virions for 30 min at 37°C. The ratio of vimentin to Ad2 virions, determined by protein concentration, ranged from 1:3 to 1:30 (10^{10} to 10^{11} virions per sample). The incubation mixtures were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. No vimentin cleavage could be evidenced, even in the presence of a large number of purified WT Ad2 particles (data not shown). This suggests that (i) a viral protease is not directly involved in vimentin processing and (ii) purified vimentin has no autolytic property which can be induced by the virus in vitro. However, it could not be excluded that the adenovirus protease has to be activated or rendered accessible by some modification of the virus particle. Thus, a similar experiment was performed using WT Ad2 virions subjected to 12 cycles of freezing and thawing (4). The 58-kDa vimentin band remained intact, as shown by analysis in an SDS-polyacrylamide gel, even after prolonged times of incubation (12 h) with broken virions (data not shown).

The total IFs were then tested in vitro for possible proteolysis in the presence of Ad2. IFs are composed of two major components, desmin (52 kDa) and vimentin (58 kDa). After labeling was performed with [^{14}C]valine, the IFs were isolated from BHK-21 cells (2) and incubated with purified WT Ad2 particles in vitro. To maintain the cohesiveness of the IF edifice during the extraction and purification process, the cells were fixed with the cleavable diimidoester dimethyl 3,3′-dithiobispropionimidate dihydrochloride (DTBP) at 30 mM in phosphate-buffered saline for 20 min at room temperature (2) before processing for IFs. The IF samples were incubated with adenovirus particles for 30 min at 37°C at a ratio of IF protein to adenovirus of 1:3 and analyzed by sedimentation through a preformed combined CsCl-glycerol gradient (2). Slower-sedimenting ^{14}C-labeled material appeared on top of the gradient in samples incubated with virus particles (Fig. 3).

Autoradiograms of SDS-polyacrylamide gels showed that the top fractions of the gradient contained [^{14}C]valine-labeled polypeptides with apparent molecular weights lower

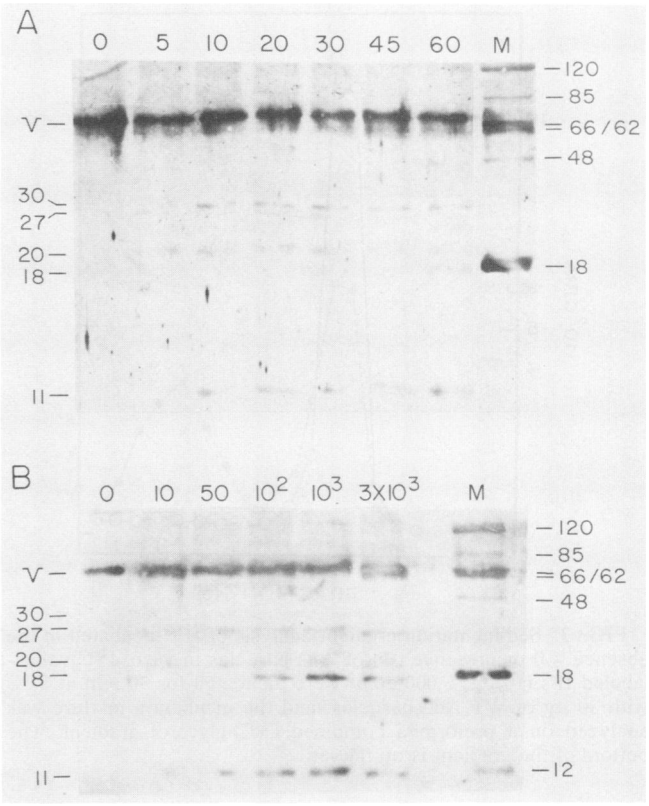

FIG. 2. (A) Kinetic analysis of vimentin cleavage products at early times of Ad2 infection. BHK-21 cells were infected with 100 FFU of Ad2 at 37°C and harvested at 5, 10, 20, 30, 45, and 60 min after infection. Vimentin (V) and vimentin products were analyzed with anti-bovine vimentin rabbit serum and ^{35}S-labeled protein A. (B) Dependence of vimentin processing on Ad2 MOI in BHK-21 cells. BHK-21 cells were infected at different input multiplicities, ranging from 10 to 3,000 FFU per cell. Lane 0, Mock-infected cells. Molecular sizes (in kilodaltons) are indicated in the margins. Lane M, Molecular size markers.

than that of vimentin. A broad band at 45 to 35 kDa suggested that vimentin was hydrolyzed and not only depolymerized (Fig. 4). Two-dimensional analysis suggested that desmin was also hydrolyzed in vitro and in vivo (results not shown). Although comparative analysis could not be done of the processing pattern of vimentin in vivo (Fig. 1 and 2) and the apparent cleavage occurring in vitro when cross-linked IFs were incubated with adenovirus particles (Fig. 4), these results suggest that cellular protease(s) was coextracted with DTBP-fixed IFs and could still be activated by adenovirions in vitro, at least to some extent.

To determine whether the protease(s) was of viral or cellular origin, DTBP-fixed isolated IFs were incubated in vitro with particles of the temperature-sensitive mutant H2 *ts*1 (5) under the same conditions as those for WT Ad2. Particles of H2 *ts*1, which has been shown to be mutated in the virus-coded protease (1, 5, 21), were produced at the permissive temperature (33°C) and then heated at 39.5°C for 1 h to inactivate a possible virus-incorporated protease. A degree of IF cleavage was obtained with H2 *ts*1 similar to that with the WT Ad2, and the profile of radioactivity of the gradient was superimposed on that of WT Ad2 (Fig. 3). These data suggest that the proteolytic cleavage of vimentin

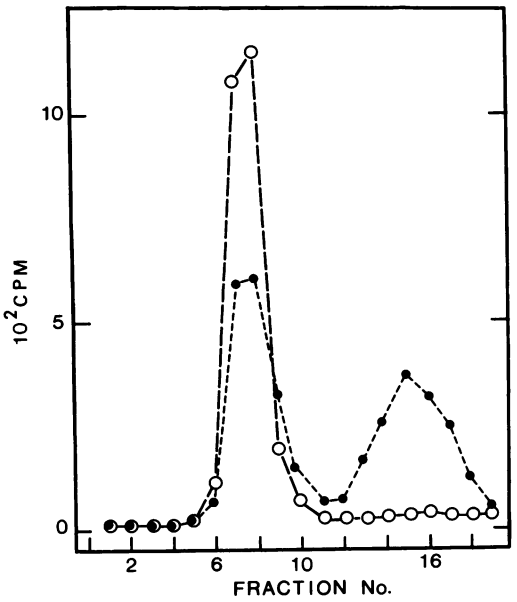

FIG. 3. Sedimentation gradient analysis of IFs incubated in the absence (○) or presence (●) of Ad2 particles in vitro. [^{14}C]valine-labeled IFs (10 μg; 6,000 cpm) were incubated for 30 min at 37°C with 30 μg of WT Ad2 particles, and the incubation mixture was analyzed on a preformed combined CsCl-glycerol gradient. The bottom of the gradient is on the left.

is not due to the virus 21-kDa protease but rather to protease(s) of cellular origin.

Activation of cellular protease(s) and processing of vimentin occurred in the absence of early adenovirus gene expression. Although cells were harvested at an early time in the adenovirus infection process, some immediate-early viral transcripts have been found as early as 30 min after infection (4). BHK-21 cells were therefore infected with UV-inactivated WT Ad2 or empty particles devoid of DNA-containing cores, and the results were compared with those of WT Ad2 infection. Empty capsids were produced at the nonpermissive temperature (39.5°C) by the assembly-defective temperature-sensitive mutant H2 *ts*112 (16, 17). The same number of physical particles (3,000 per cell) were used in the inoculum, as determined by the protein concentration of the virion or viral capsid samples. The infected cells were harvested 30 min after infection, and the fate of vimentin was analyzed by gel immunoblotting. Both H2 *ts*112 empty particles and UV-inactivated WT Ad2 induced vimentin cleavage similar to that produced by infectious WT Ad2 (data not shown). Therefore, the activation of a cellular protease responsible for vimentin processing does not require the expression of early viral genes and is not under the direct control of the viral genome.

Influence of the serotype of the infecting adenovirus on vimentin cleavage. Comparative observations of the penetration of various adenoviruses have suggested that the routes differ among the different serotypes (11, 15, 30). BHK-21 cells were therefore infected with 300 FFU per cell of at least one serotype of each of the five adenovirus subgroups (A through E) (40): serotype 2 or 5 (subgroup C), 4 (subgroup E), 9 (subgroup D), 3 or 7 (subgroup B), and 12 (subgroup A). Vimentin was analyzed comparatively in cell samples harvested 30 min after infection at 37°C.

Infection by the nononcogenic serotypes 2, 5, 4, and 9 resulted in the same degree of vimentin processing, and the pattern of antivimentin-reacting bands looked identical in all samples (Fig. 5). On the other hand, Ad7 (or Ad3) and Ad12, members of the weakly oncogenic subgroup B and the highly oncogenic subgroup A, respectively, induced no visible vimentin cleavage and their pattern resembled that of mock-infected cells (Fig. 5).

Ad7 and other members of subgroup B (e.g., Ad3) have been found to have a weak efficiency of encapsidation, and vast amounts of empty capsids (the so-called top components of CsCl gradients) are always present in Ad7 and Ad3 preparations. When these empty capsids were used to infect cells, no vimentin cleavage was detectable (results not shown). Therefore, it seems that the processing of cellular vimentin is related to the main route (quantitatively) of adenovirus penetration. Vimentin cleavage occurs to a greater extent in cells infected with serotypes for which most infecting particles are found in the cytoplasm, e.g., Ad2 and

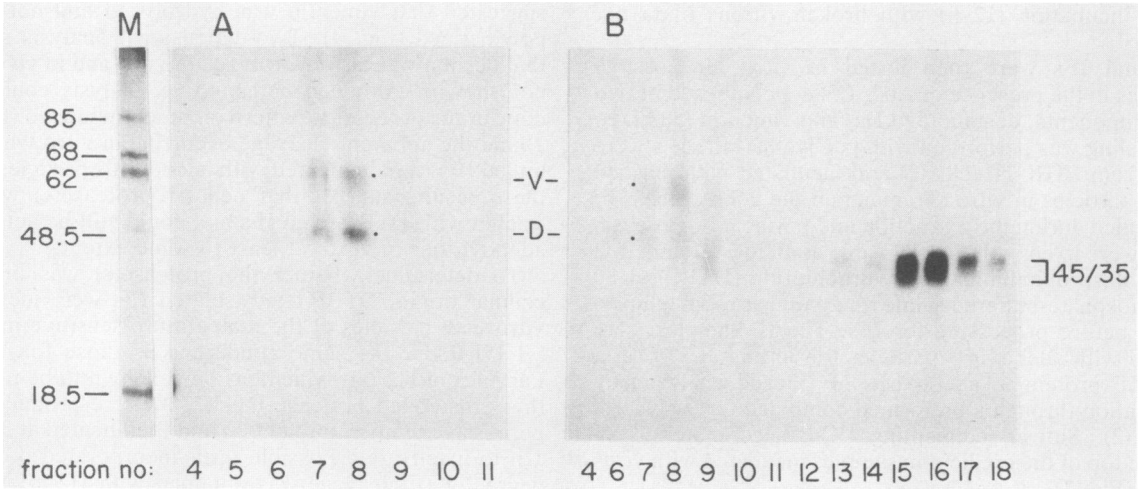

FIG. 4. Autoradiograms of SDS-polyacrylamide gels of the gradient fractions shown in Fig. 3. Shown are [^{14}C]valine-labeled IFs in the absence (A) or presence (B) of WT Ad2 virions. Lane M, Molecular size markers. V, Vimentin; D, desmin. Molecular sizes (in kilodaltons) are indicated in the margins.

Ad5 (10, 30, 38). Ad7 and Ad12 have been found to be sequestered in large numbers within phagosomes (11).

Preliminary characterization of the cellular Ad2-activated protease. (i) Identity of vimentin cleavage products after different cell treatments and in different cell lines. The cleavage of vimentin induced by adenovirus infection was reminiscent of the cleavage induced by heat shock stress or provoked by drugs such as valinomycin (6). Comparative analysis of vimentin cleavage products from (i) cells infected with WT Ad2 and harvested 30 min after infection, (ii) cells after a heat shock of 1 h at 42.5°C, and (iii) cells treated with valinomycin (10^{-5} M) for 30 min showed no detectable difference in the pattern of antivimentin-reactive polypeptide bands (results not shown). Moreover, no doublet band appeared when the different samples were mixed in pairs and electrophoresed. This suggests that the same cleavage sites were involved for all samples and, thereby, similar proteolytic activity. The protease activated in adenovirus-infected cells, heat-shocked cells, and cells treated with valinomycin might correspond to the stress-induced protease (6) or the protease of Nelson and Traub (31, 32).

An experimental result favoring the latter hypothesis was the identical pattern of vimentin-derived bands found in Ad2-infected BHK-21, HeLa, HEp-2, and HEK-293 cells (results not shown), implying a highly conserved specificity for this protease associated with cytoskeletal elements (31).

(ii) Inhibition of proteolytic cleavage of vimentin reduced adenovirus production. The protease of Nelson and Traub is known to behave as a metal-dependent protease, inhibited by 1,10-phenanthroline, leupeptin, and EGTA (31). HeLa cells were treated with *o*-phenanthroline or leupeptin at different concentrations for 30 min at 37°C before Ad2 infection. Infection was conducted for 30 min at 37°C in the presence or absence of the inhibitor. The inhibitor was then removed, the virus cycle was allowed to proceed for 30 h at 37°C, and virus production was determined by the immunofluorescent-focus assay.

Incubation with *o*-phenanthroline at 0.25 to 0.50 mM resulted in a 10-fold decrease in Ad2 production at the end of the infection cycle, and at 1 mM, a 10^3-fold decrease was observed (Table 1). Similar results, albeit less pronounced, were obtained with leupeptin, which decreased virus production to 3 to 8% of the level obtained without inhibition (Table 2). Controls showed no detectable difference in the final virus titers when incubation was conducted in the absence of inhibitor or the inhibitor was added for 1 h at a late time postinfection (19 h), suggesting that the effect on virus production was an early event.

Inactivation of metal-dependent protease(s) by *o*-phenanthroline and leupeptin resulted in a drastic decrease in virus production. [^{14}C]valine-labeled adenovirus controls showed

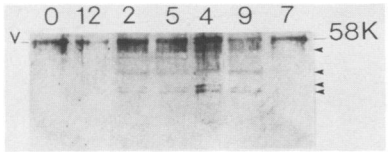

FIG. 5. Vimentin processing in BHK-21 cells infected with an equivalent input multiplicity (300 FFU per cell) of various adenovirus serotypes (indicated by lane numbers) belonging to different subgroups: 2 and 5 (subgroup C), 7 (subgroup B), 4 (subgroup D), 9 (subgroup D), and 12 (subgroup A). Lane 0, Mock-infected cells. Shown is an immunoblot with anti-human vimentin rabbit serum. The arrowheads indicate the major vimentin cleavage products. 58 K, Intact vimentin (V).

TABLE 1. Adenovirus progeny yields in HeLa cells treated with *o*-phenanthroline[a]

Serotype	Expt	Progeny yield[b] at *o*-phenanthroline concn (mM):					
		0.00[c]	0.10	0.25	0.50	0.75	1.00
Ad2	1	100	47.9	15.7	3.5	1.2	0.3
	2	100	47.2	8.4	3.1	0.6	0.05
Ad3	2	100			100		106
Ad7	2	100			103		95

[a] HeLa cells were infected with adenovirus without (experiment 1) or with (experiment 2) preincubation of the cells with the inhibitor for 30 min at 37°C. The inhibitor was maintained in phosphate-buffered saline for 30 min, the cells were washed, and the viral cycle was allowed to proceed for 30 h at 37°C. The viral progeny titers were determined on HeLa cells by fluorescent-focus assays.

[b] Percentage of that of untreated infected cell culture (control).

[c] Control (untreated adenovirus-infected cells): Ad2, 2.6×10^9 FFU; Ad3, 2.2×10^9 FFU; Ad7, 3.5×10^8 FFU.

that neither of the inhibitors altered the penetration of the virus. However, the proportion of virus particles entering the nucleus was slightly reduced in cells treated with leupeptin or *o*-phenanthroline, suggesting that the migration, but not the penetration, of Ad2 was altered (Table 3). In contrast, Ad3, Ad7, and Ad12 yields were not sensitive to either inhibitor (Tables 1 and 2).

(iii) Ad2-induced vimentin degradation is likely due to specific cleavage of X-Arg peptide bonds. [^{14}C]arginine-labeled vimentin and vimentin breakdown products were electrophoresed on SDS-polyacrylamide gels, blotted onto nylon membranes, and digested with aminopeptidase M for 15 min to 2 h. Three major ^{14}C-labeled antivimentin-reacting polypeptides with apparent molecular weights of 30 (a), 27 (b), and 20 (c) kDa were compared with undegraded vimentin with regard to the kinetics of [^{14}C]arginine liberation by aminopeptidase M. ^{14}C labeling in intact vimentin, which has proline at its N terminus (35), was stable over 2 h of digestion with aminopeptidase (Fig. 6). In contrast, the kinetics of liberation of [^{14}C]arginine from polypeptides a, b, and c appeared to be very rapid, with a plateau reached as early as 30 min. These results strongly suggest that these vimentin-derived polypeptides have arginine at or near their N termini. This is consistent with the hypothesis that the predominant proteolytic cleavages of vimentin occur at X-Arg, which is the specificity of the protease described by Nelson and Traub (31, 32).

TABLE 2. Adenovirus yields in HeLa cells treated with leupeptin[a]

Serotype	Yield[b] at leupeptin concn (mM):					
	0.00[c]	0.25	0.50	1.0	2.0	5.0
Ad2	100	92	73.6	26.3	11	8
Ad3	100					105
Ad7	100					99
Ad12	100					100

[a] HeLa cells were infected with adenovirus after preincubation of the cells with leupeptin for 30 min at 37°C. Leupeptin was maintained in the culture medium for 30 min after infection, the cells were rinsed, and the viral cycle was allowed to proceed for 30 h at 37°C. The viral progeny titers were determined on HeLa cells by a fluorescent-focus assay.

[b] Percentage of that of untreated infected cells (control).

[c] Control (untreated, adenovirus-infected cells): Ad2, 3.8×10^{10} FFU; Ad3, 2.1×10^{10} FFU; Ad7, 2.2×10^9 FFU; Ad12, 3.1×10^8 FFU.

TABLE 3. Recovery of adenovirus particles in extracts of BHK-21 cells treated with protease inhibitors at early stages after infection

Cell treatment[a]	Amt (cpm) (%) recovered							
	Whole cells		Cytoplasmic extract		Triton extract		Residual pellet[b]	
	30 min	45 min	30 min	45 min	30 min	45 min	30 min	45 min
None (control)	7,170 (2.05)	9,400 (2.68)	2,301 (0.66)	3,641 (1.04)	1,140 (0.32)	999 (0.28)	3,183 (0.91)	4,032 (1.15)
Incubation with leupeptine (5 mM)	6,800 (1.94)	8,450 (2.41)	2,475 (0.70)	4,023 (1.15)	1,067 (0.30)	1,415 (0.40)	2,323 (0.66)	2,360 (0.67)
None (control)	21,041 (6.01)	26,182 (7.48)	11,900 (3.40)	12,847 (3.67)	3,348 (0.96)	4,321 (1.23)	2,790 (0.79)	5,129 (1.46)
Incubation with *o*-phenanthroline (0.25 mM)	22,690 (6.48)	25,385 (7.25)	12,695 (3.62)	12,569 (3.59)	4,257 (1.21)	4,184 (1.18)	2,623 (0.75)	3,575 (1.02)

[a] ^{14}C-labeled Ad2 inoculum (350,000 cpm; 3,000 FFU per cell) was added to BHK-21 monolayer cells, and adsorption and penetration were allowed to occur for 30 or 45 min at 37°C. Unadsorbed virus was removed by rinsing in prewarmed medium, DTBP fixation was performed in phosphate-buffered saline, and cell extracts were obtained as previously described (2).

[b] The residual pellet contained nuclei, cell organelles, and insoluble cytoskeletal matrices.

DISCUSSION

The route of adenovirus penetration and migration to the cell nucleus, the site of its replication, has been a matter of controversy for many years. Direct engulfment by the plasma membrane and rapid transit through the cytoplasm has been described (30), whereas convincing images of vast amounts of virus within endosomes have also been published (10, 14). Later, it was found that the mechanism of penetration is different among various adenovirus serotypes (10, 15). The role of endocytotic vesicles during adenovirus internalization has recently been reinvestigated (36, 38, 39). Although it has been clearly demonstrated that Ad2 internalization involves receptor-mediated endocytosis (11, 19, 20), a process in which the penton base plays a significant role (36), it was also suggested that an alternative pathway is direct penetration of the virion into the cytoplasm, as described in earlier work (8, 30). Whatever the mechanism of penetration, there is a consensus that adenovirus migration through the cytoplasm to the nucleus involves a transient association with microtubules (2, 15, 25, 26, 28). We have also recently shown that vimentin, a major component of IFs, is associated with intracytoplasmic Ad2, along with three other cytoskeletal proteins, including alpha-tubulin (2).

Evidence is presented in this study that a significant portion of vimentin is processed into smaller polypeptides at early times after adenovirus infection (5 to 30 min). The extent of vimentin cleavage is dependent upon the MOI in the range of 5 to 500 FFU per cell and reaches a plateau above the latter value. Analysis of vimentin modifications in vivo and in vitro with WT Ad2, the adenovirus mutants H2 *ts*112 (17) and H2 *ts*1 (5), and UV-inactivated WT Ad2 suggested that vimentin is cleaved by a cellular protease activated during the early stage of the viral infection. This cleavage is different from the processing of vimentin which has recently been found to occur in dense cell cultures (3).

Preliminary characterization of this protease indicates that it shares characteristics with the calcium-dependent protease described in earlier work (31). The protease activated by adenovirus appeared to be a metal-dependent enzyme inhibited by *o*-phenanthroline and leupeptin and may have a specificity for cleavage of vimentin at X-Arg peptide bonds. These characteristics differ significantly from those of the adenovirus-coded endopeptidase, a chymotrypsinlike, nonmetal-dependent, serine protease (5, 12). *o*-Phenanthroline or leupeptin treatment of adenovirus-infected cells drastically reduced the production of viral progeny at the end of the cycle, suggesting that vimentin processing is required for efficient transport of adenovirions to the nucleus.

A recent immunofluorescence study of adenovirus-infected cells has shown a collapse of the vimentin network around the nucleus (37). This process and other cytoskeletal alterations seem to occur continuously rather than abruptly and to progress during the course of infection (37). They are not due to structural viral protein synthesis and virus assembly since they were observed in the abortive infection of monkey TC-7 cells with human WT Ad2, as well as in the permissive infection of TC-7 with Ad2HR400 (37).

The cleavage pattern of vimentin was found to be identical in cells infected with Ad2, Ad5, Ad4, or Ad9, but no detectable vimentin cleavage was observed in cells infected with Ad3, Ad7, or Ad12, three serotypes belonging to the weakly oncogenic subgroup B (Ad3 and Ad7) or highly oncogenic subgroup A (Ad12). These serotypes have been

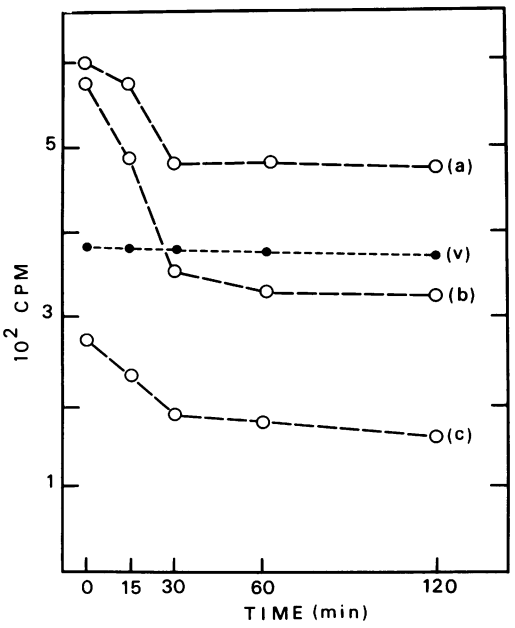

FIG. 6. Kinetics of [^{14}C]arginine release from vimentin (v) and three major vimentin-derived polypeptides (a, b, and c) by aminopeptidase. Hydrolysis was performed on blotted polypeptides at 37°C for 2 h. The plots represent the radioactivity remaining on the blots.

found to be preferentially sequestered in dense granules (11). Inhibition of vimentin cleavage by *o*-phenanthroline and leupeptin reduced the yield of viral progeny for members of subgroup C only, e.g., Ad2. No change was observed with serotypes 3, 7, and 12 (Tables 1 and 2).

Therefore, vimentin cleavage appears to be related to the serotype of the infecting virus and to the main route followed by the viral particles to gain entry into the nucleus. Vimentin breakdown occurs in cells infected with serotypes transiting in the cytoplasm (Ad2 and Ad5) but not with serotypes following the phagosomic route (Ad3, Ad7, and Ad12). Inhibitors of vimentin cleavage reduced the progeny yields of Ad2 but had no effects on Ad3 and Ad7, serotypes for which the major pathway is within vesicles. This confirmed that adenovirus can reach the nucleus by totally different routes, as previously evidenced by electron microscopic studies. For members of adenovirus subgroup C (Ad2 and Ad5) which induced proteolytic cleavage of vimentin and desmin, probably by activation of a cellular protease, the viral component responsible for the activation of the protease and the mechanism of this activation remain to be identified.

ACKNOWLEDGMENTS

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (Unité 233), the Centre National de la Recherche Scientifique (UAC 114), the Université du Droit et de la Santé de Lille (UER III), the Fondation pour la Recherche Médicale, and the Fédération Nationale des Groupements d'Entreprises Françaises dans la lutte contre le cancer.

We thank Didier Petite for providing cell cultures, Chantal Cousin for virus stocks, and Douglas Barker for revision of the English manuscript.

LITERATURE CITED

1. **Akusjärvi, G., J. Zabielski, M. Perricaudet, and U. Pettersson.** 1981. The sequence of the 3′ non-coding region of the hexon mRNA discloses a novel adenovirus gene. Nucleic Acids Res. **9**:1–16.
2. **Belin, M. T., and P. A. Boulanger.** 1985. Cytoskeletal proteins associated with intracytoplasmic human adenovirus at an early stage of infection. Exp. Cell Res. **160**:356–370.
3. **Ben-Ze'ev, A., L. E. Babis, and P. B. Fisher.** 1986. Cleavage of vimentin in dense cell cultures. Inhibition upon transformation by adenovirus 5. Exp. Cell Res. **166**:46–62.
4. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
5. **Bhatti, A. R., and J. Weber.** 1979. Protease of adenovirus type 2: partial characterization. Virology **96**:478–485.
6. **Biessmann, H., F. G. Falkner, H. Saumweber, and M. F. Walter.** 1982. Disruption of the vimentin cytoskeleton may play a role in heat shock response, p. 275–281. *In* M. J. Schlesinger, M. Ashburner, and A. Tissières (ed.), Heat shock: from bacteria to man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Boudin, M. L., J. C. D'Halluin, C. Cousin, and P. A. Boulanger.** 1980. Human adenovirus type 2 protein IIIa. II. Maturation and encapsidation. Virology **101**:144–156.
8. **Brown, D. T., and B. T. Burlingham.** 1973. Penetration of host cell membranes by adenovirus 2. J. Virol. **12**:386–396.
9. **Burnette, N. W.** 1981. "Western blotting" electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
10. **Chardonnet, Y., and S. Dales.** 1970. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. Virology **40**: 462–477.
11. **Chardonnet, Y., and S. Dales.** 1970. Early events in the interaction of adenovirus with HeLa cells. II. Comparative observations on the penetration of types 1, 5, 7 and 12. Virology **40**: 478–481.
12. **Chatterjee, P. K., and S. J. Flint.** 1987. Adenovirus type 2 endopeptidase: an unusual phosphoprotein enzyme matured by autocatalysis. Proc. Natl. Acad. Sci. USA **84**:714–718.
13. **Dales, S.** 1965. Penetration of animal viruses into cells. Prog. Med. Virol. **7**:1–43.
14. **Dales, S., and Y. Chardonnet.** 1973. Early events in the interaction of adenoviruses with HeLa cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. Virology **56**:465–483.
15. **Dales, S., and Y. Chardonnet.** 1974. Penetration and uncoating of adenoviruses. Adv. Biosci. **11**:29–40.
16. **D'Halluin, J. C., C. Cousin, and P. Boulanger.** 1982. Physical mapping of adenovirus type 2 temperature-sensitive mutations by restriction endonuclease analysis of interserotypic recombinants. J. Virol. **41**:401–413.
17. **D'Halluin, J. C., M. Milleville, P. A. Boulanger, and G. R. Martin.** 1978. Temperature-sensitive mutant of adenovirus type 2 blocked in virion assembly: accumulation of light intermediate particles. J. Virol. **26**:344–356.
18. **Dimmock, N. J.** 1982. Initial stages of infection with animal viruses. J. Gen. Virol. **59**:1–22.
19. **Hennache, B., G. Torpier, and P. Boulanger.** 1979. Freeze-fracture study of adenovirus induced KB cell surface alterations. Exp. Cell Res. **124**:139–150.
20. **Hennache, B., G. Torpier, and P. Boulanger.** 1982. Adenovirus adsorption and sterol redistribution in KB cell plasma membrane. Exp. Cell Res. **137**:459–463.
21. **Kruijer, W., F. M. A. van Schaik, and J. S. Sussenbach.** 1981. Structure and organization of the gene coding for the DNA binding protein of adenovirus type 5. Nucleic Acids Res. **9**: 4439–4459.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Lenstra, J. A., and H. Bloemendal.** 1983. The major proteins from HeLa cells. Identification and intracellular localization. Eur. J. Biochem. **130**:419–426.
24. **Lonberg-Holm, K., and L. Philipson.** 1969. Early events of virus-cell interaction in an adenovirus system. Virology **4**:323–338.
25. **Luftig, R. B.** 1982. Does the cytoskeleton play a significant role in animal virus replication. J. Theor. Biol. **99**:173–191.
26. **Lyon, M., Y. Chardonnet, and S. Dales.** 1978. Early events in the interaction of adenovirus with HeLa cells. V. Polypeptides associated with the penetrating inoculum. Virology **87**:81–88.
27. **Martin, G. R., R. Warocquier, C. Cousin. J. C. D'Halluin, and P. A. Boulanger.** 1978. Isolation and characterization of human adenovirus type 2 temperature sensitive mutants. J. Gen. Virol. **41**:303–314.
28. **Miles, B. D., R. B. Luftig, J. A. Weatherbee, R. R. Weihing, and J. Weber.** 1980. Quantitation of the interaction between adenovirus types 2 and 5 and microtubules inside infected cells. Virology **105**:265–269.
29. **Mirza, M. A. A., and J. Weber.** 1979. Uncoating of adenovirus type 2. J. Virol. **30**:462–471.
30. **Morgan, C., H. S. Rosenkranz, and B. Mednis.** 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. J. Virol. **4**:777–796.
31. **Nelson, W. J., and P. Traub.** 1982. Intermediate (10 nm) filament proteins and the Ca^{2+}-activated proteinase specific for vimentin and desmin, in the cells from fish to man: an example of evolutionary conservation. J. Cell Sci. **57**:25–49.
32. **Nelson, W. J., and P. Traub.** 1983. Proteolysis of vimentin and desmin by the Ca^{2+}-activated proteinase specific for these intermediate filament proteins. Mol. Cell. Biol. **3**:1146–1156.

33. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
34. **Philipson, L.** 1984. Adenovirus assembly, p. 309–333. *In* H. S. Ginsberg (ed.), The adenoviruses. Plenum Publishing Corp., New York.
35. **Quax-Jeuken, Y. E. F., W. J. Quax, and H. Bloemendal.** 1983. Primary and secondary structure of hamster vimentin predicted from nucleotide sequence. Proc. Natl. Acad. Sci. USA **80**: 3548–3552.
36. **Seth, P., D. Fitzgerald, H. Ginsberg, M. Willingham, and I. Pastan.** 1984. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to pepidermal growth factor. Mol. Cell. Biol. **4**:1528–1533.
37. **Staufenbiel, M., P. Epple, and W. Deppert.** 1986. Progressive reorganization of the host cell cytoskeleton during adenovirus infection. J. Virol. **60**:1186–1191.
38. **Svensson, U.** 1985. Role of vesicles during adenovirus 2 internalization into HeLa cells. J. Virol. **55**:442–449.
39. **Svensson, U., and R. Persson.** 1984. Entry of adenovirus 2 into HeLa cells. J. Virol. **51**:687–694.
40. **Wadell, G., M. L. Hammarskjöld, G. Winberg, T. W. Varsanyi, and G. Sundell.** 1980. Genetic variability of adenovirus. Ann. N.Y. Acad. Sci. **354**:16–42.
41. **Weatherbee, J. A., R. B. Luftig, and R. R. Weihing.** 1977. Binding of adenovirus to microtubules. II. Depletion of high-molecular-weight microtubule-associated protein content reduces specificity of in vitro binding. J. Virol. **21**:732–742.