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Adenovirus Inhibition of Cell Translation Facilitates Release of Virus Particles and Enhances Degradation of the Cytokeratin Network

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Infection of animal cells by a number of viruses generally results in an array of metabolic defects, including inhibition of host DNA, RNA, and protein synthesis, and morphological alterations known as cytopathic effects. For adenovirus infection there is a profound loss of cell structural integrity and a marked inhibition of host protein synthesis, the latter generally assumed necessary to enhance virus production. We examined the purpose of viral inhibition of cell translation and found that it was related in part to cytopathic wasting of infected cells. We show that viral shutoff of host translation promotes destruction of the intermediate filament network, particularly cytokeratins which are proteolysed at keratins K7 and K18 by the adenovirus late-acting L3 23-kDa proteinase. We found that if adenovirus is prevented from inhibiting cell translation, the intermediate filament network remains relatively intact, keratin proteins are still synthesized, and cells possess an almost normal morphological appearance and lyse poorly, reducing the release of nascent virus particles by several hundredfold. Remarkably, in tissue culture cells the accumulation of late viral structural proteins is only marginally reduced if host translation shutoff does not occur. Thus, a surprising major function for adenovirus inhibition of cellular protein synthesis is to enhance impairment of cellular structural integrity, facilitating cell lysis and release of progeny adenovirus particles.

Many viruses mediate cytopathic effects (CPE) in host cells through a variety of poorly understood molecular mechanisms, in some cases culminating in death of the cell (for a general review, see reference 54). On the other hand, some viruses cause persistent infections and replicate for extended periods of time without producing noticeable CPE. Cells productively infected with human adenovirus (Ad) classically demonstrate a significant CPE which becomes progressively more obvious during the late phase of the viral replication cycle. The late phase of infection is typified by replication of Ad genomic DNA, activation of the viral major late transcription unit which encodes late viral polypeptides, suppression of host and early viral mRNA translation, and preferential translation of late viral mRNAs (reviewed in reference 46). Attendant CPE is manifested by dramatic morphological alterations of infected cells, including rounding, clumping, and detachment from the monolayer (reviewed in reference 20), cessation of cellular DNA and protein synthesis (27), dissolution of the cytoskeleton (14, 55, 57, 59, 65), and eventually cell lysis.

The causes of Ad pathogenic effects are not well understood, but appear to involve a variety of different factors depending on whether in vivo infection of animals or in vitro infection of cultured cells is studied. For instance, there is limited evidence that early viral gene expression may be directly cytotoxic in some cultured cells (11, 17, 66a). There is considerably more evidence, however, that during natural infection early Ad gene expression indirectly causes extensive pathological effects by provoking a cell-mediated immune response directed against early viral proteins presented on the cell surface or by inducing elaboration of cytokines from infected cells (28, 43).

Immune attack of infected cells obviously cannot be responsible for the severe CPE typically evident at late times during Ad infection of cultured cells and may be only one factor that contributes to CPE during natural infection. Ad-mediated CPE could include an extensive collapse of cellular intermediate filament networks, which is mediated by several viral gene products. The vimentin network degenerates about the perinucleus at early times after Ad infection (14, 65), apparently by activation of a cellular protease triggered solely by viral particles (6). Collapse of vimentin and lamin networks is also mediated by the Ad early E1B 19-kDa protein in transfected cells (56, 57). In addition, it has recently been shown that during the late phase of Ad infection cytokeratins K18 and K7 are degraded by the viral late acting L3 23-kDa proteinase, leading to collapse of the keratin network (10). The disruption of cellular intermediate filament networks would be expected to contribute to CPE by impairing the structural integrity of the cell.

Few studies have systematically investigated the basis for severe pathogenic effects that occur during the late phase of Ad replication or questioned the purpose of Ad inhibition of host protein synthesis. Several inconclusive early reports studied the effect of adding high concentrations of late Ad structural polypeptides to cells in culture or to cell extracts (reviewed in reference 26). A recent study demonstrated that infection of cotton rat lungs by a replication-defective Ad virus that expressed early but not late viral genes caused extensive pathology but less than infection with wild-type (wt) Ad virus (28). Another study found that during infection of primary cultures of kerotinocytes CPE occurred only if Ad progressed into its late phase of replication (3). We therefore asked whether key events of late Ad replication are directly related to CPE, such as virus-mediated shutoff of cellular protein synthesis or the potentially toxic accumulation of late Ad structural polypeptides. Several experimental approaches were used, including infection of cell lines that are resistant to Ad-controlled translation inhibition, the ability to prevent late Ad shutoff of host protein synthesis by treatment of cells with

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2-aminopurine (2AP), and infection with a temperature-sensitive (*ts*) mutant in the viral late-acting L3 proteinase. We describe a surprising major function for late Ad inhibition of cellular protein synthesis, which is to prevent synthesis of new keratins and restoration of the cytokeratin network that is degraded during late infection. We found that viral proteolysis of keratin filaments coupled to inhibition of host translation prevents the cell from maintaining its structural integrity, which is crucial for the efficient release of infectious Ad particles.

MATERIALS AND METHODS

Viruses, cells, and plasmids. Ad 300 is a wt strain 5 isolate (H5*wt*300) originally purified by H. Ginsberg. Ad *dl*309 is a phenotypically wt strain with altered restriction enzyme sites (34). Ad2 *ts*1 was provided by D. Ornelles (Wake Forest University). 293 cells are a human embryonic kidney cell line that express the E1 region of Ad5 (31). 293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Virus stocks were grown and titers were determined on 293 cells. RD (human rhabdosarcoma) cells were obtained from the American Type Culture Collection and grown in DMEM containing 15% fetal bovine serum. 2AP was prepared and used as previously described (32). Infections of cells with viruses were typically carried out for 1 h with 50 PFU per cell unless otherwise noted.

Labeling of cells and analysis of polypeptides. Cells were labeled with [^{35}S]methionine for 1 to 2 h by using 50 μCi of *trans*[^{35}S]methionine (ICN) per ml in DMEM lacking methionine and supplemented with 2% calf serum. Cell extracts were prepared by sonication of washed cells in 10 mM KCl–10 mM Tris (pH 7.4)–1 mM EDTA at 4°C and cleared of debris by centrifugation at 10,000 × *g*; equal amounts of protein were analyzed in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Gels were fluorographed and quantitated by densitometry. Immunoprecipitation analysis was performed with equal amounts of protein in cell extracts, using specific antisera and protein A-Sepharose beads (Sigma Chemical Co.). Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Antisera consisted of a polyclonal antibody directed against the E1B 19-kDa protein (provided by E. White, Rutgers University; 59) or a monoclonal antibody directed against hsp/hsc 70 proteins (J. Thomas, New York University).

Indirect immunofluorescence staining and photography of cells. Cells were grown on coverslips, fixed with paraformaldehyde, and permeabilized with Triton X-100 as described previously (61). Mouse monoclonal antibodies directed against vimentin were purchased from Boehringer Mannheim Biochemicals. A mouse monoclonal antibody specific for keratins K18 (KS-B17.2) and K1, 5 to 8, 10, 11, and 18 (no. 8.13) were from Sigma Chemical Co. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies were purchased from Sigma. Cells were photographed under visible or ultraviolet light using a Zeiss photomicroscope.

Extraction and biochemical analysis of insoluble keratin filament proteins. Intermediate filaments were extracted by the method of Wu et al. (62) as modified by Vassar et al. (53). Briefly, cells were scraped from plates, washed twice in cold phosphate-buffered saline, resuspended in 20 mM Tris-HCl (pH 7.4)–0.6 M KCl–1% Triton X-100, and then lysed by sonication at 4°C. An insoluble fraction was derived by centrifugation for 20 min at 10,000 × *g*; the pellet was resuspended in the same buffer and centrifuged again. The insoluble pellet was then resuspended in 8 M urea–10% β-mercaptoethanol, protein levels were determined using Bio-Rad reagent, and equal amounts were subjected to SDS-polyacrylamide gel electrophoresis.

Western immunoblot analysis. Equal amounts of protein samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked, and probed with keratin K-18-specific immunoglobulin G monoclonal antibody (KS-B17.2; Sigma) or one that recognizes keratins K1, 5 to 8, 10, 11, and 18 (no. 8.13, Sigma). Antibodies were detected by binding to ^{125}I-protein A (New England Nuclear) and quantitated by densitometry of autoradiograms.

RESULTS

Absence of CPE in late Ad-infected cell lines resistant to translation inhibition. Previous studies found that several cell lines are capable of supporting productive Ad replication without permitting viral suppression of host protein synthesis at late times after infection (33, 39). One such line, a rhabdosarcoma (RD) cell line, maintains normal levels of host and somewhat reduced levels (~three- to fourfold lower) of late viral protein synthesis (39), which correlated with resistance to Ad-mediated inactivation and dephosphorylation of cap binding protein (also called eIF-4E) (33). RD cells were therefore infected with wt Ad5 virus (Ad 300), and the level of protein synthesis was monitored by metabolic labeling with [^{35}S]methionine and SDS-polyacrylamide gel electrophoresis. Plates of cells were also observed and photographed until 3 to 4 days postinfection (p.i.), at which point cells detached from the monolayer.

Figure 1A demonstrates the inability of Ad to inhibit host protein synthesis in RD cells, which was readily apparent by the diffuse background of labeled cellular polypeptides and the equal levels of actin protein present in uninfected and late Ad-infected cells. As shown previously by several laboratories (33, 39), accumulation of late Ad structural polypeptides and viral particles is reduced by ~two- to fivefold in these cells. Remarkably, few if any morphological features associated with Ad-induced CPE were found in these cells (Fig. 1B), including cell rounding and swelling. Instead, centers of infection were observed to display slight distortion immediately preceeding cell detachment from the monolayer and occasionally cell lysis, leaving large areas devoid of cells by day 3 as shown. Identical results were also obtained in GM2767A cells (data not shown), another line resistant to late Ad translation inhibition (45), suggesting but not proving that CPE might correlate with inhibition of cellular translation. However, given the inability to directly compare CPE and viral translational control in similar cells that undergo shutoff, we instead chose a different approach to this problem.

2AP prevents Ad shutoff of cell protein synthesis and induction of CPE. Previous work showed that the drug 2AP could prevent the shutoff of cell translation during late Ad infection while maintaining high rates of viral protein synthesis and accumulation (32), by blocking virus-mediated underphosphorylation of translation factor eIF-4E (33). Late Ad mRNAs continue to translate because they possess a common 5′ noncoding region called the tripartite leader (16) which apparently recruits minute amounts of remaining active factor (51) through preferential binding to an unstructured 5′ end (66; reviewed in references 46 and 50). Since most late viral polypeptides are synthesized at near-normal levels in cells treated with 2AP (32), this agent can be used to determine whether CPE results from potential cytotoxic effects of a late Ad polypeptide, from inhibition of cellular protein synthesis, or from alteration of other cellular processes.

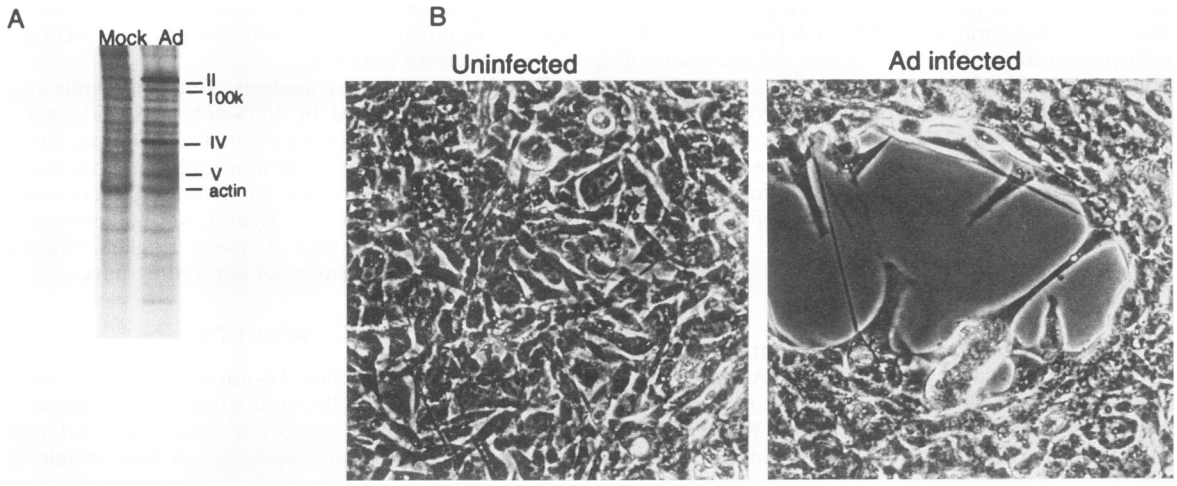

FIG. 1. Pattern of protein synthesis and changes in cellular morphology in uninfected and late Ad-infected RD cells. Cells were infected with 50 PFU of wt Ad per cell and analyzed 48 h later. (A) Cells were labeled with 50 μCi of [^{35}S]methionine per ml for 2 h, lysates were prepared, and equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and fluorography. Late Ad polypeptides are indicated. (B) RD cells were photographed at 48 h p.i. A typical area in which infected cells have detached from the monolayer is shown.

293 cells were treated with 2AP for the duration of infection, starting shortly after the addition of virus, as previously described (32). Duplicate plates of cells were then labeled with [^{35}S]methionine, and extracts were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2) or photographed at various times after infection for evidence of CPE (see Fig. 4). The ability of 2AP to prevent Ad shutoff of cell protein synthesis while maintaining high rates of translation for late Ad mRNAs was apparent (Fig. 2). Levels of cellular polypeptide synthesis (e.g., actin and background bands) as well as of Ad mRNAs which lack the tripartite leader (e.g., protein IX) were all significantly elevated by treatment of cells with 2AP. Immunoprecipitation analysis of selected polypeptides from labeled extracts indicated that preferential translation of late Ad mRNAs suppressed synthesis by severalfold of several cellular and early viral proteins that could potentially protect against cytotoxicity (Fig. 3). These include the heat shock hsp/hsc 70 proteins (Fig. 3A), which maintain normal cell viability (reviewed in references 13 and 25), and the Ad early E1B 19-kDa protein (Fig. 3B), shown to block cytotoxicity and apoptosis associated with expression of E1A proteins and tumor necrosis factor alpha (30, 44, 58, 60). Western immunoblot analysis of both hsp/hsc 70 and E1B 19-kDa proteins showed a two- to threefold decrease in steady-state levels as well (data not shown).

Most striking, however, was the large reduction in CPE in Ad-infected 293 cells treated with 2AP (Fig. 4). 293 cells are exquisitely sensitive to late Ad CPE as shown and generally displayed severe morphological alterations such as swelling, detachment from the monolayer, and lysis by 24 to 48 h after infection. Infected cells treated with 2AP demonstrated only slight CPE at 24 and 48 h. As observed with infected RD cells, by 48 h after infection some 2AP-treated 293 cells began to detach from the monolayer but there was little evidence that large numbers of cells lysed. Uninfected cells treated with 2AP did not demonstrate evidence of drug toxicity until ~3 days after treatment (data not shown; 32). Similar effects of 2AP on Ad-induced CPE were also observed for infected KB and HeLa cells (data not shown). These results therefore indicated that accumulation of abundant amounts of late Ad polypeptides was not particularly cytotoxic and was not likely to account for CPE during the late phase of infection.

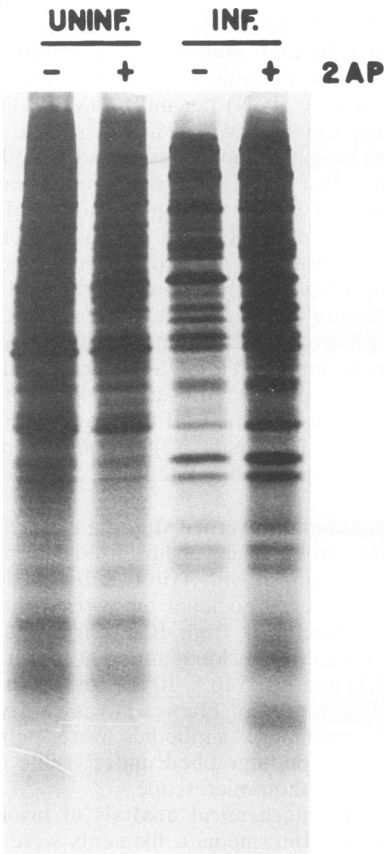

FIG. 2. 2AP prevents the shutoff of translation of cellular and early Ad mRNAs. Uninfected and wt Ad-infected 293 cells were labeled with [^{35}S]methionine at 24 h p.i., and duplicate plates were treated with 10 mM 2AP, added 1 to 2 h after infection as described previously (32). Equal amounts of protein from labeled extracts were analyzed by SDS-polyacrylamide gel electrophoresis and fluorographed. Ad late polypeptides correspond to proteins II, III, IV, V, pIV, pVIII, pVI, and IX.

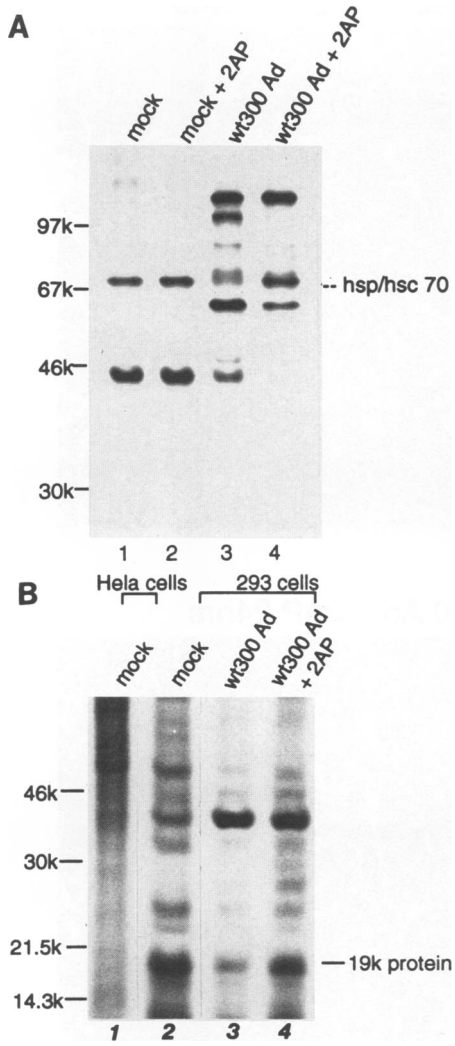

FIG. 3. Synthesis of hsp/hsc70 and E1B 19-kDa proteins is suppressed during the late phase of Ad infection. 293 cells were infected with wt Ad at 50 PFU per cell in the absence or presence of 2AP treatment. Uninfected (mock) and infected cells were labeled for 1 h with 50 μCi of [^{35}S]methionine per ml at 20 h p.i., lysates were prepared, and equal amounts of protein were used for immunoprecipitation with antibodies directed specifically against hsp/hsc 70 proteins (A) or the E1B 19-kDa protein. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Quantitation of bands was performed by densitometry. The positions of molecular size markers are indicated. A HeLa cell sample was included as a control in panel B because it is devoid of the E1B 19-kDa protein.

2AP is an inhibitor of several protein kinases (19), and it is therefore not surprising that treatment of cells results in a range of effects, including the ability to override cell cycle control checkpoints (2), inhibition of transcriptional activation by double-stranded RNA and interferon (52, 67), possible inhibition of the activity of the double-stranded RNA-activated inhibitor (DAI) kinase (35, 67), and rescue of poliovirus mutant 2A protease activity (40). It was therefore of concern that the ability of 2AP to prevent late Ad-induced CPE might be a general phenomenon, unrelated to prevention of Ad translation shutoff. This possibility was addressed by determining whether 2AP could prevent CPE caused by poliovirus and influenza virus, both of which shut off cellular protein synthesis. We found that 2AP was unable to prevent CPE caused by poliovirus and, in agreement with a previous report (40), did not prevent translation shutoff or greatly alter poliovirus replication. Viral translation and shutoff of host protein synthesis in influenza virus-infected cells was also not appreciably affected by 2AP treatment (data not shown). Thus, 2AP is not a general inhibitor of virus-mediated CPE and translation shutoff but rather acts with specificity in Ad-infected cells.

Disruption of vimentin filaments is not sufficient to cause Ad-associated CPE. Several Ad polypeptides have been shown to disrupt different components of the network of cellular intermediate filaments, either during early virus infection as shown for vimentin (14, 65) or in the late phase as shown for cytokeratins (10). We asked whether CPE results from viral disruption of different families of intermediate filaments by examining their organization in late Ad-infected 293 cells, with and without 2AP treatment (Fig. 5). Cells were grown on coverslips, infected with wt Ad, and then fixed and processed for indirect immunofluorescence by using an antibody directed against vimentin, which was shown previously to indicate the collapse of these filaments during infection (10, 56). As expected, uninfected cells possessed a highly organized network of vimentin filaments, which was found to be extensively disrupted during late Ad infection. Treatment of uninfected cells with 2AP for 48 h did not alter the normal organization of vimentin filaments. Surprisingly, infected cells treated with 2AP, which appeared morphologically similar to uninfected cells (Fig. 4), still showed extensive disruption of the vimentin network. The collapse of intermediate filaments in uninfected cells treated with 2AP was slightly less extensive than that in untreated infected cells, but still was quite significant. We can conclude that the disruption of vimentin filaments during late Ad infection was not sufficient to generate the CPE typically observed. The lack of correlation between collapse of the vimentin network and Ad-induced CPE is understandable, however. Many cells lack vimetin networks but synthesize them in culture, suggesting that the network may not be vital for the structural integrity of these cells (18).

Degeneration of the cytokeratin network requires shutoff of host translation and Ad L3 23-kDa proteinase activity. An intact cytokeratin network has been shown to be vital for the maintenance of normal cell structure, in that disruption of keratin filaments leads to cell lysis by only mild mechanical stresses (12, 53). The Ad L3 23-kDa proteinase, which is required at late times after infection for morphogenesis of viral capsid proteins (7, 64), has also been shown to cleave cytokeratin K18 and probably K7, thereby disrupting the keratin network during late infection (10). We therefore determined whether a primary function of host translation shutoff by Ad is to prevent repair of the proteolysed cytokeratin network, leading to extensive loss of cellular structural integrity and shape.

Recent work by Chen et al. (10) showed that during the late phase of Ad infection in HeLa cells, the L3 23-kDa proteinase cleaves the amino-terminal head domain of cytokeratin K18 and probably K7, resulting in disassembly of the intermediate filament network into cytoplasmic clumps. In our experiments, 293 kidney epithelial cells were used because they demonstrate a severe CPE and possess a cytokeratin network more related to bronchial epithelium, the natural host tissue for infection by Ad2 and Ad5 (22).

Cells were infected with wt Ad in the presence or absence of 2AP treatment and then fixed for indirect immunofluorescence by using an antibody directed specifically against keratin K18. Visual inspection of uninfected 293 cells demonstrated a

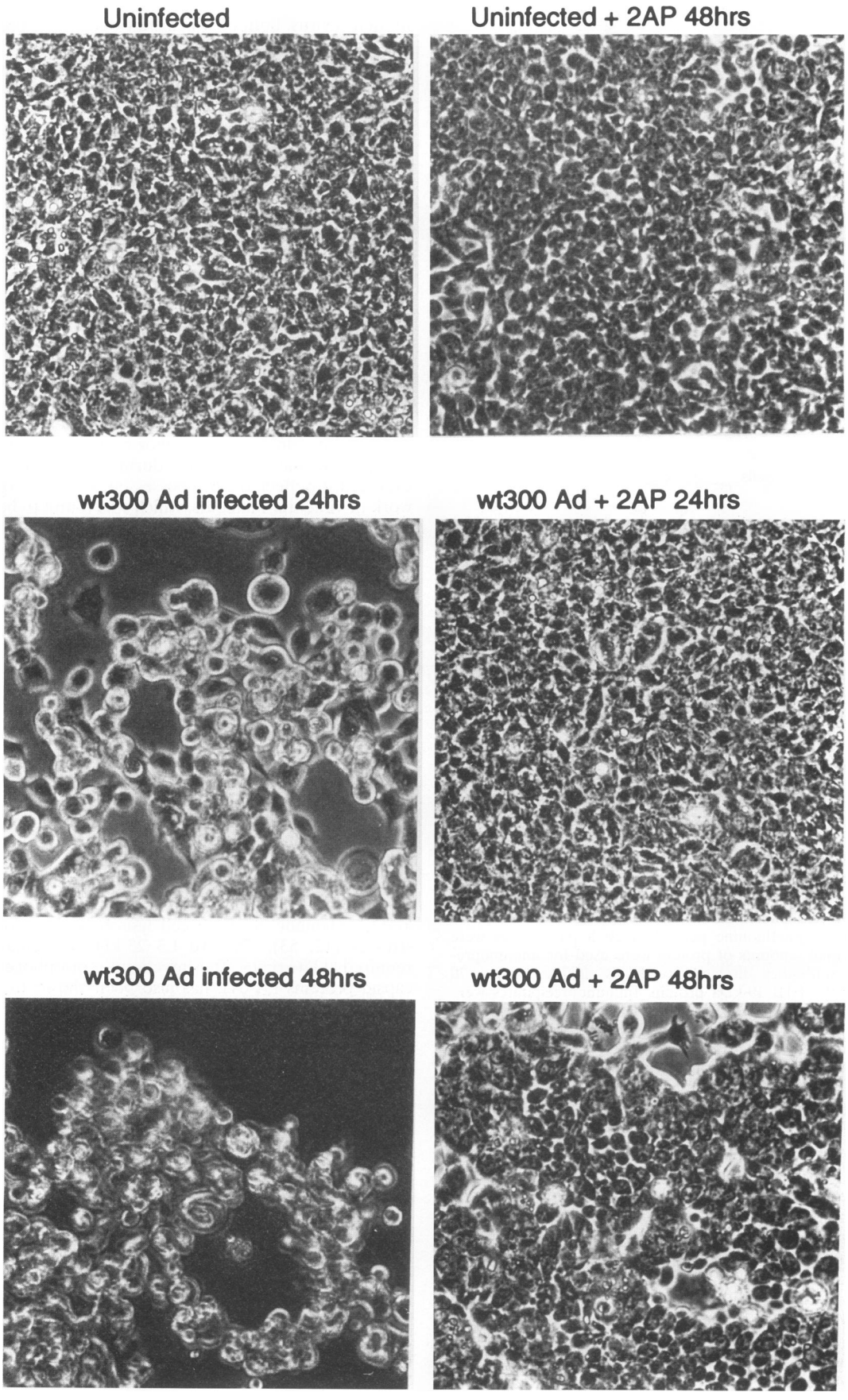
Uninfected
Uninfected + 2AP 48hrs
wt300 Ad infected 24hrs
wt300 Ad + 2AP 24hrs
wt300 Ad infected 48hrs
wt300 Ad + 2AP 48hrs

FIG. 4. Effect of 2AP on Ad-induced late CPE in 293 cells. Cells were infected at 50 PFU per cell with wt Ad, and 10 mM 2AP was added to duplicate plates 2 h p.i. for up to 48 h. Cells were photographed at 24 and 48 h. 2AP treatment of uninfected 293 cells had little detectable cytotoxicity at 48 h posttreatment as shown and largely prevented detectable CPE in wt Ad-infected cells observed as late as 48 h p.i.

normal pattern of ordered keratin cables (Fig. 6). 2AP treatment did not alter the normal appearance of keratin networks in uninfected cells. Cells infected with wt Ad for 24 h (late phase) displayed extensive disruption of keratin filaments, ranging from accumulation of large cytoplasmic aggregates to numerous spheroid globules. This pattern of disruption is typically observed after amino-terminal cleavage of keratin filaments in experimental systems (53), in epidermolysis bullosa simplex skin disorders (12), and in late Ad-infected HeLa cells (10). As expected, late Ad-infected cells also showed gross morphological changes typical of viral CPE. As shown earlier, cells infected with wt Ad but treated with 2AP possessed only slight evidence of CPE by light microscopic examination. Most striking was the pattern of the cytokeratin network in these cells. Only very limited amounts of disassembly (clumping and globules) could be detected in the presence of 2AP, despite

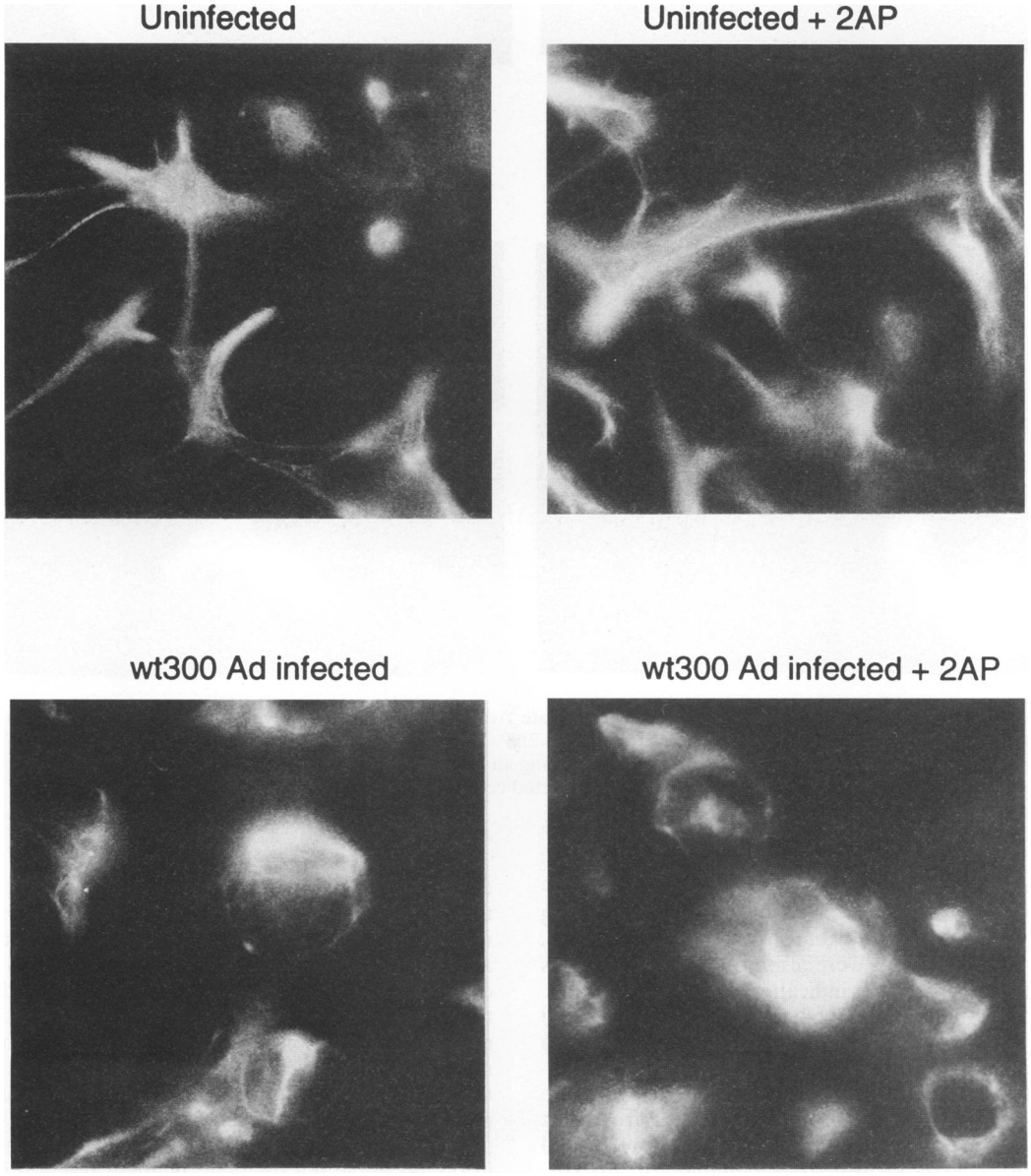

FIG. 5. Changes in vimentin filaments during late Ad infection. 293 cells grown on coverslips were infected with 50 PFU of wt Ad per cell in the absence or presence of 10 mM 2AP. At late times after infection when untreated infected cells showed extensive CPE, cells were fixed and processed for indirect immunofluorescence by using an antibody directed specifically against vimentin. Extensive disruption of vimentin filaments was observed during late infection, regardless of treatment with 2AP and the absence of CPE.

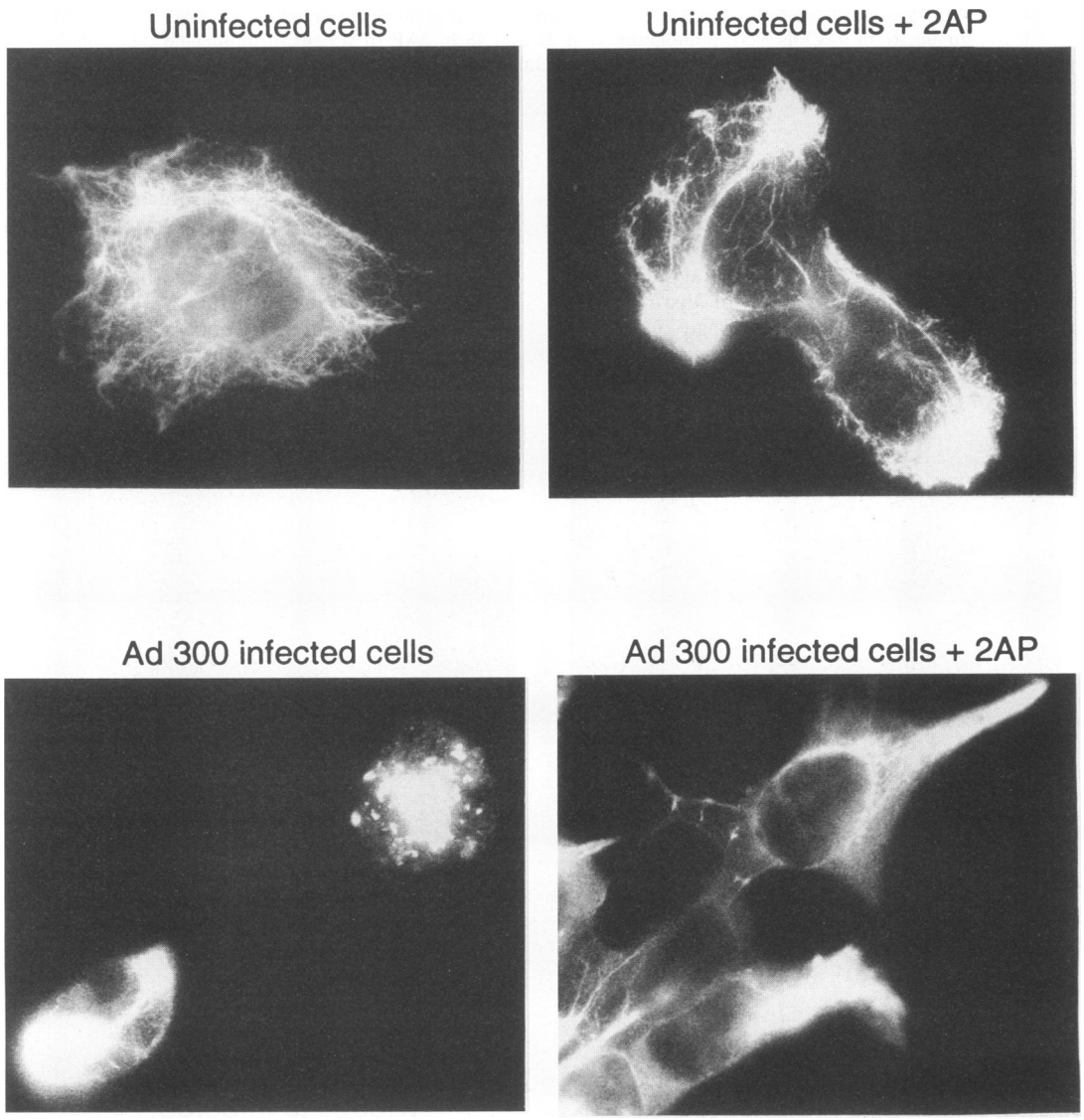

FIG. 6. The cytokeratin network is not extensively disrupted in late Ad-infected cells treated with 2AP. 293 cells grown on coverslips were infected at 50 PFU per cell with wt Ad in the absence or presence of 2AP. When untreated infected cells developed extensive CPE (~24 h p.i.), they were fixed and processed for indirect immunofluorescence by using an antibody directed specifically against keratin protein K18. Only slight disaggregation of cytokeratin filaments was observed in late Ad-infected cells treated with 2AP.

productive virus infection. Most networks were relatively intact, and only slight evidence was observed of intensely staining cytoplasmic clumps, typical of keratin proteolytic cleavage. However, most keratin cables were also not as well defined as those in uninfected cells, again indicative of some disruption.

The biochemical integrity of cytokeratins was next analyzed to determine whether blocking Ad translation shutoff also prevented accumulation of degraded keratin proteins. Insoluble keratin networks were extracted from uninfected and late Ad-infected cells in the absence or presence of 2AP treatment and resolved by SDS-polyacrylamide gel electrophoresis, and individual keratins were identified by immunoblot analysis, using specific antibodies (Fig. 7A). Blots probed for keratin K18 demonstrated roughly equal amounts of 45-kDa K18 protein in uninfected cells regardless of 2AP treatment. Cells infected with Ad for 24 h contained mostly the 41-kDa K18 cleavage product, indicating almost quantitative proteolysis of the amino-terminal head domain (10). It is also noteworthy that in late Ad-infected cells total K18 protein (proteolysed and full length) found in networks was reduced 10- to 20-fold compared with that in uninfected cells. Thus, Ad proteolysis of the K18 head domain or inhibition of host protein synthesis results in reduction of total K18 protein in polymerized networks in infected cells. Most surprisingly, infected cells treated with 2AP contained normal levels of uncleaved K18 protein and only detectable amounts of the 41-kDa cleavage product. The normal level of intact K18 polypeptides present in the insoluble intermediate filament fraction is indicative of its ongoing synthesis and rapid incorporation into structural keratin filaments.

A similar analysis was performed for keratins K1, 5 to 8, 10, 11, and 18, using an antibody that recognizes all species equally

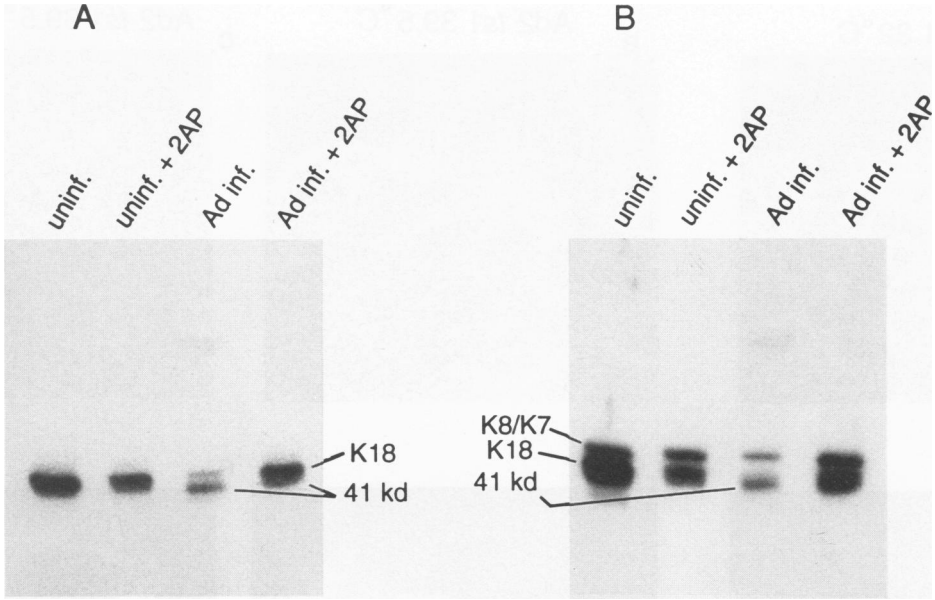

FIG. 7. Western immunoblot analysis of keratin cleavage during late Ad infection in cells treated with 2AP. 293 cells infected with 50 PFU of wt Ad per cell in the absence or presence of 2AP treatment were harvested at 24 h p.i. Whole-cell lysates were prepared, and the insoluble keratin network was purified, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed by immunoblot analysis with an antibody specific for keratin K18 and the 41-kDa degradation product (A) or an antibody that recognizes keratins K1, 5 to 8, 10, 11, and 18 (B). Specific cytokeratin polypeptides were identified by molecular size, using protein markers as indicated. The high molecular size band present in Ad-infected samples has not been identified, but might correspond to a cleavage product of a larger keratin protein.

well (Fig. 7B). The low-abundance K7 keratin is either not present in 293 cells or could not be separated from K8, which migrates in almost the same position. Keratins K1, 5, 6, 10, and 11 are not found in most cultured or transformed cells, so their absence from 293 cells is not surprising in this case (38). However, it was readily apparent that in addition to proteolysis of K18, the level of K7/K8 was also significantly reduced. It was previously shown that inhibition of cell protein synthesis with cyclohexamide did not significantly alter the pattern of keratin filaments, although the abundance of keratin proteins was not examined (37). Thus, since K8 is not a substrate for the Ad proteinase, these results suggest that viral shutoff of host protein synthesis coupled to cleavage of K18 likely reduces the abundance of a variety of polymerized keratin proteins.

Disruption of the cytokeratin network facilitates release of newly synthesized infectious virus particles. Previous studies showed that 2AP prevented Ad-mediated inhibition of host translation with only a marginal reduction in the kinetics of Ad replication and the yield of infectious virus particles (32), which at first seems to be at odds with the absence of cell lysis observed here. However, measurement of infectious virus yields involved release of particles by mechanical disruption of cells, which is typically performed to maximize recovery of mature virus regardless of whether lysis has occurred. The amount of cell-free virus released into medium in the absence of mechanical cell disruption was therefore investigated. 293 cells were infected with 50 PFU of wt Ad per cell in the presence or absence of 2AP treatment. At 3 to 4 days p.i., approximately 24 h after the majority of cells had lifted, the amount of infectious virus released into the medium was assayed before and after mechanical lysis of cells. Infection of cells and treatment with 2AP could not be extended past 3 to 4 days, because the drug itself began to cause toxicity and cell death in uninfected cells. In the absence of mechanical lysis, approximately 250-fold lower levels of infectious virus particles were released from cells treated with 2AP (Table 1). The majority of infected cells not treated with 2AP lysed within 2 to 3 days of infection, releasing large amounts of newly synthesized infectious virus particles. Thus, cell lysis and release of mature virus particles is facilitated by degradation of the cytokeratin network coupled to translation shutoff and an inability to synthesize new keratin proteins.

We next tested whether coupled shutoff of host protein synthesis and cleavage of cytokeratins truly correlates with development of CPE. Mutant Ad2 *ts*1 (temperature sensitive in the L3 proteinase) was exploited to uncouple the two effects, because it cannot cleave cytokeratins K7 and K18 at the restrictive temperature (39.5°C) but efficiently inhibits host cell protein synthesis. Thus, if the model is correct, 293 cells infected with Ad2 *ts*1 should demonstrate only partial CPE and degeneration of the cytoskeleton at the restrictive temperature for L3 proteinase activity, resulting mainly from translation shutoff.

Cells were infected with Ad2 *ts*1 at permissive (32°C) and restrictive (39.5°C) temperatures. Infection with wt Ad at

TABLE 1. Effect of CPE on release of mature Ad virus particles[a]

Treatment	Yield of wt Ad (PFU/ml)[b]	
	−2AP	+2AP
Mechanical cell lysis	1×10^9	5×10^8
No mechanical cell lysis	4×10^8	2×10^6

[a] Infected 293 cells were harvested at 3 to 4 days p.i. when most cells treated with 2AP had lifted from the monolayer, and the amount of infectious virus released into the medium was determined. Cells were either cleared from the medium by centrifugation (no mechanical disruption) or first lysed by sonication before clarification.

[b] Virus yields in medium were determined by plaque assay on 293 cells and represent the average of several independent experiments.

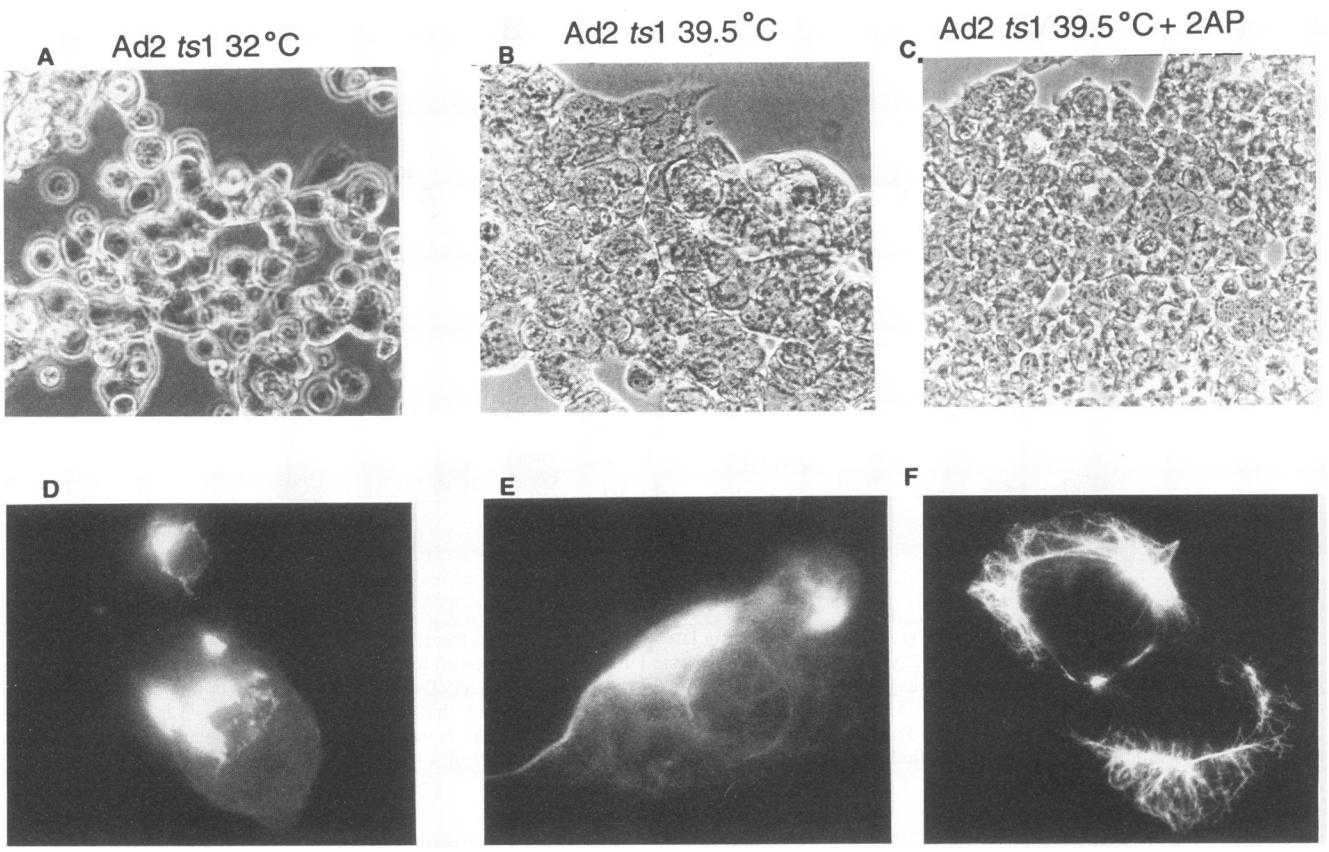

FIG. 8. Disruption of the cytokeratin network requires coupled shutoff of host translation and cleavage of keratin K18. 293 cells were infected with Ad2 *ts*1 at 4,000 particles per cell, a 25 PFU per cell equivalent, given the high particle/PFU ratio for this mutant (10), in the presence or absence of 2AP at 32 or 39.5°C. Cells infected with wt Ad at 39.5°C displayed identical viral growth kinetics as those infected at 37°C (data not shown). Cells were fixed and processed for indirect immunofluorescence, using a K18-specific antibody. Cells infected at 32°C were fixed at 40 h p.i.; cells infected at 39.5°C were fixed at 22 h p.i.

39.5°C showed a growth pattern identical to that of virus at 37°C, and therefore those data were not presented. At the permissive temperature (32°C) the Ad replication cycle is somewhat delayed, and late phase is not fully developed until approximately 36 h p.i. compared with that at 24 h at 37°C. Nevertheless, at 32°C, infection with Ad2 *ts*1 was indistinguishable from that of wt Ad (Fig. 8), causing gross morphological alterations and degeneration of the cytokeratin network as observed earlier. At the restrictive temperature, however, there was a striking difference from wt Ad-infected cells (compare Fig. 6 with 8). Cells infected with Ad2 *ts*1 possessed only partial manifestations of CPE, including slight swelling and rounding (Fig. 8B and E). Interestingly, staining of keratin filaments revealed that the cables were largely intact but poorly resolved. Thus, slight degeneration of the intermediate filament network was evident in the absence of keratin cleavage, similar to that of 2AP-treated cells infected with wt Ad. In cells infected with Ad2 *ts*1 at 39.5°C and treated with 2AP, the keratin network was remarkably well preserved (Fig. 8C and F), resembling that of uninfected cells rather than that of wt Ad-infected cells treated with 2AP. Distinct keratin cables were clearly visible despite productive Ad infection, and cells appeared almost identical to uninfected controls. Accordingly, at 39.5°C, the Ad2 *ts*1-infected cells only detached from the monolayer quite late in the infection and lysed more poorly than cells infected at the permissive temperature for L3 proteinase activity (data not shown). Precise quantitation of the number of Ad2 *ts*1 particles released from cells under these conditions was not possible, however, given the impaired ability of the virus to form plaques even at the nonrestrictive temperature (10; unpublished results).

DISCUSSION

A primary function for Ad inhibition of host translation was elucidated in this report. Through several lines of evidence it was shown in tissue culture cells that shutoff of host protein synthesis was only slightly (severalfold) involved in selective translation of late viral mRNAs and instead was necessary to facilitate cell lysis and release of infectious progeny virus particles. First, there was a striking absence of CPE in late Ad-infected cells if shutoff of host translation was prevented by 2AP (Fig. 2 and 4) or in cells which are resistant to viral translation inhibition (Fig. 1). Since only slightly reduced levels of virus particles were synthesized (~two- to fourfold), it was apparent that efficient translation and accumulation of late viral polypeptides did not require shutoff of host protein synthesis and that accumulation of late polypeptides was not necessarily cytotoxic. Second, the absence of CPE was associated with an inability to efficiently release virus particles from infected cells (Table 1). The Ad particles retained in cells were fully matured and infectious, as evidenced by the normal yields

obtained if cells were mechanically lysed. Third, the Ad L3 23-kDa proteinase was shown previously to degrade K18 and probably K7 keratin proteins late during Ad infection, leading to loss of cell structural integrity (10). In Fig. 6 and 8 we showed that degeneration of the cytokeratin network, appearance of CPE, and loss of cell structural integrity only occurred if Ad prevented host protein synthesis. Biochemically, Ad pathogenic effects correlated with proteolysis of keratin K18 and with an inability of the cell to maintain the integrity of the cytokeratin network through continued synthesis of new proteins (Fig. 7 and 8). It is important to note that synthesis of keratin K18 alone is probably insufficient to prevent CPE and cell lysis and that other host polypeptides may also be required. Additionally, although cleavage of keratin K18 coupled to translation inhibition is clearly important for CPE, it is unlikely to be solely responsible for the Ad-induced effect.

2AP was shown to prevent Ad shutoff of host protein synthesis by preventing virus-mediated dephosphorylation and inactivation of translation factor eIF-4E, also known as cap binding protein (32, 33). Unlike most cell mRNAs, late Ad mRNAs appear to require only minute amounts of this factor (16, 51). However, the basis for 2AP activity in translation shutoff is not known, and several other interpretations of our results should therefore be considered. First, we can currently exclude the possibility that the activity of 2AP was unrelated to its effect on Ad translation shutoff. For instance, it is clear that the L3 23-kDa proteinase was not simply inactivated by 2AP treatment. This proteinase is essential for morphogenesis of viral structural proteins and assembly of virus particles (7, 64), which were produced at near-normal levels despite 2AP treatment (Fig. 2 and Table 1). Thus, the L3 23-kDa proteinase retained its activity in the presence of 2AP, but large amounts of degraded keratin filaments did not accumulate. Second, it is clear that 2AP did not act to enhance the synthesis of new keratin proteins in uninfected cells (Fig. 7). Third, it is unlikely that 2AP treatment resulted in modification of keratin K18 in such a way that it was resistant to cleavage by the Ad proteinase, because the same recognition site is present in several late Ad structural proteins which were efficiently cleaved in the presence of 2AP. Finally, further evidence that 2AP prevents cytokeratin degradation by blocking late Ad translation shutoff derives from its inability to prevent shutoff of host protein synthesis when added more than 2 h p.i. (32). The later addition of 2AP failed to block both development of CPE during Ad infection and proteolysis of the cytokeratin network (data not shown). Additionally, 2AP prevented even the milder form of CPE apparent in cells infected with Ad2 *ts*1, which inhibits host translation without cleavage of keratin cables. Thus, it is doubtful that 2AP possesses an intrinsic ability to prevent Ad-mediated degradation of cytokeratin filaments apart from its ability to block translation shutoff by the virus.

The cytokeratin network is generally considered to be quite stable, with polymerized keratin proteins such as K18 possessing half-lives on the order of 3 to 4 days (15). Evidence also indicates that there is little free pool of unpolymerized, soluble cytokeratin proteins (reviewed in reference 8). Thus, the stability of the keratin network can be readily impaired by the introduction of only small amounts of an amino-truncated keratin protein (1) or cleavage of the head domain of keratin K18 by the Ad L3 23-kDa proteinase (10). Importantly, inhibition of cell protein synthesis does not cause degeneration of the keratin network (37), excluding Ad translation shutoff per se as the mitigating effect.

In contrast to the image of the cytokeratin network as static and easily disrupted, there is also good evidence that it can be a dynamic structure, disassembling and reassembling with mitosis in some cells (23, 36). Limited evidence from in vitro studies suggests that disassembly may be regulated by specific phosphorylation of certain keratin proteins (9, 63). In vivo, reorganization of the network occurred by epidermal growth factor-induced phosphorylation of a 55-kDa keratin (5), although disassembly induced by phosphorylation has not yet been observed in experimental systems in vivo. Regardless, it is unlikely that 2AP acted by altering cytokeratin phosphorylation. The viral L3 proteinase clearly mediated disassembly of the cytokeratin network and induction of CPE during late Ad infection when coupled to translation shutoff, since the L3 23-kDa mutant (Ad2 *ts*1) failed to degrade cytokeratin filaments at the restrictive temperature (10) (Fig. 8) and caused only mild CPE in 293 cells. In addition, 2AP had no effect in uninfected cells or when added later than 2 h after Ad infection. Our results are most consistent with a model in which inhibition of host translation is required to prevent continued insertion of new keratin proteins into filament networks impaired by Ad proteolysis.

Several studies support a model in which continued synthesis of keratin proteins could prevent collapse of the filament network despite cleavage of K18. The cytokeratins, like most intermediate filaments, have been shown to exist in a dynamic equilibrium in which a small free pool of unpolymerized subunits can rapidly exchange into polymerized filaments (reviewed in reference 47). For example, microinjected keratin K18 was fully incorporated into the cytokeratin network of epithelial cells by 1 to 2 h after introduction (37). The very rapid incorporation of free keratin protein into authentic filaments in epithelial cells was also observed after microinjection of K8 and K18 mRNAs, which showed a clustered pattern of new protein distribution (22, 24). It was suggested that newly synthesized keratins were preferentially or cotranslationally incorporated into existing cytokeratin filaments. Cleavage of keratins K18 and K7 during Ad infection would therefore be expected to only marginally alter the keratin network if synthesis of new keratin proteins was not prevented, since it is likely that those proteolysed would be rapidly replaced. In support of this model, wt Ad-infected cells treated with 2AP were found to possess a slightly disassembled network of cytokeratins (Fig. 6), fewer distinct cables than in Ad2 *ts*1-infected cells, and small amounts of the 41-kDa K18 cleavage product (Fig. 7).

Previous work from our laboratory suggested that Ad shutoff of host protein synthesis involves a different molecular mechanism than that used to promote selective translation of late Ad mRNAs (reviewed in reference 46). Separate control for translation of late Ad mRNAs and inhibition of host protein synthesis can be envisioned to provide flexibility in the viral life cycle. For example, it is well established that Ad can chronically infect lung bronchial lining and intestinal mucosa of humans for periods of months or years, resulting in the continuous shedding of low levels of virus (21, 49; reviewed in reference 48). It is possible that chronicity might be partly mediated by a failure of the virus to inhibit cell translation or to degrade the cytoskeleton. This would be expected to result in slower and poorer lysis of infected cells and a gradual release of smaller numbers of infectious particles. In addition, it is possible that these cells might be more efficiently cleared by cell-mediated immune mechanisms before they ultimately die since viral antigens would be presented on the surfaces of infected cells for considerably longer periods of time. It should be possible to test the role of cell structural integrity in the release of Ad particles and development of chronicity in cotton rats, an animal model for human pulmonary infection by Ad

(29, 41, 43). As shown by Ginsberg and colleagues, the pulmonary temperature of cotton rats is near the restrictive temperature for many Ad *ts* mutants (28). These studies demonstrated that Ad early gene expression was sufficient to induce viral pneumonia but that more extensive pathology developed when Ad was permitted to enter the late phase of its replication cycle. Infection of cotton rats with a mutant Ad deleted of the E1B 55-kDa protein, which is required for expression of Ad late genes and shutoff of cell protein synthesis (4, 42), was reported to produce significantly less pathology (28). These results led to the suggestion that the shutoff of host protein synthesis might be a critical part of virus-induced pneumonia. Future studies should now more directly investigate the influence of Ad translational control and virus-mediated degeneration of the cytoskeleton on the pathogenicity of infection in animal models.

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