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The Adenovirus Death Protein (E3-11.6K) Is Required at Very Late Stages of Infection for Efficient Cell Lysis and Release of Adenovirus from Infected Cells

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Adenovirus (Ad) infection is concluded by assembly of virions in the cell nucleus followed by lysis of cells by an unknown mechanism. We have described an Ad nuclear membrane glycoprotein of 11,600 kDa (E3-11.6K) which is encoded by the E3 transcription unit and which is synthesized in small amounts from the E3 promoter at early stages of infection but in large amounts from the major late promoter at very late stages of infection. We now report that E3-11.6K is required for the efficient lysis (death) of Ad-infected cells, and we propose that the function of E3-11.6K is to mediate the release of Ad progeny from infected cells. We have renamed E3-11.6K the Ad death protein (ADP). Virus mutants that lack ADP replicated as well as *adp*$^+$ Ad, but the cells lysed more slowly, virus release from the cell was retarded, and the plaques were small and developed slowly. Cells infected with *adp*$^+$ viruses began to lyse at 2 or 3 days postinfection (p.i.) and were completely lysed by 5 or 6 days p.i. In contrast, cells infected with *adp* mutants did not begin significant lysis until 5 or 6 days p.i. Cell lysis and viability were determined by plaque size, extracellular virus, cell morphology, release of lactate dehydrogenase, trypan blue exclusion, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for mitochondrial activity, RNA degradation, and DNA degradation as determined by agarose gel electrophoresis and the terminal deoxynucleotidyltransferase end labeling assay. Protein synthesis was almost nonexistent at 3 days p.i. in cells infected with *adp*$^+$ Ads, but it was still increasing in cells infected with *adp* mutants. Host cell protein synthesis was undetectable at 1 day p.i. in cells infected with *adp*$^+$ Ads or *adp* mutants. Cells infected with *adp* mutants showed Ad cytopathic effect at 1 or 2 days p.i. in that they rounded up and detached, but the cells remained metabolically active and intact for >5 days p.i. When examined by electron microscopy, the nuclei were extremely swollen and full of virus, and the nuclear membrane appeared to be intact. ADP is unrelated in sequence to other known cell death-promoting proteins.

Human adenoviruses (Ads) consist of a nonenveloped icosahedral capsid (reviewed in reference 45). The linear duplex DNA genome within the capsid is tightly associated with Ad-encoded core proteins. Infection of cultured cells is initiated via binding of the Ad fiber capsid protein to an unknown cellular receptor. This is followed by endosome-mediated internalization of the virion via interaction between the penton capsid protein and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (63). The virion is sequentially disassembled and extruded from the acidified endosome (20), and then the DNA-protein core enters the nucleus.

In the nucleus, the immediate-early E1A proteins are expressed initially, and then they induce transcription of the delayed-early genes in the E1B, E2, E3, E4, and L1 (early) transcription units (reviewed in references 2 and 34). About 25 early proteins function to usurp the cell, convert it into an efficient factory for virus replication, carry out viral DNA replication, and counteract host antiviral defenses (reviewed in reference 27). Viral DNA replication begins at about 7 h postinfection (p.i.), and then the infection moves into the late phase. Late proteins are primarily virion proteins and proteins required for assembly of infectious virions. Synthesis of cellular DNA, mRNA, and proteins is inhibited at late stages. Virions begin to assemble in the cell nucleus at about 1 day p.i. (reviewed in reference 12) and continue to increase in abundance until 2 or 3 days p.i.

The fate of Ad virions at the termination of productive infection is unclear and has not been investigated in detail. Most Ad researchers prepare Ad stocks by extracting virions from infected cells at 2 or 3 days p.i., a time when there is much less virus in the culture supernatant than within the cells. It is not known whether cells eventually lyse and release free infectious virions or whether Ad remains associated with cells or cellular remnants. If the cells do lyse, is it a nonspecific process, or is there a specific mechanism?

An 11,600-kDa protein (E3-11.6K) that is encoded by the E3 transcription unit has been described previously (64). E3-11.6K is an integral membrane glycoprotein that contains O-linked and complex N-linked oligosaccharides (41). E3-11.6K initially localizes to the endoplasmic reticulum and Golgi apparatus and then ultimately to the Golgi apparatus and nuclear membrane (41). Interestingly, although E3-11.6K is encoded within the early E3 transcription unit, it is synthesized in only very small amounts from the E3 promoter at early stages of infection (52). Rather, E3-11.6K is synthesized very abundantly from the Ad major late promoter beginning at about 20 to 25 h p.i. (52). In fact, E3-11.6K primarily is a late protein, and it represents the sixth family of major late proteins (52).

In this communication, we report that E3-11.6K is required for the efficient lysis of Ad-infected cells beginning at 2 or 3

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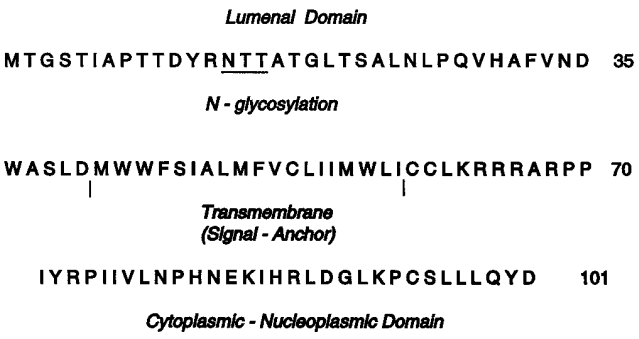

FIG. 1. Amino acid sequence of ADP from Ad2 (64). The *adp* gene is located at nucleotides 29468 to 29771 in the Ad2 genome. Shown are the single Asn-linked glycosylation site, the predicted signal-anchor transmembrane domain, and the predicted N_{lumen}-C_{exo} orientation in the membrane (41). ADP localizes primarily to the nuclear membrane at >30 h p.i., but it is not known whether localization is to the inner or outer nuclear membrane (41).

days p.i. We propose that the function of E3-11.6K is to mediate the release of virus from the infected cell. Considering that E3-11.6K is required for efficient cell lysis (death), we have renamed it the Ad death protein (ADP).

MATERIALS AND METHODS

Cells and viruses. Human A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Virus stocks were prepared in suspension cultures of human KB cells and banded in CsCl, and the titers of virus were determined using A549 cells as described previously (21). Plaques were counted at 2- or 3-day intervals until ca. 4 weeks p.i.

The viruses used in this study are Ad type 5 (Ad5) (wild type), Ad2 (wild type), and *rec*700 (wild type). *rec*700 is an Ad5-Ad2-Ad5 recombinant consisting of the Ad5 *Eco*RI A (map positions 0 to 76), Ad2 *Eco*RI D (map positions 76 to 83), and Ad5 *Eco*RI B (map positions 83 to 100) fragments (65). *rec*700 is the parental virus for *dl*712 (11), which lacks the entire *adp* gene, and for *pm*734.1 (with ADP residues 1 to 48 deleted [Δ1-48]), which has Met-1 and Met-41 in ADP mutated to Ser so that the 101-residue ADP (Fig. 1) initiates at Met-49. Construction of *pm*734.1 will be described elsewhere. *pm*734.1 does not express ADP sequences that are detectable by immunoprecipitation (53). H5*dl*309 (28), which has the genes for the E3 10.4K, 14.5K, and 14.7K proteins deleted (3), expresses ADP (53). H5*dl*327, which is isogenic with H5*dl*324 (50), has all E3 genes except the gene for the 12.5K protein deleted. H2*dl*801 (5) has all E3 genes except those for the 12.5K and 14.7K proteins deleted (24, 57).

Virus growth curves. For assay of virus growth, A549 cells (2.9×10^6 cells per 60-mm-diameter dish) were infected at 20 PFU per cell in 2 ml of serum-free DMEM. At the end of 1.5 h, the medium was removed and the cells were washed with DMEM (10% FBS), then 5 ml of DMEM (2% FBS) was added, and the cells were incubated at 37°C. At the times indicated in the figures, the supernatant was removed and centrifuged to collect cells; the cells were returned to the dish in 2 ml of DMEM (2% FBS). Monolayers were freeze-thawed three times in the dishes and then collected. Supernatant and monolayer samples were assayed by plaque assay to determine the virus titer.

LDH release assay. A549 cells (1.5×10^6 per 60-mm-diameter dish) were infected at 20 PFU per cell. At 6 h p.i., the cells were trypsinized and 1.0×10^4 cells (100-μl aliquots) were plated per well in 96-well plates in 8% FBS–DMEM. Twenty-microliter samples were removed at the times indicated in the figures and assayed for lactate dehydrogenase (LDH) release with the Cytotox 96 assay (Promega Biotec Corp., Madison, Wis.). Samples were assayed in triplicate, and results were read on a EL340 Microplate reader (BioTec Instruments, Inc.) at 490 nm.

MTT assay. A549 cells were infected at 20 PFU per cell, and 2.3×10^4 cells were plated per well in 96-well plates at 6 h p.i. At times p.i., 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) (5 mg/ml) was added to each well. After 2 h, lysis buffer (20% sodium dodecyl sulfate [SDS] in 50:50 dimethyl formamide–double-distilled water, pH 4.7) was added to each well, and the plates were incubated overnight at 37°C. Results were read on a microplate reader at 570 nm; samples were processed in triplicate.

Trypan blue exclusion assay. A549 cells were infected at 100 PFU per cell (1.3×10^6 cells per 60-mm-diameter dish) in 1 ml of serum-free DMEM; at 1 h p.i., 4 ml of DMEM (10% FBS) was added to each dish. At the times indicated in the figures, the supernatant was removed and cells were trypsinized. The supernatants and cells were combined, and trypan blue (GibcoBRL, Gaithersburg, Md.) was added to a final concentration of 0.02%. Cells were counted with a hemacytometer (a total of 600 to 1,000 cells were counted per time point). Similar results were obtained when cells were infected with 20 PFU of virus per cell.

Protein synthesis. A549 cells were mock infected or infected with 20 PFU of *rec*700 or *dl*712 per cell, metabolically labeled for 2 h with 25 μCi of Expre^{35}S^{35}S (NEN Dupont Research Products, Boston, Mass.) at different periods p.i., and then proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (52). All gels were 15% polyacrylamide. Some samples were treated with 20 μg of 1-β-D-arabinofuranosylcytosine (araC) per ml; araC inhibits viral DNA replication and prevents the transition from early to late stages of infection.

DNA and RNA degradation assay by agarose gel electrophoresis. A549 cells (1.8×10^6 cells per 60-mm-diameter dish) were infected at 25 PFU per cell. The cells were trypsinized at 4 h p.i. and then replated at 3.6×10^5 cells per 35-mm-diameter dish. On subsequent days, Hirt supernatants were prepared (65), treated with RNase, electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. RNA was analyzed in the same manner but without treatment with RNase.

DNA degradation assay by the terminal deoxynucleotidyltransferase end labeling (TUNEL) method. A549 cells were plated on no. 1 coverslips 1 day prior to infection. The cells were infected at 50 PFU per cell in 1 ml of serum-free DMEM; at 1 h p.i., 1 ml of DMEM (10% FBS) was added and the cells were incubated at 37°C. At 58 h p.i., the cells were rinsed with PBS, then fixed with paraformaldehyde (3.7% in PBS) for 10 min at room temperature, and treated with methanol for 6 min at −20°C. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (2 μg/ml) in methanol at room temperature for 2 min and then rinsed with methanol and then with 70% ethanol. Cells on coverslips were processed to detect DNA fragmentation by the addition of dUTP-digoxigenin by terminal deoxynucleotidyltransferase and detection of digoxigenin by fluorescein isothiocyanate-conjugated antibody (ApopTag in situ apoptosis detection kit; Oncor, Inc., Gaithersburg, Md.).

Light microscope cytology. A549 cells were infected at 20 PFU per cell. At daily intervals, the cells were gently trypsinized and pelleted for 1 min at 5,000 rpm in a microcentrifuge. After the supernatant was discarded, the cell pellets were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% sucrose and 1 mM calcium chloride. The tissue was washed several times in cold cacodylate buffer containing 5% sucrose and postfixed for 3 h at 4°C with 1% osmium tetroxide in cacodylate buffer containing 2% sucrose. The tissue was washed twice at room temperature with distilled water, stained en bloc for 1 h with 2.5% aqueous uranyl acetate, dehydrated through graded ethanols and propylene oxide, and infiltrated overnight with a 1:1 mixture of propylene oxide and Polybed resin (Polysciences, Inc., Warrington, Pa.). The tissue was then infiltrated for 6 h with 100% Polybed resin and embedded in fresh resin in BEEM capsules and polymerized overnight at 70°C. Sections (0.5 μm thick) were cut from the trimmed tissue blocks with a Reichert Ultracut E ultramicrotome and glass knives, dried onto glass microscope slides, stained with toluidine blue, and photographed on a Zeiss research light microscope with Kodak T-max 100 film. The film was developed with Kodak HC110 developer, dilution B, and printed on Kodak paper.

Electron microscopy. Cells at 4 days p.i. were prepared as described above. Silver-gray thin sections were cut from the trimmed tissue blocks with a Reichert Ultracut E ultramicrotome by using a diamond knife and were collected on 200-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and viewed and photographed with a JEOL 100 CX electron microscope at 60 kV.

RESULTS

Cells infected with *adp* mutants have small plaques that are slow to develop. The first clue to the function of ADP as a cell death-promoting agent came from studies of the plaque morphology of virus mutants that lack the *adp* gene. Ad5, H5*dl*309, Ad2, and *rec*700 form large, distinct plaques (Fig. 2). These viruses all express ADP. In contrast, H5*dl*327, H2*dl*801, and *dl*712 have much smaller plaques (Fig. 2). These viruses all lack the *adp* gene, and *dl*712 lacks only the *adp* gene.

The difference in plaque sizes among *adp* mutants can be quantitated by observing the rate at which the plaques develop. Figure 3 illustrates the rate of plaque development for the experiment whose results are shown in Fig. 2. The data are presented as the number of plaques observed on any given day of the plaque assay as a percentage of the final number of plaques that were observed at the end of the plaque assay (on the *y* axis) versus the number of days of the plaque assay (on the *x* axis). At 10 days p.i., 10% of the final number of plaques were observed with H5*dl*327, which lacks ADP, versus 90 to 95% of the final plaques with Ad5 and H5*dl*309, which express

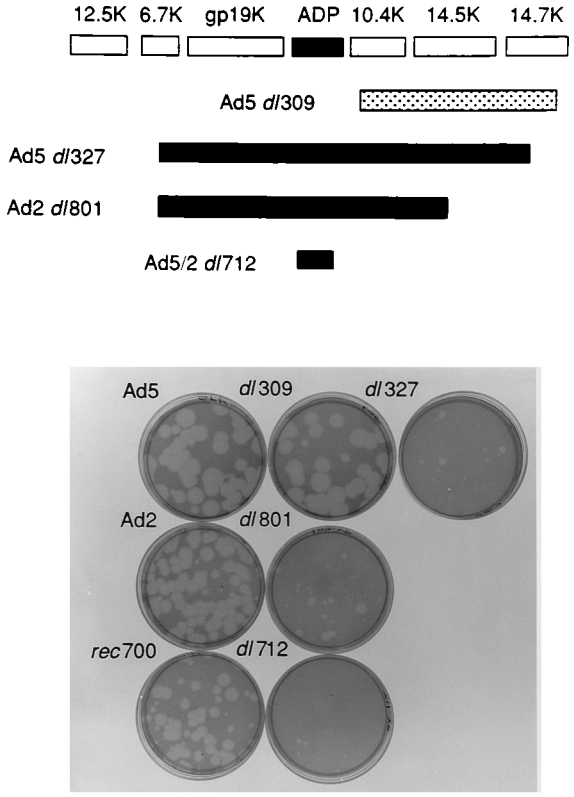

FIG. 2. Plaque morphology of wild-type Ads and Ads that have deletions in the E3 transcription unit. The plaques are on human A549 cells at 14 days p.i. *rec*700 is an Ad5-Ad2-Ad5 recombinant that, in common with Ad5 and Ad2, has a wild-type phenotype with respect to the properties of ADP. *rec*700 has the Ad2 version of ADP. The genes deleted in the mutants H5*dl*309, H5*dl*327, H2*dl*801, and *dl*712 are indicated in the schematic. All mutants that lack the *adp* gene have much smaller plaques than do the Ads that retain the *adp* gene, as shown below the diagram.

ADP (Fig. 3A). With H2*dl*801, which lacks ADP, 25% of the final plaques were observed at 10 days versus 90% for Ad2 (Fig. 3B). With *dl*712, in which the ADP gene is the only E3 gene deleted, 8% of the final plaques were observed at 10 days compared with 75% with *rec*700 (Fig. 3C). The deletion in *dl*712 changes the splicing of the E3 mRNAs at early stages of infection, and therefore the relative abundance of the E3 proteins at early stages is affected (11). The *dl*712 deletion does not have a marked effect on the E3 mRNAs at late stages of infection (data not shown), the period when ADP is synthesized abundantly. Nevertheless, we constructed *pm*734.1 (Δ1-48), a double missense mutant which does not express detectable ADP (53). With *pm*734.1 and *dl*712, 10% of the final plaques were observed at 10 days p.i. versus 85% with *rec*700 (Fig. 3D). Thus, *pm*734.1 is as defective as *dl*712 in promoting cell death, not only in the plaque development assay (Fig. 3D) but also in all other assays of cell viability (e.g., see Fig. 5).

We conclude that mutants that do not express functional ADP have small plaques that are slow to develop. (We note that in order to obtain an accurate titer for *adp* mutants, it is necessary to keep the A549 cell monolayer alive for 20 to 30 days.) The small-plaque phenotype is specific to the *adp* gene, as indicated by plaque morphology and development studies of virus mutants with individual deletions of the E3 genes (data not shown).

Mutants with alterations in the *adp* gene could have small plaques because they do not grow as well as wild-type Ad or because progeny virions are released more slowly from cells than wild-type virions. If the virions are released more slowly, then it will take longer for the virus to spread from cell to cell and form a plaque. To address these two possibilities, monolayers of A549 cells were infected with *rec*700 (wild type) or *dl*712 (ADP negative [ADP$^-$]); then the virions present in the cells and released into the culture supernatant were quantitated by plaque assay. The amounts of virus within cells increased rapidly until about 2 days p.i. with both *rec*700 and *dl*712 and then declined slightly for *rec*700 but remained stable for *dl*712 (Fig. 4). It is clear that *dl*712 grows as well as *rec*700. This is consistent with many experiments in which CsCl-banded virus stocks of wild-type Ad and *adp* mutants had similar titers (e.g., Fig. 3). When the culture supernatant was examined, a dramatic difference was seen between *rec*700 and *dl*712: from 2 to 4 days p.i., there were 10^{10} to 10^{11} *rec*700 infectious particles but only 10^7 to 10^8 *dl*712 infectious particles per ml (Fig. 4). Thus, *adp* mutant virions are released more slowly from cells than are wild-type virions. (It is unclear why 10^5 to 10^7 infectious virions were found in the culture supernatants at 12 to 30 h p.i., prior to progeny virus assembly [12 h] or cell lysis [30 h; see below], but this was a reproducible observation. Possibly, these are virions that remained adsorbed to cells during the infection and subsequent washings before addition of media. Also, 6×10^7 PFU of virus was added to each dish; if 1 to 3% of cells undergo spontaneous lysis in the period immediately following infection and these cells release virus, this could account for the background levels of virus observed in the experiment.)

Cells infected with *adp* mutants stay alive much longer than cells infected with *adp*$^+$ Ad. The data in Fig. 2 to 4 raise the possibility that cells infected with *rec*700 (wild type) are lysed more rapidly than are cells infected with *dl*712 (ADP$^-$), so several cell viability assays were employed. With *rec*700, A549 cell lysis assayed by the release of LDH began at 2 days p.i. and increased until 7 days p.i. (Fig. 5A). In marked contrast, with two *adp* mutants, *dl*712 and *pm*734.1 (Δ1-48), the cells did not release LDH until 6 days p.i.

Trypan blue exclusion was also used to assay cell viability. With *rec*700, 60% of A549 cells were dead by 3 days p.i. and 90% were dead by 5 days p.i. (Fig. 5B). With *pm*734.1 (Δ1-48) and *dl*7001 (which lacks all E3 genes, including *adp*), 90% of cells were alive at 5 days and cell death began only at 6 days p.i. The *pm*734.1-infected cells died with the same kinetics as cells infected with *dl*7001, indicating that none of the E3 genes except *adp* plays a major role in promoting cell death.

The MTT assay was carried out to monitor the mitochondrial activity in infected cells. Cells infected with *rec*700 and *dl*712 were similar until 2 days p.i., but then cells infected with *rec*700 began to lose mitochondrial activity, and all cells were dead by 5 days (Fig. 5C). Cells infected with *dl*712 retained 90% of mitochondrial activity at 5 days p.i. Human A549 cells were used in the experiment whose results are shown in Fig. 5C; similar results were obtained with monolayers of human KB cells and A431 cells (data not shown).

These results indicate that cells infected with *adp* mutants remain viable much longer than cells infected with wild-type Ad.

As another indicator of cell viability, we examined protein synthesis. Cells were labeled with [^{35}S]Met-Cys for 2 h at different periods p.i., and then proteins were resolved by SDS-PAGE. With *rec*700, Ad late protein synthesis was readily apparent at 25 and 29 h p.i. (Fig. 6A; compare the protein bands in lanes d and e with those of mock-infected cells [lane a] and mock- and Ad-infected cells treated with araC [lanes b and c]). Protein synthesis began to decline at 45 h p.i. (Fig. 6A,

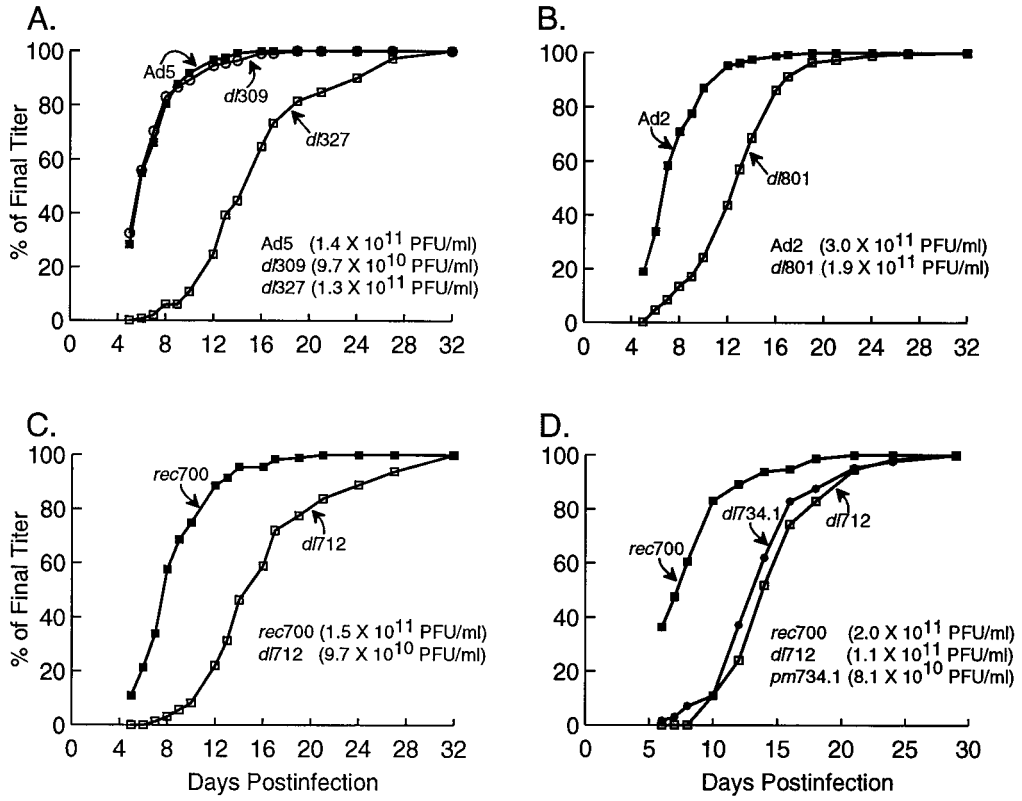

FIG. 3. Plaque development assay for mutants with alterations in ADP. Panels A to C are from the experiment whose results are shown in Fig. 2; panel D is from a separate experiment. The *y* axis shows the number of plaques observed on any given day of the plaque assay (the *x* axis), as a percentage of the number of plaques observed on the final day (day 32 or 30, as indicated) of the plaque assay. Each panel shows the final titer of the CsCl-banded virus stock that was assayed. A549 cells were used. The plaques of mutants that lack ADP (*dl*327, *dl*801, *dl*712, and *pm*734.1) developed much more slowly than adp^+ viruses, but the final titers obtained for all virus stocks were similar.

lane f) and was barely detectable at 71 h p.i. (lane h). In marked contrast, with *dl*712, the rate of protein synthesis was still increasing at 71 h p.i. (Fig. 6A, lanes j to n). In a separate experiment, protein synthesis was examined up to 6 days p.i., and this was correlated with cell viability as determined by trypan blue exclusion. The gel was overexposed in order to detect protein synthesis at very late times. With *rec*700, Ad late proteins were synthesized actively at 2 days p.i., a time when

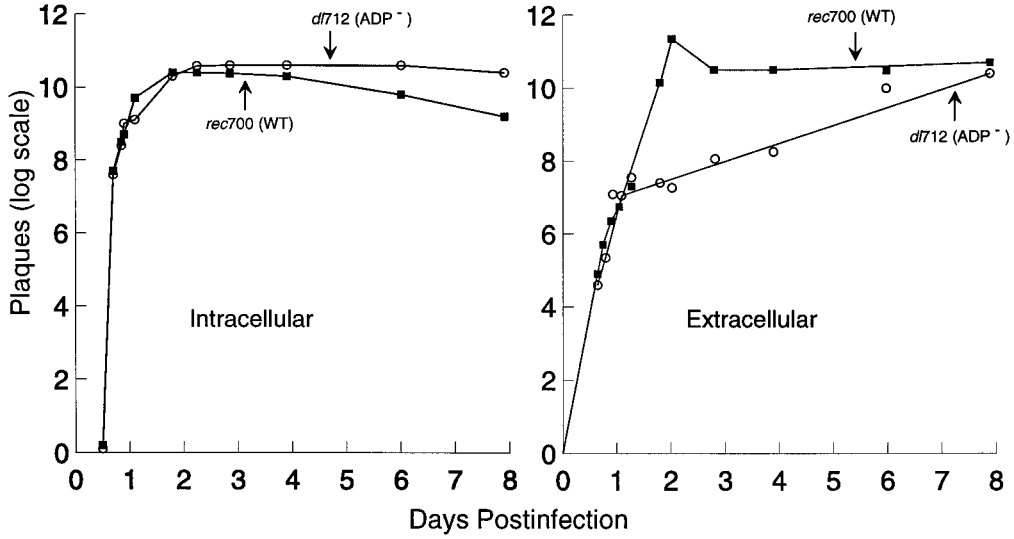

FIG. 4. Assay for the accumulation of *rec*700 (wild-type [WT]) and *dl*712 virions inside cells and in the extracellular medium. Monolayers of A549 cells were infected, and the virus present within the cells (left panel) and in the culture supernatant (right panel) was quantitated by plaque assay. The data show that the *adp* mutant grows as well as the wild-type virus within the cells but that the mutant virions are released much more slowly from the infected cells than are wild-type virions.

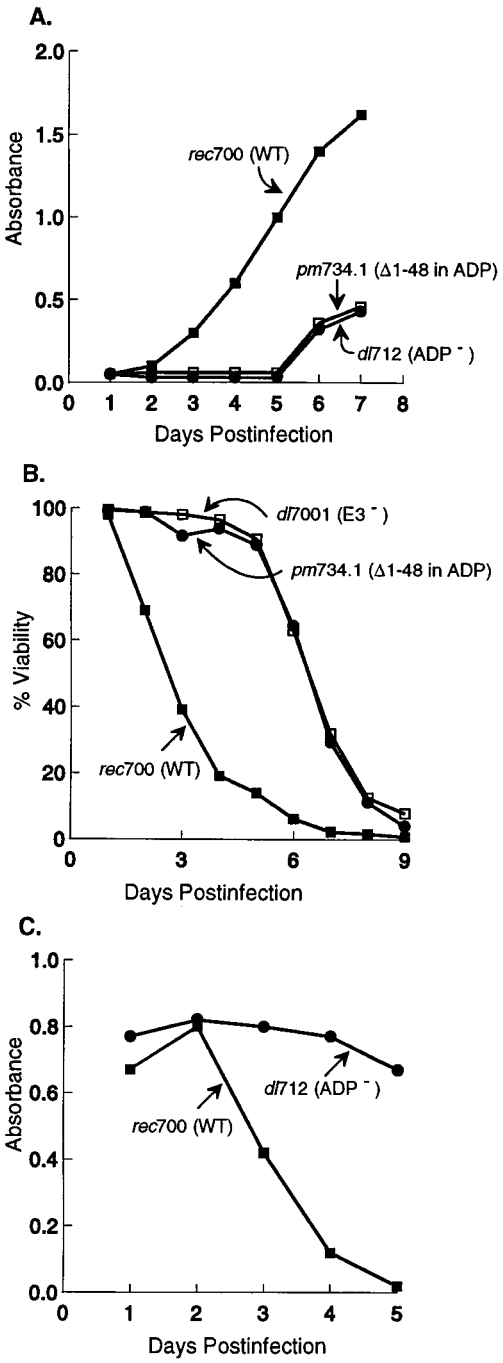

FIG. 5. Cell viability assays for A549 cells infected with *rec*700 (wild type [WT]) or mutants that lack a functional *adp* gene. *dl*712 lacks the entire *adp* gene, *pm*734.1 (Δ1-48) synthesizes only residues 49 to 101 of ADP, and *dl*7001 lack all the genes in the E3 transcription unit. (A) Cells were infected and cell lysis was measured by release of LDH into the culture medium. Cells infected with *rec*700 died (i.e., released LDH) much more rapidly than did cells infected with the two *adp* mutants. (B) Cells were infected, and the percentage of viable cells was determined on the basis of exclusion of trypan blue from the cells. Cells infected with *rec*700 died much more rapidly than did cells infected with *dl*7001 or *pm*734.1. (C) Cells were infected and cell viability was assayed (MTT assay) on the basis of mitochondrial activity within the cells. Cells infected with *dl*712 remained metabolically active much longer than cells infected with *rec*700.

66% of the cells were viable (Fig. 6B, lane c). Protein synthesis declined dramatically at 3 days and was undetectable at 5 days, when 48 and 16%, respectively, of the cells were viable (Fig. 6B, lanes d to f). With *pm*734.1 (Δ1-48), the rate of protein synthesis increased until 3 days, declined slightly at 4 days, and declined dramatically at 5 days p.i. (Fig. 6B, lanes j to m). On days 2 to 5, from 95 to 84% of the cells were viable. Thus, active protein synthesis continued for at least 2 days longer in cells infected with the two *adp* mutants than in those infected with wild-type Ad.

With *pm*734.1 (Fig. 6B, lane m) and *dl*712 (data not shown), it is interesting that protein synthesis had nearly ceased even though most of the cells were still viable (i.e., still intact, as indicated by trypan blue exclusion). This was also the case for *rec*700 (Fig. 6A, lanes f to h).

Another noteworthy result is that *rec*700 and the two *adp* mutants were equally effective in suppressing the synthesis of host cell proteins (Fig. 6). Thus, the prolonged synthesis of Ad proteins in cells infected with *adp* mutants is not due to the inability of the mutants to block host protein synthesis (e.g., it is unlikely that the host cell synthesizes antiapoptosis proteins in *adp* mutant-infected cells and not in wild-type-infected cells).

As still another indication of cell viability, cellular RNA and DNA were examined. When total cellular RNA was analyzed by agarose gel electrophoresis, the RNA was degraded by 3 days p.i. with *rec*700, but it was intact at 5 or 6 days p.i. in cells infected with *dl*712 (Fig. 7). The DNA from cells infected with *rec*700 or *dl*712 was intact at 2 days p.i., but at 3 to 5 days p.i. the DNA from *rec*700 was much more degraded than the DNA from *dl*712 (Fig. 8). A DNA ladder characteristic of apoptosis was not observed, even though DNA from cells undergoing apoptosis (dying thymocytes) yielded such a ladder on a comparable gel (data not shown). The TUNEL assay was also used to monitor DNA integrity. This assay uses a fluorescein isothiocyanate-coupled monoclonal antibody to digoxigenin to detect digoxigenin-labeled nicked DNA in fixed cells. With mock-infected cells, two nuclei contained nicked DNA (Fig. 9; compare the fields in which the DNA was stained with DAPI with the fields stained with the ApopTag kit). With *rec*700, most of the nuclei had nicked DNA, whereas with *dl*712 virtually none of them did (Fig. 9). Thus, DNA is degraded much sooner in cells infected with *rec*700 than in those infected with *dl*712.

When cells were examined by microscopy at 4 days p.i., the majority of *rec*700-infected cells were lysed, whereas nearly all *pm*734.1 (Δ1-48)-infected cells were intact (Fig. 10). The nuclei in the *pm*734.1-infected cells were extremely swollen, occupying nearly the entire cell; this is shown clearly in the electron micrograph in Fig. 11A. The nucleus was full of virus; in fact, a crystal array of virus is apparent. The nuclear membrane appeared to be intact. Figure 11B shows a *rec*700-infected cell; the cell is totally lysed. Note that the cell does not have features typical of apoptosis, i.e., condensed chromatin, cell shrinkage, membrane blebbing, and membrane-bound apoptotic bodies.

We conclude from all these data that ADP is required for the efficient lysis (death) of Ad-infected cells at very late stages of infection.

ADP is not required for Ad CPE. Although it is clear that cells infected with *adp* mutants remain viable for several days longer than those infected with adp^+ Ads, monolayer cells show typical Ad cytopathic effect (CPE). For both the wild type and *adp* mutants, CPE was observed between 1 and 2 days p.i. and was very pronounced by 3 days p.i. An example of such cells at 4 days p.i. is shown in Fig. 12. Cells infected with *rec*700

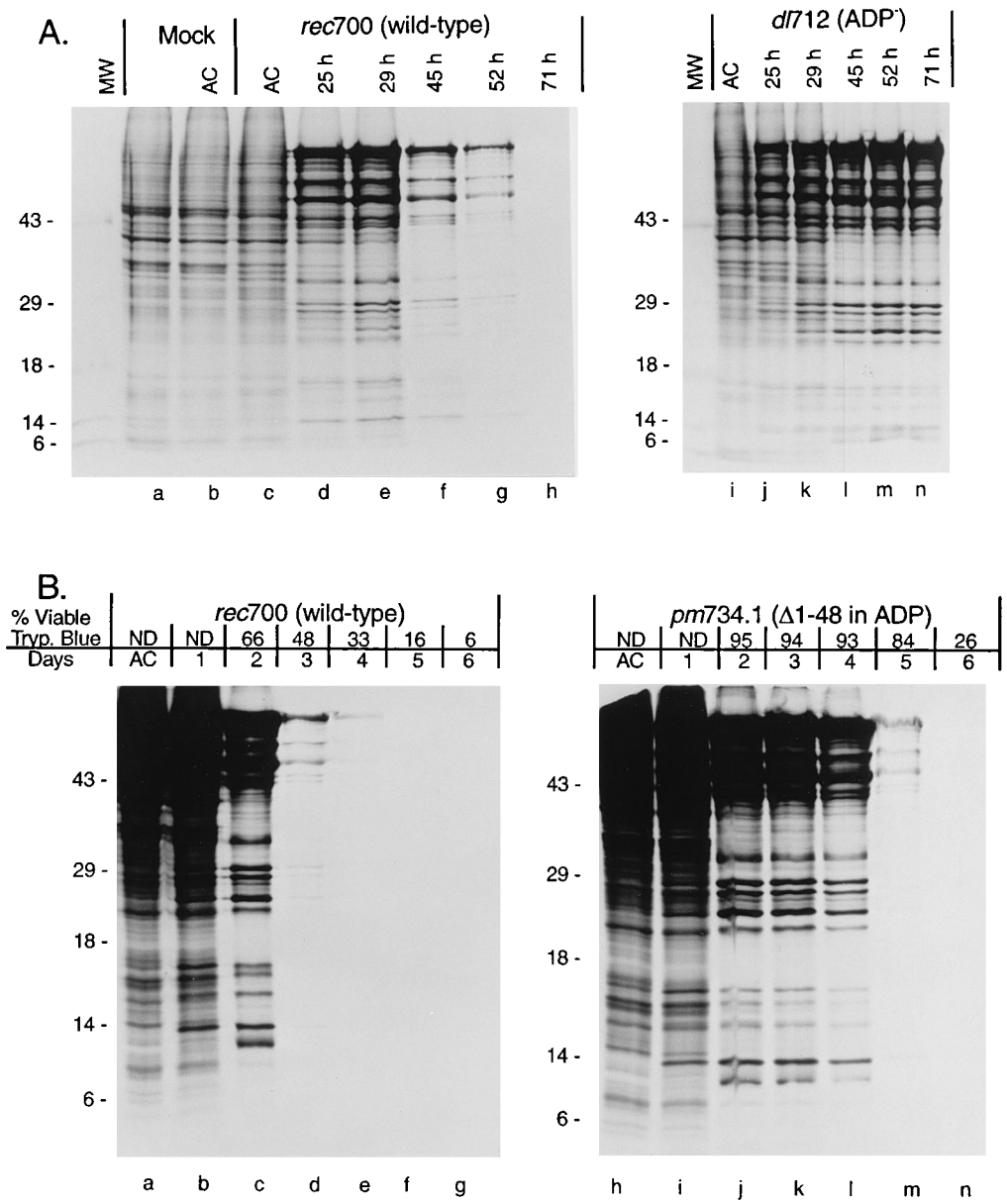

FIG. 6. Protein synthesis in cells infected with *rec*700, *dl*712, or *pm*734.1. A549 cells were mock infected or infected with *rec*700, *dl*712, or *pm*734.1 and metabolically labeled for 2 h with 25 μCi of Expre^{35}S^{35}S (NEN Dupont) at the indicated times p.i., and then proteins were analyzed by SDS-PAGE. Panels A and B are results of separate experiments. In panel B, cell viability was determined by trypan blue exclusion in parallel with labeling of the proteins. araC (AC) inhibits viral DNA replication and prevents the transition from early to late stages of infection. ND, not done; MW, molecular weight markers (weights are shown to the left of the gels, in thousands). At 25 h p.i., Ad late proteins are readily apparent, and host cell protein synthesis is curtailed.

had rounded up and detached from the plastic dishes into individual floating cells, many of which showed ballooning of the plasma membrane as if the cells had lost the ability to control osmotic pressure. On the basis of our measurements of cell viability and macromolecular synthesis and integrity, most of these cells were probably dead. With *pm*734.1 (Δ1-48), the cells had rounded up and detached into grape-like clusters. The cells were highly refractile and intact. As judged by our viability studies, most of these cells should be viable. These cells were metabolically active as indicated by the acidity (pH) of the medium (data not shown). By day 6, when many of the *adp* mutant-infected cells were dead, the grape-like clusters had dispersed into single floating cells (data not shown) in the manner of *rec*700-infected cells at day 4. That is, most of the

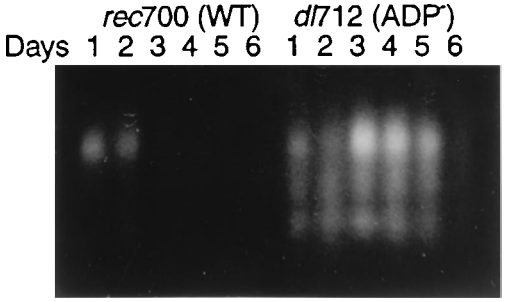

FIG. 7. Agarose gel assay for total cellular RNA in cells infected with *rec*700 (wild type [WT]) or *dl*712 at days 1 to 6 p.i.

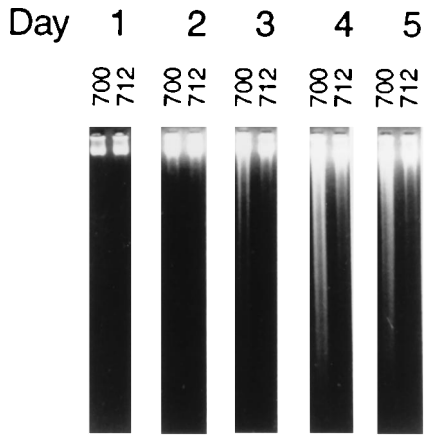

FIG. 8. Agarose gel DNA degradation assay for cells infected with *rec*700 (wild type) (lanes 700) and *dl*712 (ADP$^-$) (lanes 712). The DNA from cells infected with *rec*700 was degraded more rapidly than the DNA from cells infected with *dl*712.

clumped cells were alive, whereas most of the individual cells were dead.

We conclude that although ADP is required for efficient cell lysis, it is not required for most of the features of Ad CPE.

DISCUSSION

This study was prompted by our observation that Ad mutants that lack the *adp* gene have small plaques that are slow to develop. We then showed that mutants in the *adp* gene replicate as well as wild-type Ad, but the mutant virions are released more slowly from cells. Thus, mutant viruses spread more slowly from cell to cell, and the plaques are small.

Further studies showed that ADP is required for the efficient lysis (death) of Ad-infected cells at very late stages of infection. Cell death was demonstrated by LDH release, trypan blue exclusion, the MTT assay for mitochondrial activity, protein synthesis, DNA and RNA degradation, and cell morphology. Monolayers of permissive human cells infected with *adp*$^+$ Ad begin to die at 2 to 3 days p.i., and all the cells are typically dead by 5 to 7 days. Cells infected with *adp* mutants do not begin to die until about 6 days p.i. The cells stay intact, nuclear DNA can be readily stained with DAPI, and the cells remain metabolically active. Prior to death, cells infected with *adp* mutants have swollen nuclei that are full of virus. In recent experiments in which the medium was changed every 2 days, cells infected with wild-type Ad died with normal kinetics, but the survival of cells infected with *dl*712 (ADP$^-$) was significantly enhanced so that about 20% of the cells were still viable after 14 days p.i. (data not shown).

Our studies establish that ADP is necessary for efficient cell death, but they do not address whether ADP is sufficient for cell death. This will require experiments in which ADP is expressed autonomously.

Although Ad is one of the most prolific models in molecular biology and has been intensively studied for four decades, the mechanism by which Ad is released from cells is not understood. ADP begins to be synthesized abundantly at 1 day p.i., when virions begin to assemble in the cell nucleus, and both ADP (52) and virions (Fig. 4) continue to increase in abundance. As mentioned, cells begin to die at 2 to 3 days p.i. We propose that the function of ADP is to promote cellular lysis and the release of virus from the infected cell. To our knowledge, ADP is the first protein encoded by a mammalian DNA virus to have such a function.

Viruses encode a variety of proteins that either induce or inhibit cell death (reviewed in reference 42). Ad has several such proteins (reviewed in references 42, 58, 60, and 66 to 68). The E1A proteins induce apoptosis (59, 62), presumably by deregulating the cell cycle (10, 32, 58, 59). The E1B-19K (8, 10, 38, 40, 46, 47, 49) and E1B-55K (38, 69) proteins inhibit E1A-induced apoptosis. The E1A proteins also render cells susceptible to lysis by tumor necrosis factor (6, 13, 43), an inflammatory cytokine that is secreted by activated macrophages. Tumor necrosis factor-induced cytolysis is inhibited independently by the Ad E1B-19K protein (16, 62), the E3-14.7K protein (17, 19, 26, 37, 56), and the E3-10.4K–14.5K complex of proteins (18). These proteins presumably maintain cell viability so that virus replication can occur. ADP appears to have the opposite function: it promotes cell death so that Ad can be released from the infected cell.

In studies related to Ad-induced CPE, Chen et al. (7) observed that the Ad L3-encoded protease cleaves cytokeratin 18 and thereby disrupts the cytokeratin network of HeLa cells. Since disruption of keratin filaments renders epithelial cells more susceptible to lysis by mild mechanical stress (9, 55), Chen et al. (7) proposed that disruption of the cytoskeletal system might promote host cell lysis and release of progeny virions. Zhang and Schneider (70) extended this concept, reporting that disruption of the cytoskeletal network by the Ad protease, together with inhibition of host protein synthesis (to prevent repair of the cytokeratin network by newly synthesized keratin), is necessary for marked Ad CPE and for facilitation of lysis of infected cells and the release of mature virus particles.

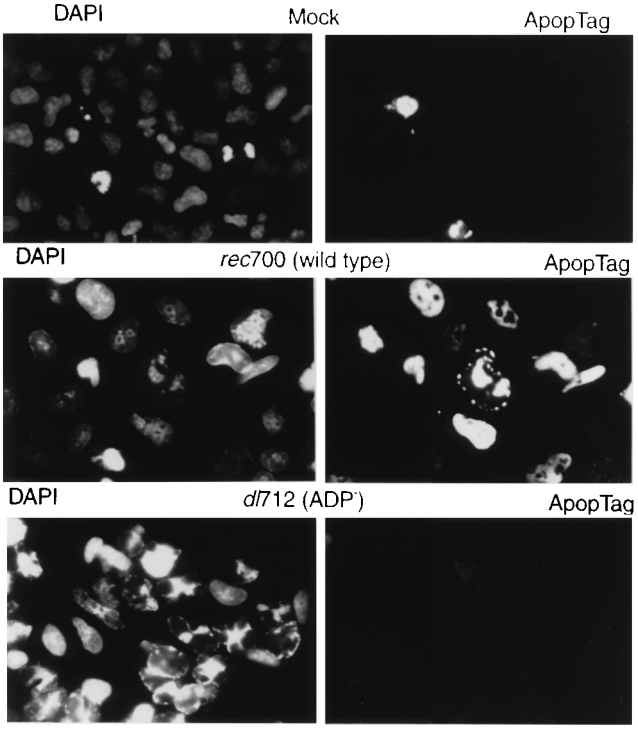

FIG. 9. TUNEL assay for nicked DNA in mock-infected A549 cells and in cells infected with *rec*700 (wild type) or *dl*712 (ADP$^-$). The cells are at 58 h p.i. DNA is stained with DAPI; fragmented DNA in the same field is labeled with ApopTag.

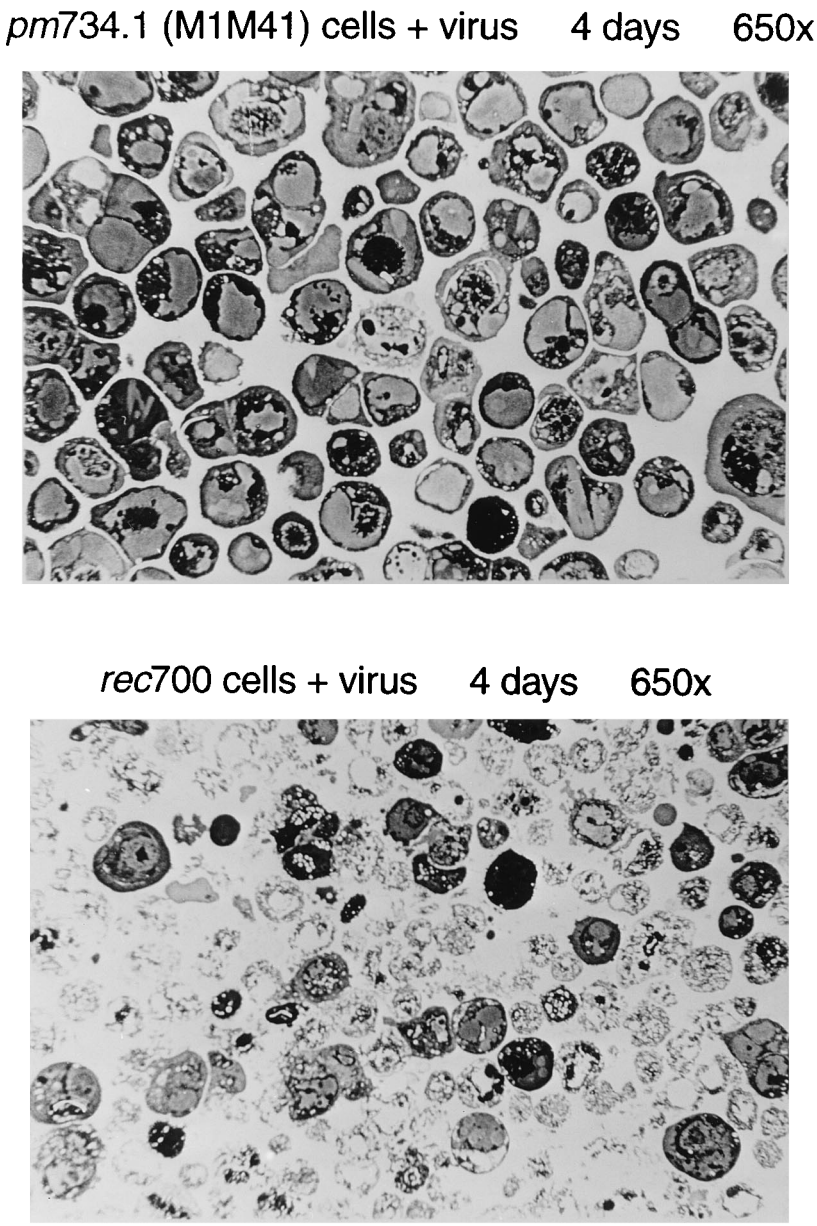

FIG. 10. Morphology at 4 days p.i. of cells infected with *rec*700 (wild type) or *pm*734.1 (lacks functional ADP). M1M41 refers to the fact that *pm*734.1 has missense mutations in Met-1 and Met-41 in ADP; thus, ADP initiates at Met-49. Most cells infected with *rec*700 are lysed, whereas most cells infected with *pm*734.1 are intact.

In our studies, cells infected with *adp* mutants showed Ad CPE in that the cells rounded up and detached from plastic dishes. Although we did not examine the Ad protease directly, it must be active in cells infected with *adp* mutants, because the protease is necessary for the production of mature infectious Ad, and deletion of the *adp* gene does not affect virus replication (Fig. 4). Also, in our studies, cellular protein synthesis was inhibited equally efficiently in cells infected with wild-type Ad or *adp* mutants (Fig. 6). Thus, the cytokeratin network was presumably equally disrupted in the infected cells. Nevertheless, our data show that *adp* is required for efficient cell lysis and progeny release. It would not be surprising if disruption of the cytokeratin network by the Ad protease and inhibition of cellular protein synthesis contribute to the fragility of infected cells, but our data show clearly that *adp* is the major determinant of cellular integrity.

At very late stages of infection, e.g., 30 to 40 h p.i., ADP becomes localized exclusively in the Golgi apparatus and nuclear membrane in A549 cells (41) and also in HeLa, A431, Hep3B, and 293 cells (53). Most likely, the site of action of ADP in promoting cell death is the nuclear membrane. Bcl-2 (15, 31) and E1B-19K (61), proteins that inhibit apoptosis, localize to the nuclear membrane. This is also true for Nip1, Nip2, and Nip3, cellular proteins that interact with Bcl-2 and E1B-19K (4). Perhaps ADP promotes cell death by directly or indirectly interacting in the nuclear membrane with these proteins and abrogating their ability to inhibit cell death. Other possibilities are that ADP forms a channel for, e.g., Ca^{2+} ions or that ADP disrupts the nuclear membrane. Whatever the mechanism of action, it is likely that ADP functions in a stoichiometric rather than catalytic manner, because the protein is made in very large amounts (52).

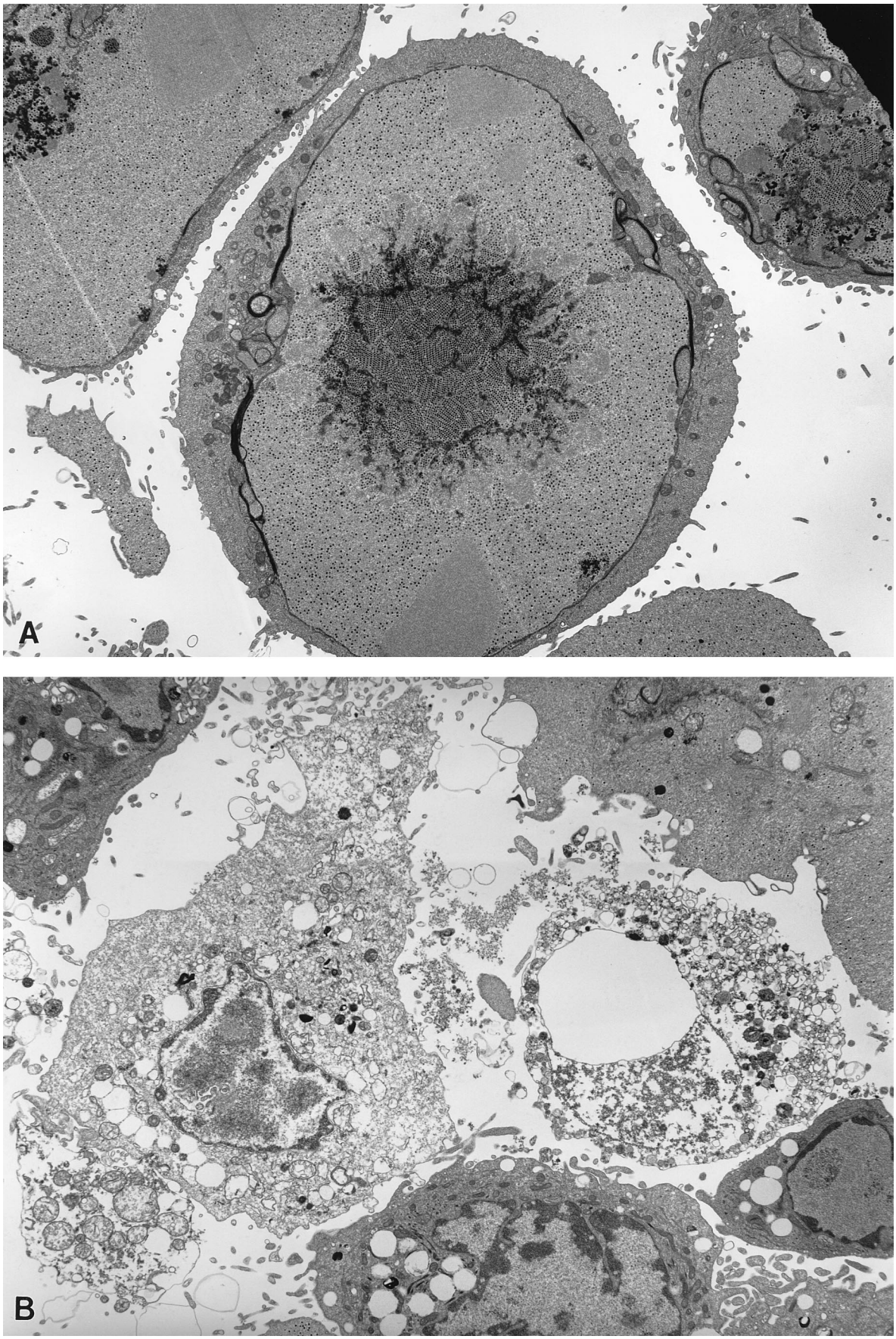

FIG. 11. Electron micrographs of typical A549 cells at 4 days p.i. with *pm*734.1 (Δ1-48) (A) or *rec*700 (wild type) (B).

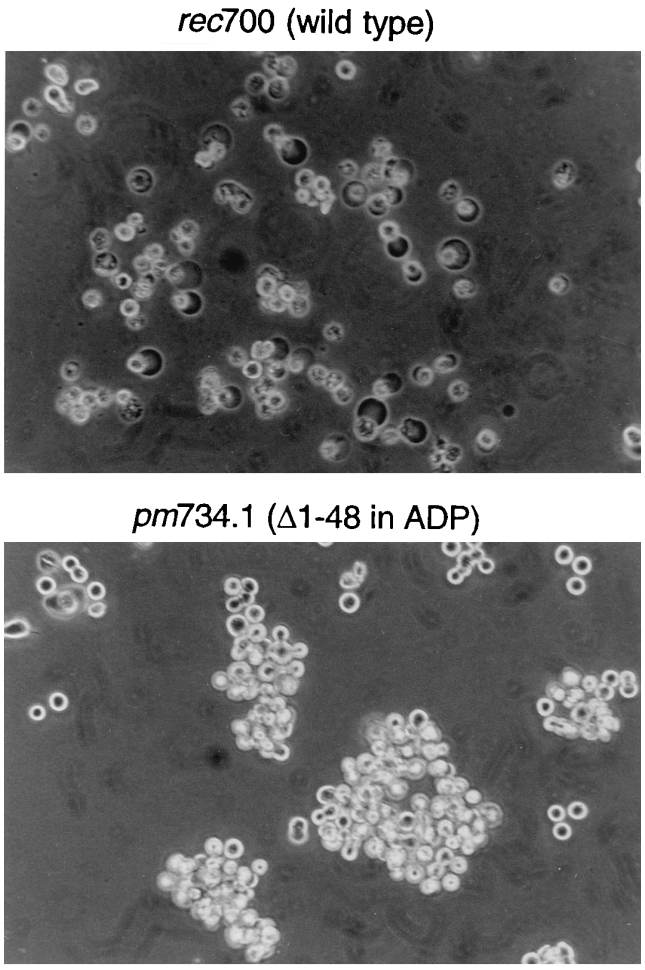

FIG. 12. Phase-contrast microscopy of A549 cells infected with *rec*700 or *pm*734.1 at 4 days p.i. With *rec*700, the cells are round, detached from the plastic dish, and dispersed, many are lysed, and many have the plasma membrane ballooned out. With *pm*734.1, the cells are round and detached into grape-like clusters, but most are intact.

There are other proteins that promote cell death (reviewed in references 30, 33, 36, 39, 44, and 51). ADP is not related to any of these proteins, including p53, c-Myc, ICE and ICE family members, Ced4, Bax, Bad, Bcl-X_S, Fas, tumor necrosis factor receptor, TRADD, MORT1, MyD118, and Reaper (14). Thus, ADP represents a new class of proteins that promote cell death. It will be interesting to determine whether ADP functions in the same pathway(s) as these other death-inducing proteins or in a novel cell death pathway. Regardless, ADP should be a useful tool for studying cell death.

Cells dying by apoptosis display distinct morphological characteristics and usually a ladder of DNA nicked between the nucleosomes. In our studies, the cells did not have the morphological characteristics of apoptosis, nor did the degraded DNA form a ladder on agarose gels. Thus, ADP may induce atypical apoptosis. It seems unlikely that the cells are dying by nonspecific necrosis, as this would not be expected to be mediated by a specific protein. Cell death in the Ad system appears to be different from the apoptosis reported to be induced by human immunodeficiency virus type 1 (1, 22), chicken anemia virus (35), Sindbis virus (54), and influenza virus (25, 48).

There is a great deal of interest in the use of Ad vectors for somatic cell gene therapy (23). Most vectors have the E3 region deleted because it is not required for virus replication in cultured cells. However, some workers are developing vectors that retain E3 in the anticipation that E3 proteins may counteract the inflammatory response to the vector. We suggest that such vectors should not contain the *adp* gene, because ADP may promote the death of the transduced cells.

Ad transcription units tend to have genes with similar functions. We have postulated that the E3 transcription unit is a cassette of genes that counteracts immunosurveillance (66–68). The cell death-promoting function of ADP is in accord with this proposal. That is, the quicker the virus is released from the infected cell, the less chance the virus has of being killed in the cell by cytotoxic T lymphocytes, NK cells, and phagocytic cells and the greater chance it has to infect another cell. Also, if the cell death induced by ADP is a form of apoptosis, then there may not be an inflammatory response, because cell fragments produced during apoptosis are rapidly phagocytosed and do not induce inflammation (29).

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