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We have reported that an 11,600-Da nuclear membrane glycoprotein named adenovirus death protein (ADP), encoded by the E3 region, is required for the efficient death (lysis) of adenovirus (Ad)-infected cells. We postulated that ADP mediates the release of virions from cells at the conclusion of replication. Here we provide further characterization of cells infected by adp and adp- Ads. Using virus mutants with deletions in the individual E3 genes, we show that only mutants that lack ADP have small plaques that are slow to develop. Mutants in the adp gene replicated as well as wild-type Ad, but the cells lysed much more slowly. Cell lysis and viability were determined by plaque size, cell morphology, trypan blue exclusion, the release of lactate dehydrogenase, and the MTT assay for mitochondrial activity. ADP is required for efficient lysis of human A549, KB, 293, and MCF-7 cells. A549 cells infected with adp Ads began to die at 2–3 days postinfection and were dead by 6 days. With adp mutants, >80% of cells remained viable for 5–6 days; when the medium was changed, >80% of cells were viable after 7 days and 10–20% after 14 days. When the MTT assay was used, there was an increase in mitochondrial activity, suggesting that Ad infection stimulates respiratory metabolism. Nearly all nuclei from wild-type Ad-infected cells lacked DAPI-stained DNA by 7 days, whereas with an adp mutant nearly all nuclei stained brightly after 15 days. Nuclei from adp mutant-infected cells were extremely swollen and full of virus, and appeared to have an intact nuclear membrane. Cells infected with wild-type Ad had many vacuoles and perhaps a disrupted nuclear membrane; they did not display features typical of apoptosis.

INTRODUCTION

Apoptosis (programmed cell death) is a regulated biochemical cell death process (reviewed in Korsmeyer, 1995; Kumar, 1995; Martin and Green, 1995; Reed, 1994; Steller, 1995). Apoptosis may be a defense against virus infections, i.e., the cell precludes virus replication by committing suicide. Viruses, in turn, might be expected to regulate cell viability, inhibiting apoptosis during early stages of replication, then promoting apoptosis (or another form of cell lysis) late in infection so that virus can be released from the cell. Indeed, there are several viral proteins that inhibit apoptosis (reviewed in Vaux et al., 1994; Shen and Shenk, 1995; Wold et al., 1995a). The cowpox virus CrmA protein, a serpin, prevents apoptosis in CrmA-transfected cells by inhibiting the interleukin-1β-converting enzyme (ICE) (Gagliardi et al., 1994; Wang et al., 1994) and/or the ICE family member Yama/CPP32 (Tewari et al., 1995). Another serpin, SPI-1, is required to inhibit apoptosis in rabbit poxvirus-infected cells (Brooks et al., 1995). The Epstein–Barr virus BHRF1 protein is structurally related to cellular Bcl-2, and in common with Bcl-2, BHRF1 inhibits apoptosis (Henderson et al., 1993; Tarodi et al., 1994). The African swine fever virus LMW5-HL gene is similar to bcl-2 and BHRF1 (Neilan et al., 1993). The herpes simplex virus-1 γ134.5 protein is required to preclude premature death in neuronal cells (Chou and Roizman, 1992; He et al., 1996). The human cytomegalovirus IE1 and IE2 proteins block apoptosis induced by tumor necrosis factor (TNF) or adenovirus (Ad) E1A expression (Zhu et al., 1995). Baculoviruses encode two proteins, p35 and IAP, that inhibit apoptosis (Clem et al., 1991; Kamita et al., 1993; Clem and Miller, 1994; Hershberger et al., 1994). p35 may block apoptosis by directly inhibiting ICE (Bump et al., 1995; Xue and Horvitz, 1995).

In Ad, the immediate early E1A proteins induce apoptosis as a consequence of their ability to deregulate the cell cycle (reviewed in Bayley and Mymryk, 1994; Moran, 1994; White, 1994). The Ad E1B-19K protein inhibits apoptosis induced by E1A (Rao et al., 1992; White et al., 1992; White, 1994) or the DNA-damaging agents cisplatin and UV light (Subramanian et al., 1993; Tarodi et al., 1993). E1B-19K may be the Ad functional equivalent of BHRF1 and Bcl-2 (Tarodi et al., 1993; Chiou et al., 1994). E1B-19K, Bcl-2, and BHRF1 physically interact with common cellular proteins (Boyd et al., 1994; Farrow et al., 1995), and E1B-19K and Bcl-2 share limited sequence similarity (Tarodi et al., 1993; Chiou et al., 1994; Subramanian et al., 1995a). E1B-19K may prevent apoptosis by alleviating repression by p53 of cellular survival proteins.

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(Sabbatini et al., 1995). The Ad E1B-55K protein also is hypothesized to inhibit E1A-induced apoptosis by binding to and inactivating p53 (Yew and Berk, 1992; Lowe et al., 1994).

Ad also has proteins that inhibit cell death induced by other agents (reviewed in Gooding, 1994; Laster et al., 1994; Wold et al., 1994, 1995a,b). E1B-19K (Gooding et al., 1991a; White et al., 1992), the E3-14.7K protein (Gooding et al., 1988, 1990; Horton et al., 1991; Ranheim et al., 1993), and the E3-10.4K/14.5K complex of proteins (Gooding et al., 1991b) protect cells from cytolysis by TNF. E1B-19K also blocks apoptosis induced by cross-linking the Fas/APO-1 antigen, a receptor related to the p55 TNF receptor (Chiou et al., 1994; Hashimoto et al., 1991). The E3-gp19K protein forms a complex with major histocompatibility class I antigens in the endoplasmic reticulum and blocks their transport to the cell surface; accordingly, E3-gp19K prevents Ad-infected cells from being killed by cytotoxic T-lymphocytes (reviewed in Wold et al., 1995a,b).

We have described a novel Ad protein that, rather than inhibiting cell death as is the case for the proteins discussed above, is required for the efficient death (lysis) of Ad-infected cells (Tollefson et al., 1996). This protein, named adenovirus death protein (ADP), was previously named E3-11.6K (Wold et al., 1984). We have proposed that ADP mediates the release of Ad from the cell after the infectious cycle is complete. ADP is synthesized in small amounts from the E3 transcription unit during early stages of infection, but in very large amounts from the major late transcription unit at very late stages of infection (Tollefson et al., 1992). ADP is a type III (N_exoC, O), bitopic N-linked O-linked glycoprotein that localizes predominantly to the nuclear membrane and Golgi at very late stages of infection (Scaria et al., 1992). Here we report further characterization of cells infected with adp mutants.

MATERIALS AND METHODS

Cells and viruses

A549, KB, 293, and MCF-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Virus stocks were prepared in suspension cultures of KB cells, banded in CsCl, and titered on A549 cells as described by Green and Wold (1979).

The viruses used in this study (see Fig. 1A), rec700 (Wold et al., 1986), dl712 (ADP−) (Deutscher et al., 1985), dl722 (12.5K−, ADP+), dl731 (12.5K−, ADP+), dl762 (14.7K−, ADP+), dl739 (6.7K−, ADP+), dl764 (14.5K−, ADP+) (Tollefson et al., 1990), dl753 (10.4K−, ADP+) (Brady and Wold, 1987), dl704 (gp19K−, ADP+) (Bhat and Wold, 1987), and dl7001 (E3+) (Ranheim et al., 1993), have been described. rec700 is an Ad5-Ad2-Ad5 recombinant consisting of the Ad5 EcoRI A (map position 0–76), Ad2 EcoRI D (76–83), and Ad5 EcoRI B (83–100) fragments (Wold et al., 1986). rec700 is the equivalent of Ad2 and Ad5 in inducing cell lysis (Tollefson et al., 1996), and it is the wild-type control for the studies described here. dl712 deletes the entire adp gene (Deutscher et al., 1985), pm734.1 (A1–48, i.e. lacking residues 1–48 in ADP) has two missense mutations at Met1 and Met41 such that only residues 49–101 in ADP (Fig. 1B) can be synthesized; these mutations were made by oligonucleotide mutagenesis, as will be described elsewhere. The presumptive 49–101 ADP polypeptide cannot be detected by immunoprecipitation with the antipeptide antiserum against residues 87–101 in ADP (unpublished results). pm734.1 and dl712 have an indistinguishable adp mutant phenotype, and so the mutants are used interchangeably. dl7001 (E3−) lacks all E3 genes (Ranheim et al., 1993).

Plaque development assay for cell viability

Plaque assays were carried out on A549 cells as described by Green and Wold (1979). Plaques were counted at 2- to 3-day intervals until 4 weeks postinfection (p.i.).

Trypan blue exclusion assay for cell viability

Cells were infected at 20, 25, or 100 PFU/cell (as indicated in figure legends) in 1 ml serum-free DMEM. At 1 hr p.i., DMEM (10% FBS) was added to each dish to a final serum concentration of 5 to 8%. At indicated times, the supernatant was removed, cells were trypsinized, the supernatants and cells were combined, and trypan blue (GibcoBRL, Gaithersberg, MD) was added to a final concentration of 0.02%. Cells were counted on a hemacytometer (a total of 600 to 1000 cells per point). For Fig. 4B, the medium was changed every 2 days during the course of infection. The floating cells were collected by centrifugation, then resuspended in DMEM containing 8% FBS.

Lactate dehydrogenase (LDH) release assay for cell viability

A549 cells (2.3 × 10^6 cells/60-mm dish), 293 cells (1.8 × 10^6 cells/35-mm dish), KB (ATCC) cells (2.2 × 10^6 cells/60-mm dish), or MCF-7 cells (9.9 × 10^5 cells/35-mm dish) were infected at 100 PFU/cell in serum-free medium except for 293 cells which were infected in DMEM (2% FBS). At 1 hr p.i., DMEM (10%) was added to a final serum concentration of 5% (MCF-7), 6% (293), or 8% (A549, KB). Twenty-microliter samples were removed at the indicated times and assayed in triplicate for LDH release by the Cytotox 96 assay (Promega Corp., Madison, WI). Samples were read on an EL340 microplate reader (BioTec Instruments, Inc.) at 490 nm.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for cell viability

A549 cells were infected at 20 PFU/cell and 2.2 × 10^4 cells were plated per well in 96-well plates at 4 hr p.i.
At indicated times p.i., 25 μl of MTT (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) (5 mg/ml) was added to each well (Mosmann, 1983; Hansen et al., 1989). After 2 hr, lysis buffer (20% sodium dodecylsulfate in 50:50 dimethyl formamide:ddH₂O, pH 4.7) was added to each well and plates were incubated overnight at 37°C. Plates were read on a microplate reader at 570 nm. Samples were in triplicate.

4,6-Diamidino-2-phenylindole (DAPI) staining

Infected cells were collected in a microfuge, washed with PBS, then resuspended in methanol containing DAPI (Sigma) (2 μg/ml) to fix and stain the cells. After 10 min, cells were pelleted and methanol/DAPI was removed. Cells were resuspended in methanol to remove excess DAPI (10 min), centrifuged in a microfuge, resuspended in a small volume of methanol, and spread on glass slides. When dry, mounting medium and coverslips were applied. Immunofluorescence was viewed by epifluorescence on a Nikon Optiphot microscope using an UV cube.

Electron microscope cytology

For electron microscopy, cells at 4 days p.i. were gently trypsinized, pelleted, and, after the supernatant was discarded, fixed with glutaraldehyde and postfixed with osmium tetroxide in sodium cacodylate buffer. After washing, the tissue was stained en bloc with uranyl acetate, dehydrated through graded ethanol and propylene oxide, and infiltrated, embedded, and polymerized in Polybed resin. Sections were cut from the tissue blocks with a Reichert Ultracut E ultramicrotome using a diamond knife, collected on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed and photographed with a JEOL 100 CX electron microscope at 60 kV.

RESULTS

Virus mutants in the adp gene, and no other E3 gene, have small plaques that are slow to develop.

The E3 region encodes seven known proteins (Fig. 1A). Mutants in the adp gene, and none of the other E3 genes, have small plaques (Fig. 2). The difference in plaque sizes can be quantitated based upon the rate at which the plaques develop, as shown in Fig. 3. The data are presented as the number of plaques seen on any given day of the plaque assay as a percentage of the final number of plaques seen at the end of the assay.

![Diagram of E3 proteins and deletions](image-url)
versus the number of days of the assay. At 10 days, 10% of the final plaques was observed with the adp mutant (dl712), whereas approximately 60–88% of the final plaques was observed with the other E3 mutants. Clearly, the dl712 plaques develop slowly compared to the other mutants.

The final titers of all these CsCl-banded virus stocks were similar (legend to Fig. 3). These data, together with wild-type Ad and adp mutant growth data (Tollefson et al., 1996), indicate that adp mutants are not defective in growth, only in efficient lysis of cells and the release of virus. The slow release of virus explains why the plaques are small.

Cells infected with adp mutants remain viable much longer than cells infected with wild-type adenovirus.

As described by Tollefson et al. (1996), when cell viability was examined using a LDH release assay, a trypan blue exclusion assay, or the MTT assay for mitochondrial activity, cells infected with rec700 (wild-type) began to die at 2–3 days p.i. and most were dead by 6 days, whereas cells infected with dl712 (ADP-) did not begin to die until 6 days p.i. Here we provide additional features of the death of Ad-infected cells. Figure 4A shows a typical trypan blue exclusion experiment with rec700 or pm734.1 (Δ1–48 in ADP) using 20 PFU/cell; about 90% of rec700-infected cells were dead at 5 days, whereas 95% of pm734.1-infected cells were alive. pm734.1-in-
Thus, cells infected with adp mutants stay alive much longer than cells infected with adp⁺ viruses.

Nuclei of adp mutant-infected cells are enlarged, full of virus, and appear to have an intact nuclear membrane.

The nuclei in dl712-infected cells were very enlarged as is apparent in electron micrographs of typical dl712-infected cells at 4 days p.i. (Figs. 5A and 5B), a time when 90% of cells were alive. The nuclei were packed with virus and the nuclear membrane appeared to be intact. Virus was not detected in the cytoplasm. Figures 5C and 5D show examples of different types of rec700-infected cells at 4 days p.i., when >80% of cells were dead. The cells contained many vacuoles and often had poorly stained structures of unknown origin (the upper cells in Figs. 5C and 5D), or they were totally lysed (bottom cell in Fig. 5D). The upper cells in Figs. 5C and 5D also contained virus, but the nuclear membrane did not seem to be intact as was the case with dl712-infected cells. Condensed chromatin and membrane blebs, diagnostic of apoptosis, were not observed.

DNA remains associated with nuclei in cells infected with an adp mutant but not with wild-type adenovirus.

We showed previously by agarose gel electrophoresis and the TUNEL (terminal deoxynucleotidyltransferase end labeling) assay that DNA is degraded sooner in cells infected with wild-type Ad compared to an adp mutant (Tollefson et al., 1996). With rec700, significant detectable DNA degradation began at 3 days p.i., but it was delayed by 2 days with dl712 (ADP⁺). When DNA was examined within nuclei using DAPI to stain DNA, a dramatic difference was seen between rec700 and pm734.1 (Fig. 6A). At 7 days p.i. with rec700, most nuclei were not stained with DAPI, giving them a ghost-like appearance (Fig. 6A). A small fraction of cells was brightly stained, and others displayed a speckled pattern. With pm734.1, most nuclei were brightly stained throughout, although some nuclei were brighter on the rim (Fig. 6B). All the rec700-infected cells and 70% of pm734.1-infected cells were lysed by 7 days. With rec700 at 9 and 13 days p.i. (data not shown), and at 15 days p.i. (Fig. 6C), there were few brightly stained or speckled nuclei, and only the rims of the nuclei were visible. With pm734.1 at 15 days (Fig. 6D), the nuclei were brightly stained, similar to the nuclei at 7 days. Thus, the DNA remains associated with the nucleus much longer in adp mutant-infected cells.

Adenovirus infection stimulates mitochondrial activity prior to inducing cell death.

Figures 7A and 7B show parallel cell viability assays using MTT and trypan blue exclusion. MTT is a tetrazolium salt whose tetrazolium ring is cleaved to formazan (dark blue) by dehydrogenases, primarily in active mito-
chondria (Mosmann, 1983; Hansen et al., 1989). The amount of formazan generated per cell is proportional over a wide range to the number of living cells. It is also proportional to the level of energy metabolism in cells, as shown by comparing resting lymphocytes with lymphocytes stimulated with concanavalin A (Mosmann, 1983). Thus, MTT can be used as an indicator of the number of viable cells as well as the energy metabolism in cells. With rec700, there was a sharp increase in mitochondrial activity from 1 to 2 days p.i. (Fig. 7A), a period when nearly all the cells were intact (Fig. 7B). Mitochondrial activity declined at 3 days as the cells began to die. With dl712 (ADP−) or pm734.1 (Δ1–48), mitochondrial activity continued to increase until 4 or 5 days (Fig. 7A), when most of the cells are alive (Fig. 7B), then began to decline at 6 days, coincident with the onset of cell death. Prolonged metabolic activity in dl712- or pm734.1-infected cells, compared to rec700-infected cells, was also indicated by the pH of the medium which was 6.5 with adp mutants and 7.2–7.4 with rec700 at 5 days p.i. (data...
FIG. 6. Immunofluorescence of DNA from cells infected with rec700 (wild-type) and pm734.1 (Δ1–48 in ADP). A549 cells were infected with 20 PFU/cell of virus, then at 7 and 15 days p.i. were fixed and stained with DAPI. With rec700-infected cells, many nuclei can be seen, but only a few were stained by DAPI. With pm734.1, most of the nuclei contained DAPI-stainable DNA.

not shown). This decrease in pH occurred even though the medium was buffered.

It is important to emphasize that the virus-infected cells ceased to divide after infection, so the increase in mitochondrial activity was not due to an increase in cell number. This contrasts with the increase in mitochondrial activity seen with mock-infected cells (Fig. 7A), where the cells continued to multiply during the experiment. At 5 days, there were 4–6 times more mock-infected cells than virus-infected cells. Despite the larger cell number, the medium from mock-infected cells was not as acidic as the medium from adp mutant-infected cells.

These results suggest that Ad has a function that stimulates mitochondrial activity. This function is manifested prior to the onset of cell death.

ADP is required for efficient cell death in the human A549, KB, 293, and MCF-7 cell lines

The experiments in Figs. 2–7 were performed in A549 cells. In Figs. 8 and 9, the LDH release and trypan blue exclusion cell viability assays were used to compare the death kinetics of rec700- and pm734.1 (Δ1–48)-infected A549 cells with three other cell lines. When infected with rec700, A549 cells died perhaps slightly more rapidly than KB, 293, or MCF-7 cells. When infected with pm734.1 or dl712, A549 cells seemed to remain viable about 1 day longer than the other cell lines. Results obtained with primary human foreskin fibroblasts were similar to those with A549 cells (data not shown). It is clear that ADP is required for efficient death in A549, KB, 293, and MCF-7 cells.

DISCUSSION

We have shown that Ad adp mutants have small plaques that develop slowly. Mutants that lack other E3 genes have essentially normal plaques. The adp mutants grow as well as wild-type Ad, but the cells lyse much more slowly so the plaques are small. A549 cells infected with wild-type Ad begin to die at 2–3 days p.i. and are dead by 6 days p.i. With adp mutants, most cells do not begin to die until 6 days p.i. Cell lysis was assayed by trypan blue exclusion, LDH release, cell morphology, DAPI staining of DNA, and the MTT assay for mitochondrial activity. Cell viability was examined previously using agarose gel electrophoresis of degraded DNA and RNA, the TUNEL assay for nicked DNA, and protein synthesis
much longer than wild-type, ADP apparently functions to promote cell death and the release of Ad from the infected cell (Tollefson et al., 1996). This ADP function represents a novel concept in viral pathogenesis, i.e., by promoting rapid cell lysis after virus replication is complete, ADP facilitates the spread of virus and reduces the chances of virus being destroyed by cell-mediated immunity.

Prior to cell lysis, the nuclei of adp mutant-infected cells were remarkable in that they were extremely swollen and full of virus. In some cells, crystals of virus were apparent. Although not investigated in detail, the nuclear membrane appeared to be intact. Consistent with this, virus was not observed in the cytoplasm. With wild-type Ad, it was difficult to discern a nuclear membrane. ADP localizes to the nuclear membrane (and Golgi) at very late stages of infection (>30 hr) (Scaria et al., 1992). Therefore, a manifestation of ADP function may be the disruption of the nuclear membrane and the liberation of virus from the nucleus. However, a mechanism must also exist that leads to lysis of the entire cell. In this regard, E1B-19K (White et al., 1984) and Bcl-2 (Lithgow et al., 1994; Reed, 1994) localize, in part, to the nuclear membrane. These proteins inhibit both apoptosis and necrosis (Subramanian et al., 1995b). Perhaps ADP induces cell lysis by abrogating the ability of E1B-19K and/or Bcl-2 to inhibit apoptosis and/or necrosis.

A number of cellular and viral proteins have been identified that inhibit apoptosis, and some cellular proteins promote apoptosis (see Introduction). ADP is not obvi-
293, and MCF-7 cells. The KB, 293, and MCF-7 cells died at a slightly faster rate than the A549 cells. This probably reflects, in part, inherent differences in the fragility of cells or their ability to survive crowded conditions. For example, uninfected KB, 293, and MCF-7 cells died more rapidly in dishes than A549 cells. This suggests that these cells overgrow to a greater extent, deplete nutrients more rapidly, or produce higher levels of toxic metabolic products. This would result in earlier death for ADP mutant-infected cells that is not directly determined by viral infection or viral proteins. Human primary foreskin fibroblasts showed a cell death pattern most similar to A549 cells (data not shown), consistent with longer term stability of the cell monolayer. On the other hand, these cell types may differ in expression of proteins, e.g., members of the Bcl-2 or ICE families, which could play a role in ADP-induced cell death.

Interestingly, as judged by the MTT assay, Ad-infection stimulates mitochondrial activity. This was particularly apparent in cells infected with adp mutants because they remain viable for so long. The increased MTT activity in Ad-infected cells at 1–2 days p.i. is consistent with the classical observation that Ad-infected cells initially produce more acid than mock-infected cells as evidenced by the media changing to an orange-yellow color. The increase in mitochondrial activity occurred in cells that had been exponentially growing in complete DME containing 10% FBS; these cells would be expected to be fully activated. Therefore, Ad may encode a function that superinduces mitochondrial activity. This should be a useful function for the virus, inasmuch as high levels of ATP must be required for the prodigious synthesis of DNA, RNA, and protein that occurs in Ad-infected cells. It will be interesting to determine whether this putative mitochondrial-stimulated function is an Ad protein, and, if so, how the protein functions.

FIG. 9. Trypan blue exclusion cell viability assay of different human cell lines infected at 100 PFU/cell of rec700 (wild-type), pm734.1 (Δ1-48 in ADP), or dl712 (ADP-). The data are from the same experiment as in Fig. 8.

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