Cell

GAPDH and Autophagy Preserve Survival after Apoptotic Cytochrome *c* Release in the Absence of Caspase Activation

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DOI 10.1016/j.cell.2007.03.045

SUMMARY

In cells undergoing apoptosis, mitochondrial outer-membrane permeabilization (MOMP) is followed by caspase activation promoted by released cytochrome *c*. Although caspases mediate the apoptotic phenotype, caspase inhibition is generally not sufficient for survival following MOMP; instead cells undergo a "caspase-independent cell death" (CICD). Thus, MOMP may represent a point of commitment to cell death. Here, we identify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a critical regulator of CICD. GAPDH-expressing cells preserved their clonogenic potential following MOMP, provided that caspase activation was blocked. GAPDH-mediated protection of cells from CICD involved an elevation in glycolysis and a nuclear function that correlated with and was replaced by an increase in Atg12 expression. Consistent with this, protection from CICD reflected an increase in and a dependence upon autophagy, associated with a transient decrease in mitochondrial mass. Therefore, GAPDH mediates an elevation in glycolysis and enhanced autophagy that cooperate to protect cells from CICD.

INTRODUCTION

Caspases orchestrate the apoptotic phenotype, including DNA fragmentation, nuclear condensation, phosphatidyl serine externalization, plasma membrane blebbing, and other events (Fischer et al., 2003), but they are not necessarily required for cell death under proapoptotic conditions (Chipuk and Green, 2005). When the mitochondrial pathway of apoptosis is engaged and cytochrome *c* is released following mitochondrial outer-membrane permeabilization (MOMP), addition of caspase inhibitors fails to rescue proliferating cells from death (Amarante-Mendes et al., 1998; McCarthy et al., 1997; Xiang et al., 1996). Similarly, cells from animals lacking Apaf-1 or caspase-9, in which caspases fail to activate following MOMP, nevertheless undergo cell death that can be inhibited by Bcl-2 (Haraguchi et al., 2000). This "caspase-independent cell death" (CICD) may occur as a consequence of mitochondrial failure and/or mediators released from the mitochondria following MOMP (Green and Kroemer, 2004). Examples of potential mediators released from mitochondria include apoptosis-inducing factor (AIF), endonuclease G, Omi/HtrA2, and others. While each of these have been shown to trigger cell death upon transient overexpression, none have been shown to be essential for CICD, and their roles remain controversial. An alternative possibility is that cells die following MOMP due to eventual collapse of mitochondrial function (Chipuk and Green, 2005).

An exception to this scenario may be found in sympathetic neurons deprived of neurotrophic factors. These cells release cytochrome *c* but can remain viable and regenerate mitochondria upon readdition of growth factors, provided that caspase activation is blocked (Martinou et al., 1999; Deshmukh et al., 2000). This requires that the mitochondria sustain function as indicated by transmembrane potential ($\Delta\Psi m$) (Deshmukh et al., 2000). Persistence of $\Delta\Psi m$ despite MOMP can be observed in many cell types, provided that caspases are blocked

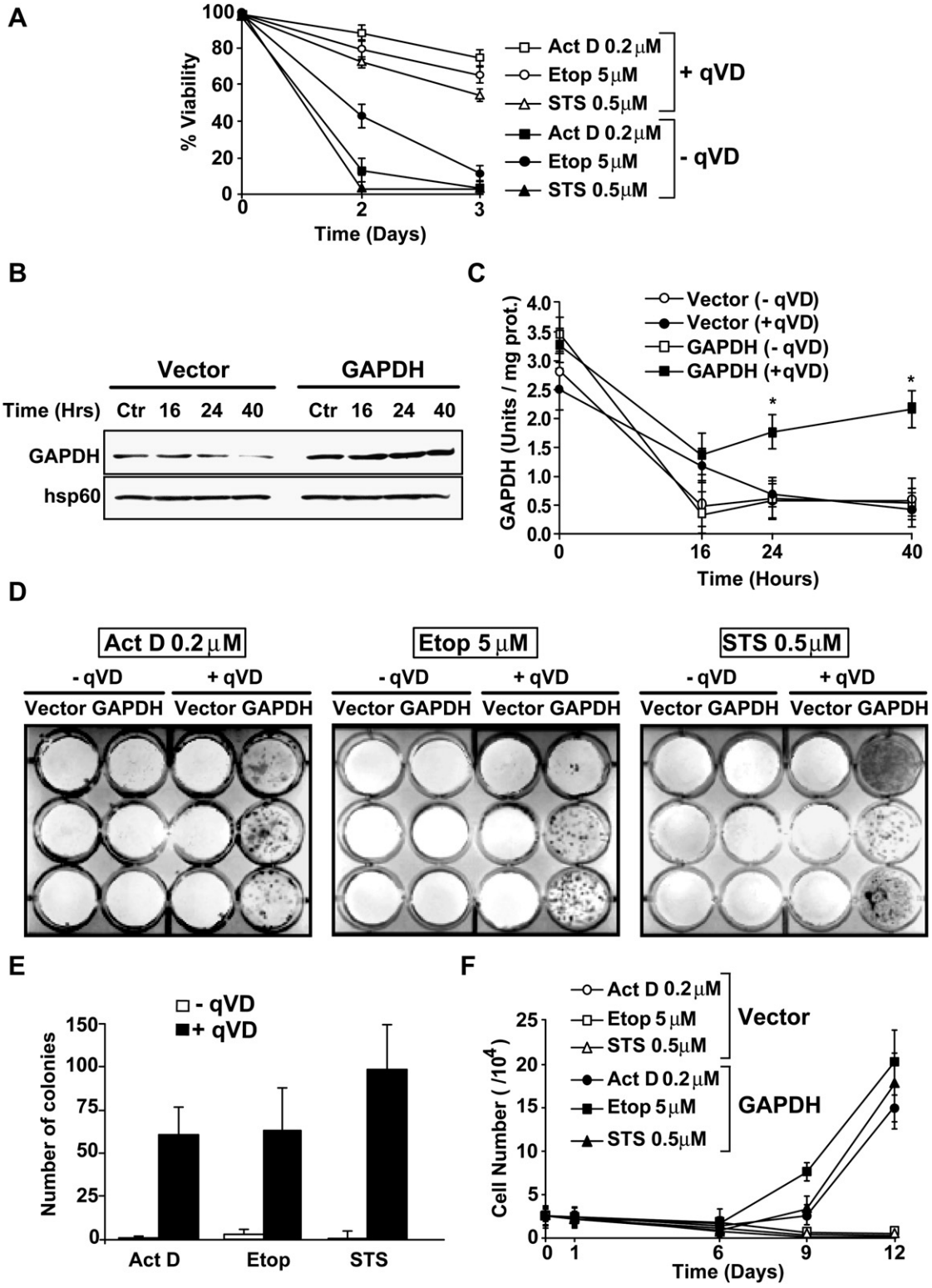

Figure 1. GAPDH Protects Cells from Caspase-Independent Cell Death but Not from Apoptosis

(A) HeLa cells transduced with a virus encoding GAPDH were treated as indicated in the presence or in the absence of qVD-oph. Percent of viability was measured by propidium iodide staining and flow cytometry analysis.

(Waterhouse et al., 2001). It therefore remains puzzling why recovery from MOMP is not a common occurrence when caspase activation is disrupted.

Recovery from MOMP would have important implications for oncogenesis, since regulation of caspase activation downstream of MOMP can therefore influence cell survival. Tumor lines often lack APAF-1 (Liu et al., 2002; Soengas et al., 2001; Wolf et al., 2001) or caspases (Devarajan et al., 2002), and inhibitor of apoptosis proteins (IAPs) that block activation of caspases are frequently elevated in cancer (Ferreira et al., 2001; Krajewska et al., 2003; Tamm et al., 2000). In one study a dominant-negative form of caspase-9 enhanced survival and proliferation of transformed cells (Schmitt et al., 2002) and therefore it remains possible that cells can undergo MOMP, recover, and proliferate if caspase activation is inhibited.

In this study, we investigated the problem of survival and proliferation following MOMP by searching for proteins that specifically protect cells from CICD but not apoptosis.

RESULTS

To understand the conditions under which proliferating cells might recover from MOMP when caspase activation fails to occur, we took advantage of an unbiased approach using a functional screen for proteins that could sustain clonogenic survival in cells induced to undergo apoptosis, but only in the presence of caspase inhibitors (Figure S1 in the Supplemental Data available online). We introduced a retroviral cDNA library into Jurkat cells that were transiently treated with a lethal concentration of staurosporine (1 μM, 6 hr) and then maintained in the presence of a pancaspase inhibitor, valine-aspartate-fluoromethylketone (VD-fmk) (Yang et al., 2003). Control cultures of uninfected cells treated in this manner consistently failed to contain surviving cells (data not shown, see Amarante-Mendes et al., 1998). Clones of infected cells that survived this treatment were retested by again inducing apoptosis, plus or minus caspase inhibitor, and those that proliferated only in the presence of the inhibitor were further evaluated. The retroviral inserts in such clones were recovered by PCR and characterized. In this way, we identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a potential inhibitor of "caspase -independent" but not caspase-dependent cell death.

We evaluated the effects of GAPDH in HeLa cells subjected to proapoptotic conditions in the presence or absence of the pancaspase inhibitor quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone (qVD-oph) (Figure 1). Under these conditions, cells rapidly underwent caspase-dependent cell death, and in the presence of a caspase inhibitor a majority of cells sustained membrane integrity for 72 hr or more (Figure 1A). GAPDH protein levels (Figure 1B) and enzymatic activity (Figure 1C) declined in the parental line upon transient treatment with a proapoptotic agent and culture with caspase inhibitor, prior to any substantial death in the cells. In contrast, enforced expression of GAPDH sustained expression and enzymatic activity at approximately normal levels. HeLa cells with or without GAPDH died upon transient treatment with staurosporine (STS), etoposide (Etop.), or actinomycin D (Act D), and addition of qVD-oph failed to protect HeLa cells from these treatments, as measured by clonogenic survival (Figures 1D and 1E). However, HeLa cells ectopically expressing GAPDH showed clonogenic growth following these treatments, provided the caspase inhibitor was present (Figures 1D and 1E). This effect was also observed in HeLa expressing GAPDH in the presence of the pancaspase inhibitor VD-fmk (Figure S2). The survival and proliferation of HeLa cells expressing GAPDH exposed to proapoptotic agents in the presence of caspase inhibitors was also observed as cell accumulation (Figure 1F) and by dilution of carboxy-fluorescein diacetate, succinimidyl ester (CFSE) (Figure S3). At substantially higher concentrations of the cytotoxic agents tested, no protection was afforded by expression of GAPDH plus caspase inhibitors (data not shown).

Oncogenes such as Myc and E1A induce cell proliferation and apoptosis, such that transformation is not seen unless the apoptotic pathway is blocked or evaded (Evan et al., 1992; Green and Evan, 2002). Tumor suppression by apoptosis proceeds predominantly via the mitochondrial pathway accounting for the cotransforming activity of Bcl-2, which blocks this pathway (Green and Kroemer, 2004). However, despite the fact that tumors frequently repress APAF-1 expression (Ferraro et al., 2003), recent studies have failed to find an effect of APAF-1 deficiency on transformation by such oncogenes in lymphocytes or early passage MEF (Scott et al., 2004). However, if enforced expression of GAPDH can allow recovery of cells following MOMP (provided caspases are not engaged), then APAF-1 deficiency might allow transformation to occur. Therefore, early passage MEF (less than or equal to passage 5) from wild-type or APAF-1 null mice, were transduced with E1A plus or minus GAPDH.

(B) HeLa cells transduced with a control retrovirus or with a virus encoding GAPDH were treated with 1 μM staurosporine (STS) in the presence of 20 μM qVD-oph. After the indicated times GAPDH expression was assessed by Western blot.
(C) Same as in (B) in the presence or absence of qVD-oph. *$p < 0.01$ versus vector with qVD-oph.
(D) HeLa cells (transduced with control or GAPDH encoding retrovirus) were treated for 6 hr with Act D, etoposide, or STS as indicated ± the caspase inhibitor qVD-oph (20 μM). The caspase inhibitor was added 30 min before the apoptotic agents and replaced periodically at 24 hr intervals for 3 days. Colonies were stained with methylene blue and assessed 12 days after treatment.
(E) Treatment as in (D). Quantitation of the number of colonies under each condition 12 days after treatment.
(F) Cells (1×10^4/well) were treated for 6 hr as indicated in the presence of qVD-oph (20 μM). Viable cells were counted by trypan blue exclusion at the indicated times (up to 12 days). All results in the figure represent mean ± SD of three to five independent experiments.

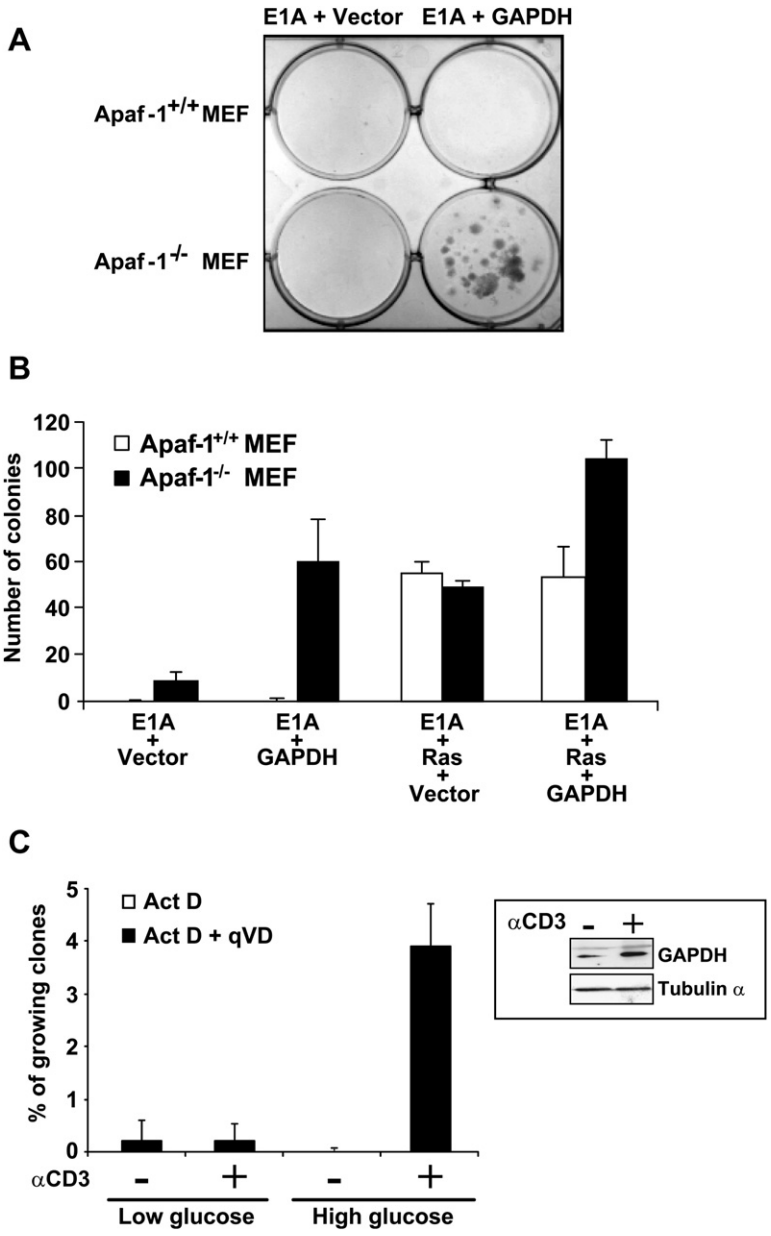

Figure 2. GAPDH Protects Cells from CICD

(A) Early passage MEFs (less than or equal to passage 5) from wild-type or Apaf-1 null mice were transduced with E1A plus a control retrovirus or with GAPDH. After 2 weeks of selection, colonies were visualized by methylene blue staining.

(B) Cells were treated as in (A) with the indicated combination of retroviruses expressing E1A, Ras, and/or GAPDH.

(C) Jurkat cells were maintained in low (0.3 mM) or high (4 mM) glucose for 2 weeks, and then activated by culture on anti-CD3 for 24 hr. GAPDH expression was determined by immunoblot (right). The cells were then treated with Act D (0.5 μM, 6 hr, ± qVD-oph) and plated at approx. 1 cell per well in low or high glucose. Wells with proliferating cells were assessed by visual inspection after 8 days.

In (B) and (C), results are mean ± SD from three independent experiments.

Wild-type MEF did not survive expression of E1A, whether or not GAPDH was expressed, and similarly, APAF-1-deficient MEF did not proliferate if transduced with E1A in the absence of GAPDH (Figure 2A). However, APAF-1 null MEF survived and proliferated following expression of E1A plus GAPDH (Figure 2A). In a similar experiment, cells were transduced with E1A alone or E1A plus Ras, ± GAPDH (Figure 2B). Ras is capable of providing anti-apoptotic signaling via AKT (Bonni et al., 1999), upstream of MOMP (Kennedy et al., 1997). While both wt and APAF-1 null MEF failed to survive expression of E1A alone, both MEF generated equivalent numbers of colonies with E1A plus Ras. Expression of GAPDH permitted colony formation in APAF-1 null MEF expressing E1A to the same extent seen with E1A plus Ras, but did not protect wt MEF.

To ensure that the surviving clones did not arise because of mutations in p53, three clones from the E1A plus GAPDH transduced group in the experiment shown in Figure 2B were expanded and the p53 status assessed by sequencing. No mutations were detected (data not shown). In addition, APAF-1 null cells expressing E1A plus GAPDH showed robust expression of the p53-responsive gene $p21^{WAF1}$ in response to DNA damage (Figure S4), indicating that p53 is functional in these cells.

Activation of T lymphocytes elevates GAPDH levels (Teague, et al., 1999), and therefore we asked if such activation can promote protection from CICD. We treated

Jurkat T cells with anti-CD3 to activate them, and observed that GAPDH expression increased (Figure 2C, right). We then treated the cells with Act D (0.5 μM, 6 hr, ± qVD-oph) prior to plating them at approx. 1 cell per well (±qVD-oph), and assessed cell survival and proliferation after 8 days. We observed that while no cells survived in the absence of the caspase inhibitor, a small proportion of activated Jurkat cells survived in the presence of the inhibitor, suggesting that activation had protected the cells from CICD (but not apoptosis). Interestingly, this protection was not seen in Jurkat cells that were maintained in low glucose, a condition under which GAPDH does not protect HeLa cells from CICD (see below and Figures 4A and 4B). These results suggest that physiological elevation of GAPDH can protect activated T lymphocytes from CICD. This may explain the intriguing finding that T cells with a memory phenotype accumulate in mice carrying a cytochrome *c* mutation that disrupts the ability of the protein to induce caspase activation following MOMP (Hao, et al., 2005).

Clearly, one way by which GAPDH might protect against cell death would be to inhibit MOMP and cytochrome *c* release in a manner analogous to Bcl-2 (Kluck et al., 1997; Kuwana and Newmeyer, 2003). We introduced cytochrome *c*-GFP into HeLa cells expressing GAPDH (Goldstein et al., 2000) and observed that cytochrome *c* release occurred in a majority of cells induced to undergo apoptosis in the presence of caspase inhibitors, whether or not the cells expressed GAPDH (Figure 3A). Strikingly, while no recovery of mitochondrial cytochrome *c* was observed in the control cells, such recovery was eventually observed in cells expressing GAPDH (data not shown). This, however, did not resolve the issue of whether or not those cells that had released cytochrome *c* were able to restore their mitochondria and proliferate.

Therefore, we plated HeLa cells expressing cytochrome *c*-GFP (± GAPDH) at approximately one cell per well in microwell cultures. Following overnight culture, cells were treated with Act D (for six hrs or overnight) in the presence of qVD-oph, washed, and the caspase inhibitor was again added. Wells were scored for cells containing mitochondrially-localized cytochrome *c*-GFP (see Table S1 for examples). Strikingly, we consistently observed wells that contained only cells showing cytochrome *c* release subsequently harboring cells displaying relocalization of cytochrome *c*-GFP to the mitochondria and evidence of cell proliferation. Examples are shown in Figures 3B and S5A. This recovery and proliferation was never observed in the absence of caspase inhibitors or in cells without enforced expression of GAPDH (data not shown). The mitochondrial localization of the cytochrome *c*-GFP was confirmed by costaining with TMRE (Figure S5B).

During apoptosis, a caspase-dependent loss of $\Delta\Psi$m quickly follows release of cytochrome c, and this loss is substantially delayed if caspase activation is blocked (Ricci et al., 2004; Waterhouse et al., 2001). Therefore, we assessed $\Delta\Psi$m using the potentiometric dye TMRE in HeLa cells treated with cytotoxic agents with or without caspase inhibitors. GAPDH expression had no effect on the maintenance of $\Delta\Psi$m during caspase-dependent apoptosis (Figure 3C) while the addition of caspase inhibitor delayed loss of $\Delta\Psi$m in control HeLa cells, this subsequently declined as previously described (Waterhouse et al., 2001). Enforced expression of GAPDH extended the maintenance of $\Delta\Psi$m in the presence of caspase inhibitors (Figures 3C and S6). To determine if this effect contributed to protection from CICD, cells were treated to induce MOMP in the presence of caspase inhibitor, and three days later sorted into populations with low (M1) or high (M2) $\Delta\Psi$m (Figure 3D). While no clonogenic growth was observed in either population of vector control cells, GAPDH-expressing cells with high $\Delta\Psi$m showed a dramatic enhancement of clonogenic survival (seen as a ''lawn'' of growing cells) as compared to those cells with low $\Delta\Psi$m (seen as individual colonies). Therefore, maintenance of $\Delta\Psi$m by GAPDH correlates with protection from CICD.

GAPDH is a multifunctional enzyme with a role in glycolysis and other less well understood roles in membrane fusion, transcriptional coactivation, and DNA repair (Nakagawa et al., 2003; Glaser et al., 2002; Kaneda et al., 1997; Zheng et al., 2003). We reasoned that a GAPDH-mediated elevation in glycolytic metabolism and a consequent maintenance of ATP generation might provide the requisite energy for mitochondrial regeneration following MOMP in the absence of caspase activation. Indeed, we found that cells with enforced GAPDH expression showed elevated ATP levels when maintained in high glucose (10 mM) but not low glucose (0.1 mM) (Figures 4A and S7). This elevation in ATP was likely due to glycolysis, since the complex V inhibitor oligomycin did not inhibit it, but did suppress ATP levels in cells maintained in low glucose. The increase in ATP appeared to have a role in protection from CICD, since such protection was seen only when cells were maintained in high glucose (Figure 4B). Untreated cells maintained in high or low glucose were equally viable, and no survival was observed in those cells treated to undergo apoptosis in the absence of caspase inhibitors (data not shown).

CICD has been suggested to occur through the generation of reactive oxygen species (ROS) (Chipuk and Green, 2005). However, we did not observe any effect of enforced GAPDH expression on ROS levels in cells undergoing CICD (Figure 4C). Three different ROS scavengers effectively reduced hydrogen peroxide levels in these cells (Figure 4C), but none protected cells from CICD nor enhanced the protection seen with GAPDH (Figure 4D). We were therefore unable to demonstrate a role for ROS scavenging in protection from CICD under the conditions used in this study.

In addition to its glycolytic function, and consistent with its C-terminal dependent nuclear localization (Brown et al., 2004; Mazzola and Sirover, 2003), GAPDH is also involved in several nuclear functions (Zheng et al., 2003; Sundararaj et al., 2004; Meyer-Siegler et al., 1991) and translocates to the nucleus upon cellular stress (Saunders et al., 1999;

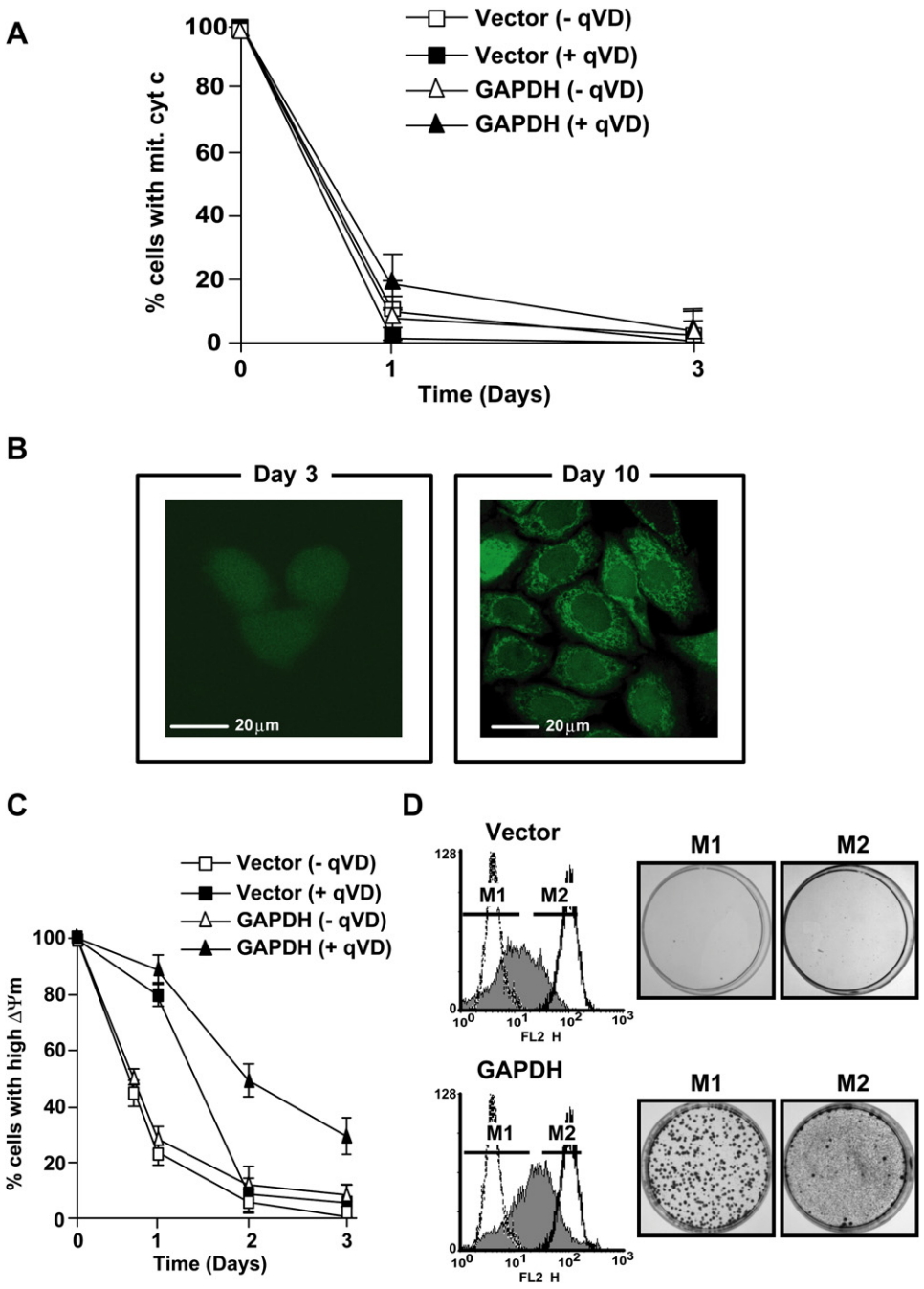

Figure 3. Cytochrome *c*-GFP Release and Persistence of $\Delta\Psi$m during Caspase-Independent Cell Death

(A) Control or GAPDH-expressing HeLa cells were transduced with a cytochrome *c*-GFP encoding vector. Mitochondrial retention of cytochrome *c*-GFP was examined by flow cytometry (see Supplemental Experimental Procedures) in cells treated for 6 hr with Act D (0.5 μM) ± qVD-oph (20 μM). Results represent mean ± SD of four independent experiments.

(B) GAPDH-expressing HeLa cells transduced with the cytochrome *c*-GFP encoding vector were individually seeded in microwells on glass bottom dishes and treated with Act D (0.2 μM) plus qVD-oph (20 μM) for 6 hr. Mitochondrial localization of cytochrome *c*-GFP was analyzed by confocal microscopy 3 and 10 days after stimulation.

(C) Cells were treated as in A and loss of $\Delta\Psi$m during caspase independent cell death was measured by flow cytometry using the fluorescent dye TMRE. Results represent mean ± SD of three independent experiments.

(D) 3 days after treatment with STS (0.5 μM) plus qVD-oph (20 μM), cells were stained using TMRE (filled curve in the FACS profile) and FACS sorted for high (M2) or low (M1) $\Delta\Psi$m. Cells were then seeded at 1×10^4 cells/dish and assessed 7 days later. In the FACS profiles, black lines represent untreated cells and dotted lines represent cells treated with the uncoupling agent FCCP (10 μM), both populations were stained with TMRE (50 nM).

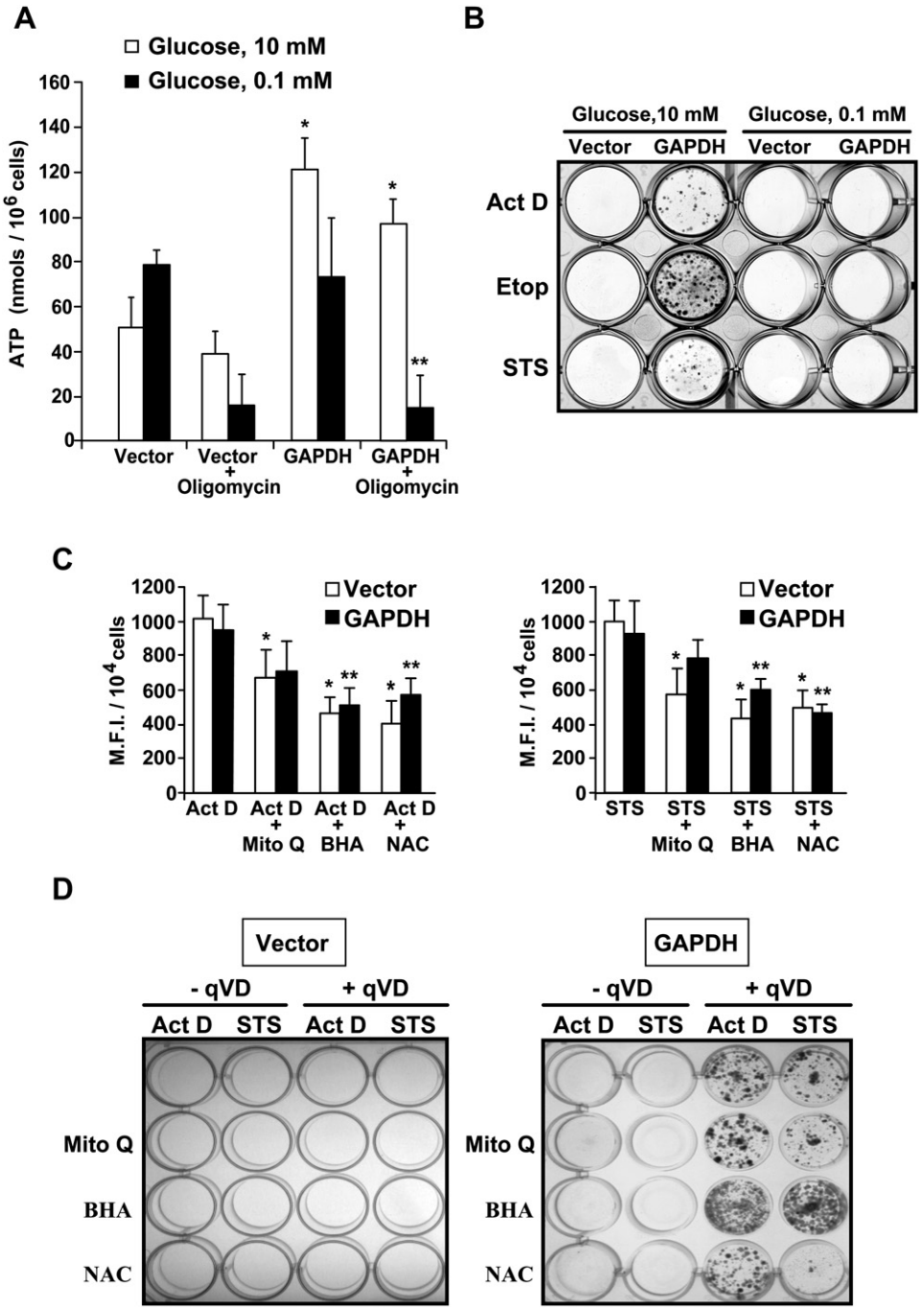

Figure 4. Increased ATP Production Induced by GAPDH but Not ROS Scavenging Is Associated with Its Protective Effect

(A) Control or GAPDH-expressing HeLa cells were cultured for a minimum of 2 weeks in glucose-free DMEM, supplemented with D-glucose (10 mM or 0.1 mM), sodium pyruvate (5 mM), uridine (5 mM), and 10% dyalized FBS. ATP was measured after 2 hr incubation with or without oligomycin (10 μM). Results are mean ± SD of three experiments. *$p < 0.05$ versus vector values, **$p < 0.05$ versus GAPDH values.

(B) Cells growing in the presence of high or low glucose and treated for 6 hr with Act D (0.5 μM), etoposide (5 μM), or STS (1 μM) ± qVD-oph (20 μM). Colonies were stained with methylene blue and assessed 12 days after treatment.

(C) Control or GAPDH-expressing HeLa cells were preincubated for 1 hr with MitoQ (1 μM), BHA (200 μM) or NAC (10 mM) and then treated with Act D (0.5 μM) or STS (1 μM) in the presence of qVD-oph (20 μM) for 6 hr. Data shown are mean of fluorescence intensity (M.F.I.) ± SD from spectrofluorimetric analyses of H_2DCFDA-stained cells. *$p < 0.05$ versus vector without antioxidant pretreatment values, **$p < 0.05$ versus GAPDH without antioxidant pretreatment values.

(D) Cells were treated as in C ± qVD-oph (20 μM). Colonies were stained with methylene blue and assessed 12 days after treatment.

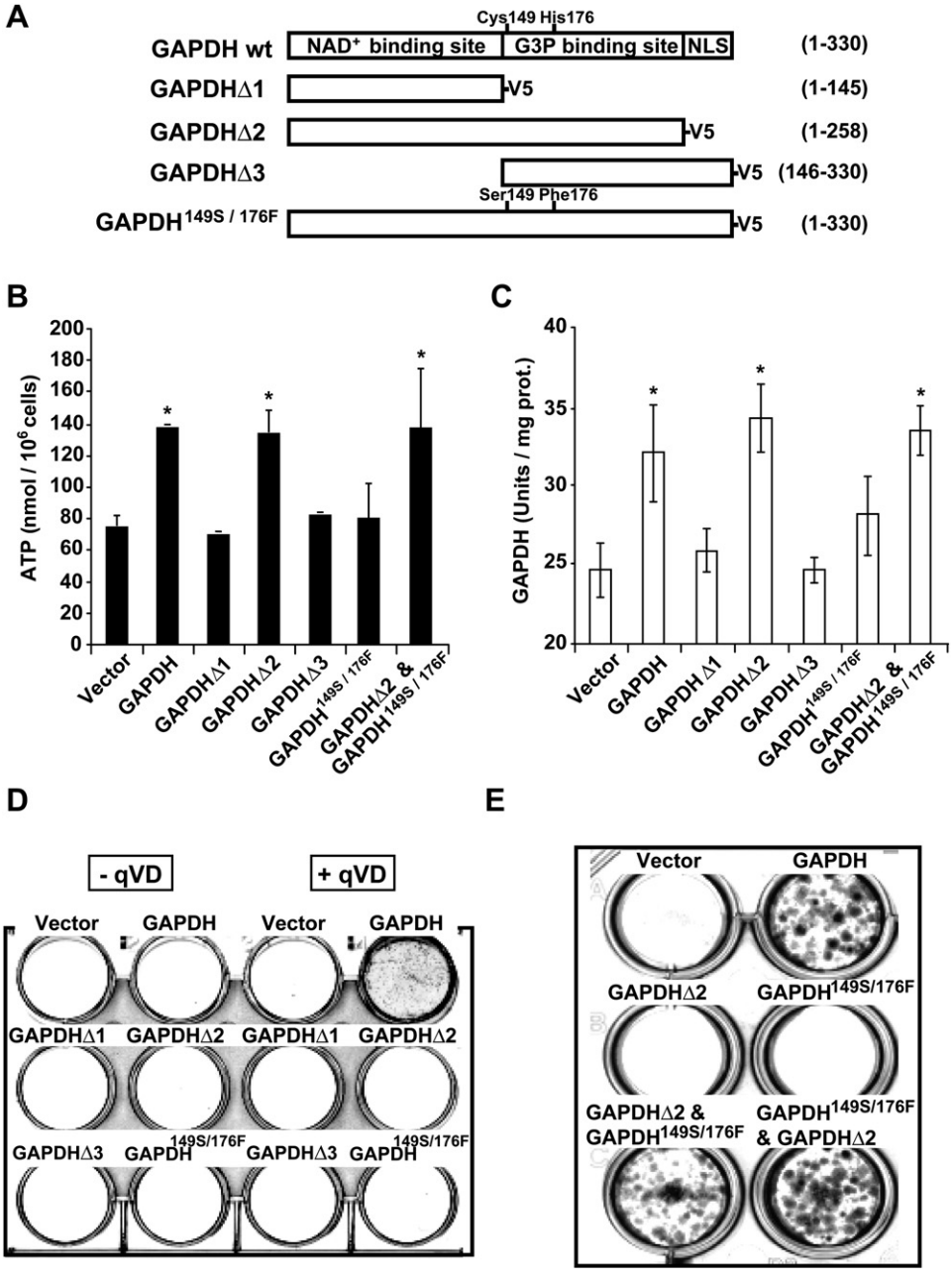

Figure 5. Effects of GAPDH Mutants

(A) Schematic representation of different GAPDH mutants, used in this study. GAPDHΔ2 lacks the putative nuclear localization sequence (NLS) of GAPDH.

(B) ATP levels in HeLa cells stably expressing each construct represented in A or with a combination, as indicated. *$p < 0.05$ versus vector values.

(C) GAPDH activity of HeLa cells stably expressing the constructs represented in A was measured as described in the Experimental Procedures. In (B) and (C), results represent mean ± SD from three independent determinations. *$p < 0.05$ versus vector values.

(D) HeLa cells expressing the mutants shown in A were treated for 6 hr with Act D (0.5 μM) ± qVD-oph (20 μM). Colonies were stained with methylene blue and assessed 12 days after treatment.

(E) HeLa cells expressing the WT, GAPDH$^{149S/176F}$, GAPDHΔ2 or a combination of both mutant forms of GAPDH were exposed to Act D (0.5 μM) + qVD-oph (20 μM). Stable double-expressing cell lines were generated by transducing each mutant in the order indicated. Colonies were stained with methylene blue and assessed 12 days after treatment.

Dastoor and Dreyer, 2001). We generated mutants of GAPDH (Figure 5A) and examined their abilities to elevate ATP and localize to the nucleus. The V5-tagged mutants were stably introduced into HeLa cells and their expression levels verified to be equivalent via immunoblot (data not shown). Of these, only one (GAPDHΔ2) elevated ATP

levels and GAPDH enzymatic activity to the same extent as that seen with wild-type GAPDH (Figures 5B and 5C). Another mutant (GAPDH$^{149S/176F}$) while failing to elevate ATP levels, localized to the nucleus upon cellular stress, as observed with wild-type GAPDH (Figure S8).

We then tested these mutants for their ability to rescue cells following MOMP in the absence of caspase activation. None of the mutants when expressed in HeLa cells were able to promote survival following treatment with cytotoxic agents in the presence of caspase inhibitors (Figure 5D) and neither were they capable of sustaining clonogenic survival in APAF-1 null MEF transduced with E1A (Figure S9). However, in both cases wild-type GAPDH was effective. We therefore cotransfected the ATP-elevating GAPDHΔ2 mutant and the GAPDH$^{149S/176F}$ mutant capable of nuclear localization. In contrast to each mutant alone, both together reproduced the effect of wild-type GAPDH such that the cells expressing both GAPDHΔ2 and GAPDH$^{149S/176F}$ recovered from cytotoxic treatment in the presence of caspase inhibitors (Figure 5E). In this experiment, stable HeLa lines expressing GAPDHΔ2 or GAPDH$^{149S/176F}$ were each stably transduced with the other GAPDH mutant to generate stable double-expressing lines (Figure S10A). Similar results were obtained following treatment with etoposide or staurosporine plus caspase inhibitor (Figure S10B). No cell survival was observed in cells treated with the apoptosis-inducing agents in the absence of caspase inhibitor (data not shown). Similarly, cointroduction of both mutants with E1A permitted transformation of APAF-1 deficient (but not wild-type) MEF, albeit with less efficiency than seen with wt GAPDH (Figure S11). Thus, both the glycolytic function and nuclear effects of GAPDH appear to be required for recovery from MOMP in the absence of caspase activation.

Autophagy is a process of cellular survival that is often associated with forms of cell death that are caspase-independent (Levine and Yuan, 2005). However, the role of autophagy in dying cells remains controversial. Examination of cells treated with Act D or plus qVD-oph showed increased numbers of autophagic vesicles in GAPDH-expressing versus vector-control cells (Figures 6A–6C). Similar observations were made in cells expressing LC3-GFP, in which punctate distribution of the fluorescent protein correlates with engagement of autophagy (Kabeya, et al., 2000) (Figure S12). These data indicated that GAPDH-mediated protection against CICD might involve the engagement of autophagy. To determine if this may be the case, GAPDH-expressing cells, ± caspase inhibitor were preincubated for 6 hr with the autophagy inhibitors 3-methyladenosine (3-MA) or Bafilomycin A1 (BafA1) followed by a 6 hr treatment with STS. While neither 3-MA nor BafA1 inhibited growth in cells not treated with STS, protection from CICD was inhibited (Figure S13). To address the role of autophagy more rigorously, we employed shRNA to stably knock down Atg5, a critical component of the autophagic pathway (Figure S14); these stable lines grew with nearly identical kinetics to control cells in terms of doubling time and clonogenicity (data not shown). However, GAPDH expression failed to protect cells from CICD when inhibition of autophagy was achieved by stable knockdown of Atg5 (Figure 6D).

Autophagy can function in the removal of damaged mitochondria, a process called "mitophagy" (Kundu and Thompson, 2005). It has previously been noted that mitophagy occurs in neurons and HeLa cells that have been treated with cytotoxic agents in the presence of caspase inhibitors, and this is blocked by Bcl-2 (Xue et al., 2001), suggesting that it may be a consequence of MOMP. Further, it has been suggested that mitochondria that have undergone a permeability transition (mPT) are removed by such mitophagy (Elmore et al., 2001). Although we have not found evidence of an mPT during MOMP (Waterhouse et al., 2001; Ricci et al., 2003, 2004), we examined the possibility that GAPDH-enhanced autophagy may promote removal of mitochondria in cells protected from CICD. We found that a subpopulation of cells displayed a reduced mitochondrial mass following MOMP in the presence of caspase inhibitors, and this was exacerbated by enforced expression of GAPDH (Figures 6E and 6F). This effect of GAPDH was not observed with the GAPDHΔ2 mutant (Figure S15) and was blocked by inhibition of autophagy by stable knockdown of Atg5 (Figures 6E and 6F). Thus, one way in which GAPDH may protect cells following MOMP is through autophagic removal of damaged mitochondria. Indeed, in cells expressing GAPDH and subjected to conditions for induction of CICD, colocalization of some (but not all) mitochondria with LC3-GFP was observed (Figures 6G and S16). We suspect that this is a dynamic process that may remove damaged mitochondria over an extended time period, as suggested by others (Elmore, et al., 2001; Xue, et al., 2001).

Nuclear GAPDH has been implicated in transcriptional regulation (Zheng et al., 2003). We therefore examined differential gene expression by RNG/MRC microarray in vector- or GAPDH-expressing cells treated with cytotoxic agents (data not shown). Of particular note was the differential expression of Atg12 that was further examined by real-time PCR (Figure 7A) and at the protein level (Figures 7B and 7C). We observed that Atg12 mRNA expression was induced by a brief (6 hr) treatment with Act D to a greater extent in those cells expressing GAPDH and not the vector control cell line. It is useful to note that upon transient (e.g., six hour) treatment with similar or higher doses of Act D, transcriptional activity rapidly recovers in cells (Sawicki and Godman, 1972), and that Act D-induced apoptosis is probably a consequence of DNA damage (Hietanen, et al., 2000; Mischo, et al., 2005). This expression resulted in sustained Atg12 protein levels in the GAPDH-expressing cells (Figures 7B and 7C). Interestingly, GAPDHΔ2 failed to have this effect on Atg12 expression (Figure 7C).

Atg12 participates in the process of autophagy, upon conjugation to Atg5 (Levine and Yuan, 2005). To determine if GAPDH-induced Atg12 expression contributes to protection from CICD, we asked if it could substitute for

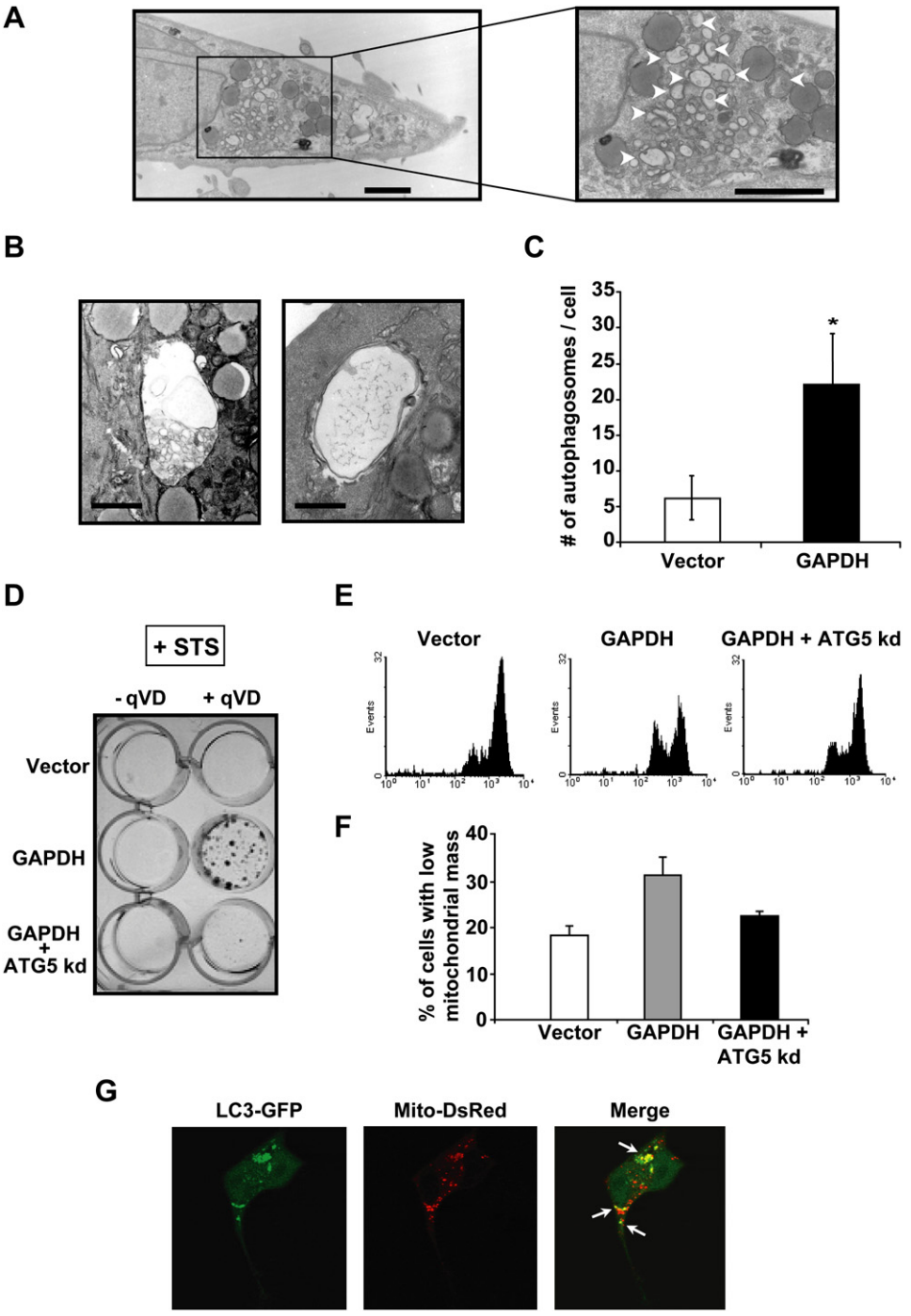

Figure 6. Autophagy Induced by GAPDH Protects Cells from Caspase-Independent Cell Death

(A) Representative electron microscopic image of GAPDH-expressing HeLa cells 4 days after Act D (0.5 μM) + qVD-oph (20 μM) exposure. Arrowheads denote autophagosomes. Scale bars represent 1 μm.

(B) Examples of autophagosomes. GAPDH-expressing HeLa cells treated cells as in (A). The double membrane characteristic of autophagy can clearly been seen. Scale bar represents 6 μm.

(C) Electron microscopic quantitation of autophagy in vector and GAPDH-expressing HeLa cells treated for 6 hr with Act D (0.5 μM) ± qVD-oph (20 μM). Results shown represent the mean ± S.D number of autophagosomes per cell, profiled for 18 cells per condition. *$p < 0.000001$ versus vector values.

(D) Control or GAPDH-expressing HeLa cells, or cells stably expressing an shRNA for Atg5 (Atg5 kd), were treated for 6 hr with STS (1 μM) + qVD-oph (20 μM). Colonies were stained with methylene blue and assessed 12 days after treatment.

nuclear GAPDH in cooperating with the glycolysis-elevating GAPDHΔ2 mutant to sustain survival. HeLa cells expressing Atg12 with or without GAPDHΔ2 were subjected to treatment with STS and cultured with or without caspase inhibitor. While Atg12 or GAPDHΔ2 did not protect cells on its own, substantial protection from CICD was observed in cells expressing both (Figure 7D). Two other components of the autophagy pathway, LC3 and ATG5, failed to protect cells from CICD, either alone or with GAPDHΔ2 (Figures S17A and S17B). These data suggest the possibility that the nuclear function of GAPDH in protecting cells from CICD is mediated by the transcriptional up-regulation of Atg12. Whether this reflects a direct or indirect effect of GAPDH remains to be determined.

DISCUSSION

During apoptosis, MOMP does not, by itself, cause a rapid loss of mitochondrial function (Martinou et al., 1999, Waterhouse et al., 2001, Ricci et al., 2003). However, with time the absence of an intact outer membrane compromises these organelles and commits the cell to death even under conditions in which caspase activation is disrupted or prevented. During this process, GAPDH levels decline. Such a loss of GAPDH has been described to occur via a chaperone-mediated degradation mechanism in nutrient-deprived cells (Majeski and Dice, 2004).

A cell lacking the downstream elements of the mitochondrial pathway (such as APAF-1) gains no growth advantage when provided with a proliferative signal (Scott et al., 2004). However, if GAPDH levels are sufficiently sustained, this disruption in caspase activation can permit cellular recovery and could contribute to oncogenic transformation. Tumors frequently have defects in APAF-1 expression (Ferraro et al., 2003) and elevated GAPDH expression (Revillion et al., 2000; Rondinelli et al., 1997; Schek et al., 1988; Tokunaga et al., 1987). Together, these may have contributed to their survival from the initial transforming event. Similarly, caspase activation downstream of the mitochondria can be antagonized by inhibitor of apoptosis proteins, such as XIAP (Deveraux et al., 1998), which is often found to be elevated in transformed cells (Ferreira et al., 2001; Krajewska et al., 2003; Tamm et al., 2000).

How does GAPDH contribute to recovery from MOMP? In part, this appears to be through maintenance of mitochondrial $\Delta\Psi m$, probably through elevation of ATP, which can sustain $\Delta\Psi m$ via F_0F_1ATPase (complex V) activity. Because HeLa cells have elevated activity of the rate-limiting glycolytic enzyme phosphofructokinase (PFK1) (Chesney et al., 1999, Hue and Rider, 1987), increased levels of GAPDH directly result in higher ATP levels. Maintenance of $\Delta\Psi m$ is required for most mitochondrial functions including protein import, and therefore any repair or dynamic processes in this organelle.

In addition, other functions of GAPDH appear necessary for full recovery from MOMP in the absence of caspase activation. In particular, the enforced expression of GAPDH enhances a process whereby mitochondria are removed in a manner that depends on Atg5. It is likely that this represents an autophagic removal of damaged mitochondria, consistent with our observed association of LC3 with mitochondria in GAPDH-expressing cells subjected to conditions for CICD. In the nucleus, GAPDH can participate in transcription, including regulation of the expression of Histone 2B (Zheng et al., 2003), cell cycle regulation (Carujo, et al., 2006), and DNA repair (Meyer-Siegler et al., 1991), among other functions (reviewed in Sirover 1999). Our studies indicate that nuclear GAPDH promotes Atg12 expression under conditions that induce MOMP, and that enforced expression of Atg12, but not LC3 or Atg5, obviated the requirement for nuclear GAPDH in protection from CICD. It remains to be seen whether this effect is due to maintenance of autophagy. It should be noted that our findings do not indicate that expression of Atg12 induces autophagy per se, but that such expression may limit autophagy occurring as a consequence of MOMP under conditions for CICD. Why this should be the case remains unclear.

While autophagy has also been proposed to promote cell death under some conditions (Levine and Yuan, 2005), our results point to a survival role for this process in cells subjected to conditions for CICD. If CICD is a form of "autophagic cell death," any accompanying autophagy is likely to represent engagement of this protective process, which fails if levels of GAPDH fall below a requisite threshold. It is tempting to speculate that autophagy contributes to protection from CICD by promoting the removal of terminally damaged mitochondria, while enhanced glycolysis and elevated ATP provide energy for this process as well as other aspects of cellular repair and survival. The approximate 7–10 day delay in restoration of proliferative potential in those GAPDH-expressing cells induced to undergo MOMP may therefore represent the time required for repair and expansion of functional mitochondria, until a critical cohort is attained.

(E) GAPDH promotes a reduction in mitochondrial mass under conditions of CICD. Mitochondrial mass was assessed by staining with nonyl-acridine orange 48 hr after treatment with Act D (6 hr) plus qVD-oph, and analyzed by FACS. Control or GAPDH-expressing HeLa with stable knockdown of Atg5 (ATG5 III) were examined.

(F) As in (D), results from three independent experiments. Values are mean ± SD (G) LC3 localizes to mitochondria under conditions of CICD. HeLa cells expressing GAPDH were transfected with constructs for expression of mitochondrially-localized DsRed and LC3-GFP, and treated with STS (1 μM, 6 hr) plus qVD-oph. Localization of fluorescent proteins was assessed 48 hr later. Additional images are shown in Figure S16. Examples of LC3-GFP and Mito-DsRed colocalization are indicated by white arrows.

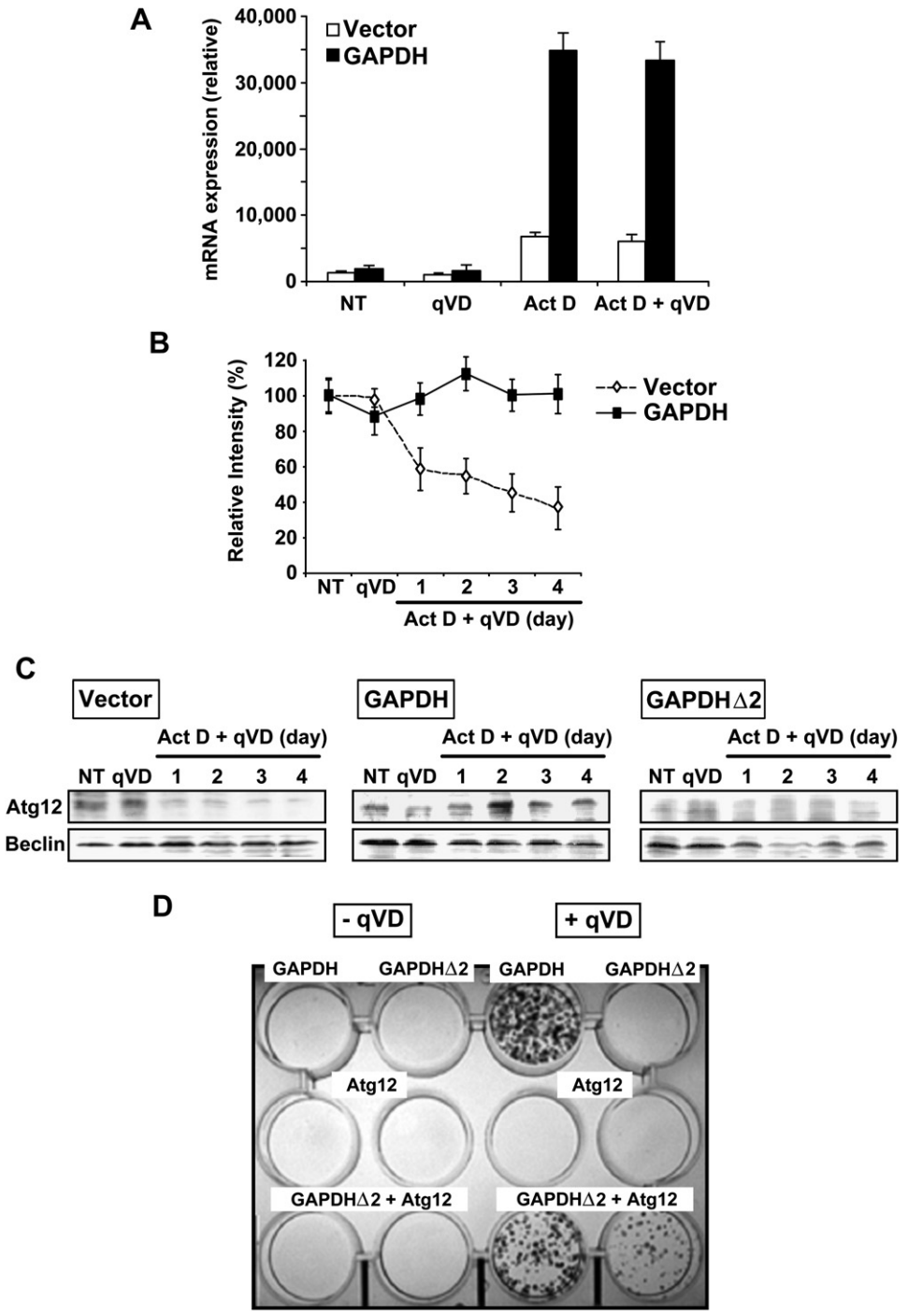

Figure 7. Atg12 Increase in GAPDH-Expressing Cells after a Cell Death Stimulus Is Sufficient to Replace the Requirement for Nuclear GAPDH for Protection from CICD

(A) HeLa cells (transduced with control or GAPDH encoding retrovirus) were exposed for 6 hr to Act D (0.2 μM) ± qVD-oph (20 μM). 24 hr after treatment, total mRNA was isolated and assessed by real-time RT-PCR for Atg12 expression. Absolute mRNA values were determined, normalized to cyclophilin A and reported as arbitrary units. Similar results were obtained with other housekeeping genes as controls (data not shown). Abbreviation: NT, nontreated cells.

(B) Cells were treated as in (A), and level of expression of Atg12 and Beclin-1 in the stable lines were assessed by immunoblot at the indicated times. The figure shows quantification of Atg12 protein levels versus the loading controls in three independent experiments.

(C) As in (B), and showing the effect of GAPDHΔ2.

(D) Cells expressing GAPDHΔ2 were transduced with a construct for expression of Atg12 and 24 hr later treated as in (A). Colonies were assessed 12 days after treatment.

EXPERIMENTAL PROCEDURES

Retroviral Library and Screening

Retroviral libraries were generated using cDNA derived from MCF-7 cells (cells expressing a defective caspase-3) in the retrovirus vector pMX (Onishi et al., 1996). The library was kindly provided by Dr. E. Rousalati (Burnham Inst., La Jolla, CA). The recombinant retrovirus library was used to infect Jurkat cells as described below. 48 hr later, those cells were subjected to selection (in the presence of staurosporine 0.5-1 μM ± 50 μM VD-fmk, the latter kindly supplied by Maxim Pharmaceuticals, San Diego, CA) and surviving clones were isolated by limiting dilution. Only cells presenting a resistance to caspase-independent death (i.e., resistance to staurosporine in the absence of VD-fmk) were further characterized. The cDNAs expressed in those cells were amplified by PCR using retroviral oligonucleotides and then sequenced.

Virus Production and Infection

Plasmids containing the wt or the mutated forms of GAPDH together with Hit 60 (MoMuLV gag-pol expression plasmid) and pCG (VSV-G envelope protein expression vector) were transfected into 293T cells using the standard Ca^{2+}-phosphate procedure. After 36 hr, the virus-containing medium was filtered (0.4 μm SFCA membrane filter; Nalgene) and supplemented with 10 μg/ml polybrene (Sigma) (first supernatant). Viruses were collected for an additional 10 hr as before (second supernatant). Target cells were plated at 50% confluence and incubated overnight. For infections, the culture medium was replaced by the appropriate first supernatant, the plate centrifuged for 10 min at 1500 rpm and then incubated at 37°C for 10 hr. The infection process was repeated using the second supernatant. Forty-eight hrs later, infected cell populations were purified using the appropriate selection: 1 μg/ml of puromycin or 1 mg/ml of G418. After 1 week expression of the tagged proteins was assessed by Western blot.

The same procedure was used for cytochrome *c*-GFP (cloned into pBabe-Puro vector; Goldstein et al., 2000) expression in HeLa cells stably expressing GAPDH.

Cell Culture and Induction of Cell Death

HeLa cells were grown in Dulbecco's Modified Essential Medium (DMEM, GIBCO BRL) and Jurkat cells (human acute T cell leukemia) were cultured in RPMI-1640 (GIBCO BRL). All media were supplemented with 2 mM glutamine, antibiotics and 10% fetal bovine serum (FBS). For experiments involving glucose limitation, cells were cultured in glucose-free DMEM, and the required amount of D-glucose added together with 2 mM glutamine, 5 mM uridine, 5 mM sodium pyruvate and 10% dialyzed FBS.

To induce death, cells were treated with actinomycin D (0.2-0.5 μM), staurosporine (0.5-1 μM), or etoposide (5-10 μM) for 6 hr then washed, fed with fresh medium and cultured for indicated periods of time. Caspase inhibition was achieved by including 20 μM of qVD-oph (Qbiogene, Irvine, CA) or 50 μM VD-fmk (Yang et al., 2003) in the medium where indicated. The caspase inhibitors were added 30 min before the apoptotic stimuli and replaced periodically at 24 hr intervals.

RNA Interference

Complementary sense and antisense oligonucleotides corresponding to nucleotides 89–107, 354–371, and 661–679 of human Atg5 were annealed and cloned into pSuper generating pSuper Atg5 I, II and III respectively. pSuper Atg5 II and Atg5 III were digested with EcoRI and XhoI and the restriction fragments encoding the H1 promoter and shRNA sequence were subcloned into pSuper RV puro via EcoRI/XhoI restriction sites. Retrovirus production and infection of GAPDH-V5 expressing Hela cells was done as described above. Stably transduced cells were selected and grown in puromycin (1ug/ml).

Atg5 mRNA expression was analyzed using the following oligonucleotides: sense 5′-TGGGATTGCAAAATGACAGA-3′ and antisense 5′-TTTCCCCATCTTCAGGATCA-3′.

To assess knockdown of Atg5 protein, human Atg5 (MGC clone number 110866) was amplified by PCR using the following oligonucleotides: sense 5′-ACGCGTCGACCATGACAGATGACAAAGATGTGCTTC-3′ encoding a SalI restriction site and antisense 5′-ATAGTTTAGCGGCCGCTCAATCTGTTGGCTGTGGGATG-3′ encoding a NotI restriction site. The PCR product was cloned into PMT2-HA via SalI/NotI restriction sites, and employed for transient expression. Expression was assessed by immunoblot for the tagged protein.

Statistics

Statistics were performed using a two tailed distribution Student's t test. All values shown in the text and figures are ±SD.

Additional methods are provided in the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Discussion, Supplemental Experimental Procedures, Supplemental References, one table, and seventeen figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/5/983/DC1/.

ACKNOWLEDGMENTS

We thank C. Munoz-Pinedo, P. Auberger, C. Bonzon, P. Colosetti, M. Mari, and A. Morales for invaluable help and discussion; P. Gounon for EM; and T. Mak and X. Wang for mice. This work was supported by NIH grant AI40646 (D.R.G.) and Association pour la Recherche Contre le Cancer (3642) and Fondation pour la Recherche Médicale (J.-E.R.) and Plan Nacional I+D grant (SAF2005-03923) (A.C.). A.C. received a fellowship from the Secretaria de Estado de Universidades e Investigacion.

Received: May 23, 2005
Revised: October 31, 2006
Accepted: March 7, 2007
Published: May 31, 2007

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