Apoptosis, or programmed cell death, occurs because of the activation of a protease cascade amplification circuit that includes the critical effector caspase-3. Previously, we identified the widely expressed actin modulatory protein gelsolin as a prominent substrate of caspase-3 and demonstrated that the N-terminal gelsolin cleavage product promotes apoptosis. Here we show that phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4-bisphosphate in pure micelles or mixed vesicles prevent caspase-3 cleavage of gelsolin. Moreover, phosphatidylinositol 4,5-bisphosphate-gelsolin strongly inhibits caspase-3 and -9 activity through the formation of a stable phosphatidylinositol 4,5-bisphosphate-gelsolin-caspase complex. In addition, phosphatidylinositol 4,5-bisphosphate-gelsolin prevents apoptotic progression mediated by caspase-3 in a cell-free system, and phosphatidylinositol 4,5-bisphosphate-gelsolin-caspase-9 and phosphatidylinositol 4,5-bisphosphate-gelsolin-caspase-3 complexes form in mouse embryonic fibroblasts during apoptosis induction when stimulated with fibronectin, to delay cell death. The results suggest that gelsolin can act as both an effector and an inhibitor of caspase-3, the latter in concert with phosphatidylinositol 4,5-bisphosphate, and other membrane phospholipids to regulate the onset and progression of apoptosis.

Regulation of cell survival is crucial to normal mammalian homeostasis, including embryonic development, injury responses, and tumor suppression (1, 2). Cells have the inherent capacity to both initiate and block apoptotic signals (3). Recent studies have delineated two primary pathways leading to apoptosis. The first involves the interaction between cytokine signals, e.g. tumor necrosis factor, and their receptors, which leads to activation of the initiator caspase-8 (4). The second pathway is a stress response pathway in which release of mitochondrial cytochrome c leads to formation of the apoptosome complex in which caspase-9 is activated (5). Both pathways then converge to activate the effector caspase-3, -2, and -7, which have multiple downstream targets, resulting in the morphological and nuclear changes of apoptosis. Bcl-2 and some related proteins inhibit the early stages in the activation of the second pathway (6). Several inhibitors of caspase-3 and -7 are known (XIAP, c-IAP1, c-IAP2, survivin), but their effectiveness in inhibiting apoptosis in vivo and tissue distribution is uncertain (7, 8).

Gelsolin was identified as a prominent substrate of caspase-3 in murine embryos in an in vitro translation assay system (9). Gelsolin is cleaved between residues Asp-352 and Gly-353, and the N-terminal gelsolin fragment contributes to the morphologic changes of apoptosis, demonstrated by the accelerated apoptosis of wild-type neutrophils in comparison with gelsolin null neutrophils. Gelsolin is also cleaved and appears to contribute to apoptotic progression in vascular smooth muscle cells (10). However, in Jurkat cells, a human T lymphocyte cell line, expression of gelsolin has been reported to inhibit apoptosis induced by any of several different stimuli, including both cytokine apoptosis inducers and stress response activators (11). Here, we explored the mechanism and cellular circumstances in which gelsolin might act as an inhibitor of apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Protein Preparation—Phospholipids were obtained from either Sigma or Fluka. Recombinant caspases were prepared as described (caspase-3), (9), the gift of Dr. Hong Lin, Harvard Medical School (caspase-8), or purchased from BioVision (caspase-9) as an unpurified 6xhis fusion protein in a bacterial cell extract. Murine mutant D352K gelsolin (aspartic acid at amino acid 352 replaced with a lysine) was made by polymerase chain reaction mutagenesis, cloned into pMW172, and confirmed by sequencing. Wild-type and D352K mutant gelsolins were expressed in Escherichia coli strain BL21(DE3) and purified as described (12). A D4-GDI expression construct was the gift of Dr. Bing Lim (Beth Israel Hospital, Boston, MA).

Caspase Assays—Caspase-3, -8, -9 activity levels were assayed by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from DEVD-AFC, IETD-AFC, or LEHD-AFC, respectively, using a Perkin-Elmer LS-5 fluorimeter (excitation 405 nm, emission 500 nm) in 50 mM HEPES buffer (pH 7.5), 10% sucrose, 10 mM DTT, and 0.1 mM peptide substrate. The inhibition rate (I) was calculated from reaction rates on progression curves performed with DEVD-AFC, 0.3 mM caspase-3, 60 mM gelsolin, plus or minus phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) or phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) at concentrations of 250 mM–2 mM.

Cell Lines and Apoptosis Induction—Primary murine embryo fibroblasts (MEFs) prepared from wild-type and gelsolin null mouse day 12.5 embryos were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin G-streptomycin sulfate. Serum-starved quiescent MEF cells were detached from culture dishes with trypsin, treated with 0.5 mg/ml soybean trypsin inhibitor, washed, and placed in serum-free media (SFM) for 8 h. Glass coverslips or tissue culture plates were coated with either 50 mg/ml fibronectin (FN) overnight at 4 °C or 10 mg/ml poly-L-lysine (PLL) at room temperature for 1 h. Cells in SFM were plated onto the coverslips in SFM. Cell death was analyzed using the ApoAlert DNA fragmentation assay (CLONTECH).

Antibodies and Immunoblotting—Rabbit anti-murine gelsolin antibody was used as described (13). Monoclonal anti-6xhis tag antibody was the gift of Hiroaki Onda (Brigham and Women’s Hospital). Rabbit anti-caspase-3 antibody was prepared using purified caspase-3 ex-

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¶ The abbreviations used are: AFC, 7-amino-4-trifluoromethyl-coumarin; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-bisphosphate; DTT, dithiothreitol; MEF, murine embryo fibroblast; SFM, serum-free media; FN, fibronectin; PLL, poly-L-lysine; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; fmk, phenylmethyl ketone.
pressed in E. coli. Anti-PI(4,5)P$_2$ antibodies were obtained from Advanced Magnetics (Cambridge, MA). Immunoblotting after SDS-PAGE was performed using ECL (13).

**Protein Binding Assays and Immunoprecipitation**—One mg of wild-type or D352K mutant gelsolin was pre-incubated with or without 0.3 mm PI(4,5)P$_2$ and added to purified 0.25 mg of caspase-3 or 1.0 mg of caspase-9 with or without caspase peptide inhibitor (1 mm z-VAD-fmk or 1 mm LEHFM-fmk, respectively) in phosphate-buffered saline with 0.2% bovine serum albumin. In some experiments, PI(4,5)P$_2$ was pre-mixed with the PIP$_2$ binding peptide rhodamine B-QRLFQVKGRR (gelsolin-60–169) (14) before adding it to the reaction mixture. After incubation at 4 °C for 60 min, anti-gelsolin antibody immobilized on protein A-agarose beads (ImmunoPure IgG, Pierce) was added and incubated for 30 min. at 4 °C. The beads were collected by centrifugation and washed, and proteins were eluted with SDS sample buffer without a reducing reagent and analyzed by immunoblotting. Cellular extracts were processed similarly after lysis with TTE buffer (10 mm Tris-Cl, pH 7.5, 1% Triton X-100, 150 mm KCl, 1 mm EDTA, 10 mg/ml aprotinin).

**A Cell-free System for Analysis of Apoptosis**—Cell extracts were prepared as described (15). Mouse hepatocyte nuclei were prepared by homogenizing fresh mouse liver in buffer A (10 mm HEPES, 2.4 mm sucrose, 15 mm KCl, 2 mm sodium-EDTA, 0.15 mm spermidine, 0.5 mm DTT, and 0.5 mm phenylmethanesulfonyl fluoride) with a Dounce homogenizer. Homogenates were centrifuged through a 10-ml cushion of buffer A at 25,000 rpm for 1 h in a SW 28 rotor at 4 °C. The nuclear pellet was resuspended in buffer C (10 mm PIPES, pH 7.4, 20 mm NaCl, 5 mm EGTA, 250 mm sucrose, and 1 mm DTT) at 5.0 × 10$^8$ nuclei/ml and stored at −80 °C (15). 50 μl (500 μg of protein) of Jurkat cell extract, 10 μl of hepatocyte nuclei, and 250 ng of caspase-3 were incubated at 37 °C for 2 h in the presence or absence of lipids and gelsolin. DNA was extracted and size-fractionated by agarose gel electrophoresis.

**Lipid Vesicle Preparation**—Bilayer vesicles were prepared by drying a chloroform mixture containing 30% phosphatidylcholine (PC), 40% phosphatidylethanolamine (PE), and 20% phosphatidylserine (PS) onto a glass surface with N$_2$ gas (16). Vesicles were swelled off the glass by the addition of buffer (5 mm HEPES, pH 7.4, 0.1 mm EDTA, and 211 mm sucrose) with or without PI(4,5)P$_2$ to make the final PI(4,5)P$_2$ concentration at 10%. Hydrated vesicles were extruded through a 100-nm membrane to make unilamellar vesicles of uniform size. Vesicles were then diluted into iso-osmotic buffers containing 120 mm KCl but no sucrose.

**RESULTS AND DISCUSSION**

Despite the rapid and complete cleavage of gelsolin by caspase-3 in vitro, we observed that some gelsolin remained uncleaved in all cells even after complete cell death (9). Because gelsolin binds to Ca$^{2+}$, actin, polyphosphoinositides (17), and lysophosphatidic acid (18) with high affinity, we examined whether these binding partners could affect caspase-3 cleavage of gelsolin. Although free Ca$^{2+}$ concentrations in the nM to mM range had no effect on the rate of gelsolin cleavage, such cleavage was blocked by bound actin (data not shown) and by bound PI(4,5)P$_2$ at all Ca$^{2+}$ concentrations (Fig. 1a). Complete inhibition of caspase-3 cleavage of gelsolin was observed with PI(4,5)P$_2$ at a molar ratio of 30:1 PI(4,5)P$_2$/gelsolin, consistent with the assembly of PI(4,5)P$_2$ into micelles of ~90 kDa and a binding stoichiometry of 1 micelle:1 gelsolin (17) (Fig. 1c). Other lipids (PS, PC) not known to bind to gelsolin had no effect on caspase-3 cleavage of gelsolin (data not shown).

We then examined whether PI(4,5)P$_2$-gelsolin or gelsolin-actin had any effect on caspase-3 activity. PI(4,5)P$_2$-gelsolin inhibited caspase-3 cleavage of the fluorogenic peptide substrate DEVD-AFC in a dose-dependent manner (Fig. 2a), and a 100:1 ratio of PI(4,5)P$_2$-gelsolin complex: caspase-3 resulted in near complete inhibition ($K_i = 1.5$ nm). No effect on caspase-3 activity was seen with PI(4,5)P$_2$ alone. Gelsolin ± PC or PS caused a mild decline in fluorescence increase, as gelsolin is a preferred substrate (9). PI(3,4)P$_2$-gelsolin also had strong inhibitory activity ($K_i = 0.9$ nm, Fig. 2b), as did gelsolin added to PI(4,5)P$_2$-PC-PS-mixed micelles (Fig. 2c). The ability of clustered PI(4,5)P$_2$ within the mixed micelles to bind to gelsolin (17) and inhibit caspase-3 suggests that PI(4,5)P$_2$-gelsolin complexes that occur in vivo (19) could inhibit caspase-3. In contrast gelsolin-actin complexes had no effect on caspase-3 activity.

A D352K mutant gelsolin (aspartic acid (D) at residue 352 changed to lysine (K)) was generated and had PI(4,5)P$_2$-sensitive actin severing activity similar to that of native gelsolin (data not shown). The D352K mutant gelsolin, however, was completely resistant to caspase-3 cleavage (data not shown), and PI(4,5)P$_2$-D352K-gelsolin did not inhibit caspase-3 (Fig. 2d). These observations suggest that the site of interaction between PI(4,5)P$_2$-gelsolin and caspase-3 involves the Asp-352 gelsolin cleavage site. The effect of PI(4,5)P$_2$ on another caspase-3 substrate, D4-GDI (20), was also examined. D4-GDI cleavage by caspase-3 was not affected by PI(4,5)P$_2$ (data not shown), and mixtures of PI(4,5)P$_2$ and D4-GDI had no effect on caspase-3 activity (Fig. 2e).

We also examined other caspases and found that caspase-7 and -9 cleaved gelsolin in a manner similar to caspase-3, whereas caspase-1, -6, and -8 did not cleave gelsolin (Fig. 3a). In addition, the PI(4,5)P$_2$-gelsolin complex inhibited caspase-9 activity on a peptide substrate (Fig. 3b), similar to its effect on caspase-3, but did not inhibit caspase-8 (Fig. 3c). Consistent with these observations, we also demonstrated that PI(4,5)P$_2$-gelsolin formed a stable complex with these caspases (Fig. 3d). For each of caspase-3 and -9, incubation with PI(4,5)P$_2$-gelsolin yielded a complex that could be isolated by immunoprecipitation with antibodies specific for gelsolin, and shown to contain the caspase. Pre-incubation with either a caspase active site covalent ligand (zVAD-fmk and LEHD-fmk, respectively, for caspase-3 and -9) or a molar excess of a PI(4,5)P$_2$ binding peptide (14) blocked formation of the ternary complex. The D352K-gelsolin did not form a complex with the caspases in parallel experiments, confirming the importance of the wild-type cleavage site within gelsolin for this interaction (Fig. 3d).

To explore the in vivo significance of PI(4,5)P$_2$-gelsolin inhibition of caspases, we began with an assay of DNA fragmentation in a cell-free system consisting of Jurkat cell cytoplasmic...
extracts and mouse hepatocyte nuclei (15) and added caspase-3. We also prepared unilamellar bilayer vesicles containing PS, PC, and PE, as well as 10% PI(4,5)P_2 to mimic native cell membrane. In this assay, both PI(4,5)P_2 micelles and PI(4,5)P_2 in vesicles without gelsolin had a slight inhibitory effect on DNA fragmentation, whereas gelsolin alone or vesicles without PI(4,5)P_2 showed no inhibition (Fig. 4). Both PI(4,5)P_2-gelsolin and PI(4,5)P_2/vesicle-gelsolin inhibited DNA fragmentation to a major extent, and at higher doses of PI(4,5)P_2-gelsolin, this inhibition appeared to be complete. Note that this occurred at relatively low concentrations of PI(4,5)P_2, possibly reflecting the contribution of cellular phos-
Gelsolin is a widely and relatively highly expressed protein that is important in the mediation of actin-based motility in several cell types (12, 13, 22). It binds to D3 and D4 polyphosphoinositides with high affinity in vitro (17, 22) and has been isolated from several cell types in complex with PI(4,5)P_2. Many substrates and inhibitors of caspases have been identified in vitro (17, 22) and has been shown to have critical roles in apoptotic progression in vivo (23–25). Bcl-2 family members bcl-2, bcl-X, and BID are all cleaved by different caspases, and in vivo, all these family members, cIAP1, cIAP2, XIAP, and survivin, all inhibit caspase activity and apoptotic progression (23–25). The IAP family members, cIAP1, cIAP2, XIAP, NAIP, and survivin, all inhibit caspase activity and apoptotic progression (23, 24). The inhibitory activity of PI(4,5)P_2-gelsolin is as strong as that of the most potent IAP proteins (23–25), although it is also novel in the requirement for bound phosphoinositide. In contrast, cleaved gelsolin has potent apoptosis promoting capability (9), showing that gelsolin can act as a double-edged sword during apoptosis, serving as a brake against apoptotic progression as well as an active effector of apoptosis. The balance between these two opposing activities of gelsolin may have significant effects on the onset and progression of apoptosis. Growth factor treatment and binding to adhesiv e substrates each prevents or delays apoptosis in many cell types, acting through phospholipid and kinase signaling pathways (29). We have shown that binding to fibronectin by observed that during apoptosis induced by either serum withdrawal (0.2% fetal calf serum) or cycloheximide (10 mg/ml), pre-treatment with a phospholipase C inhibitor (U73122) increased cellular levels of PI(4,5)P_2-gelsolin (21) and significantly delayed the onset of apoptosis in wild-type MEF compared with similarly treated gelsolin null MEF. For a model more closely approximating in vivo conditions, we induced apoptosis in wild-type and gelsolin null MEF by serum withdrawal and used plating on fibronectin-coated dishes to stimulate formation of PI(4,5)P_2-gelsolin complexes. Gelsolin null MEF had similar rates of apoptosis when plated on either fibronectin-coated or poly-l-lysine-coated dishes (Fig. 5b). In contrast, apoptosis in wild-type MEF was significantly delayed when the cells were plated on fibronectin in comparison with plating on poly-l-lysine (Fig. 5a). In addition, processing of both pro-caspase-3 and pro-caspase-9 to their active forms was delayed in the wild-type MEF plated on fibronectin, in comparison with gelsolin null MEF plated on fibronectin (Fig. 5, c–f), consistent with the presence of an inhibitory molecule or effect in the wild-type cells. Gelsolin cleavage was also delayed in the wild-type MEF plated on fibronectin compared with plating on poly-l-lysine (Fig. 5, g and h). We also observed that as gelsolin levels fell somewhat during these conditions, PI(4,5)P_2 binding to gelsolin, as assessed by immunoprecipitation of gelsolin, followed by SDS-PAGE, and immunoblotting for PI(4,5)P_2, occurred within 2 h of plating on fibronectin and persisted for up to 18 h (Fig. 5, i and j). To confirm that a complex between gelsolin and caspases formed during these treatments, we immunoprecipitated gelsolin from cell extracts and found that both caspase-3 and caspase-9 were stably bound to gelsolin at appropriate intervals after plating on fibronectin (Fig. 5, k and l). The presence of PI(4,5)P_2 in these complexes was confirmed by immunoprecipitation for each of the caspases from cell extracts, followed by SDS-PAGE and immunoblotting for PI(4,5)P_2 (Fig. 5m).

Gelsolin is a widely and relatively highly expressed protein that is important in the mediation of actin-based motility in several cell types (12, 13, 22). It binds to D3 and D4 polyphosphoinositides with high affinity in vitro (17, 22) and has been isolated from several cell types in complex with PI(4,5)P_2, the predominant cellular phosphoinositide (19, 21, 22). Here, we have shown that the PI(4,5)P_2-gelsolin complex is a high affinity inhibitor of caspase-3 and -9, forming a stable ternary complex with each caspase both in vitro and in vivo. Many substrates and inhibitors of caspases have been identified through in vitro biochemical assays, whereas only a few have been shown to have critical roles in apoptotic progression in vivo (23, 24). The IAP family members, cIAP1, cIAP2, XIAP, NAIP, and survivin, all inhibit caspase activity and apoptotic progression, but with limited induction agent specificity and uncertain in vivo distribution (23–25). Bcl-2 family members bcl-2, bcl-X, and BID are all cleaved by different caspases, and in all cases such cleavage greatly accentuates their pro-apoptotic activity (26–28). The inhibitory activity of PI(4,5)P_2-gelsolin is as strong as that of the most potent IAP proteins (23–25), although it is also novel in the requirement for bound phosphoinositide. In contrast, cleaved gelsolin has potent apoptosis promoting capability (9), showing that gelsolin can act as a double-edged sword during apoptosis, serving as a brake against apoptotic progression as well as an active effector of apoptosis. The balance between these two opposing activities of gelsolin may have significant effects on the onset and progression of apoptosis. Growth factor treatment and binding to adhesive substrates each prevents or delays apoptosis in many cell types, acting through phospholipid and kinase signaling pathways (29). We have shown that binding to fibronectin by

FIG. 4. A cell-free assay demonstrates the inhibitory effect of the PI(4,5)P_2-gelsolin complex on caspase-3 mediated apoptosis. 10 μl of mouse liver nuclei, 200 ng of caspase-3, and 50 μl of Jurkat cell extract were incubated with or without gelsolin and lipids for 2 h at 37 °C. The DNA was then extracted and analyzed by agarose gel electrophoresis. The first lane shows DNA standards. Phospholipid to this inhibition or the enhanced binding of PI(4,5)P_2-gelsolin to caspase-3 under these conditions.

To explore the in vivo significance of PI(4,5)P_2-gelsolin inhibition of caspase activity during apoptosis directly, we used MEF obtained from wild-type and gelsolin null day 12.5 embryos (12). In preliminary experiments (data not shown), we

FIG. 3. Cleavage specificity of caspases for gelsolin and formation of a PI(4,5)P_2-gelsolin-caspase complex. a, 6 μM gelsolin was incubated with 30 nM of each caspase at 37 °C for 4 h and then analyzed by SDS-PAGE. b, progress curves for caspase-9. 1 mg of caspase-9 extract with or without 60 nM gelsolin (Gsn 60 nM) and various concentrations of PI(4,5)P_2 were added to 100 μM LEHD-AFC, and the fluorescence was monitored. c, same as in b, except for caspase-8; 1 μM caspase-8 was added to 100 μM IETD-AFC. d, 1 μM of recombinant wild-type (lanes 1–4) or D352K mutant (lanes 5–8) gelsolin was incubated with 0.25 μg of caspase-3 or 1.0 μg of caspase-9 and then immunoprecipitated with anti-gelsolin antibody and assayed by immunoblotting for caspase-3 or the 6xhis tag (caspase-9). Lanes: 1 and 5, no additives; 2 and 6, + 0.3 μM PI(4,5)P_2; 3 and 7, + 0.3 μM PI(4,5)P_2 + 1 μM Z-VDAD-fmk (lane 3) or 1 μM LEHD-fmk (lane 7); 4 and 8, + 0.3 μM PI(4,5)P_2 + 10 μM PI(4,5)P_2-binding peptide.

FIG. 5. Apoptosis in wild-type and gelsolin null MEF by serum withdrawal or cycloheximide. a, 10% of mouse liver nuclei, 200 ng of caspase-3, and 50 μl of Jurkat cell extract were incubated with or without gelsolin and lipids for 2 h at 37 °C. The DNA was then extracted and analyzed by agarose gel electrophoresis. The first lane shows DNA standards.
MEF cells leads to formation of PI(4,5)P₂-gelsolin complexes that directly contribute to this anti-apoptotic pathway by complexing with active caspase-3 and -9. The full extent of involvement of PI(4,5)P₂-gelsolin in preventing or delaying apoptosis in vivo will require further study of additional cell types and models of apoptosis induction, but the induced proximity model of caspase activation (30) implies that caspase inhibitors are critical regulator of apoptosis induction. In addition, expression of gelsolin in Jurkat cells (a human T cell line) is reported to prevent apoptotic progression in response to several stimuli (18) and may be due to gelsolin inhibition of caspase activity. The balance of gelsolin activity in apoptosis (anti- versus pro-) may well be highly cell type- and stimulus-specific, similar to observations made on the role of caspases and Bcl proteins (23, 24, 31, 32).

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FIG. 5. Reduced apoptotic death with formation of PI(4,5)P₂-gelsolin- caspase-9 and -3 complexes in MEF plated on FN-coated coverslips. MEF were detached and suspended in SFM for 8 h, then plated in SFM on either FN-coated or poly-L-lysine (PLL)-coated coverslips, and harvested for analysis at the designated time points after plating. a and b, apoptosis was assessed by a DNA fragmentation assay in wild-type (WT, a) and gelsolin null (Gl−, b) MEF. c and d, caspase-3 processing in wild-type (c) and gelsolin null (d) MEF after plating on fibronectin was assessed by immunoblotting of whole cell extracts. e and f, caspase-3 processing in wild-type (e) and gelsolin null (f) MEF after plating on fibronectin was assessed by immunoblotting of whole cell extracts. g and h, gelsolin cleavage in wild-type MEF plated on poly-L-lysine (g) or fibronectin (h) was assessed by immunoblotting of whole cell extracts. i and j, gelsolin levels (i) and PI(4,5)P₂ binding to gelsolin (j) was assessed by immunoprecipitation (IP) of gelsolin, followed by immunoblotting (IB) for gelsolin and PI(4,5)P₂, respectively, in extracts from wild-type MEF plated on fibronectin. Note that PI(4,5)P₂ does not dissociate from gelsolin during SDS-PAGE and does not affect its migration (19). Therefore, the anti-PI(4,5)P₂ antibody gives a signal at the same position as gelsolin. k and l, formation of PI(4,5)P₂-gelsolin-caspase-3 (k) and PI(4,5)P₂-gelsolin-caspase-9 (l) complexes was assessed by immunoprecipitation of gelsolin from cell extracts followed by immunoblotting with anti-caspase-3 and anti-caspase-9 antibodies, respectively. Whole cell extracts of MEF without treatment (Pre) and after (Post) 30 h of serum starvation are also shown for comparison. m, formation of PI(4,5)P₂-gelsolin-caspase-3 and PI(4,5)P₂-gelsolin-caspase-9 complexes was assessed by immunoprecipitation of caspase-3 or caspase-9 from cell extracts followed by immunoblotting with anti-PI(4,5)P₂. As described above, the anti-PI(4,5)P₂ co-migrates through the gel with gelsolin, so that the reactive band has an apparent molecular weight comparable with gelsolin. Lanes 1–4 are controls with various combinations of purified caspase-3, caspase-9, gelsolin, and PI(4,5)P₂ immunoprecipitated (IP) for either caspase-3 or caspase-9. Lanes 5–10 show analyses of cell extracts immunoprecipitated using either anti-caspase-3 or anti-caspase-9 antibodies, derived from either wild-type (G⁺) or gelsolin null (G⁻) MEF, after plating on either fibronectin or poly-L-lysine.
3766

PI(4,5)P₂-Gelsolin Inhibits Caspases and Apoptosis

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