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Gelsolin Inhibits Apoptosis by Blocking Mitochondrial Membrane Potential Loss and Cytochrome c Release

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ABSTRACT

Apoptotic cell death, characterized by chromatin condensation, nuclear fragmentation, cell membrane blebbing, and apoptotic body formation, is also accompanied by typical mitochondrial changes. The latter includes enhanced membrane permeability, fall in mitochondrial membrane potential (m) and release of cytochrome c into the cytosol. Gelsolin, an actin regulatory protein, has been shown to inhibit apoptosis, but when cleaved by caspase-3, a fragment that is implicated as an effector of apoptosis is generated. The mechanism by which the full-length form of gelsolin inhibits apoptosis is unclear. Here we show that the overexpression of gelsolin inhibits the loss of m and cytochrome c release from mitochondria resulting in the lack of activation of caspase-3, -8,and -9in Jurkat cells treated with staurosporine, thapsigargin, and protoporphyrin IX. These effects were corroborated in vitro using recombinant gelsolin protein on isolated rat mitochondria stimulated with Ca2+, atractyloside, or Bax. This protective function of gelsolin, which was not due to simple Ca2+ sequestration, was inhibited by polyphosphoinositide binding. In addition we confirmed that gelsolin, besides its localization in the cytosol, is also present in the mitochondrial fraction of cells. Gelsolin thus acts on an early step in the apoptotic signaling at the level of mitochondria.

INTRODUCTION

Apoptotic cell death is a fundamental process of normal development and tissue homeostasis of multicellular organisms (1, 2). It is characterized by typical structural changes including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear DNA fragmentation (3, 4). Deregulated apoptosis can lead to human diseases such as cancer and degenerative disorders (2).

There is much evidence suggesting that apoptotic signaling is processed by highly regulated and specific proteolysis via caspases (5). Caspases are an evolutionarily conserved family of aspartic acid-specific cysteine proteases (6, 7) that are

synthesized as inactive precursor molecules and are converted to active heterodimers by proteolytic cleavage (8). Cleavage of specific substrates has been proposed to activate death effector molecules or induce structural changes characteristic of apoptotic cells (8, 9). Long prodomain caspases containing sequence motifs that promote their interaction with activator molecules (caspase-2, -8,-9,and -10) function as apoptotic initiators, generally acting upstream of small prodomain executioner caspases (caspase-3, -6,and -7) (10-12).

Recent evidence indicates that mitochondria play a prominent role in cell death as a central organelle involved in the signal transduction and amplification of the apoptotic response (13, 14). Mitochondrial dysfunction is an early event, preceding nuclear and plasma membrane alterations. It is characterized by an increase in mitochondrial membrane permeability and loss of membrane potential that is regulated by the permeability transition (PT)1 pore complex, an elusive multiprotein channel composed of voltage-dependent anion channel, adenine nucleotide translocator, cyclophilin D, peripheral benzodiazepine receptor, and probably others (13, 15). An important role of mitochondria in apoptotic signaling is the translocation of cytochrome c from the mitochondrial intermembrane compartment into the cytosol. Once released, cytochrome c binds to APAF-1 in the presence of ATP or dATP and forms a complex that processes and activates pro-caspase-9, which in turn cleaves and activates the executioner caspases, such as caspase-3 and -7(13, 16). The release of cytochrome c has been linked to loss of mitochondrial membrane potential (m) and PT (15, 17, 18), although there are also reports providing evidence that these are independent events (19). Probably m-dependent and -independent mechanisms exist, differing with specific apoptotic stimuli (19, 20). PT pores are controlled by pro- and anti-apoptotic members of the Bcl-2 family of proteins, which can bind to this channel and regulate the release of cytochrome c into the cytosol (17, 18).

Gelsolin is a Ca2+-dependent actin-regulatory protein that can sever actin filaments and cap the quickly growing ends of filaments in vitro, promoting actin disassembly (21). These functions are inhibited by polyphosphoinositides (22-24). Gelsolin has roles in organization of the cytoskeleton, cell motility, cell growth, and apoptosis (25-32). Gelsolin is a substrate for caspase-3 and the N-terminal cleavage product has been shown to accelerate morphological changes associated with apoptosis when expressed in mouse embryonic fibroblasts (32). On the other hand, the full-length form of gelsolin can inhibit apoptosis induced by various agents, including anti-Fas antibody, ceramide, and dexamethasone (30, 31). Moreover, in cells overexpressing gelsolin, caspase-3 is not activated after treatment with apoptotic stimuli, indicating that gelsolin may block apoptosis upstream of the activation of this caspase (30, 31).

Here, we extend this study to examine the mechanism of the inhibitory function of gelsolin on apoptosis by analyzing the mitochondrial system and provide evidence for a direct effect of gelsolin on these organelles. It inhibits the loss of m and cytochrome c release into the cytosol, resulting in the lack of activation of caspases. We also show that this effect is not due to Ca2+ sequestration and that binding to polyphosphoinositide can regulate this process.

EXPERIMENTAL PROCEDURES

Cells and Induction of Apoptosis - The Jurkat lymphoblastoid T-cell line was maintained in RPMI 1640medium containing 10% fetal bovine serum. Stable clones from Jurkat cells transfected with the control plasmid LK444 (JNF) or with plasmid LKCG containing human cytoplasmic gelsolin (JGF) were obtained as described (30). Brie fly, transfections were carried out using Lipofectin, and stable clones were selected in the presence of 1mg/ml G418 (Geneticin). The reagents were all purchased from Life Technologies, Inc. To induce apoptosis, cells were treated with 0.2g/ml anti-Fas antibody (clone CH-11; Medical and Biological Laboratories), 1M staurosporine (Sigma), 3M thapsigargin (Wako), or 45M protoporphyrin IX (Sigma). Nuclear condensation or fragmentation was analyzed by staining with Hoechst 33342and counting under an inverted fluorescence microscope (Olympus, IX-70). For the measurement of m of Jurkat cells, Rhodamine 123was added to the culture medium and maintained for 10min, followed by PBS wash and flow cytometric analysis (FACScalibur, Becton Dickinson). Cytochrome c release from mitochondria into the cytosol of Jurkat cells was evaluated by SDS-polyacrylamide gel electrophoresis and immunoblotting of the cytosolic fraction as described (31).

Assessment of Caspase Activation - Cells were harvested at determined time points, washed with PBS, and lysed. Equal amounts of protein (Bradford method) were boiled in SDS sample buffer (40mM Tris-HCl, pH 7.4,5% glycerol, 5% -mercaptoethanol, 2% SDS, 0.05% bromphenol blue) and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal anti-caspase-3 (Transduction), monoclonal anti-caspase-8 (Medical and Biological Laboratories), or polyclonal rabbit anti-caspase-9 (Medical and Biological Laboratories). For cell-free experiments, cell extracts (cytosolic fraction) of untreated JNF or JGF cells were obtained and incubated at 36ÅC with bovine cytochrome c protein (Sigma) and dATP and were analyzed for caspase activation as described previously (33).

Protein Purification - Human Bax was expressed as a His-tagged protein in Escherichia coli strain XL1-Blue using the Xpress System (Invitrogen) and purified on a nickel-nitrilotriacetate-agarose column (Qiagen) as described (34). Corresponding mock control protein was produced using His-tagged proteins from empty vectors (34). Human cytoplasmic gelsolin-pET-11a (Novagen) plasmid was produced as described (28) and used for gelsolin protein expression in E.coli strain BL21-DE3 (Novagen). Protein puri fi cation was performed as described previously (28), according to the method of Kurokawa et al. (36). Proteins were then dialyzed with a buffer containing 0.3M mannitol and 10mM HEPES/KOH, pH 7.4.The purity of proteins was shown to be >95% as assessed by SDS-polyacrylamide gel electrophoresis.

Isolation of Rat Liver Mitochondria, Measurement of m, and Cytochrome c Release- Mitochondria from fresh rat liver were isolated as described (34, 37). Briefly, livers of male Donryu rats suspended in ice-cold buffer A (0.3M mannitol, 0.1mM EDTA, 10mM HEPES/KOH, 0.1% fatty acid-free bovine serum albumin, pH 7.4) were homogenized with a glass-Teflon Potter homogenizer and centrifuged at 2,000Å~g for 10min at 4ÅC. The supernatant was further centrifuged at 4,500Å~g for 8min and then

10,000Å~g for 5min at 4ÅC in a new tube. The pellet was suspended in ice-cold buffer B (0.3M mannitol, 10mM HEPES/KOH, 0.1% fatty acid-free bovine serum albumin, pH 7.4) and centrifuged at 2,000Å~g for 10min at 4 ÅC. The supernatant was centrifuged at 10,000Å~g for 10min at 4ÅC in a new tube, and the resulting pellet (mitochondria) was suspended in ice-cold buffer B.For m measurement experiments, freshly isolated mitochondria (1mg of protein per ml) were incubated at 25ÅC in a buffer containing 0.3M mannitol, 10mM HEPES/KOH, pH 7.4,0.1% fatty acid-free bovine serum albumin, 0.5mM KH2PO4, 6mM succinate, and 1g/ml rotenone. Depending on the experiment, cyclosporin A (1nM), albumin (400nM), recombinant gelsolin (25to 600nM), calmodulin (400nM), PI, PIP, or PIP2 from bovine brain (5-30 M) were added, and m loss was induced by CaCl2 (25M), atractyloside (50M), or Bax (100g/ml). The doses used for induction of m loss are within those described elsewhere (37, 38-42). All chemicals were purchased from Sigma. Phosphoinositides were dissolved in distilled water, sonicated, and frozen in liquid nitrogen. Just prior to use, phosphoinositide suspensions were sonicated in a water bath sonicator for 30min at room temperature. m was assessed spectrophotometrically (Hitachi F-4500) by Rhodamine 123(Rh 123) uptake with excitation at 505nm and recording at 534nm after addition of 10M Rh 123.The solution with isolated mitochondria was centrifuged to pellet the mitochondria. The supernatants were mixed with SDS sample buffer and boiled, and aliquots of 20l were subjected to immunoblotting using anti-cytochrome c monoclonal antibody (Pharmingen). For analysis of the effect of PIP2 on interactions between gelsolin and intact mitochondria, isolated mitochondria (1mg/ml) were incubated with gelsolin (40g/ml) with or without PIP2 (20M), pelleted, washed, and lysed before immunoblotting using anti-human gelsolin monoclonal antibody.

Subcellular Fractionation - All steps below were carried out at 4ÅC. Jurkat cells (1Å~107 cells) were washed with PBS and suspended in isosmotic buffer (0.3M sucrose, 10mM Tris-HCl, 1mM EDTA, pH 7.5). After 5min of incubation, cells were Dounce homogenized using a type B (loose) pestle and centrifuged at 1,000Å~g for 10min to separate nuclei and unbroken cells. Then the supernatant was centrifuged at 8,000Å~g for 10min to pellet heavy membranes (mitochondrial fraction). The pellet was washed five times with isosmotic buffer to eliminate contamination by other subcellular fractions. The supernatant from the 8,000Å~g spin fraction was further centrifuged at 100,000Å~g to produce a supernatant corresponding to the cytosolic fraction (S100). Aliquots from each subcellular fraction in the same proportion to the initial number of cells harvested were loaded for SDS-polyacrylamide gel electrophoresis and immunoblotting. Gelsolin was detected using monoclonal anti-human gelsolin clone 2C4 (Sigma). For markers, monoclonal anti-FADD (Transduction Laboratories) and monoclonal anti-cytochrome c (Pharmingen) were used to indicate the cytosolic and mitochondrial fractions, respectively, and to assure the purity of the fractions.

Confocal Immunofluorescence Microscopy - Human dermal fibroblasts grown on coverslips in DulbeccoÅs modified EagleÅs medium supplemented with 10% fetal bovine serum and Jurkat cells immobilized on poly-L-lysine coated coverslips were incubated with 500nM chloromethyl-X-rosamine (Molecular Probes) to identify mitochondria for 30min according to the manufacturerÅs instructions. Cells were then fixed with 3.7% formaldehyde in PBS for 30min at 4ÅC and permeabilized with 0.2% Triton X-100 for 10min at room temperature. Nonspecific binding sites were blocked

by incubation with 20% normal goat serum in PBS for 1h at room temperature. Anti-gelsolin monoclonal antibody was added to the coverslips and incubated for 1h at room temperature. The coverslips were washed twice for 2min with PBS and incubated for 1h at room temperature with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma). The coverslips were then washed two times for 2min with PBS. Analysis was performed using a laser confocal microscope (MRC-1024, Bio-Rad).

RESULTS

Gelsolin Blocks m Loss and Release of Cytochrome c from Mitochondria of Jurkat Cells Treated with Apoptotic Agents

As previously reported (30), stable clones of Jurkat cells overexpressing gelsolin (JGF) were resistant to Fas-induced apoptosis, as assessed by nuclear condensation/fragmentation. In control experiments, in contrast, empty vector (control) transfected Jurkat cells (JNF) were not. We next analyzed apoptosis induced by several other agents including: staurosporine, a protein kinase inhibitor; thapsigargin, a selective inhibitor of endoplasmic reticular Ca2+-ATPase that causes an increase in the cytosolic Ca2+ concentration; and protoporphyrin IX, a direct inducer of m loss by interaction with peripheral benzodiazepine receptor present in the outer mitochondrial membrane. As shown in Fig. 1, JGF cells were resistant to apoptosis in response to all of these stimuli, which are known to induce loss of m (15, 20). Fas receptor stimulation provokes the recruitment of FADD and activation of the initiator caspase-8, which has the ability to activate directly the downstream caspases such as caspase-3 in vitro (43). However, it is believed that in Jurkat cells mitochondrial changes are necessary for the activation of downstream caspases because of reduced amounts of caspase-8 initially activated near the Fas receptor (44), implying the important role of these organelles as Åapoptotic amplifiersÅ (13). Further analysis of the m, as measured by cell loading of Rhodamine 123and flow cytometry, indicated that JGF, in comparison to control cells do not undergo signifi cant alteration in their m upon stimulation with either of the apoptosis inducing drugs tested (Fig. 2A). The effect of gelsolin implies a mechanism of direct inhibition of the m loss, as shown by its antagonistic effect on protoporphyrin IX, an agent that primarily targets mitochondria to cause m loss. Analysis of the cytosolic concentration of cytochrome c after exposure of JGF to apoptotic agents also indicated that cytochrome c release from mitochondria was blocked (Fig. 2B). These results suggest that gelsolin affects the mitochondrial changes that typically occur during apoptosis.

Lack of Activation of Caspases-3, -8,and -9in JGF Treated with Apoptotic Agents

Caspases-3, -8,and -9are known to be activated in response to apoptosis-related mitochondrial changes in Jurkat cells (33, 44, 45). Among them, the activation of the executioner caspase-3 was shown to be inhibited by gelsolin overexpression (30, 31). To explore the effect of gelsolin overexpression on other caspases, cells were treated with anti-Fas or staurosporine and harvested at various time points (Fig. 3). Consistent with the block of cytochrome c release observed in JGF, there was no activation of caspases-3, -8,and -9in JGF, in contrast to control JNF cells, after

apoptotic stimulation. To confirm that the block of cytochrome c release from mitochondria into the cytosol was the factor responsible for the lack of caspase activation, cytosolic extracts of unstimulated transfectants were incubated with cytochrome c in the presence of dATP (Fig. 4). Accordingly, caspases-3, -8,and -9were activated in both JNF and JGF extracts, indicating that gelsolin functions at or upstream of the mitochondrial release of cytochrome c into the cytosol.

Recombinant Gelsolin Protein Inhibits m Loss and Cytochrome c Release from Isolated Rat Mitochondria Stimulated with Apoptotic Agents

The inhibition of apoptosis induced by a broad range of agents seen in JGF suggests that gelsolin must affect a common point in the apoptotic pathway, which is consistent with a role at the mitochondrial step. Therefore, we sought to examine the direct effect of gelsolin on mitochondrial changes using an experimental model based on freshly purified mitochondria isolated from rat liver (34, 37). Accumulation of Ca2+ in the mitochondrial matrix is a requirement for induction of the mitochondrial permeability transition (20), and the loss of m can be provoked by Ca2+ treatment. To determine the effect of gelsolin on the m changes that occur after Ca2+ stimuli, we incubated freshly isolated mitochondria from rat liver with Ca2+. A progressive increase in the discharge of Rh 123fluorescence from the mitochondria was observed, indicating a loss of m (Fig. 5). As a positive control for the inhibition of m loss we used cyclosporin A, an immunosuppressive agent that is a well known inhibitor of PT (37, 46) (Fig. 5A). Bovine serum albumin was added to the isolated mitochondria as a negative control, and no effect on m was seen indicating that stabilization of the mitochondrial membrane was not mediated by increased protein concentration in a nonspecific manner (Fig. 5A). When recombinant gelsolin was added to the isolated mitochondria, a dose-dependent and saturable inhibition of m loss occurred (Fig. 5, B and C). The same protective effect of gelsolin was reproduced with other m loss inducers, including atractyloside, a PT pore-opening agent that binds to adenine nucleotide translocator (18, 46) (Fig. 5D), and Bax, a member of the Bcl-2 family with proapoptotic activity (13, 17, 18) (Fig. 5E). Gelsolin is an abundantly expressed protein, and its endogenous concentration in cells from a variety of tissues is estimated to be 1-4 g/mg protein (47, 50). As cells contain about 75mg/ml protein, the concentration of gelsolin that is needed for blocking m loss in our assay is within the range of gelsolin concentration in cells. These results demonstrate that gelsolin functions efficiently to stabilize mitochondrial membrane potential, inhibiting the m loss provoked by a wide range of agents to a degree similar to that seen with the anti-apoptotic Bcl-2 and Bcl-xL (37). We then examined the release of cytochrome c from isolated mitochondria treated with Ca2+, atractyloside, or Bax and found that mitochondria incubated with gelsolin did not show cytochrome c release (Fig. 5F), corroborating m experiments that show a membrane stabilizing property of gelsolin.

The Effect of Gelsolin Is Not Due to Ca2+ Sequestration

Gelsolin binds to Ca2+, and its activity on mitochondrial membrane potential could be explained as a simple consequence of Ca2+ sequestration that could prevent the entry and thus the accumulation of this ion in the mitochondria. To test this hypothesis, we incubated the isolated mitochondria with the same molar concentration of another known Ca2+-binding protein, calmodulin. The Ca2+-binding

property of gelsolin and calmodulin under the same conditions as that of the isolated mitochondria experiment was comparable, which was confirmed by measuring the free Ca2+ concentration before and after addition of the proteins with a Ca2+ electrode (before Ca2+ addition: mock=1.0Å~106 M, gelsolin=1.0Å~106 M, calmodulin=0.9Å~106 M; after Ca2+ addition: mock=5.2Å~105 M, gelsolin=4.8Å~105 M, calmodulin=4.2Å~105 M). In contrast to the results with gelsolin, calmodulin could inhibit neither the loss of m (Fig. 6A) nor cytochrome c release from mitochondria (Fig. 6B), indicating that Ca2+ sequestration per se cannot explain our results with gelsolin.

Effect of Polyphosphoinositides on Gelsolin Function

The polyphosphoinositides phosphatidylinositol 4-monophosphate (PIP) and PIP2 interact with gelsolin with high affinity and alter its structural conformation (48), inhibiting gelsolin-actin interactions that consequently inhibit the capping and severing properties of gelsolin (49). To study the effects of polyphosphoinositides on gelsolin membrane stabilizing function, gelsolin was first mixed with PIP2 and then incubated with isolated mitochondria for m analysis (Fig. 7, A and B). PIP2 itself had no effect on m. However, PIP2 significantly reduced the inhibition of m loss by gelsolin after Ca2+ treatment. Another phosphoinositide known to bind to gelsolin, phosphatidylinositol 4-monophosphate (PIP), had an effect similar to PIP2 (Fig. 7C), whereas phosphatidylinositol (PI), a phosphoinositide that does not bind to gelsolin, could not reproduce the same effect (Fig. 7D). The reversion of the inhibition of m loss by gelsolin observed with PIP2 was somewhat more efficient than with PIP treatment (Fig. 7E). The effect of PIP2 was not due to a block of gelsolin incorporation into mitochondria as pelleted and extensively washed mitochondria still retained recombinant gelsolin protein (Fig. 7F). Inhibition of the effect of gelsolin on m by PIP and PIP2 indicates that gelsolin conformation or the binding of gelsolin to actin is important for its effect on mitochondrial membranes.

Gelsolin Is Localized in Mitochondria

Gelsolin has been shown to be localized in the cytosol, but it has also been found to be associated with plasma and intracellular membranes, including endoplasmic reticulum, cortical vesicules, and mitochondria of macrophages and platelets (51, 52). To determine whether gelsolin was actually in mitochondria, JGF cells were subjected to subcellular fractionation by differential centrifugation, followed by immunoblotting. Gelsolin was mainly present in the cytosolic fraction (S100) but was also present in the extensively washed fraction to eliminate cytosolic contamination, heavy membrane fraction containing JGF cell mitochondria (Fig. 8A). In addition, confocal immunofluorescence microscopy of JGF and human dermal fibroblasts incubated with anti-gelsolin monoclonal antibody confirmed co-localization of gelsolin with mitochondria (Fig. 8B).

DISCUSSION

The caspase proteolytic cascade is a central component of the cellular machinery of the apoptotic process (5, 11). The early mitochondrial changes, i.e. the release of

apoptogenic factors, especially cytochrome c, function as a powerful trigger for the activation of the caspase cascade (13). Here we present evidence that the lack of activation of caspases seen in gelsolin-transfected cells is due to the block of cytochrome c release at the mitochondrial level. A similar mechanism of inhibition of apoptosis is seen with anti-apoptotic members of the Bcl-2 family (13, 17, 37). Diverse apoptotic stimuli induce signaling pathways that converge at the mitochondria, which serve as sensors and amplifiers of the apoptotic process, and recent discoveries point out the importance of the mitochondrial permeability transition pore complex in this process (15, 17, 18, 20). The opening of this megachannel has been linked to enhanced permeability and loss ofmitochondrial membrane potential. In addition, a model proposed for the release of mitochondrial apoptogenic factors, such as AIF or cytochrome c, is based on the opening of the PT pore and its consequences (17, 18). Our data show that gelsolin blocks apoptosis induced by anti-Fas, staurosporine, thapsigargin, and protoporphyrin IX. All of these apoptosis inducers are known to utilize mitochondria in their apoptotic signaling. Protoporphyrin IX has been reported to induce mitochondrial changes directly by binding to the mitochondrial peripheral benzodiazepine receptor, a putative component of the permeability transition megachannel, suggesting that the effect of gelsolin implies a mechanism of direct inhibition of the m loss. Thapsigargin causes a rise in the intracellular calcium concentration through an initial depletion of calcium stored in the endoplasmic reticulum by its selective inhibitory effect on Ca2+-ATPase. In this situation, the calcium overload on mitochondria induces the opening of the PT pore. In addition, we showed that gelsolin blocks the effect of Ca2+ overload, atractyloside, and Bax on isolated mitochondria. Atractyloside is a ligand for the adenine nucleotide translocator, another component of the PT pore complex that causes its opening. Bax also causes the opening of this megachannel, although an intrinsic channel activity or an independent mechanism has been proposed. Taken together, these findings indicate that gelsolin counteracts apoptotic signals that converge at the mitochondrial PT pore or at a mitochondrial step necessary for the release of cytochrome c.

The inhibition of apoptosis by gelsolin through its effects on mitochondria is possibly associated with cellular systems that necessarily depend on mitochondrial changes to initiate the apoptotic process. It has been demonstrated that apoptotic signals induced by Fas receptor stimulation can be transmitted through two pathways, depending on the cell type (44). In type I cells, caspase-8 activated at the Fas receptor-FADD complex can activate downstream caspases independent of mitochondria, whereas in type II cells, caspases are only fully activated after cytochrome c release. So it is reasonable to speculate that gelsolin could inhibit only apoptosis of type II cells but not type I cells, similar to what occurs with Bcl-2 and Bcl-xL (44).

Gelsolin is an ubiquitously expressed, founding member of the actin-severing/capping family of proteins (21). Although the full-length form of gelsolin inhibits apoptosis, gelsolin is also a substrate for caspase-3, which cleaves it between Asp352 and Gly353 sequences, generating an N-terminal fragment that contributes to morphological changes associated with apoptosis (32). A similar scenario of opposite functions promoting life and death in the same protein has been demonstrated with the anti-apoptotic proteins Bcl-2 and Bcl-xL, which when cleaved by caspase-3 release a C-terminal product that lacks the BH4 homology domain and potently induces cell death (53-55).

Physiologically, in resting cells, the cytosolic Ca+2 concentration is maintained at about 100nM, but after certain hormonal stimuli, it can rise to 500-1000 nM. However, besides the global increases in cytosolic Ca+2 concentration, recent reports indicate that localized increases in Ca+2 concentration, for example, in microdomains between clusters of inositol 1,4,5-trisphosphate receptors in the endoplasmic reticulum and the outer mitochondrial membrane, the local Ca+2 concentration can reach 10-20 M (56, 57). The opening of the PT pore is facilitated by Ca2+ signals evoked by addition of large Ca2+ pulses or inositol 1,4,5-trisphosphate-mediated cytosolic Ca2+ spikes (20, 56). Recently, it has been shown that localized increases in Ca2+ concentration in these mitochondria-endoplasmic reticulum junctions can transmit pro-apoptotic signals, causing m loss and cytochrome c release from mitochondria (56). The doses we used for the isolated mitochondrial experiments mimic the conditions found at the cellular level for transmission of pro-apoptotic signals by Ca2+. Lowering of the concentration of Ca2+ around mitochondria could contribute to the block of m loss and cytochrome c release, but as demonstrated above, the Ca2+ binding activity of gelsolin cannot explain these findings. This indicates a more specific action of gelsolin on mitochondria. Some Bcl-2 family members can interact with and regulate specific PT pore components; Bax has been shown to interact with adenine nucleotide translocator (18) or voltage-dependent anion channel (17, 34) to initiate permeability transition and cytochrome c release, and Bcl-xL interacts with voltage-dependent anion channel to close the pore (17). Because the effect of gelsolin was dose-dependent and saturable, it is probable that gelsolin targets specifically an effector on mitochondria. Furthermore, the fraction of intracellular gelsolin that is associated with mitochondrial membranes can interact with and regulate voltage-dependent anion channel.2

It has been reported that gelsolin modulates ion channel function in vivo (35), as demonstrated by its regulatory function on the voltage-dependent Ca2+ channel and NMDA receptor-coupled activity (35). The findings presented here indicate that gelsolin also regulates the function of another channel, the mitochondrial PT pore, with important repercussions in the inhibition of apoptosis. ACKNOWLEDGEMENTS

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ABBREVIATIONS

The abbreviations used are: PT, permeability transition; JGF, Jurkat cells overexpressing gelsolin; JNF, control Jurkat cells; PBS, phosphate-buffered saline; Rh 123, rhodamine 123; PIP2, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PIP, phospha- tidylinositol 4-monophosphate; FADD, Fas-associated protein with death domain. REFERENCESTOP

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Figure legends:

Fig. 1. Inhibition of apoptosis in Jurkat cells overexpressing gelsolin stimulated with anti-Fas antibody, staurosporine, thapsigargin, and protoporphyrin IX. After apoptotic stimuli, empty vector (open circle) or gelsolin overexpressing (filled circle) Jurkat transfectants were assessed for apoptotic nuclear changes by Hoechst 33342staining.

Fig. 2. m loss and cytochrome c release from mitochondria are blocked in Jurkat cells overexpressing gelsolin and stimulated with anti-Fas antibody, staurosporine, thapsigargin, and protoporphyrin IX. A, result of flow cytometry using Rh 123showing m of control (JNF) or gelsolin (JGF) Jurkat transfectants at 2h after anti-Fas antibody or staurosporine treatment and at 18h after thapsigargin or protoporphyrin IX treatment. The numbers in the figure indicate the percentages of cells with decreased m. B, cytochrome c (Cyt. c) release from mitochondria into the cytosol at 20h after treatment.

Fig. 3. Activation of caspase-3, -8,and -9are blocked in Jurkat cells overexpressing gelsolin. Control (JNF) or gelsolin (JGF) Jurkat transfectants were treated with anti-Fas (A) antibody or staurosporine (B) and analyzed for the activation of caspase-3, -8,and -9by immunoblotting. Pro., precursor procaspase; Cleav. pr., cleavage products.

Fig. 4. Addition of cytochrome c activates caspases in both control (JNF) or gelsolin (JGF) Jurkat transfectants. JNF and JGF untreated cytosolic extracts were incubated at 36ÅC with cytochrome c and dATP and analyzed for caspase-3, -8,and -9activation. Pro., proform; Cleav. pr., cleavage products.

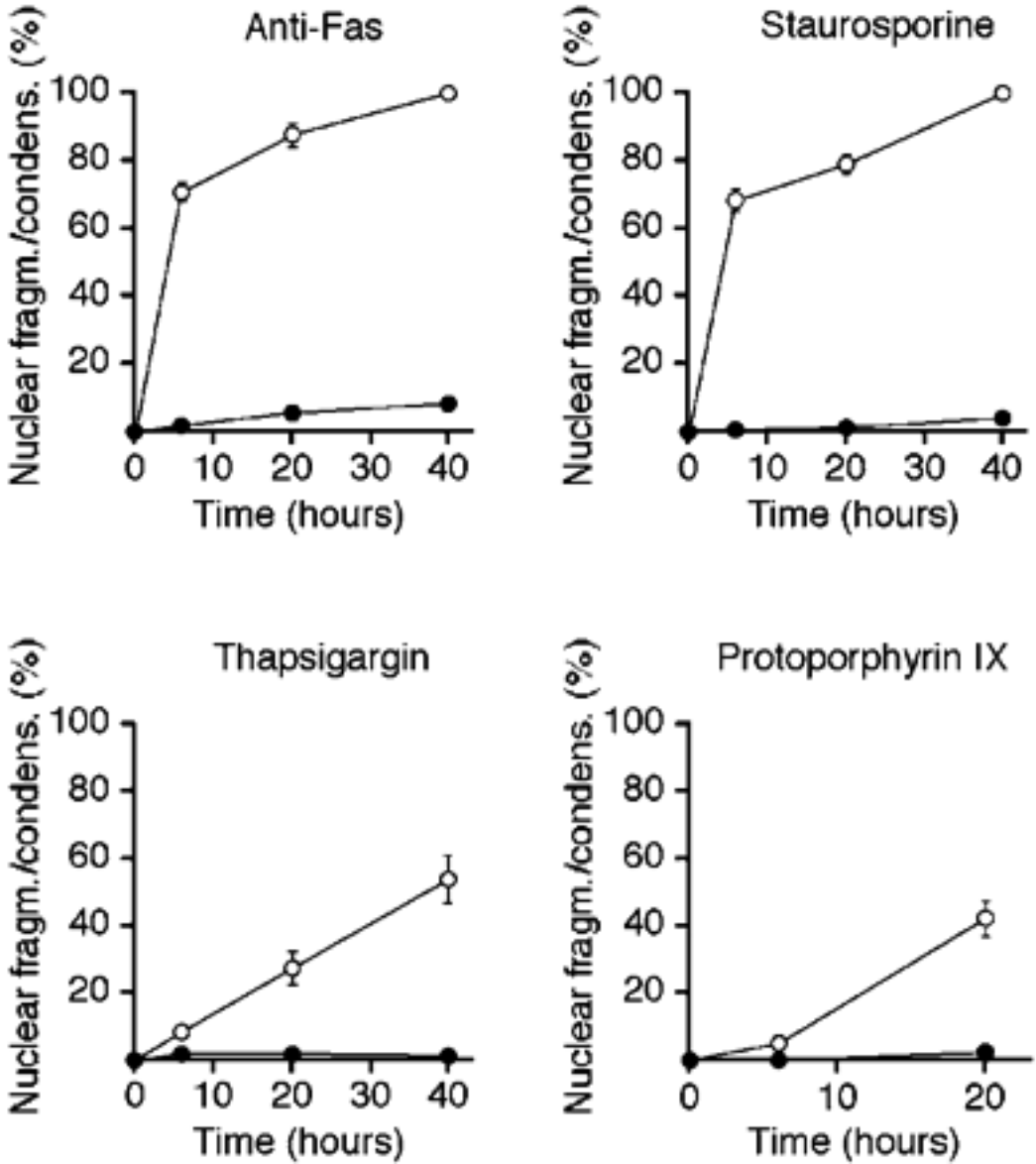
Fig. 5. Effect of gelsolin on isolated rat liver mitochondria. Mitochondria (1mg/ml) were incubated with 1nM cyclosporin A or 400nM albumin (A), 400nM gelsolin or mock control protein followed by 25M CaCl2 induction of m loss (B). m was assessed

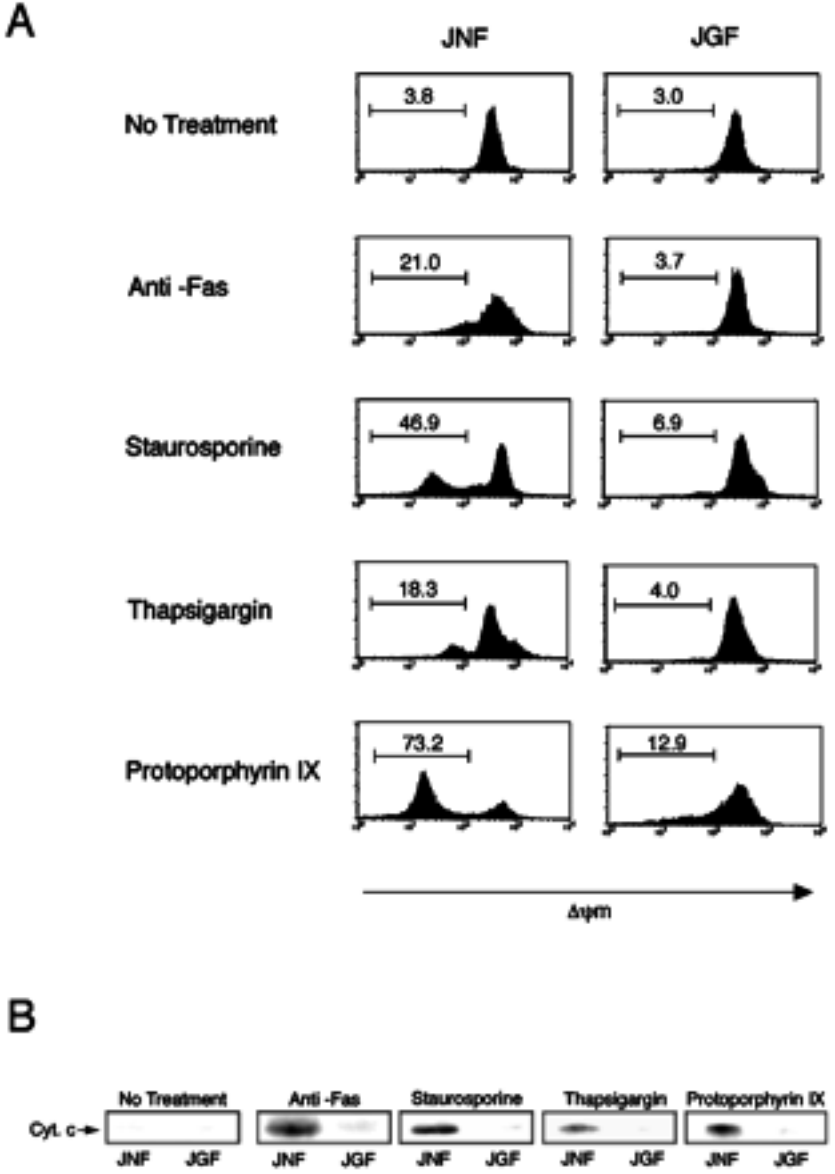
by measuring the m-dependent uptake of Rh 123(F=arbitrary units) as described under ÅExperimental Procedures.Å C, dose-dependent gelsolin inhibition of CaCl2 (25M) induced m loss as assessed by Rh 123uptake at 12min. m loss was also induced with 50M atractyloside (D) and 100g/ml Bax (E) in the presence of 400nM gelsolin or mock. F, cytochrome c (Cyt. c) release from mitochondria treated as in B, D, and E.

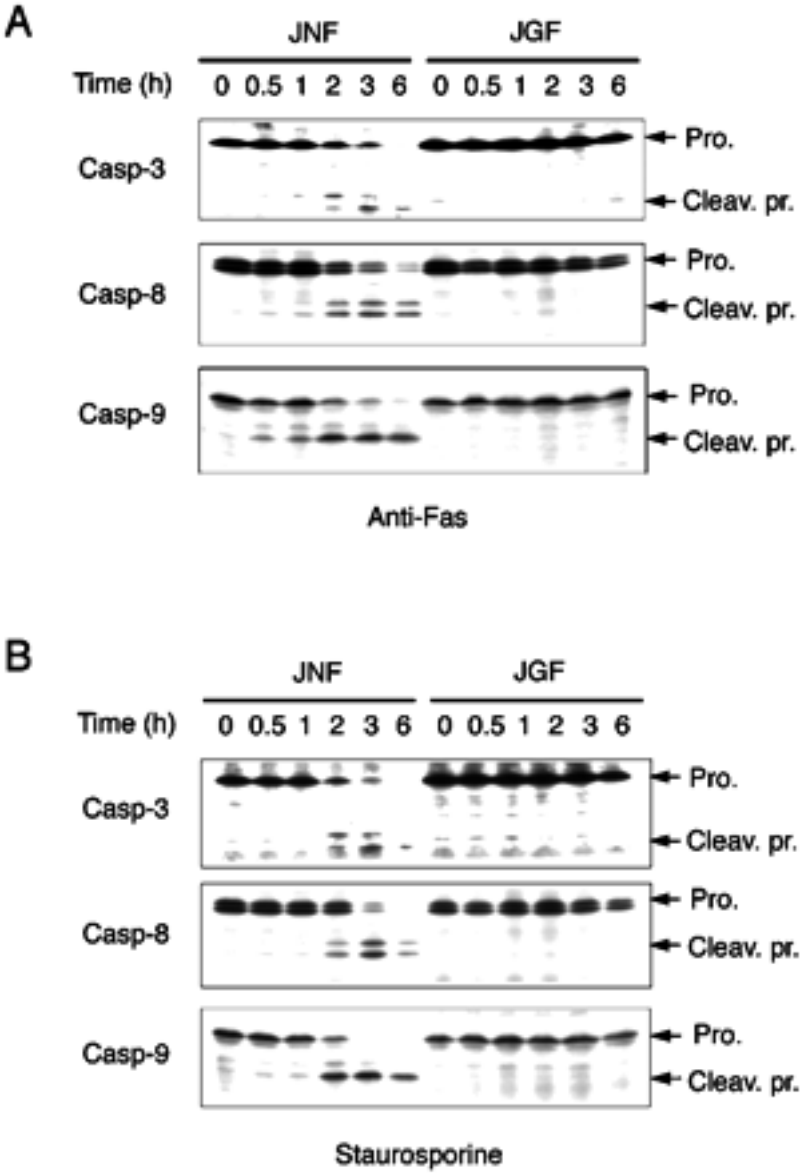
Fig. 6. Effect of calmodulin on isolated rat liver mitochondria. Calmodulin (400nM) was added to isolated mitochondria and, after stimulation with 25M CaCl2, m loss (A) and cytochrome c (cyt. c) release (B) were analyzed as described under ÅExperimental Procedures.Å

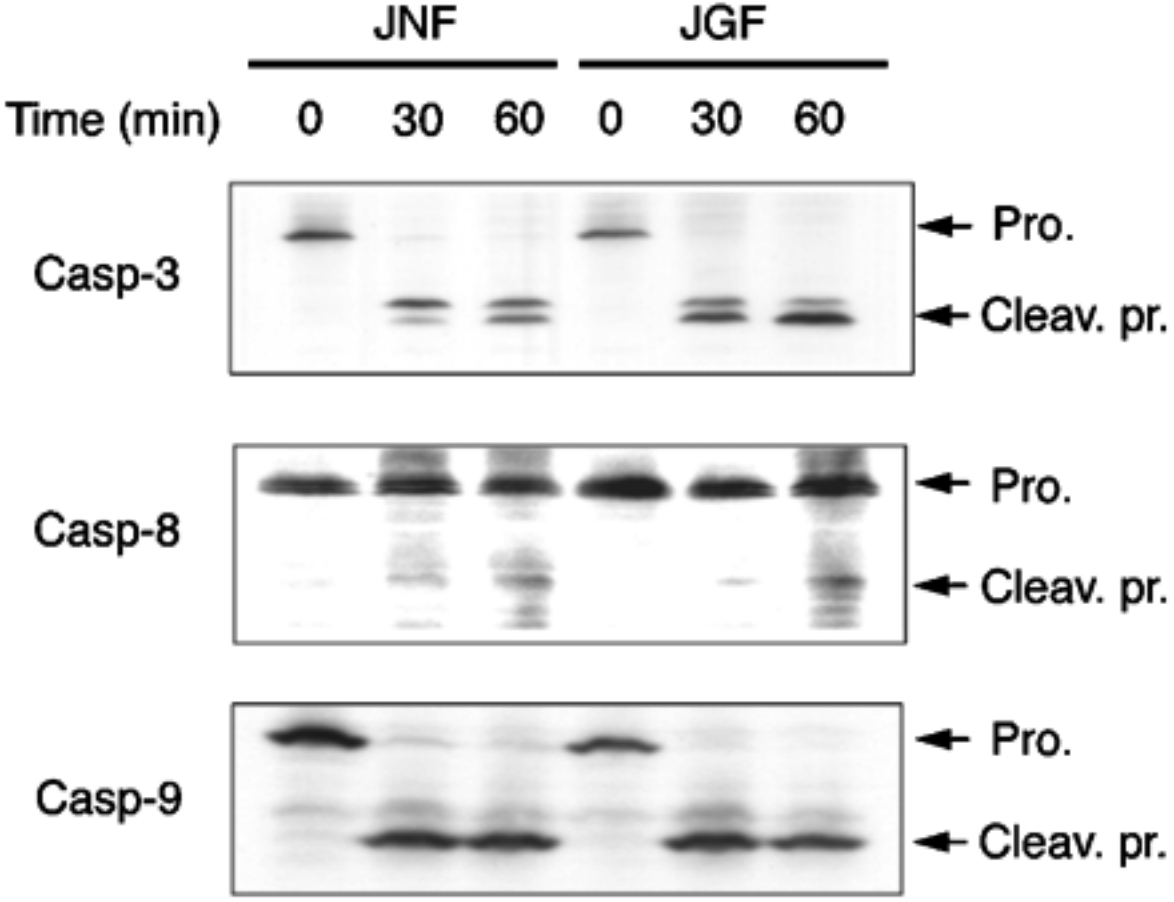
Fig. 7. Effect of phosphoinositides on the functionof gelsolin in mitochondria. A, PIP2 (15M) and gelsolin (400nM) alone or in combination were incubated with isolated rat liver mitochondria that were assessed for m loss after stimulation with CaCl2 (25M) as described under ÅExperimental Procedures.Å B, dose-dependent effect of PIP2 on gelsolin (400nM) inhibition of m loss induced by CaCl2 (25M) assessed at 10min. C and D, effects of 15M PIP (C) and 15M PI (D) treated as in A.E, the effect of equimolar concentration (15M) of PIP2, PIP, and PI on gelsolin (400nM) inhibition of m loss is compared. F, effect of PIP2 on interaction between gelsolin and intact mitochondria. Isolated mitochondria (1mg/ml) were incubated with gelsolin (400nM) with or without PIP2 (20M), pelleted, washed, and lysed, followed by immunoblotting using anti-human gelsolin monoclonal antibody, clone 2C4, that does not recognize rat gelsolin.

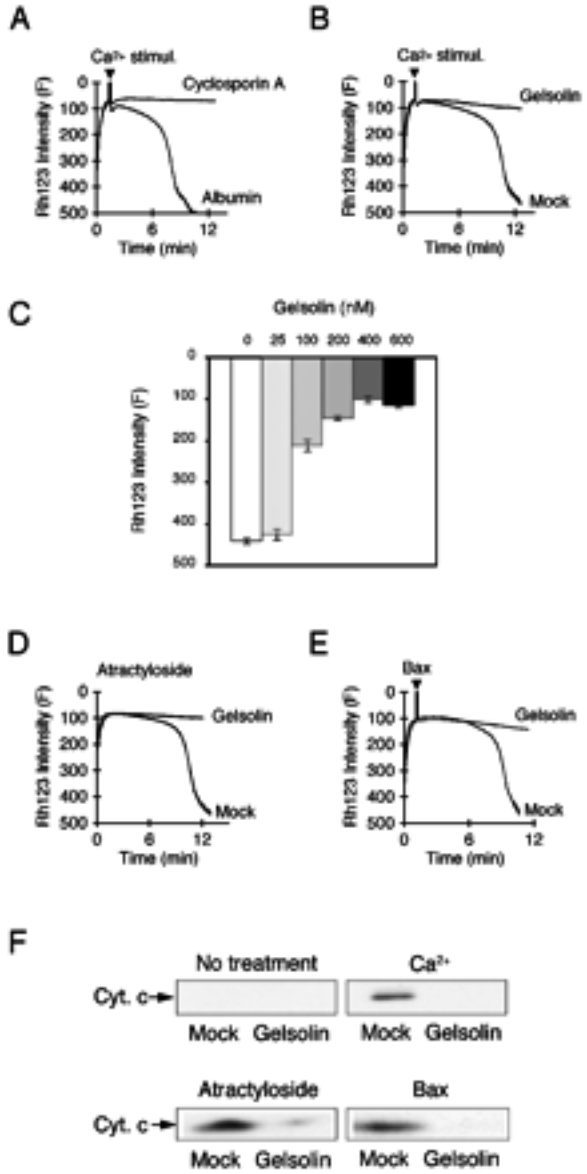
Fig. 8. Subcellular fractionation and confocal immunofluorescence microscopy showing localization of gelsolin. A, Jurkat cells overexpressing gelsolin were subjected to subcellular fractionation by differential centrifugation, followed by immunoblotting. Heavy membrane fraction is the mitochondria enriched fraction, whereas S100 fraction denotes the cytosolic fraction.Cytochrome c was used for mitochondrial marker and FADD for cytosolic marker, ensuring the purity of the fractions. B, laser scanning confocal microscopy. Normal human dermal fibroblasts (a-c) and Jurkat cells overexpressing gelsolin (d-f) were stained with chloromethyl-X-rosamine to indicate the mitochondria (a and d) and incubated withanti-gelsolin monoclonal antibody followed by FITC conjugated anti-mouse secondary antibody (b and e). Overlay of the images are shown in c and f. Bar, 50m


Anti-Fas
Staurosporine
Thapsigargin
Protoporphyrin IX
Nuclear fragm./condens. (%)
Time (hours)
0
10
20
30
40
60
80
100

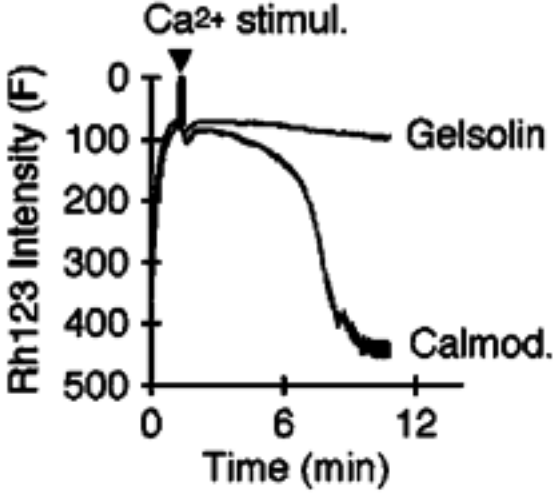

A
JNF
JGF
No Treatment
3.8
3.0
Anti -Fas
21.0
3.7
Staurosporine
46.9
6.9
Thapsigargin
18.3
4.0
Protoporphyrin IX
73.2
12.9
Δψm
B
No Treatment
Anti -Fas
Staurosporine
Thapsigargin
Protoporphyrin IX
Cyt. c
JNF
JGF


A
JNF
JGF
Time (h)
0 0.5 1 2 3 6 0 0.5 1 2 3 6
Casp-3
Pro.
Cleav. pr.
Casp-8
Pro.
Cleav. pr.
Casp-9
Pro.
Cleav. pr.
Anti-Fas
B
JNF
JGF
Time (h)
0 0.5 1 2 3 6 0 0.5 1 2 3 6
Casp-3
Pro.
Cleav. pr.
Casp-8
Pro.
Cleav. pr.
Casp-9
Pro.
Cleav. pr.
Staurosporine

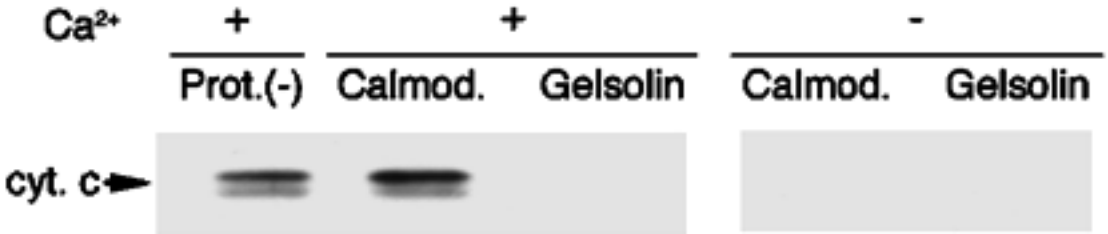

JNF
JGF
Time (min)
0
30
60
0
30
60
Casp-3
Pro.
Cleav. pr.
Casp-8
Pro.
Cleav. pr.
Casp-9
Pro.
Cleav. pr.

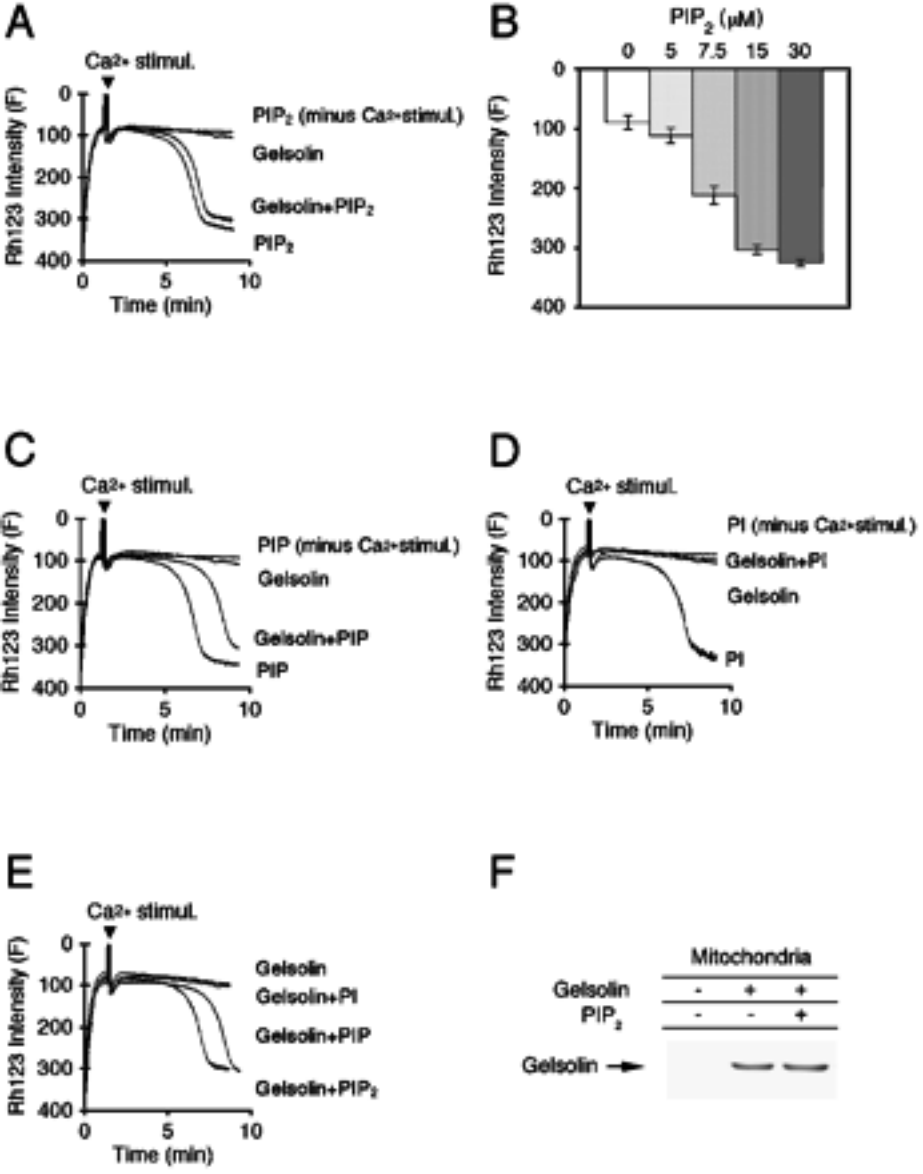

A
Ca2+ stimul.
Cyclosporin A
Albumin
Rh123 Intensity (F)
Time (min)
B
Ca2+ stimul.
Gelsolin
Mock
C
Gelsolin (nM)
0 25 100 200 400 600
D
Atractyloside
Gelsolin
Mock
E
Bax
Gelsolin
Mock
F
No treatment
Ca2+
Cyt. c
Mock Gelsolin Mock Gelsolin
Atractyloside
Bax
Cyt. c
Mock Gelsolin Mock Gelsolin

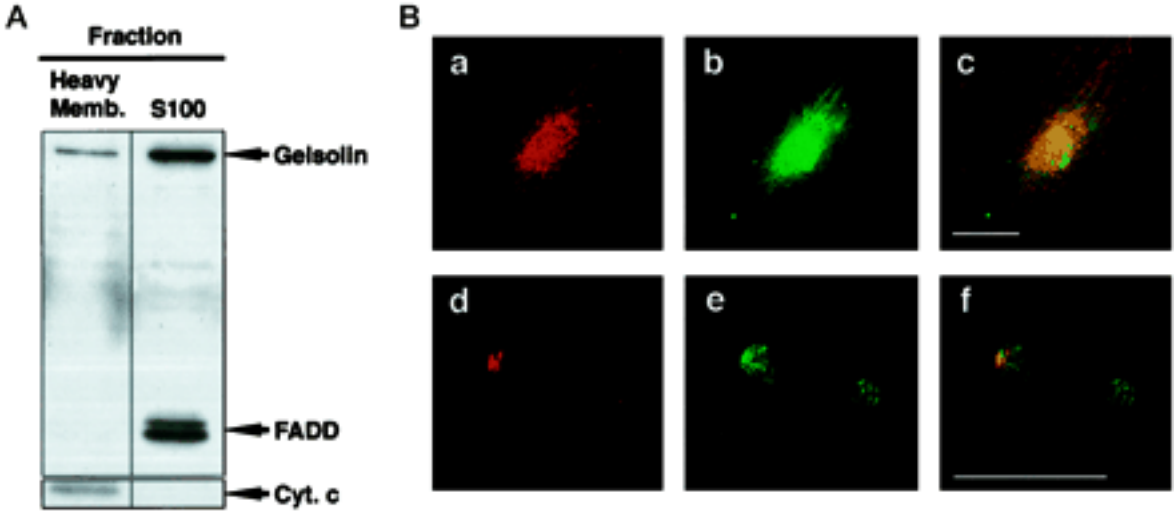
A


Ca2+ stimul.
Rh123 Intensity (F)
0
100
200
300
400
500
Gelsolin
Calmod.
0
6
12
Time (min)

B


Ca2+
+
+
-
Prot.(-)
Calmod.
Gelsolin
Calmod.
Gelsolin
cyt. c


A
Ca2+ stimul.
Rh123 Intensity (F)
Time (min)
PIP2 (minus Ca2+stimul.)
Gelsolin
Gelsolin+PIP2
PIP2
B
PIP2 (μM)
0 5 7.5 15 30
Rh123 Intensity (F)
C
Ca2+ stimul.
Rh123 Intensity (F)
Time (min)
PIP (minus Ca2+stimul.)
Gelsolin
Gelsolin+PIP
PIP
D
Ca2+ stimul.
Rh123 Intensity (F)
Time (min)
PI (minus Ca2+stimul.)
Gelsolin+PI
Gelsolin
PI
E
Ca2+ stimul.
Rh123 Intensity (F)
Time (min)
Gelsolin
Gelsolin+PI
Gelsolin+PIP
Gelsolin+PIP2
F
Mitochondria
Gelsolin - + +
PIP2 - - +
Gelsolin


A
Fraction
Heavy Memb.
S100
Gelsolin
FADD
Cyt. c
B
a
b
c
d
e
f