Caspase activation during phorbol ester-induced apoptosis requires ROCK-dependent myosin-mediated contraction

Jin-Mei Lai, Chia-Ling Hsieh and Zee-Fen Chang*
Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1 section 1 Jen-Ai Road, Taipei 100, Taiwan, Republic of China
*Author for correspondence (e-mail: zfchang@ha.mc.ntu.edu.tw)

Accepted 7 May 2003
Journal of Cell Science 116, 3491-3501 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00660

Summary
Treatment of cells with phorbol ester, phorbol-12-myristate-13-acetate (PMA), triggers differentiation or apoptosis, depending on the cell type. In this study, we used an erythroblastic cell line, TF-1, to investigate the molecular mechanism that determines the cell fate in response to PMA exposure. Upon PMA treatment in the presence of serum or lysophosphatidic acid (LPA), TF-1 cells exhibited contraction followed by apoptosis. By contrast, under serum-free conditions, cells became adherent and survived after PMA treatment. Here, we show that the pathway of Rho kinase (ROCK)/myosin light chain (MLC) phosphorylation/myosin-mediated contraction was activated in PMA-induced apoptotic cells in serum-containing medium, but not in the adherent and survived cells. Pretreatment of cells with a specific ROCK inhibitor, Y27632, not only abrogated MLC phosphorylation and membrane contraction, but also prevented PMA-induced activation of caspase-3 and subsequent cell death, indicating that ROCK-dependent myosin-mediated contraction elicits an upstream signal required for caspase-3 activation in PMA-induced apoptosis. Interestingly, we further found that caspases-8 and -10 are the initiator caspases in PMA-induced apoptosis and a ROCK-dependent enhancement of specific complex formation between the Fas-associated death domain (FADD) and pro-caspase-10 in pro-apoptotic cells. In summary, these results revealed that, following PMA treatment, the upregulation of the RhoA/ROCK pathway contributes to a cellular context that switches-on myosin-mediated contraction, which provides a mechanism for triggering apoptotic induction mediated by caspase-8 and -10.

Key words: Rho kinase, Myosin light chain, Phosphorylation, Apoptosis, Caspase

Introduction
Coordination and balance between cell survival and apoptosis is crucial for the normal development and homeostasis of multicellular organisms throughout adult life. In hematopoiesis, some cells commit to differentiation and others undergo renewal or apoptosis to maintain homeostasis. It is still a challenge to understand what mechanisms are used to produce a cell that behaves in one way rather another. When exposed to external stimulation, such as phorbol-12-myristate-13-acetate (PMA), which is an activator of protein kinase C (PKC), hematopoietic cells are induced to differentiate or undergo apoptosis depending on the cell type (Collins, 1987; Day et al., 1994; Garzotto et al., 1998; Gunji et al., 1992; Kizaki et al., 1989; Lotem et al., 1991). We have previously shown that lysophosphatidic acid (LPA) or serum promotes PMA-induced apoptosis in TF-1 cells by the RhoA-dependent pathway, whereas following PMA stimulation in serum-free medium, TF-1 cells survive and attach to the culture flask (Lai et al., 2001). We have also found that the RhoA signaling pathway acts as a molecular switcher that affects extracellular signal-regulated kinase (ERK)-regulated gene expression and determines the cell fate in response to PMA exposure (Lai et al., 2002). In this study, we used this system to investigate further the detailed molecular mechanism by which the RhoA signal pathway is utilized to trigger the apoptotic process during PMA treatment.

Similar to other members of the Rho family of small GTPases, RhoA cycles between a GDP-bound inactive form and a GTP-bound active form that is necessary for interaction with and activation of their effectors. When cells are stimulated with LPA or serum, RhoA is converted to the GTP-bound form (Goetzl and An, 1998), which binds to specific effectors and exerts its biological functions, including cell adhesion, motility, enhancement of the contractile response, cytokinesis and transcriptional regulation (Bishop and Hall, 2000). The best-characterized function of RhoA is the regulation of the architecture of the actin cytoskeleton. Activated RhoA mediates the formation of stress fibers, which are elongated actin bundles that traverse the cells and promote cell attachment to the extracellular matrix through focal adhesions. Unlike that in fibroblasts, activated RhoA in hematopoietic cells prevents cell adhesion induced by PMA (Aepfelbacher, 1995; Kaibuchi et al., 1999; Lai et al., 2001). For TF-1 cells, we have established that the PMA treatment induces apoptosis
in cells that do not display adhesion, which involves upregulation of RhoA activity (Lai et al., 2001). Two forms of serine/threonine kinase (Rho kinases, ROCK1 and ROCKII) have been identified as effectors of RhoA (Amano et al., 2000; Matsui et al., 1996). RhoA in GTP-bound form interacts with ROCK proteins and activates their kinase activity by disrupting their autoinhibition. Activation of ROCK regulates the phosphorylation of myosin light chain (MLC) by directly phosphorylating MLC at Thr18 and Ser19, and by the inactivation of myosin phosphatase (Amano et al., 1996; Kimura et al., 1996). Phosphorylation of MLC activates myosin ATPase activity, which couples with actin-myosin filaments to the plasma membrane, thus increasing the actin-myosin force generation and cell contractility. In addition, recent studies have linked this contraction event to membrane blebbing and apoptotic body formation observed in apoptotic cells. ROCK1, a substrate of caspase-3, is activated by cleavage in its C-terminal region, which is involved in its negative regulation (Coleman et al., 2001; Sebbagh et al., 2001). The cleaved form of ROCK1 activates MLC phosphorylation, thus resulting in formation of membrane blebbing in apoptotic cells. These studies suggest that caspase-3 activation mediates Rho-independent ROCK activation, which generates contraction force as the characteristic of apoptotic phenotype. Distinctly, in this study, we demonstrated that ROCK-mediated MLC phosphorylation acts as an upstream event required for membrane contraction and the subsequent caspase-3 activation during PMA stimulation.

Caspases comprise a family of different cysteine proteases that are synthesized as inactive zymogens and are activated by proteolysis. It is well established that different initiator pathways can activate caspase-3 in response to various apoptotic stimuli (Thornberry and Lazebnik, 1998). In general, a pro-apoptotic signal generated from a death receptor or mitochondria can activate an initiator or upstream caspase, which usually possesses a long NH2-terminal prodomain such as found in caspases-8, -9 and -10 (Muzio et al., 1996; Strasser et al., 2000; Yang et al., 1998). In turn, these initiator caspases can activate the effector caspases, such as caspases-3 and -6, which result in apoptotic execution. One of the best-defined apoptotic pathways is mediated by death receptors such as CD95 or tumor necrosis factor receptors (TNFRs). Upon ligand binding, the intracellular death domain of the death receptor recruits Fas-associated death domain (FADD) through protein-protein interaction. FADD links the receptor to the apoptotic caspase, procaspase-8 or -10, through homotypic interactions of death effector domains (DED), to form a death-inducing signaling complex (DISC); this, in turn, leads to oligomerization and activation of these two zymogens by self cleavage and the subsequent apoptotic cascade (Ashkenazi and Dixit, 1998; Kischkel et al., 2001; Vincenz and Dixit, 1997; Wang et al., 2001).

In this study, we also provided the first evidence that PMA-induced activation of caspase-8 and -10 in TF-1 cells is controlled by ROCK-mediated activation of myosin motor activities, by which effector caspase-3 is activated to trigger this apoptotic pathway. In particular, we showed that DISC formation is enhanced in PMA-induced apoptotic cells. On the basis of these data, we propose that activation of the RhoA/ROCK/MLC phosphorylation pathway in cooperation with PMA signaling in cells provides a cellular context that generates an initial membrane contraction, which in turn leads to activation of caspase-8 and -10 through a mechanism involving membrane receptor-mediated signaling.

Materials and Methods

Chemicals

1-oleoyl-2-stearoyl-glycerol-3-phosphate (LPA) was from Fluka (Switzerland), and was dissolved in phosphate-buffered saline (PBS) containing 10 mg/ml fatty acid-free albumin. PMA was from Sigma Chemicals (St Louis, MO), and was dissolved in dimethyl sulfoxide (DMSO). Y-27632 [(R)-(+)−trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride, monohydrate], ML-7, ML-9, latrunculin B and caspase inhibitors, Benzylpyoxy-carbonyl-DEVD-fluorometyl-ketone (z-DEVD-fmk), z-IEHD-fmk, z-LEHD-fmk and Boc-D-fmk, were from CalbioChem-Novabiochem (San Diego, CA). z-AEVD-fmk was from AnaSpec (San Jose, CA). 2,3-butanedione monoxime (BDM) was from Sigma Chemicals and was dissolved in DMSO. Rabbit polyclonal anti-phospho-p44/p42 MAP kinase (ERK1/2) was from Cell Signaling Technology (Beverly, MA). Monoclonal anti-MLC antibody was from Sigma. Anti-phosphoMLC antibody (kindly supplied by J. M. Staddon, Eisai London Research Laboratories, London, UK) has been described previously (Ratcliffe et al., 1999). Monoclonal ROCK1 antibody was from BD Bioscience. The antibodies for detecting pro-caspase and active caspase were from: MBL (caspase-10), Upstate (Lake Placid, NY; caspase-8) and Imgenex (San Diego, CA; caspase-3).

Cell culture

TF-1 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, 100 μl streptomycin and 1 ng/ml of granulocyte-macrophage colony stimulation factor (GM-CSF). Human GM-CSF was purchased from R & D Systems (Minneapolis, MN).

Constructs

RhoAV14 in the pcDNA3 vector was constructed as described previously (Lai et al., 2001). The cDNA of ROCK(CAT) in pEF-BOS-myc vector was from K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan) and was further subcloned to pcDNA3 vector. The pEGFP expression plasmid was purchased from Clontech Laboratories (Palo Alto, CA). The protease-inactive mutant of procaspase-10c, C401S was a gift from M. J. Lenardo (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, USA). The fusion construct of procaspase-10c/S-FLAG was generated by inserting procaspase-10c, C401S into the pRK5 expression vector to have flag-tag in-frame fused to the C-terminus of procaspase-10c, C401S.

Caspases-3 assay

Caspases-3 activity was assayed in 100 μl of reaction mixtures with Acetyl-DEVD-pNA (CalbioChem-Novabiochem). Brieﬂy, cells were lysed in the buffer [20 mM Hepes, pH 7.8, 100 mM NaCl, 1 mM EDTA, 10 mM EGTA, 1 mM DTT, 0.1% Chaps, 10% sucrose, 1 mM phenyl methylsulfonic fluoride, 1 μg/ml each of leupeptin and aprotinin], after which equal amounts of lysates containing 100 μg protein were incubated with the substrate peptide (0.4 mM) at 37°C for 2 hours, followed by reading the absorbance at 405 nm with a spectrophotometer.

SDS-PAGE and immunoblotting

Samples containing equal amounts of proteins (50 μg) were separated
by 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore). The antibodies used and their dilutions were as follows: rabbit polyclonal anti-ROCK1 (Santa Cruz) at 1:1000, anti-MLC (Sigma) at 1:5000, anti-phosphoMLC at 1:250, anti-caspases-3, -8, and -10 at 1:1000 dilution, anti-FADD (CalbioChem-Novabiochem) at 1:1000. Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG or anti-mouse IgG antibody (Santa Cruz) was used for the detection of the primary antibodies. The ECL detection for HRP reaction was performed according to the vendor’s instruction.

Detection of apoptosis by DNA fragmentation assay
2x10^6 cells were collected following treatment and the cell pellets were then lysed in 100 µl of lysis buffer [50 mM Tris- HCl, pH 7.4, 10 mM EDTA, 1% SDS, 0.5 mg/ml protease K] and incubated at 50°C for 3 hours. DNase-free RNase A (final 0.5 mg/ml) was added, and incubated for another 3 hours. After phenol/chloroform extraction, samples were mixed with loading buffer [50% glycerol, 0.25% bromophenol blue] and were resolved on a 1.8% agarose gel.

Transient transfection
5x10^6 cells were washed twice with STBS buffer [25 mM Tris-HCl, pH 7.4, 5 mM KCl, 0.7 mM CaCl2, 137 mM NaCl, 0.6 mM Na2HPO4, 0.5 mM MgCl2]. The cell pellet was resuspended in 250 µl of STBS containing 600 µg DEAE-dextran and 6 µg of plasmid DNA consisting of 4.5 µg of expression plasmid plus 1.5 µg of pEGFP. After incubation for 20 minutes at 37°C, 5 ml of STBS buffer was added to the transfection mixture. Cells were then centrifuged and resuspended in 10 ml of RPMI-1640 containing 10% heat-inactivated FBS and GM-CSF, and incubated for 48 hours at 37°C prior to the treatment.

Fluorescence microscopy
After transfection for 48 hours, cells were pelleted and resuspended in fresh RPMI-1640 medium. Following treatment without or with PMA for 8 hours, cells that remained in suspension were collected by centrifugation at 800 g for 5 minutes. Cells expressing GFP were counted using an Olympus AX-70 fluorescence microscope.

DISC analysis by immunoprecipitation
D2 or TF-1 cells were transfected with the expression plasmid procaspase-10 C/S-flag as described above. After transfection for 48 hours, 1-3x10^7 cells with different treatment were washed with ice-cold PBS, and collected for the subsequent lysis with buffer [30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail (Sigma)] for 30 minutes at 4°C. The post-nuclear supernatants were obtained by centrifugation at 15,000 g for 20 minutes at 4°C, and then were rotated at 4°C overnight in the presence of 25 µl of anti-flag M2 beads (Sigma) to immunoprecipitate procaspase-10 C/S-flag-containing complex. After six washes with lysis buffer, the immunocomplexes were analyzed by SDS-PAGE followed by immunoblotting analysis using antibody against FADD (CalbioChem-Novabiochem).

Results
PMA-induced membrane contraction and MLC phosphorylation precede caspase-3 activation in TF-1 cells
When exposed to PMA in serum-containing medium for 12 hours, TF-1 cells became shrunk and non-viable. We then measured caspase-3 activity by monitoring proteolysis of the corresponding colorimetric substrates. Caspase-3 activity was significantly elevated at 6 hours and peaked at 10 hours followed by cell death at 12 hours (Fig. 1A). Pretreatment of cells with cycloheximide did not affect the extent of PMA-induced cell death (Fig. 1B). By contrast, pretreatment of cells with a kinase inhibitor, staurosporine, prevented PMA-induced apoptosis. These results suggest that a signal pathway involving a post-translational control plays a crucial role in this apoptotic process, which does not require newly synthesized protein.

Membrane contraction was observed for TF-1 cells as early as 1 hour after PMA treatment (Fig. 1C). It has been extensively studied that MLC phosphorylation plays a crucial role in actomyosin formation and contributes to membrane contractility. Therefore, we used antibody specific for phosphorylated MLC (Ratcliffe et al., 1999) to examine the phosphorylation status of MLC after PMA treatment. The level of MLC phosphorylation became detectable after 3 hours of PMA treatment and was further increased, accompanied with the subsequent cleavage of procaspase-3 at 6 hours (Fig. 1D). It has been shown that, by removing its C-terminal inhibitory domain, caspase-3-mediated cleavage of ROCKI activates its intrinsic kinase activity, resulting in myosin-driven membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001). However, in our case, a short form of ROCKI, corresponding to a cleaved active form, was not detected until 9 hours in PMA-induced apoptotic cells, where procaspase-3 was already extensively cleaved. Consistently, MLC became further heavily phosphorylated concomitantly with ROCKI cleavage. Probably, the PMA treatment induces initial MLC phosphorylation and membrane blebbing prior to the onset of caspase-3 activation.

Correlation between effects of serum or LPA on ROCK-mediated MLC phosphorylation and cell death during PMA treatment
When TF-1 cells were incubated in serum-free medium, cells were attached to the plate and remained viable following PMA treatment. By contrast, the presence of serum or LPA promotes PMA-induced apoptosis, which can be prevented with pretreatment of cells with a specific inhibitor of ROCK, Y27632 (Narumiya et al., 2000) (Fig. 2A). We next tested whether the ROCK/MLC phosphorylation pathway is absent in TF-1 cells incubated in serum-free medium. As shown in Fig. 2B, MLC phosphorylation was indeed undetectable in these survived and adherent cells, and could be seen in the cells incubated in the medium containing either LPA or serum, which underwent subsequent cell death. Treatment of cells with Y27632 abrogated PMA-induced apoptosis and MLC phosphorylation for those cells incubated in LPA or serum-containing medium (Fig. 2B). Whereas the extent of PMA-induced phosphorylation of ERK was similar in the cells incubated in the medium regardless of the presence or absence of serum or LPA (Fig. 2B), it is likely that MLC phosphorylation is specifically associated with upregulation of the LPA/RhoA pathway in a ROCK-dependent manner and correlates well with PMA-induced apoptosis.

MLC phosphorylation and membrane blebbing occur upstream of caspase activation during PMA induction
Since PMA-induced apoptosis is associated with early...
contraction, we then addressed the questions of whether membrane contraction is a direct result of caspase activation. We found that cells pretreated with a general caspase inhibitor (Boc-D-FMK) still displayed the contraction phenotype following PMA treatment, whereas cell death was significantly inhibited. This result implied not only that caspase activity is responsible for the major execution of PMA-induced cell death, but also that membrane contraction is not a downstream event of caspase activation (Fig. 3A). The involvement of ROCK in cell contraction and cell death was further assessed by pretreatment of cells with Y27632. Following PMA treatment, these Y27632-pretreated cells remained rounded or spread without the appearance of membrane contraction and were viable. Pretreatment of cells with latrunculin B, an actin polymerization inhibitor (Spector et al., 1989), also abolished contraction and resulted in cell survival during PMA stimulation, confirming the necessary role of actin in generating contraction. Clearly, a ROCK-dependent contraction force during the early stage of PMA treatment is necessary for activation of the apoptotic signal.

In addition to ROCK, MLC kinase (MLCK) is the major kinase that phosphorylates MLC (Gallagher et al., 1997; Kohama et al., 1996). To ascertain whether ROCK plays a necessary role for MLC phosphorylation during PMA-induced apoptosis, we then tested the effects of ML-7, an inhibitor of MLCK (Itoh et al., 1992), and Y27632 on MLC phosphorylation in TF-1 cells during PMA treatment. As expected, PMA-induced MLC phosphorylation was diminished by Y27632 pre-treatment, but not by the MLCK inhibitor ML-7, ranging from 1 to 20 μM (Fig. 3B). In addition, DNA fragmentation analysis also showed that PMA-induced apoptosis is inhibited by Y27632 but not ML-7 (Fig. 3C). Similar results could be observed for the cells treated with another MLCK inhibitor, ML-9 (Ishikawa et al., 1988) (data not shown). Thus, these experimental results exclude the possibility of MLCK in generating a contraction force for PMA-induced apoptosis.

PMA-induced activation of caspase-3 is controlled by myosin motor activity
To prove the necessary role of myosin motor activity in caspase-3 activation, we further examined the effect of Y27632, latrunculin B and 2,3-butanedione monoxime (BDM), a myosin ATPase inhibitor (Soeno et al., 1999), on PMA-induced activation of caspase-3 activity in TF-1 cells. Similar to the results obtained from cells treated with Y27632, latrunculin B treatment also abolished the increase in caspase-
Inhibition of ROCK abrogates the requirement of adhesion for survival

It should be mentioned that TF-1 cells in serum-free medium always survive with the adhesion property during PMA exposure. We have previously proven that adhesion can act to prevent the occurrence of the apoptotic signal triggered by PMA treatment, and RhoA signaling from serum or LPA may interfere with the adhesion process to allow PMA-induced apoptosis to be triggered (Lai et al., 2001). Therefore, it is possible that inhibition of ROCK may switch cells to become adherent, thereby preventing apoptosis. We then plated cells onto a hydrogel-coated dish to prevent cell adhesion during PMA treatment in serum-free medium, as compared with those plated onto the regular culture dish to allow adhesion. It appeared that pretreatment of cells with Y27632 rescued cells from PMA-induced apoptosis even when cell adhesion was blocked (Fig. 5), indicating that the inhibition of ROCK that abolishes the apoptotic process is not through the adhesion event. Most importantly, this result also implied that ROCK-mediated contraction occurs downstream of loss of adhesion to exert its effect in PMA-induced apoptosis.

Effects of expression of dominant-active forms of RhoA and ROCK on PMA-induced apoptosis in serum-free conditions

We have previously shown that over-expression of RhoAV14, a dominant-active form of RhoA, causes cells to become apoptotic in response to PMA in serum-free medium. As the effector pathways of the RhoA signal are diverse, we then assessed whether activation of the ROCK-mediated signal is a solely effector pathway of RhoA involved in PMA-induced apoptosis. For this purpose, the expression vector of ROCK(CAT), which contains only an NH2-terminal kinase domain (catalytic domain, amino acids 6 to 553) (Amano et al., 1997), or RhoAV14 was co-transfected with the EGFP expression vector into TF-1 cells. Cells were incubated in serum-free medium during PMA treatment. The successfully transfected cells were revealed by fluorescent microscopic observation. The suspension cells before and after PMA treatment were collected, and GFP-positive cells were counted. All PMA-treated cells in suspension were apoptotic, as judged by Trypan Blue staining after 15 hours of PMA treatment. Consistent with our previous observation, expression of RhoAV14 significantly increased PMA-induced apoptosis under serum-free conditions. However, expression of the dominant-active form of ROCK(CAT) did not increase PMA-induced apoptosis in serum-free medium to an extent similar to that by RhoAV14 transfection (Fig. 6). As the effect of RhoAV14 on PMA-induced apoptosis in serum-free medium can be consistently abolished by Y27632 treatment (data not shown), it is clear that the RhoA/ROCK pathway is necessary for this apoptotic induction. Given the fact that activation of RhoA interferes with phorbol ester-induced adhesion, it is conceivable that another downstream effector pathway of RhoA is involved in prevention of adhesion, and that a ROCK-mediated signal by itself is not sufficient to result in membrane contraction and to turn cells apoptotic.

Activation of initiator caspases requires ROCK-dependent contraction in PMA-induced apoptotic cells

We next determined which caspase initiator is responsible for caspase-3 activation in this apoptotic process. By western blot analysis, we could clearly see the processed form of caspase-8 and -10 at 6 hours after PMA treatment (Fig. 7A). To know whether caspase-8 and -10 are the apical caspases responsible for the PMA-induced activation of caspase-3, we further pretreated cells with different caspase inhibitors. We found that caspase-3 activity in PMA-treated cells was completely blocked by pretreatment of cells with caspase-3 inhibitor (z-DEVD-fmk). Activation of caspase-3 in PMA-treated cells was
clearly decreased by caspase-8 inhibitor (z-IETD-fmk) or caspase-10 inhibitor (z-AEVD-fmk), as compared with a slight decrease by caspase-9 inhibitor (z-LEHD-fmk) (Fig. 7B). Consistent results could be seen in D2 cells, a cytokine-independent derivative from TF-1, which also displayed apoptosis and differentiation in response to PMA treatment (Lai et al., 2002).

We further incubated cells with caspase-3 inhibitor (z-DEVD-fmk) during PMA treatment to examine whether activation of caspase-8 and -10 is a secondary event of caspase-3 activation. As shown in Fig. 7C, neither caspase-8 nor -10 activation was affected by inhibition of caspase-3, whereas ROCKI cleavage was prevented. This result excludes the possibility that the activation of these two caspasas is a result of caspase-3 activation and provides further evidence that ROCKI does serve as a substrate of caspase-3 in the latter stage of PMA-induced apoptosis. Taken together, caspase-8 and -10 are likely the initiator caspases in PMA-induced apoptosis.

We further tested whether the cleavage of caspase-8 and -10 in PMA-treated TF-1 cells depends on the ROCK-mediated activation of actin-myosin motor activity. As expected, pretreatment of cells with either Y27632 or latrunculin B prevented PMA-induced cleavage of caspases-10, -8 and -3. As a comparison, pretreatment of cells with MLCK inhibitor, ML-9, did not affect this activation process (Fig. 8). Clearly, ROCK-mediated MLC phosphorylation and the subsequent myosin-actin interaction is a requisite process for activation of caspase-8 and -10 that act as the initiator caspases in this apoptotic process.
To substantiate the role of ROCK in PMA-induced DISC but not in the attached and survived population (Fig. 9A). procaspase-10 C/S-flag expressed in D2 and TF-1 cells in increase of FADD is associated with the immunocomplex of response to PMA treatment. It appeared that a significant increased association between FADD and procaspase-10 in co-immunoprecipitation to examine whether there is an protease-dead mutant, in D2 and TF-1 cells to perform DISC caspase-8 and -10, we expressed procaspase-10 C/S-flag, an adaptor role in death receptor-mediated activation of formation in PMA-induced apoptosis. Since FADD often plays their activation, we then assessed the involvement of DISC by which a DISC at the cell membrane is formed to facilitate the cytoplasmic death domain of the death receptor via FADD, given the fact that caspase-8 and -10 are always recruited to the membrane receptor-mediated death pathway can be activated in cells where a cellular context allows ROCK-mediated contraction determines the cellular fate in response to PMA induction. Here, we found that this process does occur at the late stage in membrane blebbing in apoptosis (Coleman et al., 2001; Sebbagh et al., 2002). In other words, the signal flow of RhoA/ROCK/MLC phosphorylation/myosin-mediated contraction acts as an upstream signal flow required for caspase-8/-10 activation, which are the initiator caspases in this PMA-induced apoptosis. Second, prevention of adhesion and ROCK-mediated contraction were two downstream events of the RhoA signaling that cooperate for PMA-induced apoptosis, and activation of ROCK is necessary, but insufficient, for PMA-induced apoptosis. Third, we provide the first evidence that DISC formation is involved in PMA-induced activation of caspase-10, indicating the participation of the death receptor-mediated pathway in this apoptotic stimulation. Taken together, we hypothesize that the membrane receptor-mediated death pathway can be activated in cells where a cellular context allows ROCK-mediated membrane contraction to occur during PMA treatment. In other words, the signal flow of RhoA/ROCK/MLC phosphorylation/myosin-mediated contraction determines the cellular fate in response to PMA induction. Membrane blebbing has been considered as a morphological change in the execution phase of apoptosis. In particular, it has been demonstrated that ROCK activation by caspase-3-mediated cleavage at its C-terminus inhibitory domain results in MLCK or the Rho-dependent pathway is increased in the PMA-induced apoptotic cells; however, the initial membrane blebbing in apoptosis (Coleman and Olson, 2002). The role of ROCK in PMA-induced apoptosis formation, D2 cells with procaspase-10 C/S-flag transfection were treated with PMA for 30 minutes. Cells remained in suspension, representing the early pro-apoptotic stage, were collected and re-plated into a new culture dish in the presence or absence of Y27632 and incubated for another 2.5 hours in the PMA-containing medium. Data shown in Fig. 9B revealed that Y27632 treatment diminished complex formation between pro-caspase-10 C/S-flag and FADD, indicating that inhibition of ROCK reduced the recruitment of FADD to form the DISC complex in PMA-treated cells. Taken together, there is a good possibility that ROCK-mediated membrane contraction can provide a mechanism to stimulate a death receptor-mediated pathway during PMA induction.

Discussion

Cell proliferation, promotion of differentiation and cell death are the processes that interplay to maintain homeostasis during hematopoiesis. It remains elusive as to how cells display differential cell fates in response to one stimulus. Following clues from our previous observations that RhoA plays a switcher role in determining whether cells undergo PMA-induced differentiation or apoptosis, in this report we further detail the molecular events involved in this apoptotic stimulation and establish several new points. First, the pathway of RhoA/ROCK/MLC phosphorylation/myosin-mediated contraction acts as an upstream signal flow required for caspase-8/-10 activation, which are the initiator caspases in this PMA-induced apoptosis. Second, prevention of adhesion and ROCK-mediated contraction were two downstream events of the RhoA signaling that cooperate for PMA-induced apoptosis, and activation of ROCK is necessary, but insufficient, for PMA-induced apoptosis. Third, we provide the first evidence that DISC formation is involved in PMA-induced activation of caspase-10, indicating the participation of the death receptor-mediated pathway in this apoptotic stimulation. Taken together, we hypothesize that the membrane receptor-mediated death pathway can be activated in cells where a cellular context allows ROCK-mediated membrane contraction to occur during PMA treatment. In other words, the signal flow of RhoA/ROCK/MLC phosphorylation/myosin-mediated contraction determines the cellular fate in response to PMA induction. Membrane blebbing has been considered as a morphological change in the execution phase of apoptosis. In particular, it has been demonstrated that ROCK activation by caspase-3-mediated cleavage at its C-terminus inhibitory domain results in MLCK phosphorylation (Coleman et al., 2001; Sebbagh et al., 2001), which contributes to myosin motor activity and membrane blebbing in apoptosis (Coleman and Olson, 2002). Here, we found that this process does occur at the late stage in PMA-induced apoptotic cells; however, the initial membrane contraction in PMA-induced apoptotic cells is via the RhoA/ROCK pathway rather than through caspase-3-dependent cleavage of ROCKI. Relevant to our results, it has been shown that membrane blebbing occurs in Rat-1 and PC-12 cells after serum withdrawal (McCarthy et al., 1997; Mills et al., 1998), and that the extent of MLC phosphorylation by MLCK or the Rho-dependent pathway is increased in the population of blebbing cells when caspase is inhibited. However, in our system, serum or LPA provides signal via

Involvement of FADD in PMA-induced apoptosis

Given the fact that caspase-8 and -10 are always recruited to the cytoplasmic death domain of the death receptor via FADD, by which a DISC at the cell membrane is formed to facilitate their activation, we then assessed the involvement of DISC formation in PMA-induced apoptosis. Since FADD often plays an adaptor role in death receptor-mediated activation of caspase-8 and -10, we expressed procaspase-10 C/S-flag, a protease-dead mutant, in D2 and TF-1 cells to perform DISC co-immunoprecipitation to examine whether there is an increased association between FADD and procaspase-10 in response to PMA treatment. It appeared that a significant increase of FADD is associated with the immunocomplex of procaspase-10 C/S-flag expressed in D2 and TF-1 cells in PMA-induced pro-apoptotic cells that remained in suspension, but not in the attached and survived population (Fig. 9A). To substantiate the role of ROCK in PMA-induced DISC formation, D2 cells with procaspase-10 C/S-flag transfection were treated with PMA for 30 minutes. Cells remained in suspension, representing the early pro-apoptotic stage, were collected and re-plated into a new culture dish in the presence or absence of Y27632 and incubated for another 2.5 hours in the PMA-containing medium. Data shown in Fig. 9B revealed that Y27632 treatment diminished complex formation between pro-caspase-10 C/S-flag and FADD, indicating that inhibition of ROCK reduced the recruitment of FADD to form the DISC complex in PMA-treated cells. Taken together, there is a good possibility that ROCK-mediated membrane contraction can provide a mechanism to stimulate a death receptor-mediated pathway during PMA induction.
the ROCK pathway to stimulate MLC phosphorylation independent of MLCK. In addition, it should be noted that MLC phosphorylation is a PMA-induced event. It has been shown that activity of myosin phosphatase can be inhibited by CPI (PKC-potentiated inhibitory phosphoprotein of myosin phosphatase), whose inhibitory activity is potentiated by PKC-mediated phosphorylation (Watanabe et al., 2001). From this view, it is possible that MLC phosphorylation in PMA-induced pro-apoptotic cells is a result of ROCK and PKC activation. Still, it remains to be determined whether CPI is involved in MLC phosphorylation in PMA-treated TF-1 cells or whether activation of ROCK requires PKC activation.

It has been reported that MLCK-mediated MLC phosphorylation increases translocation of tumor necrosis factor receptor (TNFR) to the plasma membrane independently of a TNF signal, and this in turn activates caspase-8 to initiate the apoptotic pathway (Jin et al., 2001). Another study has also shown that treatment of cells with the cytoskeleton-disturbing reagent cytochalasin B increases clustering of the CD95 receptor to activate caspase-8 and enhances UV-induced apoptosis (Kulms et al., 2002). Here, our results indicate that both caspase-8 and -10 are the apical caspases in PMA-induced apoptosis and their activation is a result of membrane contraction dependent on ROCK. The obligatory role of caspase-8 and -10 in apoptosis initiation by a death receptor-mediated pathway has prompted us to examine whether the PMA-induced death signal involves the enhancement of the receptor-mediator adaptor recruitment. Indeed, a complex containing endogenous FADD with procaspase-10 is preferentially formed in PMA-induced pro-apoptotic cells, but not the survived cells.

Fig. 7. Activation of caspase-8 and -10 in PMA-induced apoptosis. (A) TF-1 cells were treated with PMA for different time intervals (0-12 hours). Whole cell lysates (50 µg) were resolved by SDS-PAGE followed by immunoblotting with antibodies specific for caspase-8 and caspase-10. (B) TF-1 cells were pre-incubated with different caspase inhibitors (5 µM) for 30 minutes as indicated prior to PMA treatment. The corresponding inhibitor of each caspase is z-DEVD-fmk for caspase-3, z-IETD-fmk for caspase-8, z-LEHD-fmk for caspase-9 and z-AEVD-fmk for caspase-10. After 8 hours, cells were analyzed for caspase-3 activity as described in the legend to Fig. 4. (C) Following pretreatment with z-DEVD-fmk (10 or 50 µM) for 30 minutes, TF-1 cells were treated with PMA for 9 hours and harvested for immunoblot analysis using antibodies specific for ROCK1, caspase-8 and -10.

Fig. 8. ROCK-mediated signal is required for processing of procaspases-8 and -10 in PMA-treated TF-1 cells. TF-1 cells were either left untreated or pre-incubated with specific ROCK inhibitor (Y27632, 20 µM), Latrunculin B (L.B, 0.5 µM) or MLCK inhibitor (ML-9, 20 µM), as indicated. Eight hours after PMA treatment, cells were harvested and analyzed by immunoblotting with antibodies specific for caspase-3, -8 and -10. NT, not treated.
Distinct from our result, one study (Meng et al., 2002) has demonstrated that PMA inhibits Fas-mediated apoptosis by disturbing the receptor-mediated adaptor molecule recruitment, suggesting a mechanism conferred by PMA-induced signal for survival. Again, together with our results, it is conceivable that the different concurrent pathways can cooperate with the diverse signals from PMA stimulation to have different cellular fates. Previously, another study has shown that phorbol ester induces apoptosis in U937 cells, in part through a pathway that requires endogenous production of TNF-α depending on activation of MEK/ERK during stimulation (Takada et al., 1999).

Since PMA-induced apoptosis in TF-1 cells did not require newly synthesized protein, it is unlikely that induction of a particular gene expression is required for the apoptotic signal in this case. To test the possibility that release of the death receptor ligand TNF-α is involved, we have pre-incubated cells with recombinant TNF receptor R1 protein prior to PMA induction. However, this pre-treatment experiment did not affect PMA-induced apoptosis, excluding the possibility that TNF ligand binding is involved in this apoptotic process (data not shown).

In this study, we also found that PMA-induced apoptosis was decreased, but was not completely abolished by expression of a dominant-negative form of FADD (data not shown), implying that the death receptor-

**Fig. 9.** Involvement of FADD in PMA-induced apoptosis. (A) D2 or TF-1 cells transfected with procaspase-10 C/S-flag were either untreated or treated with PMA for 3 hours (D2) or 5 hours (TF-1) as indicated. For DISC analysis, the post-nuclear supernatant from transfected cells was immunoprecipitated with anti-flag M2 beads or with normal mouse IgG followed by addition of protein A-Sepharose beads. The resulting protein complexes were separated by SDS-PAGE, and analyzed by immunoblotting with antibodies for flag and FADD. (B) D2 cells transfected with procaspase-10 C/S-flag were treated with PMA for 30 minutes, the pro-apoptotic suspension cells were then transferred to a new dish without or with Y27632 for another 2.5 hours. DISC analysis was then performed as described above. The lysates without co-immunoprecipitation were directly subjected to the same immunoblotting analysis and indicated as ‘Input’. Abbreviations: NT, non-treated; S, cells in suspension representing PMA-induced apoptotic cells; A, attached cells representing PMA-induced pro-differentiation or survived cells; IgG H, heavy chain; IgG L, light chain; IP, immunoprecipitation.

**Fig. 10.** Model for PMA-induced apoptosis involving the signal from the RhoA/ROCK pathway. During PMA treatment, a concurrent signal from LPA or serum upregulates the RhoA/ROCK/MLC phosphorylation pathway, which cooperates with the PMA/PKC signal to generate a membrane contraction force that leads to activation of caspase-8 and -10. In this model, we propose that additional pathways including blocking myosin phosphatase by PKC-mediated upregulation of CPI, the negative control on MLC phosphorylation by cell adhesion, and the subsequent death-receptor-dependent or-independent pathway for activation of caspases-8 and -10, interplay with RhoA/ROCK pathway in PMA-induced apoptosis. Dashed lines represent the pathways that remain to be verified.
mediated pathway probably only plays a partial role in PMA-induced apoptosis. It has been demonstrated that unligated integrins recruit caspase-8 to the membrane and form the DISC without FADD, suggesting the presence of a death receptor-independent caspase-8 activation mechanism (Stupack et al., 2001). Therefore, it is possible that another pathway independent of the death receptor is also involved in this apoptotic process. In summary, we propose that the first phase of membrane contraction leads to the subsequent random clustering of cell-surface death receptors or other membrane receptors, such as unligated integrins, which in turn activate the apoptotic signaling via caspase-8 and -10.

Our previous study has demonstrated that expression of a dominant-active form of RhoA14 increases PMA-induced apoptosis significantly in TF-1 cells under serum-free conditions (Lai et al., 2001). We proposed that, in serum-free medium, PMA induces only adhesion, which provides the survival signal, whereas activation of RhoA by LPA or serum may promote PMA-induced apoptosis by interfering with the adhesion process. In this study, we further found that inhibition of ROCK abrogates the requirement of adhesion for cell survival following PMA treatment by preventing myosin-mediated contraction, so that the death signal is abolished; under this circumstance, the survival signal from adhesion is no longer required. However, the effect of expressing a dominant-active form of ROCK(CAT) on PMA-induced apoptosis in serum-free medium was not as dramatic as that of expressing RhoAV14 (Fig. 5). Probably, membrane contraction induced by PMA in TF-1 cells indeed requires cells remaining in the suspension status, and adhesion can provide a signal to prevent the occurrence of ROCK-dependent contraction. This finding led us to propose that another downstream pathway of RhoA is involved in preventing TF-1 cells from PMA-induced adhesion, by which the concurrent activation of ROCK can confer contraction force. In other words, under serum-free conditions, cell adhesion may still provide an inhibitory mechanism to prevent membrane contraction despite the presence of a dominant-active form of ROCK. According to these results, we propose a model for PMA-induced apoptosis in TF-1 cells as depicted in Fig. 10, in which there is an interplayed relationship between cell adhesion, PKC activation and the serum/RhoA/ROCK pathway in MLC phosphorylation that results in myosin-mediated contractility, thus switching-on the death receptor-mediated or death receptor-independent activation of the caspase cascade.

A growing number of examples have shown that the coordinated activation and functional cooperation between members of the Ras and Rho GTPase families regulate cellular proliferation and actin-based cell motility (reviewed by Bar-Sagi and Hall, 2000). Specifically, it has been shown that Rho suppresses the induction of the cell-cycle inhibitor p21Cip1/Waf1, thus enabling Ras to stimulate cell-cycle progression in 3T3 fibroblasts (Olson et al., 1998), and this mechanism is important for the role of Rho in uncontrolled proliferation during Ras-induced transformation (Qiu et al., 1995; Sahai et al., 2001). Interestingly, we have recently shown that a ROCK-mediated signal may cause cytosolic retention of activated ERK in PMA-induced apoptotic cells, thus impairing ERK-mediated gene expression such as p21Cip1/Waf1 (Lai et al., 2002). The enhancement of PMA-induced apoptosis by the LPA/RhoA/ROCK pathway in TF-1 cells intriguingly exemplifies a situation in which upregulated ROCK from RhoA signaling in some cells not only impairs gene expression required for growth arrest or differentiation, but also has a potential in changing the extent of myosin-mediated contraction to trigger apoptosis. Thus, the RhoA/ROCK pathway can act as a molecular switcher by cooperating with other pathways depending on the cellular context to determine cell fate during hematopoiesis.

We are grateful to H.-F. Yang-Yen (Academia Sinica, Taipei, Taiwan) for providing TF-1 cells, T.-S. Jou (National Taiwan University, Taipei, Taiwan) for expression plasmids of RhoAV14 and ROCK(CAT), which were originally obtained from K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan), and J. M. Staddon (University College London, London, UK) for the antibody against ppMLC. This research is supported by grants NSC90-2320-B-002-074 and NSC91-3112-B-002-012 from the National Science Council, Taiwan, Republic of China.

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