

Actin-myosin–based contraction is responsible for apoptotic nuclear disintegration

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Membrane blebbing during the apoptotic execution phase results from caspase-mediated cleavage and activation of ROCK I. Here, we show that ROCK activity, myosin light chain (MLC) phosphorylation, MLC ATPase activity, and an intact actin cytoskeleton, but not microtubular cytoskeleton, are required for disruption of nuclear integrity during apoptosis. Inhibition of ROCK or MLC ATPase activity, which protect apoptotic nuclear integrity, does not affect caspase-mediated degradation

of nuclear proteins such as lamins A, B1, or C. The conditional activation of ROCK I was sufficient to tear apart nuclei in lamin A/C null fibroblasts, but not in wild-type fibroblasts. Thus, apoptotic nuclear disintegration requires actin-myosin contractile force and lamin proteolysis, making apoptosis analogous to, but distinct from, mitosis where nuclear disintegration results from microtubule-based forces and from lamin phosphorylation and depolymerization.

Introduction

Apoptosis leads to the death and subsequent removal of damaged or redundant cells. Cysteine-proteases called caspases are responsible for the apoptotic “execution” phase, which is characterized by morphological changes including cell contraction, dynamic membrane blebbing, and nuclear disintegration. Contractile force generated by actin-myosin cytoskeletal structures is the driving power behind cell contraction and the formation of membrane blebs (Coleman and Olson, 2002). In the apoptotic cell, the disintegrated nucleus is found within blebs and packaged into membrane-clad apoptotic bodies that facilitate uptake by neighboring cells or by specialized phagocytic cells. The release of nuclear fragments from apoptotic cells is believed to be the source of antigens in autoimmune diseases such as systemic lupus erythematosus (Rosen and Casciola-Rosen, 1999; Stollar and Stephenson, 2002).

The dynamic contraction and membrane blebbing seen in apoptotic cells are dependent on intracellular force generated by the actin-myosin cytoskeleton. These morphological

events are controlled by the Rho effector ROCK I, a serine/threonine kinase that plays a key and central role in the regulation of actin cytoskeletal structures. We and others showed that caspase-mediated cleavage of ROCK I results in constitutive activation and consequent myosin light chain (MLC) phosphorylation leading to contraction and membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001). Inhibition of ROCK activity with the small molecule inhibitor Y-27632 attenuated blebbing in a variety of cell types, independent of the type of apoptotic stimulus. Inhibition of ROCK activity also prevented the relocalization of fragmented DNA into membrane blebs and apoptotic bodies (Coleman et al., 2001), suggesting additional roles for ROCK in the morphological changes that occur during apoptosis.

In addition to the gross external morphological responses, there are significant effects on the morphology and integrity of organelles, the most obvious being nuclear disintegration. Separating the nucleus from the cytoplasm is the nuclear envelope, which is comprised of outer and inner nuclear membranes. Giving the nucleus form, structure, and rigidity is a filamentous meshwork called the lamina, which is made up from intermediate filament A-type (A and C, alternately spliced products from the *Lmna* gene) and B-type (B1, B2, and B3) lamins. Caspase-mediated cleavage of lamins A/C and B1 is thought to contribute to nuclear fragmentation during apoptosis (Neamati et al., 1995; Rao et al., 1996; Broers et al., 2002).

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Abbreviations used in this paper: 4-HT, 4-hydroxytamoxifen; CHX, cycloheximide; LAP, lamin-associated protein; LIMK, LIM kinase; MEF, mouse embryo fibroblast; MLC, myosin light chain; PARP, poly-ADP ribose polymerase; TEM, transmission EM.

Ultrastructural analysis has shown that the nucleus is surrounded by a meshwork of actin (Clubb and Locke, 1998b), with “knots” of actin physically associated with the nuclear envelope (Clubb and Locke, 1998a). Disruption of the actin cytoskeleton alters nuclear morphology (Zhen et al., 2002), while mutations to Anc-1/Syne family actin-binding proteins result in aberrant nuclear anchoring (Starr and Han, 2003), indicating that the actin cytoskeleton influences nuclear positioning, shape, and structure. Therefore, one possibility is that during apoptosis, active caspase-cleaved ROCK I leads to shortening of actin-myosin filaments that are tethered to the nucleus at one end, resulting in nuclear envelope tearing and disintegration, thereby allowing for the relocalization of fragmented DNA to membrane blebs and apoptotic bodies (Coleman et al., 2001). Mitotic nuclear envelope breakdown also requires weakening of the nuclear lamina and a pulling force, but is mediated by phosphorylation-induced depolymerization of the nuclear lamina (Heald and McKeon, 1990) and microtubule-anchored pulling force generated by the minus-end-directed motor, cytoplasmic dynein, and components of its associated regulatory complex, dyactin (Beaudouin et al., 2002; Salina et al., 2002).

In this work, we examined the contribution of ROCK activity and MLC phosphorylation to nuclear disintegration during apoptosis. We found that ROCK activity, intact actin filaments, MLC phosphorylation, and MLC ATPase activity are each required for the breakdown of nuclear structure, whereas intact microtubules are dispensable. Caspase-mediated cleavage of lamins A/C and B1 is not sufficient for nuclear disintegration in the absence of ROCK and MLC ATPase activity. In addition, conditional activation of ROCK I induces nuclear breakdown in nonapoptotic cells only in the absence of lamin A/C expression. These results indicate that apoptotic nuclear breakdown requires weakening of the nuclear lamina by proteolytic cleavage and the contractile force generated by ROCK on actin-myosin filaments. Thus, apoptotic nuclear breakdown parallels mitotic nuclear breakdown in the requirements for lamina disassembly and generation of pulling force, but differs in the means by which these events are achieved.

Results

ROCK activity and nuclear disintegration

We previously observed by TUNEL that in apoptotic cells treated with the ROCK inhibitor Y-27632, fragmented DNA was located within discrete foci and not in membrane blebs or apoptotic bodies as in untreated apoptotic cells (Coleman et al., 2001). Therefore, in addition to plasma membrane blebbing, we hypothesized that ROCK activation by caspase-mediated cleavage promotes apoptotic nuclear disintegration, thereby allowing fragmented DNA to be released from the nuclear compartment.

We used transmission EM (TEM) to obtain fine-detailed photomicrographs of nuclear morphology during apoptosis. NIH 3T3 mouse fibroblasts were treated with 25 ng/ml TNF α plus 10 μ g/ml cycloheximide (CHX) for 2 h as the apoptotic stimulus, and then processed for TEM. A typical apoptotic cell is shown in Fig. 1 A, with membrane blebs, chromatin condensation, and a significantly fragmented nucleus with large invag-

inations of the nuclear envelope and evidence of electron-dense heterochromatin within the cytoplasm and in blebs (Fig. 1 A, arrows). In contrast, cells given the ROCK inhibitor Y-27632 (10 μ M) in conjunction with TNF α /CHX did not bleb and the nucleus remained intact, although chromatin condensation and nuclear envelope dilation were evident (Fig. 1 B). Consistent with our previous observations (Coleman et al., 2001), heterochromatin was retained within the nucleus, indicating that the nuclear envelope remained a barrier to macromolecules.

Phosphorylation of ROCK substrates

We and others previously demonstrated that ROCK I becomes cleaved and activated in apoptotic cells (Coleman et al., 2001; Sebbagh et al., 2001). The cleavage of ROCK I (Fig. 1 C, cleaved form indicated by gray arrow and Δ) is accompanied by increased phosphorylation of type II MLC on residues Thr18 and Ser19 (Fig. 1 D), which is critical for association of MLC with actin filaments and generation of contractile force. MLC phosphorylation could be blocked with the ROCK inhibitor Y-27632 (Fig. 1 D) or by blocking ROCK I cleavage (Fig. 1 C) by pretreating cells with the caspase inhibitor z-VAD-fmk before the induction of apoptosis with TNF α . The myosin binding subunit (MYPT1) of the MLC phosphatase has been reported to be phosphorylated by ROCK on Thr696, which results in inhibition of MLC dephosphorylation (Feng et al., 1999; Kawano et al., 1999). Using a phospho-specific antibody against Thr696, no significant change in phosphorylation was observed (Fig. 1 E, top); however, one major and one minor (Fig. 1 E, gray arrows) proteolytic fragment were observed. MYPT1 cleavage was not apparently changed by Y-27632 but could be prevented with z-VAD-fmk. An antibody raised against the amino terminus of MYPT1 revealed a loss of immunoreactivity in apoptotic cells, indicating that the cleavage occurred at the amino-terminal end (Fig. 1 E, bottom). Analysis of protein mobility on SDS-PAGE revealed that \sim 80 amino acids were removed from the cleaved fragment. Deletion analysis of MYPT1 has shown that the amino-terminal region is responsible for binding both MLC and the catalytic subunit of the phosphatase complex. Therefore, cleavage of the \sim 80 amino acids from the MYPT1 amino terminus would likely inactivate the MLC dephosphorylating activity and commit the apoptotic cell to irrevocable MLC phosphorylation.

Additional downstream substrates of ROCK that may contribute to actin-myosin contractility are the LIM kinases (LIMK) 1 and 2 (Ohashi et al., 2000; Sumi et al., 2001). Overexpression of LIMK2 has been reported to induce the formation of stress fibers and membrane blebs (Amano et al., 2001), suggesting that LIMK1/2 activation contributes to the apoptotic morphology. Using an antibody that detects phosphorylation of a Thr residue in the conserved activation loops of LIMK1 (Thr508) and LIMK2 (Thr505), we observed increased phosphorylation following the induction of apoptosis, which could be blocked with ROCK inhibitor Y-27632 or by inhibition of ROCK I cleavage with z-VAD-fmk (Fig. 1 F). These results indicate that LIMK activation may contribute to ROCK-mediated morphological effects in apoptotic cells.

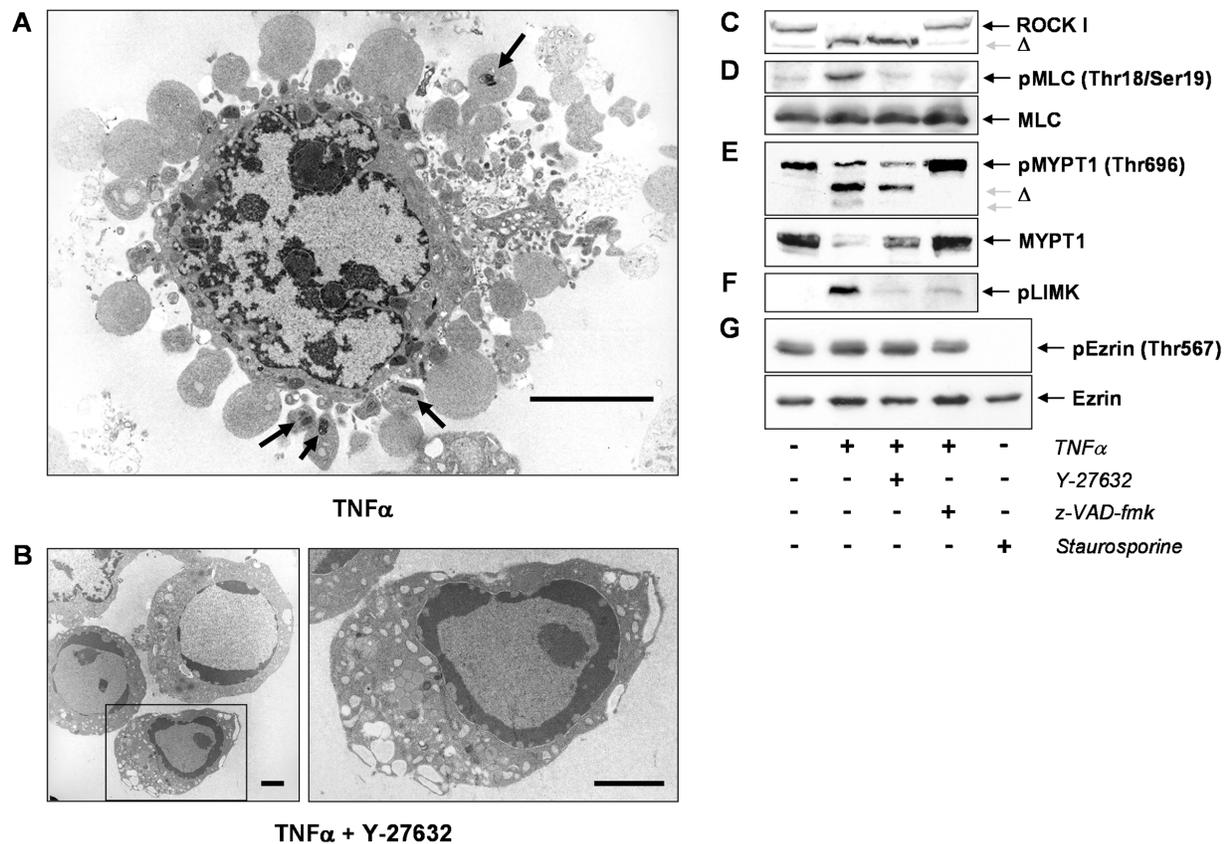
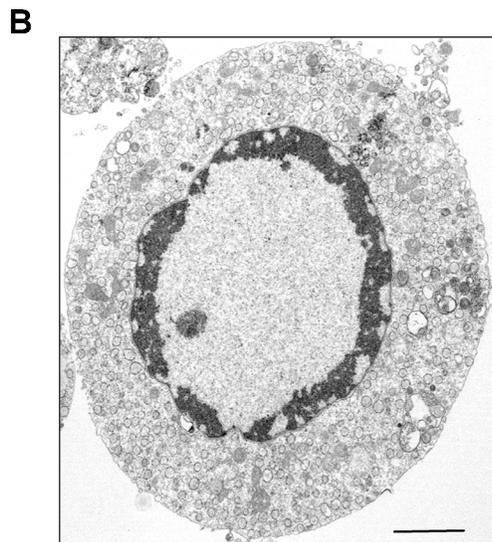
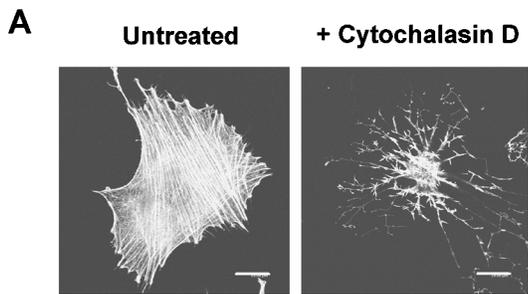


Figure 1. Apoptotic nuclear breakdown is blocked following ROCK inhibition. (A) Transmission electron micrograph of an NIH 3T3 fibroblast treated with 25 ng/ml TNF α plus 10 μ g/ml cycloheximide (CHX) for 2 h to induce apoptosis, showing large plasma membrane blebs, nuclear disintegration, and heterochromatin within blebs (arrows). Bar, 5 μ m. (B) TEM of cells treated with 10 μ M Y-27632 to block ROCK activity along with TNF α /CHX showing an absence of blebs and preservation of nuclear envelope integrity. Inset in left panel shown in right panel. Bars, 2 μ m. (C–G) Serum-starved NIH 3T3 fibroblasts were left untreated or treated with 25 ng/ml TNF α plus 10 μ g/ml CHX for 2 h to induce apoptosis, either in the absence or presence of 10 μ M Y-27632 or 50 μ M z-VAD-fmk as indicated. Lysates were prepared by direct lysis in 1 \times Laemmli sample buffer and samples run on 10% SDS-PAGE. Western blotting with antibodies against ROCK I (C), myosin light chain (MLC; D, bottom), and dually phosphorylated Thr18 Ser19 MLC (D, top), myosin binding subunit of the MLC phosphatase (MYPT1; E, bottom), and phosphorylated Thr696 MYPT1 (E, top), the common epitope of phospho-LIMK1 (Thr508; F) and phospho-LIMK2 (Thr505; F), and Ezrin (G, bottom) and phosphorylated Ezrin (Thr567; G, top). Phosphorylation of MLC and LIMK1/2 in apoptotic cells was blocked by ROCK inhibitor Y-27632 and by preventing caspase-mediated ROCK I cleavage with z-VAD-fmk. No differences in MYPT1 or Ezrin phosphorylation were evident, although MYPT1 was cleaved in a z-VAD-fmk-sensitive manner in apoptotic cells, removing \sim 80 amino acids from the amino-terminal end.

We also examined how the induction of apoptosis affected the phosphorylation state of Ezrin, which acts as a linker between actin filaments and transmembrane proteins such as CD44, thereby providing an anchoring point in the plasma membrane for actin-myosin fibers. ROCK-induced contraction of NIH 3T3 cells was reported to be dependent on phosphorylation of Ezrin Thr567 (Tran Quang et al., 2000), which allows for dissociation of inter- and/or intramolecular interactions and association with actin filaments (Gautreau et al., 2000). Using an antibody specific for Ezrin phosphorylated on Thr567, no changes in the phosphorylation status were observed in apoptotic cells or after Y-27632 treatment (Fig. 1 G), indicating that ROCK-mediated phosphorylation of Ezrin on Thr567 does not contribute to apoptotic cell contraction. Treatment of cells with the general kinase inhibitor staurosporine (0.2 μ M) for 30 min eliminated Ezrin T567 phosphorylation, suggesting that kinases other than ROCK are responsible for basal phosphorylation.

Nuclear disintegration and the actin and microtubule cytoskeletons

Next, we determined whether or not, in addition to ROCK activity, an intact actin cytoskeleton was required for nuclear disruption. Pretreatment with the actin-destabilizing compound cytochalasin D (2 μ M; Sampath and Pollard, 1991) for 2 h before treatment with TNF α /CHX disrupted the actin cytoskeleton (Fig. 2 A) and blocked fragmentation of the nucleus (Fig. 2 B). Similar results (unpublished data) were obtained when the actin cytoskeleton was disrupted with swinholide A (0.1 μ M; Bubb et al., 1995) or latrunculin B (0.5 μ M; Spector et al., 1983). These results indicate that an intact actin cytoskeleton is necessary to mediate nuclear disintegration. Disruption of the cytoskeleton with cytochalasin D did not affect caspase 3 activation (Fig. 2 C) and cleavage of ROCK I (Fig. 2 D) or poly-ADP ribose polymerase (PARP; Fig. 2 E), which is consistent with the lack of effect of Y-27632 on caspase activation (Fig. 1, C and E).



TNF α + cytochalasin D

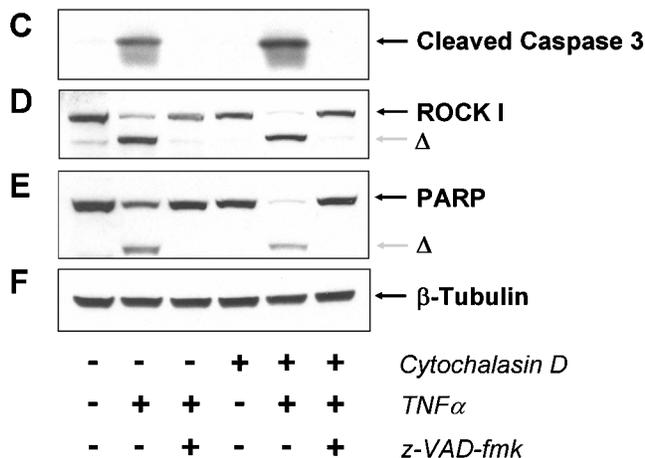


Figure 2. Apoptotic nuclear breakdown is blocked following actin filament disruption. (A) Actin filaments were apparent in untreated NIH 3T3 cells (left), but cell morphology and actin filaments were profoundly affected by treatment with 2 μ M cytochalasin D (right). Bars, 20 μ m. (B) TEM of cell pretreated with 2 μ M cytochalasin D to disrupt actin structures before induction of apoptosis, showing intact nucleus. Bar, 2 μ m. (C) Activation of Caspase 3 and cleavage of ROCK I (D) and PARP (E) were not affected by cytochalasin treatment and disruption of the actin cytoskeleton before the induction of apoptosis. (F) Blotting for β -tubulin indicates equal loading across lanes.

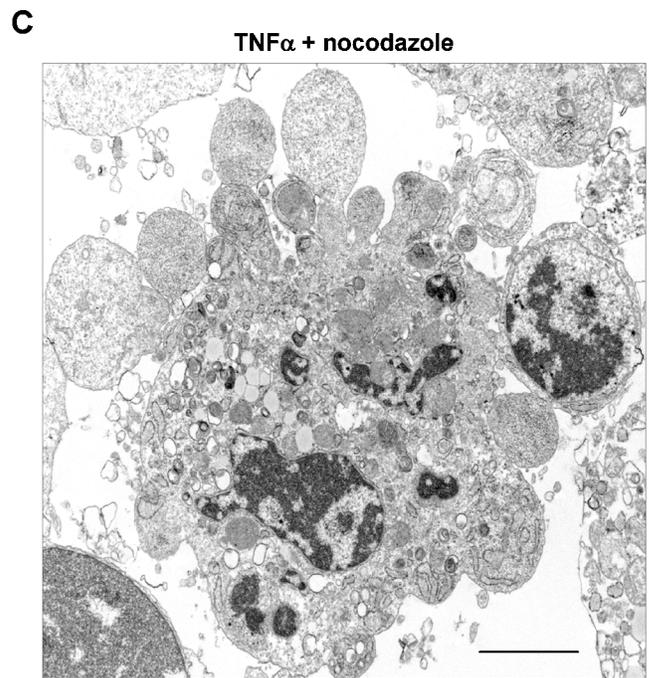
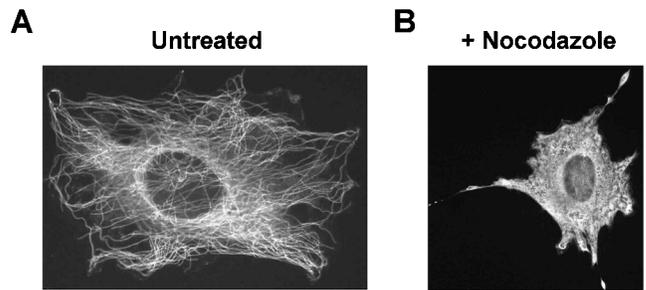


Figure 3. Disruption of microtubular structures does not affect apoptotic nuclear breakdown. Microtubular structures were visualized with antibody against anti- β -tubulin antibody in untreated (A) or nocodazole (1 μ M; B)-treated NIH 3T3 cells. (C) TEM of cell pretreated with 1 μ M nocodazole to disrupt microtubules, as in B, before induction of apoptosis, showing marked blebbing and nuclear disintegration. Bar, 2 μ m.

To determine whether or not an intact microtubule network is required for apoptotic nuclear breakdown, microtubules were disrupted by pretreatment with nocodazole (1 μ M) for 2 h before treatment with TNF α plus CHX for an additional 2 h. Despite efficient disruption of the microtubule network (Fig. 3 B), nocodazole treatment did not prevent apoptotic membrane blebbing or nuclear breakdown (Fig. 3 C). These data indicate that nuclear disruption in apoptotic cells differs from mitotic nuclear breakdown in that the actin, and not microtubular, cytoskeleton provides the framework for mechanical force that leads to nuclear tearing and rupture.

MLC phosphorylation and ATPase activity

Intact actin filaments and MLC phosphorylation have been shown to be required for plasma membrane blebbing (Coleman and Olson, 2002). Given that ROCK I is cleaved and MLC phosphorylation is elevated in apoptotic cells, the requirement

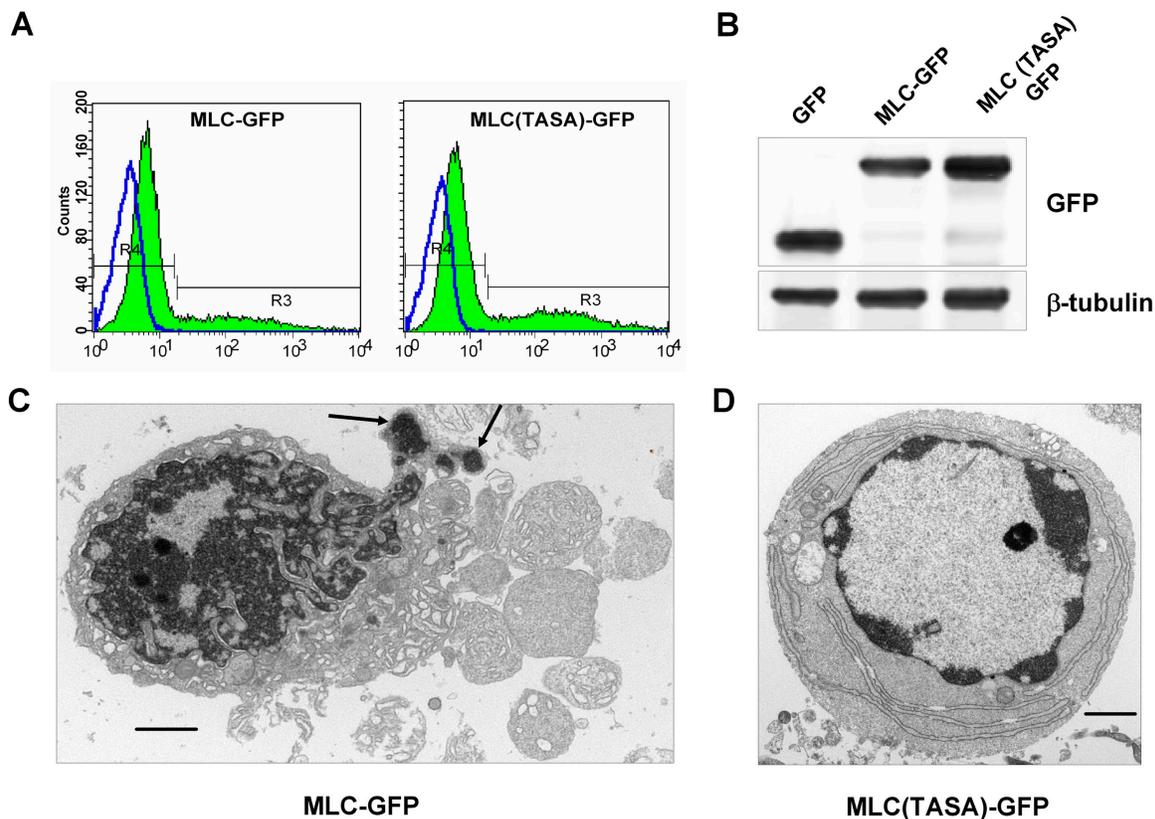


Figure 4. MLC phosphorylation is required for apoptotic nuclear disintegration. NIH 3T3 fibroblasts were transfected with a plasmid encoding GFP, myosin light chain fused to GFP (MLC-GFP), or MLC doubly mutated to Ala at the phosphorylation sites Thr18 Ser19 (MLC(TASA)-GFP). After allowing 24 h for protein expression, cells were sorted for GFP expression. (A) Autofluorescence of untransfected cells is indicated as R4, sorting was gated such that only GFP-positive cells with fluorescence intensity greater than all cells in R4 were collected (indicated as R3 in MLC-GFP and MLC(TASA)-GFP sorting profiles; GFP profile not depicted). Post-sorting analysis indicated that sorted cells were 99% GFP-positive. (B) Lysates prepared from equal numbers of GFP-positive cells were Western blotted for GFP to confirm equal protein expression (top) and for β -tubulin to confirm equal protein loading (bottom). (C) Cells were plated in medium containing 10% FCS for 5 h, and then placed in serum-free medium for 18 h. Apoptosis was induced with 25 ng/ml TNF α plus 10 μ g/ml CHX for 2 h, and then nonadherent apoptotic cells were collected, fixed, and processed for TEM. Expression of GFP (not depicted) or MLC-GFP did not affect nuclear disintegration in apoptotic cells (arrows indicate heterochromatin in blebs). Bar, 2 μ m. (D) Expression of nonphosphorylatable MLC(TASA)-GFP inhibited plasma membrane blebbing and nuclear fragmentation, although typical apoptotic chromatin condensation was evident. Bar, 2 μ m.

for MLC phosphorylation in apoptotic nuclear disintegration was examined. Expression of MLC with alanine substitutions at Thr18 and Ser19 has previously been shown to block actin bundling and contractility (Iwasaki et al., 2001). NIH 3T3 cells were transfected with plasmids encoding GFP, wild-type rat MLC with carboxyl-terminally fused GFP, or the MLC(TASA) double mutant fused with GFP (Fig. 4 A). Approximately 10–12% of GFP-positive cells were selected (Fig. 4 A, R3), all of which had greater fluorescence intensity than the autofluorescence of untransfected cells (Fig. 4 A, R4, blue peak). Post-sorting analysis revealed the GFP-positive population to be 99% pure (unpublished data). Equal aliquots of GFP-, MLC-GFP-, and MLC(TASA)-GFP-positive cells were Western blotted with GFP and β -tubulin antibodies (Fig. 4 B) revealing approximately equal protein expression. Sorted cells were plated in medium containing 10% fetal calf serum for 5 h, placed in serum-free medium for 18 h, and apoptosis induced with TNF α and CHX treatment, and then cells were collected, fixed, and processed for TEM. Cells expressing GFP (not depicted) or MLC-GFP (Fig. 4 C) were typically apoptotic, with plasma membrane blebs and disintegration of nuclei, often with portions of hetero-

chromatin visible within blebs (Fig. 4 C, arrows). In marked contrast, cells expressing MLC(TASA)-GFP were typified by an absence of plasma membrane blebs and possessed intact nuclei (Fig. 4 D). In addition, MLC(TASA)-GFP-expressing cells contained long, apparently membrane enclosed, structures. The origin of these membranous structures is currently under investigation. These results demonstrate that MLC phosphorylation is required for nuclear disintegration in apoptotic cells.

Cell contraction is dependent on the myosin ATPase, which through an ATP-dependent process catalyzes the shortening of actin-myosin filaments. Blebbistatin is a recently developed inhibitor of nonmuscle myosin II function that will most likely supplant the nonselective inhibitor butanedione monoxime (BDM) as the inhibitor of choice. We tested whether the ATPase activity of myosin was required for apoptotic nuclear disintegration by coadministering blebbistatin (50 μ M) along with the TNF α plus CHX apoptotic stimulus for 2 h. As can be seen in Fig. 5 A, inhibition of myosin ATPase activity inhibited apoptotic nuclear breakdown and resulted in the formation of elongated cytoplasmic structures, similar to those seen in MLC(TASA)-GFP-expressing cells. Blebbistatin did

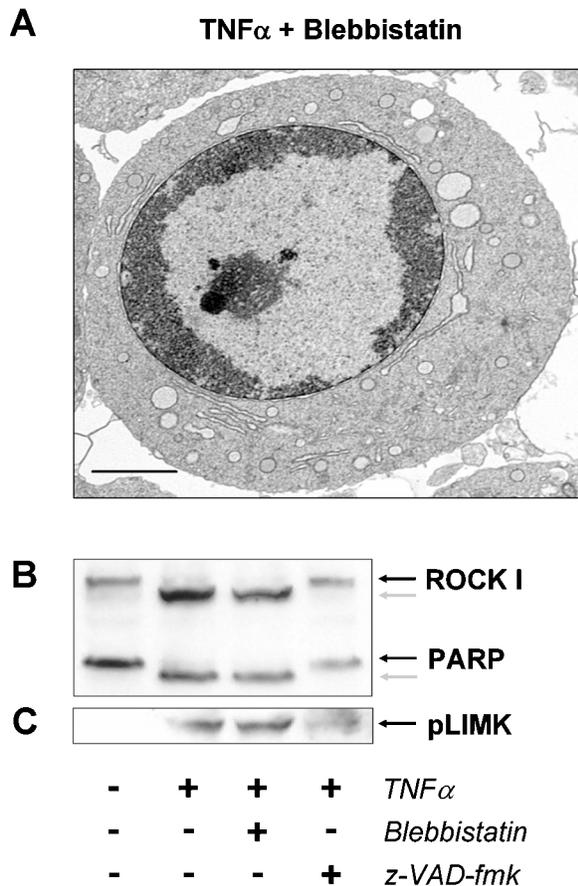


Figure 5. Inhibition of myosin ATPase activity protects apoptotic nuclear integrity. (A) NIH 3T3 cells were coadministered blebbistatin (50 μ M) along with the TNF α plus CHX apoptotic stimulus for 2 h before collection and processing for TEM. Bar, 2 μ m. NIH 3T3 fibroblasts were untreated or treated with 50 μ M blebbistatin to inhibit myosin ATPase activity or 50 μ M z-VAD-fmk to inhibit caspase activity as indicated. Apoptosis was induced with TNF α /CHX where indicated. Lysates were prepared and Western blotted with antibodies against ROCK I and PARP (B) and phospho-LIMK1 and 2 (C). Blebbistatin affects apoptotic nuclear morphology without inhibiting caspase-mediated proteolysis or ROCK activity.

not affect ROCK I cleavage (Fig. 5 B) or LIMK phosphorylation (Fig. 5 C), which is consistent with the protective effect on nuclear integrity being the result of myosin ATPase inhibition.

Caspase cleavage of nuclear proteins

The nucleus is separated from the cytoplasm by a nuclear envelope composed of outer and inner nuclear membranes that are joined at nuclear pore complexes (for review see Burke and Ellenberg, 2002). Underlying the inner nuclear membrane is the nuclear lamina, composed of A-type and B-type lamins that form a fibrous meshwork. Chromosomes associate with the lamina via lamin-associated proteins (LAP), which are either integral membrane proteins or soluble chromatin-associated proteins such as LAP2 α . Apoptotic nuclear disintegration is associated with caspase-mediated proteolysis of nuclear proteins, particularly the lamins B1 and A/C, which leads to the destabilization of the structural elements that preserve the integrity of the nucleus (Neamati et al., 1995). Given that lamin phosphorylation by protein kinases C α (Shimizu et al., 1998), β II (Chiarini et al., 2002),

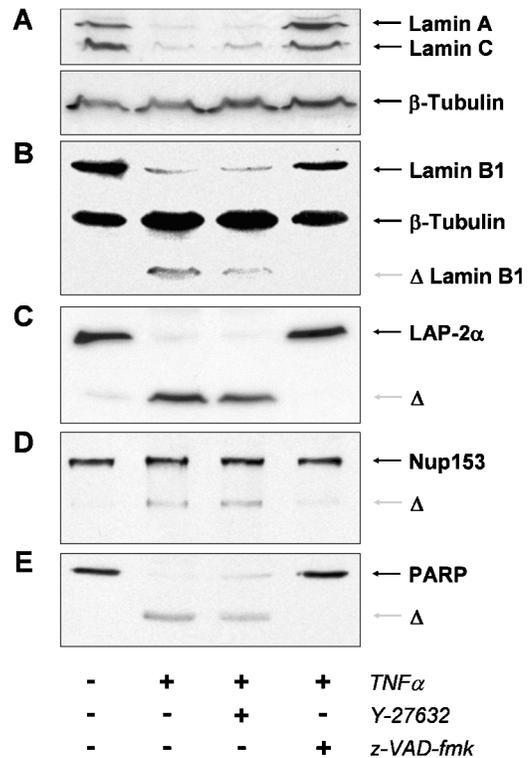


Figure 6. Degradation of nuclear lamins A/C and B1, LAP2 α , Nup153, and PARP during apoptosis is unaffected by ROCK inhibition. NIH 3T3 fibroblasts were left untreated or treated with 10 μ M Y-27632 to inhibit ROCK activity or 50 μ M z-VAD-fmk to inhibit caspase activity as indicated. Apoptosis was induced with TNF α /CHX where indicated. Lysates were prepared and Western blotted with antibodies against lamins A and C (A), lamin B1 (B), LAP-2 α (C), Nup-153 (D), and PARP (E). Equal loading was verified with an antibody against β -tubulin.

or δ (Cross et al., 2000) has been reported to be required for subsequent caspase-mediated cleavage, we wished to determine if inhibition of ROCK activity with Y-27632 prevented apoptotic nuclear disintegration by interfering with nuclear protein cleavage. Lysates from NIH 3T3 cells that were untreated, treated with TNF α /CHX either without or with Y-27632, or treated with TNF α /CHX after a 2-h pretreatment with the general caspase inhibitor z-VAD-fmk (50 μ M) were prepared and analyzed by Western blotting. Lamins A/C (Fig. 6 A) and B1 (Fig. 6 B) levels were significantly reduced after TNF α treatment with the generation of a faster mobility fragment of lamin B1 (indicated by Δ), even in the presence of Y-27632. Inhibition of caspase activity with z-VAD-fmk fully protected lamins A/C and B1 from cleavage. Treatment with cytochalasin D or blebbistatin also had no effect on lamin B1 cleavage (unpublished data). We also observed that the generation of cleaved forms (Δ) of LAP2 α (Fig. 6 C), nuclear pore protein Nup153 (Fig. 6 D), and PARP (Fig. 6 E) were unaffected by Y-27632 administration but fully protected by z-VAD-fmk. These results indicate that degradation of the constituent proteins of the nuclear lamina and nuclear pore complexes, as well as soluble nuclear proteins, does not require ROCK activity. By inference, these results indicate that degradation of nuclear proteins is not sufficient for apoptotic nuclear disintegration in the absence of contractile force induced by ROCK.

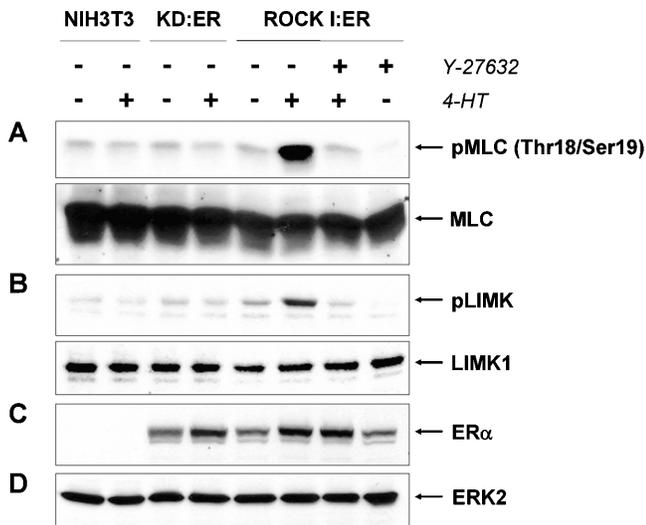


Figure 7. Conditionally active ROCK I:ER induces MLC and LIMK1 and 2 phosphorylation when stimulated with 4-HT. NIH 3T3 cells were left untreated or transduced with retrovirus encoding the conditionally active ROCK I:ER or kinase-dead KD:ER fusion proteins as indicated. After selection, stable pools were left untreated or treated with 1 μ M 4-hydroxytamoxifen (4-HT) for 18 h as indicated, either in the absence or presence of ROCK inhibitor Y-27632 (10 μ M). The phosphorylation status was examined with antibodies against MLC (A; dual Thr18/Ser19 phosphorylation, top panel; total MLC, bottom panel) and LIMK1 and 2 (B; LIMK1 Thr508, LIMK2 Thr505 phosphorylation, top panel; total LIMK 1, bottom panel). (C) Expression of KD:ER and ROCK I:ER confirmed by blotting with ER α antibody. (D) Equal protein levels confirmed by blotting with ERK2 antibody.

Active ROCK I affects nuclear morphology

We wished to determine whether or not the contractile forces generated by active ROCK I were sufficient to induce nuclear disintegration in nonapoptotic cells. NIH 3T3 cells were transduced either with a retroviral vector encoding amino acids 2–537 of human ROCK I fused to the hormone-binding domain of the estrogen receptor (ROCK I:ER) or with a kinase-dead version (KD:ER) in which Lys105 was mutated to glycine, similar to ROCK II:ER fusion proteins we have used previously (McMullan et al., 2003; Croft et al., 2004). Estrogen receptor fusion proteins have been previously used to produce conditionally active kinases (McMahon, 2001) that can be stimulated by estrogen analogues such as tamoxifen or 4-hydroxytamoxifen (4-HT). The addition of 1 μ M 4-HT for 18 h specifically activated ROCK I:ER resulting in MLC (Fig. 7 A) and LIMK1/2 phosphorylation (Fig. 7 B), which could be blocked by the ROCK inhibitor Y-27632 (10 μ M). Further confirmation that the effect of 4-HT on MLC and LIMK1/2 phosphorylation resulted from activation of ROCK I:ER was the lack of effect in parental NIH 3T3 or KD:ER-expressing cells (Fig. 7, A and B). Expression of the ROCK I:ER fusion protein was confirmed by Western blotting with anti-ER α antibody, revealing no expression in parental NIH 3T3 cells and comparable levels in KD:ER- and ROCK I:ER-expressing cells that were elevated approximately twofold by 4-HT treatment (Fig. 7 C), whereas blotting with anti-ERK2 antibody confirmed equal protein loading (Fig. 7 D). These results indicate

that conditional ROCK I:ER activation resulted in phosphorylation of proteins also found to be phosphorylated in apoptotic cells in a Y-27632-sensitive manner, which is consistent with the conclusion that these modifications during apoptosis are a consequence of ROCK I cleavage and activation.

We next used ROCK I:ER to examine the effects of ROCK activation on nuclear morphology and integrity. Consistent with previous findings (Coleman et al., 2001; Sebbagh et al., 2001), conditional activation of ROCK I:ER with 4-HT, in the absence of apoptosis (cells were pretreated with z-VAD-fmk), resulted in contraction and induction of membrane blebbing in \sim 50% of ROCK I:ER-expressing NIH 3T3 cells (Fig. 8 A, F-Actin left panels), which could be blocked by coadministration of ROCK inhibitor Y-27632 (Fig. 8 A, F-actin bottom panels). Treatment of parental NIH 3T3- or KD:ER-expressing cells with 4-HT did not affect actin structures or cell morphology (not depicted), which is consistent with the lack of effect on MLC or LIMK1/2 phosphorylation (Fig. 7). Staining with anti-lamin B1 antibody revealed that nuclei in contracted, ROCK I:ER-activated cells were smaller and occasionally distorted, similar to previous findings that expression of active ROCK II can alter gross nuclear morphology (Leung et al., 1996). These morphological changes were blocked by Y-27632. Although sufficient to induce contractility and membrane blebbing, these data illustrate that ROCK activation is not sufficient for nuclear disruption.

Next, we tested whether or not ROCK I:ER-induced contractility was sufficient for nuclear disruption under conditions in which the tensile strength provided by an intact lamina is reduced. Lamin A/C null mouse embryo fibroblasts (MEFs), which have a significant frequency of distorted nuclei with herniations (Sullivan et al., 1999), and wild-type MEFs were transduced with ROCK I:ER or KD:ER retrovirus. Treatment of ROCK I:ER-expressing cells with 4-HT plus z-VAD-fmk resulted in increased actin stress fibers, and frequent cell contraction accompanied by membrane blebbing, which could be blocked by the ROCK inhibitor Y-27632 (Fig. 8, B and C, F-actin). Similar to NIH 3T3 cells, KD:ER-expressing MEFs did not display changes in actin structures or morphology after 4-HT treatment (unpublished data). Nuclei in contracted blebbing wild-type MEFs were smaller than spread or untreated cells (Fig. 8 B), similar to nuclei in 4-HT-treated ROCK I:ER-expressing NIH 3T3 cells (Fig. 8 A). In marked contrast, lamin A/C null MEFs treated with 4-HT plus z-VAD-fmk that contracted and blebbed had significantly disrupted nuclei (Fig. 8 C), an effect that was blocked by Y-27632. Staining by TUNEL showed no detectable DNA fragmentation (unpublished data), which is consistent with the nuclear disruption being a direct effect of ROCK and not resulting from the induction of apoptosis. These results indicate that nuclei weakened by deletion of an important component of the lamina are disrupted by ROCK-induced cell contraction. Therefore, two elements are required for nuclear disruption, contractile force generated by active ROCK and weakening of the nuclear lamina, both of which occur in apoptotic cells through caspase-mediated cleavage of ROCK I and nuclear lamins.

Cell contraction, blebbing, and nuclear disruption in lamin A/C null MEFs occurred after 4-HT-induced activation

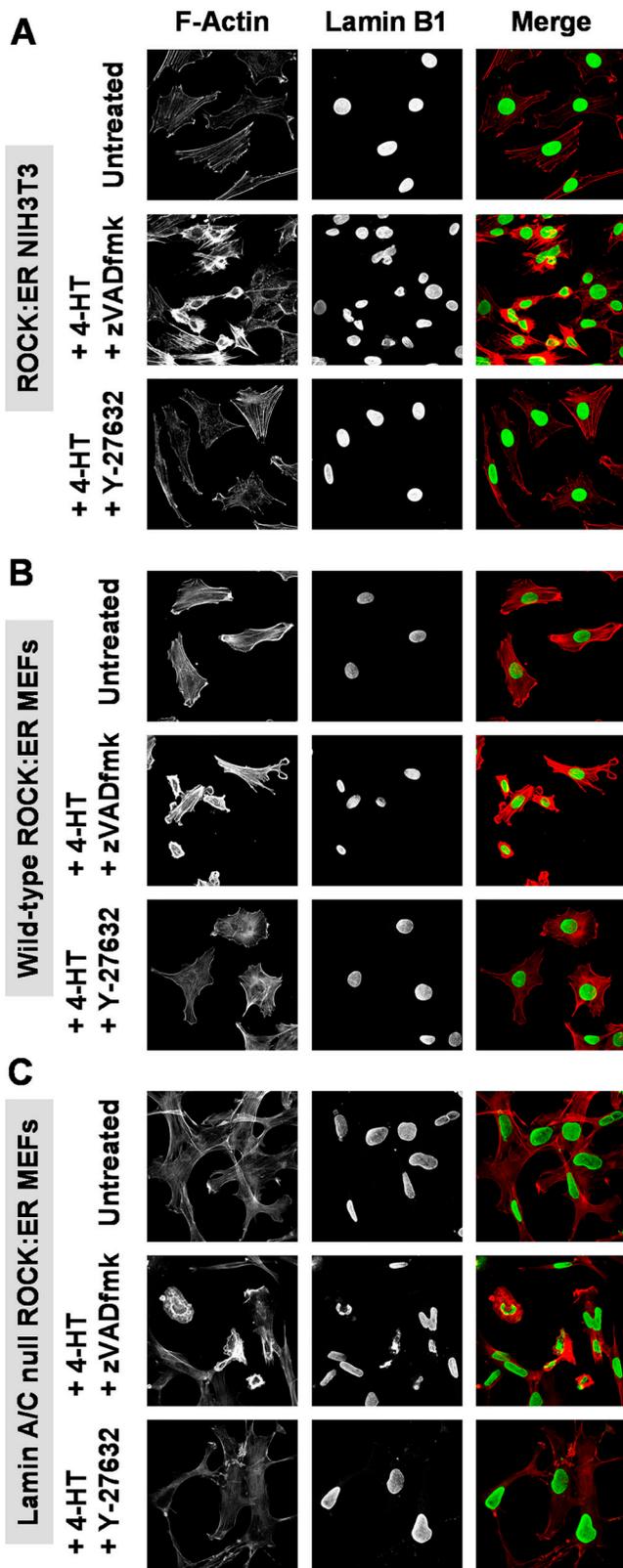


Figure 8. Active ROCK I induces contraction and membrane blebbing, but nuclear disruption only in lamin A/C null cells. Activation of ROCK I:ER with 4-HT resulted in increased filamentous actin structures and profound morphological responses. (A) ROCK I:ER-expressing NIH 3T3 cells had increased actin structures accompanied by cell contraction and membrane blebbing in ~50% of cells after treatment with 1 μ M 4-HT, either in the presence (middle panels) or absence (not depicted) of z-VAD-fmk.

of ROCK I:ER in the presence of caspase inhibitor z-VAD-fmk, which is consistent with these effects being directly induced and not secondarily through the induction of apoptosis. Western blotting of lysates prepared from ROCK I:ER- and KD:ER-expressing wild-type and lamin A/C null fibroblasts revealed no evidence of lamin B1 (a caspase 6 substrate; Fig. 9 A) or PARP (a caspase 3 substrate; Fig. 9 B) cleavage, which were readily detectable in cells made apoptotic by TNF α /CHX treatment. Western blotting for lamin A/C confirmed the absence of expression in the lamin A/C null cells (not depicted), whereas blotting for β -tubulin and ERK2 showed protein loading (Fig. 9, A and C). These results reinforce the conclusion that ROCK I:ER-mediated contraction is sufficient to induce nuclear disruption in nuclei with compromised lamina structures independent of caspase activation.

Discussion

Nuclear fragmentation is one of the classical hallmarks of apoptosis originally described by Kerr et al. (1972) three decades ago. Early studies concluded that caspase-mediated cleavage of nuclear structural proteins was sufficient for apoptotic nuclear disintegration (Neamati et al., 1995). However, we now show that in the absence of ROCK activity, MLC phosphorylation, or an intact actin cytoskeleton, the proteolysis of lamins, lamin-associated, and pore proteins is not sufficient for nuclear disintegration during apoptosis. Caspase-mediated cleavage of lamins B1 and A/C, Nup153, LAP2 α , and PARP occurred to the same extent in apoptotic cells with disintegrated nuclei or in ROCK inhibitor-treated cells with intact nuclei, indicating that proteolysis of nuclear proteins is not sufficient to rupture the nucleus during apoptosis. In addition, expression of conditionally active ROCK I:ER induced membrane blebbing and distortion of nuclear morphology without causing nuclear disintegration in NIH 3T3 and primary MEFs. However, genetic deletion of lamin A/C, which weakens the nuclear lamina occasionally resulting in spontaneous herniations (Sullivan et al., 1999), allowed ROCK-induced contraction to disrupt nuclear integrity. We propose that during apoptosis, cleavage of nuclear lamins A/C and B1 weakens the lamina and makes the nuclear envelope susceptible to tearing

Coadministration of ROCK inhibitor Y-27632 blocked the effects on actin structures and morphological responses (bottom panels). Nuclear morphology and integrity was assessed by staining for lamin B1 (middle column), ROCK-induced contraction and blebbing also resulted in contracted and distorted nuclei that remained intact (middle row), which could be blocked by ROCK inhibitor Y-27632 (bottom row). (B) Primary wild-type mouse embryo fibroblasts (MEFs) transduced with ROCK I:ER responded to 4-HT treatment with increased filamentous actin structures and many contracted, blebbing cells (middle row), responses that could be blocked by ROCK inhibitor Y-27632 (bottom row). ROCK-induced contraction and blebbing also resulted in contraction of nuclei (middle column) as determined by lamin B1 staining. (C) Primary lamin A/C null MEFs transduced with ROCK I:ER also responded to 4-HT with increased filamentous actin structures, cell contraction, and membrane blebbing (middle row), which could be blocked with ROCK inhibitor Y-27632. Nuclei (middle column) in contracted blebbing cells were severely distorted and often disrupted, even in the presence of z-VAD-fmk (middle row), which could be blocked by ROCK inhibitor Y-27632.

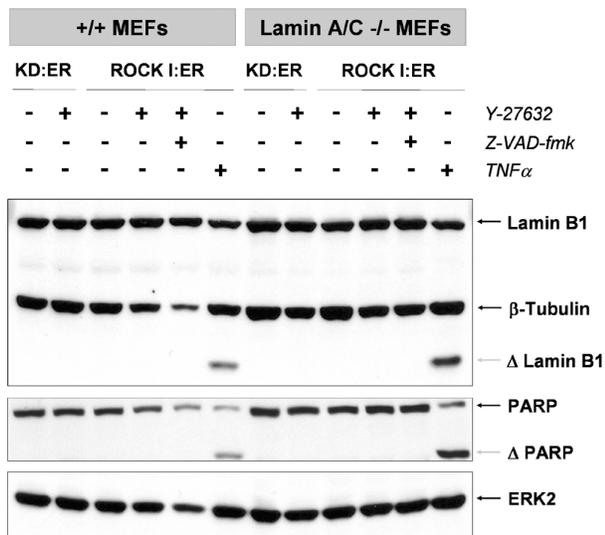


Figure 9. **ROCK I does not activate caspases.** Wild-type and lamin A/C null MEFs expressing ROCK I:ER or kinase dead KD:ER were left untreated or treated with 1 μ M 4-HT, either in the absence or presence of z-VAD-fmk as indicated. As a positive control for caspase activation, cells were treated with 25 ng/ml TNF α plus 10 μ g/ml CHX. Western blotting revealed that 4-HT treatment of KD:ER- or ROCK I:ER-expressing wild-type and lamin A/C null fibroblasts did not induce cleavage of lamin B1 (A) or PARP (B) although each was cleaved in response to TNF α treatment. (C) Western blotting for ERK2 reflects protein levels across samples.

by actin-myosin filaments, which contract due to caspase-activation of ROCK I. This proposed mechanism is analogous to, but distinct from, mitotic nuclear breakdown, which is mediated by phosphorylation-induced depolymerization of the nuclear lamina (Heald and McKeon, 1990) and microtubule-anchored pulling force generated by the minus-end-directed motor, cytoplasmic dynein, and components of its associated regulatory complex, dynactin (Beaudouin et al., 2002; Salina et al., 2002).

One unresolved question is the identity of the protein or proteins that couple actin-myosin filaments to the nuclear envelope, thereby facilitating the disruption of nuclear integrity during apoptosis. The leading candidates for this function are the spectrin family proteins Syne-1 and Syne-2 (of which there are numerous alternative splice variants and names including Myne-1/Nesprin-1/Enaptin/CPG2 and Myne-2/Nesprin-2/NUANCE) that have been shown to associate with actin filaments at their amino-terminal ends (Zhang et al., 2001, 2002; Zhen et al., 2002; Padmakumar et al., 2004) and insert into the nuclear membrane at the carboxyl-terminal ends (Apel et al., 2000; Zhang et al., 2001; Mislów et al., 2002b; Zhen et al., 2002; Padmakumar et al., 2004), where they may directly bind A-type lamins (Mislów et al., 2002a,b). Previous research has demonstrated that disruption of the Syne homologue ANC-1 in *Caenorhabditis elegans* leads to defects in nuclear tethering, which result in aberrant spacing of nuclei in multinucleated syncytial cells (Starr and Han, 2002). Therefore, we propose that during apoptosis the Syne proteins contribute to nuclear disintegration by serving as the physical link that couples the contraction of actin-myosin filaments to the physical tearing of

the weakened nuclear lamina and membrane. Efforts in our laboratory are currently underway to test this proposal.

Data from the *lmna* knockout mouse (Sullivan et al., 1999) and from RNAi studies (Harborth et al., 2001) have shown that Lamin A/C is not essential for viability. However, data from the knockout mouse and from human laminopathies (Burke and Stewart, 2002) indicate that Lamin A/C function is critical in specific tissues, most notably in skeletal and cardiac muscle where actin-myosin contractile forces are considerable. Therefore, we speculate that the viability of cells with mutated or deleted *lmna* is compromised in these tissues because actin-myosin contractile forces are sufficient to disrupt nuclear integrity, leading to cell death. Other tissues are apparently unaffected, likely due to actin-myosin contractile force being insufficient to significantly disrupt nuclear integrity.

Certain autoimmune diseases such as systemic lupus erythematosus are believed to arise due to immunological responses to nuclear autoantigens released from apoptotic cells. Treatment with ROCK inhibitor Y-27632 reduced the translocation of the 681 (Shiratsuchi et al., 2003) and D56R/S76R (Cocca et al., 2002) nuclear autoantigens to plasma membrane blebs and apoptotic bodies without affecting phosphatidylserine externalization (Coleman et al., 2001). The results of this work indicate that ROCK activity is required for actin-myosin contractile force generation and consequent disruption of nuclear integrity, which is required for the movement of DNA and proteins from the nucleus into blebs and apoptotic bodies. Therefore, ROCK inhibition may have some prophylactic and/or ameliorative benefits for the treatment of specific autoimmune diseases.

Materials and methods

Cell culture and plasmids

NIH 3T3 and primary MEFs were cultured in DME containing 10% FCS (Sigma-Aldrich) at 37°C in a 10% CO₂ atmosphere. To induce apoptosis, cells were placed in serum-free medium for 18 h, and then treated with 25 ng/ml TNF α (R&D Systems) plus 10 μ g/ml CHX (Sigma-Aldrich) for 2 h. For EM, cells were scraped in media and processed as described in the section TEM. For Western blotting, cells were scraped in media, lysed in 1 \times Laemmli buffer, briefly sonicated to shear DNA, and heated to 100°C for 5 min before loading equal portions onto SDS-PAGE.

Rat MLC cDNA (provided by L. Machesky, The University of Birmingham, Edgbaston, Birmingham, UK) was subcloned into the EcoRI restriction site of pEGFP-N3 (CLONTECH Laboratories, Inc.). Mutation of Thr18 Ser19 sites to Ala was done using a QuikChange site-directed mutagenesis kit (Stratagene) according to instructions. Human ROCK I:ER was made by subcloning a blunt-ended PCR product encoding amino acids 2–537 into filled-in *ScaI*-EcoRI sites of pBABE puro EGFP-Raf-1:ER (Woods et al., 1997; provided by M. McMahon, University of California San Francisco Comprehensive Cancer Center, San Francisco, CA). Mutation of Lys105 to Gly was done with QuikChange.

BOSC 293 ecotropic retroviral packaging cells were transfected with pBABE puro ROCK I:ER or KD:ER plasmid using Effectene (QIAGEN) according to instructions. After 36 h, supernatant was collected, centrifuged at 14,000 rpm for 15 min, and aliquots were stored at –80°C. Exponentially growing NIH 3T3 or MEFs were infected with undiluted retroviral supernatant mixed with 4 μ g/ml polybrene (Sigma-Aldrich) and selected with 2.5 μ g/ml puromycin (Sigma-Aldrich) to establish transduced pools.

Cytochalasin D, 4-HT, and swinholide A were from purchased from Sigma-Aldrich, z-VAD-fmk and staurosporine were purchased from BIOMOL Research Laboratories, Inc., and Y-27632 and Latrunculin B were purchased from Calbiochem.

TEM

Cell suspensions were pelleted by centrifugation, resuspended in 1 ml of fixative in an Eppendorf tube and immediately spun down again to form a pellet, and stored overnight at 4°C. Samples were postfixed in 2% osmium tetroxide, dehydrated through a graded series of ethanols, infiltrated, and embedded in Epon. For light microscopy, 1.0- μ m sections were cut, dried onto microscope slides, and stained with toluidine blue. For EM, 75-nm sections were collected onto copper grids, double stained with uranyl acetate and bismuth subnitrate, and examined in a transmission electron microscope (model 1010 Jem; JEOL) at 80 Kv accelerating voltage. Digital images were collected using a CCD camera (model 2k; Hamamatsu) aided with AMT 12-HR software.

Western blot analysis

After treatment as described in the figure legends, cells were lysed in 1 \times Laemmli sample buffer, sonicated, and electrophoresed on 10% SDS-PAGE before electro-transfer to nitrocellulose membranes. Blots were probed with antibodies against lamins B1 and A/C (Santa Cruz Biotechnology, Inc.), PARP (BD Biosciences), β -tubulin (Amersham Biosciences), GFP (CLONTECH Laboratories, Inc.), phospho-MLC (Thr18/Ser19), cleaved caspase 3 (Asp 175; Cell Signaling), MLC (Sigma-Aldrich), phospho-MYPT1 (Thr696; Upstate Biotechnology), MYPT1 (Covance), ROCK I (BD Biosciences), phospho-Ezrin (Thr567; Cell Signaling), Ezrin (Upstate Biotechnology), ER α (Santa Cruz Biotechnology, Inc.), phospho-LIMK1(Thr508)/LIMK2(Thr505) (Cell Signaling), LIMK1 (Cell Signaling), ERK2 (provided by C.J. Marshall, The Institute for Cancer Research, London, UK), Nup153 (provided by B. Burke, University of Florida College of Medicine, Gainesville, FL), or LAP2 α (provided by R. Foisner, Vienna Biocenter, Vienna, Austria) and appropriate HRP-conjugated secondary antibodies (Pierce Chemical Co.) followed by visualization with ECL (Amersham Biosciences) or SuperSignal West Femto (Pierce Chemical Co.) according to instructions and exposure to BioMax autoradiographic film (Kodak).

FACS sorting and analysis

NIH 3T3 cells were transfected with pEGFP-N3 (CLONTECH Laboratories, Inc.), pEGFP-N3 MLC-GFP, or pEGFP-N3 MLC(TASA)-GFP using Lipofectamine as described previously (Coleman et al., 2001). GFP-expressing cells were detached with trypsin, sorted, and collected using a FACSVerse cell sorter (BD Biosciences). The machine has a Coherent 90 C-4 argon ion laser that is tuned to 488 nm. This light was used to excite GFP, which was measured at 515–545 nm.

Immunofluorescence

NIH 3T3 and primary MEFs were fixed in 4% PFA, permeabilized with 0.5% Triton X-100, and stained in PBS with goat anti-lamin B1 antibody (Santa Cruz Biotechnology, Inc.) at 1:500 dilution followed by FITC-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories) at 1:200 dilution, or with mouse anti- β -tubulin (Sigma-Aldrich) at 1:200 dilution and FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories) at 1:200 dilution. Filamentous actin structures were stained with 1:250 dilution of Texas red-conjugated phalloidin (Molecular Probes). DNA fragmentation by TUNEL was done with a DeadEnd Fluorometric TUNEL kit (Promega) according to the manufacturer's instructions. Coverslips were mounted in Mowiol and visualized using a confocal microscope (model MRC1024; Bio-Rad Laboratories).

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