Myosin light chain kinase plays a role in the regulation of epithelial cell survival

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Summary

Myosin II activation is essential for stress fiber and focal adhesion formation, and is implicated in integrin-mediated signaling events. In this study we investigated the role of acto-myosin contractility, and its main regulators, i.e. myosin light chain kinase (MLCK) and Rho-kinase (ROCK) in cell survival in normal and Ras-transformed MCF-10A epithelial cells. Treatment of cells with pharmacological inhibitors of MLCK (ML-7 and ML-9), or expression of dominant-negative MLCK, led to apoptosis in normal and transformed MCF-10A cells. By contrast, treatment of cells with a ROCK inhibitor (Y-27632) did not induce apoptosis in these cells. Apoptosis following inhibition of myosin II activation by MLCK is probably meditated through the death receptor pathway because expression of dominant-negative FADD blocked apoptosis. The apoptosis observed after MLCK inhibition is rescued by pre-treatment of cells with integrin-activating antibodies. In addition, this rescue of apoptosis is dependent on FAK activity, suggesting the participation of an integrin-dependent signaling pathway. These studies demonstrate a newly discovered role for MLCK in the generation of pro-survival signals in both untransformed and transformed epithelial cells and supports previous work suggesting distinct cellular roles for Rho-kinase- and MLCK-dependent regulation of myosin II.

Supplementary material available online at
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Key words: Acto-myosin contractility, MLCK, ROCK, Apoptosis

Introduction

The interaction of normal epithelial cells with the extracellular matrix (ECM), is necessary for cell growth and survival (Frisch and Francis, 1994; Meredith et al., 1993; Meredith et al., 1996). When epithelial or endothelial cells are detached from the extracellular matrix they undergo a specialized form of apoptosis termed anoikis (Frisch and Francis, 1994). Anoikis is thought to function as a safeguard against cell relocalization to the wrong locations. During cell transformation a number of normal cellular functions are altered, including unregulated proliferation, immortalization and the ability of cells to grow outside their local environment (metastasize). The ability of tumor cells to bypass anoikis is believed to have an important role in metastasis (Bogenrieder and Herlyn, 2003; Frisch and Screaton, 2001; Hood and Cheresh, 2002; Rytomaa et al., 2000).

Our understanding of the signaling pathways activated by cell attachment to the ECM has been advanced by recent studies (reviewed by DeMali et al., 2003; Ginsberg et al., 2005; Guo and Giancotti, 2004). Cell adhesion to the extracellular matrix is mediated through integrins, which are a family of heterodimeric transmembrane proteins that transmit signals from outside the cell to inside the cell (DeMali et al., 2003; Ginsberg et al., 2005; Schwartz and Shattil, 2000). These signals originate from sites called focal adhesions, clusters of activated integrins in the cell membrane, which are essential elements of cell proliferation and survival. The changes in cellular architecture associated with the formation of focal adhesions are accompanied by activation of various signal transduction pathways accompanied by tyrosine phosphorylation of focal adhesion kinase and paxillin and activation of cell cycle regulators, resulting in cell proliferation and survival signals (Assoian, 1997; Bershadsky et al., 2003; Geiger et al., 2001). Adhesion-dependent signals cooperate with mitogen-activated pathways whose effects are mediated by the Rho family of small GTPases (Burridge and Wennerberg, 2004; Ridley, 2001).

Transformation of cells is characterized by anchorage-independent cell growth, i.e. growth in soft agar, and activation of integrins is sufficient for anchorage-independent cell survival in some cases (Marconi et al., 2004; Meredith et al., 1993; Zahir et al., 2003). For example, a role for integrins in cell survival was demonstrated in studies in which function-blocking integrin antibodies caused epithelial cells to undergo apoptosis (Frisch and Francis, 1994; Meredith et al., 1996). Furthermore, MDCK cells expressing a constitutively active focal adhesion kinase (FAK), which is normally activated in response to integrin engagement, prevents cells from undergoing apoptosis when placed in suspension (Frisch et al., 1996b). These studies, as well as many others, demonstrate that integrin engagement and subsequent activation of downstream signaling pathways is essential for survival in epithelial cells.

The actin cytoskeleton and myosin II are key components of focal adhesion formation and recent studies have shown that an intact actin cytoskeleton is required for cell survival (Bharadwaj et al., 2005; Celeste Morley et al., 2003; Cheng et al., 2004; Kulms et al., 2002; Martin and Leder, 2001; Martin et al., 2004). In addition, expression of a mutant FAK that disrupts actin filaments causes apoptosis and loss of adhesion-independent growth in transformed cells (Westhoff et al., 2004), further implicating the cytoskeleton in pathways required for anchorage-independent cell survival. As described, myosin II is needed for the formation of stress fibers and focal adhesions. Since adherent cells depend upon activation of integrins for survival signals, we hypothesized that myosin II is a target for cell survival regulators. The formation of focal adhesions and stress fibers is dependent upon activation of myosin II (Chrzanowska-Wodnicka and Burridge, 1996). Activation of myosin II through light chain phosphorylation allows myosin to bind to actin filaments. The best characterized pathways for phosphorylation of myosin regulatory light chains are through Rho kinase (ROCK), and myosin light chain kinase (MLCK) (Kamm and Stull, 2001; Sorokina and Chernoff, 2005). In addition, ZIP kinase and DAP kinase have been shown to phosphorylate myosin light chain and have a role in cell motility and apoptosis (Bialik and Kimchi, 2004; Kawai et al., 1999; Komatsu and Ikebe, 2004) Why cells use multiple kinases to phosphorylate and activate myosin II is still poorly understood, however, the kinases probably function to regulate diverse biological processes dependent on myosin II.

Here, we show that inhibition of MLCK induces apoptosis in both untransformed and transformed MCF-10A cells, in agreement with recent findings that MLCK induces apoptosis in vivo (Fazal et al., 2005). Moreover, expression of dominant-negative FADD prevents apoptosis in these cells, suggesting that the involvement of the death receptor pathway in apoptosis is due to MLCK inhibition. We also show that activation of β_1 integrin is markedly decreased after inhibition of MLCK or actin polymerization, and addition of a β_1-activating antibody to adherent epithelial cells protected them from apoptosis, and this protection correlates with activation of FAK, suggesting that these agents induce apoptosis through suppression of integrin-mediated signaling. Interestingly, treatment with Y-27632, an inhibitor of ROCK, had little effect on cell survival, suggesting discrete roles for ROCK- and MLCK-dependent functions in the regulation of myosin II.

Results

The actin cytoskeleton has been recently implicated in cell survival and we sought to determine the possible role of myosin II and its regulators in cell survival using a mammary epithelial cell line, MCF-10A. We first determined if normal and Ras-transformed MCF-10A cells are susceptible to anoikis by plating the cells on polyHEMA. Plating of cells on polyHEMA does not allow cells to adhere to the substratum, thus inducing apoptosis in cells sensitive to loss of adhesion signaling. Cells were plated on polyHEMA for 16 hours in 2% BSA, and assayed for apoptosis using two compounds, annexin V and propidium iodide (PI), that bind to apoptotic cells. This allows the cells to be separated into three populations: live, early apoptotic and late apoptotic. MCF-10A cells were serum starved in all of the following experiments, and this was necessary to prevent cell clumping, which can lead to increased numbers of live cells because of cell-cell interactions (Rytomaa et al., 2000). In addition, because many survival pathways activated in response to cell adhesion are also activated by growth factors (Howe et al., 1998; Meredith et al., 1993; Plopper et al., 1995), we attempted to isolate only those pathways activated as a result of cell adhesion by serum starvation of the MCF-10A cells. After 72 hours, serum starved MCF-10A cells began to display signs of apoptosis (data not shown). All of the assays performed here are well under this time point, and therefore serum starvation did not have an impact on the number of apoptotic cells quantified in these assays. In addition, in all of the assays performed, the untreated cells have been subject to serum starvation as a control.

The FACS analysis results are summarized in the bar graph shown in Fig. 1 as the percentage of apoptotic cells. Approximately 70% of the untransformed MCF-10A cells undergo apoptosis when plated on polyHEMA for 16 hours (Fig. 1A). If MCF-10A cells plated on polyHEMA were cultured for an additional 36 hours, virtually all of the untransformed MCF-10A cells undergo apoptosis (data not shown). By contrast, the Ras-transformed MCF-10A cells were relatively resistant to anoikis when plated on polyHEMA, exhibiting less than 5% difference in apoptosis compared with adherent cells after 16 hours. In agreement with previous studies (Wang et al., 1997; Wang et al., 2002), we found that untransformed MCF-10A are sensitive to anoikis, whereas MCF-10A Ras cells are relatively insensitive (Wang et al., 1997). In addition, we performed the same experiments with another epithelial cell line, MDCK and MDCK Ras cells, and found similar results (Fig. 1B). A typical representation of the FACS results is shown in Fig. 1C. In the plot shown on the left, untreated cells are found mainly in the lower left quadrant, meaning there is minimal staining of annexin V and/or PI. In the plot shown on the right, apoptosis is induced, and the cells are separated into three populations: live, early apoptotic (stained with annexin V, lower right quadrant) and late apoptotic (stained with both annexin V and PI, upper right quadrant). The results above support previous studies demonstrating that normal epithelial cells require adhesion to the ECM for survival, whereas transformed cells are resistant to anoikis following the loss of cell attachment (Frisch and Francis, 1994; Meredith et al., 1993; Rytomaa et al., 2000).

Recent studies have underscored the importance of an intact actin cytoskeleton for cell survival in MCF-10A and HeLa cells, demonstrating that disruption of actin filaments by treatment with actin polymerization inhibitors (latrunculin A and B) or by actin filament disruption (cytochalasin B and D) induces apoptosis (Cheng et al., 2004; Kulms et al., 2002; Martin and Leder, 2001; Martin et al., 2004; Rubtsova et al., 1998). Similar experiments were repeated in this study to confirm these results in our cell lines. Apoptosis of MCF-10A and MCF-10A Ras was observed after treatments with latrunculin A (data not shown). To determine whether cells were undergoing apoptosis as a result of detachment from the substrate following disruption of the actin cytoskeleton, the activation state of caspases was examined in adherent cells. Untransformed and Ras-transformed MCF-10A cells were grown on coverslips and treated with 5 μM latrunculin A. The cells were then fixed and permeabilized, and stained with phalloidin, Topro-3 and an anti-caspase-3 antibody that

specifically recognizes the active form of caspase-3. MCF-10A and MCF-10A Ras cells treated with latrunculin A exhibit a complete disruption of actin filaments and activation of caspase-3 (supplementary material Fig. S1A). Staining with Topro-3, a nuclear stain, demonstrates the chromatin condensation of MCF-10A cells, another hallmark of apoptosis (supplementary material Fig. S1A). These experiments support previous studies suggesting that actin polymerization and/or an intact actin cytoskeleton are needed for epithelial cell survival, and these effects are observed in adherent cells.

Inhibition of MLCK induces apoptosis in adherent cells

Inhibitors of MLCK and ROCK were then used to determine the role myosin II activation might have in cell survival. Previous studies in epithelial cells suggesting a pro-survival role for the actin cytoskeleton (Kulms et al., 2002; Martin and Leder, 2001; Martin et al., 2004; Rubtsova et al., 1998), led us to the question of whether inhibition of myosin II light chain phosphorylation would induce apoptosis in epithelial cells. Both untransformed and Ras-transformed MCF-10A cells were treated with ML-7, ML-9 and Y-27632. ML-7 and ML-9 inhibit the catalytic activity of MLCK (Saitoh et al., 1987) and Y-27632 inhibits ROCK activity (Narumiya et al., 2000). The number of apoptotic cells was determined using FACS analysis as described above. Briefly, the cells were treated with MLCK and ROCK inhibitors for 16 hours, and cells were then collected, stained with annexin V FITC and PI and sorted by FACS analysis. As shown in Fig. 1, both MCF-10A and MCF-10A Ras undergo apoptosis in response to MLCK inhibition. After 16 hours ~50-60% of normal and transformed MCF-10A cells undergo apoptosis. This effect of MLCK inhibitors on apoptosis is time- and dosage-dependent (data not shown), and the concentration and duration of the inhibitor treatment used in this investigation was based on our previous studies of the effects of time and dosage. To ensure that the above results are not specific to MCF-10A cells, we then tested another epithelial cell line, MDCK, and similar results were obtained. However, MCF-10A and MCF-10A Ras cells treated with the ROCK inhibitor Y-27632 did not display significant apoptosis. Again, similar results were found in MDCK and MDCK Ras cells (Fig. 1B). A control to test for decrease in myosin light chain phosphorylation after treatment with inhibitors for 1 hour is demonstrated by western blotting in Fig. S1B in the supplementary material.

As with the treatment of latrunculin A, in order to determine whether cells were detaching from the substrate following

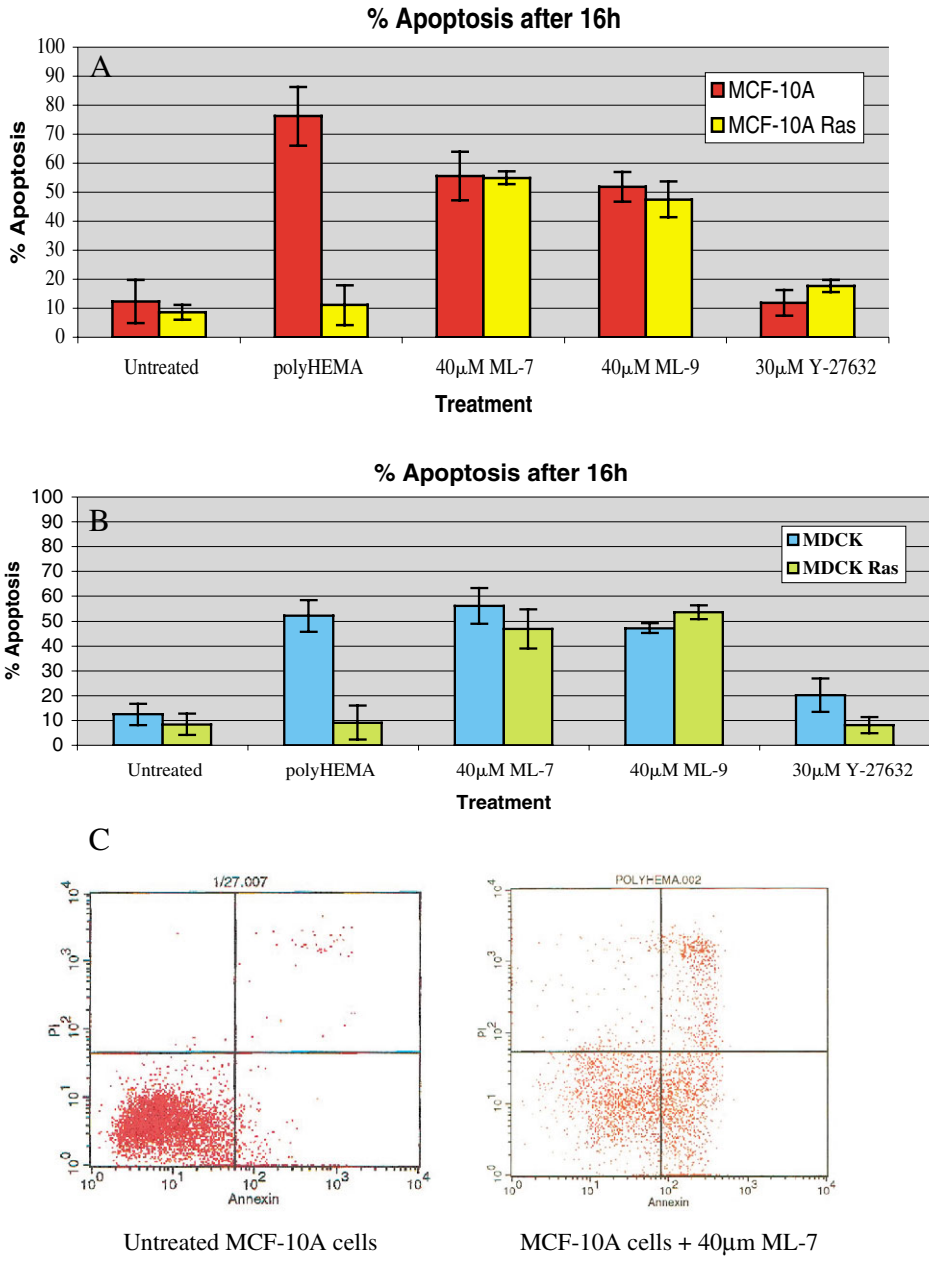

Fig. 1. FACS analysis of cells treated with MLCK and ROCK inhibitors. (A) Percentage apoptosis of untransformed and Ras-transformed MCF-10A cells grown on polyHEMA or treated with inhibitors of MLCK or ROCK. Cells were plated on polyHEMA and adherent cells were treated with ML-7 (40 μM), ML-9 (40 μM) and Y-27632 (30 μM) for 16 hours. Cells were then collected, stained with annexin V FITC and propidium iodide and subjected to FACS analysis. Results presented are the mean ± s.e.m. of at least three experiments. (B) Percentage apoptosis of MDCK and MDCK Ras cells grown on polyHEMA or treated with inhibitors of MLCK or ROCK. (C) Example plot of untreated MCF-10A cells, and MCF-10A cells treated with ML-7.

inhibition of MLCK, and then undergoing apoptosis owing to loss of MLCK activity, adherent cells were monitored for signs of apoptosis. Untransformed and Ras-transformed MCF-10A cells were grown on coverslips and treated with MLCK and ROCK inhibitors. The cells were then fixed and permeabilized, and stained with phalloidin, Topro-3 and an anti-caspase-3 antibody that specifically recognizes the active form of this caspase. Figs 2 and 3 show activation of caspase-3 in adherent cells treated with the MLCK inhibitors ML-9 or ML-7 after 16 hours of treatment. In agreement with the FACS analyses, cells treated with 30 μM Y-27632 for 16 hours exhibited little or no activation of caspase-3 above untreated cells (Fig. 4).

It is noteworthy that the morphology of cells treated with MLCK or actin filament polymerization inhibitors seems to disrupt cell-cell adhesions, whereas ROCK inhibition does not. Whether this is a result of apoptosis (cell rounding) or a factor contributing to apoptosis is unknown at this stage. These results demonstrate that inhibition of MLCK causes apoptosis in both normal and transformed epithelial cells, suggesting a role for MLCK in cell survival. Furthermore, these results support and extend previous studies implicating distinct cellular functions for MLCK and ROCK (Totsukawa et al., 2004; Totsukawa et al., 2000).

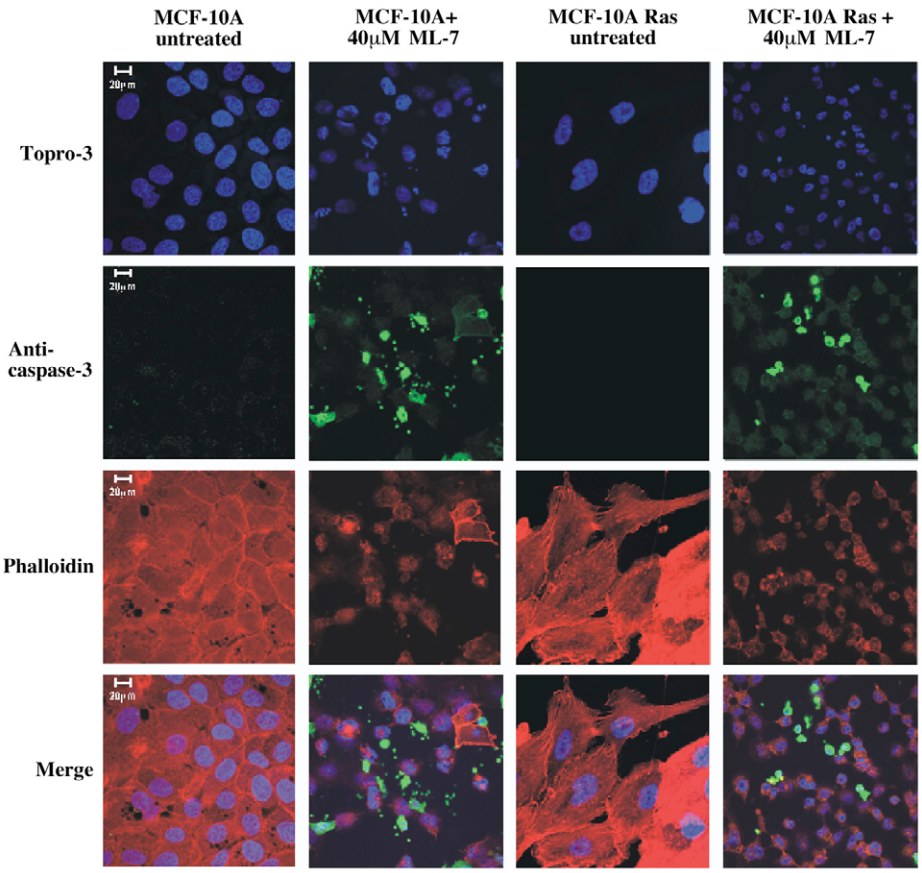

Fig. 2. Inhibition of MLCK by ML-7 induces activation of caspase-3 in adherent MCF-10A and MCF-10A Ras-transformed cells. Cells were grown on coverslips and treated with ML-7 (40 μM) for 16 hours. Cells were then fixed with 3% paraformaldehyde, then permeabilized and stained with Topro-3, anti caspase-3 (Cell Signaling) and anti-actin (Rhodamine-phalloidin).

Table 1. Percentage apoptosis after transfection of wild-type MLCK

	MCF10A Parental NM MLCK wt	MCF10A Parental SM MLCK wt
16 hours	7±2	6±1
24 hours	7±2	8±2
36 hours	8±2	8±2
48 hours	10±2	9±1

Expression of dominant-negative MLCK induces apoptosis in untransformed and Ras-transformed MCF-10A cells

The above data demonstrate that treatment of cells with pharmacological agents that inhibit myosin light chain kinase induces apoptosis. In order to provide additional evidence supporting a role for MLCK in cell survival, MCF-10A cells were transiently transfected with wild-type and kinase-dead non-muscle and smooth muscle (short) MLCK. As shown in Fig. 5, cells transfected with the dominant-negative proteins undergo apoptosis, visualized by Topro-3 staining. Table 1 displays the percentage of apoptotic cells in the total cell population, as well as the percentage of apoptotic cells that were FLAG tagged. Table 2 summarizes the percentage of apoptosis in transfected cells. The numbers of apoptotic cells were counted by scoring cells for chromatin condensation at 16, 24, 36 and 48 hours. For each time point, three independent sets of transfections were carried out and random fields, totaling 100 cells were counted. The total number of transfected cells was between 30% and 40%. As shown, the number of apoptotic cells transfected with kinase-dead MLCK increases with time, and by 72 hours, there are no detectable transfected cells (Table 2). We attribute the inability to detect transfected cells on the coverslips after 72 hours to the apoptosis observed after expression of a kinase-dead MLCK, resulting in loss of adhesion. As a control, wild-type smooth muscle MLCK was transfected into MCF-10A cells, and these transfected cells displayed 5-10% apoptosis (Table 1), the same as control untransfected cells. Owing to the fact that the cells underwent apoptosis within 72 hours, stable cell lines could not be produced, and the state of myosin light chain phosphorylation could not be tested. These studies further indicate that MLCK has a role in the survival of both untransformed and transformed epithelial cells.

Apoptosis induced by inhibition of MLCK is suppressed by expression of dominant-negative FADD

Next, we investigated the apoptotic

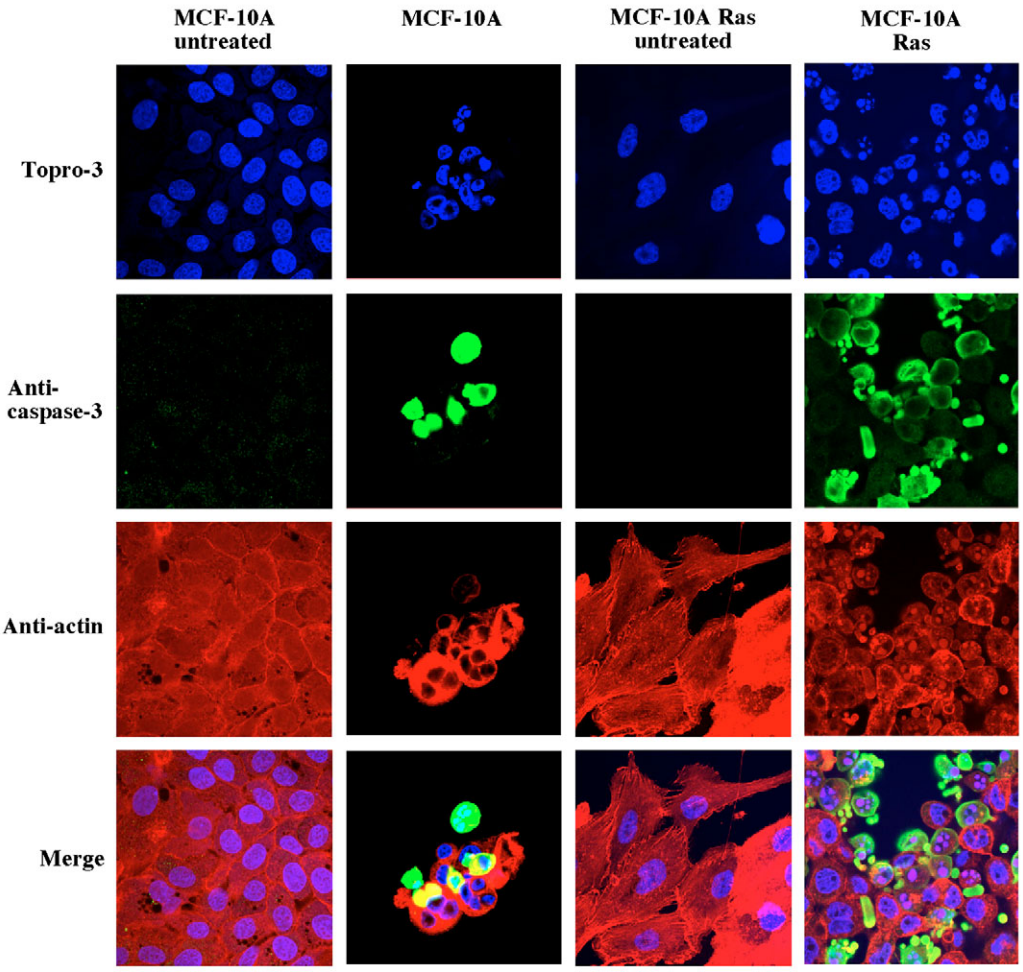

Fig. 3. Inhibition of MLCK by ML-9 induces activation of caspase-3 in adherent MCF-10A and MCF-10A Ras-transformed cells. Cells were grown on coverslips and treated with ML-9 (40 μM) for 16 hours. Cells were then fixed with 3% paraformaldehyde, permeabilized and stained with Topro-3, anti caspase-3 (Cell Signaling) and anti-actin (Rhodamine-phalloidin).

pathway that is activated in response to MLCK inhibition. Apoptosis can be initiated through an intrinsic or extrinsic pathway (reviewed by Fridman and Lowe, 2003). The intrinsic pathway is characterized by mitochondrial release of cytochrome c, whereas the extrinsic pathway is activated through transmembrane death receptors. Overexpression of a dominant-negative component of the CD95/Fas death receptor, FADD, was shown to inhibit anoikis in MDCK cells (Frisch, 1999; Frisch et al., 1996a). We used this dominant-negative FADD vector and stably transfected MCF-10A cells. FADD initiates caspase activation by recruiting caspase-8 to the cell membrane, resulting in self-activation of caspase-8, and subsequent activation of downstream caspases. A dominant-negative FADD prevents activation of caspase-8 when apoptosis is initiated through the death receptor pathway, resulting in protection from apoptosis (Zhang et al., 1998). As shown in Fig. 6, cells transfected with DN FADD are protected from apoptosis when treated with ML-7 or ML-9. Stably transfected MCF-10A cells were tested in an anoikis assay, and we found that they were resistant to anoikis (Fig. 6), similar to

Table 2. Percentage apoptosis after transfection of DN MLCK

	% Apoptosis of total cells		% Apoptosis of transfected cells	
	MCF-10A Parental NM MLCK KD	MCF-10A Parental SM MLCK KD	MCF-10A Parental NM MLCK KD	MCF-10A Parental SM MLCK KD
16 hours	39±5	30±6	79±6	83±6
24 hours	39±5	33±7	87±5	85±5
36 hours	49±8	38±5	90±5	88±6
48 hours	56±6	47±5	92±8	96±4
	MCF-10A Ras NM MLCK KD	MCF-10A Ras SM MLCK KD	MCF-10A Ras NM MLCK KD	MCF-10A Ras SM MLCK KD
16 hours	25±4	27±5	66±7	69±4
24 hours	41±6	30±8	80±8	78±6
36 hours	49±6	38±4	94±6	85±5
48 hours	53±7	43±3	93±5	94±6

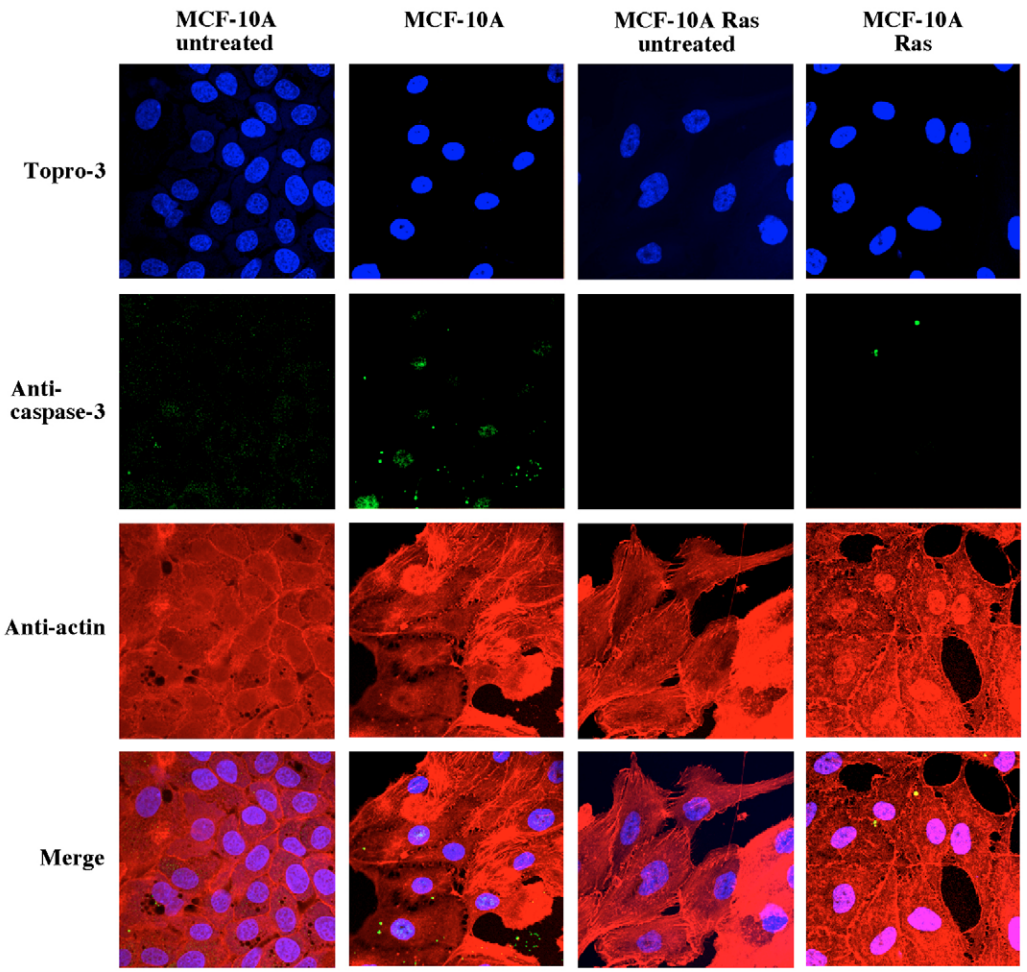

Fig. 4. Inhibition of ROCK by Y-27632 does not activate caspase-3 in adherent MCF-10A and MCF-10A Ras-transformed cells. Cells were grown on coverslips and treated with Y27632 (30 μM), for 16 hours. Cells were then fixed with 3% paraformaldehyde, permeabilized and stained with Topro-3, anti caspase-3 (Cell Signaling) and anti-actin (Rhodamine-phalloidin).

the results found previously in MDCK cells expressing DN FADD (Frisch, 1999). DN FADD expressing MCF-10A cells were characterized by the expression of truncated FADD protein (data not shown). The MCF-10A cells expressing DN FADD also exhibited a different morphology from untransfected cells, with decreased cell-cell contacts, and a more fibroblast-like morphology. However, stably transfected MCF-10A Ras cells did not appear to have a different morphology to untransfected MCF-10A Ras cells. Thus, it appears that apoptosis induced following inhibition of MLCK is mediated through the death receptor pathway, and this apoptosis is not dependent on expression of the Fas ligand, which was observed by western blot after treatment of MCF-10A cells (data not shown).

Inhibition of MLCK results in decreased activation of β_1 integrin in untransformed and Ras-transformed MCF-10A cells

Integrins and soluble growth factors cooperate to promote cellular response to ECM components to generate signals required for cell survival and proliferation (DeMali et al., 2003; Geiger et al., 2001). To determine if activation of integrins might be involved in mediating the apoptotic response of cells to inhibition of actin polymerization, or MLCK, we first analyzed the active state of integrins using a β_1 integrin antibody that specifically recognizes the integrin is in its active state. MCF-10A and MCF-10A Ras cells were treated with MLCK inhibitors ML-7 and ML-9, the actin polymerization inhibitor latrunculin A, and a myosin II specific inhibitor, blebbistatin (BB) (Kovacs et al., 2004) for 2 hours, before any caspase activation is detected. Blebbistatin was used in this experiment to determine if the results observed with MLCK inhibitors are consistent with the results of inhibition of myosin II activity. MCF-10A and MCF-10A Ras cells were stained with a β_1-integrin antibody that recognizes β_1 integrin in its active state called HUTS21 (Fig. 7). This antibody was used in a similar assay by Vial and colleagues to demonstrate suppression of β_1 signaling by Fra-1 (Vial et al., 2003). All treatments resulted in a significant decrease in levels of active β_1 integrin, supporting a role for integrin activity downstream of MLCK-dependent pathways. MCF-10A and MCF-10A Ras cells were also treated with the ROCK inhibitor, Y-27632, and no significant decrease in active β_1 integrin was detected, although the distribution of active β_1 integrin was different (data not shown).

Interestingly, the localization of active β_1 integrin in treated cells changes, from cortical regions and at the ends of stress fibers to a more perinuclear and cytoplasmic distribution. Furthermore, to demonstrate the decreased activity levels of β_1 integrin was not due to a decrease in the amount of total integrin protein on the cell surface, cells were stained with a β_1-integrin antibody that recognizes total β_1 integrin (supplementary

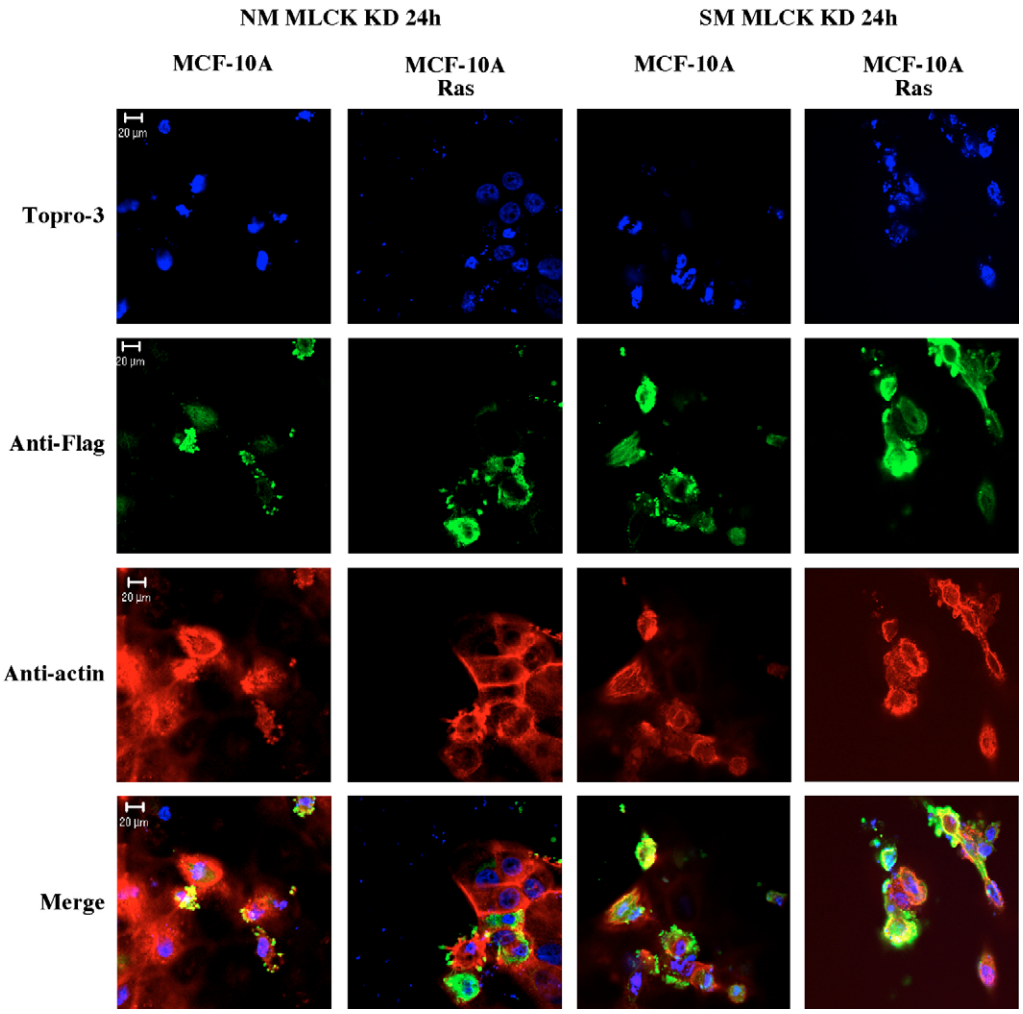

Fig. 5. Expression of kinase-dead MLCK activates caspase-3 in MCF-10A cells. (A) Normal and Ras-transformed MCF10A cells transfected with a kinase-dead MLCK undergo apoptosis. Cells were grown on coverslips, transfected with a FLAG-tagged kinase-dead non-muscle (NM MLCK KD) or kinase-dead smooth muscle MLCK (SM MLCK KD) using Lipofectamine 2000 (Invitrogen). Cells were grown for 24 hours, fixed in 3% paraformaldehyde and stained with anti-FLAG polyclonal antibody (Sigma), Topro-3 and anti-actin (Rhodamine-phalloidin).

material Fig. S2) and a western blot was performed after 2 hours of treatment (Fig. 7C). These results suggest that inhibition of actin polymerization, myosin II phosphorylation, or MLCK is associated with the inactivation of integrin β_1 in untransformed and Ras-transformed MCF-10A cells.

A β_1-integrin-activating antibody can protect untransformed and Ras-transformed MCF-10A cells from apoptosis by inhibition of MLCK or disruption of actin filaments through a FAK-dependent mechanism

Previous studies have shown that HMT-3522 mammary epithelial cell lines are dependent upon activation of β_1 integrin for survival (Weaver et al., 1996). The results presented above (Fig. 7) suggested that β_1 integrin inactivation could be a prerequisite for inducing apoptosis via inhibition of myosin activation through MLCK or actin filament polymerization. To test this hypothesis, cells were treated with a β_1-integrin-activating antibody before addition of latrunculin A, ML-7, and ML-9, and tested for rescue from apoptosis following inhibition of MLCK, myosin II and actin filament polymerization. Accordingly, a β_1-integrin-activating antibody, 9EG7, which can maintain activation of β_1 integrin, was added to cells before drug treatment. Untransformed and Ras-transformed MCF-10A cells were grown in the presence of 20 μg/ml 9EG7 as described in the Materials and Methods. Cells were then treated with the inhibitors above, stained with Annexin V FITC and propidium iodide and subjected to FACS analysis. As shown in Fig. 8, both untransformed and transformed MCF-10A cells are protected from apoptosis by addition of 9EG7; however, there appears to be a greater protective effect with 9EG7 in the MCF-10A parental cell lines.

To address the issue of which signaling pathways are involved in MCF-10A cell rescue of apoptosis by 9EG7, we investigated the components downstream of integrin activation. Since overexpression of a constitutively active FAK can rescue epithelial cells from anoikis (Frisch et al., 1996b), we investigated whether FAK had a role in the rescue of apoptosis by 9EG7. Cell extracts were made of serum-starved MCF-10A cells pre-treated for 24 hours with 9EG7 and subsequently treated with blebbistatin, latrunculin A and ML-7 for 1 hour. As shown in Fig. 8C, cells treated with inhibitors of MLCK, actin filament polymerization or myosin II alone for 1 hour display a decrease in phosphorylation of FAK. However, suppression of phospho-FAK was not observed in cells pre-treated with the β_1-integrin-activating antibody, 9EG7 (Fig. 8C). In addition, the total FAK levels are unchanged (Fig. 8C).

These results suggest that activation of myosin II through MLCK is required for the generation of anti-apoptotic signals by activating integrin-dependent pathways in both

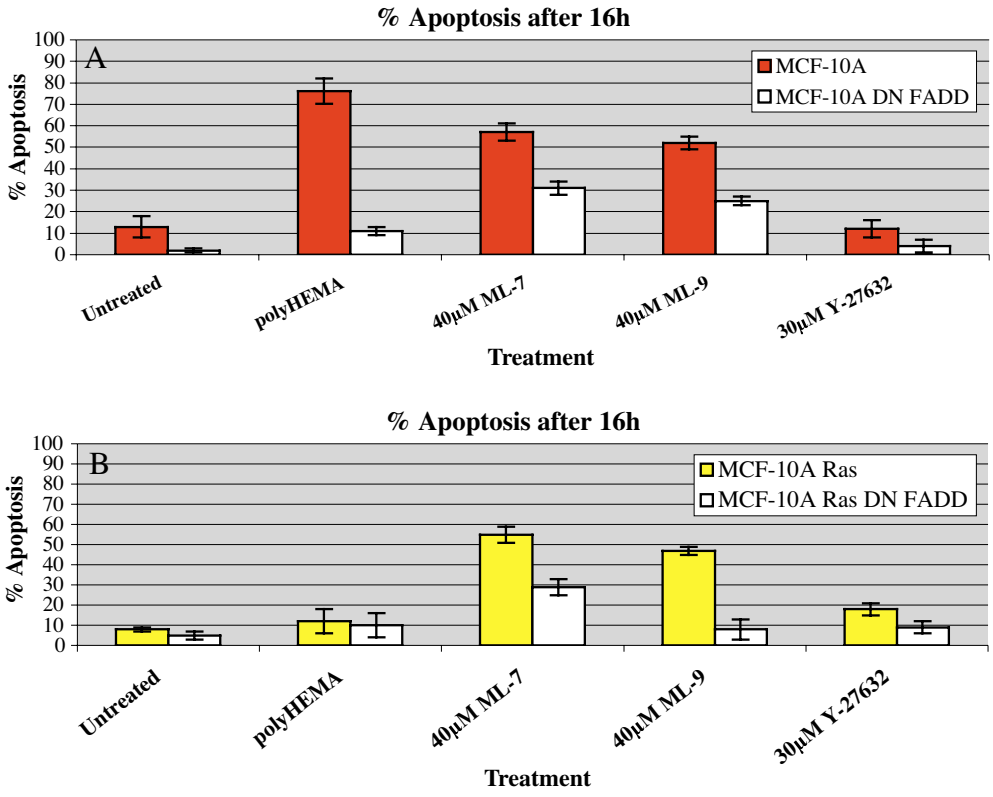

Fig. 6. Overexpression of dominant-negative FADD can inhibit apoptosis in MCF-10A cells treated with MLCK or actin polymerization inhibitors. FACS analysis of MCF-10A (A) and MCF-10A Ras-transformed (B) cells expressing pBabe DN FADD after plating on polyHEMA or treatment with ML-7, ML-9 or Y-27632 for 16 hours. Results are the mean ± s.e.m. of at least three experiments.

untransformed and Ras-transformed cells. Furthermore, addition of an α_3- or α_5-integrin-blocking antibody, which does not allow activation of either α_3 or α_5 integrins, causes MCF-10A Ras cells to undergo apoptosis (data not shown). This effect was specific for α_3 and α_5 integrin subunits because addition of blocking antibodies for α_2 or α_6 subunits had no effect (data not shown). Both α_3 and α_5 integrins can bind with β_1 integrin, supporting the above data that MCF-10A Ras cells are rescued from apoptosis through activation of β_1 integrin.

Discussion

Based on the hypothesis by Burridge and others, activation of acto-myosin contractility is an indispensable step in the process of formation of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Delanoe-Ayari et al., 2004; DeMali et al., 2003; Helfman et al., 1999). In this study we investigated the role of acto-myosin contractility and its main regulators, i.e. MLCK and Rho-kinase. We found that untransformed and Ras-transformed epithelial cells are dependent upon MLCK activity for survival, and this survival is mediated through integrin activation. This study indicates that survival signals in both untransformed and transformed cells are dependent on MLCK activity, and not ROCK activity. Adhesion of untransformed cells to matrix results in cytoskeletal remodeling, including myosin II activation via MLCK resulting in integrin clustering and adhesion-dependent signaling (Fig. 9, model 1). Interestingly, our studies also demonstrated that Ras-transformed cells are also dependent on MLCK and integrin activation for survival signals (Fig. 9). Thus, although one might expect Ras-transformed cells to bypass the requirement for cytoskeletal remodeling and acto-myosin contractility for survival signals, our data demonstrate that acto-myosin contractility under the control of MLCK is required for cell survival (Fig. 9, model 2).

Interestingly, several groups have proposed that an autocrine loop exists in transformed cells involving constitutive activation of integrins. Lee et al. (Lee et al., 2004) have shown that transformed mammary epithelial cells can secrete a protein that associates with integrins and promotes cell adhesion and cell survival. In addition, Weaver and colleagues, investigated breast tumors that express large amounts of laminin-5, a matrix protein (Weaver et al., 1996). Their findings demonstrate that an autocrine LM-5 mediates anchorage-independent survival in breast tumors through ligation of a particular integrin. This integrin mediates tumor survival through activation of basal and epidermal-growth-factor-induced RAC activity, and RAC mediates tumor survival (Zahir et al., 2003). These studies support the idea that a transformed cell escapes apoptosis through constitutive activation of integrins, and therefore adhesion-mediated signaling. As shown in Fig. 9, we propose a model (model 2) in which Ras-transformed cells could target upstream regulators of myosin II, i.e. MLCK, leading to formation of integrin-dependent signaling complexes and

Fig. 7. Decreased activation of β_1 integrin in MCF10A cells after inhibition of myosin II, actin polymerization and MLCK. Untransformed MCF-10A (A) or Ras-transformed MCF-10A cells (B) were treated with MLCK, myosin II and actin polymerization inhibitors, as indicated. Cells were grown on coverslips and treated for 2 hours, then fixed in 3% paraformaldehyde and stained with an anti-β_1-integrin antibody (clone HUTS-21), which recognizes integrins in their active state, Topro-3 and anti-actin (Rhodamine-phalloidin). (C) Western blot showing level of β_1 integrin after 2 hours of treatment with inhibitors.

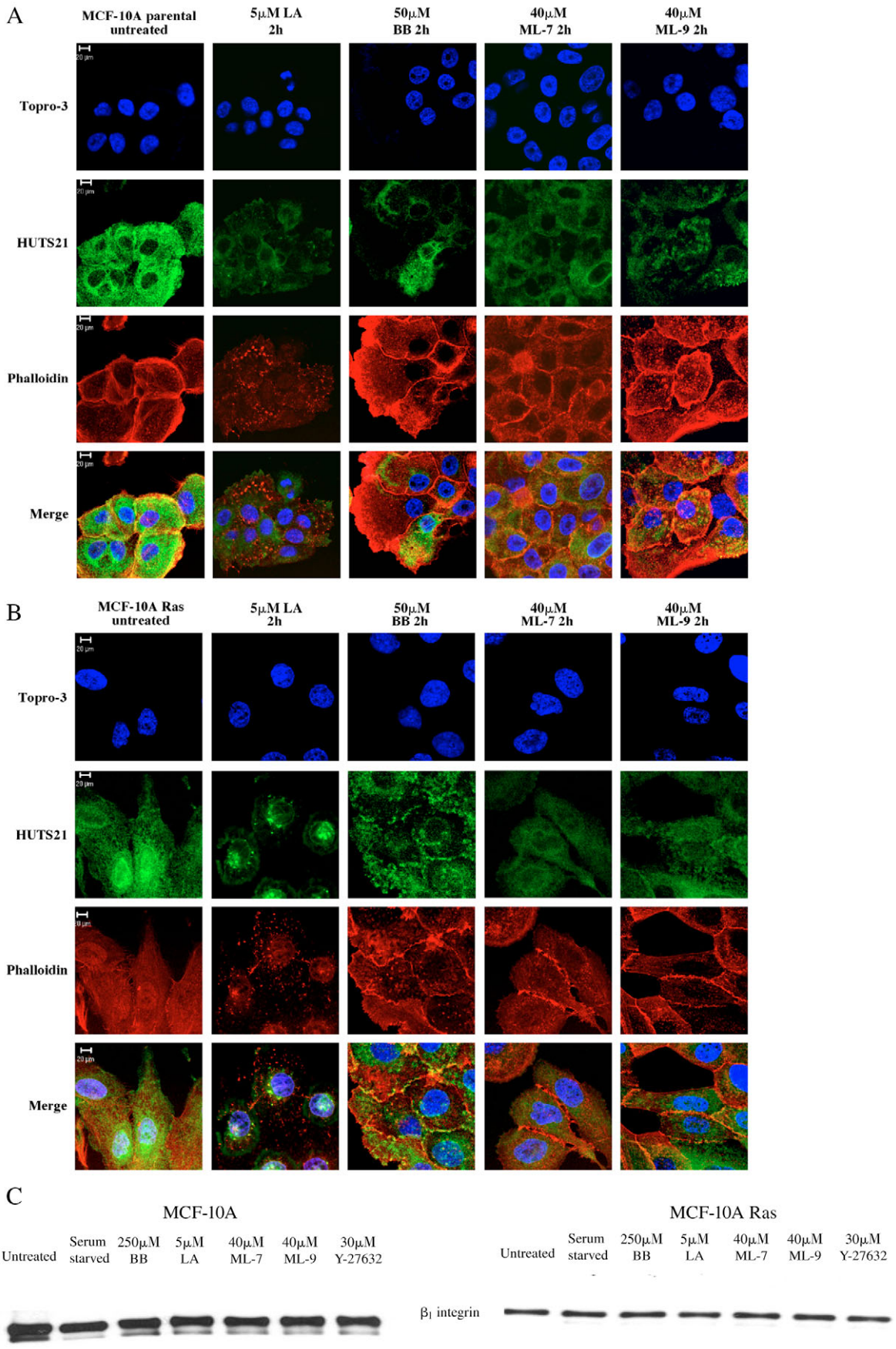

Fig. 7. See previous page for legend.

initiation of survival signals even in the absence of cell adhesion. This would allow a transformed cell to bypass the requirement for cell adhesion.

Several studies have indicated that MLCK or ROCK have a pro-apoptotic role in the apoptotic program (Bisson et al., 2003; Coleman et al., 2001; Jin et al., 2001; Petrache et al., 2003; Petrache et al., 2001; Sebbagh et al., 2001). MLCK and ROCK were both found to be cleaved and in a constitutively active state when cells were treated with tumor necrosis factor (TNF), an inducer of apoptosis (Petrache et al., 2003; Petrache et al., 2001). The constitutively active MLCK or ROCK is thought to contribute to membrane blebbing observed during late apoptosis (Mills et al., 1998; Song et al., 2002). In addition, Gallagher and colleagues found that inhibition of MLCK decreased apoptosis in TNF-treated cells in the early stages of apoptosis (Jin et al., 2001). Myosin light chain phosphorylation status was found to correlate with localization of TNFR to the cell membrane, linking myosin II activation to TNF-induced apoptosis. These results are not necessarily in conflict with our data demonstrating a requirement for MLCK activity for cell survival. Myosin II has multiple functions within the cell, and our studies demonstrate a previously uncharacterized role for myosin II and myosin light chain kinase in preventing apoptosis in cells. It is possible that activation of actomyosin contractility is needed in pathways that both prevent and facilitate the apoptotic program. An adherent cell may depend upon activation of actomyosin contractility for survival, and once apoptosis is initiated, these same proteins contribute to the new cell program. In addition, a recent study by de Lanerolle and colleagues found that inhibition of MLCK induces apoptosis in mammary cells in vivo (Fazal et al., 2005).

Here, we have shown that inhibition of ROCK has no effect on cell survival, but inhibition of MLCK induces apoptosis. There are several possible explanations for these observations. One scenario is that ROCK inhibition by Y-27632 blocks myosin light chain phosphorylation (as shown in

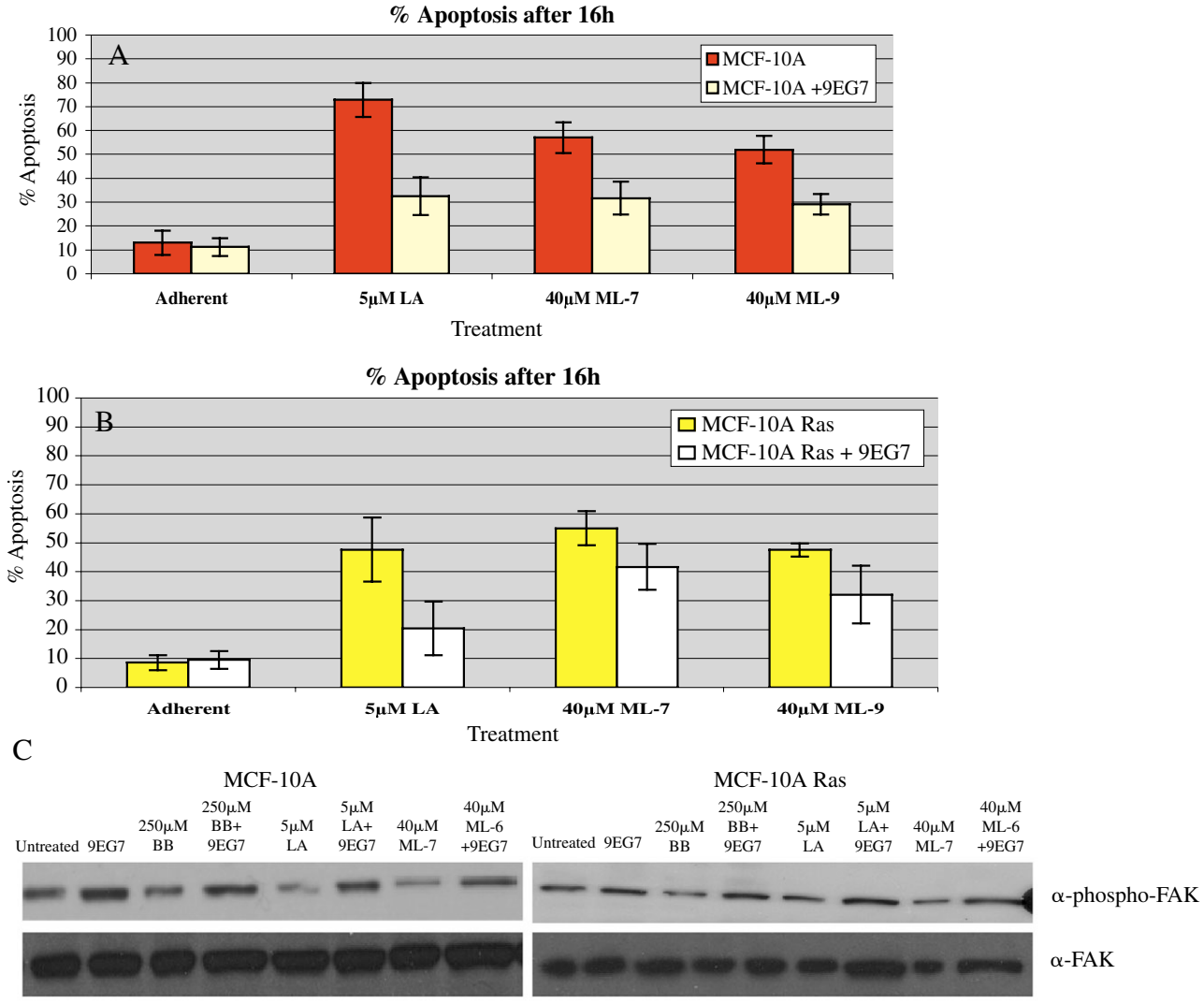

Fig. 8. Addition of a β_1-integrin-activating antibody, 9EG7, rescues cells from apoptosis as a result of MLCK or actin polymerization inhibition. MCF-10A (A) and MCF-10A Ras-transformed (B) cells were plated with 20 μg/ml 9EG7 for 24 hours and subsequently treated with ML-7, ML-9 and LA. (C) Phospho- FAK is decreased in cells treated with inhibitors, and restored in MCF-10A and MCF-10A Ras cells pre-treated with 9EG7, then incubated with Blebbistatin (BB), LA or ML-7 for 1 hour. Total FAK levels remain the same in each cell line with treatments (C).

supplementary material Fig. S1B), but other targets downstream of ROCK remain unaffected, leading to cell survival, whereas the effects of MLCK inhibition are on myosin light chain phosphorylation alone. This is probably not the case, because the inhibition of ROCK catalytic activity by Y-27632 should block all ROCK activity. However, the possibility remains that unknown functions or targets of ROCK exist. A second explanation for the results presented here is that the pharmacological agents used are affecting other unknown pathways. We have attempted to use control experiments to ensure that this has not occurred.

The observation made here that ROCK is unnecessary for MCF-10A cell survival, and that MLCK has a role in the maintenance of epithelial cells, supports the view that these two kinases have distinct functions within the cell. It was previously shown that ROCK and MLCK have distinct functions in the spatial regulation of myosin light chain phosphorylation in 3T3 fibroblasts, with MLCK responsible for the formation of stress fibers at the periphery of the cell and ROCK necessary for the assembly of stress fibers in the center of the cell (Totsukawa et al., 2000). Further evidence for the separate roles of MLCK and ROCK shows that during cell migration MLCK and ROCK have specialized functions within the cell, with MLCK necessary for membrane ruffling, and ROCK discovered to be more important for efficient cell migration (Totsukawa et al., 2004). Recently we reported that activation of the ERK cascade by Ras was dependent on MLCK and not ROCK (Helfman and Pawlak, 2005). ROCK activation is also required for survival in some cell types (Li et al., 2002), indicating that the effects seen here are cell-type specific.

We show that activation of β_1 integrin is markedly decreased in MCF-10A and MCF-10A Ras cells after treatment with agents that inhibit myosin, myosin light chain kinase and actin polymerization (Fig. 9). Furthermore, addition of a β_1-activating antibody to epithelial cells protected them from apoptosis through a FAK-dependent mechanism, suggesting that these agents induce apoptosis through suppression of integrin-mediated signaling. However, the activating antibody shows a greater protective effect on MCF-10A parental cells compared with the Ras-transformed MCF-10A cells treated with MLCK inhibitors (Fig. 8), suggesting that the Ras-transformed cells may be undergoing apoptosis in response to more than one cellular cue. MCF-10A Ras cells were tested for their dependence on active integrins for survival, and we found that blocking α_3 or α_5 integrin using specific antibodies induces significant apoptosis in MCF-10A Ras cells (data not shown). This supports the notion that Ras-transformed cells are dependent on active integrins for survival, and also supports the model in Fig. 9 showing that regulation of myosin II downstream of Ras in transformed cells could generate survival signals.

Although both ROCK and MLCK are implicated in the formation of integrin-containing adhesive structures, further studies will be required to determine how these kinases differentially mediate integrin-dependent function. Finally, the signaling pathways involved in apoptosis through the suppression of integrin activation remain to be determined.

In conclusion, we have shown that disruption of actin filaments or inhibition of MLCK in untransformed and Ras-transformed MDCK or MCF-10A cells induces apoptosis. Our results are supported by studies demonstrating that inhibition of MLCK activity induces apoptosis in vivo (Fazal et al., 2005). Apoptosis following inhibition of MLCK is mediated through the death receptor pathway because expression of dominant-negative FADD, or treatment of cells with zIETD-FMK (data not shown) – both inhibitors of caspase-8 activation – blocked apoptosis. In addition, apoptosis induced by disruption of actin filaments and inhibition of MLCK is probably due to inhibition of integrin-mediated signaling because treatment of cells with integrin-activating antibodies prevented apoptosis. These studies also demonstrate that MLCK, but not ROCK, has an essential role in the survival of both untransformed and transformed epithelial cells and indicate distinct cellular roles for Rho-kinase- and MLCK-dependent functions involving the regulation of cell survival.

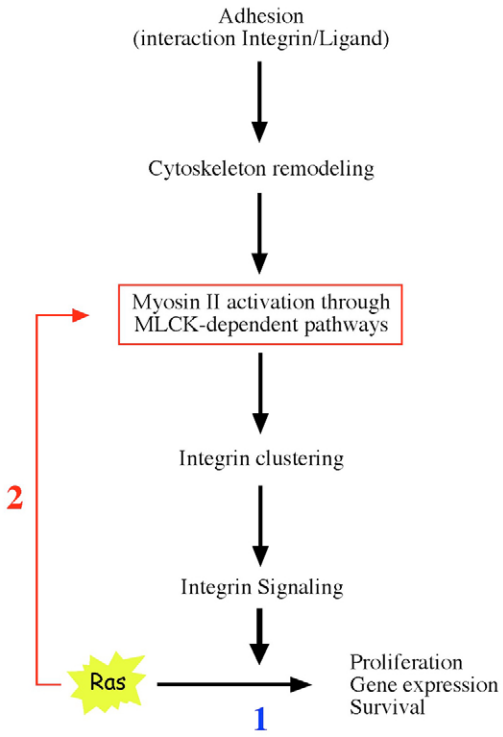

Fig. 9. Model for adhesion-dependent and adhesion-independent integrin signaling. In model 1, survival signals are generated by both adhesion and growth factors, as seen in untransformed cells. Model 2 illustrates a possible mechanism of eliminating the requirement for adhesion by Ras-transformed cells, but signaling events that are still dependent on myosin II. We propose that Ras-transformed cells can activate myosin II through MLCK, leading to integrin activation and associated survival signals.

Materials and Methods

Antibodies and reagents

Cleaved caspase-3 polyclonal antibody was purchased from Cell Signaling (Beverly, MA). Trevigen TACS apoptosis kit was purchased from Trevigen (Gaitherburg, MD). Phalloidin was purchased from Molecular Probes (Eugene, OR). Blebbistatin, Y-27632, ML-7 and ML-9 were purchased from Calbiochem (San Diego, CA). latrunculin A was purchased from Molecular Probes. β_1-activating antibody 9EG7 and β_1 HUT21 antibodies were purchased from BD Pharmingen (San Diego, CA). P5D2 β_1 antibody was obtained from Santa Cruz. β_1 clone LM534 was purchased from Chemicon (Temecula, CA). Phospho S19-MLC antibody was obtained from Abcam (Cambridge, MA) and MLC antibody from Chemicon (Temecula, CA).

Cell culture and drug treatments

MCF-10A cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 5% donor horse serum, 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 10 μg/ml insulin (Sigma, St Louis, MO), 1 ng/ml cholera toxin (Sigma), 100 μg/ml hydrocortisone (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). MDCK cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin. MCF-10A cells were a generous gift from David Soloman (NIH, Bethesda, MD). MDCK cells were obtained from ATCC. They were maintained in DMEM containing 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin MDCK Ras cells were a gift from Linda Van Aelst (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Cells were treated with vehicle alone, Blebbistatin, ML-7, ML-9, latrunculin A or Y-27632 in media without serum at varying concentrations and time points before being fixed or prepared for FACS analysis. Untreated cells were also serum starved for the duration of the inhibitor treatment. Stock solutions of each agent were made in DMSO (50 μM Blebbistatin, 5 mM LA), 50% ethanol (10 mM ML-7), 70% ethanol (20 mM ML-9) or water (20 mM Y-27632) and were maintained at –20°C.

Cell extracts and immunoblotting

Cells were washed with ice-cold PBS containing 2 mM sodium orthovanadate before direct extraction in 2% SDS Laemmli sample buffer. Lysates were clarified by centrifugation (16,000 ***g***, 15 minutes at 4°C) and protein concentrations were measured by bicinchoninic acid protein assay (BioRad, Hercules, CA). Equal amounts of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell Bioscience). The membranes were incubated with primary antibodies overnight at 4°C. After incubation with appropriate HRP-conjugated secondary antibodies, the immunoreactive bands were detected by chemiluminescence (NEN) according to the manufacturer's instructions.

Immunofluorescence

Cells were grown on glass coverslips and treated with indicated drug. The cells were then fixed in 3% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and blocked with 5% BSA at room temperature for 30 minutes. Incubations with primary antibodies against active caspase-3 (1:50), or anti β_1 integrin HUTS21 or P5D2 (1:100) were conducted at room temperature in 1% BSA for 1 hour. Incubations with secondary antibody and Rhodamine-phalloidin were conducted in 1% BSA for 45 minutes. Cells were then stained with Topro-3 1:100 in PBS at room temperature for 15 minutes and coverslips were mounted using Prolong Antifade (Molecular Probes). Coverslips were examined with a Zeiss confocal microscope and analyzed by LSM 5 Image Examiner software.

Fluorescence-activated cell sorting

The Trevigen ApoTACS kit was used to stain cells before cell sorting. Cells were grown on 24-well plates and treated with actin or myosin inhibitors for various times and concentrations. Cells were trypsinized and media was collected, washed twice in ice-cold PBS and stained with annexin V FITC and propidium iodide at room temperature for 15 minutes. Cells were then sorted by a Becton Dickinson LSRII and analyzed by FACS Diva software.

Anoikis assays

Tissue culture dishes were incubated with 300 mg/ml polyHEMA (Sigma) in 95% ethanol, and allowed to dry overnight at room temperature. Dishes were rinsed three times with PBS before use. For anoikis assays, cells were trypsinized, washed twice with 1× PBS and replated on polyHEMA in DMEM with 1% BSA to prevent clumping of cells.

Transfection

The non-muscle and smooth muscle MLCK wild-type and dominant-negative plasmids were a generous gift of Patricia Gallagher (Indiana University, Indianapolis, IN). Transient transfection of MCF10A cells with these constructs was performed with Lipofectamine 2000 (Invitrogen). The dominant-negative FADD vector was kindly provided by Martin Schuler (Johannes Gutenberg University, Mainz, Germany). pBabe Hygro DN FADD cell lines were produced by transfecting MCF10A cells with Lipofectamine 2000 and selecting with 750 μg/ml G418. Following selection, cells were pooled, expanded, and tested for the expression of DN FADD proteins by western blotting, using antibodies for FADD (Calbiochem).

Integrin activation assays

MCF-10A cells were trypsinized and washed twice with 1× PBS with 2% BSA. Cells were then resuspended with 15 μg/μl of β_1-activating antibody clone 9EG7 and incubated on ice for 30 minutes. Cells were then replated, allowed to spread, and treated with the appropriate agents.

Statistical analysis

All experiments were performed at least three times. The student's *t*-test was used statistical analysis of data. Values are expressed as mean ± s.e.m. Data were considered statistically significant at $P<0.01$.

We wish to thank Patricia Gallagher for providing non-muscle and smooth muscle MLCK cDNA constructs, and Martin Schuler for supplying us with pBabe Hygro DN FADD. We also thank Linda Van Aelst for providing Ras-transformed MDCK cells. Special thanks Yuri Lazebnik, Senthil Muthuswamy and Geraldine Pawlak for critical comments and helpful discussions. D.M.H. is supported by a grant from National Cancer Institute (CA83182).

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