Disruption of α-actinin-integrin interactions at focal adhesions renders osteoblasts susceptible to apoptosis

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Triplett, Jason W., and Fredrick M. Pavalko. Disruption of α-actinin-integrin interactions at focal adhesions renders osteoblasts susceptible to apoptosis. Am J Physiol Cell Physiol 291: C909–C921, 2006. First published June 28, 2006; doi:10.1152/ajpcell.00113.2006.—Maintenance of bone structural integrity depends in part on the rate of apoptosis of bone-forming osteoblasts. Because substrate adhesion is an important regulator of apoptosis, we have investigated the role of focal adhesions in regulating bone cell apoptosis. To test this, we expressed a truncated form of α-actinin (ROD-GFP) that competitively displaces endogenous α-actinin from focal adhesions, thus disrupting focal adhesions. Immunoﬂuorescence and morphometric analysis of vinculin and tyrosine phosphorylation revealed that ROD-GFP expression dramatically disrupted focal adhesion organization and reduced tyrosine phosphorylation at focal adhesions. In addition, β-catenin protein levels were reduced in ROD-GFP-expressing cells, but caspase 3 cleavage, poly-(ADP-ribose) polymerase cleavage, histone H2A.X phosphorylation, and cytotoxicity were not increased due to ROD-GFP expression alone. Increases in both ERK and Akt phosphorylation were also observed in ROD-GFP-expressing cells, although inhibition of either ERK or Akt individually or together failed to induce apoptosis. However, we did find that ROD-GFP expression sensitized, whereas α-actinin-GFP expression protected, cells from TNF-α-induced apoptosis. Further investigation revealed that activation of TNF-α-induced survival signals, specifically Akt phosphorylation and NF-κB activation, was inhibited in ROD-GFP-expressing cells. The reduced expression of antiapoptotic Bcl-2 and inhibited survival signaling rendered ROD-GFP-expressing cells more susceptible to TNF-α-induced apoptosis. Thus we conclude that α-actinin plays a role in regulating cell survival through stabilization of focal adhesions and regulation of TNF-α-induced survival signaling.

osteoPorsis is a bone wastinG disease in which loss of bone results in compromised skeletal strength (37). Several factors influence the maintenance of bone shape and structural integrity, including remodeling rate, loading intensity and frequency, and calcium metabolism (36, 40, 51). Central to the formation of new bone is the maintenance of an active bone-forming osteoblast population. However, it has been proposed that >70% of osteoblasts at sites of bone remodeling undergo apoptosis, whereas others differentiate into bone lining cells or osteocytes (21). Because alterations in osteoblast apoptosis rates could affect both physiological and pathophysiological conditions, understanding the cellular mechanisms that regulate osteoblast apoptosis is important.

Focal adhesions are protein complexes composed of clustered integrin dimers and related proteins that mediate the connection between the extracellular matrix (ECM) outside the cell and the actin cytoskeleton inside the cell (50). In many cell types, important survival signals from the ECM are transmitted through proteins localized to focal adhesions (3). Anoikis, or detachment-induced apoptosis, occurs as a natural process in maintenance of tissue homeostasis and has been documented in multiple cell and tissue types (13). Whereas classic anoikis involves cellular detachment from the substrate, recent studies have shown that changes in cell shape (12) or the presence of unoccupied integrins (44) also can induce anoikis. The established role of integrins in regulating survival signals (14, 26, 55) and the role of focal adhesion-associated proteins in anoikis resistance (2, 52) suggest that maintenance of focal adhesion organization is important for cell survival.

In addition to survival signals initiated at focal adhesions, there is evidence of cross talk between focal adhesion signaling pathways and receptor-mediated pathways (38, 47, 53). One receptor-mediated pathway that plays a key role in osteoblast physiology is the tumor necrosis factor-α (TNF-α) pathway. TNF-α is thought to play a central role in postmenopausal bone loss by stimulating osteoclastogenesis, inhibiting osteoblast precursor differentiation, and increasing osteoblast apoptosis (27). When TNF-α binds its receptor, it stimulates receptor trimerization, the recruitment of death domain-containing proteins, and the activation of a caspase cleavage cascade, eventually leading to apoptosis (6). In addition, TNF-α activates a survival pathway mediated by Akt and the nuclear translocation and increased transcriptional activity of nuclear factor-κB (NF-κB) (56). Recently, evidence of cross talk between the TNF-α pathway and focal adhesion signaling has been noted; specifically, TNF-α-induced NF-κB nuclear translocation is inhibited in focal adhesion kinase (FAK)-deficient cells (16).

In this study we manipulated osteoblast focal adhesion organization by expressing green fluorescent protein (GFP)-tagged versions of truncated or full-length α-actinin. First described as an actin bundling protein, α-actinin can also localize to focal adhesions through binding to the cytoplasmic tails of β-integrins (29, 32). At focal adhesions, α-actinin primarily functions to link β-integrins to filamentous actin, but it also interacts with several focal adhesion proteins, including vinculin, zyxin, palladin, FAK, extracellular signal-related kinase (ERK), and cysteine-rich protein (4, 8, 18, 23, 30, 34). These interactions potentially position α-actinin to play a central role in both focal adhesion organization and signaling. To disrupt the function of endogenous α-actinin in focal adhesions, we expressed a truncated version of α-actinin that lacks the actin binding domain but retains the central “rod” region, containing spectrin repeats responsible for dimerization and integrin binding (29). Therefore, this construct, which we
term ROD-GFP, is thought to be able to localize to focal adhesions but not to recruit or stabilize actin or, potentially, α-actinin’s other interacting partners therein. Thus ROD-GFP is thought to function as a competitive inhibitor of endogenous α-actinin, specifically at focal adhesions.

Presently, we report that expression of ROD-GFP in primary cells obtained from rat calvaria (Rcob) and UMR106 osteoblast-like cells disrupts focal adhesion organization, as evidenced by vinculin immunofluorescence and decreased total cellular tyrosine phosphorylation, and specifically, FAK phosphorylation. In addition, ROD-GFP-expressing cells have reduced levels of the antiapoptotic protein Bcl-2 but do not exhibit increased levels of apoptosis or cytotoxicity. Other basal effects of ROD-GFP expression include increased phosphorylation of both isoforms of the ERK mitogen-activated protein kinase (MAPK) and Akt. Inhibition of either of these pathways individually or together does not induce apoptosis in ROD-GFP-expressing cells. Interestingly, analysis of receptor-mediated apoptosis revealed that cells expressing ROD-GFP are more sensitive and α-actinin-GFP-expressors are more resistant to TNF-α-induced apoptosis. We propose that increased TNF-α-induced apoptosis in ROD-GFP-expressing cells results from decreased survival signaling through the TNF receptor, given that these cells have less TNF-α-induced NF-κB nuclear translocation and activity, as well as inhibited TNF-α-induced Akt phosphorylation. Together, these results suggest that α-actinin plays an important role in focal adhesion organization, regulation of Bcl-2 expression, and TNF-α-induced survival signaling.

**MATERIALS AND METHODS**

**Cell culture.** The rat osteosarcoma cell line UMR106.01 (UMR106) was cultured in minimal essential medium (MEM; Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and antibiotics. Osteoblast-like cells were isolated from rat calvarium as described previously (19) and are referred to as Rcob throughout this text. Briefly, calvaria from 10–13 neonatal rats (birth to day 2) were isolated aseptically and minced and digested consecutively for 5, 20, and 45 min at 37°C in 0.2% collagenase P-0.25% trypsin. Cells released in the second and third digests (20- and 45-min digests) were pooled and cultured in MEM supplemented with 10% FCS and antibiotics. Experiments were performed on UMR106 passages 23–35 and Rcob passages 2–5.

**Immunofluorescence and morphometric analysis.** Cells grown on glass slides expressing GFP, ROD-GFP, or α-actinin-GFP were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Immunofluorescent labeling was performed using a colorimetric assay for lactate dehydrogenase (LDH) release into the medium (Roche, Indianapolis, IN).

**Dual-luciferase assay.** UMR106 (3.0 × 10^5 cells) or Rcob (1.0 × 10^5 cells) were seeded in six-well dishes. On the following morning, cells were transfected with 1 µg of appropriate construct (pShuttle vector from Clontech, Mountain View, CA), 0.5 µg of pNFkB-LUC reporter construct (BD Biosciences), and 0.5 µg Renilla luciferase reporter gene (a gift from B. P. Herrin, Indiana University School of Medicine) using the FuGene transfection reagent (Roche). At 24 h posttransfection, cells were switched to serum-free medium. At 48 h postransfection, cells were treated with vehicle (0.1% BSA in PBS)
or 10 ng/ml murine TNF-α (Calbirochem) for 4 h. Cells were collected and analyzed on a Centro LB 620 luminometer (Berthold Technologies, Bad Wilbad, Germany) using the dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s instructions. Results are reported as relative luciferase activity (±SD). Statistical significance was determined using Student’s t-test, with a P value <0.01 considered significant.

RESULTS

Expression of truncated α-actinin results in disorganization, but not complete disruption, of F-actin stress fibers. Using an adenoviral vector, we expressed a truncated form of α-actinin, which lacks the actin binding domain. Endogenous α-actinin exists as a homodimer in cells and localizes to focal adhesions via its spectrin repeat domain binding to β1-integrin cytoplasmic tails (32). The truncated version we expressed (ROD-GFP) retains the central spectrin repeats, allowing the protein to localize to focal adhesions but not to recruit or stabilize actin therein. Previously, we reported that microinjection of a similar protein fragment into fibroblasts resulted in complete disruption of the actin cytoskeleton and disassembly of focal adhesions (31). To verify that expression of ROD-GFP had similar effects in cultured osteoblasts, we infected Rcob and UMR106 osteoblast-like cells with GFP, ROD-GFP, and a GFP-tagged full-length α-actinin construct (α-actinin-GFP). Using phalloidin immunofluorescence, we observed that in both UMR106 and Rcob control cells, F-actin was well organized, with bundled stress fibers formed primarily around the cell periphery (Fig. 1, A and C, top). In control Rcobs, GFP appeared to colocalize with actin stress fibers as well as in the nucleus, although stress fiber colocalization was not seen in UMR106 cells or other cell types (Fig. 1, A and B, and data not shown). Actin stress fibers appeared more centrally localized and less bundled in UMR106 cells expressing ROD-GFP (Fig. 1A, middle). Stress fibers in Rcobs expressing ROD-GFP appeared condensed in the cell center but stained brightly with phalloidin, indicating bundled filaments (Fig. 1C, middle). Together, these data suggest that ROD-GFP expression leads to a disorganization of actin in both UMR106 cells and Rcobs, but not to a complete collapse of the actin cytoskeleton. However, it is important to note that very high levels of ROD-GFP expression achieved through the use of higher MOIs led to complete disruption of the actin cytoskeleton (data not shown). For the purposes of this study, we chose to use lower expression levels to avoid dramatic cytoskeletal disruption.

Under the infection conditions used in this experiment, we achieved comparable levels of GFP, ROD-GFP, and α-actinin-GFP expression in both UMR106 cells and Rcobs (Fig. 1, B and D, top). To determine the relative level of exogenous protein to endogenous α-actinin, we performed Western blot analysis using an anti-α-actinin antibody raised against the actin-binding domain of α-actinin. The antibody used recognized α-actinin-GFP (~130 kDa) and endogenous α-actinin (~100 kDa) but not ROD-GFP (~100 kDa). We found that α-actinin-GFP expression levels were ~1.25-fold that of endogenous α-actinin in UMR106 cells, and they were ~0.2-fold that of endogenous α-actinin in Rcobs (Fig. 1, B and D, bottom). Because anti-GFP blots showed relatively equal expression of all three constructs, all three appear to be expressed at approximately similar levels relative to endogenous α-actinin. These modest levels of truncated α-actinin expression differ significantly from studies using microinjected proteolytic fragments, in which relative protein levels were between ~2.5- and 13-fold (31). We observed that 70% of UMR106 cells and 52% of Rcobs expressed exogenous protein in our system (data not shown). Because higher levels of expression of ROD-GFP could be achieved and a higher percentage of cells could be infected in UMR106 cells, these cells were used to further assess the role of α-actinin.

Expression of ROD-GFP disrupts focal adhesion organization. To determine the effect of ROD-GFP expression on focal adhesion organization, we quantitatively analyzed the focal adhesion protein vinculin by using immunofluorescent microscopy. Vinculin staining in cells expressing ROD-GFP indicated that focal adhesions were smaller in these cells compared with focal adhesions in GFP-expressing controls (Fig. 2, D and J). In addition, focal adhesions appeared to be more diffusely distributed throughout the cell in ROD-GFP expressors, as opposed to being concentrated at the cell periphery in controls (Fig. 2, B, D, H, and J). Similar results were seen in cells stained for the focal adhesion protein paxillin (data not shown).

Using the morphometric software Scion Image, we quantified the area, major axis length, and minor axis length of vinculin-stained focal adhesions in GFP-, ROD-GFP-, and α-actinin-GFP-expressing UMR106 and Rcob cells. In control UMR106 cells, focal adhesion area was 5.3 μm², with a major axis length of 4.15 μm and a minor axis length of 1.64 μm on average; and in control Rcobs, focal adhesion area was 12.9 μm², with a major axis length of 9.3 μm and a minor axis length of 1.7 μm (n > 400 focal adhesions in 10 cells) (Fig. 2, M and N, solid bars). In ROD-GFP-expressing UMR106 cells, we observed significant 44.0, 30.4, and 19.5% decreases in focal adhesion area, major axis length, and minor axis length, respectively (Fig. 2M, open bars). Interestingly, we also observed a significant 10.3% decrease in focal adhesion area in UMR106 cells expressing full-length GFP-tagged α-actinin but saw no significant difference in major or minor axis length in these cells compared with controls (Fig. 2, shaded bars). In ROD-GFP-expressing Rcobs, we observed significant 51.5 and 47.0% decreases in focal adhesion area and major axis length, respectively (Fig. 2N, open bars). We did not observe any changes in minor axis length due to ROD-GFP expression in Rcobs and did not observe changes in any focal adhesion dimension measured in Rcobs expressing α-actinin-GFP compared with controls (Fig. 2N, shaded bars).

To further assess focal adhesion organization and signaling capability, we assessed the basal tyrosine phosphorylation state of cells expressing GFP, ROD-GFP, or α-actinin-GFP. First, we immunofluorescently labeled phosphotyrosine residues in UMR106 cells. In control UMR106 cells, phosphotyrosine staining is distributed throughout the cell in ROD-GFP expressors, as opposed to being concentrated at the cell periphery in controls (Fig. 3, A, B, D, H, and J). Similar results were seen in cells stained for the focal adhesion protein paxillin (data not shown).

Using an anti-phosphotyrosine antibody (PeproTech) and anti-α-actinin antibody (C911), we performed Western blot analysis of global phosphotyrosine levels in cell lysates of UMR106 cells. Again, we observed a decrease in total tyrosine phosphorylation in cells expressing ROD-GFP (Fig. 3B, top). Furthermore, we also observed a specific decrease in FAK phosphorylation in cells expressing ROD-GFP.
Together, these data indicate that expression of ROD-GFP effectively disrupts focal adhesion organization and basal activation state of proteins therein.

**ROD-GFP expression decreases Bcl-2 expression without inducing apoptosis.** Analysis of apoptotic endpoints such as the activation of the effector protease caspase 3, its substrate PARP, and phosphorylation of histone H2A.X indicated that expression of ROD-GFP did not induce apoptosis (Fig. 4A). Cells treated with 10 ng/ml TNF-α and 10 μg/ml CHX for 4 h were used as a positive control for apoptosis (Fig. 4A, right lane). A colorimetric assay for LDH release into the medium revealed no increase in cellular cytotoxicity in ROD-GFP-expressing cells compared with GFP-expressing or mock-infected controls (Fig. 4B). Cells treated with 1% Triton X-100 were used as a positive control for LDH release. Importantly, no significant changes in total protein were observed in cells expressing ROD-GFP (data not shown), suggesting that early apoptosis or loss of cells did not occur.
To determine whether ROD-GFP expression affected the expression of BH3 family proteins, we performed Western blot analysis of the antiapoptotic Bcl-2 and Bcl-XL proteins. We observed that cells expressing ROD-GFP showed a significant decrease in Bcl-2 protein level and a trend toward a decrease in Bcl-XL (Fig. 4, C and D). Together, these data indicate that disruption of focal adhesions via ROD-GFP expression significantly reduces the expression of Bcl-2 without inducing apoptosis or cell death.

ERK MAPK and Akt phosphorylation are increased in ROD-GFP-expressing cells. Because survival signals from the ECM are known to be transmitted through MAPKs and Akt (10, 11), we assessed the activation state of the ERK MAPK and Akt. Surprisingly, in UMR106 cells expressing ROD-GFP, both phosphorylation of tyrosine 204 of ERK and serine 473 of Akt were increased greater than twofold compared with GFP-expressing control cells (Fig. 5, A and B). To determine whether the increased ERK activation may allow ROD-GFP
cells to survive despite having disrupted focal adhesions and reduced Bcl-2 protein, we specifically inhibited ERK with the MEK1 inhibitor PD-98059 or Akt with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002. Neither treatment with 25 μM PD-98059 for 12 h (Fig. 5C) nor treatment with 50 μM LY-294002 for 6 h was able to induce apoptosis in either control or ROD-GFP-expressing cells (Fig. 5D). In addition, treatment with both drugs for a period of 6 h did not induce apoptosis, indicating that increased activity of these proteins is not likely responsible for survival in ROD-GFP-expressing cells (Fig. 5E).

Fig. 3. Analysis of global tyrosine phosphorylation and focal adhesion kinase phosphorylation. A: UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP were fixed, permeabilized, and stained with an anti-phosphotyrosine antibody (PY20). Bars, 50 μm. B: Western blot analysis of tyrosine phosphorylation in UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP. Top, anti-PY20 blot; middle, anti-phospho-focal adhesion kinase (pFAK) blot; bottom, anti-glycogen synthase kinase-3β (GSK-3β) blot to verify equal loading. Experiments were performed in triplicate on 2 occasions, and representative examples are shown. IB, immunoblot.

Fig. 4. Analysis of apoptotic end points and cytotoxicity. A: Western blot analysis of caspase 3 (Casp 3), poly(ADP-ribose) polymerase (PARP), phospho-histone H2A.X (ph-H2A.X), and GSK-3β in UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP. Cells treated with 10 ng/ml TNF-α and 10 μg/ml cycloheximide (CHX) for 4 h were used as a positive control for apoptosis. cl-PARP, cleaved PARP. B: medium samples from UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP were analyzed for cytotoxicity using a colorimetric assay for lactate dehydrogenase (LDH). Experiment was performed in triplicate, and results are expressed as percentages of control (cells treated with 1% Triton X-100 for 1 h). *P < 0.01 vs. GFP; bars represent SD. C: Western blot analysis of Bcl-2, Bcl-XL, and GSK-3β levels in UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP. D: quantification of Bcl-2:GSK-3β and Bcl-XL:GSK-3β densitometric ratios in UMR106 cells expressing GFP, ROD-GFP, or α-actinin-GFP. Experiment was performed in triplicate. *P < 0.01 vs. GFP; bars represent SD.
Receptor-mediated apoptosis is augmented in cells expressing ROD-GFP and inhibited in cells expressing α-actinin-GFP. Because inhibition or downregulation of antiapoptotic BH3 family members can sensitize cells to receptor-mediated apoptosis (9, 39), we determined whether the reduced levels of Bcl-2 observed in cells expressing ROD-GFP rendered these cells more sensitive to receptor-mediated apoptosis. We treated UMR106 cells with increasing concentrations of TNF-α in the presence of CHX, which inhibits new protein synthesis and expedites the apoptotic process, for a period of 4 h. In control cells, treatment with 10 ng/ml TNF-α and 10 μg/ml CHX induced cleavage of caspase 3 and its substrate PARP, as well as increased histone H2A.X phosphorylation (Fig. 6, A, C, and E, left lanes). A titration of TNF-α up to 100 ng/ml showed
increasing amounts of caspase 3 and PARP cleavage, as well as histone H2A.X phosphorylation (Fig. 6, B, D, and F, open circles). Cells expressing ROD-GFP showed increased levels of PARP cleavage when treated with 1, 10, and 100 ng/ml TNF-α, increased caspase 3 cleavage at 1 and 10 ng/ml TNF-α, and increased H2A.X phosphorylation at 10 ng/ml TNF-α compared with control cells (Fig. 6, B, D, and F, filled squares). Cells treated without TNF-α were incubated with CHX alone to confirm that CHX itself did not induce apoptosis, suggesting the effect of ROD-GFP expression is on receptor-mediated apoptosis (Fig. 6, −TNF lanes).

In a separate experiment, cells expressing α-actin-GFP showed a resistance to TNF-α-induced apoptosis, TNF-α-induced PARP cleavage was reduced in α-actin-GFP-expressing cells at 1, 10, and 100 ng/ml TNF-α, and caspase 3 cleavage was reduced at the 10 ng/ml dosage compared with control cells (Fig. 7, B and D, closed triangles). No significant changes in TNF-α-induced H2A.X phosphorylation were observed. Together, these data suggest that disruption of focal adhesions and subsequent downregulation of Bcl-2 sensitizes cells to TNF-α-induced apoptosis.

TNF-α-induced survival signals are inhibited in cells expressing ROD-GFP. Because TNF-α also initiates a survival pathway after binding to its receptor, we determined whether cells expressing ROD-GFP or α-actin-GFP showed altered TNF-α-induced survival signaling. We treated UMR106 cells with TNF-α for 15 min and assessed Akt phosphorylation using Western blot analysis. As expected, ROD-GFP expression itself results in increased Akt phosphorylation, and there was no change in cells expressing α-actin-GFP (Fig. 8A, −TNF-α lanes). No increase in Akt phosphorylation in response to TNF-α was observed in cells expressing ROD-GFP, contrary to what was seen in control cells (Fig. 8, A and B, +TNF-α lanes and shaded bars). Paradoxically, TNF-α-induced Akt phosphorylation was not increased in cells expressing α-actin-GFP compared with control cells; in fact, it was not observed at all, despite the fact that these cells are slightly more resistant to TNF-α-induced apoptosis than control cells.

To further assess the role of α-actin in TNF-α survival signaling, we treated Rcob or UMR106 cells with TNF-α or vehicle for a period of 1 h. Immunofluorescent labeling of NF-κB was used to assess the nuclear translocation of this important survival signal. In vehicle-treated cells, NF-κB was diffusely localized throughout the cytoplasm under all expression conditions (Fig. 9, A and J). Treatment with TNF-α induced a robust nuclear translocation of NF-κB in GFP- and α-actin-GFP-expressing cells, indicating that the TNF-α-induced survival signaling pathway remains intact in cells expressing α-actin-GFP (Fig. 9, D–F and L). Interestingly, in cells expressing ROD-GFP, the TNF-α-induced nuclear translocation was reduced (Fig. 9K). To confirm these results, we utilized a dual-luciferase assay using a reporter construct with the NF-κB promoter to assess TNF-α-induced survival signaling. Rcob or UMR106 cells were transfected with the NF-κB reporter construct, a reference luciferase reporter, and GFP, ROD-GFP, or α-actin-GFP. In GFP control cells and α-actin-GFP-expressing cells, treatment with TNF-α for a period of 4 h in serum-free medium induced an approximately three- to fourfold increase in reporter luciferase activity (Fig. 9M). In ROD-GFP-expressing cells in both cell types tested, basal and TNF-α-stimulated NF-κB reporter activity were significantly inhibited compared with
GFP controls. In Rcobs, we observed small but significant decreases in basal and TNF-α-stimulated NF-κB reporter activity in cells expressing α-actinin-GFP. Importantly, cells transfected with a nonspecific promoter-luciferase reporter, the reference luciferase reporter, and GFP did not show any increase in luciferase activity in response to TNF-α (Fig. 9M, negative control).

DISCUSSION

Survival signals from the ECM are transmitted through proteins localized at focal adhesions (43). In most studies of focal adhesion function, attempts to disrupt the link between integrin dimers and the extracellular matrix have been made by plating cells on inhibitory matrices or incubating cells with function-blocking antibodies or soluble RGDS fibronectin-mimetic peptides (20, 22, 35). In the present study we have taken a novel approach by disrupting focal adhesion organization from the cytoplasmic face of the complex by expressing a truncated form of α-actinin, which displaces endogenous α-actinin from focal adhesions. This approach allowed us to examine the role of focal adhesion organization in physiological processes apart from dramatic...
changes in the actin cytoskeleton or cell shape. We observed that GFP itself localized in the nucleus, as expected, and also with actin stress fibers in Rcobs. Stress fibers are prominent in Rcobs, and GFP likely localizes nonspecifically with these structures because of their relative abundance in these cells. We do not propose that GFP has the ability to bind actin, because this phenomenon was not observed in UMR106 or other cell types, which have less abundant stress fibers.

Bcl-2 family proteins are major regulators of apoptosis in many cell types, and the relative expression levels of pro- and antiapoptotic members may determine the sensitivity of cells to apoptotic stimuli (42). In cells expressing ROD-GFP, we found decreased expression of the important antiapoptotic protein Bcl-2 and a trend toward a decrease in the related protein, Bcl-XL. Similarly, previous studies have shown that disruption of endothelial cell focal adhesions by chymase, an enzyme that degrades fibronectin, leads to a reduction in Bcl-2 mRNA (24). The mechanism by which focal adhesion organization, or α-actinin itself, may regulate the expression of Bcl-2 is unclear, but Bcl-2 has been shown to be a target of NF-κB activation (17, 48). We found that ROD-GFP expression alone reduced basal NF-κB activity, potentially explaining the reduction in Bcl-2 expression. More specifically, we showed that disruption of focal adhesions by ROD-GFP resulted in decreased FAK activation, which was recently shown to play an important role in NF-κB activity (54).

Because anoikis, or detachment-induced apoptosis, can be executed through the initiator and effector caspases as well as through the BH3-domain proteins localized to the mitochondria (25), we hypothesized that the ROD-GFP-induced reduction in Bcl-2 could result in anoikis. However, the reduction in expression of Bcl-2 in ROD-GFP-expressing cells did not result in cell death by itself. Although our studies do not rule out the possibility that expression of ROD-GFP may be inducing apoptosis at early time points, we did not observe significant differences in total protein amounts between ROD-GFP-expressing and control cells, indicating that these cells are proliferating normally. Although it may be surprising that ROD-GFP expression alone induced no changes in cell death, other factors may play a role. UMR106 cells are derived from an osteosarcoma, and other cancer-derived cells have been shown to have increased Bcl-2 levels (33). Therefore, the
reduction in Bcl-2 level, although dramatic, may not be to a level low enough to induce apoptosis in these cells. Alternatively, we observed concurrent increases in Akt and ERK phosphorylation in cells expressing ROD-GFP. Both of these proteins have been shown to have potent effects on cell survival and proliferation (10, 11) and therefore may be activated in ROD-GFP-expressing cells as a mechanism to counter the disruption of focal adhesions. However, inhibition of either Akt or ERK individually or together was not sufficient to induce apoptosis in these cells. ERK requires phosphorylation of both threonine 202 and tyrosine 204 for activation (45), and Akt requires phosphorylation of both threonine 308 and serine 473 for full activation (1). Because we used antibodies against only one of these phosphorylation sites in the case of both kinases (Y204 for ERK and S473 for Akt), we cannot confirm that full activation of either protein is induced by ROD-GFP expression. The upstream ERK kinase MEKK1 interacts directly with α-actinin (7), and the potential mislocalization of this protein may explain the alterations in ERK phosphorylation.

The reduced Bcl-2 expression seen with ROD-GFP expression suggested that cells with disrupted focal adhesions may be more sensitive to apoptotic stimuli. To assess this, we challenged cells with TNF-α in the presence of CHX to activate an apoptotic program that is executed through effector caspases, such as caspase 3. Therefore, the cleavage and activation of caspase 3, as well as its substrate PARP, signals a point of no return for the cell on the road to apoptosis (6). We observed increased levels of cleaved caspase 3, cleaved PARP, and histone H2A.X phosphorylation in ROD-GFP-expressing cells treated with TNF-α/CHX compared with GFP controls. Similarly, Frisch et al. (15) showed that when Madin-Darby canine kidney cells were grown to confluence, Bcl-2 protein levels were decreased. This decrease did not induce apoptosis, but it rendered the cells sensitive to anoikis (15). Our results are significant considering the fact that TNF-α is thought to play a central role in postmenopausal bone loss through the stimulation of osteoclastogenesis and osteoblast apoptosis (27). In addition, Bucar et al. (5) have demonstrated that exposure of osteoblasts to modeled microgravity results in decreased Bcl-2 and Akt protein levels and a sensitization to staurosporine-induced apoptosis.

Cells expressing α-actinin-GFP had lower levels of caspase 3 and PARP cleavage when treated with TNF-α compared with GFP controls, suggesting that intact, endogenous α-actinin itself has a protective role against apoptosis in cells. To assess how α-actinin may exert these protective effects, we measured activation of components of the survival pathway activated by TNF-α. In addition to the stimulation of apoptosis in UMR106 cells, TNF-α initiates survival signals through its receptor, which involves Akt activation in a PI3K-dependent manner and ultimately results in nuclear translocation and transcriptional activity of NF-κB (6). First, we examined Akt activation in response to TNF-α, which peaked at 15 min in UMR106 cells (data not shown). Surprisingly, an increase in Akt phosphorylation following exposure to TNF-α was not observed in cells expressing α-actinin-GFP or ROD-GFP. Although the results observed in α-actinin-GFP-expressers are confusing, these cells do show the ability to translocate NF-κB in response to TNF-α, indicating that the survival pathway remains intact in these cells.

FAK has previously been suggested to play a role in TNF-α-induced survival signaling (16). Because ROD-GFP expression resulted in decreased FAK phosphorylation, it follows that TNF-α survival signals are also inhibited in these cells. We also analyzed the activation of NF-κB nuclear translocation using both immunofluorescence microscopy and a dual-luciferase activity assay. We found that TNF-α-stimulated nuclear translocation of NF-κB was inhibited in cells expressing ROD-GFP. Also, ROD-GFP expression led to significant decreases in both basal and TNF-α-stimulated NF-κB reporter activity. NF-κB acts to increase the expression of antiapoptotic proteins to promote cell survival (46); however, in this study we used CHX to inhibit new protein synthesis. Therefore, the lack of NF-κB activation cannot completely explain the increased apoptosis in cells expressing ROD-GFP. However, together with the lack of TNF-α-activated Akt phosphorylation, these data suggest that inhibition of survival signaling initiated by TNF-α may be part of the mechanism leading to increased apoptosis in cells with ROD-GFP-disrupted focal adhesions. In support of this, Zhang, et al. (54) recently demonstrated that increased expression of dominant negative FAK increased sensitivity to TNF-α-induced apoptosis and inhibited NF-κB activity, suggesting that cross talk between focal adhesion and TNF-α signaling pathways may be a common cellular phenomenon. However, the exact mechanism by which expression of ROD-GFP interrupts TNF-α-induced survival signaling remains unknown. Potentially, cells with disrupted focal adhesions have a limited ability to recruit/activate cytoplasmic proteins of the TNF-α cascade. Alternatively, disruption of FAK signaling by ROD-GFP expression could have a direct impact on TNF-α-induced survival signaling. Further analysis is needed to determine the molecular link between ROD-GFP-induced focal adhesion disruption and TNF-α signaling. We conclude that reduced TNF-α-induced survival signaling, in combination with reduced Bcl-2 levels, rendered ROD-GFP-expressing cells more sensitive to TNF-α-induced apoptosis.

In summary, we took the novel approach of perturbing focal adhesion function by expressing a truncated version of α-actinin, which localized to and disrupted focal adhesions. Although disruption of focal adhesions by ROD-GFP expression alone did not induce apoptosis, the expression of Bcl-2 was decreased. We propose that this phenomenon, in combination with reduced survival signaling, rendered ROD-GFP-expressing cells more sensitive to TNF-α-induced apoptosis. This study highlights the important role of α-actinin function and focal adhesion signaling in bone cell survival. Overall, expression of ROD-GFP is an effective approach to disrupt both the endogenous function and focal adhesion organization of α-actinin without directly altering integrin-ECM interactions or dramatic disruption of the cytoskeleton. Future studies using this method could shed light on the role of focal adhesions in many cellular processes.

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