THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 35, pp. 25501–25508, August 31, 2007
Printed in the U.S.A.

A Novel Ligand-independent Function of the Estrogen Receptor Is Essential for Osteocyte and Osteoblast Mechanotransduction*

Received for publication, March 14, 2007, and in revised form, July 3, 2007 Published, JBC Papers in Press, July 3, 2007, DOI 10.1074/jbc.M702231200

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Bone senses and adapts to meet mechanical needs by means of an extensive mechanotransduction network comprising osteocytes (former osteoblasts entrapped in mineral) and their cytoplasmic projections through which osteocytes communicate with osteoblasts and osteoclasts on the bone surface. Mechanical stimulation promotes osteocyte (and osteoblast) survival by activating the extracellular signal-regulated kinases, ERKs. Estrogens have similar effects and, intriguingly, the adaptive response of bone to mechanical forces is defective in mice lacking estrogen receptor (ER) α or ERβ. We report that ERKs are not activated by stretching in osteocytic and osteoblastic cells in which both ERα and ERβ have been knocked out or knocked down and this is reversed partially by transfection of either one of the two human ERs and fully by transfection of both receptors. ERK activation in response to stretching is also recovered by transfecting the ligand-binding domain (E) of either receptor or an ERα mutant that does not bind estrogens. Furthermore, mechano-responsiveness is restored by transfecting the Eα targeted to the plasma membrane, but not to the nucleus, whereas ERα mutants with impaired plasma membrane localization or binding to caveolin-1 fail to confer ERK activation in response to stretching. Lastly, the ER antagonist ICI 182,780 abrogates ERK activation and the anti-apoptotic effect of mechanical stimulation. We conclude that in addition to their role as ligand-dependent mediators of the effects of estrogens, the ERs participate in the transduction of mechanical forces into pro-survival signaling in bone cells, albeit in a ligand-independent manner.

That the skeleton adapts to meet mechanical needs was first recognized by Wolff (1) and later expanded by Frost (2) in the mechanostat hypothesis. Bone adjusts to load by changing its mass, shape, or microarchitecture (3, 4), and it responds differently depending on the magnitude of strain. Whereas insufficient or excessive levels of strain induce bone resorption, physiological levels of strain maintain bone mass (5). Osteocytes (former osteoblasts buried in the mineral) are thought to be the cells acting as mechanosensors. Osteoblasts and osteoclasts, the executive cells for bone formation and resorption, are present on bone for relatively short periods and occur in low number and only in locations that undergo remodeling at a given time point, which represent ~10% of the bone surface. On the other hand, osteocytes are by far the most abundant resident cells and are present throughout the entire bone tissue. Importantly, osteocytes are the core of a functional syncytium that extends from the mineralized bone matrix to the bone surface and the bone marrow, all the way to the blood vessels. This strategic location permits the detection of variations in the level of strain as well as the dispersion of the signals leading to adaptive responses. Through such network, osteocytes might continually compare present mechanical strains to usual levels of strain (the "set point" of the mechanostat) and send signals to osteoblasts or osteoclasts that result in bone gain or loss, as needed (4).

Changes in osteocyte viability evidently influence the mechanical competence of the skeleton. Indeed, the increased bone fragility resulting from glucocorticoid excess or sex steroid deficiency in animals and humans is associated with increased prevalence of osteocyte apoptosis (7–9). Conversely, bisphosphonates, intermittent parathyroid hormone administration, and sex steroids all prevent osteocyte apoptosis, suggesting that preservation of osteocytes contributes to the antifracture efficacy of these agents that is disproportional to their effects on bone mass (9–11). Moreover, blockade of glucocorticoid action on osteocytes in a transgenic mouse model preserved bone strength despite loss of bone mass, directly demonstrating that osteocyte viability is indeed an independent determinant of bone strength (12).

The life span of osteocytes is greatly influenced by mechanical forces. Indeed, lack of mechanical loading promotes osteocyte apoptosis, whereas mechanical stimulation maintains osteocyte (and osteoblast) viability (13–16). We have recently shown that the anti-apoptotic effect of mechanical stimulation requires integrin signaling as well as intact caveolae and the kinase activity of Src and focal adhesion kinase and downstream phosphorylation and nuclear translocation of the extra-

* This work was supported by National Institutes of Health Grants KO2-AR02127 and P01-AG13918) and the U. S. Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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cellular signal-regulated kinases (ERKs)[4] (15). In addition to the kinase domain of Src, the Src homology 2 domain is necessary for the anti-apoptotic effect of stretching (15). This evidence strongly suggests that the scaffolding properties of caveolin-1 and Src interaction with other proteins might be crucial for the formation of a pro-survival signalsome in osteocytes.

Estrogen receptor (ER) α interacts with the Src homology 2 domain of Src (17) and also binds to caveolin-1 (18, 19). Moreover, mice lacking ERα or ERβ exhibit a poor osteogenic response to bone loading (20, 21). Based on these lines of evidence, we have investigated herein the participation of the ERs in mechanotransduction in osteocytes and osteoblasts. We report that cells lacking ERα and ERβ are unresponsive to mechanical stimulation and that both ERα as well as ERβ rescue ERK activation in response to stretching. In addition, the ligand-binding domain (E) of either receptor is sufficient to confer responsiveness to mechanical stimuli, albeit in a ligand-independent fashion. Furthermore, both plasma membrane localization of ERα and its interaction with caveolin-1 are required for stretching-induced ERK activation and anti-apoptosis. These findings reveal a novel function of the membrane-associated ERs that is independent of the ligand and is essential for the transduction of mechanical forces into intracellular survival signaling in osteocytes and osteoblasts. Moreover, our findings suggest a mechanism by which bone adaptation could be modulated by changes in the molecular makeup of osteocytes.

EXPERIMENTAL PROCEDURES

Cells—MLO-Y4 osteocytic cells and MLO-Y4 cells stably expressing green fluorescent protein (GFP) targeted to the nucleus (MLO-GFP) were obtained and cultured as previously described (10, 22). Osteoblastic cells were obtained from calvaria of wild type or ERα- and ERβ-deficient mice (ER$\alpha\beta^{-/-}$) (23) and cultured as previously described (24).

Plasmids—Vector pBlueScript (pBSC) II was purchased from Stratagene (La Jolla, CA). Wild type human (h) ERα and hERα L525A were provided by B. Katzenellenbogen (University of Illinois at Urbana-Champaign, Urbana, IL) (25, 26). The constructs containing the ligand-binding domain of ERα (Eα) fused to cyan fluorescent protein and targeted to the nucleus (Eα nuc) or to the cell membrane (Eα mem) were previously described (9). Wild type hERβ was provided by T. C. Spelsberg (Mayo Clinic College of Medicine, Rochester, MN) (27). The ligand-binding domain of ERβ (Eβ) was generated by PCR amplification of the wild type hERβ, using the following primer pair, 5′-CAAGGTCGACAAAATGGAGTTGGTACAC-3′ and 5′-GCGGGATCCTTAAAGCACGTGGGCATTC-3′. PCR products were digested using SalI and BamHI and inserted into the same sites of the pCMV5 vector as previously published for Eα (9). Murine ERα and ERβ were provided by D. L. Bodenner (University of Arkansas for Medical Sciences). Murine ERα S522A and human C447A were provided by E. R. Levin (University of California, San Francisco, CA) (28) and M. Marino (University of Rome, Italy) (29), respectively. ERK2 fused to GFP (GFP-ERK2) and ERK2 fused to red fluorescent protein (RFP-ERK2) were provided by R. Seger (The Weizmann Institute of Sciences, Rehovot, Israel) (30) and L. Luttrell (Medical University of South Carolina, Charleston, SC) (31), respectively. Wild type MEK was provided by N. G. Ahn (University of Colorado, Boulder, CO) (32). The construct encoding the nuclear red fluorescent protein was previously described (9, 33).

Knock Down and Rescue of ERα and ERβ Expression—The expression of ERα, ERβ, or the irrelevant protein lamin A/C was knocked down in MLO-Y4 cells using small interfering RNAs. Cells were incubated with Oligofectamine reagent and small interfering RNA oligonucleotides (Dharmacon Research Inc., Lafayette, CO) for 4 h at 37 °C. Two days later, the expression of ERα and/or ERβ was rescued by transient transfections with wild type or mutated receptors along with ERK2-GFP or ERK2-RFP and wild type MEK.

Mechanical Stimulation of Cell Cultures—Cells were plated on flexible bottom wells coated with collagen type I. 16–24 h later cells were stretched at 5% elongation for 10 min using different regimens of biaxial stretching (Fig. 1*A*) in a FX-4000 Flexercell Strain Unit (Flexcell International Corp., Hillsborough, NC). Regimen 1 was performed using 20-s stretching and 0.1-s resting periods. Regimens 2–6 were performed using equal periods of stretching and resting. The frequency for regimens 1 and 2 was 3 cycles/min (equivalent to 0.05 Hz); for regimen 3, 6 cycles/min (0.1 Hz); for regimen 4, 12 cycles/min (0.2 Hz); for regimen 5, 24 cycles/min (0.4 Hz); and for regimen 6, 60 cycles/min (1 Hz). The optimal conditions for ERK activation for each cell type (Fig. 1, *B–D*) were used in subsequent experiments: regimen 1 for MLO-Y4 cells and regimen 2 for osteoblastic cells. For the experiments using pharmacological inhibitors, cells were treated with 10^{-7} M ICI 182,780, 10^{-7} M AG1478, 10^{-4} M AG538 (Sigma), or 5×10^{-5} M PD98059 (New England Biolabs, Beverly, MA) for 30 min before mechanical stimulation.

Western Blot Analysis—Immediately after stretching, cells were lysed and proteins were separated by SDS-polyacrylamide electrophoresis as previously described (15). Immunoblotting was performed using antibodies recognizing phosphorylated or total ERKs, lamin A/C, ERα (Santa Cruz Biotechnology, Santa Cruz, CA), ERβ (Affinity BioReagents, Golden, CO) or β-actin (Sigma), followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Blots were developed by ECL, and the intensity of the bands was quantified by scanning and densitometry using a VersaDoc TM imaging system (Bio-Rad Laboratories).

Real-time PCR—RNA was isolated using Ultraspec reagent and treated with DNase (Worthington Biochemical Corp., Lakewood, NJ) to remove contaminating plasmid DNA. Reverse transcription was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). Primers and probes for mERβ (probe, 5′-ACAAAGCCAGGGATTTT-3′, forward

[4] The abbreviations used are: ERK, extracellular signal-regulated kinase; ER, estrogen receptor; hER, human ER; E, ligand-binding domain of the ER; GFP, green fluorescent protein; EGF, epidermal growth factor; IGF, insulin-like growth factor; Eα nuc, E from the ERα targeted to the nucleus; Eα mem, E from the ERα targeted to the cell membrane; RFP, red fluorescent protein; AR, androgen receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ANOVA, analysis of variance.

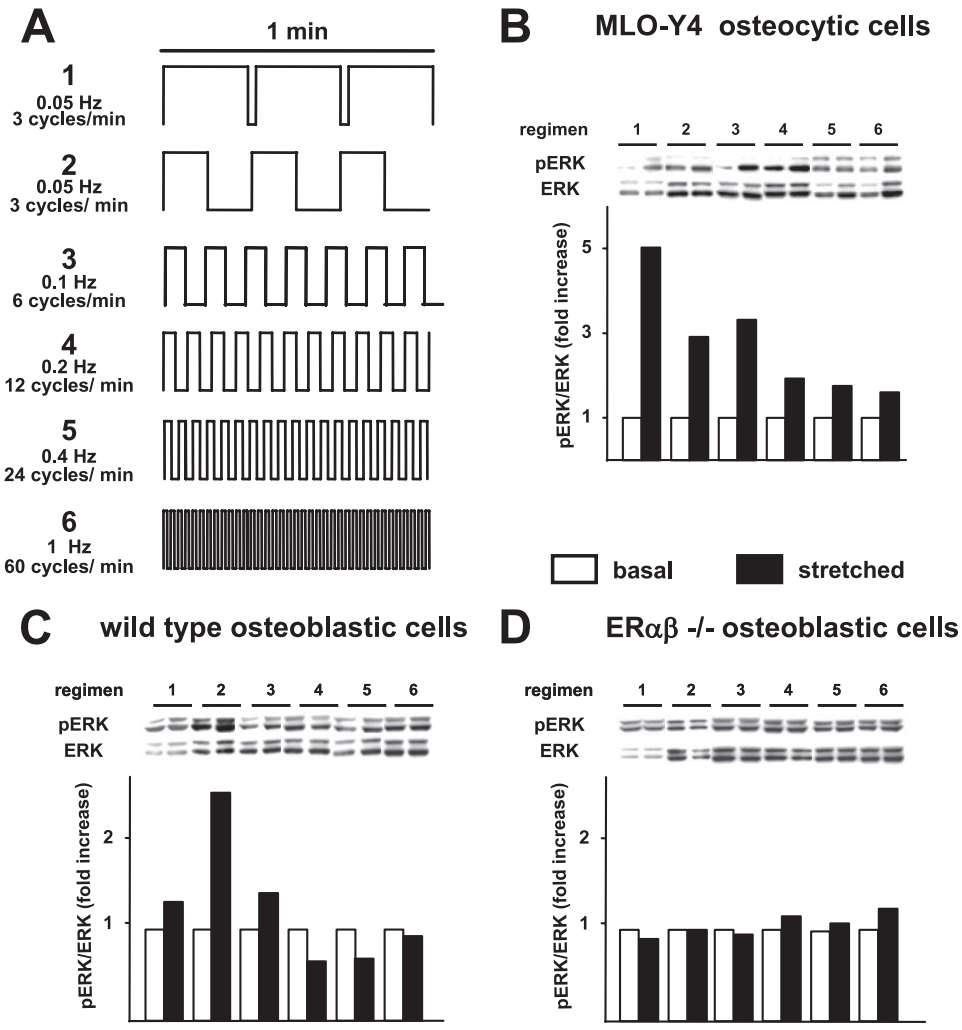

FIGURE 1. **Mechanically induced ERK phosphorylation depends on ERα and ERβ expression.** MLO-Y4 osteocytic cells or osteoblastic cells derived from wild type or ERαβ-deficient (−/−) mice were stretched for 10 min at 5% elongation, as detailed under "Experimental Procedures." *A*, schematic representation of stretching regimens applied to the cell cultures. For regimen 1, each cycle consisted of 20 s of stretching, followed by 0.1 s of resting. For regimens 2–6, each cycle consisted of periods of stretching and resting of the same duration. The frequency in Hz and number of cycles/min is indicated on the *left* of each scheme. ERK phosphorylation induced by stretching in MLO-Y4 osteocytic cells (*B*), osteoblastic cells derived from wild type (*C*), or ERαβ-deficient (−/−) (*D*) mice was analyzed by Western blotting. Representative images of two independent experiments for each cell type are shown. *Bars* depict -fold increase in the ratio pERK/ERK over the respective basal control group, designated as *1*.

Quantification of Apoptosis—MLO-Y4 cells were stretched for 10 min at 5% elongation using regimen 1 (Fig. 1*A*) followed by treatment with 6×10^{-5} M etoposide (Sigma) for 9 h. Caspase 3 activity was determined in cell lysates by measuring the degradation of the fluorogenic substrate Ac-DEVD-AFC (Biomol, Plymouth Meeting, PA) as previously reported (10). Caspase 3 units/μg protein were calculated using a standard curve prepared with recombinant caspase 3 (Biomol) assayed together with the samples. Apoptosis was also quantified by enumerating MLO-GFP cells exhibiting chromatin condensation and nuclear fragmentation under a fluorescence microscope, as previously published (10). Cells were stretched and treated with etoposide for 6 h. At least 250 cells from fields selected by systematic random sampling were examined for each experimental condition.

Subcellular Localization of ERK2—MLO-Y4 cells or osteoblastic cells derived from wild type and ERαβ$^{-/-}$ mice were transiently transfected with ERα, ERβ, or the different ER mutants together with GFP-ERK2 or RFP-ERK2 to visualize the subcellular localization of ERKs. Cells were also co-transfected with wild type MEK to anchor inactive ERK2 in the cytoplasm. Twenty-four hours after transfection, cells were incubated with medium without serum for 20 min and stretched for 10 min at 5% elongation or treated for 5 min with 10 ng/ml epidermal growth factor (EGF) (Sigma) or 5 ng/ml insulin-like growth factor-1 (IGF-I) (Genetech, South San Francisco, CA). Cells were immediately fixed. ERK2 nuclear accumulation was quantified in more than 250 cells/condition by enumerating the percentage of cells exhibiting increased GFP or RFP in the nucleus compared with the cytoplasm, as previously reported (33).

Image Acquisition—Fluorescent images were collected on an inverted microscope (Axiovert 200; Carl Zeiss Light Microscopy, Gottingen, Germany) with an LD A-Plan, ×32/0.40 lens and a low light camera (Polaroid DMC Ie; Polaroid Corp., Cambridge, MA), using a filter set for GFP. The acquisition software was Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Statistical Analysis—Data were analyzed by one-way analysis of variance (ANOVA). The Student-Newman-Keuls method

primer, GGAACTGGTGCACATGATTGG, reverse primer, GCTTTCCAAGAGGCGGACTT); hERα (probe, 5′-CACAAAGCCTGGCACC-3′, forward primer, CATGATCAACTGGGCGAAGAG, reverse primer, CCTGATCATGGAGGGTCAAATC); hERβ (probe, 5′-CACAAAGCCGGGAAT-3′, forward primer, TGATCAGCTGGGCCAAGAAG, reverse primer, GAGCCGCACTTGGTCGAA); and ChoB (probe, 5′-TCCAGAGCAGGATCC-3′, forward primer, CCCAGGATGGCGACGAT, reverse primer, CCGAATGCTGTAATGGCGTAT) and for mERα (mm00433149_m1) were from Applied Biosystems. The PCR reaction was performed using 20 μl of Gene Expression Assay Mix TaqMan Universal Master Mix containing 80 ng of each cDNA template in triplicates, using an ABI 7300 Real-time PCR system (Applied Biosystems). The -fold change in expression was calculated using the ΔCt or ΔΔCt comparative threshold cycle method.

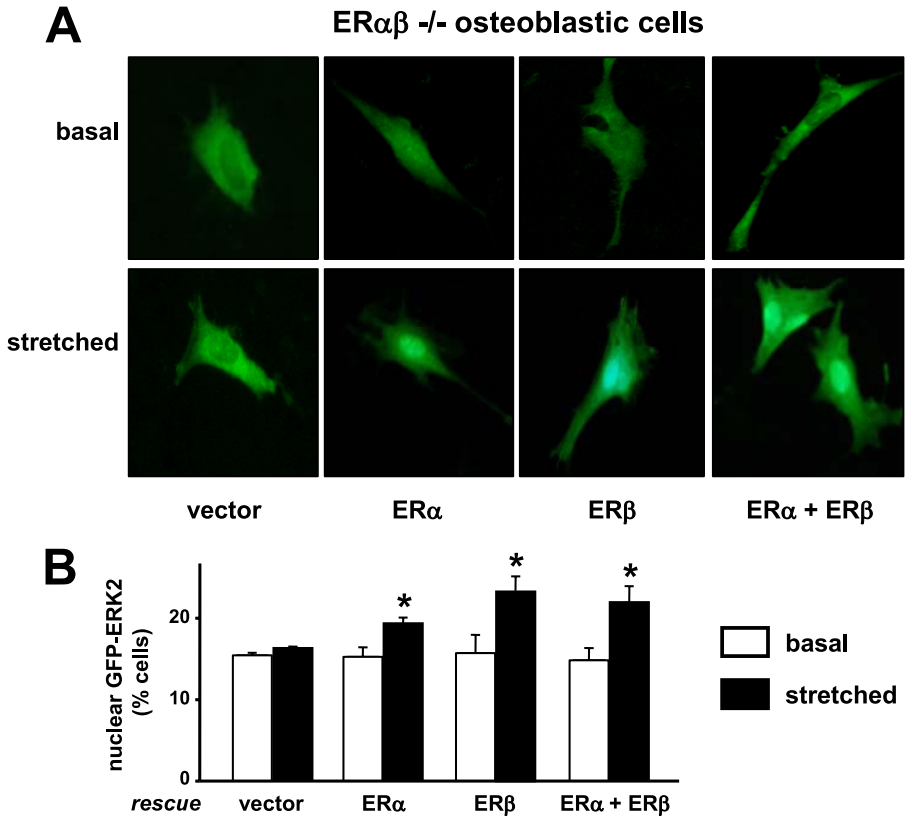

FIGURE 2. **Expression of ERα or ERβ confers ERK nuclear accumulation by stretching in ERαβ-deficient osteoblastic cells.** Cells from ERαβ-deficient mice were transiently transfected with pBSC II (*vector*), murine ERα, murine ERβ, or both receptors along with GFP-ERK2 to allow quantification of nuclear ERK accumulation as detailed under "Experimental Procedures." Twenty-four hours after transfection, cells were plated on collagen I-coated flexible bottom wells and cultured overnight. Cells were then left unstretched (*basal*) or stretched for 10 min at 5% elongation using regimen 2 (indicated in Fig. 1), followed by fixation. *A*, representative images of ERαβ$^{-/-}$ osteoblastic cells exhibiting either cytoplasmic or nuclear localization of GFP-ERK2. *B*, nuclear accumulation of GFP-ERK2 was quantified as described under "Experimental Procedures." *Bars* represent mean ± S.D. of triplicate determinations. *, $p < 0.05$ by one-way ANOVA *versus* unstretched (*basal*) cells.

was used to estimate the level of significance of differences between means, or by Student's *t* test.

RESULTS

Expression of ERα and ERβ Is Required for Stretching-induced ERK Activation in Osteocytic and Osteoblastic Cells—To establish whether the ERs participate in mechanotransduction, we compared responsiveness to mechanical stimulation of MLO-Y4 osteocytic cells or osteoblastic cells derived from wild type mice to osteoblasts derived from mice lacking both ERα and ERβ (ERαβ$^{-/-}$ osteoblastic cells). To this end, cells grown on flexible bottomed culture plates were subjected to biaxial stretching. Unlike MLO-Y4 or wild type control cells, ERαβ$^{-/-}$ osteoblastic cells did not exhibit ERK phosphorylation in response to mechanical stimulation under various regimens of stretching (Fig. 1). Moreover, stretching did not induce ERK nuclear accumulation in ERαβ$^{-/-}$ cells, as assessed by determining the subcellular localization of a GFP-ERK2 fusion protein by epifluorescence microscopy (Fig. 2, *A* and *B*). However, when ERαβ$^{-/-}$ cells were transfected with ERα, ERβ, or both receptors, stretching significantly increased the percentage of cells exhibiting nuclear GFP-ERK2 accumulation.

We then proceeded to examine the response to stretching of MLO-Y4 cells in which the expression of ERα, ERβ, or both was knocked down using oligonucleotides specific for each receptor or for the nonessential protein lamin, used as a negative control. Cells transfected with small interfering RNAs for murine ERα or ERβ exhibited significantly reduced expression of the respective receptors compared with cells in which lamin was silenced, as determined by quantitative PCR and by Western blotting (Fig. 3*A*). Transfection of the human receptors to cells that had been silenced for ERα, ERβ, or both receptors successfully restored receptor expression as indicated by the levels of human ERα or human ERβ, quantified by PCR (Fig. 3*B*). Stretching induced an increase of ~100% in the number of cells exhibiting ERK nuclear accumulation when the control protein lamin was silenced (Fig. 3*C*). In contrast, cells in which ERα or ERβ was silenced exhibited a reduced response to stretching with an increase of only ~40–60% compared with unstretched cells; full responsiveness to stretching was restored by transfection of the corresponding human receptors. Furthermore, stretching failed to induce ERK nuclear translocation in cells in which both ERα and ERβ were silenced. Whereas transfection of ERα or ERβ partially restored the response, transfection of both receptors conferred full responsiveness to mechanical stimulation. Taken together, these findings demonstrate that the expression of both ERα and ERβ is required for transduction of mechanical stimulation into ERK activation.

The Ligand-binding Domain of the ERs Is Sufficient to Mediate Stretching-induced ERK Activation—We have previously shown that the ligand-binding domain (designated E) of ERα is sufficient to mediate the anti-apoptotic effect of estrogens (9). In agreement with these earlier observations, we found that stretching-induced nuclear ERK accumulation was rescued in cells in which ERα and ERβ were silenced by transfecting the E domain of ERα, the E domain of ERβ, or both E domains (Fig. 4, *A* and *B*). However, in contrast to estrogen-induced ERK activation that requires binding of the ligand to the receptor (9), stretching-induced ERK nuclear accumulation was also rescued by the ERα mutant receptor L525A that does not bind estrogens. These results indicate that the ligand-binding domain of ERα or ERβ is sufficient for the activation of ERKs by stretching and strongly suggest that binding of the ligand is not required.

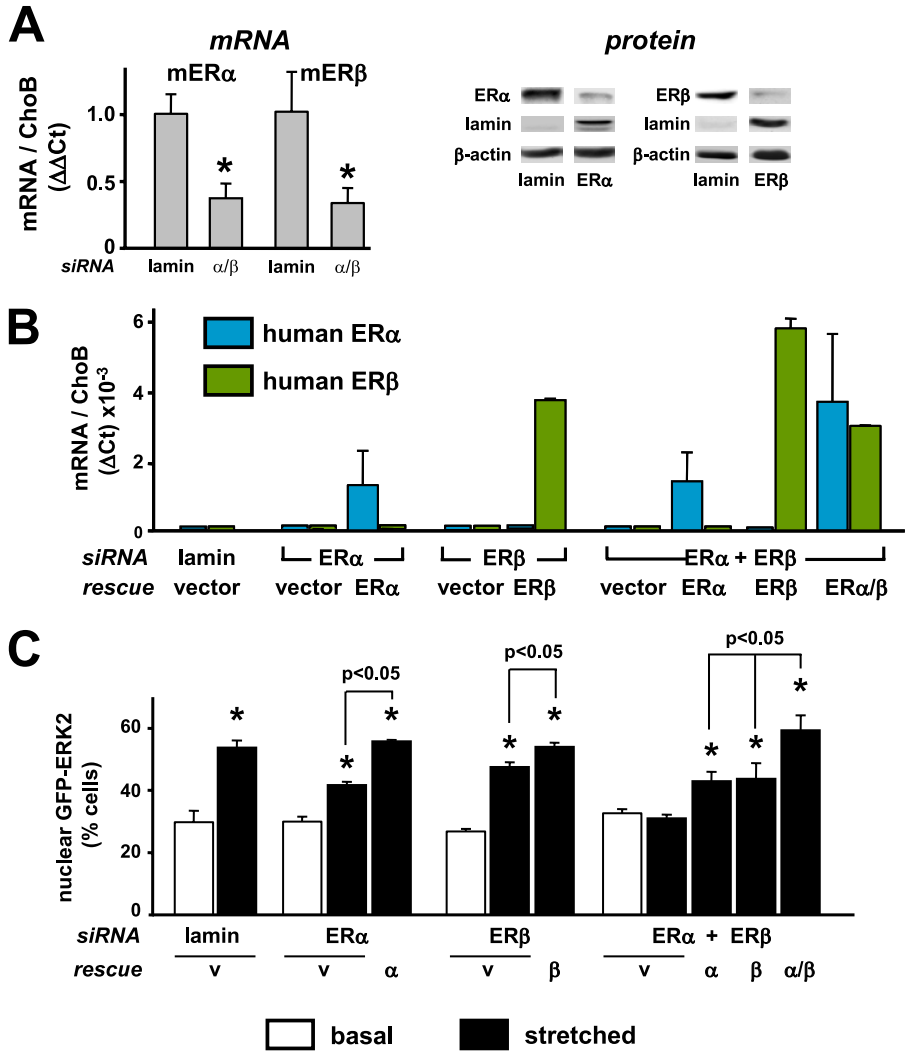

FIGURE 3. **Stretching-induced ERK nuclear accumulation is abolished by knocking down ERα and ERβ expression in osteocytic MLO-Y4 cells.** The expression of ERα, ERβ, or the control protein lamin A/C was silenced using the respective small interfering RNAs. Forty-eight hours later, cells were transfected with pBSC II (*vector*) or with ERα, ERβ, or both receptors along with GFP-ERK2 to allow quantification of nuclear ERK accumulation. *A*, murine ERα and ERβ levels were determined by real-time reverse transcription PCR and normalized by the levels of the housekeeping gene ChoB (*left panel*) and by Western blotting using anti-ERα and ERβ antibodies (*right panel*). The expression of lamin was determined by Western blotting. Membranes were re-blotted using an anti-β-actin antibody as control for equal sample loading. *, $p < 0.05$ by *t* test *versus* cells silenced for lamin. *B*, human ERα and ERβ levels were determined by real-time reverse transcription PCR and normalized by the levels of the housekeeping gene ChoB. *C*, cells silenced for lamin or for the ERs and transfected with vector (*v*), human ERα, ERβ, or both receptors along with GFP-ERK2 were stretched for 10 min at 5% elongation using regimen 1 (from Fig. 1). *, $p < 0.05$ by ANOVA *versus* vector transfected and unstretched (*basal*) cells for each silencing condition.

Plasma Membrane Localization of ERα and Its Interaction with Caveolin-1 Are Required for Stretching-induced ERK Activation—We next investigated whether the ability of the E domain of ERα to confer responsiveness to stretching was dependent on its localization to a particular subcellular compartment. The response to stretching in ERα/ERβ-silenced cells was recovered to a similar extent by transfecting the wild type E domain or the E domain targeted to the plasma membrane (Eα mem), but not by the E domain targeted to the nucleus (Eα nuc) (Fig. 4, *A* and *C*). Appropriate expression of the Eα mutants was confirmed by epifluorescence microscopy of MLO-Y4 osteocytic cells transfected with the constructs (which are fused to enhanced cyan fluorescent protein) along with nuclear red fluorescent protein to mark the nucleus (Fig. 4*D*). As we have previously shown in HeLa cells (9), the construct containing the wild type nontargeted E domain was evenly distributed throughout the cell. In contrast, Eα nuc accumulated in the nuclear compartment, whereas Eα mem localized in membranes and was excluded from the nucleus. Consistent with the findings with the targeted E domain constructs, transfection of two different ERα mutants that exhibit decreased localization at the plasma membrane and deficient interaction with caveolin-1 (S522A and C447A) failed to confer ERK activation in response to stretching.

Stretching-induced Anti-apoptosis Is Abolished by the ER Antagonist ICI 182,780, but Not by Inhibiting EGF or IGF Receptor Activation—In agreement with the requirement of ER expression for ERK activation by stretching, the estrogen receptor antagonist ICI 182,780 abolished ERK nuclear accumulation as effectively as the inhibitor of ERK activation PD98059 (Fig. 5*A*). Moreover, osteocytic cells subjected to 10 min of stretching were protected from etoposide-induced apoptosis, assessed by measuring caspase 3 activity or by quantifying cells exhibiting chromatin condensation or nuclear fragmentation (Fig. 5, *B* and *C*). In contrast, cells treated with ICI 182,780 were refractory to the anti-apoptotic effect of stretching.

ERK activation induced by ligand binding to the ER can result from transactivation of growth factor receptors (34, 35). However, we found that the ERK-dependent anti-apoptotic effect of stretching was not affected by the specific inhibitors of the EGF receptor AG1478 or the IGF receptor AG538 (Fig. 5*D*), although these agents effectively blocked EGF or IGF-induced ERK nuclear translocation, respectively (Fig. 5*E*).

DISCUSSION

Mounting evidence indicates that the life span of osteocytes and osteoblasts is a critical determinant of bone strength and that the viability of these cells is controlled not only by systemic hormones and local factors but also by mechanical forces (36). Indeed, recent studies indicate that lack of mechanical stimulation leads to increased prevalence of osteocyte apoptosis followed by loss of bone mass and decreased bone strength (13).

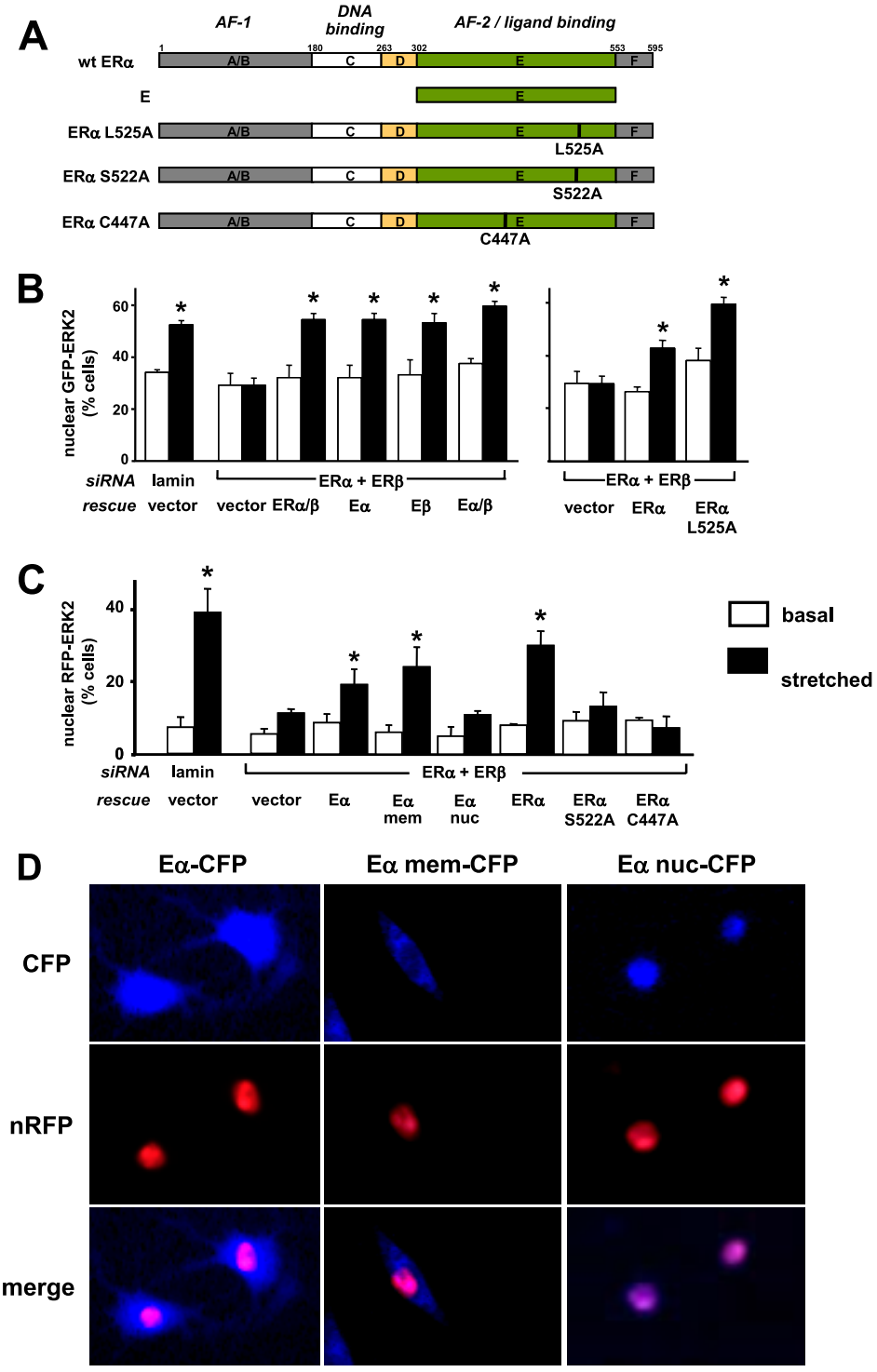

FIGURE 4. **The ligand-binding domains of ERα and ERβ are sufficient, whereas membrane localization and binding to caveolin-1 of ERα are required for stretching-induced ERK nuclear accumulation.** *A*, schematic representation of the structure of wild type and mutated forms of ERα used in the experiments. *B* and *C*, MLO-Y4 cells silenced for lamin or for the ERs and transfected with vector, human ERα, and human ERβ, the ligand-binding domain (E) of each receptor, or the indicated ERα or Eα mutants, along with RFP-ERK2, were stretched for 10 min at 5% elongation using regimen 1 (from Fig. 1). *, $p < 0.05$ by ANOVA *versus* vector transfected and unstretched (*basal*) cells for each silencing condition. *D*, representative images of MLO-Y4 osteocytic cells expressing wild type nontargeted Eα (*Eα-CFP*), Eα targeted to the membrane (*Eα mem-CFP*), or targeted to the nucleus (*Eα nuc-CFP*) along with nuclear RFP.

Conversely, mechanical stimulation by stretching activates ERKs, which in turn inhibit the effect of pro-apoptotic agents (15). The effect of mechanical stimulation by stretching requires integrins and the kinases focal adhesion kinase and Src. Disruption of caveolae abolishes mechanically induced ERK activation and survival, strongly suggesting that mechanotransduction depends on the proximity and likely the physical interaction of structural and signaling molecules localized within caveolae. The results of the present report indicate that the ERs are also critical components of a signalsome that perceives and transduces mechanical cues into cytoplasmic kinase signaling in osteocytes and osteoblasts.

The presence of ERα and ERβ in caveolae has been shown to mediate a variety of cellular functions in response to estrogens (37–39). Palmitoylation of the ligand-binding domains of the ERs appears to be responsible for the plasma membrane localization of a fraction of these receptors (40), probably facilitating their interaction with molecules localized in caveolae. Indeed, ERα associates with Src by the Src homology 2 domain of the kinase (17). ERα also binds to caveolin-1, and this binding increases the transcriptional activity of the receptor (18, 19). The inability of ERα mutants with decreased localization at the plasma membrane or defective interaction with caveolin-1 to confer ERK activation in response to stretching strongly suggests that membrane localization and binding to caveolin-1 is necessary for the ER to mediate ERK activation. This conclusion is supported by the fact that ERK activation induced by Eα in ERα$\beta^{-/-}$ cells is maintained by targeting this fragment to the membrane and it is eliminated by targeting it to the nucleus. Binding of the ER to both caveolin-1 and Src suggests a potential mechanism by which the receptors contribute to the assembly of the signalsome involved in mechanotransduction. The requirement of both Src and the ER for the transduction of mechanical forces into intracellular signaling raises the possibility that the scaffolding protein modulator of non-genomic activity of estrogen receptor, which mediates the activation of the Src/ERK signaling pathway in

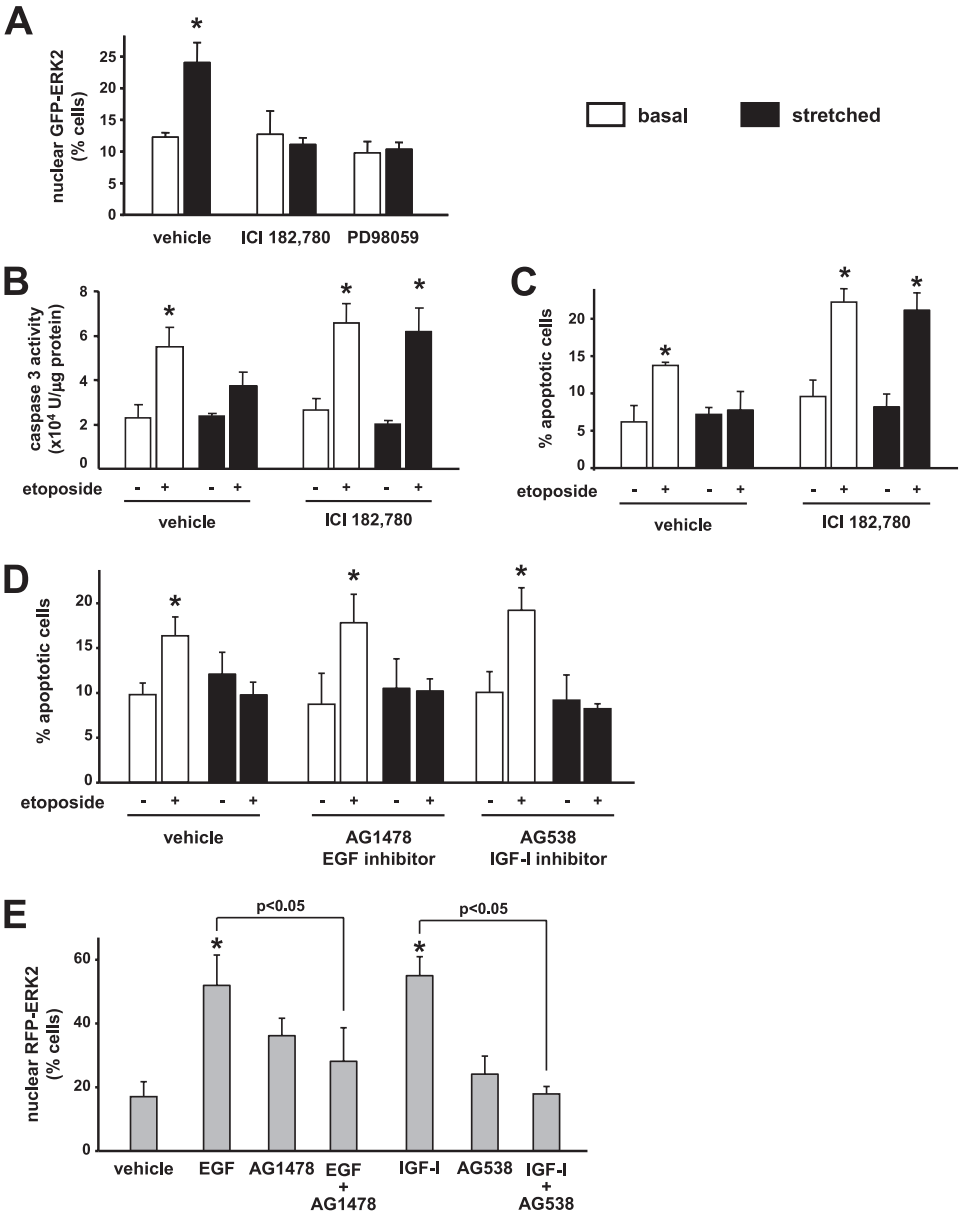

FIGURE 5. **Stretching-induced anti-apoptosis is abolished by the ER antagonist ICI 182,780, but not by inhibiting EGF or IGF receptor activation.** *A*, MLO-Y4 cells transiently transfected with GFP-ERK2 were treated with ICI 182,780 or PD98059 and left unstretched (*basal*) or stretched for 10 min at 5% elongation using regimen 1 (from Fig. 1). MLO-Y4 cells (*B*) or MLO-GFP cells (*C* and *D*) were treated with ICI 182,780, AG1478, or AG538 and stretched. Apoptosis was induced by addition of etoposide and quantified by measuring caspase 3 activity (*B*) or by evaluating nuclear morphology (*C*). *, $p < 0.05$ by ANOVA *versus* unstretched (*basal*), vehicle-treated cultures. *E*, MLO-Y4 cells transiently transfected with RFP-ERK2 were treated with AG1478 or AG538 followed by addition of EGF or IGF-I. *, $p < 0.05$ by ANOVA *versus* vehicle.

response to estrogen binding to the ER (41–43), may be also involved in mechanotransduction. Nevertheless, future studies are required to examine this possibility.

Unlike the anti-apoptotic effect of sex steroids that is mediated by either the ERs or the androgen receptor in bone cells (9), the androgen receptor appears not to be required for the ERK-activating effect of mechanical stimulation because the ERα/β-deficient cells express normal levels of androgen receptor. This is in agreement with previous observations of ours demonstrating that transfection of a Src kinase mutant lacking the ability to interact with the ER abrogated stretching-induced survival, whereas transfection with a Src mutant that cannot interact with androgen receptor had no effect (15). Remarkably, the requirement of the ERs for ERK activation and anti-apoptosis by mechanical forces seems to be independent of the presence of ligand, as evidenced by the fact that in our experiments cells were cultured and stretched in phenol red-free medium. Furthermore, an ERα mutant unable to bind estrogens was as effective as wild type ERα in recovering the response to mechanical stimulation, although it failed to confer responsiveness to estrogens (9). However, the results of the current studies have not ruled out the possibility that mechanotransduction causes clustering of the ER leading to ER activation through the dimerization domains previously identified in the ligand-binding domain of the ER (44).

Consistent with the present findings, Lanyon and coworkers (45, 46) showed that cyclic strain induces proliferation and activates ERE-mediated transcription in osteoblastic cells and that both responses are abrogated by the ER antagonist ICI 182,780. We found that ICI 182,780 also abolished stretching-induced ERK activation and anti-apoptosis of osteocytic cells. This compound is an antagonist of both ERα and ERβ action. ICI binds to ERα with similar affinity to 17β-estradiol but impairs receptor dimerization and blocks its nuclear translocation, therefore acting as a competitive inhibitor. In addition, ICI accelerates the proteasomal degradation of ERα, leading to reduction in the half-life of the protein from 5 to 0.5 h (47). ICI also competes with 17β-estradiol for binding to the ERβ and abolishes its actions (48), although the mechanism of this antagonism is not completely understood. Therefore, it is likely that abrogation of stretching-induced ERK activation and anti-apoptosis by ICI in our experiments results from decreased levels of ERs due to hastened proteolysis or to displacement of the receptors from the mechanotransduction signalsome to a different intracellular compartment.

In agreement with the requirement of the ER for osteoblast and osteocyte responses to mechanical forces *in vitro*, the new bone formation in the cortex of wild type mice subjected to mechanical loading is absent in mice lacking functional ERα and significantly lower in mice null for ERβ (20, 21). Taken

together, these findings strongly suggest that the inherent molecular composition of the bone cells, and in particular the levels of expression of the ERs, determines the adaptive response of bone to mechanical forces at the tissue level. Moreover, the present work suggests that changes in the level of expression of components of the mechanotransduction signalsome represent means by which systemic hormones or non-mechanical factors modulate the response of bone to mechanical stimuli. For example, lower or higher expression of a crucial component of the signalsome in osteocytes (or osteoblasts) would result in decreased or increased sensitivity to mechanical forces, respectively, resulting in an alteration of the threshold or set point of the mechanostat (2, 3, 6).

In conclusion, we have demonstrated herein that both ERα and ERβ are able to mediate the ERK-activating function of mechanical forces leading to preservation of osteocyte and osteoblast viability. Furthermore, the ligand-binding domain of each receptor is sufficient to confer responsiveness to mechanical stimuli, albeit in a ligand-independent fashion, and both plasma membrane localization of the ERα and its interaction with caveolin-1 are required for mechanotransduction. These findings reveal a novel function of the ERs and suggest a mechanism by which changes in the molecular composition of bone cells might modulate the response of bone to mechanical forces.

Acknowledgments—We thank Kanan Vyas for technical assistance, Dr. Laurie McCauley for providing osteoblastic cells from ERα- and ERβ-deficient mice, Dr. John L. Fowlkes for providing IGF-I, and the members of the UAMS Center for Osteoporosis and Metabolic Bone Diseases for insightful discussions.

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Mechanisms of Signal Transduction:

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J. Biol. Chem. 2007, 282:25501-25508.
doi: 10.1074/jbc.M702231200 originally published online July 3, 2007