Mechanical stress-activated integrin α5β1 induces opening of connexin 43 hemichannels

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The connexin 43 (Cx43) hemichannel (HC) in the mechanosensory osteocytes is a major portal for the release of factors responsible for the anabolic effects of mechanical loading on bone formation and remodeling. However, little is known about how the Cx43 molecule responds to mechanical stimulation leading to the opening of the HC. Here, we demonstrate that integrin α5β1 interacts directly with Cx43 and that this interaction is required for mechanical stimulation-induced opening of the Cx43 HC. Direct mechanical perturbation via magnetic beads or conformational activation of integrin α5β1 leads to the opening of the Cx43 HC, and this role of the integrin is independent of its association with an extracellular fibronectin substrate. PI3K signaling is responsible for the shear stress-induced conformational activation of integrin α5β1 leading to the opening of the HC. These results identify an unconventional function of integrin that acts as a mechanical tether to induce opening of the HC and provide a mechanism connecting the effect of mechanical forces directly to anabolic function of the bone.

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echanical loading plays a critical role in maintaining skeletal integrity and remodeling of the bone (1). Osteocytes are dispersed throughout the mineralized matrix of the bone where, in addition to being the most abundant cell type, they function as mechanosensors. Mechanical forces applied to the bone cause fluid flow through the lacunar–canalicular network surrounding the osteocyte (2). These forces stimulate cellular responses that involve different types of receptors and multiple intracellular signaling pathways (3). Our laboratory and others have shown that signaling generated from fluid shear stress in osteocytes is likely to be transmitted between cells via gap-junction channels located at the tips of the connecting dendritic processes and through the hemichannel (HC) between the osteocyte cell body and dendrites and their lacunar–canalicular network (4, 5). The signaling cascade activated by mechanical forces leads to the expression and release of important bone anabolic molecules, such as prostaglandins and ATP, through connexin 43 (Cx43) HCs expressed on the cell surface (5, 6). Extracellular prostaglandins are critical anabolic modulators that act in an autocrine or paracrine manner to promote remodeling in response to mechanical stimulation (7). Therefore, the HC provides an important means for regulating the anabolic responses of osteocytes to mechanical stress.

Osteocytes interact with the extracellular matrix (ECM) in the pericellular space through integrins, focal adhesion proteins, and transverse elements that bridge osteocyte processes to the canalicular wall (8, 9). Integrins comprised of heterodimers of α and β subunits serve as the major receptors/taxaductors that connect the cytoskeleton to the ECM. Fibronectin (FN) in ECM is a ligand for integrin α5β1 recognized through arginine-glycine-aspartic acid (RGD) sequences in FN (10). Upon interaction, integrins frequently form focal adhesions where they recruit proteins such as vinculin and paxillin (11). In addition to focal adhesions, integrins also form fibrillar adhesions that are characterized by elongated/bead-like structures across the basal surface of the cell (12). Integrins are reported to be mechanical sensors on the cell surface (13) and have been proposed as candidate mechanosensors in bone cells (14, 15). Mechanical stimulation is thought to invoke various signaling pathways that are known to be activated by integrins (16). Integrin α5β1 is expressed in bone and cartilage and can induce responses to mechanical stimuli (17, 18). Hence, integrins not only provide support to the cell through focal and fibrillar adhesions but also function as mechanosensors. There is some evidence that integrins are involved in connexin expression and gap-junction communication (19–21). However, the association of connexins with integrins has not been reported, and the molecular mechanisms by which integrins regulate connexins to affect channel functions are also unknown.

Results

Cx43 Interacts Directly with Integrin α5β1, and Fluid Flow Enhances the Interaction. Integrin α5 and Cx43 colocalized in osteocytic MLO-Y4 cells (Fig. L4 and Fig. S1 A and B) and in primary osteocytes (Fig. S1C). However, colocalization of α5 with vinculin or paxillin was not observed even under fluid flow (Fig. S2), suggesting that α5 was not present at the focal adhesions. Instead, α5 appeared to be located at fibrillar adhesions. Interaction between Cx43 and α5 was demonstrated by immunoprecipitation of α5 by Cx43 antibody (Fig. LB, lane 3), but not by preimmune serum (Fig. LB, lane 2). Likewise, reciprocal experiments showed immunoprecipitation of Cx43 (Fig. LB, lane 5) and β1 (Fig. LB, lane 8) by α5 antibody, but not by preimmune serum (Fig. LB, lane 5). The cytoplasmic C terminus of Cx43 (Cx43CT) is known to interact with several intracellular proteins (22). The peptide spanning the entire C terminus of α5 was able to pull down the GST-fusion protein containing the C terminus of Cx43 (GST-Cx43CT), suggesting that the C termini of Cx43 and α5 interact (Fig. 1C). There is no interaction between Cx43 extracellular loop domains (E1 and E2) and α5 (Fig. S3). Further support for a direct interaction was obtained from surface plasmon resonance (SPR) experiments in which we detected a concentration-dependent binding of soluble α5 C-terminal peptide to immobilized GST-Cx43CT but not to GST (Fig. 1D, Upper). Using the GST-only surface as background, Kd estimated from both kinetic (Fig. 1D, Upper) and equilibrium (Fig. 1D, Lower) analyses were in good agreement (1.8 mM). The interaction with scrambled...
Integrin α5 and Its Interaction with Cx43 Are Required for the Opening of the HC. The effect of fluid flow on the Cx43 HC function was studied by measuring the uptake of the tracer dye, Lucifer yellow (LY) dye uptake was similar in cells with and without bound FN-coated beads, further confirming that binding to FN or RGD is not required for HC opening induced by fluid flow (Fig. 3D). Although cells were cultured on collagen matrix, we could not exclude the possible involvement of FN secreted by the cells. Hence, we cultured the cells for 8 h, a time period during which minimal extracellular FN was accumulated (Fig. 3F). No change in HC opening was noticed when the cells were cultured for short time period (8 h) or for the usual 48 h (Fig. 3E). Furthermore, cells cultured for 8 h in an FN-depleted medium had a similar degree of HC opening (Fig. 3G). Together, these data exclude the involvement of FN, suggesting that the association of integrin α5 with its FN substrate is not essential for the fluid flow-induced opening of the HC.

Conformational Activation of Integrin α5β1 Through PI3K Activated by Mechanical Stimulation Opens the Cx43 HC. To establish a role for direct perturbation of integrin α5β1 in a force-dependent regulation of the Cx43 HC, we used magnetic beads coated with either FN, which is the primary substrate for integrin α5, or an anti-α5 antibody against α5, because both bind directly to α5. The diameter of the HC determined by Thimm et al. (23) is around 1.8 nm when closed and 2.5 nm when open; thus the magnetic beads are likely to bind to multiple HCs. The HC opening observed through magnetic bead induced dye uptake could be a collective action of multiple HCs. Application of a magnetic field induced dye uptake in cells with attached magnetic beads coated with anti-α5 antibody or the α5 substrate FN (Fig. 4A). As controls, we examined beads coated with Alexa Fluor 488 antibody, or polylysine. CD44, a receptor for hyaluronic acid, is expressed on the surface of MLO-Y4 cells as well as in primary osteocytes (24). Control beads
failed to induce dye uptake (Fig. 4A, Right). These results indicate that forces on integrin α5, but not other cell-surface molecules, induce the opening of the HC. This effect could be a result of a change in conformation of α5, because force is known to trigger conformational activation of this integrin (13, 25).

To examine this hypothesis, we treated cells with TS2/16, an integrin β1-activating antibody (26), in the absence of FN (FN-depleted serum was used) and observed Cx43 HC opening without mechanical stimulation (Fig. 4B). Indeed, α5P1 was activated from 5 min to 30 min of fluid flow as observed by the increase in the binding of GST-FNIII9-11, a reporter that specifically binds the activated form of α5P1 (Fig. 4C) (27). We then examined whether fluid flow activates α5β1 directly or whether another component might influence the force that leads to the conformational activation of α5P1. PI3K has been reported to mediate activation of integrins after fluid flow in endothelial cells (27–29), and we demonstrated the activation of PI3K signaling by fluid flow in osteocytes (Fig. S5) (30). Fluid flow-induced Cx43...
HC opening, as detected by dye uptake, was inhibited significantly by LY294002, a PI3K signaling inhibitor (Fig. 4D, Right). LY294002 also blocked the interaction between Cx43 and α5 (Fig. 4D, Lower). The activation of α5β1 was reduced effectively in the presence of the PI3K inhibitors LY294002 (LY, 10 μM) or wortmannin (WM, 100 nM) or were not treated as control (C) before fluid flow for 15 min. Cell lysates were immunoblotted for bound GST-FNIII9–11 or β-actin. Bound GST-FNIII9–11 was normalized with β-actin. **P < 0.01. (F) Schematic diagram of the role of integrin α5β1 in regulating Cx43 HC opening. (Left) In the absence of mechanical loading (static condition), the association between α5β1 and Cx43 exists through their C termini; however, the HCs remain closed. (Right) Upon fluid flow, PI3K signaling is activated, leading to activation of α5β1, promoting conformational change of its extended extracellular domain, and subsequently to the opening of the HC, which allows the passage of small bone anabolic factors, such as prostaglandin E2 (5), that are essential for bone formation and remodeling.

**Discussion**

Mechanical forces regulate skeletal remodeling through a wide range of biochemical signals. Osteocytes, in particular, are well positioned in the bone to sense the magnitude of mechanical strain and are important for the skeleton’s adaptive response to load (2). The shear stress activation of the Cx43 HC is a key element of this response. Here, we show that the integrin α5β1 interacts directly with the Cx43 HC through their respective C termini to promote HC opening in response to shear stress. The opening of the HC is likely a direct consequence of the activation and conformational change of the interacting integrin. Interestingly, recent data showed that mechanical force can trigger the conversion of integrin α5β1 to an activated state that is competent to signal (25). Here we give another dimension to the study by showing that integrin activation can induce Cx43 HC opening, as detected by dye uptake, was inhibited significantly by LY294002, a PI3K signaling inhibitor (Fig. 4D, Right). LY294002 also blocked the interaction between Cx43 and α5 (Fig. 4D, Lower). The activation of α5β1 was reduced effectively in the presence of the PI3K inhibitors LY294002 (LY, 10 μM) or wortmannin (WM, 100 nM) or were not treated as control (C) before fluid flow for 15 min. Cell lysates were immunoblotted for bound GST-FNIII9–11 or β-actin. Bound GST-FNIII9–11 was normalized with β-actin. **P < 0.01. (F) Schematic diagram of the role of integrin α5β1 in regulating Cx43 HC opening. (Left) In the absence of mechanical loading (static condition), the association between α5β1 and Cx43 exists through their C termini; however, the HCs remain closed. (Right) Upon fluid flow, PI3K signaling is activated, leading to activation of α5β1, promoting conformational change of its extended extracellular domain, and subsequently to the opening of the HC, which allows the passage of small bone anabolic factors, such as prostaglandin E2 (5), that are essential for bone formation and remodeling.

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opening through a process that involves PI3K signaling stimulated by mechanical force. However, the process is independent of integrin binding to its extracellular substrate, FN. This study, therefore, establishes a physical and molecular mechanism involving integrin α5β1 in the regulation of mechanotransduction via direct manipulation of HC function, as summarized in Fig. 4E.

In our study, the role of integrin α5 was addressed using siRNA against α5, which abolished the opening of the Cx43 HC in response to fluid flow. Moreover, uncoupling of the interaction between α5 and Cx43 using the C-terminal domain of Cx43 inhibited fluid flow-induced HC opening. Although our results essentially show that Cx43 is not located at the focal adhesion, a previous study showed that Cx43 colocalizes and interacts between the cells cultured on FN matrices. Moreover, neither application of FN or RGD peptide to Cx43 nor the HC-blocking Cx43 antibody abolished the opening of the Cx43 HC.

Materials and Methods

Cell Culture and Reagents. MLO-Y4 osteocytic cells derived from murine long bones were cultured on rat tail collagen type I-coated surfaces and were grown in α-modified essential medium with 2.5% (vol/vol) FBS and 2.5% (vol/vol) BCS (24). FN from the serum was depleted using a gelatin Sepharose column (GE Healthcare). Antibodies against integrin α5 (CD49e; R&D Systems), FN (BD Biosciences), and RGD peptides (Biomol) were used in this study.

Immunofluorescence Labeling and 3D Scanning Confocal Fluorescence Microscopy.

Total cell labeling. The cells cultured with FN or poly-lysine were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, permeabilized with 0.25% Triton X-100, and blocked with 3% BSA. The cells were incubated overnight at 4 °C with affinity-purified antibodies against Cx43CT (1:300), integrin α5 (1:50), integrin β1 (1:500), vinculin (1:300), or paxillin (1:300) and with 1 h for the appropriate secondary antibody.

Surface labeling. Cultured cells were washed in PBS followed by incubation for 1 h with α5 and FN antibody (1:30). The cells were fixed in 4% PFA and labeled with secondary antibodies in succession for 1 h each at room temperature. Confocal fluorescence with 3D z-scanning was performed on mounted slides using a confocal laser-scanning microscope (Fluoview; Olympus Optical) at a thickness of 0.5 μm.

Immunoprecipitation, Protein Pull Down, and Immunoblotting. Cultured MLO-Y4 cells were lysed in lysis buffer (5 mM Tris, 0.5 mM EDTA/EGTA, pH 8.0). Supernatants were incubated with Cx43 or α5 antibodies overnight at 4 °C, followed by incubation on beads for 2 h. Two biotinylated peptides (each 27 amino acids in length; >95% purity) were synthesized to cover the α5 C terminus (LGFKKORSPLYGTAMEKAQLQPAATSDA-biotin) and a scrambled peptide (LGKSATPYAQGMNLKTASEPRPK-AAT). Biotinylated peptides were conjugated to streptavidin-coupled Dynabeads and were incubated with GST-Cx43CT overnight at 4 °C. Bound proteins were eluted in 0.1% SDS buffer. Immunoprecipitates and elutes from pull down were immunoblotted with anti-Cx43CT (1:300) (35), anti-α5 (1:1,000), or anti-β1 (1:10,000) antibody.

Fluid Flow. Fluid flow was created by parallel-plate flow chambers separated by a gasket of defined thickness with gravity-driven fluid flow using a peristaltic pump. The thickness of the gasket determined the channel height, which was adjusted along with flow rate to generate stress levels of 16 dyn/cm². The circulating medium was SMEM. The entire flow system was encased within a CO2 incubator at 5% CO2 and 37 °C.

Dye-Uptake Assay. Cells were cultured on collagen type I, FN, and polylysine matrices for 4 h and were subjected to fluid flow at 16 dyn/cm² for 10 min. Dye-uptake experiments were performed as described previously (5). Briefly, cells were incubated with 0.2% LY (M, ~547 Da) and 0.2% rhodamine dextran (RD) (M, ~10 kDa) dye mixture for 5 min, and a ratio of fluorescent cells to total cells per image was determined using Image J software (National Institutes of Health). Cells cultured on collagen for 48 h were incubated with RGD peptide (1 mM)- and FN (10 μg)-coated Dynabeads for 30 min and 1 h and were subjected to fluid flow followed by dye uptake. Cells cultured on collagen for 8 h using FN-depleted medium or FN-containing medium were subjected to fluid flow for 10 min followed by dye uptake. Dye uptake also was analyzed after incubation with mouse IgG (50 μg/mL) or TS2/16 (50 μg/mL) diluted in SMEM for 30 min alone or after coincubation with carbenoxolone (100 μM) or HC-blocking Cx43 (E2) antibody (1:400). MLO-Y4 cells transfected with Cx43CT-GFP constructs were assayed for dye uptake using a mixture of Alexa Fluor 350 (1 μM) and RD (2%) after fluid flow.

SPR Analysis. SPR experiments were performed on a Biacore T100 instrument using CMS sensor chips (GE Healthcare), and the response was measured in resonance units. GST-Cx43CT and GST were immobilized on the chip surface. Binding analysis was performed by injecting in duplicate a concentration series of the purified α5 peptide (Genescript) over the chip, with concentrations...


Fig. S1. Integrin α5β1 consistently colocalizes with connexin 43 (Cx43). (A) MLO-Y4 cells were dual-immunostained with α5 and Cx43 antibodies and with FITC- and rhodamine-conjugated secondary antibodies, respectively. Confocal images from 3D Z-stack focal planes ranging from the medium (Top) to the matrix (Bottom) side were generated. Colocalization is shown in merged images. (Scale bar, 10 μm.) (B) Colocalization of α5 and β1 subunits in MLO-Y4 cells was detected by immunofluorescence. (Scale bar, 10 μm.) (C) Osteocytes isolated from chicken calvarias were dual-immunostained with Cx43 and integrin α5 antibodies. (Scale bar, 10 μm.)

Fig. S2. Integrin α5 does not colocalize with focal adhesion proteins in the absence or presence of fluid flow shear stress (FFSS). MLO-Y4 cells subjected to static (A and B) or FFSS (C) conditions were dual-immunolabeled with anti-integrin α5 (green) and vinculin (red) antibodies (A and C) or with anti-integrin α5 (green) and paxillin (red) antibodies (B). The 3D z-stack confocal images were captured from the top (to the left) to the bottom (to the right) of the cell. The integrin α5 subunit does not localize at focal adhesions under static or FFSS conditions, as evidenced by the absence of yellow signals in the merged images toward the bottom side of the cell.
**Fig. S3.** Integrin α5β1 does not interact with extracellular domains of Cx43. The eluted fractions of integrin α5 in the MLO-Y4 lysates were pulled down using GST (lane 1), C-terminal GST-Cx43 (GST-Cx43CT) (lane 2), GST-Cx43E1 (lane 3), or GST-Cx43E2 (lane 4) conjugated to glutathione beads and immunoblotted with α5 antibody (Top) or anti-GST antibody (Middle). Inputs (lysates) for the pull down were probed with α5 antibody (Bottom).

**Fig. S4.** Integrin α5 does not colocalize with Cx43 in cells cultured on polylysine matrix. MLO-Y4 cells were dual-immunostained with α5 and Cx43 antibodies and FITC- and rhodamine-conjugated secondary antibodies, respectively. Confocal and merged images were generated.

**Fig. S5.** Fluid flow (FF) activates PI3K/Akt signaling. MLO-Y4 cells were subjected to fluid flow at 16 dyn/cm² for 0, 10, 30, and 60 min. (Left) Cell lysates were immunoblotted with anti-total Akt and anti-phospho-Akt specific for position 308 or 473. (Right) The normalized ratio of pAkt308 or pAkt473 to total Akt from densitometric measurements of three separate Western blots. **P < 0.01 and ***P < 0.001, pAkt308 or pAkt473 under 10, 30 and 60 min of fluid flow versus non-fluid flow controls (0 min FF).**

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