Integrin Activation and Matrix Binding Mediate Cellular Responses to Mechanical Stretch*

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Mechanical tension is a critical determinant of cell growth, differentiation, apoptosis, migration, and development. Integrins have been implicated in sensing force but little is known about how forces are transduced to biochemical signals. We now show that mechanical strain stimulates conformational activation of integrin αvβ3 in NIH3T3 cells. Integrin activation is mediated by phosphoinositol 3-kinase and is followed by an increase in integrin binding to extracellular matrix proteins. Mechanical stretch stimulation of JNK was dependent on integrin binding to extracellular matrix, indicating that integrins can transfer external force through the plasma membrane (10). Mechanical tension locally applied to integrins induced the translocation of mRNA and ribosomes to points of force application (11), and a number of responses to strain are specific to particular ECM proteins (12). Mechanical stretch has been proposed to change the conformation or clustering of integrins (9) but this hypothesis has not been directly tested. We therefore sought to determine the role of integrin conformation and matrix binding in mechanotransduction.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma-Aldrich unless otherwise noted. WOW-1, LIBS-6, and LM609 were generous gifts from Drs. Sanford J. Shattil, Mark H. Ginsberg, and David A. Cherehesh, respectively (Scrivps Research Institute). Anti-FN antibodies 16G3 and 11E5 were generous gifts from Dr. Kenneth M. Yamada (National Institute of Health). Anti-PDGF β5 rabbit antibody (Santa Cruz Biotechnology) biotinylate (Lake Placid, NY). Phospho-Akt (Ser473) monoclonal antibody, phospho-JNK (Thr183/Thr185) polyclonal antibody and JNK polyclonal antibody were from Cell Signaling Technology (Beverly, MA). Anti-Akt1 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). PYY20 was from BD Bioscience. Wortmannin and LY-294002 were from Calbiochem. Signal PIP™ kits was purchased from Echelon Biosciences Inc. (Salt Lake City, UT). Transparenci siliccone elastic membrane was purchased from Specialty Manufacturing, Inc. (Saginaw, MI).

Cell Culture and Stretch Procedures—NIH3T3 cells were maintained as described previously (13). The equibiaxial stretch devices were described previously (14). Briefly, a silicone membrane is attached to the membrane holder to form the bottom of the device. Indentation of the ring against the membrane results in a homogenous planar equibiaxial stretch of the membrane. Strain along circumferential and radial axes were equal in magnitude and homogenously distributed (14). The strain was applied over ~10 s, which is the time required to manually turn the rings (13). Strain remained constant for the duration of the experiments. Membranes were coated with either 20 μg/ml fibronectin (FN) for 1 h at 37 °C, or with 10 μg/ml fibrogen (FBG) in the presence of 5 μg/ml aprotinin for 16 h at 4 °C.

Detection of Activated or Occupied Integrins—NIH3T3 cells were plated on silicone elastic membranes attached to an equibiaxial stretch device and coated with either FN or FBG, then incubated overnight in 0.5% FBS/DMEM. Cells were then left untreated or stretched to increase cell area by 15% and strain was maintained for the duration of the experiment. Cells were incubated with His-tagged recombinant WOW-1 Fab (30 μg/ml) or LM609 (12 μg/ml) for 30 min (15). Cells were washed in PBS and lysed in SDS sample buffer with 50 mM DTT. Lysates were sonicated for 10–15 s to shear DNA and analyzed by Western blotting using His-probe (rabbit polyclonal antibody against His tag, horseradish peroxidase conjugate, Santa Cruz Biotechnology) to detect bound WOW-1. For the experiments with wortmannin or LY-294002, cells were preincubated with either 100 nM wortmannin or 5 μM LY-294002 for 1 h at 37 °C before stretching. For the experiments with intracellular delivery of phosphatidylinositol 3,4,5-trisphosphate (PIP3) (16), NIH3T3 cells were plated on FN-coated plastic dishes and incubated overnight in 0.5% FBS/DMEM. Cells were then incubated with WOW-1 and 5 μM histone H1 as a carrier, with or without 3 μM dipamitoyl (di-C16)-PIP3 for 30 min, and bound WOW-1 was quantified as above.

It is well known that mechanical forces including tension and compression are critical to the normal growth and function of many tissues (1) and to pathological states such as cardiac hypertrophy (2) and atherosclerosis (3). Classical studies of cellular responses to mechanical stimuli using cells on elastic substrata demonstrated that fibroblasts orient along the axis of stretch (4), whereas cardiac myocytes elongate and orient perpendicular to the direction of stretch (5). Tension also induced neurite growth in the direction of applied force (6), whereas rearward tension applied to cells suppressed protrusive activity at the leading edge and caused alignment of actin microfilaments parallel to the tension (7). Gene expression in early Drosophila embryos can be modulated by both exogenously and endogenously applied mechanical strains (8). However, the molecular mechanisms by which cells transduce mechanical forces into biological responses are poorly understood.

Integrins are of particular interest in mechanotransduction because they both function as signaling receptors and physically connect the cytoskeleton to the extracellular matrix, thus transmitting physical forces (9). Magnetic beads coated with a β1-integrin ligand showed force-dependent focal adhesion formation and stiffening, indicating that integrins can transfer external force through the plasma membrane (10). Mechanical tension locally applied to integrins induced the translocation of mRNA and ribosomes to points of force application (11), and a number of responses to strain are specific to particular ECM proteins (12). Mechanical stretch has been proposed to change the conformation or clustering of integrins (9) but this hypothesis has not been directly tested. We therefore sought to determine the role of integrin conformation and matrix binding in mechanotransduction.

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1 The abbreviations used are: PI3K, phosphoinositol 3-OH-kinase; ECM, extracellular matrix; ERK, extracellular regulated kinase; FBG, fibrogen; FN, fibronectin; JNK, c-Jun N-terminal kinase; PIP3, phosphoinositol 3,4,5-trisphosphate; PBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DTT, dithiothreitol.
Integrin Activation by Mechanical Stretch

**RESULTS**

Mechanical Stretch Increases High Affinity αβ3 Integrin—An engineered Fab fragment, WOW-1, reacts selectively with high affinity αβ3 integrin that is not bound to ECM ligands (17). Since WOW-1 is monovalent, it is relatively insensitive to changes in integrin clustering and reports mainly changes in affinity of αβ3 integrin. To test whether mechanical strain alters affinity of integrin αβ3, subconfluent monolayers of NIH3T3 cells plated on an elastic membrane coated with fibronectin were subjected to biaxial stretch by 15% or kept untreated. Strain was maintained for the duration of the experiment. Cells were incubated with WOW-1 for 30 min, rinsed, and lysed, and the amount of bound WOW-1 was quantified by Western blotting (Fig. 1A). Strain increased WOW-1 binding by ~8-fold (Fig. 1A). Similar results were obtained with cells on fibrinogen (data not shown). As a control to test the specificity of binding, EDTA was added. As expected, EDTA abolished binding of WOW-1 to stretched cells (Fig. 1A). LM609, an antibody against αβ3 integrin that is insensitive to integrin conformation, showed no change in binding after stretch (Fig. 1B), excluding changes in integrin surface expression. These results indicate that mechanical stretch induced conformational activation of integrin αβ3.

**Stretch Increases Integrin Binding to ECM**—We then investigated whether stretch increases αβ3 integrin binding to ECM proteins. The antibody LIBS-6 has higher affinity for ligand-occupied compared with unoccupied β3 integrins (18). LIBS-6 also stabilizes the ligand-occupied conformation of integrins and can therefore activate them, but at the low concentration and short times used in these experiments, it serves primarily as a read-out for integrin ligand to ECM (15). NIH3T3 cells plated on elastic substrata were stretched and the binding of LIBS-6 was assayed (Fig. 1C). Stretch increased LIBS-6 binding to NIH3T3 cells that had been plated on FBG (Fig. 1C) or FN (data not shown). Thus, integrin activation by stretch is associated with increased integrin-ligand binding.

**Activation of PI3K/Akt Pathway by Stretch**—In many cellu-
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FIG. 2. Effects of mechanical stretch on PI3K and Akt. A, phosphorylation of PI3K. Upper panel, NIH3T3 cells were stretched for the indicated time. Phosphorylation on tyrosine of the p85 subunit of PI3K was assayed as described under "Experimental Procedures." Lower panel, quantitation of phosphorylation of p85 normalized to total p85. Values are means ± S.E. (n = 3) relative to non-stretched controls. * denotes p < 0.05 according to Student’s t test. IP, immunoprecipitation; IB, immunoblot. B, phosphorylation of Akt. Upper panel, NIH3T3 cells were stretched for the indicated times, and cell lysates were probed with antibodies against phospho-Akt or total Akt. Lower panel, quantitation of phosphorylation of Akt normalized to total Akt. Values are means ± S.E. (n = 3) relative to non-stretched controls. * denotes p < 0.05 according to Student’s t test. C, inhibition of WOW-1 binding by PI3K inhibitors. Upper panel, NIH3T3 cells were preincubated with 100 nM wortmannin (black), 5 μM LY-294002 (gray), or without inhibitors (white). Cells were then stretched (S) or not stretched (N) and incubated with WOW-1 to probe activated αvβ3 integrin. Blots were reprobed with anti-actin antibody to assess protein loading. Lower panel, histograms showing WOW-1 binding normalized to actin. Values are means ± S.E. (n = 3) relative to non-stretched controls. * denotes p < 0.05 according to Student’s t test. D, αvβ3 integrin activation by intracellular delivery of PIP3. Upper panel, NIH3T3 cells were plated on FN-coated plastic dishes and then incubated overnight in 0.5% FBS/DMEM. Cells were then incubated with 30 μg/ml WOW-1 and 5 μg histone H1 as a carrier with or without 3 μM dipalmitoyl (di-C16)-PIP3 for 30 min, and bound WOW-1 was quantified. Lower panels, histograms showing WOW-1 binding normalized to actin. Values are means ± S.E. (n = 3) relative to non-stretched controls. * denotes p < 0.05 according to Student’s t test.
Integrin Activation by Mechanical Stretch

Previous studies have provided evidence that integrins are involved in cellular responses to mechanical forces (reviewed in Ref. 12). For example, some responses to mechanical strain depend on the ECM to which cells are adhered and inhibitors of focal adhesion signaling can block mechanotransduction. However, mechanisms of integrin-dependent mechanotransduction have not been elucidated. In this study, we demonstrate that integrin $\alpha$V$\beta$3 is activated by strain. This effect is due to affinity modulation as shown by increased binding to the monovalent, conformation-sensitive Fab fragment, WOW-1. Stretch also induced an increase of LIBS-6 binding after stretch, evidence that conformational activation leads to new binding of integrins to the ECM. PI3K and Akt are both rapidly activated by strain, and PI3K inhibition decreases integrin activation. In addition, the delivery of PIP3 into cells induces $\alpha$V$\beta$3 integrin activation. These data strongly suggest that PI3K mediates $\alpha$V$\beta$3 integrin activation. How PI3K stimulates integrin activation is unknown; however, it may be significant that the small GTPase Rap1, which has been shown to contribute to integrin activation in several cell lines, can be activated by a PI3K-dependent mechanism in platelets (23) and has been implicated in cell response to strain (24). Further investigation of a possible link may therefore be warranted. The blocking anti-FN antibody 16G3 strongly inhibited the stretch-induced activation of JNK under conditions where existing adhesions did not appear to be disrupted, indicating that new integrin-ECM is required for JNK activation.

Taken together, these data define a pathway by which early activation of PI3K, through induction of integrin activation and ECM binding, stimulates a cytoplasmic signaling pathway implicated in cellular responses. It is likely that this mechanism explains the observation that mechanotransduction in a number of systems is sensitive to short term treatment with integrin antagonists that would not be expected to disrupt existing integrin-ECM bonds (12, 25, 26). This mechanism is likely to be relevant to many systems where mechanical forces influence ECM accumulation and organization, cytoskeletal organization, gene expression, and cell motility. Furthermore, identification of PI3K as an early target of mechanical strain should facilitate future investigation into more proximal mechanisms of mechanotransduction.

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REFERENCES

1. Ingber, D. E. (1997) Annu. Rev. Physiol. 59, 575–599
2. Sadoshima, J., and Izumo, S. (1997) Annu. Rev. Physiol. 59, 551–571
3. Thonbrink, M. J., and Robicsek, F. (1995) Ann. Thorac. Surg. 59, 1584–1603
4. Haston, W. S., Shields, J. M., and Wilkinson, P. C. (1983) Exp. Cell Res. 146, 117–126
5. Terracio, L., Miller, B., and Borg, T. K. (1988) In Vitro Cell Dev. Biol. 24, 53–58
6. Bray, D. (1986) Dev. Biol. 102, 379–389
7. Kolega, J. (1986) J. Cell Biol. 102, 1400–1411
8. Farge, E. (2003) Curr. Biol. 13, 1365–1377
9. Schwartz, M. A., and Ingber, D. E. (1994) Mol. Biol. Cell 5, 389–393
10. Wang, N., Butler, J. P., and Ingber, D. E. (1993) Science 260, 1124–1127
11. Chicheurel, M. E., Singer, R. H., Meyer, C. J., and Ingber, D. E. (1998) Nature 392, 730–733
12. Katsumi, A., Orr, A. W., Tsima, E., and Schwartz, M. A. (2004) J. Biol. Chem. 279, 12001–12004
13. Katsumi, A., Milamini, J., Kisses, W. B., del Pozo, M. A., Kaunas, R., Chien, S., Hahn, K. M., and Schwartz, M. A. (2002) J. Cell Biol. 153, 153–164
14. McCulloch, A. D. (1996) Am. J. Physiol. 271, C1400–C1408
15. Tsima, E., del Pozo, M. A., Shattil, S. J., Chien, S., and Schwartz, M. A. (2001) EMBO J. 20, 4639–4647
16. Ozaki, S., DeWald, B. D., Shoie, J. C., Chen, J., and Prestwich, G. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11286–11291
17. Papporti, N., Hato, T., Stupack, D. G., Aidoudi, S., Chereshe, D. A., Nemirov, G. R., and Shattil, S. J. (1999) J. Biol. Chem. 274, 21609–21616
18. Hughes, P. E., Renshaw, M. W., Paff, M., Forsyth, J., Keivens, V. M., Schwartz, M. A., and Ginsberg, M. H. (1997) Cell 88, 521–530
19. Shimizu, Y., Rose, D. M., and Ginsberg, M. H. (1999) Adv. Immunol. 72, 325–380
20. Kisses, W. B., Shattil, S. J., Papporti, N., and Schwartz, M. A. (2001) Nat. Cell Biol. 3, 316–320
21. Suzumma, T., Kuzuma, S., Ueki, K., Hata, Y., Feener, E. P., King, G. L., and McCulloch, A. D. (1996) Ann. Thorac. Surg. 61, 152–158
22. MacKenna, D. A., Dolfi, F., Vuori, K., and Ruoslahti, E. (1998) Curr. Opin. Cell Biol. 10, 1572–1578
23. Shyy, J. Y., and Chien, S. (1997) J. Biol. Chem. 272, 23382–23390
24. Sawada, Y., Nakamura, K., Ito, K., Takeda, K., Tohiume, K., Morita, M. S. K., Komuro, I., Bein, D. O., Sheshe, M., and Ichijo, H. (2001) J. Cell Sci. 114, 1221–1227
25. Davis, M. J., Wu, X., Nukiewicz, T. R., Kawasaki, J., Davis, G. E., Hill, M. A., and Meininger, G. A. (2001) Am. J. Physiol. 280, H1427–H1433
26. Shyy, J. Y., and Chien, S. (1997) Curr. Opin. Cell Biol. 9, 707–713

Fig. 3. A, activation of JNK. Upper panel, NIH3T3 cells were stretched for the indicated times and cell lysates analyzed by Western blotting using antibodies against phospho-JNK or total JNK. Lower panel, histogram showing phosphorylation of JNK normalized to total JNK (white, p54; black, p46). Values are means ± S.E. (n = 3) relative to non-stretched controls. * denotes p < 0.05 according to Student's t test. B, blocking of new integrin-FN connection. Upper panel, NIH3T3 cells were serum-starved overnight and then incubated with 20 μg/ml blocking anti-FN antibody 16G3, non-blocking anti-FN antibody 11E5, or without antibody for 15 min. Cells were rinsed with PBS twice, and 0.5% FBS/DMEM was added except for no shear control. Cells were then stretched for 15% for 10 min (S) or not stretched (N). Cell lysates were analyzed by Western blotting using antibodies against phospho-JNK or total JNK. Lower panel, histogram showing phosphorylation of JNK normalized to total JNK (white, p54; black, p46). Values are means ± S.E. (n = 4) relative to cells with non-stretch control. * and ** denote p < 0.05 and p < 0.02, respectively, according to Student's t test. Ab, antibody.