Mechanical Stressing of Integrin Receptors Induces Enhanced Tyrosine Phosphorylation of Cytoskeletally Anchored Proteins*

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Physical forces play a fundamental role in the regulation of cell function in many tissues, but little is known about how cells are able to sense mechanical loads and realize signal transduction. Adhesion receptors like integrins are candidates for mechanotransducers. We used a magnetic drag force device to apply forces on integrin receptors in an osteoblastic cell line and studied the effect on tyrosine phosphorylation as a biochemical event in signal transduction. Mechanical stressing of both the β1 and the α2 integrin subunit induced an enhanced tyrosine phosphorylation of proteins compared with integrin clustering. Application of cyclic forces with a frequency of 1 Hz was more effective than a continuous stress. Using Triton X-100 for cell extraction, we found that tyrosine-phosphorylated proteins became physically anchored to the cytoskeleton due to mechanical integrin loading. This cytoskeletal linkage was dependent on intracellular calcium. To see if mechanical integrin stressing induced further downstream signaling, we analyzed the activation of mitogen-activated protein (MAP) kinases and found an increased phosphorylation of MAP kinases due to mechanical stress. We conclude that integrins sense physical forces that control gene expression by activation of the MAP kinase pathway. The cytoskeleton may play a key role in the physical anchorage of activated signaling molecules, which enables the switch of physical forces to biochemical signaling events.

Application of physical forces to cells induces gene expression and proliferation in a variety of cell types (1–5, 9). Therefore, mechanical forces are a fundamental physiological factor in regulating structure and function in many tissues. In bone, mechanical loading stimulates the increase of bone mass (6–8) and plays an important role in the therapy of osteoporosis. The cellular mechanisms of mechanically induced signal transduction are largely unknown. Above all, it has remained elusive how cells are able to sense physical forces. Indications exist that integrin receptors may serve as mechanotransducers and plays an important role in the therapy of osteoporosis. The method enables the application of physical forces to defined integrins, which allows the evaluation of the relevance of specific integrin subunits in mechanical signal transduction.

Because mechanical strain may act in different frequencies and strength, which appears to have relevance in regulating cell physiology (21–23), an important question is whether perception of physical forces by integrin receptors induces a differential intracellular signal transduction. Recently, we developed a method to mechanically stress cell surface receptors (14). Using magnetic beads, drag forces in defined strength and frequency can be applied to receptors of cells in a monolayer. The method enables the application of physical forces to defined integrins, which allows the evaluation of the relevance of specific integrin subunits in mechanical signal transduction. Herein, we report that in the osteosarcoma cell line U-2 OS, mechanical stressing of integrins induces an increased tyrosine phosphorylation of proteins, including the MAP1 kinase, compared with integrin clustering and depending on whether a permanent or intermittent stress is applied. We also observed an increased physical anchorage of tyrosine-phosphorylated proteins at the cytoskeleton, which suggests that the cytoskeleton may serve as a structure where mechanical signals can switch into a chemical-signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—The osteosarcoma U-2 OS cell line was obtained from American Type Culture Collection (Rockville, MD). 96-well Fluoro Nunc modules from Nunc A/S (Roskilde, Denmark) were used for plating the cells. Paramagnetic microbeads (size 2.5 μm, coated with streptavidin) were purchased from Dynal (Hamburg, Germany). For coating of the microbeads, biotinylated anti-β1 (clone 2A4) and anti-α2 (clone AK7) integrin antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, AL); biotinylated anti-CD71 (transferrin receptor) antibody was from Oncogene Science, Inc. (Uniondale, NY). For immunoprecipitation of MAP kinases, anti-ERK-1 (p44), which also reacts with ERK-2 (p42), was used from Santa Cruz Biotechnology. Protein A-agarose was also purchased from Santa Cruz Biotechnology. Recombinant anti-phosphotyrosine antibody (clone RC-20) conjugated with alkaline phosphatase was from Transduction Laboratories. CDP-star for chemiluminescence was obtained from Boehringer Mannheim.

Cell Culture—U-2 OS cells were cultured in Dulbecco’s modified Eagle’s medium and supplemented with 10% fetal calf serum at 37 °C and in 5% CO2 atmosphere. For the experiments, 100 μl of cells in complete medium containing 106 cells were seeded into wells of a 96-well culture module and grown to near confluence. 2 h before mechanical strain was applied, the cells were depleted of serum.

Mechanical Receptor Stressing—The procedure to strain integrin receptors was described in detail elsewhere (14). In brief, the cell monolayer was incubated with paramagnetic microbeads coated with anti-β1 or anti-α2 antibodies. This is termed here as clustering. In average, five beads bound at the surface of one cell. To apply mechanical

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The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; BAPTA-AM, 1,2-bis(O-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, acetoxymethyl ester.
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For chelating intracellular calcium, the cells were preincubated with 5 μM of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, acetoxymethyl ester (BAPTA-AM) for 15 min. Mechanical strain was then applied in the presence of 5 μM of BAPTA.

RESULTS

The osteosarcoma cell line expressed the β1 as well as the α2 integrin subunits on the cell surface (14). Therefore, we examined the effect of mechanical stress applied to both integrin subunits on tyrosine phosphorylation of proteins as a mechanism in integrin-mediated signal transduction. First, we were interested in the time course of tyrosine phosphorylation due to clustering of the β1 integrin subunit by incubation of the cells with anti-β1-coated microbeads. We observed an increase of phosphorylation during the time of incubation, which reached the maximum after 60 min (Fig. 1). Based on this finding, mechanical stress was applied to integrins for 30 min after an incubation time of 20 min to bind the beads to the receptors. Application of forces to the β1 as well as to the α2 subunit induced an increased tyrosine phosphorylation of proteins compared with integrin clustering alone (Fig. 2). Stressing the β1 chain, the effect was more pronounced than with α2. To prove whether the mechanically induced cellular reactions are specific for integrins, we stressed the transferrin receptor (CD71) for comparison. Although a slightly increased tyrosine phosphorylation was observed compared with untreated cells, the effect was distinctly lower than after stressing an integrin receptor (Fig. 3). Next we compared the effect of permanent mechanical loading with an intermittent stress of 1 Hz on tyrosine phosphorylation. Application of a stress with a frequency of 1 Hz induced a more profound phosphorylation than permanent drag forces (Fig. 2). To exclude the possibility that the influence of different modes of magnetic field application alone and not the mechanical receptor stress provoked the differences in tyrosine phosphorylation, we compared controls in which pure cells were subjected to a permanent and a cyclic magnetic field. This experiment clearly demonstrated that the magnetic field alone did not influence tyrosine phosphorylation, independent of the mode of the magnetic field (Fig. 3).

To evaluate the role of the cytoskeleton in mechanically induced tyrosine phosphorylation, we examined whether tyrosine-phosphorylated proteins are anchored to the cytoskeleton. After clustering and mechanically loading of the integrins, cells were extracted with Triton X-100, and the detergent insoluble fraction analyzed for tyrosine-phosphorylated proteins (Fig. 4). Both clustering and additional stress induced a linkage of tyrosine-phosphorylated proteins. Again, mechanical load was more effective than clustering. This observation concerned the β1 subunit, whereas the linkage of α2 to the cytoskeleton was similar comparing the effect of clustering and additional mechanical load. Furthermore, cytoskeletonally anchored phosphorylated proteins were preferably detected in the higher molecular weight range. The anchorage of phosphorylated proteins to the cytoskeleton was further studied by disruption of...
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Fig. 3. Comparison of tyrosine phosphorylation due to mechanical stress to integrins, mechanical load to the transferrin receptor, and application of the magnetic field alone to the cells. Cells in a monolayer were mechanically stressed at the β1 integrin with 1 Hz as described above (lane 2) or stressed in the same manner at the transferrin receptor (CD71) (lane 3). Cells in a monolayer without magnetic beads were subjected to a permanent magnetic field (lane 4) or a cyclic magnetic field with 1 Hz (lane 5) for 30 min. Untreated cells were also examined (lane 1). Total cell lysates were electrophoresed and blotted for anti-phosphotyrosine. Compared with application of stress to the β1 integrin, mechanical stress to the transferrin receptor induced a detectable but significantly lower level of tyrosine phosphorylation. Different modes of the magnetic field applied demonstrated that application of physical forces to integrin structures appears to play an essential role in integrin-mediated signal transduction because its inhibition blocks gene expression (24).

Fig. 4. Cytoskeletal anchorage of tyrosine-phosphorylated proteins due to mechanical stress. After treatment of the cells by integrin stressing (1 Hz) (s), clustering (c), or without treatment (−), the cells were extracted with Triton X-100 to obtain the detergent-insoluble fraction. This cytoskeletal fraction was then processed for anti-phosphotyrosine immunoblotting. For α2, similar quantities of phosphorylated proteins were found after mechanical stress and clustering. For β1, mechanical stress induced a significant enhancement of cytoskeletonally linked tyrosine-phosphorylated proteins compared with clustered integrins. The anchorage of tyrosine-phosphorylated proteins was observed in the region of 130 kDa but not in the lower molecular weight range.

Fig. 5. Inhibition of the cytoskeletal anchorage of tyrosine-phosphorylated proteins by cytochalasin D. Cells were treated by mechanical stressing of the β1 integrin subunit (1 Hz) (s), clustering of β1 (c), or without treatment (+) in the presence of cytochalasin D (+). The cells were then extracted with Triton X-100 to obtain the insoluble cytoskeletal fraction. This fraction was then processed for anti-phosphotyrosine immunoblotting. Cytochalasin D dramatically blocked the cytoskeletal linkage of tyrosine-phosphorylated proteins. In the absence of cytochalasin D, mechanical stressing of integrins induced a distinctly enhanced anchorage of phosphorylated proteins. This represents the typical results of four independent experiments. (To obtain a background in the cytochalasin-treated samples, the blots in these experiments were exposed longer to the film.)

Fig. 6. Influence of the calcium chelator BAPTA-AM on the anchorage of tyrosine-phosphorylated proteins. Cells were treated by mechanical stressing of the β1 integrin subunit (1 Hz) (s), clustering of β1 (c), or without treatment (−) in the presence of BAPTA-AM (+BAPTA) to chelate intracellular calcium. The cells were then extracted with Triton X-100, and the insoluble fraction was processed for anti-phosphotyrosine immunoblotting. BAPTA-AM significantly inhibited the anchorage of tyrosine-phosphorylated proteins to the cytoskeleton, which is most obvious in the higher molecular weight range. For comparison, in the absence of BAPTA-AM (−BAPTA), the most profound tyrosine phosphorylation of cytoskeletonally linked proteins was found due to mechanical stressing of the β1 integrin subunit. The results are representative of four independent experiments.

distinctly higher degree of activation of the MAP kinases compared with integrin clustering (Fig. 7). This suggests that regulation of gene expression by physical forces is controlled by differential activation of MAP kinases and mediated by integrins.

DISCUSSION

Tyrosine phosphorylation of several cellular proteins appears to play an essential role in integrin-mediated signal transduction because its inhibition blocks gene expression (24). The mechanisms by which extracellular interactions of integrins regulate tyrosine phosphorylation remains elusive. We demonstrate that application of physical forces to integrin receptors enhanced the tyrosine phosphorylation of proteins com-
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Mechanical stress to the β subunit provoked a significant anchorage of tyrosine-phosphorylated proteins to the cytoskeleton, which was increased compared with integrin clustering. Tyrosine phosphorylation of cytoskeletal anchored proteins could be a prerequisite to form the cytoskeletal complex (38), and a higher degree of phosphorylation may be a prerequisite for the higher strengthening between receptors and cytoskeleton. Regarding the factors that determine the association of activated signaling molecules to the cytoskeleton, we have found that intracellular calcium is obviously an important regulator of the immobilization of proteins to the cytoskeleton. This concerns not only intracellular-signaling proteins but also the cytoskeletal anchorage of integrins to the cytoskeleton (28). The role of calcium for a mechanically induced signal transduction is also stressed by data that have shown that intracellular calcium concentrations correlated with increasing force levels applied to integrins (39). However, our previous experiments suggest that the differential cytoskeletal anchorage of tyrosine-phosphorylated proteins and integrin subunits due to stimulation of β1 compared with the α subunit is not controlled by differences in the magnitude of the calcium response. Incubation of cells with anti-integrin antibodies prior to mechanical stimulation of the cells (13), as well as preliminary results concerning the comparison of the calcium responses due to mechanical stress applied with magnetic beads to β1 and α2, revealed no quantitative differences in calcium signaling.

Concerning downstream signaling, we argue that the cytoskeleton could represent a structure where physical forces are transformed into a biochemical signal pathway. The differential anchorage of tyrosine-phosphorylated proteins due to physically stimulated integrins may regulate downstream intracellular-signaling events.

One of these events is the activation of MAP kinases as a key mechanism to control the activation of transcription factors, which therefore mediates gene expression. The involvement of this pathway in integrin signaling has been established (40, 41). We found that activation of the MAP kinases was significantly increased due to physical forces compared with integrin clustering. Due to the key role of the MAP kinases, our finding emphasizes that physical forces transduced by integrins differentially regulate cell proliferation and the expression of genes through the MAP kinase cascade. The fact that activation of MAP kinase by integrins depends on an intact cytoskeleton (41, 42) and the involvement of cytoskeletally associated signaling molecules like focal adhesion kinase (43) highlights the significance of a controlled cytoskeletal anchorage of tyrosine-phosphorylated proteins for consequences in cell behavior. Because the integrin-mediated MAP kinase pathway converges with growth factor-induced pathways (44), our result suggests a synergistic effect of mechanical forces and cytokines in the regulation of cell function.

In conclusion, integrins mediate physical forces and may regulate physiological consequences in the cell by a well tuned induction of the degree of tyrosine phosphorylation of proteins. A significant aspect is the cytoskeletal anchorage of activated signaling proteins, which depends on the mobilization of intracellular calcium. The functional relevance of these mechanisms is supported by the result of an enhanced activation of MAP kinases due to mechanical integrin stimulation.

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