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 (10–14). Integrins are heterodimeric transmembrane molecules by which cells adhere to the extracellular matrix (15). The β subunit is combined with one of the different α subunits, and both extracellular domains are involved in ligand binding. Engagement and clustering of these receptors induce signal transduction, which involves integrin linkage to the cytoskeleton and the generation of second messengers and biochemical events (13, 16, 17). Activation of protein tyrosine kinases appears to play a central role in integrin-mediated signaling (18–20).

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 microparticles (size 2.5 μm, coated with streptavidin) were purchased from Dynal (Hamburg, Germany). For coating of the microparticles, 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) antibody (clone C-16), 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 105 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 microparticles 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(2-aminophenoxy)ethane-N,N,N’,N’'-tetraacetic acid, acetoxymethyl ester.

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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 differences in tyrosine phosphorylation, we compared controls with clustering and additional stress induced a linkage of drag forces (β1/P/s). These samples are compared with clustering of the integrins by incubation with the microbeads without subsequent mechanical loading (c), and untreated control cells (-). Total cell lysates were electrophoresed and blotted for anti-phosphotyrosine. Most pronounced differences are observed in the 40-kDa region. In all cases, physical forces induced a significantly enhanced tyrosine phosphorylation compared with clustering and controls. Mechanical stressing of β1 induced a higher response than loading the α2 subunit (compare β1/Hz/s versus α2/1 Hz/s; or β1/P/s versus α2/P/s). A cyclic stress with a frequency of 1 Hz was more effective than a permanent stress in both integrin subunits (compare β1/Hz/s versus β1/P/s; and α2/1 Hz/s versus α2/P/s). The results are representative of four independent experiments.

FIG. 1. Time dependence of tyrosine phosphorylation after clustering of the β1-integrin subunit. Cells were incubated with anti-β1-coated microbeads for the indicated periods in minutes. A total cell lysate was then electrophoresed and blotted against an anti-phosphotyrosine antibody. Tyrosine phosphorylation increased up to 60 min and then decreased.

FIG. 2. Tyrosine phosphorylation induced by mechanical stressing of integrins. Cells in a monolayer were incubated with microbeads to bind at the β1 or α2 integrin subunit followed by application of drag forces (s). These samples are compared with clustering of the integrin subunit by incubation with the microbeads without subsequent mechanical loading (c), and untreated control cells (-). Total cell lysates were electrophoresed and blotted for anti-phosphotyrosine. Most pronounced differences are observed in the 40-kDa region. In all cases, physical forces induced a significantly enhanced tyrosine phosphorylation compared with clustering and controls. Mechanical stressing of β1 induced a higher response than loading the α2 subunit (compare β1/Hz/s versus α2/1 Hz/s; or β1/P/s versus α2/P/s). A cyclic stress with a frequency of 1 Hz was more effective than a permanent stress in both integrin subunits (compare β1/Hz/s versus β1/P/s; and α2/1 Hz/s versus α2/P/s). The results are representative of four independent experiments.
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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 a 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 to untreated cells had no effect on tyrosine phosphorylation.

Fig. 4. Cytoskeletal anchorage of tyrosine-phosphorylated proteins due to mechanical stress. After treatment of the cells by integrin stressing (1 Hz) (α), 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 150 kDa but not in the lower molecular weight range. Treatment with cytochalasin D (Fig. 5). Treatment with cytochalasin D abolished the cytoskeletal linkage of tyrosine-phosphorylated proteins indicating the requirement of actin polymerization.

Because intracellular calcium is induced by integrin stimulation, we determined whether intracellular calcium plays a role in the association of tyrosine-phosphorylated proteins to the cytoskeleton. During mechanical loading of integrins, cells were treated with the intracellular calcium chelator BAPTA-AM. The following analysis of cytoskeletonally anchored proteins, which are tyrosine-phosphorylated, revealed that BAPTA significantly reduced the physical anchorage of these proteins to the cytoskeleton (Fig. 6). This indicates that calcium regulates the linkage of activated proteins to the cytoskeleton during integrin-mediated mechanically induced signal transduction.

Last, we were interested in whether the increased tyrosine phosphorylation of proteins due to mechanical integrin stressing may lead to downstream signaling, which could be relevant for gene expression. Therefore, we examined whether among the tyrosine-phosphorylated proteins the MAP kinases are also activated. Immunoprecipitation of MAP kinases and analysis of tyrosine phosphorylation demonstrated that mechanical loading of both the β1 and the α2 integrin subunits induced a 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 cytoskeletonally 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 findings emphasize 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 cytoskeletonally 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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