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Short communication

Mechano-chemical signaling in F9 cells

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Abstract

We investigated the molecular mechanism by which cells recognize and respond to physical forces in their local environment. Using a model system, to study wild type mouse F9 embryonic carcinoma cells, we examined how these cells sense mechanical stresses and translate them into biochemical responses through their cell surface receptor integrin and via the focal adhesion complex (FAC). Based on studies that show that many signal transducing molecules are immobilized on the cytoskeleton at the site of integrin binding within the focal adhesion complex, we found a time-dependent increase of focal adhesion kinase (pp125^{FAK}) phosphorylation possibly due to protein kinase C (PKC) activation as well as protein kinase A (PKA) activity increase upon cell adhesion/spreading. These studies provide some insight into intracellular mechano-chemical signaling.

Keywords: Cellular signal transduction; pp125^{FAK}; PKC; PKA; pCREB; F9Wt; Mouse embryonic carcinoma cells

1. Introduction

Cell adhesion is a pre-requisite for proper metabolism, protein synthesis, and cell survival (Chen et al., 2004). It provides the physical structure that allows important biochemical signals to initiate fundamental changes in cell behavior, architecture, and mechanical properties (Ingber, 2002; Kumar et al., 2006). An important group of adhesive transmembrane receptors that physically link the extracellular matrix (ECM) with the internal cytoskeleton are integrins (Hynes, 1992; Xiong et al., 2001; Goldmann, 2002a; Brakebusch and Fässler, 2003). These are intimately connected with the focal adhesion (FA) proteins like talin, vinculin, alpha-actinin, paxillin, and focal adhesion kinase (pp125^{FAK}), etc. whose mechanical and structural changes affect cell signaling (Alenghat and Ingber, 2002; Fonseca et al., 2005; Ballestrem et al., 2006). Applying mechanical stress at FAs leads to important signaling events through pp125^{FAK} and its downstream partners and causes cell proliferation (Leopoldt et al., 2001; Sawada and Sheetz, 2001; Goldmann, 2002b).

It was shown in bovine capillary endothelial cells that the focal adhesion complex (FAC) acts as a key mediator for mechano-chemical signal transduction that regulates cell growth and survival (Plopper et al., 1995). Protein kinase C (PKC) activation was closely associated with tyrosine phosphorylation of pp125^{FAK} linking mechanical external stimulation to the internal environment. Other structural and functional proteins within FAC were also assumed to be critically important for stress-induced cellular remodeling (Plopper et al., 1995). Interestingly, integrins, pp125^{FAK}, and PKC co-localize in focal adhesion contacts (Plopper et al., 1995; Goldmann, 2002c), which may facilitate cross-talks between signal pathways that have long been viewed as separate systems.

The close relationship of integrin receptor and the focal adhesion complex suggests that it could be a "*site of mechanical sensor*". pp125^{FAK} is intimately associated with a number of other key cytoskeletal proteins, including vinculin, talin, paxillin, and critical kinases such as rac (Goldmann and Ingber, 2002), rho (Machesky and Hall, 1996) mitogen

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activated protein kinase (MAPK) (Bhalla et al., 2002), and src (Goldmann, 2002c; Gunst et al., 2003). It is likely that a mechanical signal from the extracellular environment is transduced through the focal adhesion complex during mechanical deformation triggering a signaling cascade that could activate PKC for mechanical cytoskeleton re-organization (Chicurel et al., 1998). In the present study we examined the influence of F9 wild type cell adhesion and spreading on biochemical signaling focusing on three intracellular kinases: pp125^{FAK}, PKC, and protein kinase A (PKA).

2. Materials and methods

2.1. Culture of wild type mouse F9 embryonic carcinoma cells

The F9 cells were maintained on 0.1% gelatin-coated charged plastic culture dishes in high glucose (4 g/L) DMEM, 10% calf serum, 20 mM HEPES, 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin (Goldmann et al., 1998a,b). Prior to experiments, the cells were suspended with trypsin, washed in 1% BSA/DMEM and re-cultured in DMEM containing 4 g/L glucose, 2% calf serum, 2 mM glutamine, 100 U/ml streptomycin, 100 U/ml penicillin.

2.2. Cell adhesion, spreading, and lysis

Approximately 1×10^5 F9 wild type cells were plated on 5 μg/ml fibronectin-coated 35 mm dishes that had been serum-starved for 24 h prior to experimentation. After 0, 10, 30, 60, and 240 min of adhesion, F9 cells were exposed for 15 min to CSK(−) buffer containing: 10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$ and the following protease inhibitors: 1 μg/ml pepstatin A, 20 μg/ml leupeptin, 20 μg/ml aprotinin (final), and 1 mM PMSF; and to CSK(+) buffer containing: CSK(−) buffer plus 0.5% Triton X-100 (final); and then extracted by RIPA-buffer (Boston Bioproducts Inc, Worcester, USA) containing: 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris at pH 7.2. Specifically, F9 cells were lysed *in situ* on ice for 10 min with 0.2 ml of buffer that contained 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris at pH 7.2. (Rp-cAMP and GF 109203X were supplied by Calbiochem, Nottingham, UK; IBMX was purchased from A.G. Scientific Inc, San Diego, CA, USA and 2A7 and 4G10 monoclonal antibodies were obtained from UBI Lake Placid, NY, USA.)

2.3. SDS-Page and Western blotting

Equal amounts of proteins from cell extracts were subjected to 10% SDS-Page. Resolved proteins were electroblotted at 4 °C by applying a constant 100 V for 2 h onto nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS-T (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2% Tween 20) for 1 h at room temperature. The blots were incubated at 4 °C overnight with anti-pp125^{FAK} (1:1000) monoclonal antibody or antibodies directed against pCREB and CREB (courtesy of Dr. S. Kaufmann, TU Munich) in 0.5% BSA, TBS-T. After thorough washing with 0.5% BSA, in TBS-T, the membranes were incubated with anti-mouse IgG conjugated with anti-goat IgG (1:5000). The blots were washed, further developed, and analyzed with an enhanced chemo-luminescence. The stoichiometry of phosphorylation was determined by densitometry using NIH-image software.

2.4. Immunofluorescence nuclear staining

For PKA-c staining, F9 cells were permeabilized in a cytoskeletal stabilizing buffer (300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 mM PIPES, pH 6.8) containing protease inhibitors (20 μg/ml aprotinin, 1 μg/ml pepstatin, 20 μg/ml leupeptin, 100 μM AEB-SF). The cells were then fixed in ice-cold methanol for 7 min and washed in immunofluorescence buffer containing 0.15% Triton X-100, 10% calf serum in PBS and incubated with mouse anti-PKA-c antibody for 1 h at room temperature. The primary antibody was detected using a Texas Red-labeled donkey anti-rabbit IgG secondary antibody. Fluorescent images from 15 to 20 cells/condition were digitally recorded using a DAGE MTI camera and the nuclear staining intensity was quantified using Oncor image analysis software.

3. Results and discussion

Previously, we had looked at the influence of cell adhesion on the phosphorylation of focal adhesion kinase (pp^{125FAK}) and mitogen activated protein kinase (MAPK) in F9 cells. Results from these experiments showed that the activity of pp^{125FAK} started to increase 20–30 min after cell plating and had doubled after 240 min, whilst the MAP-kinase activity increased by a factor of 1.3 over the same period (Goldmann, 2002b).

Here, we investigated the activation and phosphorylation of pp^{125FAK} in F9 cells in more detail. We examined cell adhesion at various time points between $t = 0$ and $t = 240$ min after replating. Experimentally, F9 cells were exposed to CSK(−) and CSK(+) buffer for 15 min and then extracted by RIPA-buffer. The lysates from F9 cells were immunoprecipitated with anti-pp^{125FAK} serum, pp^{125FAK} monoclonal antibody 2A7, or with anti-Tyr(P) monoclonal antibody 4G10, or separately with other control antibodies. The immunoprecipitates were then either subjected to immune kinase reactions or analyzed directly by SDS-Page and Western blotting. Fig. 1 (a, upper panel) shows that F9 cell adhesion increased tyrosine phosphorylation of pp^{125FAK} (arrow); and (a, lower panel) demonstrates that equal amounts were immunoprecipitated

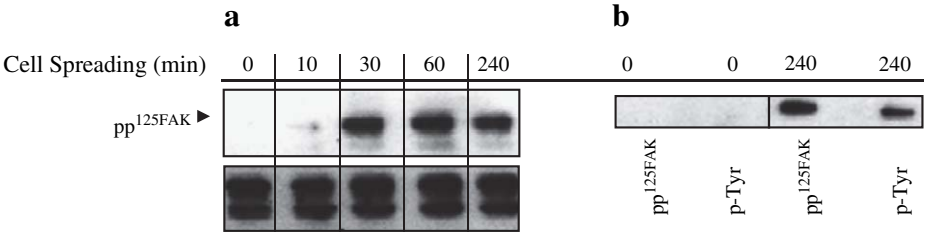

Fig. 1. Stimulation of pp125^{FAK} tyrosine phosphorylation due to cell spreading of F9 wild type cells. F9 cells were collected at various times after spreading (see Section 2) and incubated with antiserum or mAb for immunoprecipitation. The immunoprecipitates were separated by SDS-Page, transferred to immobilon-P membrane, and Western blot analysis was used against phosphotyrosine antibody 4G10 after immunoprecipitation with pp125^{FAK} antiserum (a, upper panel). The sample from the blot was stripped and probed against pp125^{FAK} antiserum to demonstrate whether equal amounts immunoprecipitated (a, lower panel). The immunoprecipitation with the aforementioned monoclonal antibody from F9 wild type cells at $t = 0$ and $t = 240$ min of spreading was blotted against pp125^{FAK} antiserum (b). pp125^{FAK} was immunoprecipitated with monoclonal antibodies against phosphotyrosine indicated by the bands.

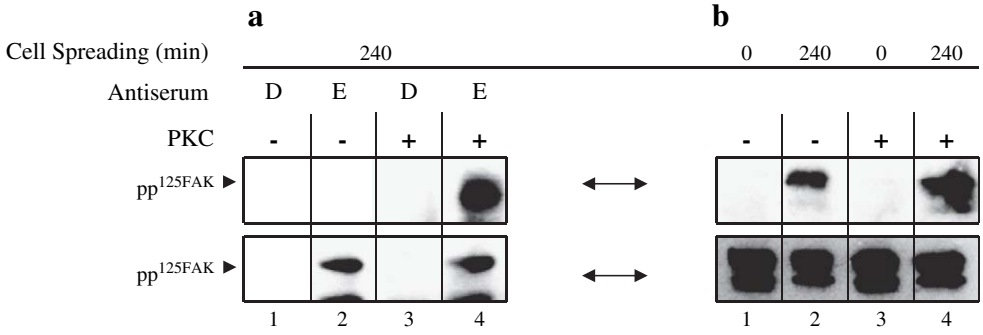

Fig. 2. *In vitro* phosphorylation of pp125^{FAK} by PKC. pp125^{FAK} was immunoprecipitated from F9 wild type cells at $t = 0$ and $t = 240$ min of spreading, and *in vitro* kinasing reactions were performed in the presence and absence of PKC. The product of the *in vitro* kinase reaction was analyzed by SDS-Page after transferring the proteins to the immobilon-P membrane. (a) Autoradiogram and (b) phosphotyrosine blot. D, normal mouse antiserum (NMS); and E, pp125^{FAK} antiserum. Lower panels (1–4) demonstrate the anti-pp125^{FAK} blot (a) and equal loading (b).

indicating two bands at ~120 kDa and ~125 kDa. The immunoprecipitation with the above monoclonal antibody from F9 cells at $t = 0$ and $t = 240$ min time of spreading was blotted against pp^{125FAK} antiserum, and pp^{125FAK} was immunoprecipitated with monoclonal antibody 4G10 against phosphotyrosine (Fig. 1b). The results from these experiments show that cell adhesion induces activation and tyrosine phosphorylation of pp^{125FAK}.

To assess the role of PKC during F9 cell adhesion, we had a closer look at the phosphorylation of pp^{125FAK}. Fig. 2a shows the autoradiogram of pp^{125FAK} after 240 min of cell attachment (D, normal mouse antiserum; and E, pp^{125FAK} antiserum). In the upper panel, lane 4, pp^{125FAK} was only phosphorylated when PKC was present, compared with lane 2 in the absence of PKC, although equal amounts of protein were loaded (Fig. 2a, lower panel).

To determine whether phosphorylation by PKC regulates the tyrosine phosphorylation of pp^{125FAK}, we performed phosphotyrosine blotting on the immunoprecipitates after treatment with and without PKC. The results presented in Fig. 2b (phosphotyrosine blot; arrow, upper panel) strongly suggest that, although PKC phosphorylates adhesion-activated pp^{125FAK}, it probably has no direct influence on the (tyrosine) phosphorylation of pp^{125FAK} *in vitro*. This was recently demonstrated using the inhibitor (GF 109203 X) of PKC that stopped activation of pp^{125FAK} by PMA (Hunger-Glaser et al., 2003). It seems, therefore, plausible that the pathway that leads to the activation of pp^{125FAK} through adhesion does require PKC activity, though its main role is indirect; i.e. either PKC makes pp^{125FAK} a more suitable substrate for another kinase, or PKC activates other kinases that (tyrosine)-phosphorylate and activate pp^{125FAK} (Hunger-Glaser et al., 2003). The lower panel in Fig. 2b indicates that equal amounts of pp^{125FAK} were immunoprecitated indicating two bands at ~120 kDa and ~125 kDa.

Previous reports also showed that cell attachment/spreading on ECM results in a marked increase in intracellular cAMP levels (Fong and Ingber, 1996). To establish whether cell adhesion-induced changes in cAMP were physiologically relevant and specific for integrins, we looked at a downstream effector of cAMP, i.e. protein kinase A (PKA) in F9 cells. We determined the PKA activation (due to integrin binding to ECM) in experiments where we used the phosphodiesterase inhibitor IBMX to control G-protein-coupled transmembrane activation according to the protocol by Meyer et al. (2000). To gauge the PKA activity, we took advantage of the fact that when four molecules of cAMP bind PKA, its catalytic subunit (PKA-c) dissociates from its regulatory subunit (PKA-r) and can freely translocate to the nucleus by passive diffusion (Bhalla and Iyengar, 1999), where it phoshorylates and activates the transcriptional regulator CREB (Nigg et al., 1985; Gonzalez and Montminy, 1989; Harootunian et al., 1993). Western blot analysis of whole cell lysates from F9 cells at $t = 0$ and $t = 240$ min shows that the presence of IBMX increases pCREB phosphorylation (Fig. 3, lane 2 + 3), and the presence of the cAMP inhibitor Rp-cAMP blunts the phosphorylation (Fig. 3, lane 1 + 4). Quantifying the immunofluorescent staining intensity of PKA-c in the nuclei of F9 cells, we indirectly measured its activation by cAMP using phase-contrast microscopy. Upon fixation and staining for PKA-c in F9 cells, we found a diffuse pattern of cytoplasmic staining after 240 min compared to a brighter nuclear stain at zero time (Fig. 4), confirming that this effect on cAMP signaling was probably dependent on both adhesion (strain energy) and integrins (Meyer et al., 2000).

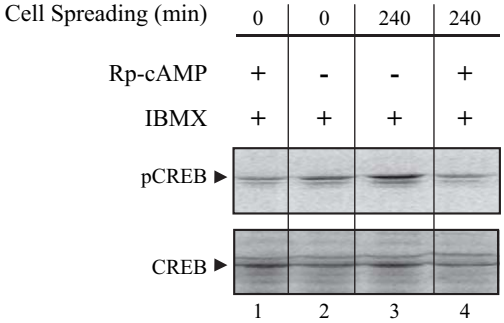

Fig. 3. Western blots stained with antibodies against phosphorylated CREB (pCREB) and total CREB using total cell proteins isolated from F9 wild type cells (see Section 2) at $t = 0$ (upper panel, lane 1 + 2) and $t = 240$ min (upper panel, lanes 3 + 4), which were cultured with IBMX in the presence/absence cAMP inhibitor Rp-cAMP. Lower panels (lanes 1–4) show equal loading of CREB.

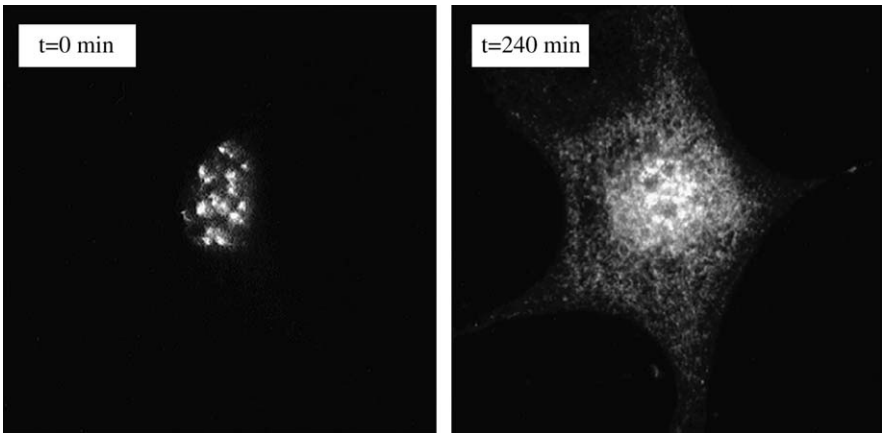

Fig. 4. Visualization of nuclear PKA-c staining. Fluorescent micrographs of F9 wild type cells at $t = 0$ and $t = 240$ min of adhesion/spreading following 15 min pretreatment with 100 μM IBMX (in the absence of cAMP inhibitor Rp-cAMP) and incubation at 37 °C.

In summary, all of the above data contribute to the growing body of evidence implicating PKC and PKA as mediator for cell remodeling after environmental stress. The present study does not indicate a direct association between PKC and pp^{125FAK}, nor does it indicate whether the two signaling kinases reside in the same signaling pathway. More work needs to be done to determine the effect pp^{125FAK} has on other supporting proteins during cell remodeling. However, with the evidence we have provided, we can conclude that pp^{125FAK} plays an important role in cellular response to mechanical stimulation.

Although signaling cascades have been proposed to be important for mechanoregulation, it was never clear how external mechanical stresses activate this response. In addition, stress-sensitive regulatory elements have been identified in the promoters of certain mechanosensitive genes (Resnick et al., 1993; Khachigian et al., 1997), but the mechanism by which mechanical signals that elicit these effects are transmitted across the cell surface and the influence of adhesion (energy) remains to be elucidated.

Recent measurements using traction microscopy showed that F9 wild type cells generate about 0.3×10^{-15} J adhesion energy within 20 min (Jaafar et al., 2005), a time course which compares to pp^{125FAK} phosphorylation demonstrated here. This raises the following questions: whether this energy is needed to activate mechano-chemical signaling in cells and whether mechanotransduction is preceded or followed by phosphorylation?

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References

Alenghat FJ, Ingber DE. Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. Sci STKE; 2002. PE6 pp.

Ballestrem C, Erez N, Kirchner J, Kam Z, Bershadsky A, Geiger B. Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. J Cell Sci 2006;119:866–75.

Bhalla US, Iyengar R. Emergent properties of networks of biological signaling pathways. Science 1999;283:381–7.

Bhalla US, Ram PT, Iyengar R. MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. Science 2002; 297:1018–23.

Brakebusch C, Fässler R. The integrin-actin connection, an eternal love affair. EMBO J 2003;22:2324–33.

Chen CS, Tan J, Tien J. Mechanotransduction at cell-matrix and cell–cell contacts. Annu Rev Biomed Eng 2004;6:275–302.

Chicurel ME, Singer RH, Meyer CJ, Ingber DE. Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. Nature 1998;392:730–3.

Fong JH, Ingber DE. Modulation of adhesion-dependent cAMP signaling by echistatin and alendronate. Biochem Biophys Res Commun 1996;221:19–24.

Fonseca PM, Inoue RY, Kobarg CB, Crosara-Alberto DP, Kobarg J, Franchini KG. Targeting to C-terminal myosin heavy chain may explain mechanotransduction involving focal adhesion kinase in cardiac myocytes. Circ Res 2005;96:73–81.

Goldmann WH. Mechanical aspects of cell shape regulation and signaling. Cell Biol Int 2002a;26:313–7.

Goldmann WH. The coupling of vinculin to the cytoskeleton is not essential for mechano-chemical signaling in F9 cells. Cell Biol Int 2002b;26:279–86.

Goldmann WH. p56(lck) controls phosphorylation of filamin (ABP-280) and regulates focal adhesion kinase (pp125(FAK)). Cell Biol Int 2002c;26: 567–71.

Goldmann WH, Galneder R, Ludwig M, Kromm A, Ezzell RM. Differences in F9 and 5.51 cell elasticity determined by cell poking and atomic force microscopy. FEBS Lett 1998a;424:139–42.

Goldmann WH, Galneder R, Ludwig M, Xu M, Adamson ED, Wang N, et al. Differences in elasticity of vinculin-deficient F9 cells measured by magnetometry and atomic force microscopy. Exp Cell Res 1998b;239:235–42.

Goldmann WH, Ingber DE. Intact vinculin protein is required for control of cell shape, cell mechanics, and rac-dependent lamellipodia formation. Biochem Biophys Res Commun 2002;290:749–55.

Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 1989;59: 675–80.

Gunst SJ, Tang DD, Opazo Saez A. Cytoskeletal remodeling of the airway smooth muscle cell: a mechanism for adaptation to mechanical forces in the lung. Respir Physiol Neurobiol 2003;137:151–68.

Harootunian AT, Adams SR, Wen W, Meinkoth JL, Taylor SS, Tsien RY. Movement of the free catalytic subunit of cAMP-dependent protein kinase into and out of the nucleus can be explained by diffusion. Mol Biol Cell 1993;4:993–1002.

Hunger-Glaser I, Salazar EP, Sinnett-Smith C, Rozengurt JE. Bombesin, lysophosphatidic acid, and epidermal growth factor rapidly stimulate focal adhesion kinase phosphorylation at Ser-910: requirement for ERK activation. J Biol Chem 2003;278:22631–43.

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11–25.

Ingber DE. Mechanical signaling and the cellular response to extracellular matrix in angiogenesis and cardiovascular physiology. Circ Res 2002;91: 877–87.

Jaafar L, Mierke CT, Paranhos-Zitterbart D, Kollmannsberger P, Pauli J, Goldmann WH, et al. Role of vinculin in cytoskeletal dynamics and regulation. ASCB Meeting San Francisco 2005; Abstract #1697, Poster B185.

Khachigian LM, Anderson KR, Halnon NJ, Gimbrone Jr MA, Resnick N, Collins T. Egr-1 is activated in endothelial cells exposed to fluid shear stress and interacts with a novel shear-stress-response element in the PDGF A-chain promoter. Arterioscler Thromb Vasc Biol 1997;17: 2280–6.

Kumar S, Maxwell IZ, Heisterkamp A, Polte TR, Lele TP, Salanga M, et al. Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. Biophys J 2006;90:3762–73.

Leopoldt D, Yee Jr HF, Rozengurt E. Calyculin-A induces focal adhesion assembly and tyrosine phosphorylation of p125(Fak), p130(Cas), and paxillin in Swiss 3T3 cells. J Cell Physiol 2001;188:106–19.

Machesky LM, Hall A. Rho: a connection between membrane receptor signalling and the cytoskeleton. Trends Cell Biol 1996;6:304–10.

Meyer CJ, Alenghat FJ, Rim P, Fong HJ, Fabry B, Ingber DE. Mechanical control of cyclic AMP signalling and gene transcription through integrins. Nat Cell Biol 2000;2:666–8.

Nigg EA, Schafer G, Hilz H, Eppenberger HM. Cyclic-AMP-dependent protein kinase type II is associated with the Golgi complex and with centrosomes. Cell 1985;41:1039–51.

Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol Biol Cell 1995;6:1349–65.

Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF, Gimbrone Jr MA. Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. Proc Natl Acad Sci USA 1993;90:4591–5.

Sawada Y, Sheetz MP. Force transduction by Triton cytoskeletons. J Cell Biol 2001;156:609–15.

Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL, et al. Crystal structure of the extracellular segment of integrin alpha Vbeta3. Science 2001;294:339–45.