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 Fassler, 2003). These are intimately connected with the focal adhesion (FA) proteins like talin, vinculin, alpha-actin, 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...
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: pp125FAK, 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, 1 mM t-glutamate, 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 × 10⁵ 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 MgCl₂ 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 electrobotted 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-pp125FAK (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 MgCl₂, 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 DMG EMTI camera and the nuclear staining intensity was quantified using Onco image analysis software.

3. Results and discussion

Previously, we had looked at the influence of cell adhesion on the phosphorylation of focal adhesion kinase (pp125FAK) and mitogen activated protein kinase (MAPK) in F9 cells. Results from these experiments showed that the activity of pp125FAK 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 pp125FAK in F9 cells in more detail. We examined cell adhesion at various time points between t = 0 and t = 240 min after re-plating. 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-pp125FAK serum, pp125FAK 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 pp125FAK (arrow); and (a, lower panel) demonstrates that equal amounts were immunoprecipitated.
indicating two bands at $\sim$120 kDa and $\sim$125 kDa. The immunoprecipitation with the above monoclonal antibody from F9 cells at $t = 0$ and $t = 240$ min of spreading was blotted against pp$^{125\text{FAK}}$ antiserum, and pp$^{125\text{FAK}}$ 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$^{125\text{FAK}}$.

To assess the role of PKC during F9 cell adhesion, we had a closer look at the phosphorylation of pp$^{125\text{FAK}}$. Fig. 2a shows the autoradiogram of pp$^{125\text{FAK}}$ after 240 min of cell attachment (D, normal mouse antiserum; and E, pp$^{125\text{FAK}}$ antiserum). In the upper panel, lane 4, pp$^{125\text{FAK}}$ 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$^{125\text{FAK}}$, 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$^{125\text{FAK}}$, it probably has no direct influence on the (tyrosine) phosphorylation of pp$^{125\text{FAK}}$ in vitro. This was recently demonstrated using the inhibitor (GF 109203 X) of PKC that stopped activation of pp$^{125\text{FAK}}$ by PMA (Hunger-Glaser et al., 2003). It seems, therefore, plausible that the pathway that leads to the activation of pp$^{125\text{FAK}}$ through adhesion does require PKC activity, though its main role is indirect; i.e. either PKC makes pp$^{125\text{FAK}}$ a more suitable substrate for another kinase, or PKC activates other kinases that (tyrosine)-phosphorylate and activate pp$^{125\text{FAK}}$ (Hunger-Glaser et al., 2003). The lower panel in Fig. 2b indicates that equal amounts of pp$^{125\text{FAK}}$ were immunoprecipitated indicating two bands at $\sim$120 kDa and $\sim$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 phosphorylates 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).

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**Fig. 2. In vitro phosphorylation of pp$^{125\text{FAK}}$ by PKC.** pp$^{125\text{FAK}}$ 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, pp$^{125\text{FAK}}$ antiserum. Lower panels (1–4) demonstrate the anti-pp$^{125\text{FAK}}$ blot (a) and equal loading (b).

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**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.
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 pp125FAK, 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 pp125FAK has on other supporting proteins during cell remodeling. However, with the evidence we have provided, we can conclude that pp125FAK 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 $0.3 \times 10^{-15}$ J adhesion energy within 20 min (Jaafar et al., 2005), a time course which compares to pp125FAK 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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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.
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