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Short communication

Tyrosine Phosphorylation of Paxillin during Cell Adhesion

Jong-Soo Chang[†,*], Hongmie Lee[‡] and Do Sik Min[§]

[†]Department of Life Science, [‡]Department of Food Science and Nutrition, Daejin University, Pochon-gun, Kyeonggido 487-800, [§]Department of Physiology, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

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Proteins that are involved in cellular signal cascade experience phosphorylation and dephosphorylation cycles in their tyrosine residue(s) during cell adhesion. In order to identify the protein(s), which tyrosine desidues are specifically phosphorylated when the cells attached to the substrate, we compared the tyrosine phosphorylation level of proteins between suspension and adhered culture condition in rat fibroblast 3Y1 cells. We found that a cluster of 70 kDa protein was specifically phosphorylated when the cells adhered to the substrate, but did not effect the cells held in suspension. The phosphorylated protein is identified as paxillin, a focal adhesion protein in immunoprecipitation and immunobloting analysis. These results suggest that the tyrosine phosphorylation of paxillin may play a role in cell-substrate adhesion.

Key words: Cell adhesion, Paxillin, Phosphorylation.

Introduction

The interactions between a cell and its extracellular matrix (ECM) regulate its morphology, migration, proliferation and differentiation (Burridge and Chrzanowska-Wodnika, 1996). One of the model systems for the study of cell adhesive interaction is the focal adhesion (FA). That is where the specialized zone of adhesion is emerged by many cells in the culture. The FA consists of integrin receptors where discrete areas of the cell membrane forms links between the cytoskeleton and the extracellular matrix by coupling to integrin. During the assembly of FA, many phosphorylated proteins are condensed to FA. These include: paxillin, p125 focal adhesion kinase (FAK), Src, Csk, Crk, Grb-2, p130cas, and PI-3 kinase (Burridge and Chrzanowska-Wodnika, 1996). Among these proteins, paxillin is a protein involved in the signal transduction from the plasma membrane to FA and actin cytoskeleton.

*To whom correspondence should be addressed.
Tel: 82-31-539-1853; Fax: 82-31-539-1850
E-mail: jchang@road.daejin.ac.kr

In order to gain a better understanding of the FA formation, we examined the level of tyrosine phosphorylation in the cellular proteins, which are involved in FA formation in rat fibroblast 3Y1 cells. We found paxillin to be phosphorylated in tyrosine residues in the adhesion cells, but not in the suspension cells. Paxillin is originally identified as a substrate for v-src tyrosine kinase and shown to be localized at FA (Glenny and Zokas, 1989). Furthermore, it has been shown recently that several protein tyrosine phosphatases are involved in the dephosphorylation of FAK and paxillin (Ouwens *et al.*, 1996; Shen *et al.*, 1998), as well as in the overexpression of the phospholipase C-γ1 reduced tyrosine phosphorylation level of paxillin (Chang *et al.*, 1999). In this paper, we describe how paxillin is tyrosine phosphorylated when the cells adhered to the substratum, but there is no phosphorylation after the cells have become rounded.

Materials and Methods

Materials The antibodies used were anti-phosphotyrosine antibody (PY20, Transduction Laboratory), anti-paxillin antibody (Z035, Zymed) and rhodamine-conjugated anti-mouse IgG (115-087-003, Jackson Immuno Research Laboratory). Phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and sodium orthovanadate were from Sigma. Fibronectin (F4759), bovine serum albumin (BSA, A9418) and poly-L-lysine (P6282) were from Sigma. The enhanced chemiluminescence detection system was purchased from Amersham.

Cell culture and adhesion to substrate The rat embryonic fibroblast 3Y1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% bovine calf serum (HyClone). The assays of cell adhesion to various substrates were performed by precoating 100-mm plastic culture dishes with 15 μg/ml fibronectin, 50 μg/ml poly-L-lysine, or 1% BSA for 4 hr at 37°C followed by washings in phosphate-buffered saline (PBS) and blocking with 1% BSA for 2 hr. Transformed cells after culture of 3-4 days were trypsinized, washed twice with PBS containing 0.5 mg/ml soybean trypsin inhibitor, and resuspended in DMEM without serum. Then, the cells were added

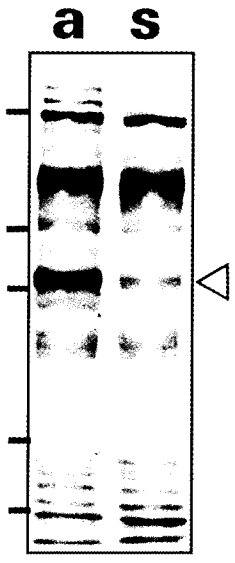

Fig. 1. Tyrosine phosphorylation of 65-70 kDa protein during cell adhesion. Tyrosine phosphorylation of paxillin was examined in extracts of the 3Y1 cells after trypsinization and of cells adhering to the plastic culture dish (a) and held in suspension (s) by immunoblot analysis using anti-phosphotyrosine antibody. The bars on the left of the panel represent MW markers of 220, 97, 66, 46, and 30 kDa, respectively. An open triangle represents tyrosine dephosphorylated proteins of 65-70 kDa.

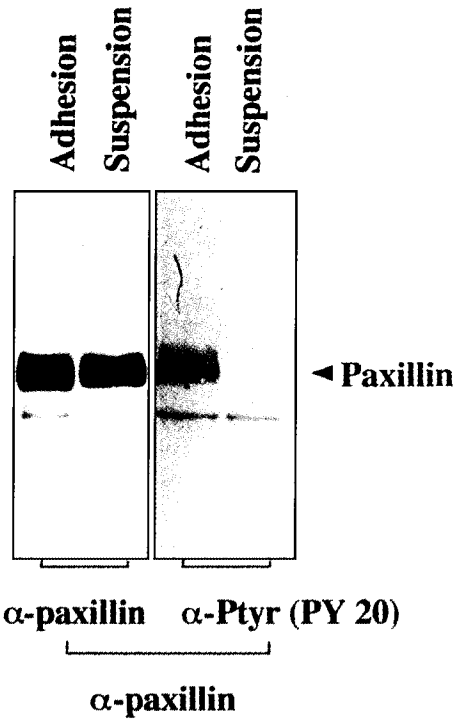

Fig. 2. Identification of 65-70 kDa protein as a paxillin. Cell extracts prepared exactly the same way as those in Fig. 1 were subjected to immunoprecipitation with anti-paxillin antibody and immunoblotted with anti-paxillin (left) and anti-phosphotyrosine (right)

to dishes precoated with the various substrates and incubated at 37°C in the absence of serum for 1 hr. The cells were then washed and lysed. The lysates were used for immunoprecipitation and immunoblot analysis with appropriate antibodies.

Immunoprecipitation and immunoblot analysis Cells were cultured in 100-mm or 150-mm dishes (Falcon), lysed in 500 μl of lysis buffer [10 mM HEPES-NaOH (pH7.4), 200 mM NaCl, 2% SDS, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate] and the extracts boiled for 3 min. The lysates were then sonicated for 10 sec and centrifuged at 10,000 × g for 20 min. Equal amounts of the cleared lysates (50-100 μg of protein per lane) were subjected to 7.5 or 10% SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted as described previously (Chang *et al.*, 1995). For immunoprecipitation, the cells were lysed in a RIPA buffer containing protease inhibitors. Each extract (0.5-1 mg of protein) was incubated with a pre-formed complex of *Staphylococcus aureus*-goat anti-mouse IgG (Pansorbin, Calbiochem) complex and either a preimmune serum or anti-paxillin antibody. After a 12-14 hr incubation at 4°C, pellets were obtained by centrifugation at 6,000 × g for 3 min, washed three times with RIPA buffer, and boiled for 3 min. An immunoblot analysis was carried out as described previously (Chang *et al.*, 1995), except that it took 12-14 hrs of incubation with the anti-paxillin antibody in order to obtain a good signal.

Immunofluorescence For immunofluorescence experiments, cells were cultured in glass slides (Lab-Tek) and processed as follows. Cells were first extensively washed with PBS, and then fixed with 4% formaldehyde in PBS for 15 min. After three additional washes of 5 min in PBS, permeabilization was performed with 1% Triton X-100 for 10 min, followed by three additional PBS washes. Blocking of non-specific sites was done with 1% BSA in PBS for 60 min, and followed by incubation with primary antibodies for 60 min in PBS-BSA. Monoclonal anti-paxillin and anti-phosphotyrosine antibodies were used at 1 : 200 and 1 : 250 dilutions respectively. After four washes in PBS-BSA, cells were incubated with secondary Rhodamine-coupled anti-mouse antibody in PBS-BSA for 30 min and extensively washed with PBS before mounting. Fluorescence was recorded with a confocal microscope (Bio-Rad).

Results and Discussion

Cells held in suspension show tyrosine dephosphorylation of paxillin We investigated the tyrosine phosphorylation level of the total cellular protein from 3Y1 fibroblasts in the adhesion and suspension by using an anti-phosphotyrosine antibody. The cells that were held in suspension showed bands of 65-70 kDa protein species, whose tyrosine phosphorylation seemed depressed in comparison to the control cells (Fig. 1).

Paxillin is a well-known focal adhesion protein. It is comprised of a family of proteins with molecular masses around 65-70 kDa, which are substrates for tyrosine kinases

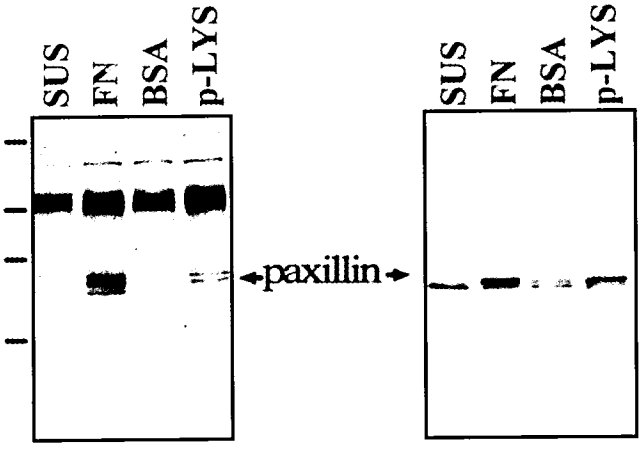

Fig. 3. Fibronectin restores tyrosine dephosphorylation of paxillin. The 3Y1 cells were collected immediately after trypsinization. The cells were then held in suspension for 1 hr and transferred for 1 hr onto fibronectin (FN), BSA, and poly-L-lysine (p-LYS) precoated dishes without serum. The tyrosine phosphorylation of paxillin was determined for extracts of the above cells by anti-phosphotyrosine (left) and anti-paxillin (right) immunoprobing. The bars on the left of the panel represent MW markers of 220, 117, 78, and 46 kDa, respectively.

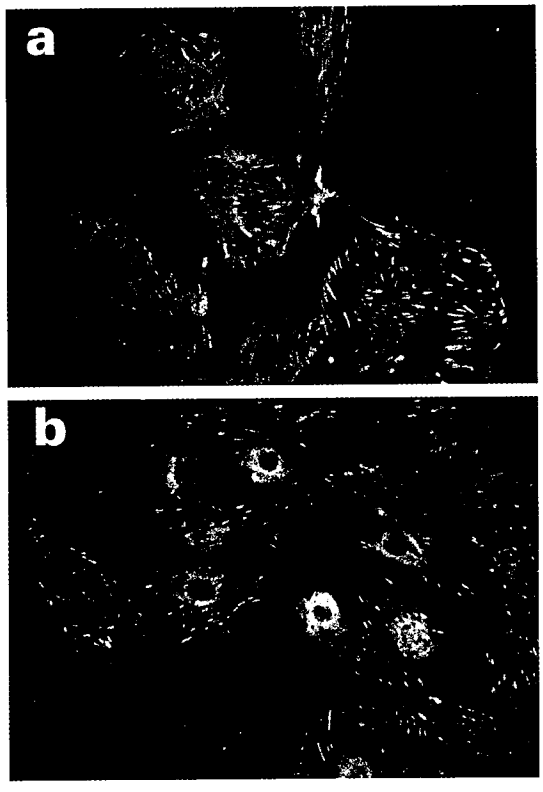

Fig. 4. Immunofluorescent labeling of paxillin and phosphotyrosine. Immunolabeling of paxillin and phosphotyrosine was performed on 3Y1 cells cultured on a glass slide. Cells were held in suspension for 1 hr and transferred for 1 hr onto a fibronectin-precoated glass. An anti-paxillin (a) and anti-phosphotyrosine (b) antibody were used for immunostaining.

such as p125FAK, p60src, and Csk (Sabe *et al.*, 1994; Richardson and Parsons, 1996) and also serine/threonine kinases (Brown *et al.*, 1998). Therefore, we tried to examine whether or not these proteins were paxillins. Fig. 2 shows that a major band could be recovered by immunoprecipitation with an anti-paxillin antibody and that it was tyrosine dephosphorylated in suspension cultured cells, but not in adhered cells (Fig. 2). We also detected very little paxillin in immunoprecipitates with an anti-phosphotyrosine antibody of suspension cultured cells. There were, however, plenty of paxillin molecules in adhered cells (data not shown). We thus concluded that the tyrosine dephosphorylated 65-70 kDa protein in the transformed cells is indeed paxillin. The question remains as to which molecule is responsible for the dephosphorylation of paxillin cells held in suspension. Recently, several protein tyrosine phosphatases, such as PTP-1D (Ouwens *et al.*, 1996), PTP-PEST (Shen *et al.*, 1998), Sph-2 (Yu *et al.*, 1998) and PTP-1B (Liu *et al.*, 1998), were shown to dephosphorylate adhesion molecules.

Cell adhesion to fibronectin stimulates paxillin tyrosine phosphorylation We next investigated the level of tyrosine phosphorylation on paxillin using various substrates. Since fibronectin and poly-L-lysine are known to provide extracellular matrices for cell adhesion, we precoated plastic dishes with fibronectin, poly-L-lysine, or BSA, which is a control for suspension culture. We then seeded round cells immediately after trypsinization into the prepared dishes. As shown in Fig. 3, paxillin on fibronectin (FN) and poly-L-lysine (p-LYS) were phosphorylated within 1 hr, while those on bovine serum albumin (BSA), and those held in suspension (SUS), were not. Although the cells in the fibronectin and poly-L-lysine precoated dishes tightly adhered to the substratum, the cells adhering to the fibronectin precoated dish were much flatter in form (data not shown). On the other hand, the cells in the BSA precoated dish did not attach to the substrate, that is, they stayed in suspension for up to 1 hr. The total number of proteins that were extracted from the cells cultured under the above conditions, and immunoblotted using anti-paxillin antibody and anti-phosphotyrosine antibody, are shown in Fig. 3. The cells that attached to the fibronectin, or poly-L-lysine precoated dishes, showed a restored tyrosine phosphorylation of paxillin. Immoprecipitation that was followed by an immuno blot analysis also showed similar results (data not shown). Paxillin is a major intracellular protein that exists in three isomers (α, β, and γ) and exhibits heterogeneity in the phosphorylation on serine, threonine, and tyrosine residues (Mazaki *et al.*, 1997; Brown *et al.*, 1998). This explains why there are several bands seen on the anti-phosphotyrosine immunoblot (Fig. 3).

To further investigate tyrosine phosphorylation and cellular localization of paxillin during cell adhesion, we performed

immunofluoresence experiments with an antibody against paxillin and phosphotyrosine (Fig. 4). Since both antibodies are monoclonal, we independently stained cells with each antibody. In cells grown on fibronectin, paxillin is detected in a FA structure, which is shown as white spots in Fig. 4. Also, a tyrosine-phosphorylated protein was detected as a punctuate structure. These results indicate that paxillin exists in a tyrosine phosphorylated form during cell adhesion. In this context, it is possible to speculate that paxillin acts as an adapter protein for the recruitment of signaling molecules to FA, including FAK, Src, and Crk. However, it has been shown that the serine/threonine phosphorylation of paxillin enhances the translocation of paxillin to focal adhesions while increasing the ability of cells to adhere to the substrate (Brown *et al.*, 1998). Therefore, we cannot exclude the possibility that serine/threonine kinase activity might be involved during cell adhesion. It is certain that phosphorylation of paxillin, in response to integrin-mediated cell adhesion, regulates protein-protein interaction in FA. These changes in protein interactions lead to cytoskeletal rearrangement and cell proliferation.

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