Transformation of rat fibroblasts by phospholipase C-γ1 overexpression is accompanied by tyrosine dephosphorylation of paxillin

Jong-Soo Chang[a,*], Shintaro Iwashita[b], Young Han Lee[c], Myung Jong Kim[d], Sung Ho Ryu[d], Pann-Ghill Suh[d]

[a] *Department of Biology, Daejin University, Pochon-gun, Kyeonggido 487-800, South Korea*
[b] *Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan*
[c] *Department of Biochemistry, College of Medicine, Yeungnam University, Taegu 705-717, South Korea*
[d] *Department of Life Science, Pohang University of Science and Technology, POSTECH, Pohang 790-784, South Korea*

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Abstract We previously have shown that the overexpression of phospholipase C-γ1 (PLC-γ1) in rat 3Y1 fibroblasts results in malignant transformation (Chang, J.-S., Noh, D.Y., Park, I.A., Kim, M.J., Song, H., Ryu, S.H. and Suh, P.-G. (1997) Cancer Res. 57, 5465–5468). The transformed cells, which initially are in an elongated and flat form after seeding in plastic dishes, become rounded during continued culture. We found that tyrosine dephosphorylation of paxillin accompanies this morphological change of the transformed cells and that PLC-γ1 co-immunoprecipitates together with paxillin and vice versa, but not after the cells have become round. Transformed cells growing on fibronectin-pre-coated dishes regain their flat morphology and this is accompanied by paxillin tyrosine phosphorylation. Furthermore, immunoprecipitation analysis showed that paxillin forms a heteromeric complex with PLC-γ1 in cells grown on fibronectin. These results suggest that a complex formation between paxillin and PLC-γ1 may play a role in cell-substrate adhesion.

Key words: Phospholipase C-γ1; Paxillin; Cell adhesion; Phosphorylation

1. Introduction

Phosphoinositide-specific PLC-γ1 plays a critical role in cellular signaling by triggering the formation of two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate [1]. It has also been revealed that PLC-γ1 functions in mitogenic signaling [2,3] and cytoskeletal rearrangement [4–7]. Furthermore, it has recently been shown that overexpression of PLC-γ1 can result in cellular transformation of rat 3Y1 cells [8] and NIH 3T3 cells [9].

In order to gain a better understanding of the mechanism underlying the PLC-γ1-mediated cell transformation, we first examined the level of tyrosine phosphorylation on the cellular proteins in transformed 3Y1 cells, because transformed cells exhibit a different level of protein tyrosine phosphorylation in comparison to wild-type. We unexpectedly found paxillin tyrosine to be dephosphorylated in PLC-γ1-transformed cells. Paxillin is found enriched in focal adhesions, where discrete areas of the cell membrane form links between the cytoskeleton and the extracellular matrix by coupling to integrin. During the assembly of focal adhesions, phosphorylated paxillin acts as an adapter in the recruitment of signaling molecules containing SH2 domains, such as p125 focal adhesion kinase (FAK), Src, Csk, Crk, Grb-2, p130cas and PI-3 kinase [10]. PLC-γ1 binds to FAK [11] and has also been suggested to be a focal adhesion protein [7,12]. Furthermore, it has been shown recently that several protein tyrosine phosphatases are involved in the dephosphorylation of FAK and paxillin [13,14]. In this paper, we describe how tyrosine-phosphorylated paxillin associates with PLC-γ1, when transformed cells adhered to the substrate, while there is no such association after the cells have become rounded.

2. Materials and methods

2.1. Materials

The antibodies used were anti-human PLC-γ1 antibody (monoclonal antibody F-7) [15], anti-phosphotyrosine antibody (PY20, Transduction Laboratory) and anti-paxillin antibody (Z035, Zymed). Hygromycin B was purchased from Calbiochem. 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.

2.2. Cell culture and adhesion to substrate

The rat embryonic fibroblast 3Y1 cells, originally a gift from Dr Kimura (Kyushu University, Fukuoka, Japan), the PLC-γ1-transformed cells and the vector-transfected cells (control cells) were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% bovine calf serum (HyClone). The two latter cell lines were cultured in the presence of 50 μg/ml hygromycin B [8]. 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 h at 37°C, followed by washes in phosphate-buffered saline (PBS) and blocking with 1% BSA for 2 h. Transformed cells on day 3–4 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 to dishes precoated with the various substrates and incubated at 37°C in the absence of serum for 1 h. The cells were then washed and lysed and the lysates were used for immunoprecipitation and immunoblot analysis with appropriate antibodies.

2.3. Immunoblot analysis and immunoprecipitation

Cells cultured in 100 or 150 mm dishes (Falcon) were lysed in 500 μl of lysis buffer (10 mM HEPES-NaOH (pH 7.4), 200 mM NaCl, 2% sodium dodecyl sulfate (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 s and centrifuged at $10\,000\times g$ for 20 min. Constant amounts of the cleared lysates (50–100 μg protein per lane) were subjected to 7.5 or 10% SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot-analyzed as described previously [16]. For immunoprecipitation, the cells were lysed in RIPA buffer containing protease inhibitors [16]. Each extract (500–1000 μg protein) was al-

*Corresponding author. Fax: (82) (357) 539-1850.
E-mail: jchang@road.daejin.ac.kr

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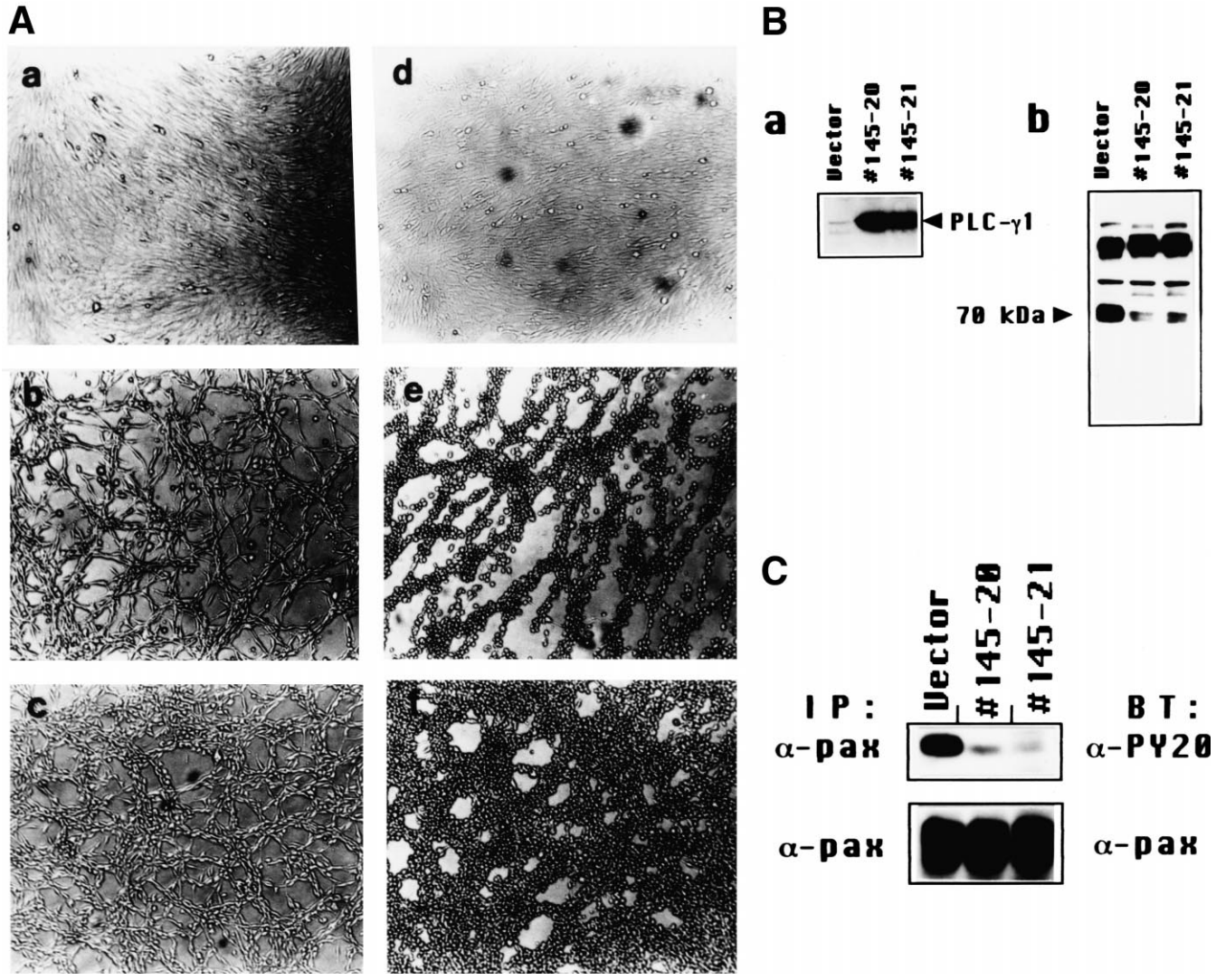

Fig. 1. Morphological alteration and paxillin tyrosine dephosphorylation in PLC-γ1-transformed cells. (A) Control, vector-transfected 3Y1 cells (a and d) and PLC-γ1-transformed cells #145-20 (b and e) and #145-21 (c and f) were plated at a density of 5×10^6 cells per 100 mm dish. Photographs were taken on day 2 (a, b and c) and day 4 (d, e and f) without change of medium. In contrast to the quiescent phase of the control cells (d), PLC-γ1-transformed cells (e and f) exhibit a proliferative stage on day 4. (B) Whole cell lysates of vector-transfected cells and PLC-γ1-transformed cells (# 145-20 and -21) were prepared and aliquots of 50 μg protein of each lysate were resolved by 10% SDS-PAGE and transferred to a nitrocellulose filter for immunoblot analysis with anti-PLC-γ1 antibody (a) and anti-phosphotyrosine antibody, PY20 (b). (C) Vector-transfected control, #145-20 and #145-21 cells were cultured for 3 days, lysed in RIPA buffer and proteins were immunoprecipitated with anti-paxillin antibody. The immuno-complexes were resolved by 10% SDS-PAGE, blotted and analyzed with anti-phosphotyrosine antibody (upper) and anti-paxillin antibody (lower).

lowed to react with a pre-formed complex of *Staphylococcus aureus* goat anti-mouse IgG (Pansorbin, Calbiochem) complex and either pre-immune serum or anti-paxillin antibody. After a 12–14 h incubation at 4°C, pellets were obtained by centrifugation at $6000\times g$ for 3 min, washed three times with RIPA buffer and boiled for 3 min. Immunoblot analysis was carried out as described above, except that it took 12–14 h of incubation with the anti-paxillin antibody to obtain a good signal.

3. Results

3.1. Tyrosine dephosphorylation of paxillin in PLC-γ1-transformed cells

Rat fibroblasts 3Y1 subclones overexpressing PLC-γ1 exhibit a transformed phenotype which includes anchoring-independent growth in culture dishes and tumor formation in nude mice [8]. In Petri dishes, these clones take on an elongated morphology after seeding. The cells maintain this form when the medium is replaced every 2 days with fresh medium and serum (Fig. 1A, b and c). If the medium is not changed, however, the cells become round on day 3–4 (Fig. 1A, e and f). If the cells are cultured in the absence of serum, they become round within 20 h (data not shown). In order to identify the pattern of total cellular protein tyrosine phosphorylation that parallels the above morphological changes, we immuno-analyzed blots of the whole cell lysates with anti-phosphotyrosine antibody. Unexpectedly, the transformed cells showed bands of 65–70 kDa protein species whose tyrosine phosphorylation seemed depressed in comparison to control cells (Fig. 1B).

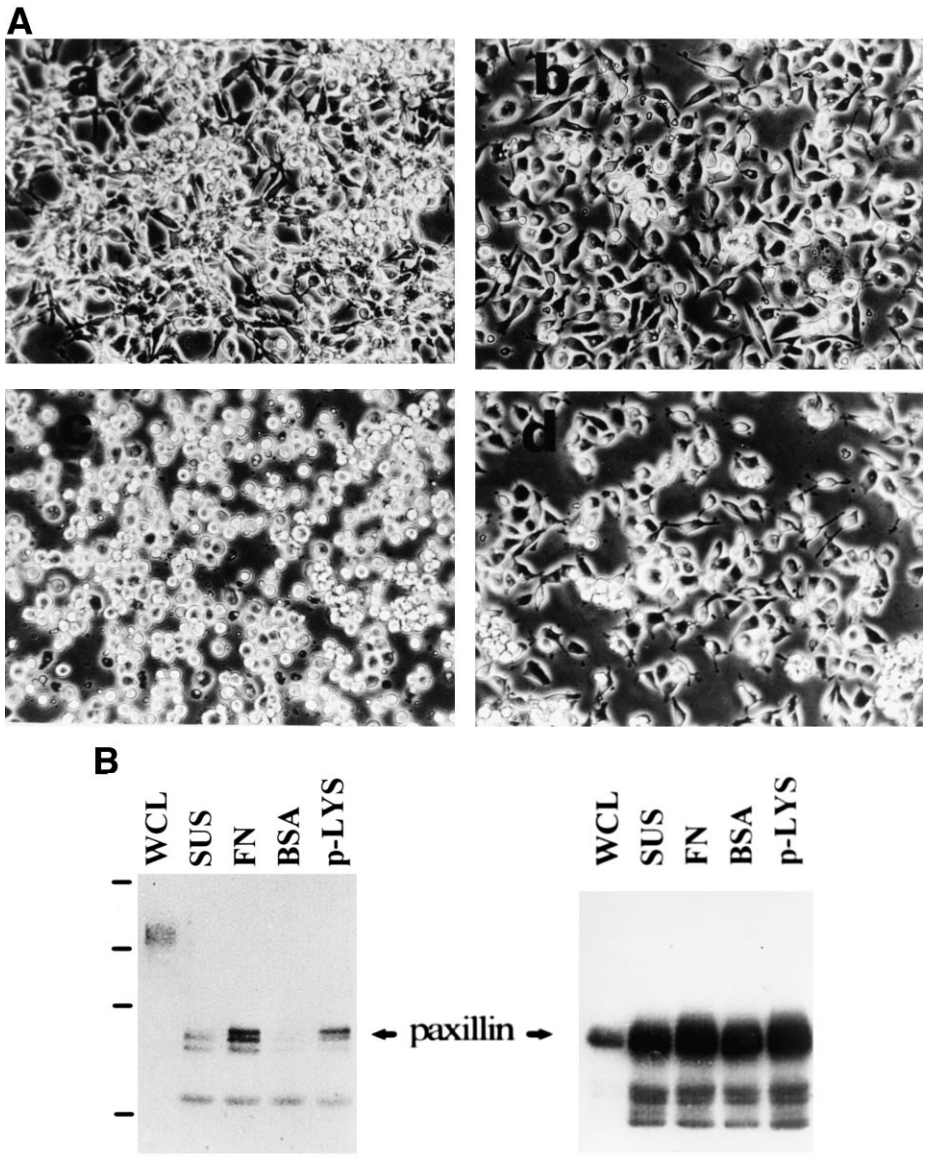

Fig. 2. Fibronectin restores paxillin tyrosine dephosphorylation and flattening of the cells. (A) Images of cells adhering to various substrates. PLC-γ1-transformed cells on day 3 (a) were collected immediately after trypsinization. The cells were then transferred for 1 h onto fibronectin- (b), BSA- (c) and poly-L-lysine- (d) pre-coated dishes without serum. (B) Tyrosine phosphorylation of paxillin was determined for extracts of the above cells after trypsinization (SUS) and of cells adhering to the substrate by immunoprecipitation of paxillin followed by anti-phosphotyrosine (left) and anti-paxillin (right) immunoprobing. WCL stands for whole cell lysate. The bars on the left of the panel represent molecular weight (MW) markers of 220, 117, 78 and 46 kDa, respectively.

Paxillin, a well known focal adhesion protein, comprises a family of proteins with molecular masses around 65–70 kDa, which are substrates for tyrosine kinases such as p125FAK, p60src and Csk [17,18] and also serine/threonine kinases [19]. We therefore examined whether these proteins were paxillins. Fig. 1C shows that a major band could be recovered by immunoprecipitation with anti-paxillin antibody and that it was tyrosine-dephosphorylated in transformed cells but not in control cells. We also detected only little paxillin in immunoprecipitates with anti-phosphotyrosine antibody of transformed cells, while there were plenty paxillin molecules in control cells (data not shown). We thus concluded that the tyrosine-dephosphorylated 65–70 kDa protein in the transformed cells is indeed paxillin.

3.2. Correlation between paxillin tyrosine dephosphorylation and cell morphology

As mentioned above, PLC-γ1-transformed 3Y1 cells become rounded if the medium is not changed in a timely manner. We looked, therefore, for a relationship between the morphological alteration and the level of tyrosine dephosphorylation on paxillin using various substrates. Since fibronectin and poly-L-lysine are well known to provide extracellular matrices for cell adhesion, we pre-coated 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. 2A, the cells on fibronectin (b) and poly-L-lysine (d) were flat within 1 h, while those on BSA (c) were not. Although both, the cells in the fibronectin and those in the poly-L-lysine-pre-

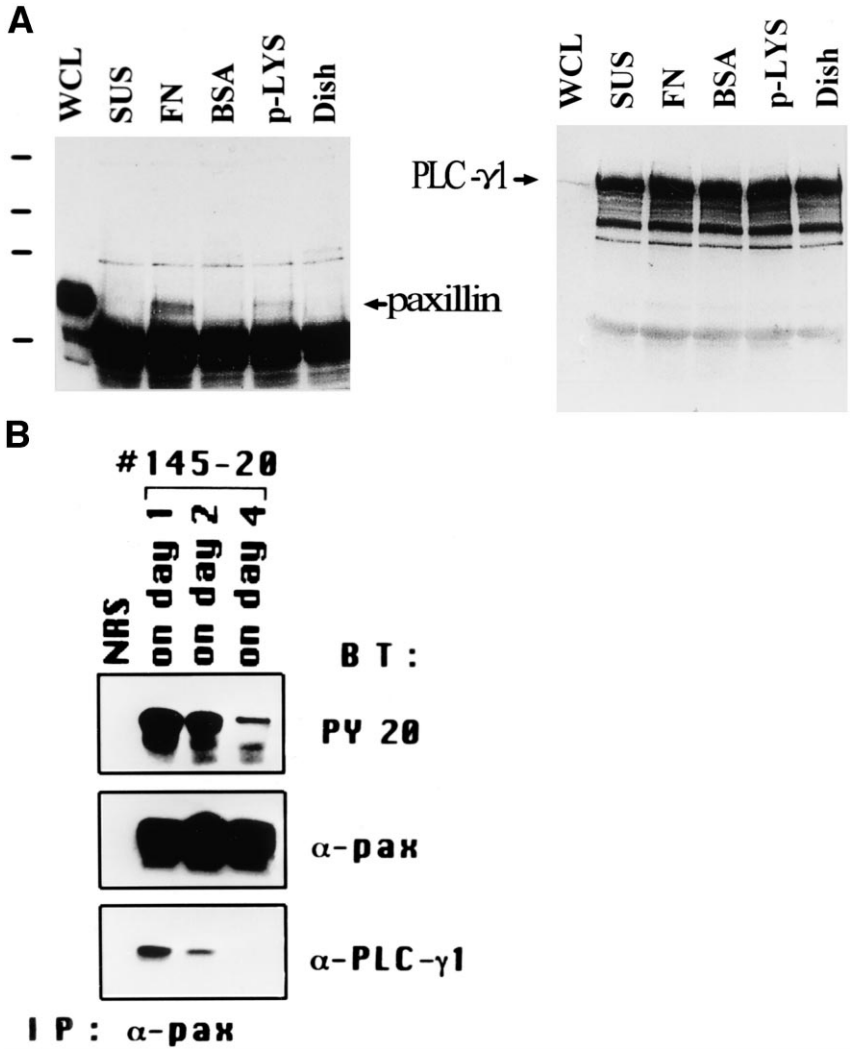

Fig. 3. PLC-γl co-immunoprecipitates with paxillin. (A) Cell extracts prepared exactly the same way as those in Fig. 2B were subjected to immunoprecipitation of PLC-γl with anti-PLC-γl antibody and analyzed by anti-paxillin (left) and anti-PLC-γl (right) immunoblot analysis. WCL and Dish indicate whole cell lysate and plastic cell culture dish, respectively. The bars on the left represent MW markers of 220, 117, 78 and 46 kDa, respectively. (B) PLC-γl-transformed cells (#145-20) were cultured and harvested at different time intervals. The cell extracts were used for paxillin immunoprecipitation with anti-paxillin antibody. The immunoprecipitates were resolved by 7.5% SDS-PAGE and analyzed with anti-phosphotyrosine antibody (PY20, upper panel), anti-paxillin antibody (middle panel) and anti-PLC-γl antibody (lower panel). NRS stands for normal rabbit serum.

coated dishes, adhered to the substrate tightly, the cells adhering to the fibronectin-pre-coated dish were much flatter in form. On the other hand, the cells in the BSA-pre-coated dish did not attach to the substrate, i.e. stayed in suspension, for up to 1 h. We extracted total protein from cells cultured under the above conditions and immunoprecipitated paxillin using anti-paxillin antibody. We then followed up by immunoblot analysis with anti-phosphotyrosine antibody (Fig. 2B). The cells that had attached to the fibronectin- or poly-L-lysine-pre-coated dishes showed restored tyrosine phosphorylation of paxillin. This result demonstrates that paxillin tyrosine dephosphorylation went hand in hand with the round morphology of the transformed cells. 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 [18,20], which explains the several bands seen on the anti-phosphotyrosine immunoblot (Fig. 2B).

3.3. Paxillin co-immunoprecipitates with PLC-γl

Since many signaling molecules are associated with paxillin [10,11], we examined the possibility that the extra amounts of the overexpressed PLC-γl might bind paxillin in the transformed cells. Using cell extracts prepared exactly the same way as those shown in Fig. 2, we precipitated PLC-γl with anti-PLC-γl antibody. We found that paxillin co-immunoprecipitated with PLC-γl from cell extracts of cells grown on the fibronectin- or poly-L-lysine-pre-coated dishes (Fig. 3A). The amount of paxillin which co-precipitated with PLC-γl was calculated as 0.5% of total paxillin for fibronectin- and 0.1% of total paxillin for poly-L-lysine-attached cells by an image analysis system (Quantity One, Bio-Rad). No such precipitation of paxillin was obtained from extracts of cells recovered from BSA-pre-coated dishes or from any round cells.

Alternatively, we also plated transformed cells in medium containing 10% bovine calf serum and we prepared cell extracts in a time-course. Paxillin was then immunoprecipitated

from each extract and subjected to immunoblot analysis with anti-phosphotyrosine antibody. As was observed before, the tyrosine phosphorylation level of paxillin decreased from day 1 to day 4 (Fig. 3B). We also immuno-detected PLC-γ1 in the paxillin immunoprecipitate of day 2 (elongated form), but only little on day 4 (round form) (Fig. 3B). The results, therefore, indicate that those two proteins exist in a complex before the cells round-up.

4. Discussion

Our present study revealed that the rounding morphology of PLC-γ1-transformed rat 3Y1 fibroblasts is accompanied by tyrosine dephosphorylation of paxillin. The observations made by us differ from those described in reports of transient tyrosine dephosphorylation of paxillin induced by extracellular ligands such as insulin and insulin-like growth factor [13,21,22], cAMP [23] and adrenocorticotropic hormone [24]. The two latter cases are also accompanied by transient morphological alterations. With regard to the relationship between cell morphology and paxillin tyrosine dephosphorylation, we observed contradictory results regarding the rounding of cells. While we saw heavy tyrosine phosphorylation of paxillin in *v-src*-transformed 3Y1 cells, we saw tyrosine dephosphorylation of paxillin in mitotic 3Y1 cells (data not shown) [17,25,26]. These observations, therefore, suggest that the paxillin tyrosine dephosphorylation in PLC-γ1-transformed cells is not a transformation-specific phenomenon.

The question remains as to which molecule is responsible for the dephosphorylation of paxillin in the PLC-γ1-transformed 3Y1 cells. Although the precise role played by protein tyrosine phosphatases in focal adhesion has not yet been conclusively established, several protein tyrosine phosphatases such as PTP-1D [13], PTP-PEST [14], Sph-2 [27] and PTP-1B [28] have been shown to dephosphorylate adhesion molecules. We also found that pervanadate, a tyrosine phosphatase inhibitor, affects the cell morphology causing cells to become flatter (data not shown). Therefore, it is possible to speculate that cells overexpressing PLC-γ1 experience some protein tyrosine phosphatase activation that affects tyrosine phosphorylation of paxillin, resulting in less tyrosine-phosphorylated paxillin.

Alternatively, since it has been shown that the serine/threonine phosphorylation of paxillin enhances translocation of paxillin to focal adhesions, increasing the ability of cells to adhere to the substrate [19], we cannot exclude the possibility that PLC-γ1 affects serine/threonine kinase activity in transformed cells. In any way, it is certain that ectopically expressed PLC-γ1 modifies the phosphorylation and dephosphorylation of adhesion molecules, which activity may be an important factor in facilitating cell spreading and cell motility.

Our present data also show paxillin/PLC-γ1 association in PLC-γ1-transformed cells. Although the amount of associated protein (PLC-γ1 co-immunoprecipitate with paxillin or vice versa) is restricted, it is meaningful to consider that PLC-γ1 might be involved in cell adhesion. To test whether the association is a direct one, we used a glutathione S-transferase-fused SH2 domain of PLC-γ1 in vitro. However, the amount of paxillin associated with the SH2 domain in vitro was extremely small and thus too low to support the notion that paxillin might bind to PLC-γ1 directly. We, therefore, suggest that the paxillin/PLC-γ1 complex formation during cell adhesion might be mediated by other adhesion molecules such as perhaps p125FAK [11,29].

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