Possible role of protein kinase C in the regulation of intracellular stability of focal adhesion kinase in mouse 3T3 cells

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Abstract Effects of various types of protein kinase inhibitor on the adhesion and spreading of BALB/c mouse 3T3 cells and on the phosphorylation and stability of focal adhesion kinase (FAK) in the cells were studied. Inhibitors of protein tyrosine kinases, methyl 2,5-dihydroxycinnamate and herbimycin A, inhibited tyrosine-phosphorylation of FAK and the adhesion of 3T3 cells to fibronectin. Among inhibitors of serine/threonine kinases tested, calphostin C, a specific inhibitor of protein kinase C, inhibited cell spreading rather than cell adhesion, and it induced the decrease of intracellular FAK within 30 min. Inhibitors of tyrosine kinase, A kinase, G kinase, and myosin light chain kinase did not induce such a rapid and specific decrease of FAK. When calphostin C (20 μM) was added to sub-confluent monolayer cultures, serine-phosphorylation of FAK was inhibited by 67% within 2 h, and decrease in the amount of FAK and rounding up of the cells began after 4 h. Label-chase experiments indicated that about 60% of ^{35}S-labeled FAK degraded within 1–2 h after addition of calphostin C to monolayer cultures. These results indicated that serine-phosphorylation of FAK induced by protein kinase C was important in the regulation of metabolic stability of FAK.

Key words: Calphostin C; Focal adhesion kinase; FAK; Protein kinase C; Fibronectin; Phosphorylation

1. Introduction

Focal adhesion kinase (FAK, pp125^{FAK}) is a member of the family of protein tyrosine kinases, which localizes to focal adhesion plaques in cells (reviewed in [1]). Phosphorylation of tyrosine residues of FAK is stimulated in the cells transformed by pp60$^{v\text{-}src}$, or by attachment of cells to fibronectin or other cell-adhesive proteins [1–6]. Tyrosine kinase activity of FAK is regulated, at least in part, by the phosphorylation of tyrosine residues of FAK [7]. Schaller et al. [8] reported that the major autophosphorylation site in FAK was Tyr-397, and that phosphorylation of this site might direct SH2-dependent binding of pp60$^{v\text{-}src}$ and pp59^{fyn}. Recently, Tyr-407, Tyr-576, and Tyr-577 were also identified as the phosphorylation sites in FAK [9]. Furthermore, it was reported that integrin-mediated cell adhesion activated MAP (mitogen-activated protein) kinases and led to an SH2-mediated binding of FAK to GRB2 adapter protein, and that the phosphorylation of FAK at Tyr-925 was involved in the activation of Ras/MAP kinase signal transduction pathway [10,11]. Thus, tyrosine-phosphorylation of FAK seems to regulate its interaction with other proteins in the signal transduction pathways, as well as its protein kinase activity.

On the other hand, it has been established that serine, not only tyrosine, residues in FAK are phosphorylated by the attachment of cells to fibronectin [9,12]. Activation of protein kinase C causes the stimulation of cell attachment, spreading, and tyrosine-phosphorylation of FAK [13,14]. Attachment of cells to fibronectin causes rapid and transient activation of protein kinase C, so serine-phosphorylation might be a prerequisite for tyrosine-phosphorylation of FAK [14]. These findings suggest that phosphorylation of serine residues in FAK may regulate the biological activities of FAK, but the mechanism and physiological roles of serine-phosphorylation of FAK are not fully understood.

In the present study, we have examined the effects of various types of protein kinase inhibitor on the adhesion and spreading of BALB/c mouse 3T3 cells, and on the phosphorylation and stability of FAK in the cells. Here, we reported that the serine-phosphorylation of FAK induced by protein kinase C might be important in the regulation of intracellular stability of FAK.

2. Materials and methods

2.1. Materials

The following protein kinase inhibitors were used: calphostin C, herbimycin A, methyl-2,5-dihydroxycinnamate (2,5-MeC), K-252a, K-252b, KT5720, KT5823, and KT2926 (Kyowa Medex Co.) Poly-L-lysine was purchased from Sigma. Polyclonal anti-FAK antibodies were raised in rabbits against bacterially expressed C-terminal fragment of mouse FAK.

2.2. Immunoblot analysis and cell adhesion assay

BALB/c mouse 3T3/K3 cells were cultivated in Dulbecco's modification of Eagle's minimum essential medium (D-MEM; Nissui Pharmaceutical Co.) that contained 10% calf serum. Sub-confluent cells were dispersed by trypsin treatment and suspended in a small volume of D-MEM that contained 0.5 mg/ml soybean trypsin inhibitor (Sigma). After appropriate dilution, 5×10^6 cells were suspended in 2.5 ml of serum-free D-MEM and put in Corning tissue culture dishes (10 cm) that were previously coated at room temperature for 2 h with 100 μg/ml human plasma fibronectin or 1 mg/ml poly-L-lysine. After incubation of the cells at 37°C for an indicated period, attached cells were washed twice with phosphate-buffered saline (PBS) that contained 100 μM sodium orthovanadate, and lysed with 0.5 ml of RIPA buffer; 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 158 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethane-sulfonyl fluoride (PMSF) [15]. An equal amount of proteins (10–20 μg) were sepa-

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Abbreviations: FAK, pp125^{125}, focal adhesion kinase; 2,5-MeC, methyl-2,5-dihydroxycinnamate; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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rated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Laemmli's buffer system [16]. Immunoblot analysis was carried out using an Amersham ECL immunoblot detection system with anti-phosphotyrosine (PY69; ICN), anti-pp125^{FAK} (UBI), anti-vinculin (Sigma), anti-talin (Sigma) monoclonal antibodies, or anti-FAK polyclonal antibodies. Relative amount of FAK was determined by densitometry using the scanning densitometer and data analysis system (The Discovery, Quantity One, pdi, New York).

For the cell adhesion assay, adhered cells were washed twice with PBS, and then they were dispersed by trypsin treatment. The cell numbers were counted with a cell counter Model D (Coulter Electronics).

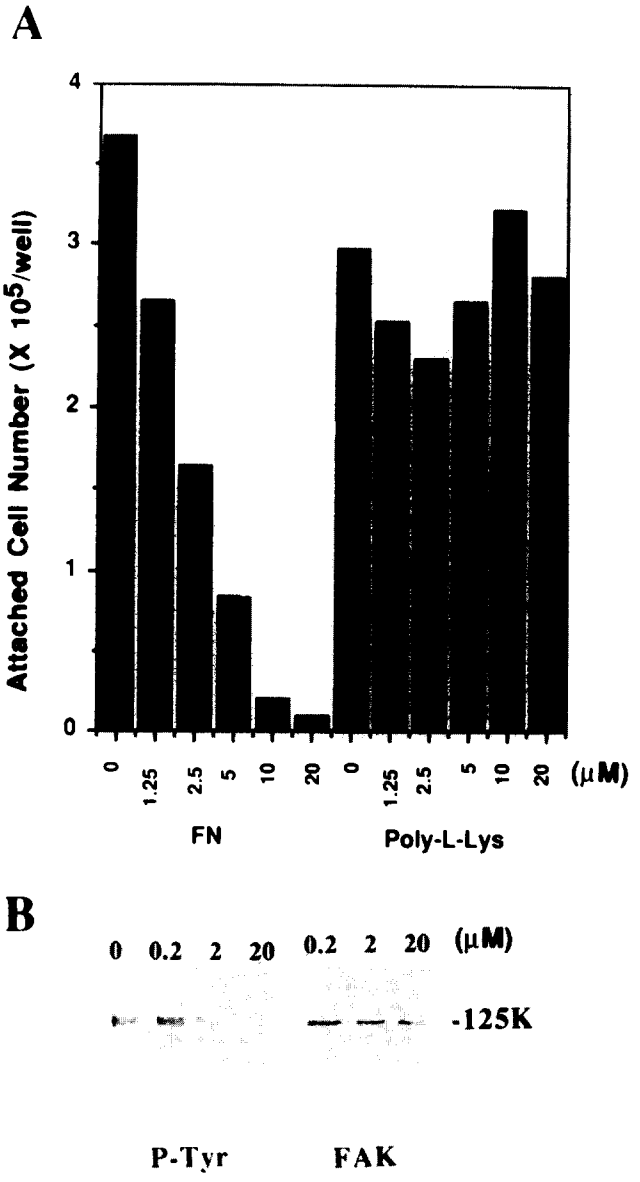

Fig. 1. Effects of 2,5-MeC on the adhesion of 3T3 cells and phosphorylation of FAK. (A) Trypsinized mouse 3T3 cells were inoculated in the dishes coated with fibronectin (FN) or poly-L-lysine (Poly-L-Lys) in the presence of various concentrations of 2,5-MeC, and the numbers of attached cells were counted after incubation at 37°C for 2 h. (B) Trypsinized mouse 3T3 cells were inoculated in the dishes coated with fibronectin in the presence of 0, 0.2, 2 and 20 μM 2,5-MeC, respectively. Cell lysate was prepared after incubation of the dishes at 37°C for 2 h, and subjected to SDS-PAGE, followed by immunoblot analysis using anti-phosphotyrosine (P-Tyr) and anti-FAK (FAK) monoclonal antibodies.

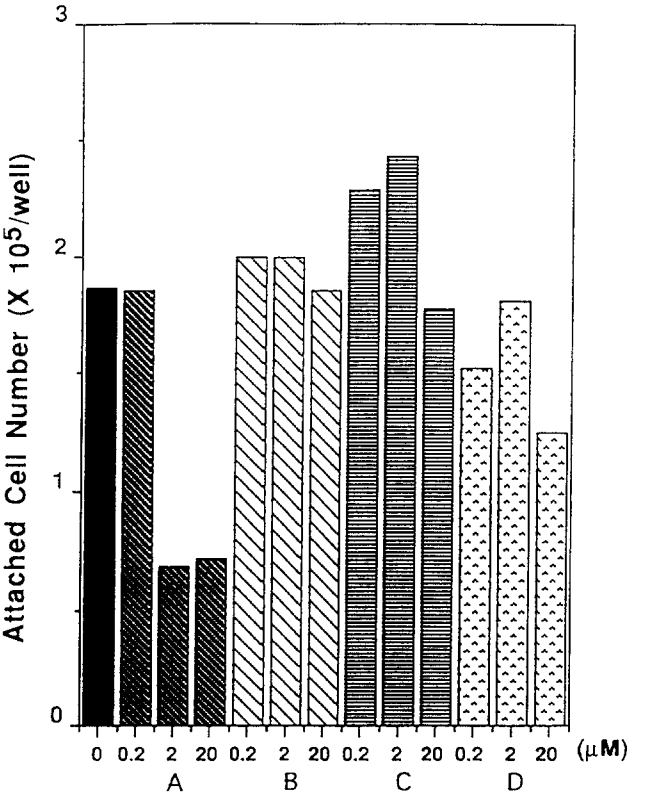

Fig. 2. Effects of protein kinase inhibitors on the adhesion of 3T3 cells. Trypsinized mouse 3T3 cells were inoculated in the dishes coated with fibronectin in the presence of various concentrations of calphostin C (A), KT5720 (B), KT5823 (C), or KT5926 (D), and the numbers of attached cells were counted after incubation of the dishes at 37°C for 2 h.

2.3. Analysis of phosphoamino acids

Sub-confluent 3T3 cells were labeled 2 mCi/ml ortho-[^{32}P]phosphate (NEN) at 37°C in phosphate-free D-MEM (GIBCO) that contained 10% dialyzed bovine serum, in the presence or absence of 20 μM calphostin C. Cells were lysed and FAK was precipitated with anti-FAK polyclonal antibodies. The immunoprecipitate was separated by SDS-PAGE in 6% gels, and the region of the gel corresponding to FAK was excised. Proteins were extracted from the gel, hydrolysed with 6 N HCl, and phosphoamino acids were separated by one-dimensional thin layer electrophoresis in 5% acetic acid/0.5% pyridine (pH 3.5) [17]. Radioactive phosphoamino acids were detected and quantitated by autoradiography using the FUJIX Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co.).

2.4. Label-chase experiments

Sub-confluent 3T3 cells were labeled with 100 μCi/ml [^{35}S]methionine/[^{35}S]cysteine mixture (Tran^{35}S-label, ICN Pharmaceuticals Inc.) at 37°C for 2 h. After washing, the cells were incubated in the presence or absence of 20 μM calphostin C, and cell lysate was prepared at intervals. FAK was immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography as described above.

3. Results

Fig. 1A shows the effects of 2,5-MeC, an inhibitor of tyrosine kinases, on the adhesion of 3T3 cells to fibronectin and poly-L-lysine. 2,5-MeC inhibited the adhesion of cells to fibronectin in a dose-dependent manner, but did not inhibit cell adhesion to poly-L-lysine. Similar results were obtained with another tyrosine kinase inhibitor, herbimycin A (data not shown). Immunoblot analysis using anti-phosphotyrosine and anti-FAK

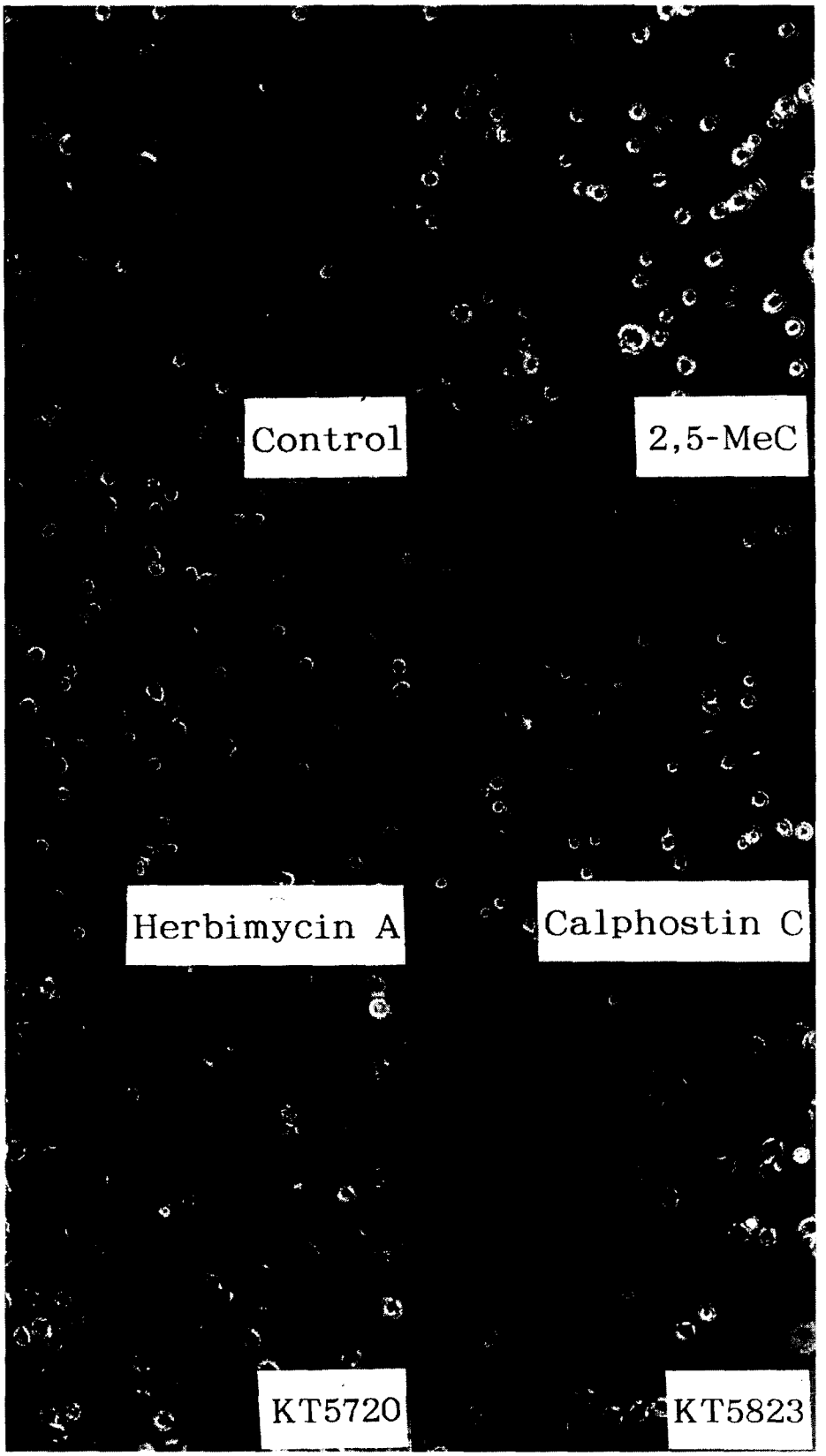

Fig. 3. Effects of protein kinase inhibitors on the adhesion and spreading of 3T3 cells. Trypsinized mouse 3T3 cells were inoculated in the dishes coated with fibronectin in the presence of various kinase inhibitors, and photographs were taken after incubation of the dishes at 37°C for 2 h.

antibodies revealed that tyrosine-phosphorylation of FAK was inhibited by 2,5-MeC at 2 to 20 μM, but the amount of FAK was not significantly reduced by 20 μM 2,5-MeC (Fig. 1B).

Effects of the inhibitors of serine/threonine kinases on the adhesion of 3T3 cells to fibronectin are shown in Fig. 2. Calphostin C, an inhibitor of protein kinase C, inhibited the adhesion of cells by about 60% at 2–20 μM. KT5720 (an inhibitor of cyclic AMP-dependent kinase), KT5823 (an inhibitor of cyclic GMP-dependent kinase), and KT5926 (an inhibitor of myosin light chain kinase) did not significantly affect the adhesion of cells. Fig. 3 shows the spreading of the cells on fibronectin in the presence or absence of 20 μM kinase inhibitors. Cells did not adhere to fibronectin in the presence of 2,5-MeC or herbimycin A. Inhibition of cell adhesion by calphostin C

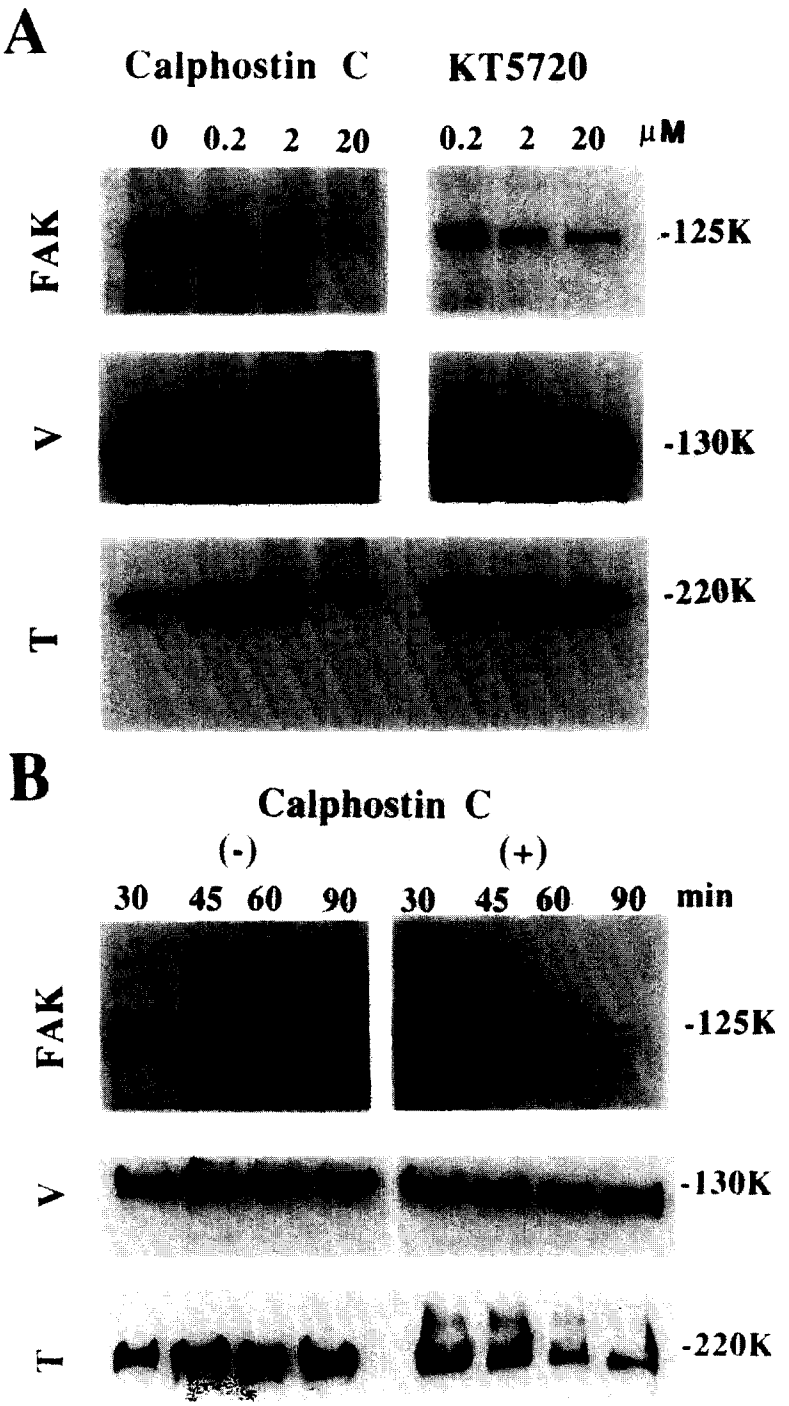

Fig. 4. Effects of calphostin C and KT5720 on the stability of FAK, vinculin, and talin. Cell lysate was analyzed by the immunoblot using anti-FAK (FAK), anti-vinculin (V), and anti-talin (T) monoclonal antibodies. (A) Trypsinized mouse 3T3 cells were inoculated in the dishes coated with fibronectin in the presence of 0, 0.2, 2 and 20 μM each of calphostin C or KT5720. Cell lysate was prepared after incubation of the dishes at 37°C for 2 h. (B) Mouse 3T3 cells were inoculated in the dishes coated with fibronectin with (+) or without (−) 10 μM calphostin C, and cell lysate was prepared after incubation of the dishes at 37°C for 30, 45, 60 and 90 min, respectively.

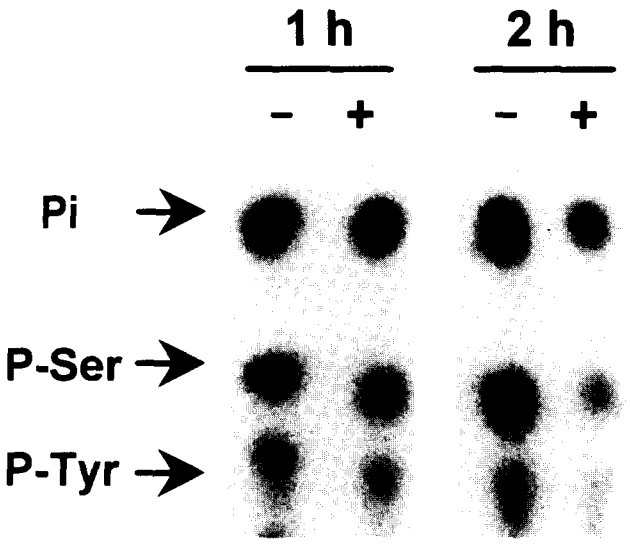

Fig. 5. Analysis of phosphoamino acids of FAK. Sub-confluent cultures of 3T3 cells were labeled with 2 mCi/ml of ortho-[^{32}P]phosphate in the presence (+) or absence (−) of 20 μM calphostin C at 37°C for indicated periods. FAK was precipitated from cell lysate, separated by SDS-PAGE, and hydrolyzed. Phosphoamino acids were analyzed by one-dimensional thin layer electrophoresis and autoradiograhed.

was not so striking as 2,5-MeC (Figs. 1 and 2), but the spreading of adhered cells was severely limited by this inhibitor. KT5720 or KT5823 did not affect the adhesion and spreading of cells.

Fig. 4 shows the results of immunoblot analysis using anti-FAK, anti-vinculin, and anti-talin monoclonal antibodies. Calphostin C (20 μM) caused a significant decrease in the amount of FAK (Fig. 4A). Overall staining patterns of the gels by Coomassie blue were not affected by calphostin C, indicating that most cellular proteins remained stable in the presence of calphostin C. Only a small decrease of FAK was induced by 20 μM KT5720. The amount of vinculin (130-kDA) was not influenced by calphostin C, while some decrease of talin (220-kDa) was observed in the presence of 20 μM calphostin C.

Fig. 4B shows the time course of the changes in the amount of FAK, vinculin, and talin induced by 10 μM calphostin C. Decrease of FAK began within 30–45 min in the presence of calphostin C. The amount of vinculin remained unaffected, while talin decreased gradually.

When calphostin C (20 μM) was added to a sub-confluent monolayer culture of 3T3 cells, the cells began to round up after 4 h, and tended to detach themselves from the dishes after 6–8 h without any changes in the viability of cells as judged by the vital staining with Trypan blue. 2,5-Mec (20 μM) did not induce such a morphological change of cells after addition of 8 h. Effects of calphostin C on the phosphorylation of serine and tyrosine in FAK are shown in Fig. 5. Calphostin C (20 μM) inhibited serine- and tyrosine-phosphorylation in FAK by 20% and 39% at 1 h, and 77% and 52% at 2 h, respectively.

Immunoblot analysis showed that the intracellular FAK began to decrease from 4 h after addition of calphostin C to monolayer cultures, and reached to 50% of control at 7 h (Fig. 6A). This decrease in FAK coincided with the time course of the morphological changes of cells. In another set of experiments, cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and further incubated in the presence or absence of 20 μM calphostin C. FAK was immunoprecipitated from cell lysates, separated by SDS-PAGE, and detected by autoradiography (Fig. 6B). Densitometric analysis indicated that the amount of labeled FAK decreased to 43% of control at 1 h and 40% at 2 h after addition of calphostin C, and the decreased level was maintained thereafter.

4. Discussion

Several lines of evidence have indicated that activation of protein kinase C (PKC) by phorbol esters or increased calcium concentration stimulated cell adhesion to fibronectin [18–20] and tyrosine-phosphorylation of FAK [13,14]. Woods and Couchman [20] reported that a kinase inhibitor HA1004, which inhibited PKC more severely than other kinases, reduced cell adhesion to fibronectin and formation of stress fibers in the cells. Shattil et al. [21] reported that tyrosine-phosphorylation of FAK in platelets was blocked by inhibition of PKC with bis-indolylmaleimide. Vuori and Ruoslathi [14] reported that calphostin C, another specific inhibitor of PKC [22], effectively blocked cell adhesion, spreading, and migration on fibronectin, and that PKC activity in the cell membrane fraction transiently increased after the adhesion of cells to fibronectin. Thus, activation of PKC seemed to be required for enhanced tyrosine-phosphorylation of FAK and formation of focal adhesion

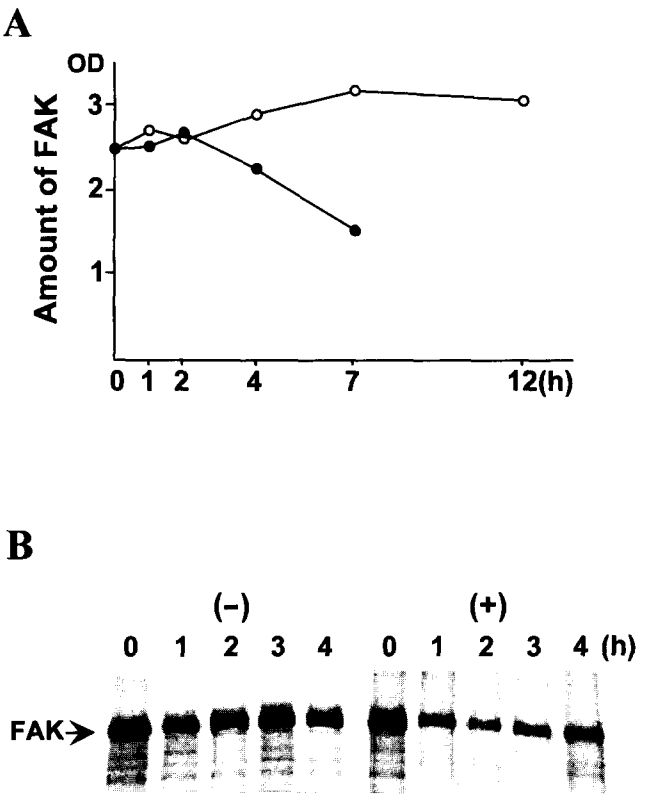

Fig. 6. Degradation of FAK in monolayer cultures induced by calphostin C. Sub-confluent cultures of 3T3 cells were pre-labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine, and then incubated without radioactive amino acids in the presence (closed circles in A and (+) in B) or absence (open circles in A and (−) in B) of 20 μM calphostin C at 37°C for indicated periods. FAK was precipitated from cell lysate and separated by SDS-PAGE. (A) Total amount of FAK determined by immunoblot using anti-FAK antibodies, and (B) autoradiography.

plaques. However, FAK was not directly phosphorylated by PKCα, so other types of protein kinase might be responsible for the serine-phosphorylation of FAK.

Our data confirmed these observations. In addition, we found that calphostin C induced rapid degradation of FAK in trypsin-dispersed cells. Most cellular proteins and vinculin were not degraded, while talin was gradually broken down in the presence of calphostin C. Although inhibitors of tyrosine kinases, such as 2,5-Mec, effectively blocked cell adhesion to fibronectin, they did not induce such a rapid degradation of FAK, indicating that the dephosphorylation on tyrosine residues of FAK by trypsinization of cells or inhibition of tyrosine-phosphorylation did not lead to the degradation of FAK. Inhibitors of myosin light chain kinase, A kinase, and G kinase did not significantly affect cell adhesion, spreading, and stability of FAK.

When trypsinized cells were plated on fibronectin-coated dishes in the presence of calphostin C, the degradation of FAK began within 30 min. In contrast, when calphostin C was added to sub-confluent monolayer cultures, degradation of FAK and rounding-up of cells began after 4 h. This discrepancy in the time course of FAK degradation is likely due to the differences in the intracellular localization of FAK. FAK in monolayer cultures seems to be mostly associated to focal adhesion plaques, but in trypsinized cells it might be present diffusely in the cytoplasm, and the latter may be more sensitive to the calphostin C-induced degradation than the former. Supporting this assumption, ^{35}S-labeled FAK in monolayer cultures decreased to about 40% of control within 1–2 h after addition of calphostin C, while total amount of FAK did not change at that time. We suppose that a large proportion of newly synthesized FAK molecules were present in cytoplasm and they are more sensitive to calphostin C-induced degradation than those associated to adhesion plaques.

Taken together, these results suggest that PKC-induced serine-phosphorylation regulates the stability of FAK molecules, especially those present diffusely in the cytoplasm. Stabilization of protein molecules would be a novel physiological function of PKC. Nishizawa et al. [23] reported that the c-*mos* proto-oncogene product, Mos, which regulates meiotic maturation in *Xenopus* oocytes, was broken down by the ubiquitin-dependent pathway in the early stage of meiosis, but it was stabilized by autophosphorylation at serine-3 in the late stage. Thus, FAK seems to be another protein whose metabolic stability is regulated by phosphorylation of serine residues. Control of FAK concentration might be important in the regulation of adhesive and locomotory activities of cells as well as in the intracellular signal transduction systems. Identification of serine kinase which directly phosphorylates FAK molecules, and determination of the sites of serine-phosphorylation in FAK would be of interest.

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