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# Unphosphorylated and tyrosine-phosphorylated forms of a focal adhesion protein, paxillin, are substrates for calpain II in vitro: implications for the possible involvement of calpain II in mitosis-specific degradation of paxillin

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**Abstract** Cell-to-substratum adhesion becomes weakened during mitosis of the cell cycle in fibroblasts. The level of one focal adhesion protein, paxillin, is greatly reduced in mitotic-arrested cells. We show here the possible involvement of calpain II, known to be localized in focal adhesion plaques, in the degradation of paxillin. Paxillin is tyrosine-phosphorylated during interphase of the cell cycle by protein tyrosine kinases (PTK) such as c-Src and Csk, and becomes dephosphorylated during mitosis. Our data, however, indicate that tyrosine phosphorylation of paxillin does not affect the rate of paxillin degradation by calpain in vitro.

*Key words:* Calpain; Calpastatin; Cell to substratum adhesion; Mitosis; Paxillin

## 1. Introduction

Adherent cells contact the extracellular matrix (ECM) through distinct clusters of integrins. Complex structures of proteins, called focal adhesion plaques, appear when cells are spread on ECM-coated culture dishes. Proteins in this complex include paxillin, focal adhesion kinase, talin, tensin, and vinculin. During mitosis, most focal adhesion plaques are disassembled. When we examined the expression of focal adhesion proteins in NIH3T3 cells arrested in the M-phase by nocodazole, we saw a reduction in the level of paxillin [1]. In contrast, other focal adhesion proteins did not show altered expression ([1]; R.Y. and H.S., unpublished).

In well-spread fibroblasts, paxillin is multiply phosphorylated during interphase (Sabe et al., submitted). Phosphorylation of paxillin occurs soon after cells are plated on tissue culture dishes [2]. Both c-Src and Csk protein tyrosine kinases are known to phosphorylate paxillin in vitro, although as different isoforms ([1]; Sabe et al., submitted). In mitotic cells, as well as cells that are trypsinized and suspended in medium, only the dephosphorylated form of paxillin is recovered [1,2].

Beckele et al. have found that calpain II localizes at focal adhesion plaques [3]. Furthermore, they found that among several focal adhesion proteins tested, talin was a good in vitro substrate for calpain II. Vinculin and α-actinin could not be digested by calpain II in vitro [3]. The existence of paxillin was unknown at the time.

In this report, the possible involvement of calpain II and a closely related protease, calpain I, in the degradation of paxillin was examined. We report that paxillin is a good substrate for calpains in vitro. We also found that paxillin phosphorylated in vitro by either c-Src or Csk kinase is just as efficiently degraded by calpain II.

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## 2. Materials and methods

### 2.1. Materials

Calpain I and calpain II were purified from human erythrocytes and human placenta, respectively, as described [4]. Recombinant human calpastatin domain 1 was prepared as described [5]. Recombinant Csk and recombinant c-Src were described previously [1,6]. An anti-paxillin antibody was purchased from Zymed (San Francisco, CA).

### 2.2 Cell culture

NIH3T3 cells were cultured as described [1]. Mitotic cells were prepared by incubating cells with 0.4 μg/ml of nocodazole (Sigma) for 7.5 h at 37°C [7] and harvested by shaking off from culture dishes. To keep the cells in suspension, the cells were trypsinized, washed twice with Dulbecco's modified Eagle's medium containing soybean trypsin inhibitor (0.5 mg/ml), suspended in culture medium, and loaded onto culture dishes pre-coated with agar (1.8% agar in culture medium).

### 2.3. Immunoblotting analysis

Cell lysates were prepared in RIPA buffer [8], and each 50 μg of lysate protein was separated by SDS-PAGE (8%), transferred to Immobilon-P filter membranes (Millipore), and subjected to immunoblotting analysis using an anti-paxillin antibody (Zymed) as described [1]. Antibody retained on filters was visualized by ECL (Amersham). To visualize cellular proteins transferred onto filters, filters were stained with Coomassie brilliant blue R-250 (0.1% in 50% methanol) for 10 min at ambient temperature, followed by destaining with a methanol/acetic acid/water (5:1:4) solution.

### 2.4. Paxillin and its digestion by calpain in vitro

Paxillin was immunoprecipitated from cell lysates prepared with RIPA buffer using an anti-paxillin antibody coupled with protein G-Sepharose beads. After washing, paxillin was eluted from beads with 0.1 M glycine-HCl (pH 2.5) and neutralized with 70 mM Tris-HCl (pH 8.8) containing 0.2% β-mercaptoethanol and 1 mg/ml acetylated bovine serum albumin as described [9,10], then dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.4), 0.14 M NaCl and 1 mM EDTA. In vitro phosphorylation of paxillin was carried out in a kinase buffer (50 mM Tris-HCl (pH 7.4), 0.1 mM $Na_3VO_4$, 0.1% Nonidet P-40, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mg/ml acetylated bovine serum albumin, 10 μM ATP) in the presence of each 0.1 μg of recombinant Csk or recombinant c-Src, and incubated for 30 min at 37°C.

In vitro digestion of paxillin by calpain was carried out in a buffer (20 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 5 mM β-mercaptoethanol, 50 mM NaCl) containing paxillin and calpain for 30 min at 30 °C. Samples


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were separated by SDS-PAGE (8%) and analyzed by immunoblotting using an anti-paxillin antibody.

## 3. Results

To confirm that the decrease in the level of paxillin in mitotic-arrested cells [1] is not due to the general degradation of cellular proteins, cellular proteins from the mitotic-arrested cells were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. As shown in Fig. 1, little change in the amount of each cellular protein was observed in the mitotic-arrested cells compared with that in cells growing on culture dishes, although probing of the same filter with an anti-paxillin antibody revealed a marked decrease in levels of paxillin in mitotic-arrested cells (Fig. 1).

Cell adhesion to the ECM in fibroblasts is greatly reduced during mitosis. We therefore examined whether the decrease in the level of paxillin is due merely to the loss of cell adhesion. Cells grown on tissue culture dishes were harvested by trypsinization and kept in suspension. As shown in Fig. 2, no change in the level of paxillin was seen for up to 10 h in cells kept in suspension. Therefore, the loss of cell to substratum adhesion per se did not affect the level of paxillin.

Since calpain II localizes at focal adhesion plaques, the possible degradation of paxillin by calpain II was examined. As shown in Fig. 3, paxillin was a good substrate for calpain II in vitro. Calpain I, is closely related to calpain II in its substrate specificity in vitro, but requires a much lower concentration of calcium (1–10 $\mu$M) for its activation. Calpain I does not localize to the adhesion plaques [3]. We found that Calpain I also degraded paxillin (Fig. 3). Degradation of paxillin by both calpains was totally inhibited by the inhibitor calpastatin (Fig. 3).

Paxillin is dephosphorylated during mitosis [1]. We postulated that tyrosine phosphorylation may be a signal that protects paxillin from degradation by protease(s) during interphase of the cell cycle. We examined whether tyrosine phosphorylation of paxillin could affect its degradation by calpains. Previously we have shown that paxillin is tyrosine-phosphorylated at multiple sites in normal fibroblasts, and Csk and c-Src are involved in this multiple tyrosine phosphorylation ([1]; Sabe et al., submitted). Thus, paxillin was phosphorylated in vitro by

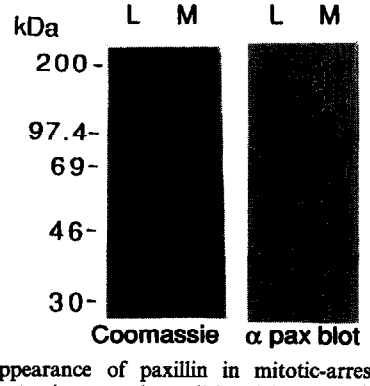


Fig. 1. Disappearance of paxillin in mitotic-arrested fibroblasts. NIH3T3 cells growing on culture dishes (L) or arrested at mitosis by nocodazole treatment (M) were lysed and cellular proteins were separated by SDS-PAGE (8%) and transferred onto a filter for immunoblot analysis. The filter was then probed with an anti-paxillin antibody (αPax). Proteins on the filter were also visualized by staining the filter with Coomassie brilliant blue R250 (Coomassie).

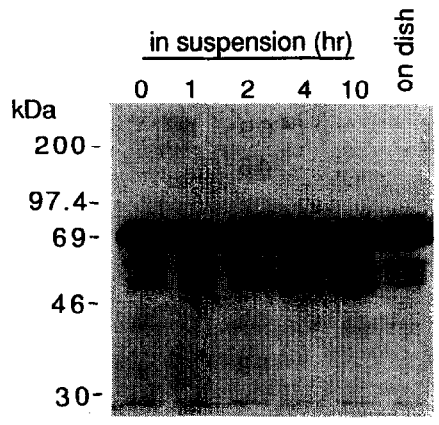


Fig. 2. Levels of paxillin were unaffected by the loss of cell to substratum adhesion. Cell lysates were prepared from NIH 3T3 cells kept in suspension for up to 10 h as indicated, and levels of paxillin were analyzed as in Fig. 1 after proteins were separated by SDS-PAGE. As a control, NIH 3T3 cells were grown on a tissue culture dish (on dish) for the same period.

Csk or by c-Src, and subjected to incubation with calpain II. As seen in Fig. 3, tyrosine phosphorylated paxillin migrated more slowly on SDS-PAGE than the unphosphorylated form. Tyrosine phosphorylation of these slow-migrating forms of paxillin was confirmed by immunoblotting using an anti-phosphotyrosine antibody (data not shown). As shown in Fig. 3, tyrosine-phosphorylation of paxillin either by Csk or by c-Src did not protect it from digestion by calpain II in vitro.

## 4. Discussion

We believe that the degradation of paxillin seen in nocodazole-treated NIH3T3 cells is a mitosis-specific event. In other studies, we have treated rat 3Y1 fibroblasts, CHO cells as well as mouse NIH3T3 cells with nocodazole or other pharmacological agents, and found that paxillin is specifically degraded in mitotic cells, but not in the similarly treated interphase cells (R.Y. and H.S., unpublished). In cells treated with a low dose of nocodazole, so that they can recover and exit mitosis, paxillin is nevertheless degraded during mitotic arrest, yet reappears as cells enter the interphase (R.Y. and H.S., unpublished).

Calpain II has been proposed to be involved in the metaphase/anaphase transition. Micro-injection of labeled calpain II into synchronized PtK cells revealed that calpain II translocates from the plasma membrane to the nucleus during mitosis [11]. Indeed, micro-injection of calcium during metaphase stimulates the transition to anaphase [12], and conditions that lower the cytoplasmic calcium concentration delay the onset of anaphase [13].

Our in vitro study suggested that calpain II might be responsible for the mitotic-specific degradation of paxillin. Talin can also be digested by calpain II in vitro [3]. We have detected a proteolytic fragment of talin, as has been reported [14], in both cells at interphase and in cells arrested at mitosis (R.Y. and H.S., unpublished). However, levels of intact talin and the proteolytic fragment did not change during mitosis when compared to the levels in interphase cells (R.Y. and H.S., unpublished). Further analysis will be required to confirm the involvement of calpain in the decreased level of paxillin during mitosis as well as in proteolysis of talin.

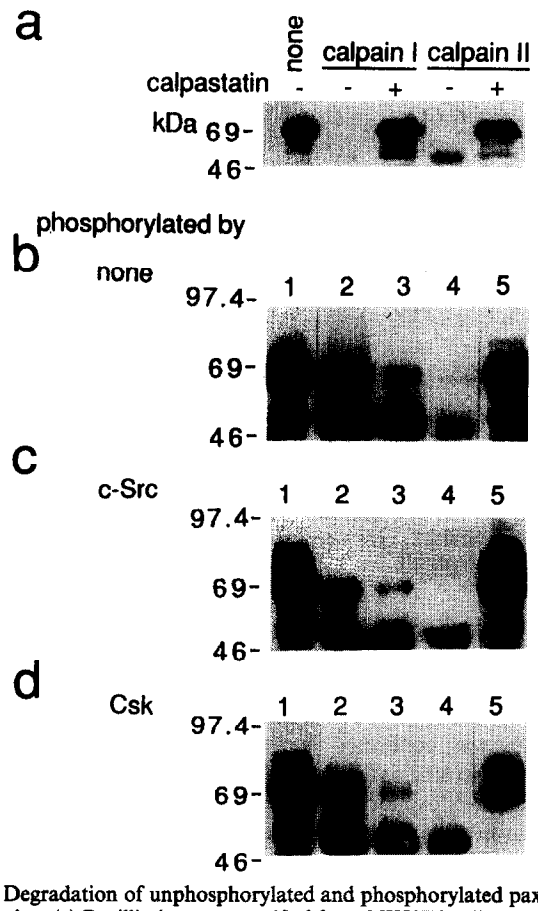


Fig. 3. Degradation of unphosphorylated and phosphorylated paxillin by calpains. (a) Paxillin immuno-purified from NIH3T3 cells was subjected to calpain treatment in vitro either with 30 mU of calpain I or calpain II, in the absence or presence of 0.2 μg of recombinant calpastatin. Samples were then separated by SDS-PAGE and subjected to immunoblotting analysis using an anti-paxillin antibody. (b–d) Paxillin immuno-purified (b) or tyrosine-phosphorylated in vitro by c-Src (c) or by Csk (d) were treated with various concentrations of calpain II: lane 1, none; lane 2, 6 mU; lane 3, 15 mU; lane 4, 37 mU; lane 5, 37 mU plus 0.2 μg of calpastatin. Samples were separated by SDS-PAGE and analyzed by immunoblotting with anti-paxillin antibody as in Fig. 1.

It is not known whether levels of cellular calcium at focal contacts prior to or during mitosis are high enough to activate calpain II. A rapid elevation of the free calcium concentration (up to 500–800 nM) at the metaphase/anaphase transition [15], and an elevation of the calcium concentration at the mitotic spindle poles during anaphase [16] has been reported. However, this level of calcium is still far below that which is required to activate calpain II in vitro (200–300 μM of calcium). Other mechanisms such as a decrease in the calcium requirement of calpain by its autoproteolysis [17–19] or the presence of phospholipids including phosphatidylinositol [20–22] may participate in its activation in vivo. In this regard, it is noteworthy that cell to substratum adhesion triggers an increase in the cellular free calcium concentration up to several μM [23,24]. Phospholipid turnover can also be enhanced by cell to substratum adhesion [25].

Lastly, our in vitro study showed that calpain II degraded paxillin regardless of whether paxillin is phosphorylated by c-Src or Csk or not phosphorylated. Of course, this may not reflect what happens in vivo. Notably tyrosine phosphorylation of paxillin is known to induce its association with SH2 domains of Csk [1] and v-Crk [26]. Thus it is still possible that formation of complexes with other proteins as a result of tyrosine phosphorylation of paxillin may induce sequestering of cleavage sites of paxillin from calpain.

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