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# The tyrosine phosphatase HD-PTP (PTPN23) is degraded by calpains in a calcium-dependent manner

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ABSTRACT

HD-PTP (PTPN23) is a non-transmembrane protein tyrosine phosphatase which contributes to the signal transduction pathways involved in the regulation of cell migration and invasion. We here demonstrate in T24 bladder carcinoma cells that HD-PTP undergoes calcium-dependent degradation which can be prevented by specific calpain inhibitors. In addition, treatment of the cells with the calpain inhibitor calpeptin results in the redistribution of endogenous HD-PTP to the periphery of the cells.

Since (i) calpains are overexpressed in some tumors and (ii) the downregulation of HD-PTP enhances cell migration and invasion, we propose that HD-PTP degradation by calpains might result in the acquisition of a more aggressive phenotype in neoplastic cells.



## 1. Introduction

Calpains are members of a large family of intracellular calcium (Ca)-dependent cysteine proteases consisting of 14 different typical or atypical, tissue specific or ubiquitous enzymes [1,2]. Upon activation, these proteases cleave a broad spectrum of functionally important intracellular proteins that regulate cytoskeletal organization, cell adhesion and spreading, and cell migration [1,2]. By cleaving so many substrates, calpains are involved in a large number of physiological and pathological phenomena, from embryogenesis to cell adhesion, from diabetes to Alzheimer's disease to cancer development and progression [1,2]. The best characterized calpains, μ-calpain and m-calpain (calpain I and calpain II, respectively), are two typical and ubiquitous isoforms. They are cytosolic, heterodimeric enzymes containing an identical regulatory 28-kDa subunit and an 80-kDa catalytic subunit that shares 55–65% sequence homology between the two proteases [1,2]. *In vitro* studies of μ-calpain and m-calpain indicate that they are activated by micro- and milli-molar levels of Ca, respectively, but the presence of phospholipids, small endogenous proteins or even specific substrates can substantially reduce the level of Ca needed for activity [3]. *In vivo* their endogenous specific inhibitor is calpastatin, which is expressed ubiquitously and coexists within cells. Currently, it is unclear how the calpain–calpastatin system is regulated. Several possible modes of regulation have been proposed, such as local Ca transients, differential localization, post-translational modifications, and association with the membranes [3,4].

HD-PTP (PTPN23) is a non-transmembrane protein tyrosine phosphatase which possesses a tyrosine phosphatase domain. Because of an evolutionary conserved amino acid divergence of a key residue located in its phosphatase domain, there has been a debate about whether HD-PTP has a catalytic activity. While HD-PTP was shown to be a catalytically inert protein phosphatase when evaluated using the highly sensitive DiFMUP substrate and a panel of different phosphatidylinositol phosphates [5], in flag/HD-PTP COS-7 transfected cells we detected phosphatase activity [6]. Recently, Src, E-cadherin and β-catenin have been identified as direct substrates of HD-PTP [7]. HD-PTP has been shown to contribute to the signal transduction pathways involved in the regulation of cell migration and invasion [6–9].

HD-PTP contains a PEST motif (rich in proline, glutamic acid, serine and threonine) in the C-terminal region. PEST sequences are found in many short-lived eukaryotic proteins and act as targets for calpains or the proteasome [10,11]. Accordingly, in endothelial cells we have shown that the angiogenic molecule basic fibroblast growth factor (FGF) downregulates HD-PTP *via* proteasome, whereas this multi-enzyme complex is not involved in the post-transcriptional regulation of HD-PTP after treatment with vascular endothelial growth factor. We therefore hypothesize that proteases other that the proteasome might participate to the regulation of HD-PTP degradation.

Because (i) calpains cleave numerous substrates implicated in the regulation of cellular motility and (ii) HD-PTP is a tyrosine phosphatase controlling cell migration and invasion, we evaluated whether HD-PTP is a substrate of calpains.

## 2. Materials and methods

### 2.1. Cell culture and lysis

Bladder carcinoma T24 cells from American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing

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10% fetal bovine serum. Subconfluent cells were scraped in ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, without protease inhibitors. Lysates were passed several times through a 21-gauge needle and incubated for 30 min on ice. Cellular debris were pelleted by centrifugation at 14,000 rpm for 15 min at 4 °C. The soluble fraction was used.

### 2.2. In vitro and in vivo HD-PTP degradation

Cells lysates were supplemented with 3 mM $CaCl_2$, 3 mM $MgCl_2$ or 3 mM $ZnCl_2$ and incubated for 30 min at room temperature. In some experiments, cell lysates were incubated with increasing concentrations of $CaCl_2$ or with 1 mM $CaCl_2$ for different times.

In another set of experiments, cells lysates were incubated with 10 mM EDTA, 10 μM calpastatin (Calbiochem, Nottingham, UK) or 30 μg/ml calpeptin (Calbiochem) for 10 min, then supplemented with 3 mM of $CaCl_2$ and incubated for additional 30 min at room temperature. Reactions were stopped by adding sample buffer. Samples were analyzed by western blot.

To study *in vivo* HD-PTP degradation, T24 cells were treated with ionomycin (1 μM) or calpeptin (15 μg/ml). After the indicated incubation times, the cells were lysed for western blot analysis.

### 2.3. Western blot analysis

Samples (75 μg/lane) were separated on a 8% SDS–polyacrylamide gel, transferred to nitrocellulose sheets at 150 mA for 16 h, and probed with polyclonal anti-HD-PTP IgGs (10 μg/ml) [12]. Antibody against actin was from Santa Cruz (Tebu-bio, Magenta, Italy). Secondary antibodies were labelled with horseradish peroxidase (Pierce, Rockford, IL, USA). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins. All the western blots were repeated at least three times.

### 2.4. Measurement of intracellular free Ca with Fura-2/AM

T24 cells were seeded in 24 well-plates and incubated with ionomycin (1 μM) and Fura-2/AM (10 μM) for 60 min. The monolayers were then washed with a buffer containing NaCl 125 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2 mM, glucose 6 mM, Hepes-NaOH buffer 25 mM (pH 7.4), trypsinized, suspended in the aforementioned buffer and analyzed using a spectrophotometer with excitation wavelengths of 360 nm and emission at 450 nm. Results were normalized in relation to the baseline fluorescence of the cells and data were expressed as a percentage of increase in relation to the baseline.

### 2.5. Immunofluorescence

Cells were cultured on coverslips for 24 h and treated with 15 μg/ml calpeptin for 2 or 4 h in DMEM. The cells were then washed with PBS, fixed in 3% paraformaldehyde/2% sucrose in PBS for 10 min at room temperature, washed with PBS, permeabilized with HEPES Triton X-100 buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) and washed again with PBS. The samples were incubated at 37 °C in 2% bovine serum albumin in PBS for 40 min and then with the antibody against HD-PTP for 30 min. After extensive washing, HD-PTP was detected with FITC-labelled anti-rabbit (Sigma, St. Louis, MO, USA) secondary antibody using a fluorescence microscopy (Olympus AX70) at 20X magnification.

## 3. Results

### 3.1. Ca induced HD-PTP degradation in vitro

Because cations are protease cofactors, we investigated whether they influenced the stability of HD-PTP. Cell lysates were incubated in the presence of 3 mM of $CaCl_2$, $MgCl_2$ or $ZnCl_2$ for 30 min at room temperature. While the addition of $ZnCl_2$ and $MgCl_2$ had little effects on HD-PTP levels, the addition of $CaCl_2$ induced a significant reduction of the amounts of HD-PTP, indicating that it is a substrate for Ca-dependent proteases (Fig. 1A). When cell lysates were incubated with EDTA, a Ca chelator, the degradation of HD-PTP levels was prevented (Fig. 1A), demonstrating that it is the presence of Ca that triggers the degradation event. Fig. 1B and C show that Ca induced a dose- and time-dependent reduction of HD-PTP levels. In particular, HD-PTP degradation was almost complete at 3 mM $CaCl_2$. With 1 mM $CaCl_2$, the maximum effect was observed within 10 min.

### 3.2. Ca induced HD-PTP degradation in vivo

To demonstrate that the Ca-regulated degradation of HD-PTP occurs in living T24 cells, ionomycin, a Ca ionophore, was added to the culture medium. Fig. 2A shows a marked increase of intracellular Ca in T24 cells exposed to ionomycin (1 μM) for 60 min.

We then incubated the cells with ionomycin for increasing times and observed a concomitant increase in the degradation of

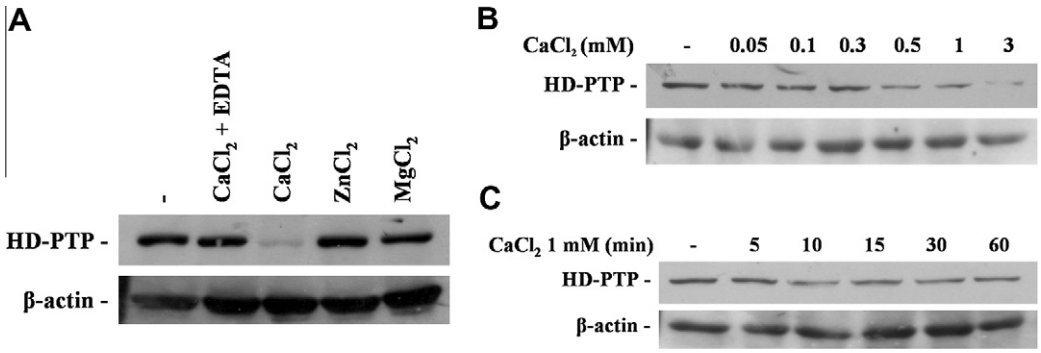


**Fig. 1.** Ca promotes HD-PTP degradation *in vitro*. (A) Cell extracts were incubated in a buffer containing 3 mM of $CaCl_2$, $MgCl_2$ or $ZnCl_2$ for 30 min at room temperature. The samples were separated by SDS–PAGE and immunoblotted with antibody against HD-PTP. (B–C) T24 cells extracts were incubated in a buffer containing increasing amounts (0–3 mM) of $CaCl_2$ for 30 min (B) or in a buffer containing 1 mM $CaCl_2$ for increasing times (0–60 min) (C). The extracts were separated by SDS–PAGE and immunoblotted with antibody against HD-PTP. In A, B and C, actin was used to show that equal amounts of proteins were loaded per lane.

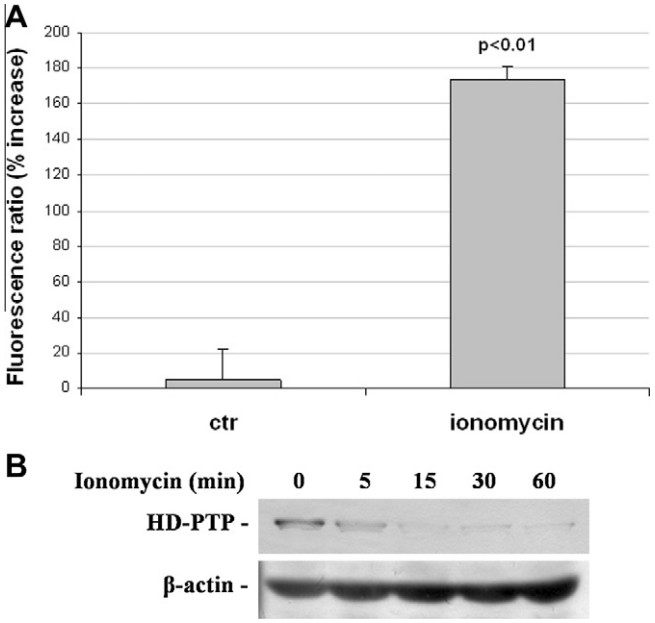


**Fig. 2.** Ca degrades HD-PTP *in vivo*. (A) After 60 min culture in the presence of Fura-2/AM and ionomycin (1 μM), intracellular Ca was measured as described. Values are means ± SD from 4 independent experiments in triplicate. (B) Cells were exposed to ionomycin (1 μM) for increasing times. The extracts were separated by SDS–PAGE and western blot was performed with anti-HD-PTP antibody. Actin staining was used as a control of sample loading.

HD-PTP, with the maximal effect occurring as early as 15 min after the addition of ionomycin (Fig. 2B).

We conclude that HD-PTP is degraded in a Ca dependent fashion both *in vivo* and *in vitro*.

### 3.3. HD-PTP degradation is mediated by calpains

Since Ca activates calpains, we investigated whether HD-PTP degradation was mediated by these enzymes. Calpeptin and calpastatin, two specific calpain I and calpain II inhibitors, were utilized. Calpeptin is a dipeptide aldehyde that was designed to bind specifically to the critical cysteine residue in the active site of calpain, preventing the binding and subsequent proteolysis of calpain substrates [13]. Calpastatin is the endogenous calpain inhibitor [1,2]. Calpeptin and calpastatin were added to the cell lysates in the presence of 3 mM $CaCl_2$ and prevented HD-PTP degradation (Fig. 3A).

We then treated T24 cells with calpeptin for 30 and 60 min and observed that calpeptin prevented HD-PTP degradation (Fig. 3B). These results indicate a role of calpains in the regulation of the amounts of HD-PTP.

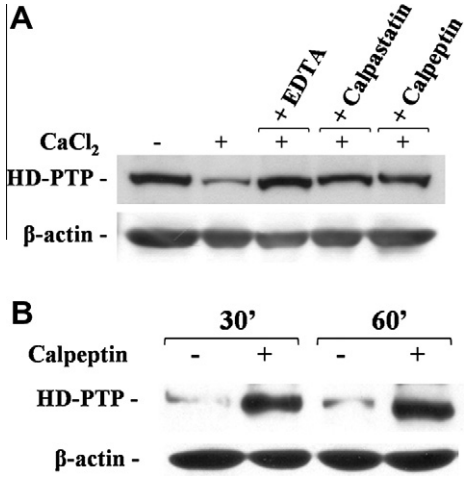


**Fig. 3.** HD-PTP degradation is prevented by calpain inhibitors. *In vitro* (A): cell lysates were exposed to EDTA (10 μM), calpastatin (10 μM) or calpeptin (30 μg/ml) for 10 min at room temperature and then supplemented with 3 mM of $CaCl_2$ for additional 30 min at room temperature. *In vivo* (B): cells were treated with calpeptin for 30 and 60 min at 37 °C and then lysed in the specific buffer. All the extracts were separated by SDS–PAGE and western blot was performed with anti-HD-PTP antibody. Actin was used to show that equal amounts of proteins were loaded per lane.

### 3.4. HD-PTP is differently distributed in T24 cells treated with calpeptin

We investigated the localization of HD-PTP in T24 cells treated with calpeptin for 2 and 4 h. HD-PTP is disperse in the cytosol of untreated cells. After calpeptin treatment, HD-PTP localized also at the cell periphery (Fig. 4). The redistribution of HD-PTP is detected after 2 h and becomes more evident after 4 h of exposure to calpeptin.

## 4. Discussion

HD-PTP was mapped to the human chromosome 3p21, a hot spot for mutation in cancer [14,15]. Interestingly, HD-PTP has been implicated in the regulation of endothelial and T24 cell migration as well as in the control of breast cancer cell invasive behavior [6–9]. These regulatory roles of HD-PTP are likely to be due to its enzymatic activity, first demonstrated in flag/HD-PTP COS-7 transfected cells [6], and then confirmed and broadened by the identification of Src, E-cadherin and β-catenin as its direct substrates [7]. Because of HD-PTP role in balancing the phosphorylation of tyrosine residues in proteins involved in crucial cell activity, it is relevant to get insights into the mechanisms involved in the regulation of its amounts. We have previously shown that HD-PTP is degraded via proteasome in endothelial cells treated with FGF. Here we report that calpains target and completely degrade HD-

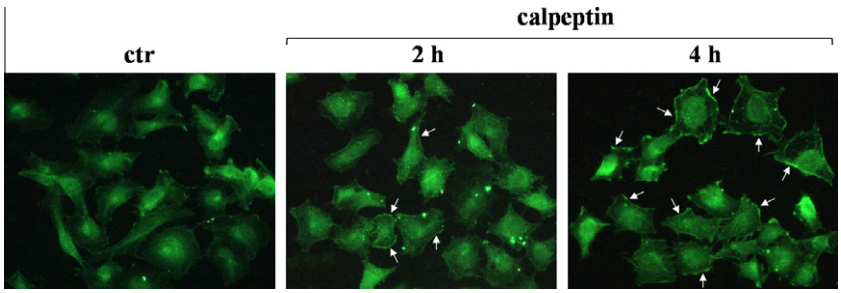


**Fig. 4.** HD-PTP is localized at the cell periphery in calpeptin-treated cells. The cells were treated with calpeptin for 2 and 4 h and processed for immunofluorescence with antibody against HD-PTP as described in the methods. The arrows indicate the presence of HD-PTP at the cell periphery.

PTP by a Ca-dependent mechanism. There is no consistent amino acid consensus sequence in proteins that defines the calpain cleavage site [10]. Previous studies have demonstrated that calpains mediate the limited proteolysis of many of their substrates, thus generating fragments that might have distinct intracellular distributions and functions. A typical example is the proteolysis of talin into a head and rod domain, where the talin head domain binds to integrin with high affinity and modulates its ligand binding affinity [16]. In contrast, calpain-mediated proteolysis of HD-PTP seems to perform a degradative function since we were unable to detect degradation products of HD-PTP *in vitro* and *in vivo*. This is likely to be due to HD-PTP digestion by calpains into fragments too small to be detected by western blot. Similar observations were made with DNA ligase III [17], Rab coupling protein [18], and cortactin [19], among others.

While m-calpain is activated by millimolar Ca (0.1–1 mM), micromolar concentrations (5–50 μM) of Ca are necessary to activate μ-calpain. In our system, the high Ca concentrations requirement for HD-PTP proteolysis *in vitro* suggest an involvement of m-calpain. In addition, since erk activates m-calpain by phosphorylating it [20], it is noteworthy that the treatment of T24 cells with erk inhibitors increased the total amounts of HD-PTP in T24 cells (not shown). Nevertheless we can not rule out the participation of μ-calpain, because it is known that the two enzymes cleave the same substrates [3].

In T24 cells treated with the calpain inhibitor calpeptin, we also show that HD-PTP is redistributed to the cell periphery. This result suggests that, when calpains are inactivated, HD-PTP is targeted to the membrane. We also hypothesize that calpain cleavage of HD-PTP might occur at the plasma membrane since, upon an increase of cellular Ca, calpains translocate to the membrane where they exert their enzymatic activity [1,2]. Moreover, we have previously shown the co-localization at the cell periphery of HD-PTP and Src in T24 cells [8]. It is therefore possible that, once at the cell periphery, HD-PTP might modulate the activity of Src, which is a major regulator of signalling pathways controlling cell migration and invasion, by dephosphorylating it [7].

We conclude that HD-PTP can be targeted and degraded by calpains. Since (i) calpains are overexpressed in some tumors [1] and (ii) decreased levels of HD-PTP enhance cell migration and invasion, partly by the increased phosphorylation of Src, we speculate that HD-PTP degradation by these enzymes might confer a more aggressive phenotype to the cells.

## Acknowledgments

The authors declare no conflict of interest.

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