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Expression and proteolytic processing of mammalian purple acid phosphatase in CHO-K1 cells

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Abstract

Rat recombinant purple acid phosphatase (PAP) stably expressed in fibroblast-like CHO-K1 cells was purified and characterized with respect to post-translational modifications such as N-glycosylation and proteolytic processing in order to elucidate subcellular and molecular pathways for proteolytic activation. In these cells, proteolytically processed PAP was more abundant than the monomeric form. PAP-transfected CHO-K1 cells were expressing active cathepsin K intracellularly, which was partially co-localized with PAP. However, neither cathepsin K nor trypsin digestion of the purified monomeric PAP in vitro did result in a two-subunit form with kinetic and electrophoretic properties resembling the endogenous cellular two-subunit form. Treatment of PAP-transfected CHO-K1 cells with the cysteine proteinase inhibitor E-64 suggested that only a minor fraction of secreted PAP is processed intracellularly by cysteine proteinases. These data do not support a dominant or critical role for cathepsins or trypsin-like serine proteinases in the proteolytic activation of PAP in CHO-K1 cells, implicating yet unidentified proteinases in the proteolytic processing of both intracellular and secreted PAP in this cell line.
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Purple acid phosphatases (PAPs),[1] also referred to as tartrate-resistant acid phosphatases (TRAPs) (EC 3.1.3.2), uteroferrin (Uf) or type 5 acid phosphatases, are acidic metallohydrolases that contain a binuclear Fe^{3+} M^{2+} center in their active site, where M is Fe or Zn [1–4]. Mammalian PAPs typically contain a redox-sensitive Fe–Fe metal center, where the iron center can exist in an oxidized purple form that contains two ferric irons (FeIII–FeIII) and in a reduced pink form that contains one ferric and one ferrous iron (FeIII–FeII) [5]. The enzymatically inactive purple form can be converted to the enzymatically active pink form by treating the enzyme with mild reducing agents such as ferrous iron or ascorbic acid. In mammals, PAP is abundantly expressed in macrophage lineage cells such as bone-resorbing osteoclasts, dendritic cells and macrophages as well as in neurons and epithelial lining cells [6–9]. PAP participates in bone remodeling, immune regulation and lysosomal catabolism [10–16]. Mammalian PAPs are synthesized as monomeric N-glycosylated polypeptides of 35–37 kDa. The enzyme can be proteolytically processed in an exposed loop region, generating a two-subunit structure with the N-terminal glycosylated 21–23 kDa fragment disulfide-linked to the C-terminal 16 kDa fragment [17–19]. Limited proteolysis of the repressive loop domain potentiate enzyme activation by reductants [20]. PAPs contain two potential N-glycosylation sites at Asn97 and Asn128 and the oligosaccharides appear to influence the conformation and/or orientation of the

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[1] *Abbreviations used:* PAPs, purple acid phosphatases; TRAPs, tartrate-resistant acid phosphatases; Uf, uteroferrin; CHO, Chinese hamster ovary; PMSF, phenylmethylsulfonyl-fluoride; pNPP, *p*-nitrophenylphosphate; NBT, nitro blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; RT, room temperature.

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repressive loop domain [21,22]. Recombinant PAP has been shown to be proteolytically cleaved in vitro by the serine proteinases trypsin and chymotrypsin or the cysteine proteinases cathepsins K, L and B [17,23–30], and it has been proposed that cathepsin K and L may process PAP in osteoclasts and macrophages, respectively [23].

It has been reported that macrophages and endometrial cells preferentially secrete the uncleaved monomeric form [31,32], whereas bone-resorbing osteoclasts secrete the monomeric form towards the bone and targets the cleaved two-subunit form into the circulation [23,33,34]. It therefore appears that proteolytic processing is tightly regulated and coupled to intracellular trafficking and secretion of PAP.

With regard to post-translational modifications, native PAPs isolated from mammalian tissues or recombinant PAPs expressed in Baculovirus-infected insect cells have been extensively characterized [19,21,23,25,26,35–38]. These sources of the PAP enzyme are however not suitable for dynamic studies of turnover and targeting of PAP. In order to dissect the pathways and regulatory mechanisms involved in post-translational modifications of PAP, e.g., N-glycosylation and proteolytic processing, we sought to establish a mammalian cell line with stable expression of both the monomeric and two-subunit forms of PAP. As recently shown by Janckila et al. stable expression of different isoforms of PAP can be achieved by Chinese hamster ovary (CHO) cells [39]. Moreover, CHO cells can process pro-cathepsin K to an active form intracellularly and target the active enzyme to lysosomes [40].

In the present study, data are presented suggesting that the proteolytic processing of intracellular and secreted PAP involves several proteolytic events, with a minor role attributable to cysteine proteinases.

Materials and methods

Materials

Restriction enzymes and DNA purification system were purchased from Life Technologies (Carlsbad , CA, USA).

Construction of expression vectors and stable expression of recombinant purple acid phosphatase in CHO-K1 cells

A 1397 bp cDNA fragment encoding the purple acid phosphatase from rat bone [19], was ligated into the *Eco*RI site of the pTZ18R-based expression vector, CMV5, carrying a CMV promoter, a SV40 enhancer, a hGH polyA signal and ampicillin-resistance marker (Promega, Madison, WI, USA). The CMV5-PAP plasmid was subsequently amplified in HB 101 cells. The orientation of the inserts was examined by restriction mapping. Clones with correctly oriented inserts were selected and used for transfection. For selection of co-transfected cells, the pLTR-Neo plasmid derived from pML containing the Harvey Murine Sarcoma Virus promoter was used, enhancing the expression of the neomycin phosphotransferase gene, and a SV40 polyA signal.

Approximately 400,000 CHO-K1 cells per 35 mm culture dish were maintained in RPMI 1640 culture medium containing 10% FCS, 1% non-essential amino acids and 50 mg/l Gentamicin over night at 37 °C. The cells were washed twice with Opti-MEM. Two milliliters of transfection mix containing Opti-MEM, 20 μl Lipofectamine, 2.5 μg CMV-TRAP and 0.25 μg pLTR-Neo was added per dish and incubated together with the cells over night at 37 °C. On day 1 post-transfection, 2 ml selection medium (culture medium containing 0.8 mg/ml G418) was added. On day 7, cells were trypsinized and seeded 1:6. On day 14, clones were picked and transferred to a 96-well plate. Positive clones were detected following TRAP assay of the conditioned medium, and were propagated to $5–8 \times 10^6$ cells. Of a total of 23 positive clones, the cell-associated TRAP activity of clone 12 was estimated to around 20 mU/10^6 cells and was used for further purification and characterization of the enzyme.

Purification of recombinant purple acid phosphatase from CHO-K1 cells

Approximately 1×10^9 cells from clone 12 were harvested and lysed with three volumes (v/v) 0.15 M KCl, 0.1% Triton X-100 containing 100 μM phenylmethylsulfonyl-fluoride (PMSF) for 1 h at 4 °C followed by homogenization using a Polytron for 3×10 s at 4 °C. Protamine sulfate was added to final concentration of 0.5%, and the suspension was cleared by centrifugation at 3200*g* for 15 min. CHO-K1-PAP was further purified on a CM-cellulose column, a Phenyl-Sepharose column and a HiTrap 5 ml Heparin HP column according to published procedures [19,24].

Protein quantification and TRAP activity assay

Protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA). TRAP activity was measured using *p*-nitrophenyl-phosphate (pNPP) as the substrate as described previously [24].

1 U corresponds to 1 μmol pNPP formed/min.

Kinetic analysis of PAP

For PAP kinetic analysis, pNPP was used as the substrate described in [24]. In short, all reactions were performed in duplicate. 0.05–50 mM substrate (final concentration) was incubated at 37 °C. K_M and K_{cat} values were calculated by non-linear regression analysis and plotted as Lineweaver–Burk plots using Origin software (Microcal Software, Inc. Northampton, MA, USA). The pH optima of CHO-K1-PAP were measured using 0.1 M NaAc, pH 4.5–6.5. All measurements were repeated twice and results were averaged from two different enzyme preparations.

Proteolytic digestion of PAP

The cleavage of CHO-K1-PAPs with recombinant human mature cathepsin K kindly provided by Dr. Robert Dodds, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA, (12.6 U/mg protein) [42] was performed with final concentrations of 16.7 ng/μl PAP, 0.2 ng/μl cathepsin K , 2 mM dithiothreitol, 50 mM sodium acetate, pH 5.5 and 1 mM EDTA in a final volume of 30 μl, and incubated at 37 °C for 2 h. The reaction was stopped by the addition of the proteinase inhibitor cocktail Complete (Boehringer Mannheim, Germany).

CHO-K1-PAPs (7 ng/μl) were digested with 20 ng/μl of trypsin (bovine pancreas 10,800 U/mg protein, Sigma) in incubation solution (10 mM Tris–HCl, pH 7.5, 20 mM NaCl and 20 mM $CaCl_2$). Incubation was performed at room temperature for 45 min. The reaction was stopped by the addition of the inhibitor cocktail Complete.

Western blot analysis and silver staining

Protein samples were separated by 15% SDS–PAGE gel under reducing conditions according to Laemmli [41]. Proteins were transferred to PVDF membranes and blocked by TBST (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1% Tween 20). Immunoblots were probed with polyclonal rabbit anti-rat recombinant PAP antiserum (1:500 dilution) at room temperature for 1 h [19] or polyclonal rabbit anti-mouse cathepsin K (1:500 dilution) antiserum [23]. After washing with 0.05% TBST, goat

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