The tartrate-resistant purple acid phosphatase of bone osteoclasts—a protein phosphatase with multivalent substrate specificity and regulation

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Tartate-resistant purple acid phosphatases (TRAP/PAP) are iron-containing, cationic glycoproteins with molecular weights of around 35 kDa and a monomeric peptide structure (Vincent and Averill, 1990; Andersson et al, 1992). When isolated and sufficiently concentrated, these enzymes exhibit a characteristic purple color. The purple acid phosphatases of spleen and uterus have been extensively characterized, in particular with respect to the unique active site containing two iron atoms, which are essential for catalytic activity (Vincent and Averill 1990). This binuclear iron center can exist in two interconvertible states: in the purple enzyme as a diferric pair which is catalytically inactive or in the reduced, catalytically active pink species where the di-iron cluster exist as a mixed-valent Fe(II)-Fe(III) couple (Wang et al. 1992). Low levels of TRAP/PAP enzymes can be detected in most tissues, whereas much higher expression levels are detected in bones of growing animals (Ek-Rylander et al. 1991a). In bones, the tartrate-resistant purple acid phosphatase is abundantly and selectively expressed in actively resorbing osteoclasts (Andersson and Marks, 1989), which is the reason for the wide-spread use of the TRAP/PAP as a histochemical marker for the identification of osteoclasts. Moreover, TRAP/PAP has been tried as an indicator for bone resorption in pathological conditions involving increased bone turnover (Kraenzlin et al. 1990). Despite the fact that the TRAP/PAP enzyme has been recognized as an osteoclast marker enzyme for more than a decade (Minkin, 1982), until recently almost nothing was known concerning the mechanistic role of TRAP/PAP in bone metabolism. Studies employing enzyme histochemical detection of the TRAP/PAP activity as well as immunohistochemical detection of the protein at the ultrastructural level have shown a predominant localization of the enzyme to the resorption vacuole (Andersson et al. 1992; Reinholt et al. 1990a), suggesting that the enzyme is secreted by the osteoclast to the site of active resorption. Moreover, inclusion of an antibody against TRAP/PAP blocks resorption in calvarial explants (Zaidi et al. 1989), suggesting that the enzyme has a promotive role in the resorption process. TRAP/PAP enzymes catalyze the hydrolysis of phosphate monoesters (Vincent et al. 1992), and among the known substrates for these enzymes are certain acidic phosphoserine-containing proteins, e.g casein and phosvitin (Andersson et al. 1992). In addition to these extraskeletal phosphoproteins, it was recently demonstrated (Ek-Rylander et al. 1994) that purified bone TRAP/PAP can dephosphorylate the bone matrix phosphoproteins osteopontin (OPN), known as an anchor for the binding of osteoclasts via integrin receptors to bone (Heinegård et al. 1995), and bone sialoprotein. Dephosphorylation of OPN was shown to alter the function of the protein, since the modification abolished the capacity of OPN to promote osteoclast attachment in vitro (Ek-Rylander et al. 1994). This observation suggested that one potential physiological function of TRAP/PAP could be to regulate osteoclast attachment to bone. These initial observations on the substrate specificity of the osteoclast TRAP/PAP enzyme has been extended in the present study, where another abundant phosphorylated protein present in the bone matrix , i.e. osteonectin, as well as a phosphotyrosine-containing gastrin peptide were identified as substrates for the osteoclast TRAP/PAP enzyme. Thus, these studies indicate that the TRAP/PAP enzymes, in contrast to most other protein phosphatases with regulatory functions, exhibit broad specificity both with regard to individ-

ual phosphoserine-proteins as well as to preferred phosphoamino acid residues.

Material and methods

Materials

Bovine milk ß-casein, p-nitrophenylphosphate (ditris salt), phospho-serine, phospho-threonine and phospho-tyrosine was purchased from Sigma Chemical Co. Tyrosine phosphatase non-radioactive assay kit was obtained from Boeringer-Mannheim. The colloidal gold protein staining kit came from BioRad. Osteonectin purified from bovine bone was generously provided by Drs Mikael Wendel and Dick Heinegård, Dept. of Cell-and Molecular Biology, Lund University, Sweden.

Purification of TRAP/PAP from rat bone

TRAP/PAP was purified from long bones of 3-week old rats (Andersson et al. 1984; Ek-Rylander et al. 1991b). In short, the purification method involved homogenization of bones in the presence of 0.15 M KCl / 0.1% Triton X-100, precipitation with protamine sulfate, CM-cellulose ion-exchange chromatography and Sephacryl S-300 gel permeation chromatography. The specific activity of the preparation was 130 U/mg protein, where 1 U is defined as 1 µmole p-nitrophenol (or phosphate) formed/ min at 37 °C.

Acid phosphatase assay

Tartrate-resistant acid phosphatase activity was measured in 96 well plates using p-nitrophenylphosphate (pNPP) as the substrate in an incubation medium (150 µl) containing (in final concentrations): 10 mM pNPP, 0.1 M sodium acetate buffer, pH 5.8, 0.2 M KCl, 0.1 % Triton X-100, 10 mM sodium tartrate, 1 mM ascorbic acid and 0.1 mM $FeCl_3$. The liberated p-nitrophenol after 1 hour of incubation at 37 °C was converted to p-nitrophenylate by the addition of 100 µl 0.1 M NaOH and absorbance was read using a Titer-Tek Multiscan Plus (Flow Laboratories) spectrophotometer.

Phosphotyrosyl-peptide phosphatase assay

Protein-tyrosine phosphatase activity was measured using a commercially available kit (Boeringer-Mannheim) employing ELISA for the detection of phospho-tyrosyl peptides. In short, the method detects biotinylated phospho-tyrosyl peptides bound to streptavidin-coated wells using an anti-phosphotyrosine monoclonal antibody conjugated to peroxidase. The standard incubation condition was the same as when measuring the acid phosphatase activity using pNPP as the substrate as described above. The incubations were carried out in a final volume of 50 µl with 1 µmole PPS2 (amino acids 1–17 of human gastrin phosphorylated on tyrosine-12) as the substrate. Detection of remaining phosphopeptide was performed exactly according to the manufacturer's instructions.

Protein phosphatase assays

The phosphoproteins ß-casein or osteonectin were incubated with aliquots of the purified TRAP/PAP enzyme in an incubation medium containing 10 mM sodium acetate buffer, pH 5.8 and 0.05 mM EDTA for 1 hour at 37 °C. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min. Proteins were subjected to 12% SDS-PAGE using the Laemmli-protocol. Following electrophoresis, proteins were transferred to nitrocellulose filters and visualized using colloidal gold (Bio-Rad) according to the manufacturer's instructions. The positions of the protein bands were compared to those of molecular weight standards (Low molecular weight standards from Pharmacia).

Phosphoamino acid analysis

The phosphoamino acid content of ß-casein following incubation in the presence or absence of the purified TRAP/PAP enzyme under the conditions described above was carried out essentially according to Cooper et al. (1983). In short, incubations were lyophilized, dissolved in 6 M HCl and heated to 110 °C for 1 hr. The hydrolysate was lyophilized over NaOH tablets, and the residues then dissolved in the buffer (formic acid/glacial acetic acid/ H_2O, pH 1.9) used for the thin-layer electrophoresis. Electrophoretic separation of phospho-serine from phospho-threonine/ phospho-tyrosine was achieved using precoated cellulose F thin-layer plates (Merck) at 2000 V for ~1 hr. The individual phosphoamino acids were detected by spraying the plate with 0.2% ninhydrin and identified by comparison with phosphoamino acid standards.

Results

TRAP/PAP purified from rat bone was recently shown to be capable of dephosphorylating the bone matrix phosphoproteins osteopontin and bone sialoprotein (Ek-Rylander et al. 1994). To extend these observations, two other phosphoproteins, ß-casein from bovine milk and bovine bone osteonectin, were tested as potential substrates for the TRAP/PAP enzyme (Figure 1). Incubation of the phosphopro-

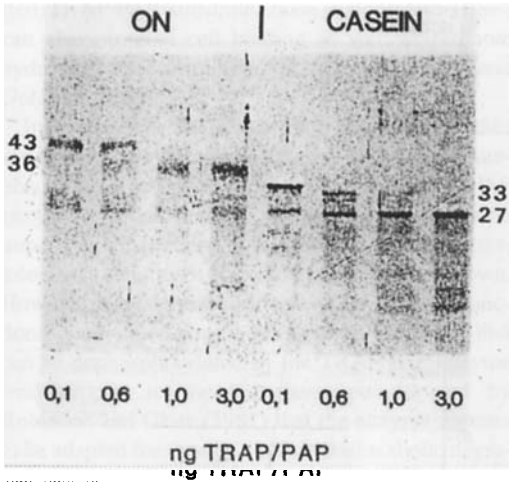

Figure 1. TRAP/PAP alters the electrophoretic mobility of osteonectin (ON) and ß-casein. Different amounts of purified rat bone TRAP/PAP were incubated with 100 ng ON and 75 ng ß-casein using conditions described under Materials and Methods, and the incubations were subjected to 12% SDS-PAGE followed by transfer of proteins to nitrocellulose filters. The proteins were visualized by colloidal gold staining.

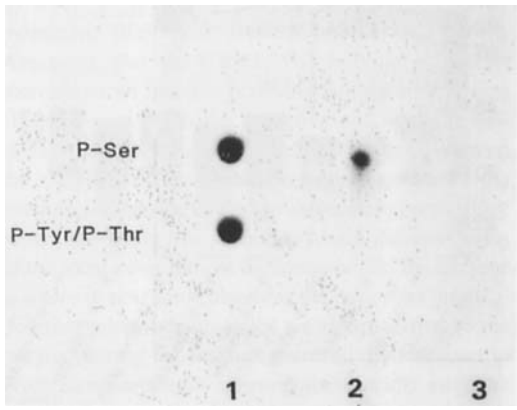

Figure 2. Phosphoamino acid analysis of ß-casein incubated in the absence or presence of rat bone TRAP/PAP. Following incubation of 50 µg ß-casein with or without 1.2 µg enzyme protein and acid hydrolysis, the phosphoamino acids were separated using thin-layer electrophoresis as described under Materials and Methods. Lane 1; phosphoamino acid standards (0.5 µg each of P-Ser = phospho-serine, P-Thr = phospho-threonine, P-Tyr = phospho-tyrosine), Lane 2; ß-casein – TRAP/PAP, Lane 3; ß-casein + TRAP/PAP.

teins with 1 ng of enzyme protein caused a shift in the electrophoretic mobility of the proteins, indicating that the enzyme caused modification of the proteins. A control incubation replacing the phosphoproteins with bovine serum albumin showed in this case no alteration in the electrophoretic mobility or fragmentation of this protein, suggesting that proteolytic activity was absent or minimal in the enzyme preparation (data not shown).

To verify that the change in electrophoretic mobility was due to removal of phosphate groups from the proteins, ß-casein was incubated in the absence or presence of the TRAP/PAP enzyme, and following acid hydrolysis the incubations were subjected to phosphoamino acid analysis using thin-layer electrophoresis (Figure 2). In control incubations without enzyme, the only detectable phosphoamino acid was phospho-serine. In the TRAP/PAP-treated sample, the spot representing phospho-serine was absent, showing that the enzyme indeed removed phosphate associated with serine residues.

To exclude the possibility that a contaminating phosphatase in the preparation other than TRAP/PAP could be responsible for the observed dephosphorylation, osteonectin was incubated with the enzyme in the presence of different phosphatase inhibitors (tartrate, vanadate) or substrates (pNPP, ATP) (Figure 3). Orto-vanadate, a non-competitive inhibitor of the TRAP/PAP-enzyme (Andersson et al. 1984), as well as the substrates pNPP and ATP inhibited the modification of osteonectin, whereas tartrate, as expected, had no effect. This pattern of inhibition strongly indicates that modification of osteonectin, presumably the removal of phosphate groups, is due to the action of the TRAP/PAP enzyme.

It has recently been reported that a TRAP enzyme purified from hairy cell leukemia exhibits phospho-tyrosyl protein phosphatase activity (Janckila et al. 1992). The osteoclast enzyme can hydrolyze phospho-tyrosine (Andersson et al. 1989), and it was therefore of interest to verify that the osteoclast-derived TRAP/PAP enzyme also could dephosphorylate phospho-tyrosyl peptide substrates. For this purpose, a tyrosine-phosphorylated gastrin peptide was incubated with the purified bone TRAP/PAP at different pH values (Figure 4). Similar to the hydrolysis of other phosphomonoester substrates by the TRAP/PAP enzyme (Andersson et al. 1984), dephosphorylation of the gastrin peptide exhibited an optimum at a pH of approximately 5.5. No activity could be observed above pH 6.0. To ensure that the phospho-tyrosyl phosphatase activity of the preparation was due to the TRAP/PAP enzyme and not to a contaminating phospho-tyrosine phosphatase, various TRAP/PAP inhibitors were included in the incubations (Table 1). All three inhibitors tested, e.g. molybdate, tungstate and arsenate, inhibited the enzyme reaction with the same rank order of potency as when pNPP

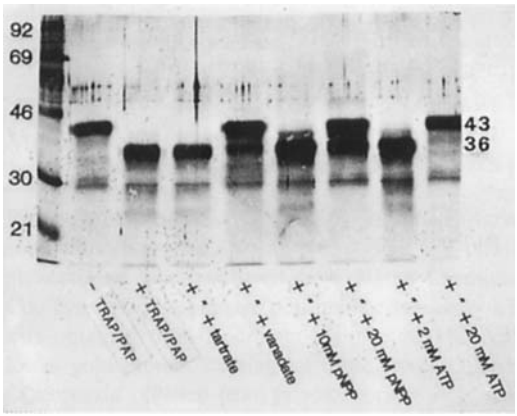

Figure 3. Effect of various TRAP/PAP inhibitors and substrates on the TRAP/PAP-dependent modification of osteonectin. 20 ng of purified TRAP/PAP was incubated with 1 µg osteonectin in the absence or presence of the different compounds indicated in the figure. The effect of the compounds was monitored as described in the legend to Figure 2.

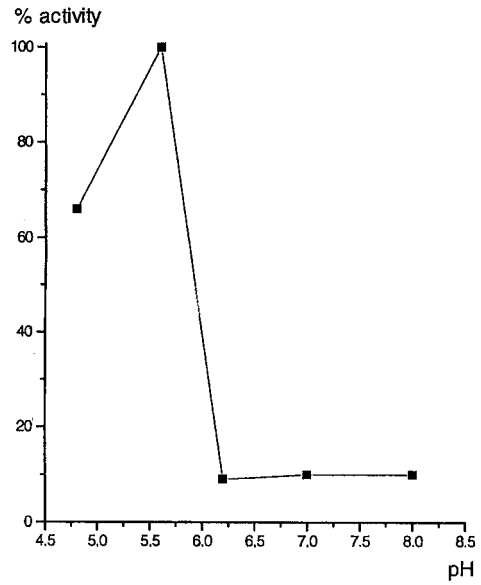

Figure 4. pH-dependence of the phospho-tyrosyl protein phosphatase activity of the purified rat bone TRAP/PAP. Hydrolysis of phosphate from phosphotyrosylated human gastrin peptide was measured as described under Materials and Methods.

Table 1. Inhibitor constants for tetrahedral oxyanions on the TRAP/PAP activity towards different substrates. The inhibition constant IC50 is defined as the concentration of inhibitor which give 50% inhibition compared to the activity in the absence of inhibitor (µM)

Inhibitor	p-NPP	Phosphotyrosyl peptide
Molybdate	25	3
Tungstate	60	10
Arsenate	575	100

was used as the substrate for the enzyme. Interestingly, half-maximal inhibition of the phospho-tyrosyl peptide phosphatase activity of the TRAP/PAP enzyme required significantly lower concentrations of inhibitors compared to when pNPP was used as the substrate. Most protein-tyrosine phosphatases require the inclusion of a sulfhydryl reducing agent during incubation in order to keep an essential cysteine residue of the active site in the reduced state (Stone and Dixon 1994). However, the phosphotyrosyl-peptide activity of the purified TRAP/PAP enzyme was not increased in the presence of ß-mercaptoethanol (data not shown). Furthermore, a computer-assisted comparison of the TRAP/PAP amino acid sequence to other protein-tyrosine phosphatases did not reveal any significant sequence homology. Importantly, the active site motif—(I/V)-HCXAGXGR(S/T)G—common to all protein-tyrosine phosphatases (Stone and Dixon 1994) was not present in the TRAP/PAP sequence.

Discussion

One of the hydrolytic enzymes secreted by the osteoclast into the resorption area underneath the cell is the tartrate-resistant acid phosphatase (Andersson et al. 1992). Despite several observations providing circumstantial evidence that this enzyme participates in the resorption process (Zaidi et al. 1989, Reinholt et al. 1990a), it was only recently that extracellular substrates, localized to the bone matrix, for this enzyme could be identified (Ek-Rylander et al. 1994). One of these TRAP/PAP substrates is osteopontin (OPN), with a well-characterized role in mediating the attachment of the osteoclast to the bone surface (Reinholt et al. 1990b, Heinegård et al. 1995). The action of the osteoclast TRAP/PAP on OPN, resulting in removal of phosphate bound to serine residues, was interestingly found to have functional consequences, since the dephosphorylated OPN, in contrast to its phosphorylated counterpart, could not promote the attachment of osteoclasts in vitro (Flores et al. 1992, Ek-Rylander et al. 1994). The other identi-

fied TRAP/PAP substrate, bone sialoprotein (BSP) can also promote cell binding as well as promote hydroxyapatite formation in vitro (Hunter and Goldberg 1993).

In the present study, we have identified another serine-phosphorylated bone matrix component, osteonectin, as a substrate for the osteoclast TRAP/PAP enzyme. It is still unclear whether this modification imparts on the function of this protein, since the physiological function of osteonectin is unknown. However, the fact that an increasing number of functionally unrelated phosphoproteins are identified that can be dephosphorylated by the TRAP/PAP enzyme lend support to the hypothesis put forward by Robinson and Glew (1981) that the enzyme appears to be adapted for serving a role in the catabolic degradation of phosphoproteins. This feature may be particularly relevant for the intracellular forms of TRAP/PAP, for instance the spleen enzyme, which seems to participate in lysosomal degradation processes (Schindelmeiser et al. 1987). The idea of a general role of TRAP/PAP in the catabolism of phosphoproteins is further supported by the observations in the second part of the present study, where in addition to the phosphoserine-containing proteins also a phospho-tyrosyl peptide was found to be effectively dephosphorylated by the TRAP enzyme at an acidic pH. Although the enzymatic removal of phosphate groups by the TRAP enzyme obviously may have functional implications for individual phosphoproteins, as observed for OPN and BSP, the broad substrate specificity of this enzyme suggest that other factors at the site where the enzyme is localized could be even more important than substrate specificity to control the action of the enzyme. One such factor is the local pH. The enzyme is active against all substrates so far tested only at a pH of between 5-6 (Andersson et al. 1992). This is the ambient pH in acidic intracellular compartments such as the lysosomes, but the same acidic conditions is also prevalent in the extracellular resorption lacuna of the osteoclast (Baron et al. 1985;, Silver et al. 1988).

The TRAP/PAP enzymes are invariably isolated in their oxidized, catalytically inactive form, and require reduction of one ferric atom to attain enzymatic activity. Thus, the redox conditions in the vicinity of the enzyme, altering the valency of the binuclear iron cluster could theoretically be another mechanism important for the local regulation of TRAP/PAP activity (Vincent and Averill 1990). One of many electron donors that can activate the purple form of the enzyme is superoxide anion (Sibille et al. 1987). With regard to osteoclast function, it has recently been observed that superoxide is produced by an NADPH oxidase present on the ruffled border membrane (Key et al. 1994, Steinbeck et al. 1994). Assuming that the TRAP/PAP enzyme is in fact secreted as an inactive precursor with a diferric iron cluster, exposure to the reductive and acidic conditions of the resorption vacuole would render and keep the enzyme selectively active in an area where the enzyme is intended to serve catabolic and regulatory functions. Thus, this scenario, where the conditions of the local environment dictates whether the enzyme is active or not, could also bear the important implication that potential substrates are not modified to the enzyme during for instance intracellular transport to acidic compartments. Even at these acidic sites the enzyme is kept silent until an appropriate stimulus such as production of oxygen radical formation is initated. Besides the substrate specificity of the TRAP/PAP enzyme, we propose that the local conditions in the vicinity of the enzyme could provide an important means of regulation necessary for activation the TRAP/PAP enzyme to participate in physiological events, e.g. phagocytosis or bone resorption, or in pathological states involving excessive destruction of the extracellular matrix.

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