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Research Article

Differential expression of monomeric and proteolytically processed forms of tartrate-resistant acid phosphatase in rat tissues

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Abstract. Purple acid phosphatase (PAP), also known as tartrate-resistant acid phosphatase (TRAP), uteroferrin or type 5 acid phosphatase (Acp5) is synthesized as an N-glycosylated monomeric latent precursor, which can be processed by limited proteolysis to a disulfide-linked two-subunit form with increased enzyme activity. In this study, we disclosed that the proteolytically processed two-subunit form constitutes the major PAP/TRAP variant in monocytic cells in spleen, thymus, liver and colon. In addition significant expression of the monomeric PAP/TRAP, indicating a non-enzymatic function, was detected in epithelial cells of colon, lung and kidney. Interestingly, proteolytic processing alone did not activate the enzyme but rendered the enzyme more susceptible to activation by reductants. Thus, beside limited proteolysis, the subcellular redox state could also be a determinant of enzyme action in vivo. The co-localization of PAP/TRAP and the cysteine protease cathepsin L could suggest a role for cathepsin L in the in vivo proteolytic processing of PAP/TRAP in monocytic cells.

Key words. Tartrate-resistant acid phosphatase; purple acid phosphatase; uteroferrin; cathepsin B; cathepsin L.

Purple acid phosphatase (PAP), also referred to as tartrate-resistant acid phosphatase (TRAP) (EC 3.1.3.2), uteroferrin (Uf) or type 5 acid phosphatase (Acp 5) in mammals, is an acidic metallohydrolase that contains a binuclear Fe(III)-M(II) center in its active site where M can be Fe or Zn [1–5]. Mammalian PAP/TRAP typically contains a redox-sensitive Fe-Fe metal center [6, 7] and is isolated as the enzymatically inactive purple Fe(III)-Fe(III) form, which can be converted to the enzymatically active pink Fe(III)-Fe(II) form in vitro by treating the enzyme with mild reductants such as ferrous iron or ascorbic acid [3, 8]. PAP/TRAP has been isolated and characterized from mammalian [9–18] and plant [19–21] sources. In mammals, the enzyme is abundantly expressed by bone-resorbing osteoclasts, certain populations of monocytes/macrophages, dendritic cells, neurons and in the porcine endometrium during pregnancy [22–27]. The enzyme is considered to be involved in bone and cartilage resorption and in immune defense mechanisms, since PAP/TRAP deleted/over-expressing mice exhibit disturbed bone mineralization and resorption [27–30] and an altered macrophage response to cytokine stimulation and microbial challenge [31]. PAP/TRAP is a potent phosphoprotein phosphatase acting on both phosphotyrosine- and phosphoserine-containing proteins [32, 33]. The phosphoserine-containing protein osteopontin (OPN), also known as Eta-1, has been proposed as one potential physiological substrate [28, 34, 35], since dephosphorylation of OPN by PAP/TRAP has been

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shown to modulate OPN-mediated cell adhesion and migration [34, 36]. PAP/TRAP could thus serve a role in regulating the bioactivity of OPN [35]. The enzyme can also produce reactive oxygen species (ROS) and ROS generated from PAP/TRAP have been suggested to participate in the intracellular degradation of phagocytosed material in osteoclasts and macrophages [37, 38]. A role for PAP/TRAP in lysosomal catabolism in liver, spleen and kidney was also recently established [28]. The porcine PAP/TRAP homologue Uf has been invoked in fetal iron transport [39, 40] and as a hematopoetic growth factor [41].

At the subcellular level, the enzyme is translated in the endoplasmic reticulum (ER) as a monomeric N-glycosylated polypeptide of approximately 35–37 kDa [4, 42]. However, when purified from tissues, e.g. bone and spleen, the enzyme is predominantly isolated as a two-subunit form consisting of two fragments of approximately 20–23 kDa and 15–17 kDa joined by a disulfide bridge [4, 43]. In vitro, the monomeric form can be converted into the two-subunit form by proteolytic processing in the sequence between Ser145 and Val161, known as the repressive loop domain [33, 44]. Limited proteolysis of the loop domain results in increased PAP/TRAP activity comparable to the activity of endogenous two-subunit PAP/TRAP, and could provide a means of regulating its activity in vivo [33]. Both cysteine proteases, e.g. cathepsin B, K and L, and the serine proteases trypsin and chymotrypsin [43, 45, 46] have been shown to cleave monomeric recombinant rat, bovine and human PAP/TRAP to generate a two-subunit PAP/TRAP form. However, only the cysteine proteases cleaved and activated rat and human recombinant PAP/TRAP to physiological activity levels [33, 45] indicating that these proteases could potentially be responsible for the proteolytic processing of the enzyme in vivo. Whereas cathepsin K seems to have a restricted tissue expression pattern, the major sites being osteoclasts and ovarian follicular cells [47, 48] with lower levels detected in lung, brain, colon, pancreas and monocyte-derived macrophages [49–51], cathepsin L and B appear to be ubiquitous in vertebrate cells [52–54].

Interestingly, some forms of PAP/TRAP, e.g. Uf from porcine endometrium [12] and recombinant PAP/TRAP expressed in insect [4, 55, 56] or mammalian [57] cell systems, appear not to be proteolytically processed but rather secreted as the monomeric protein. The aims of this study were to explore the distribution and significance of proteolytic processing and redox regulation of PAP/TRAP in rat tissues, by assessing the expression of monomeric and two-subunit forms at the tissue and cellular levels. We also attempted to identify putative proteases involved in the proteolytic processing of the monomeric PAP/TRAP to the active two-subunit form in these tissues.

Materials and methods

Experimental animals

Three to six Spraque-Dawley rats of different ages were used in each experiment. Animals were kept under controlled light/dark conditions with food and water available ad libitum. The use of the animals in this study was approved by the local animal ethical committee of the Karolinska Institutet (S 170/98 and S 159/01).

Preparation of polyclonal antibody against monomeric PAP/TRAP

A synthetic peptide corresponding to amino acids 167–183 (DDFASQQPKMPRDLGVA) in the mouse TRAP sequence (NP 031414) was conjugated to keyhole limpet hemocyanin through a C-terminal cysteine residue using maleimide as the cross-linker (Innovagen AB, Lund, Sweden). Two rabbits were immunized subcutaneously with 200 µg of the peptide conjugate mixed with Freund's complete adjuvant at week 0, 3 and 6 followed by a final booster injection with 100 µg peptide conjugate in Freund's incomplete adjuvant at week 12. Serum was collected 2 weeks after the last booster injection.

Immunohistochemistry

Anesthetized animals were fixed by perfusion with 4% paraformaldehyde in 0.1 M Sörensen's phosphate buffer, pH 7.4. Tissues were dissected out and embedded in paraffin. Sections (4.5 µm thick) were adhered to ChemMate Capillary Slides (Dako, Glostrup, Denmark). Sections were treated for antigen retrieval using microwave treatment for 10 min at 1100 W in 1 mM EDTA pH 8.0. Light microscopy sections were stained using the automated staining system Horizon (Dako) using rabbit anti-rat total PAP/TRAP antibody, recognizing both monomeric and two-subunit PAP/TRAP [4], diluted 1:400, rabbit anti-mouse monomeric PAP/TRAP antibody 1:800 or goat anti-mouse cathepsin L antibody 1:50 (Santa Cruz Biotechnology, Santa Cruz, Calif.). As secondary antibodies and developing solutions, ChemMate Detection Kit Peroxidase/DAB rabbit/mouse (Dako) or donkey anti-goat HRP (Jackson Immunoresearch, West Grove, Pa.) were used. Double-label immunohistochemistry sections were stained as follows. After blocking in 0.1% BSA (diluted in TBST; 100 mM Tris-HCl pH 7.6, 154 mM NaCl, 0.01% Tween-20), sections were incubated with primary antibodies [rabbit anti-rat total TRAP 1:400, goat anti-mouse cathepsin L 1:50 (Santa Cruz Biotechnology)] for 1 h at room temperature (RT). After washing in TBST for 10 min, sections were incubated with secondary antibodies [donkey anti-rabbit FITC 1:100 (Jackson Immunoresearch), donkey anti-goat ALEXA 488 1:100 (Molecular Probes, Eugene, Ore.)] for 30 min at RT. After washing for 10 min in TBST, sections were mounted using Fluorescent Mounting Media (Dako)

and examined in a Leica TCS NT ArKr laser confocal microscope (Leica Microsystems AG, Wetzler, Germany). Antibodies were diluted in ChemMate Antibody Diluent (Dako). Negative controls consisted of replacing primary antibody with normal rabbit serum/normal goat serum (Dako) or primary antibody incubated with recombinant rat PAP/TRAP as previously described [23].

Purification of PAP/TRAP from rat tissues

Partial purification of PAP/TRAP for enzyme activity measurements was performed as previously reported [23, 58]. PAP/TRAP used for cleavage analysis was partially purified as follows: buffer exchange was made on partially purified PAP/TRAP from 0.5 M NaAc pH 6.5 to 0.1 M NaCl, 10 mM Tris pH 7.2. Sample was loaded onto a HiTRAP heparin 5 ml column (Amersham Bioscience, Uppsala, Sweden) and eluted using a gradient from 0.1 M NaCl, 10 mM Tris pH 7.2 to 1 M NaCl, 10 mM Tris pH 7.2 using an ÄktaPurifier (Amersham Bioscience). PAP/TRAP from rat tissues used for Western blots was purified as follows: samples were loaded on a SP Sepharose FF column after the protamine sulfate precipitation and the column was washed with 0.1 M NaAc pH 6.5. Proteins were eluted using a gradient from 0.1 to 0.5 M NaAc pH 6.5. Fractions containing PAP/TRAP enzyme activity were pooled and loaded on a HiPrep 16/10 Phenyl (high sub) column. The column was washed with 1.1 M NH_4SO_3 in 50 mM NaAc pH 6.5 and protein was eluted with a gradient from 1.1 M NH_4SO_3 in 50 mM NaAc pH 6.5 to 50 mM NaAc pH 6.5 using an ÄktaPurifier. All purifications were performed in the presence of the protease inhibitors Pefabloc (10 mg/ml) and Complete, Protease Inhibitor Cocktail Tablets (1 tablet/50 ml solution, Boehringer Mannheim, Mannheim, Germany).

Enzyme assay for PAP/TRAP

PAP/TRAP enzyme activity was assayed in 96-well plates using p-nitrophenylphosphate (pNPP) as substrate as previously reported [23]. One unit of PAP/TRAP activity corresponds to 1 µmol of p-nitrophenol liberated per minute at 37 °C. In partially purified tissue samples, PAP/-TRAP activity is defined as the activity sensitive to 100 µM MoO_4. In the IC_{50} experiments, molybdate concentrations between 1–200 µM and tungstate concentrations between 100–1000 µM were used. In the experiments with recombinant rat PAP/TRAP, V_{max} and K_m were calculated from Lineweaver-Burk plots using 0.1–20 mM pNPP.

Cleavage of recombinant rat PAP/TRAP with cathepsin K or L

Recombinant rat PAP/TRAP (5.5 µg) was cleaved with human liver cathepsin L (Calbiochem, La Jolla, Calif.) or recombinant human cathepsin K (kindly provided by Dr. R. Dodds, SmithKlineBeecham, King of Prussia, Pa.) in 5 mM NaAc pH 5.5, 1 mM EDTA and 10 mM DTT using a 1:1 molar ratio. The reaction was incubated at 37 °C for 40 min and then terminated by addition of E-64.

Electrophoresis and immunoblot analysis

Partially purified PAP/TRAP (30 mU) from spleen, liver and thymus or 50 ng each of recombinant rat PAP/TRAP and rat bone PAP/TRAP were subjected to SDS-PAGE on 12% ReadyGels (Bio-Rad, Hercules, Calif.) according to the manufacturer's protocol and transferred, stained and developed as previously reported [23] with the exception that membranes were blocked in 1% TBST for 1 h at RT. Primary antibodies were rabbit anti-recombinant rat TRAP 1:1500 or rabbit anti-monomeric TRAP 1:1500 diluted in TBST. As secondary antibody, goat anti-rabbit HRP 1:10,000 (Calbiochem) was used. The blots were developed using ECL.

Total RNA purification from rat tissues and reverse transcriptase reaction

Total RNA was extracted using ToTALLY RNA, Total RNA Isolation Kit (Ambion, Austin, Tex.) or Purescript

Table 1. Primers and probes used for RT-PCR.

Accession number/ name	Species	Oligo name/ kit name	Sequence (5′– 3′)	Position	Concentration (nM)	Annealing temperature (°C)	Construction tool
M76110	rat/ mouse	TRAP sense	TGCCTACCTGTGTGGACATGA	799	50	60	Primer Express
	rat/ mouse	TRAP antisense	CACATAGCCCACACCGTTCTC	846	50	60	Primer Express
	rat/ mouse	TRAP MGB probe TaqMan Rodent GAPDH	FAM-ACCTGCAGTATCTTC-MGB	826	100	60	Primer Express
	rat/ mouse	Control Reagents*	–	–	–	60	–

* Applied Biosystems, Foster City, Calif.

(Gentra, Minneapolis, Minn.) according to the manufacturer's protocol. RNA was treated with 5 U DNase I (Life Technologies, Rockville, Md.) for 30 min at in RT and quantified using Ribogreen RNA Quantitation Kit (Molecular Probes, Eugene, Ore.). Reverse transcription was performed on total RNA using Superscript II RNase H^- Reverse Transcriptase (Life Technologies, Rockville, Md.) or Omniscript (Qiagen, Hilden, Germany) according to the manufacturer's protocol using oligo $d(T)_{12–18}$ (Life Technologies) as primer. Negative controls included reactions where the reverse transcriptase was excluded from the reaction mixture.

Relative quantification of PAP/TRAP mRNA using real-time qPCR

Real-time qPCR was carried out for rat PAP/TRAP (primers present in exon 4 and 5, see table 1) and GAPDH using the TaqMan chemistry (for primers and probes see table 1). cDNA was amplified for PAP/TRAP and GAPDH as triplets in separate tubes containing (apart from primers and probe) 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif.) or 1* iQ Supermix (Bio-Rad) to a final volume of 25 µl. Relative quantification of PAP/TRAP was calculated using the 'two standard curve method' as described in User Bulletin 2 from Applied Biosystems.

Results

Biochemical characterization of PAP/TRAP in rat tissues

To validate the specificity of the TRAP enzyme assay in the different semi-purified organ extracts, certain biochemical properties characteristic for PAP such as pH optimum, K_m and the inhibition constants (IC_{50}) of the oxyanions molybdate and tungstate were determined. The assays were performed in the presence of 0.1 mM ferric ion and 1 mM ascorbate to maintain the iron center in the active mixed-valent state. The optimal pH for mixed-valent PAP/TRAP activity in the different tissues ranged between 5.5–5.8 (table 2), which is similar to what has previously been reported for the purified two-subunit, mixed-valent rat recombinant and bone PAP/TRAP [33]. The K_m for spleen, thymus and kidney was in a similar range (0.4–0.5 mM) as for bone, which is known to express mainly the two-subunit form [4]. Liver, colon and lung exhibited slightly higher K_m values, between 1–2 mM. The IC_{50} values of the semi-purified PAP/-TRAPs for molybdate and tungstate were in a similar range as for the recombinant enzyme [4]. Thus, the biochemical properties were consistent with expression of the structurally defined PAP enzyme [4]. To improve the specificity of the PAP/TRAP enzyme assay by minimizing contribution of unspecific phosphatases present in some of the semi-purified preparations, the samples were assayed in duplicate with and without 100 µM molybdate, and the TRAP activity sensitive to 100 µM molybdate was used as a measure of PAP/TRAP activity. Upon exposure to oxidizing agents, such as hydrogen peroxide, the mixed-valent Fe(III)-Fe(II) metal center in PAP is rapidly converted to the inactive diferric center [3, 8]. If the M(II) site were substituted with a divalent metal other than the redox-active Fe, e.g. Zn or Mn, enzyme activity would not be affected by either reduction or oxidation [7]. This characteristic property of the redox-sensitive Fe-Fe metal center was employed to assess whether PAP/TRAP in the different tissues contained a redox-sensitive or a redox-insensitive metal center. Comparison of themolybdate-sensitive PAP/TRAP activity in samples subjected to oxidation by H_2O_2 to the activity in reduced samples indicated that 97–99% of the activity in all tissues tested could be accounted for by PAP/TRAP with a redox-sensitive metal center.

Table 2. Enzymatic characterization of partially purified TRAP from different tissues of young rats.

Tissue	pH optimum	K_m	IC_{50} molybdate (µM)	IC_{50} tungstate (µM)	Activity after oxidation* (% of reduced activity‡)
Bone	5.8	0.23± 0.04‡	39 ± 8‡	239 ± 63‡	0.3
Spleen	5.5-5.8	0.45 ± 0.07	15 ± 7	276 ± 55	0.3
Liver	5.8	1.80 ± 0.17	13 ± 4	383 ± 43	0.5
Thymus	5.8	0.51 ± 0.35	19 ± 10	443 ± 27	1.0
Colon	5.8	1.32 ± 0.25	28 ± 10	398 ± 87	0.5
Lung	5.8	1.12 ± 0.09	25 ± 14	372 ± 121	1.0
Kidney	5.5-5.8	0.42 ± 0.42	19 ± 9	ND	3.3

The PAP/TRAP enzyme was partially purified from the indicated tissues of 3-week-old rats, and enzyme activity was determined in the presence of reductants using pNPP as the substrate as described in Materials and methods.

‡ Reference 23. ND, Not determined; n=3.

* Oxidation was achieved by adding 5 mM H_2O_2 to a reduced sample.

‡ reduction was achieved by adding 1 mM ascorbic acid and 0.1 mM $FeCl_3$.

Table 3. Expression of TRAP mRNA and TRAP activity in different tissues of young rats.

Tissue	PAP/TRAP mRNA normalized to GAPDH (± SD)	n	PAP/TRAP mRNA relative to spleen (%)	PAP/TRAP units/g tissue (± SD) (unreduced conditions)	PAP/TRAP units/g tissue (± SD) (reduced conditions)	n	PAP/TRAP enzyme activity relative to spleen (%)(unreduced conditions)	PAP/TRAP enzyme activity relative to spleen (%) (reduced conditions)
Bone	1.60 ± 0.04	4	117	0.38 ± 0.300	10.5 ± 2.67	4	950	452
Spleen	1.36 ± 0.02	4	100	0.04 ± 0.020	2.32 ± 1.86	3	100	100
Liver	0.14 ± 0.01	4	10	0.02 ± 0.030	0.34 ± 0.16	3	50	15
Thymus	0.16 ± 0.01	6	12	0.01 ± 0.080	0.15 ± 0.08	5	25	7
Colon	0.15 ± 0.003	4	11	0.006 ± 0.004	0.13 ± 0.07	5	15	6
Lung	0.43 ± 0.02	3	32	0.004 ± 0.006	0.12 ± 0.04	4	10	5
Kidney	0.13 ± 0.003	2	9	0.006 ± 0.003	0.10± 0.03	3	15	4

PAP/TRAP mRNA was determined using qPCR and TRAP enzyme activity using pNPP as the substrate in extracts from indicated tissues of 3-week-old rats.

Organ distribution of PAP/TRAP mRNA and enzyme activity

The distribution of PAP/TRAP mRNA and enzyme activity in different organs from 3-week-old rats is shown in table 3. Using qPCR to quantitate the mRNA levels of PAP/TRAP normalized to GAPDH mRNA, the highest expression levels were detected in bone and spleen, an intermediate level in lung, and lower levels (≥ 10% of the levels relative to bone and spleen) in liver, thymus, colon and kidney. In the absence of reductants, low enzyme activity was detected in all tissues (table 3). Addition of ferric iron and ascorbate increased the activity 15–60-fold in the different tissues, suggesting that the active-site metal center of the enzyme in the semi-purified preparations is in the oxidized, diferric state. Especially in spleen, PAP/TRAP activity was strongly dependent on the addition of reducing agents (table 3). The relative expression levels of PAP/TRAP mRNA correlated well with that of the relative activities of PAP/TRAP assayed under reducing conditions in spleen, liver, thymus, colon and kidney. In contrast, bone exhibited a relatively higher enzyme activity compared to mRNA whereas lung exhibited a relatively higher mRNA expression compared to enzyme activity.

Interestingly, the PAP/TRAP expression in some organs varied with age. In bone and liver, both mRNA and enzyme activity were decreased in adult compared to newborn rats (table 4). In contrast, in the lymphoid organ, the expression was either constant (spleen) or increased (thymus). In lung, the enzyme activity, but not mRNA expression, was decreased in adult compared to newborn animals.

Distribution of proteolytically processed two-subunit PAP/TRAP

In rat bone and spleen, the majority of the PAP/TRAP protein is isolated as the proteolytically processed two-subunit form [4]. To determine the relative distribution of the monomeric and two-subunit form in other PAP/TRAP-expressing organs, partially purified enzyme preparations were subjected to SDS-PAGE under reducing and non-reducing conditions followed by immunoblot analysis. Recombinant rat monomeric PAP/TRAP purified from baculovirus-infected Sf9 cells served as a control. Under non-reducing conditions, a single 37-kDa PAP/TRAP was detected in spleen, liver and thymus (fig. 1A). After disulfide reduction, the majority of the PAP/TRAP in spleen, liver and thymus was recovered as the two-subunit

Table 4. Age-dependent expression of PAP/TRAP: PAP/TRAP mRNA and TRAP enzyme activity in tissue extracts of 3-day (newborn) and 10-week (adult) old rats.

Tissue	Newborn		Adult		Adult/ Newborn	
	PAP/TRAP mRNA normalized to GAPDH ± (SD)	PAP/TRAP units/g tissue (± SD) (reduced conditions)	PAP/TRAP mRNA normalized to GAPDH (± SD)	PAP/TRAP units/g tissue ± SD (reduced conditions)	Enzyme activity	mRNA
Bone	2.26 ± 0.479	6.50 ± 0.240	0.32 ± 0.120	0.35 ± 0.020	0.05	0.14
Spleen	1.63 ± 0.108	1.70 ± 0.418	1.43 ± 0.033	1.40 ± 0.200	0.82	0.88
Liver	0.25 ± 0.016	0.88 ± 0.279	0.04 ± 0.041	0.15 ± 0.035	0.17	0.16
Thymus	0.03 ± 0.001	0.11 ± 0.027	0.69 ± 0.002	0.21 ± 0.066	1.91	23.0
Lung	0.42 ± 0.072	0.24 ± 0.042	0.58 ± 0.022	0.02 ± 0.003	0.08	1.38

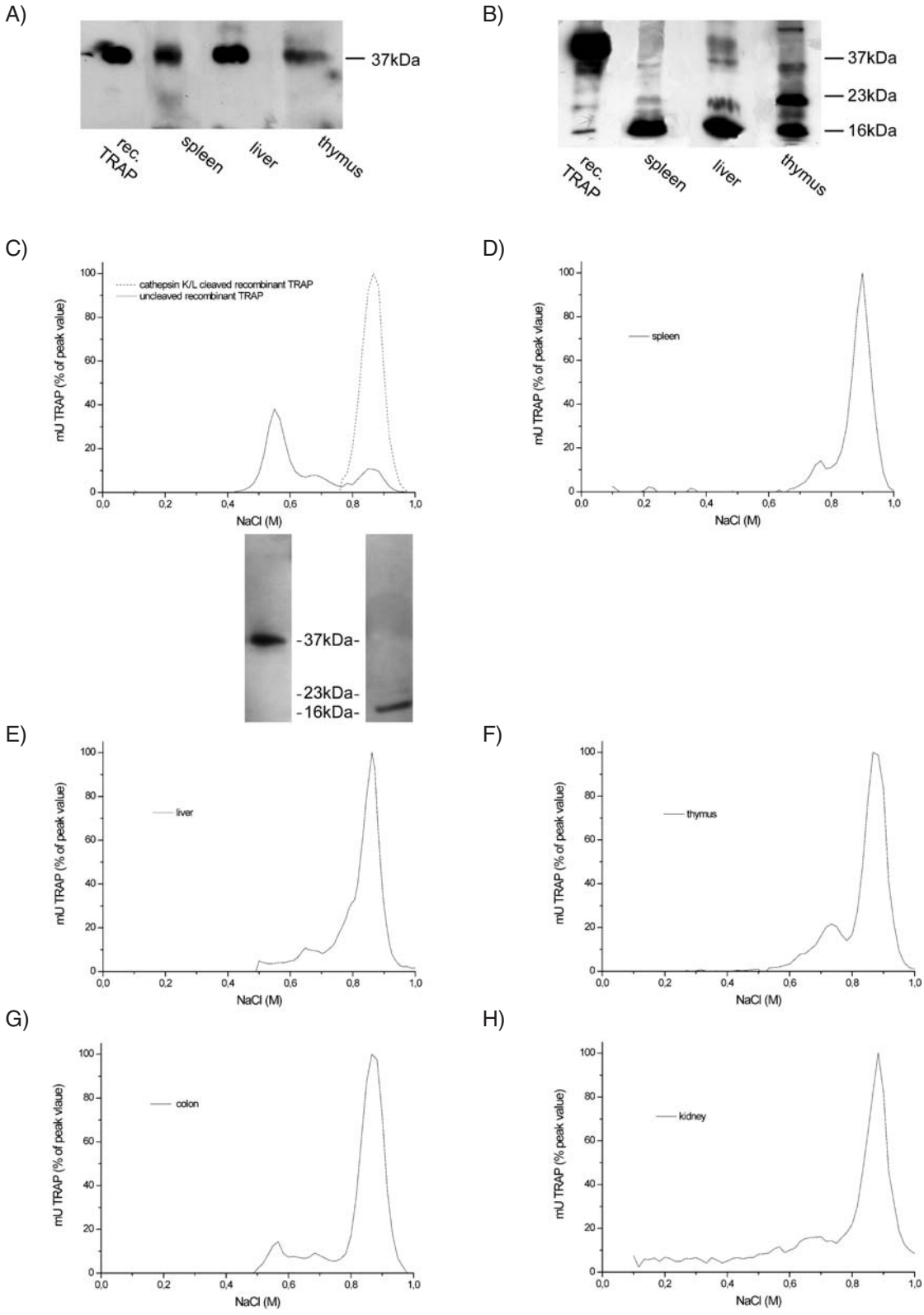

Figure 1. Proteolytic analysis of PAP/TRAP from different tissues. Partially purified PAP/TRAP from indicated tissues was subjected to SDS-PAGE under non-reducing (*A*) or reducing (*B*) conditions and probed with anti-total TRAP polyclonal antibody. The protein band with an Mr of 37 kDa corresponds to monomeric PAP/TRAP and the two bands at 23 and 16 kDa correspond to the two-subunit proteolytically processed PAP/TRAP. (*C*) Heparin-sepharose chromatography analysis of recombinant PAP/TRAP. The PAP/TRAP eluting at 0.55 M NaCl was shown by Western blot analysis to represent the monomeric 37-kDa form. Cathepsin K- or L-cleaved PAP/TRAP eluting at 0.85 M NaCl represents the two-subunit form. (D–H) Analysis of partially purified PAP/TRAP from indicated tissues by heparin chromatography.

form, consisting of the 23- and 16-kDa fragments (fig. 1B). Only minor amounts of the monomeric PAP/TRAP were detected in liver and thymus.

Heparin ion exchange chromatography to separate the monomeric and two-subunit forms was employed to assess proteolytic processing of partially purified PAP/TRAP (fig. 1C–H). As a control, monomeric rat recombinant PAP/TRAP digested with cathepsin L or K was bound to the heparin column and eluted with a linear 0.1–1.0 M NaCl gradient (fig. 1C). While the untreated, monomeric PAP/TRAP activity and protein co-eluted at 0.55 M NaCl, the digested sample eluted later at around 0.85 M NaCl. Western blot confirmed that the activity peak of the cathepsin-digested samples contained the two-subunit form (fig. 1C). The eluates from spleen, liver, thymus, colon and kidney showed a major peak of TRAP activity eluting at 0.8–0.9 M NaCl corresponding to the two-subunit form, with a smaller shoulder at 0.7 M (fig. 1D–H). In the sample from colon, a minor peak at 0.55 M NaCl corresponding to the monomeric form was present (fig. 1H).

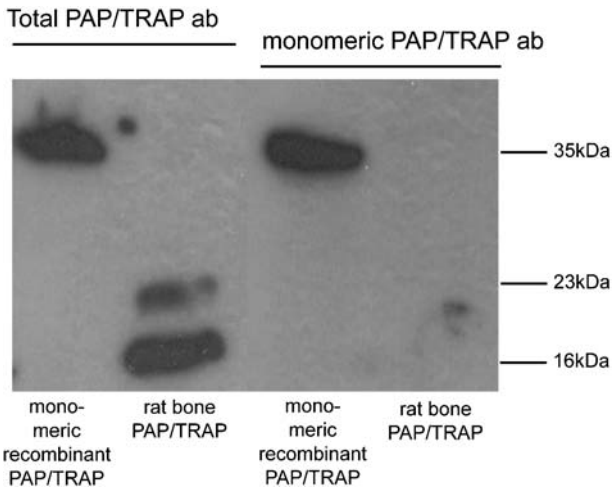

Figure 2. Antibody specificity against total and monomeric PAP/TRAP. The antibodies against the rat recombinant PAP/TRAP (total PAP/TRAP ab) and the cleavable loop sequence (monomeric PAP/TRAP ab) were probed against the monomeric recombinant PAP/TRAP and the two-subunit PAP/TRAP purified from rat bone.

In situ distribution of monomeric and two-subunit PAP/TRAP protein

A polyclonal antibody directed against the cleavable loop sequence of the monomeric form of PAP/TRAP was generated in rabbits following immunization with a peptide covering the amino acids 146–162 in the mouse TRAP sequence. The rabbit anti-mouse monomeric PAP/TRAP recognized the 35-kDa band of monomeric recombinant PAP/TRAP, but failed to detect the 23 and 16-kDa fragments of the two-subunit rat bone PAP/TRAP (fig. 2). The antibody raised against the full-length rat recombinant PAP/TRAP protein recognized as expected both the monomeric and two-subunit forms (total PAP/TRAP). To elucidate the in situ distribution of the monomeric and two-subunit forms, immunohistochemistry was carried out on paraffin-embedded rat tissue sections. The presence of predominantly the two-subunit form would be indicated by more intense labeling with the total PAP/TRAP antibody compared with the anti-monomeric PAP/TRAP antibody, whereas expression of predominantly the monomeric form would be suggested by strong staining with the anti-monomeric PAP/TRAP antibody.

In the spleen, strongly PAP/TRAP positive cells were found scattered in the red pulp (RP) (fig. 3A). A few PAP/TRAP-positive cells were also detected in the marginal zone (MZ) and in the germinal center (GC) of the white pulp. Labeling for monomeric PAP/TRAP showed a similar distribution, albeit with a lower intensity (fig. 3B).

In the thymus, PAP/TRAP-positive cells could be found both in the medulla and the cortex (fig. 3C). The morphology of the positive cells resembles that of epithelioreticular or dendritic cells with long protrusions. In thymus, also, the staining intensity was lower with the antibody recognizing the monomeric PAP/TRAP form (fig. 3D).

In the liver, staining was most intense in sinusoidal cells resembling Kupffer cells in proximity to the central vein (fig. 3E). No cells positive for monomeric PAP/TRAP were detected (fig. 3F).

In the lung, alveolar macrophages were positive for both total and monomeric PAP/TRAP antibodies (fig. 3G, H). The staining intensity was markedly stronger for monomeric PAP/TRAP. Monomeric PAP/TRAP was also detected in bronchiolar epithelial cells resembling Clara cells (fig. 3J), but absent from ciliated respiratory epithelium (fig. 3I).

In the kidney, epithelial cells of the distal tubules were positive for both total and monomeric PAP/TRAP (fig. 3K, L). The staining was more intense for monomeric compared to total PAP/TRAP.

In the colon, macrophages in the lamina propria of the submucosa were strongly positive for the total PAP/TRAP (fig. 3M). A clearly detectable signal was observed for monomeric PAP/TRAP in certain surface epithelial cells (fig. 3N).

Co localization of PAP/TRAP and cathepsin L

In spleen, cathepsin L staining was noted in vesicle-like structures in cells of the RP (fig. 4A). In these cells, cathepsin L was co-localized (yellow) with total PAP/TRAP (fig. 4B). The larger PAP/TRAP-positive cell population (green) was negative for cathepsin L.

In liver, cathepsin L staining (red) was seen in vesicular structures in a majority of the hepatocytes (fig. 4C, D).

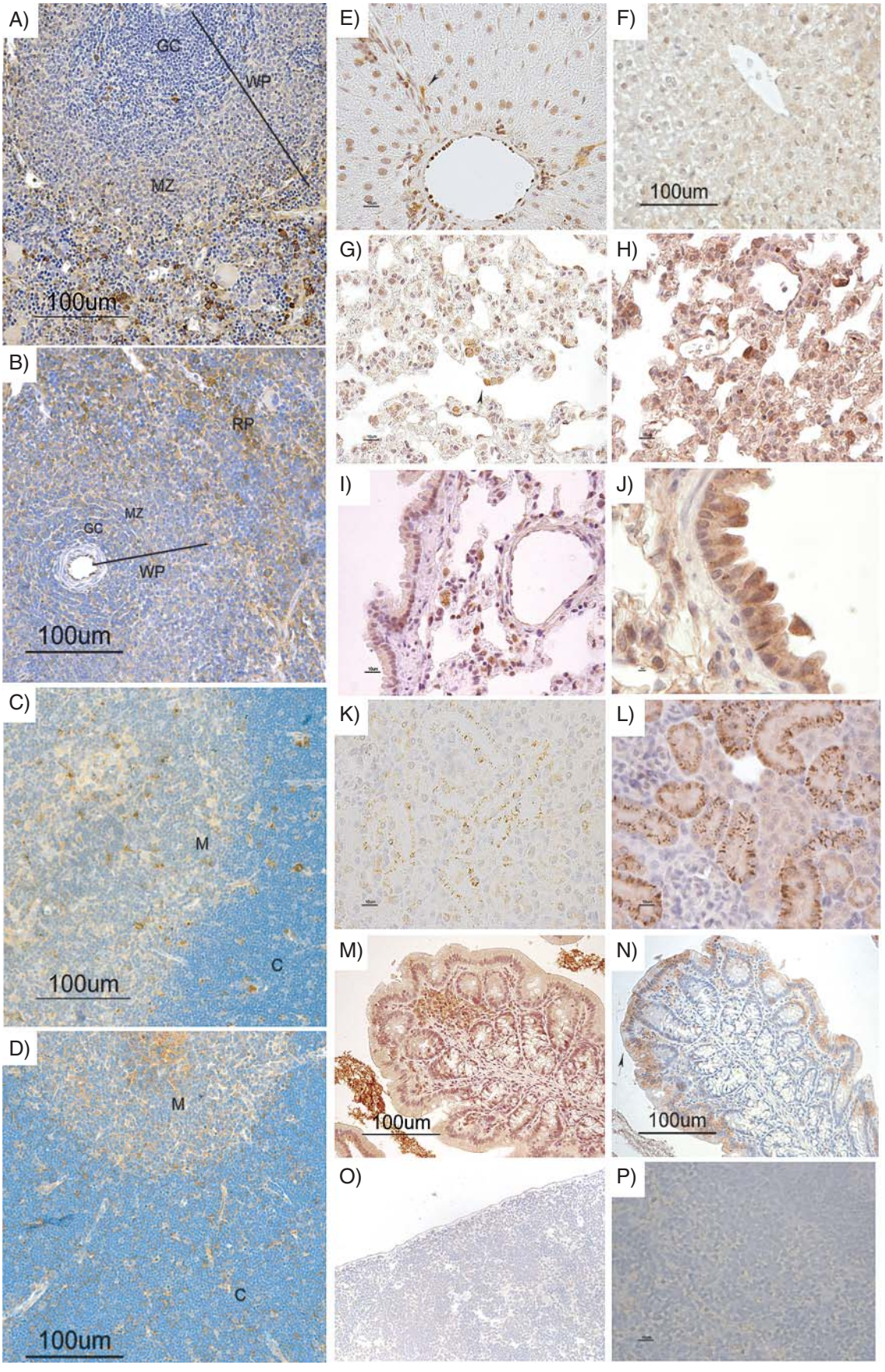
A)
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Table 5. Effect of proteolytic cleavage and reduction of the active site on PAP/TRAP activity.

PAP/TRAP form	pH	K_m (mM)	Specific activity (U/mg protein)
Without reducing agents			
Uncleaved PAP/TRAP	6	5.25 ± 2.47	26 ± 15
Cathepsin K-cleaved PAP/TRAP	6	2.00 ± 0.07	17±10
Cathepsin L-cleaved PAP/TRAP	6	1.75 ± 0.63	43 ± 50
With reducing agents			
Uncleaved PAP/TRAP	6	3.50 ± 0.89	202±10
Cathepsin K-cleaved PAP/TRAP	6	0.54 ± 0.20	1552 ± 215
Cathepsin L-cleaved PAP/TRAP	6	0.52 ± 0.20	1127 ± 179

* reduction was achieved by adding 0.1 mM Fe3+ and 1 mM ascorbic acid.

PAP/TRAP was detected in a subset of sinusoidal cells (green) and no co-localization between cathepsin L and PAP/TRAP could be observed.

In thymus, staining for cathepsin L was seen in dendritic-like cells both in the medulla and cortex (fig. 4E). The majority of PAP/TRAP-positive cells were positive also for cathepsin L (fig. 4F).

In colon, cathepsin L staining was found in goblet cells but also co-localized with PAP/TRAP in cells in the subepithelial part of the lamina propria (fig. 4G, H).

In the kidney, cathepsin L and PAP/TRAP were co-localized in the apical region of tubular epithelial cells (fig. 4E, K).

pH-dependence and activation of recombinant rat TRAP by proteolytic cleavage and reduction of the di-iron active site

Since the proteolytically processed (two-subunit) form of PAP/TRAP is widely distributed among rat tissues, we then assessed whether proteolytic processing as such is sufficient to generate an active enzyme. As the activity of the PAP/TRAP enzyme recovered in the different tissue extracts was strongly enhanced by the addition of reductants, we also considered the possibility that proteolytic processing sensitized the enzyme to subsequent reduction of the metal center. By comparing the enzyme activity of monomeric and cathepsin K- or L-cleaved recombinant PAP/TRAP at different pHs in the absence or presence of reductants (fig. 5A), we noted that the enzyme activity in the absence of reductants in the proteolytically digested samples showed a small shift in optimal pH from 4.7 to 5.2 associated with a slight decrease in enzymatic activity compared to the monomeric form. In contrast, in the presence of reducing agents, a clear shift to an optimal pH 6 and a significant increase in enzymatic activity was apparent (fig. 5B). As shown in table 5, at pH 6.0 without reducing agents, a similar V_{max} and a lower K_m was noted in the cathepsin K- or L-digested compared to the monomeric samples. Reduction of monomeric PAP/TRAP was associated with an increased V_{max}. This increase in V_{max} by reduction as well as an additional lowering of the K_m., was significantly augmented after cathepsin K or L cleavage. These data suggest that proteolytic cleavage in the repressive loop domain leads to minimal increases in optimal pH for catalysis and in affinity for the pNPP substrate, but has no effect on catalytic activity. Moreover, the effect of proteolysis on pH, K_m and in particular V_{max} was not fully executed unless the two-subunit enzyme was exposed to reducing agents.

Figure 3. In vivo distribution of total and monomeric PAP/TRAP in rat tissues. (*A*) Total PAP/TRAP distribution in spleen. PAP/TRAP-expressing cells are scattered in the red pulp (RP). A few labeled cells are also seen in the outer part of the marginal zone (MZ) as well as in the germinal center (GC) of the white pulp (WP). (*B*) Monomeric PAP/TRAP distribution in spleen. Weak labeling with a similar distribution as in *A*. (*C*) Total PAP/TRAP distribution in thymus. Total PAP/TRAP-positive cells resembling epithelioreticular or dendritic cells with long protrusions are present both in the cortex (C) and the medulla (M). (*D*) Monomeric PAP/TRAP distribution in thymus. Weak labeling with a similar distribution as in *C*. (*E*) Total PAP/TRAP distribution in liver. Total PAP/TRAP is found in sinusoidal cells resembling Kupffer cells often located close to the central vein (arrowhead). (*F*) Monomeric PAP/TRAP distribution in liver. Monomeric PAP/TRAP is not detected. (*G*) Total PAP/TRAP distribution in lung. Total PAP/TRAP is expressed in alveolar macrophages (arrowhead). (*H*) Monomeric PAP/TRAP distribution in lung. Monomeric PAP/TRAP is expressed in alveolar macrophages. (*I*) Total PAP/TRAP distribution in lung. No total PAP/TRAP is observed in respiratory epithelial cells. (*J*) Monomeric PAP/TRAP distribution in lung. Monomeric PAP/TRAP is found in bronchiolar epithelial cells resembling Clara cells. (*K*) Total PAP/TRAP distribution in kidney. Weak total PAP/TRAP labeling is found in distal tubular cells of the kidney cortex. (*L*) Monomeric PAP/TRAP distribution in kidney. Strong labeling for monomeric PAP/TRAP is noted in distal tubular cells of the kidney cortex. (*M*) Total PAP/TRAP distribution in colon. Focal PAP/TRAP staining is seen in the lamina propria. (*N*) Monomeric PAP/TRAP distribution in colon. Monomeric PAP/TRAP is found in the lamina propria and in some epithelial cells (arrowhead). (*O*) Control. Absence of staining of spleen with primary antibodies (total PAP/TRAP) preadsorbed with recombinant rat PAP/TRAP. (*P*) Control. Absence of staining of spleen with monomeric PAP/TRAP antibodies preadsorbed with recombinant rat PAP/TRAP.

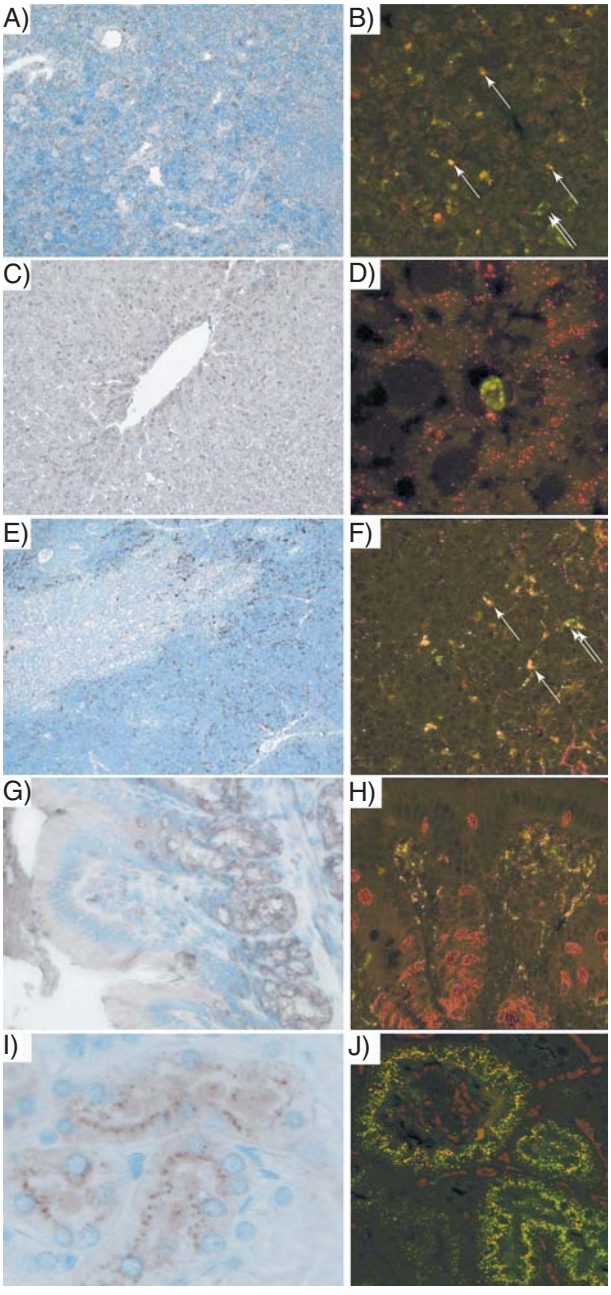

Figure 4. Distribution of cathepsin L (A, C, E, G, I) and co-localization with PAP/TRAP (B, D, F, H, J) in rat tissues. (*A*) Spleen (magnification ×20). Cathepsin L is detected in vesicle-like structures both in the red and the white pulp. (*B*) Spleen (magnification ×63). PAP/TRAP and cathepsin L are co-localized. (*C*) Liver (magnification ×20). Cathepsin L staining is seen in vesicle-like structures in the hepatocytes. (*D*) Liver (magnification ×63). PAP/TRAP and cathepsin L are not co-localized. (*E*) Thymus (magnification ×20). Cathepsin L staining is noted in vesicle-like structures both in the cortex and the medulla. (*F*) Thymus (magnification ×63). Cathepsin L and PAP/TRAP are co-localized. (*G*) Colon (magnification ×20). Cathepsin L staining is detected in both some epithelial cells and in the lamina propria. (*H*) Colon (magnification ×63). Co-localization between PAP/TRAP and cathepsin L is observed only in the lamina propria. (*I*) Kidney (magnification ×20). Cathepsin L staining in certain tubuli cells. (*J*) Kidney (magnification ×63). Cathepsin L and PAP/TRAP are co-localized in tubuli cells.

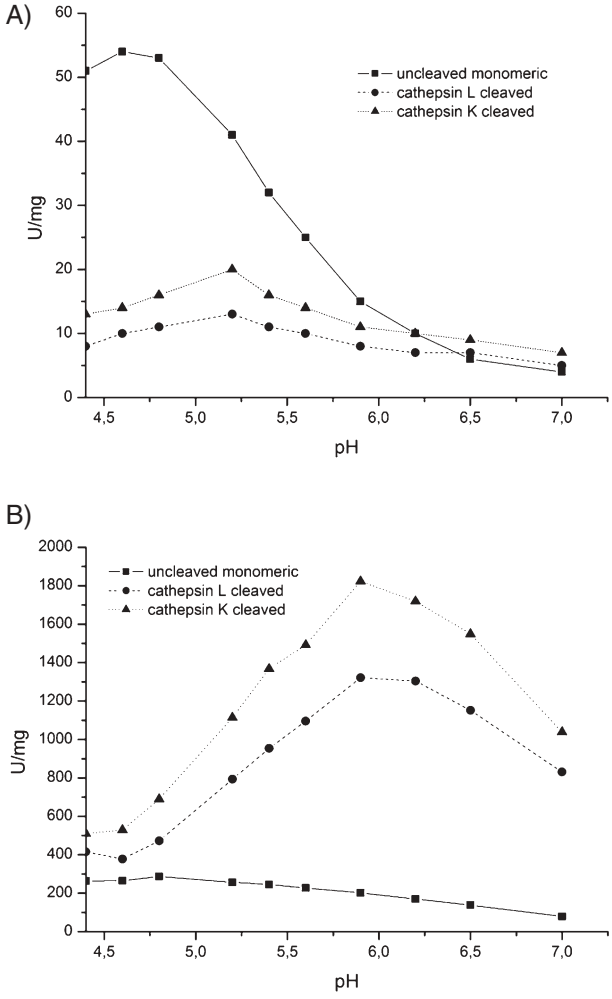

Figure 5. Effect of proteolytic cleavage and reduction on PAP/TRAP enzyme activity. The enzyme activity of recombinant rat PAP/TRAP was assayed using p-NPP as the substrate at 37 °C. (*A*) pH dependence of PAP/TRAP activity in the absence of reducing agents before and after cleavage with cathepsin K or L. (*B*) pH dependence of PAP/TRAP activity in the presence of reducing agents before and after cleavage with cathepsin K or L.

Discussion

The PAP/TRAP enzyme is widely distributed in human and rodent tissues [22, 25, 27, 59, 60]. In the present study, the levels of PAP/TRAP mRNA expression as well as the corresponding PAP/TRAP enzyme activity were quantitatively assessed in tissue extracts from organs of differently aged rats. Consistent with previous studies [25, 27, 51, 59], significant expression of both mRNA and enzyme activity was detected in bone, spleen, thymus, liver, colon, lung and kidney. The biochemical properties of the PAP/TRAP in those organs exhibited the hallmarks of the structurally defined di-iron PAP enzyme [61]. Thus, using molybdate-sensitive TRAP activity as the measure of PAP activity, we could not find evidence for significant expression of redox-insensitive forms of PAP

in rat organs as was observed in preparations of bovine spleen PAP [7]. Instead, addition of reducing agents increased enzyme activity considerably in all organs, suggesting that the enzyme as isolated predominantly exists in the oxidized, diferric state. The relative distribution at the mRNA level was comparable to that of the active enzyme, except in bone and lung. In bone, the relative expression of enzyme activity was considerably higher than the mRNA level, whereas the opposite pattern was noted in the lung. In bone, osteoclasts show a considerable intracellular expression of the enzyme and the enzyme is furthermore secreted at the apical part toward the resorption lacuna [62] as well as at the basal domain facing the blood circulation [46, 63]. Conceivably the rate of enzyme secretion as well as transcriptional regulation determine the level of PAP/TRAP activity in osteoclasts. In lung, significant expression of the less active monomeric form was noted both in alveolar macrophages and in certain bronchiolar epithelial cells, presumably explaining the relatively higher expression levels of mRNA compared to enzyme activity. In bone and liver, the expression of PAP/TRAP declined with advancing age, whereas in the lymphoid organs, e.g. spleen and thymus, the level of expression was constant or even increased. In bone, the higher expression in young compared to adult rats correlates to the diminished number of osteoclasts as a result of reduced longitudinal growth/bone modeling in adulthood [30]. In the liver, the mechanism underlying the reduced expression of the enzyme with age is unclear. The constant or increased expression of the enzyme in the lymphoid tissues may be related to the proposed role of this enzyme in innate immune reactions [31, 38].

In this study, we addressed whether proteolytic processing is a general feature of the biological turnover of PAP/TRAP in rat tissues. Except for bone [58] and spleen [43, 64], whether the enzyme in other tissues is proteolytically processed to the active two-subunit form or alternatively present as the unprocessed monomeric form was not known. As shown by Western blot analysis, the proteolytically processed, two-subunit form predominated in spleen, liver and thymus. Heparin affinity chromatography to separate monomeric and two-subunit forms corroborated the Western analysis and, furthermore, provided evidence that colon and kidney express significant levels of proteolytically processed PAP/TRAP. Since both the native and cathepsin L-generated two-subunit forms exhibit more than tenfold higher specific enzyme activity compared to the monomeric form toward a range of synthetic and natural substrates [33, 45], biochemical assays based on enzyme activity favor detection of the two-subunit form and are less useful for identifying and quantifying the monomeric form in tissues. Thus, a novel antibody generated against the cleavable loop sequence in PAP/TRAP was employed as a probe to specifically assess the expression of the uncleaved monomeric form by immunohistochemistry. The absence or low abundance of immunohistochemical labeling for the anti-monomeric PAP/TRAP in spleen, liver, thymus and the lamina propria of colon, coupled with a significant labeling using the total PAP/TRAP antibody, support the conclusion that the majority of PAP/TRAP in these organs exists as the proteolytically processed two-subunit form. The residual labeling for the monomeric PAP/TRAP in these sites is probably derived from a biosynthetic precursor pool. The two-subunit form was also expressed in kidney as shown by heparin chromatography. In the studies of Angel et al. [27], TRAP enzyme histochemical staining was noted in the glomeruli of the kidney cortex of mice. However, no glomerular staining was observed by immunohistochemistry in the present study. Since even small amounts of the two-subunit protein yield significant enzyme activity, the possible minor expression of the two-subunit form in glomerular cells may have escaped detection in the present study.

The cells expressing the proteolytically processed, two-subunit form belong to the monocytic lineage of macrophages and/or dendritic cells as they also express the rat macrophage marker ED-1 and the MHC class II antigen OX-6 [P. Lång and G. Andersson, unpublished data]. In the liver**,** the PAP/TRAP-positive hepatic sinusoidal cells were unevenly distributed in the liver lobule with enrichment around the central vein, corroborating previous findings [25]. In the spleen, a population of large cells in the RP presumably identical to iron-laden phagocytic macrophages [65, 66] expressed the PAP/-TRAP protein, as well as a few smaller cells in the marginal zone and in the GC, possibly dendritic cells [25]. The presence of an active PAP/TRAP in phagocytic macrophages of spleen and liver is particularly interesting since mice doubly deficient for both lysosomal and purple acid phosphatases exhibit a severe progressive hepatospenomegaly associated with increased numbers of hypertrophic macrophages with prominent signs of lysosomal storage disease [28]. Thus, phagocytic macrophages in liver and spleen appear to utilize PAP/TRAP for lysosomal degradation. In the thymus, the morphology of the PAP/TRAP-positive cells resembles that of epithelioreticular or dendritic cells, consistent with a role in antigen presentation. In the colon, the presence of PAP/TRAP expressing monocytic cells in a subepithelial location of the lamina propria suggests a role in the mucosal defense system.

In a study of PAP/TRAP expression in the mouse embryo, Hayman et al. [24] noted that the epithelial lining of the alimentary canal expressed low TRAP activity despite the presence of abundant expression at the protein and mRNA levels [22]. Consistent with their data, we now provide direct evidence for expression of the enzymatically latent monomeric PAP/TRAP in intestinal epithelia. Moreover, respiratory epithelial cells resembling Clara

cells in the bronchiolar epithelium expressed significant amounts of the uncleaved monomeric PAP/TRAP. In kidney, distal tubular epithelium was strongly reactive to the loop antibody, suggesting expression of the monomeric form. Proteolytic processing thus appears as a distinctive feature of PAP/TRAP expressed by monocyte-macrophage lineage cells, except for alveolar macrophages of the lung. Biochemical studies have demonstated an efficient cleavage of PAP/TRAP to the active two-subunit form by cathepsin L [this study and ref. 45]. A putative role for cathepsin L in the proteolytic processing of TRAP in monocytes/macrophages in spleen, thymus, kidney and colon was corroborated in the present study by the demonstration of co-localization between cathepsin L and TRAP. Expression of cathepsin K was not detectable in spleen, liver, thymus, colon and kidney by immunohistochemistry (data not shown), consistent with the study of Chiu et al. [51]. In liver, neither cathepsin L nor K could be implicated in PAP/TRAP processing. However, Kupffer cells are known to synthesize cathepsin B [54, 67, 68] which can process TRAP in vitro [33]. Whether the absence of processing in epithelial cells is due to the lack of expression or intracellular co-localization of processing enzymes with PAP/TRAP remains to be elucidated.

With regard to function, the presence of a proteolytically processed form with increased catalytic activity towards putative natural protein substrates e.g. osteopontin [33, 45] strongly indicate a potential role as a protein phosphatase whereas non-enzymatic functions, e.g. generation of ROS [37, 46], iron delivery [39] or growth regulation [69], could be associated with the monomeric form. An involvement of epithelial PAP/TRAP in generation of ROS is supported by co-localization of TRAP and NOS-1 in the apical region of superficial cells of the urothelium [70]. Interestingly, the ROS-generating capacity of PAP/TRAP is much less enhanced by proteolytic processing compared to its phosphatase activity [46].

Proteolytic processing of PAP/TRAP has been associated with enzyme activation in vitro [33, 45]. As shown here, proteolytic processing per se was not sufficient to activate the enzyme, as the Fe(III)-Fe(III) forms of monomeric and proteolytically processed PAP/TRAP exhibited comparable and low specific activity. Addition of reductants to form the mixed-valent Fe(III)-Fe(II) species enhanced the activity of proteolytically processed PAP/TRAP to a significantly greater extent compared to the monomeric form, suggesting that enzyme activation following proteolytic processing in vivo depends on the redox status of the divalent iron. As a practical consequence, biochemical and histochemical assays on tissues and serum for PAP/TRAP in the presence of reductants such as β-mercaptoethanol, dithiothreitol, ascorbate or ferrous iron predominantly favor detection of the proteolytically processed form over a wide range of pH values. In a physiological context, one can anticipate that the local, i.e. a suitably acidic and reducing, environment at the subcellular sites of the proteolytically processed enzyme will strongly influence its phosphatase activity. Consequently, redox regulation in conjunction with proteolytic processing thus emerge as important regulatory mechanisms to activate the enzyme in vivo.

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