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Structure, Function, and Regulation of Tartrate-Resistant Acid Phosphatase

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Introduction: Biochemistry of Tartrate-Resistant Acid Phosphatase Enzymes

The tartrate-resistant acid phosphatases (TRAPs) are a class of metalloenzymes that catalyze the hydrolysis of various phosphate esters and anhydrides under acidic reaction conditions. Because the bound metal ions confer an intense color on these enzymes they are also known as purple acid phosphatases (PAPs). Resistance to inhibition by high concentrations of the competitive inhibitor L + tartrate distinguishes TRAP from acid phosphatases of lysosomal or prostatic origin present in many mammalian cells and tissues.[58]

TRAP enzymes have been isolated from many mammalian sources, including: bovine[24,89] and rat spleen[49]; the spleens of patients affected with hairy cell leukemia[63] and Gaucher's disease[99]; human[2,53] and rat bone[3]; and human lungs[28] and placenta.[64] The TRAP purified from porcine allantoic fluid, which is also known as uteroferrin, was originally recognized as an abundant basic protein in uterine secretions induced by progesterone.[21] The catalytic mechanism, structure, and properties of the iron center of porcine TRAP have been studied extensively by our group.[10,18,61,62,122]

Mammalian isolated TRAP enzymes all have similar physical properties, including a molecular weight of about 35 kDa, a basic isoelectric point (pI 7.6–9.5), and optimal enzyme activity at an acidic pH. The enzyme can be isolated as a single chain polypeptide, but a dimeric nicked form arises from posttranslational cleavage of the single chain enzyme. Cleavage occurs in an exposed loop that is conserved in all mammalian TRAP enzymes and leads to an increase in V_{max}/k_{cat} of the enzyme by an unknown mechanism.[29,51,59,80,89] Several proteolytic enzymes are able to cleave the exposed loop, but only the cysteine proteinases papain and cathepsin B have been able to cause activation among several tested. Ljusberg et al.[75] put forth the view that TRAP, like several other hydrolases, is synthesized as a relatively inactive proenzyme, and cleavage is the physiological mechanism of proenzyme activation in osteoclasts.

Mammalian TRAP enzymes are glycoproteins and, like most lysosomal enzymes, possess the mannose-6-phosphate lysosomal targeting sequence, which must presumably be cleaved or modified to permit secretion. TRAP isolated from allantoic fluid of the pig showed a single, unphosphorylated, high-mannose-type oligosaccharide composed of five or six mannose residues and two *N*-acetylglucosamine residues.[8,98] In contrast, recombinant porcine TRAP secreted by Chinese hamster ovary (CHO) cells possessed N-linked, high-mannose oligosaccharide chains that were phosphorylated and could not be dephosphorylated by alkaline phosphatase treatment in vitro. This suggests that the uteroferrin oligosaccharide phosphates were not exposed, perhaps as a result of blocking by an *N*-acetylglucosamine residue.[74] The glycoprotein structure of human bone TRAP was analyzed by lectin binding and, in agreement with the prior analysis of native uteroferrin, contained only N-linked high-mannose carbohydrates,[41] implying that the native secreted protein is normally dephosphorylated. Analysis of TRAP activity present in electrophoretically separated human serum revealed two isoforms, termed 5a and 5b, with each isoform having a different pH optimum (5a: pH 4.9; 5b: pH 5.5–6.0).[69] The carbohydrate content of the isoforms also differed with only isoform 5a containing sialic acid.[68]

TRAP contains two iron atoms at its active site, and the intense purple color of the enzyme results from a tyrosinate Fe(III) charge transfer. Reduction of the active site binuclear center to a mixed valency Fe(III)-Fe(II) form is required for activation and this corresponds to a shift in color from purple to pink. Further reduction or the presence of iron chelators can lead to reversible inactivation and formation of a colorless form of the enzyme.[44] The enzyme is also inhibited noncompetitively by incubation with vanadate[22] or simply following more extended incubation at 37°C.[44] The latter case at least, produced a "yellowish" form of the enzyme.[44] TRAP may become irreversibly inactivated by oxidation in the presence of ascorbate.[9] The recent availability of monoclonal antibodies against TRAP[20,41,43,44,51,57] has permitted the identification of an inactivated "yellowish" form of the enzyme as the major form in the circulation.[44]

Enzyme Mechanism and Conservation of TRAP-like Enzymes from Nonmammalian Sources

Purple acid phosphatase from the red kidney (RKBPAP) was the first member of this family for which a crystal structure became available.[116] RKBPAP is a larger enzyme than the mammalian PAPs (111 kDa dimer) and contains Fe(III)-Zn(II) at the active site instead of Fe(III)-Fe(II). Only 3.1 Å separate the two metal ions at the active site of RKBPAP and the amino acid residues that provide the metal ligands are conserved across all known members of the PAP family. The octahedral coordination of the metals was presumed to be completed by a bridging hydroxo group, a hydroxyl group coordinated to Fe(III), and a water

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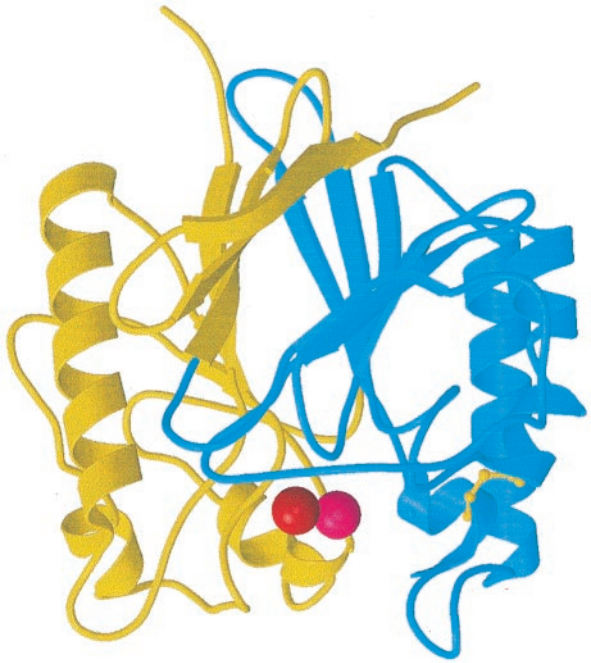

Figure 1. Crystal structure of porcine TRAP.[39] The Fe(III) atom is shown as a purple sphere, and the redox-active Fe atom as a red sphere. The disulfide bond between Cys 141 and Cys 2000 is shown as a yellow ball-and-stick model. To highlight the pseudo-symmetry of the porcine TRAP molecule one half (residues 1–115 and 270–303) is colored yellow and the other half (residues 116–269) is blue.

molecule coordinated to Zn(II). These ligands were not visible on the electron density map. A reaction mechanism of RKBPAP was proposed in which initial binding of the phosphate ester to Zn(II) displaces the bound H_2O followed by displacement of the alcoholic product by an Fe(III)-bound hydroxide ion.[66]

Sequence alignment of RKBPAP with porcine TRAP suggested that RKBPAP is a good model for the structure and mechanism of other acid phosphatases.[67] We recently confirmed this overall prediction with solution of the crystal structure of the porcine enzyme at 1.5 Å resolution[39] (Brookhaven Database, Access Code 1ute). Together with other groups we produced substantial amounts of recombinant mouse, rat, and human TRAP in baculovirus expression systems.[29,51,80] Although preliminary crystallization appeared promising there was a considerable delay between the availability of recombinant enzyme and production of crystals of sufficient size and quality to permit structure determination. In our studies, progress was assisted greatly by the availability of abundant native porcine enzyme from allantoic fluid and was expedited by prior knowledge that it was possible to produce a very stable oxidized enzyme phosphate complex.[61] The crystal structure shown in **Figure 1** reveals a symmetry that was less evident in the plant enzyme structure, and suggests that the binuclear iron center could have evolved from the combination of two mononuclear metal-ion-binding sites. The high-resolution structure revealed strong electron density between the two irons in the active site, indicating the presence of a bridging oxo/hydroxo group. Coincident solution of the structure of recombinant rat TRAP at 2.3 Å resolution by another group of investigators confirmed the overall conclusions from the porcine structure.[119]

The combination of structural and mechanistic studies provides insight into the catalytic mechanism of PAPs. In the initial step, the phosphate group of the substrate is coordinated to the divalent metal ion. Stopped-flow measurements on the pig enzyme have indicated a rapid binding of phosphate to Fe(II),[6] suggesting that this step is not rate-limiting. Formation of the enzyme-substrate complex is followed by a nucleophilic attack on the phosphorus leading to the release of the product alcohol. The nature of the attacking group remains uncertain. Three possible candidates have been proposed: (i) a terminal Fe(III)-bound hydroxide[66]; (ii) a bridging hydroxide[120]; or (iii) a hydroxide residing in the second coordination sphere of Fe(III).[81] The absence of burst kinetics in bovine TRAP indicates that the nucleophilic attack is probably rate-limiting.[82] In the final step of catalysis the metal-bound phosphate group is released. Metal replacement studies have indicated that the k_{cat} value of the hydrolysis is not affected by the nature of the trivalent metal ion.[81] This observation indicates that phosphate release is not rate-limiting and suggests that no ligand substitution reaction takes place at the trivalent site. The latter conclusion argues against the proposition that the nucleophile is located in the first coordination sphere of the binuclear center (see earlier).

With the rapid accumulation of DNA sequence information from a diversity of organisms, sequences of cDNAs from many different sources have become available. Alignment of some known TRAP-like sequences is shown in **Table 1**. TRAP-like enzymes are found in organisms ranging from *Aspergillus* sp., certain *Mycobacteria*, through primitive animals to higher plants and mammals. The presence of related enzymes in intracellular pathogens that infect macrophages could be related to virulence.[96] Otherwise, a detailed search of the complete *Saccharomyces* genome, completed *Drosophila* sequences, and several bacterial genomes reveals no genes with conserved metal ion-binding motifs.[105] This suggests either that the function(s) performed by TRAP are not absolutely required for all eukaryotic cells, or that a nonpurple enzyme that is not detected with the search motif performs the same function. In prokaryotes, TRAP enzymes may not be required because these organisms lack an acidic subcellular compartment in which such enzymes would be active. TRAP enzymes are found in invertebrates such as *C. elegans*, so the role TRAP carries out in animals is not restricted to those creatures with bones. The alignment defines a signature motif that can be used to identify new members of the gene family, as they become evident in sequence databases. Among the genes shown in Table 1 is a newly discovered class of plant enzymes more closely resembling mammalian enzymes in terms of signature motif and size;[105]—the archetype and first discovered being from the Easter lily. Purification and characterization of these mammalian-type plant enzymes is proceeding in our laboratory. The function of purple plant phosphatases is unknown, the only real clue being the fact that they are inducible in some plants by phosphate deprivation.[25,71] It is possible that elucidation of their function may give insight into the role of TRAP in mammals. Novel structural features of recently discovered plant TRAP enzymes include membrane localization via a GPI anchor in an enzyme purified from duckweed.[85]

Substrate Specificity of TRAP

TRAP can catalyze the hydrolysis of a wide range of phosphate monoesters and anhydrides, including the widely used substrates β-umbelliferylphosphate, *p*-nitrophenolphosphate, and pyrophosphate, with K_m values at pH 4.9 in the millimolar range.[80] In our studies,[86] and those of others,[44] pig, mouse, and human enzymes had specific protein tyrosine phosphatase activity. With phosphotyrosylpeptide substrates, the apparent K_m was decreased to the micromolar range at pH 4.9 by flanking charged amino acids and by a large hydrophobic group (Fmoc) attached to the amino terminus of the peptide sub-

Table 1. Conserved sequences in plant, animal, and microbial tartrate-resistant acid phosphatases (TRAPs)[a]

	Source	1		2		3		4		5	Length	Accession
Animals	Human	G**D**WG	34	G**D**NF**Y**	34	G**N**HD	87	VAG**H**	33	G**H**D**H**	325	P13686
	Mouse	G**D**WG	34	G**D**NF**Y**	34	G**N**HD	89	VAG**H**	33	G**H**D**H**	327	Q05117
	Rat	G**D**WG	34	G**D**NF**Y**	34	G**N**HD	89	VAG**H**	33	G**H**D**H**	327	P29288
	Pig	G**D**WG	34	G**D**NF**Y**	34	G**N**HD	89	VAG**H**	33	G**H**D**H**	338	P09889
	Cow[b]	G**D**WG	--	G**D**NF**Y**	--	G**N**HD	--	VAG**H**	33	G**H**D**H**	—	Not available
	Zebrafish[c]	G**D**WG	34	G**D**NF**Y**	33	G**N**HD		----		----	—	AI415792
	C. elegans	G**D**TG	33	G**D**NI**Y**	33	G**N**HD	98	ISG**H**	32	G**H**D**H**	382	Z81494
Plants (A)	Easter lily	G**D**WG	29	G**D**NF**Y**	33	G**N**HD	89	VGH**H**	33	G**H**D**H**	329	Not available
	A. thaliana	G**D**WG	29	G**D**NF**Y**	33	G**N**HD	89	VVG**H**	33	G**H**D**H**	315	AF200827
	Sweet potato	G**D**WG	29	G**D**NF**Y**	33	G**N**HD	89	VIG**H**	33	G**H**D**H**	313	AF236107
	Soybean	G**D**WG	29	G**D**NF**Y**	33	G**N**HD	89	VIG**H**	33	G**H**D**H**	333	AF236108
	Red kidney bean	G**D**WG	29	G**D**NF**Y**	33	G**N**HD	89	VVG**H**	33	G**H**D**H**	332	AF236109
Plant (B)	*A. thaliana*	G**D**LG	25	G**D**LS**Y**	32	G**N**HE	79	VLV**H**	35	G**H**V**H**	469	Q38924
	Sweet potato	G**D**IG	25	G**D**LS**Y**	32	G**N**HE	79	VLV**H**	35	G**H**V**H**	473	AF200825
	Soybean	G**D**LG	25	G**D**LS**Y**	32	G**N**HE	79	VLM**H**	35	G**H**V**H**	464	AF200824
	Red kidney bean	G**D**LG	25	G**D**LS**Y**	32	G**N**HE	79	VLM**H**	35	G**H**V**H**	459	P80366
Microorganisms	*Aspergillus* sp.	N**D**MG	25	G**D**LS**Y**	84	G**B**HE	151	VMS**H**	33	G**H**I**H**	614	U18554[d]
	Mycobacteria	G**D**QS	40	G**D**LC**Y**	31	G**N**HE	89	VCM**H**	35	G**H**E**H**	529	Z77724[e]
	Synechocystis sp.	G**D**IA	38	G**D**NQ**Y**	27	G**N**HE	72	AYW**H**	32	G**H**D**H**	326	D90900[f]

[a]Five conserved blocks of residues surround the seven metal-ligating residues (in bold). The numbers of residues between these blocks and total length are indicated. Note the clear distinction between the small (A) and large (B) forms of enzyme in plants.
[b]Determined by amino acid sequencing (incomplete).
[c]Only partial sequence available.
[d]Sequence from *A. ficuum*.
[e]Sequence from *M. tuberculosis*.
[f]Sequence from *Synechocystis 6803.*

strate.[86] These findings suggest that TRAP could have a much greater degree of substrate specificity than previously envisaged, and also indicate that specific active-site antagonists are likely to be possible. Indeed, a series of noncleavable phosphotyrosine analogs of the active peptide substrates were found to be inhibitors, with K_i values at pH 4.9 in the micromolar range (unpublished). The K_i values for these inhibitors were reduced still further at pH 3.3. This is lower than is normally used in TRAP assays, but the enzyme is actually a more efficient catalyst at this pH.[80] There is no direct evidence that such a low pH is attained in the resorptive vacuole of osteoclasts, which has been measured directly by micropuncture[32] and microelectrode[112] to be approximately pH 5. Osteoclasts possess a high-activity adenosine triphosphate (ATP)-dependent proton pump, which is absolutely required for normal bone resorption.[73] With vectorial secretion of large numbers of protons, the local pH may be much lower than is evident from bulk-phase measurements with pH-sensitive dyes.[56] The kidney bean enzyme was also shown to be susceptible to pH-dependent inhibition by the same peptide inhibitors (unpublished), supporting the view that it provides a useful model for the mammalian enzyme. Others have reported on the identification of a series of hydroxynaphthalene phosphonate inhibitors of TRAP with K_i values in the micromolar range, which further supports the view that the enzyme is a viable target for active-site antagonists.[110]

A separate class of substrates for TRAP may be sugar phosphates, possibly in the specific context of glycoproteins. Bresciani and von Figura[16] provided evidence that TRAP is a major enzyme responsible for hydrolysis of phosphate from the mannose-6-phosphate targeting sequence attached to lysosomal enzymes. The physiological importance of this finding is difficult to assess given the high levels of enzyme activity that were required to observe hydrolysis of mannose-6-phosphate.

Control of TRAP Gene Expression

Gene Structure and Transcription Control

In contrast to most plants studied, Southern blot experiments on human,[76] pig,[74] and mouse[19] have detected a single gene, and no related sequences exist in any mammalian EST databases. The human TRAP gene is located on chromosome 19 (19p13.2-13.3)[70,76] and in the syntenic region on mouse chromosome 9.[38] The overall intron-exon structure of the mouse and human TRAP genes is conserved; they contain five exons with the ATG ("start") codon at the beginning of exon 2.[19,34] The axis of symmetry of the protein structure lies at the boundary between the exons in the mouse and human genes, which suggests that a gene duplication event may have occurred during the evolution of the TRAP gene. Transcription gives rise to a 1.5 kb mRNA with an open reading frame of 969–975 bp encoding a 323–325 amino acid protein.[19,34] The porcine gene is quite different in intron-exon structure with only three exons.[19]

TRAP Expression in Osteoclasts

The promoter of the TRAP gene, flanking the noncoding exon 1, has been characterized in the human and mouse genes. The sequences of the mouse and human proximal promoters are aligned in **Figure 2**. Several contradictory locations with regard to the transcription start sites in mouse and human cell lines have been reported,[19,95] including a possible initiation within the first intron.[93] We have not been able to detect any transcripts initiating within the first intron in macrophages or osteoclasts by reverse transcription-polymerase chain reaction (RT-PCR) or RNase protection, but uncharacterized elements within the intron are absolutely required for maximal activity of the upstream promoter in transfected RAW264 cells (A.I.C., unpublished observations). Recently, we revisited the issue of transcription

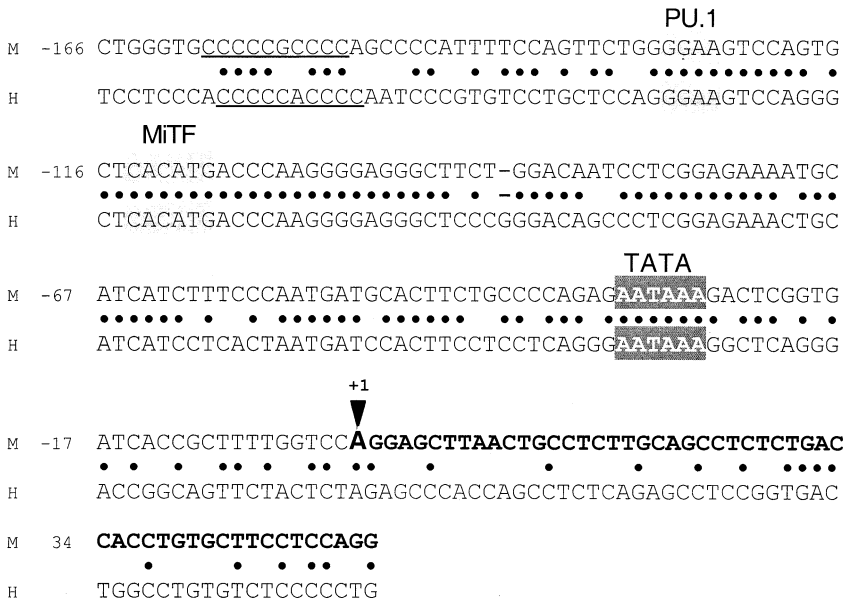

Figure 2. An alignment of the sequences of the murine and human TRAP proximal promoters. The proximal 5′ flanking region of the mouse and human TRAP genes are aligned and sequence identity is marked with a bullet. The variant TATA box is boxed with white text; the osteoclast transcription start point, as determined by 5′-RACE, is marked; the murine exon 1 sequence is shown in bold; the PU.1 binding site and the MiTF binding site are boxed. Candidate Sp1 sites are underlined.

initiation using purified mouse osteoclasts, which express the highest levels of TRAP mRNA compared with many murine tumor cell lines, all of which express TRAP mRNA at detectable levels. The data indicate that osteoclasts initiate transcription at a single point, downstream of a variant TATA-like element that is conserved between mouse and human, whereas nonosteoclasts initiate transcription from a cluster of sites up to 100 bp upstream (Figure 2).[19] The same TATA variant (AATAAA) is found upstream of the transcription start site of another osteoclast-specific gene, cathepsin K.[72] The results indicate that the gene is controlled separately by a typical "house keeping"-type promoter-driving expression in most cells, and a more active TATA-containing promoter used to direct high-level expression in osteoclasts and probably also in subsets of macrophages. There is relatively little sequence conservation upstream of the proximal promoter region between mouse and human.[94] Within the conserved region, there are multiple purine-rich elements containing the GGAA core required for recognition by members of the Ets transcription factor family. Ets family transcription factors, particularly the macrophage-specific factor PU.1, have been ascribed in control of many macrophage-specific genes.[90,100,101] Targeted disruption of the PU.1 gene also causes osteopetrosis and failure of the osteoclast differentiation.[117] In keeping with such a role, we found that the TRAP promoter can be *trans*-activated in transfections of the macrophage cell line RAW264 by both PU.1 and Ets-2, and that the former factor acts through the conserved Ets element highlighted in Figure 2 (unpublished data). Immediately 5′ of this element there is an E-box motif (CAnnTG), which we have found is a functional response element for the *microphthalmia* transcription factor (MiTF).[77] Mutations in the MiTF gene cause osteoclast functional deficiencies[115,121] such as selective downmodulation of both TRAP mRNA expression and TRAP promoter activity in culture-derived osteoclasts.[77] MiTF could interact with PU.1 bound to the adjacent site, because the factors can bind directly to each other.[104] Reddy et al.[95] noted the presence of multiple binding sites for the transcription factor C/EBPβ (NF-IL6) in the mouse promoter, a finding of particular interest given the abundant evidence of the effects of interleukin-6 (IL-6)-type cytokines in osteoclastic differentiation and function.[79] We found that C/EBPβ *trans*-activates the TRAP promoter in transiently transfected RAW264 macrophages (A.I.C. and D.A.H., unpublished observations) although the *cis*-acting elements involved have not been located.

TRAP Expression at Extraskeletal Sites

The TRAP from porcine uterus (uteroferrin) may contribute to iron transport and metabolism.[17] The pig has a noninvasive epitheliochorial placenta, so this precise mode of iron transport is unique to this class of mammals. Nevertheless, we have recently discovered that both TRAP mRNA and protein are present at very high levels in the earliest identifiable invasive trophoblasts in the hemochorial placenta of the mouse (N.W., unpublished observations). The enzyme was purified from human placenta,[64] so the function and regulation defined in the pig could be partly conserved in other mammals despite the differences in placental architecture. The possibility that TRAP from other tissues is involved in iron transport or storage has led several groups to examine the regulatory influence of intracellular iron on TRAP expression. Decreased levels of TRAP mRNA, resulting from either decreased transcription or increased degradation, have been observed in human peripheral mononuclear cells treated with desferrioxamine, a potent iron-chelating agent.[1,12] In addition, TRAP promoters of both species have been reported to be regulated by iron in some cell lines,[1,12,92] although we have not been able to reproduce this finding in the murine macrophage line RAW264 (A.I.C., unpublished observations). Reddy et al.[91] reported that mouse TRAP promoter can be downmodulated by hemin, and characterized the responsive element, GAGGC. Recently, Fleckenstein et al.[35] analyzed iron responsiveness in the human TRAP promoter and confirmed that GAGGC tandem repeat sequences represent hemin-responsive elements. Regulation by iron would be consistent with the involvement of a

redox-sensitive loop in TRAP regulation, which is relevant to the roles of oxidants in bone resorption. It is not clear whether iron or hemin regulation occurs in osteoclasts in vivo, nor whether this contributes to the regulation of bone resorption.

During embryonic development of the mouse, TRAP mRNA has been detected by whole-mount in situ hybridization in trophoblasts, and at low levels in hepatic parenchymal cells, but nowhere else until osteogenesis commenced (N.A., N.W., and D.A.H., unpublished observations). As bones start to develop, TRAP mRNA is detectable on unidentified cells associated with early stages of endochondral ossification, later becoming progressively restricted to mononuclear and multinuclear cells that coexpress other markers of osteoclasts.[58] There is a detailed report on the development expression of TRAP in cells associated with early stages of tooth formation in the mouse,[103] with TRAP-expressing cells appearing by E12, around bud-stage molar teeth. Aside from intensely stained osteoclasts, TRAP has been detected at high levels in the dental follicle and dental pilla in developing molar teeth.[58] A separate study documented the increase in TRAP mRNA in embryonic rat calvariae from E5 to E20.[30]

Within adult tissues, the cellular sites of TRAP expression have been difficult to determine with confidence, because the histochemical assay is not definitive for TRAP when used at the limits of sensitivity. Angel et al.[6] and Hayman et al.[50] analyzed TRAP in adult mouse tissues. Northern blot detected TRAP mRNA in spleen, thymus, liver, lung, skin, kidney, stomach, and small intestines, whereas transcripts were absent from testis, brain, heart, and skeletal muscle. A combination of in situ hybridization, immunohistochemical staining, and TRAP-activity staining of tissue sections established the nature of the expressing cells.[49] In bone, osteoclasts were strongly TRAP-positive, but chondrocytes within the femoral metaphysis also expressed high levels of mRNA. Overall, results have shown that, apart from macrophages and osteoclasts, TRAP is selectively expressed in Langerhans cells/dendritic cells, and in a number of other cell types of nonhematopoietic origin, including selected chondrocytes, alveolar cells, renal tubular cells, and some hepatic parenchymal cells. These abundant nonhematopoietic cells probably contribute a significant proportion of the total activity/mRNA levels in the major organs studied.

We have made transgenic mice overexpressing TRAP from its own promoter.[5] The transgenes elicited a copynumber-dependent increase in both mRNA and protein levels in all tissues. Apart from osteoclasts, tissue macrophages, particularly alveolar and splenic macrophages, expressed abundant TRAP protein that was elevated further in the transgenic animals. Among nonmembers of the mononuclear phagocyte system, hepatic parenchymal cells and renal glomerular cells (probably mesangial cells) constitutively expressed detectable TRAP enzyme activity, increasing in a graded fashion with increased transgene copynumber. With the exception of glomerular mesangial expression, which is clear in both wild-type and transgenic animals, the patterns of expression were similar to those observed by Hayman et al.[49] Independently, a TRAP promoter transgene driving the *src* protooncogene has also been shown to target expression to liver and kidney.[109] The generation of TRAP transgenic mice expressing the SV40 large T antigen, with or without the *bcl*-XL antiapoptotic gene, did not transform cells outside the osteoclast lineage.[14,54] The TRAP promoter has also been used to target overexpression of the c-*fos* gene to osteoclasts to generate a mouse model with some characteristics of Paget's disease.[11]

Although Langerhans cells in the skin express TRAP,[49] the gene is also expressed in keratinocytes, and was shown to be elevated in the transgenic animals.[6] The enzyme has been localized to keratohyalin granules in the granular layer and cornified cells in the lower part of the stratum corneum, where it was considered an important phosphoprotein phosphatase.[88] The reported detection of TRAP activity in the urothelium[106] suggests that expression of TRAP in cornified epithelium also occurs in humans. TRAP expression in all these sites outside the mononuclear phagocyte system could be simply an indication of high levels of lysosomal activity, or the enzyme could have a specific function that has not yet been determined. No overt defect in any organ other than bone has been detected in TRAP null mice, or in our TRAP overexpressing mice, but without knowing the physiological substrate(s) the nature of such a defect is impossible to predict. Furthermore, some functions of TRAP might be carried by an unrelated enzyme, lysosomal acid phosphatase.

Function of TRAP in Osteoclastic Bone Resorption

Correlation Between TRAP Activity and Bone Resorption

By far the greatest interest in the biochemistry of mammalian TRAP relates to its use as a marker for osteoclasts, the cells that resorb bone. The relative specificity of TRAP as an osteoclast marker, its proposed involvement in the resorptive process, and its abundance have suggested that it could be used as a serum marker for bone resorptive activity in pathological states such as osteoporosis. Measurement of serum enzyme activity was confounded by the difficulty of excluding other phosphatases. After an initial enzyme capture assay using antibodies against the porcine enzyme[27] was used, several groups have developed monoclonal antibodies and enzyme-linked immunosorbent assays (ELISA).[20,45–47,51,57] In all of these studies there was evidence of elevation of total immunoreactive TRAP in a range of states where osteoclastic activity is elevated, including growing children and postmenopausal women, as well as in Gaucher's and Paget's diseases. Serum TRAP however, was shown to be a poor indicator of bone turnover in response to antiresorptive therapy.[48] Brehme et al.[15] noted that much of the TRAP in the circulation forms inactive complexes with the proteinase inhibitor α2-macroglobulin, which may confound the use of TRAP as a marker of bone turnover. Recently, a new serum TRAP assay has been developed that combines enzyme capture with the monoclonal antibody O1A and isoform 5b-specific assay conditions.[42] This assay indicates that the TRAP 5b isoform is osteoclastic in origin, whereas the 5a isoform is nonosteoclastic and that isoform 5b does in fact provide an indicative marker of osteoclastic resorptive activity.[42]

Requirement for TRAP Activity in Osteoclastic Bone Resorption

The most direct in vitro evidence supporting a role for TRAP in bone resorption has come from osteoclasts cultured on cortical bone slices. Addition of anti-TRAP antibodies reduced the in vitro resorption of bone,[84,123] an observation we have reproduced using antiserum raised against recombinant murine TRAP (A.I.C., unpublished observations). Addition of bisphosphonate drugs or molybdate to the culture medium was also found to reduce both TRAP activity and bone resorption.[84] In the somewhat more complex mouse calvarial calcium-release assay, hydroxynaphthalene-phosphonate inhibitors of TRAP also inhibited bone resorption.[110]

Mice with even a heterozygous null mutation introduced into the TRAP gene showed evidence of mild osteopetrosis, which was presumed to be due to defective osteoclastic remodeling activity.[52] Homozygous TRAP-deficient mice also exhibited abnormal endochondral ossification, which probably reflects the

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high expression of TRAP in chondroclasts at the chondro-osseus boundary during development,[87] and indicates that TRAP is required for normal mineralization of developing bone as well as for maintaining adult bone integrity through continual remodeling.

As noted earlier, we produced transgenic animals in which tissue-specific overexpression of the TRAP gene was observed in the multiple cell lineages that normally express the gene, including osteoclasts. The animals were mildly osteoporotic, with decreased density of trabeculae with the bone space in long bones, but examination of the rate of bone formation and other indices of osteoblast activity has shown that compensation for increased osteoclast activity occurs through an increase in bone mineralization rate.[5] By analogy, the TRAP knockout mouse almost certainly ameliorates the potential phenotypic effects of the mutation and consequent osteoclast defect by decreasing the rate of mineralization and/or increasing the number of osteoclasts to compensate for their lack of function. To some extent, compensation also occurs through the ability of lysosomal acid phosphatase to substitute for TRAP. In contrast to the TRAP knockout, targeted disruption of lysosomal acid phosphatase causes a lysosomal storage disease,[102] together with minor bone abnormalities. The double knockout in which both genes are mutated was claimed to have a more severe osteopetrotic phenotype,[102] but the data have not been published. The combined evidence from regulation, inhibition, and transgenic studies indicates that TRAP is a "rate-limiting" enzyme in bone resorption, but overexpression or absence of the gene during development can be partly compensated by homeostatic processes. It must be recognized that the transgenic animal data do not argue against TRAP as a drug target. Currently, we have no information on the effects of specific TRAP inhibitors on bone homeostasis in an intact animal, but inhibitor studies in vitro remain promising.

Mechanistic Role of TRAP in Bone Resorption

TRAP as a Phosphoprotein Phosphatase

The bone matrix phosphoprotein, osteopontin, has been shown to bind to osteoclast cell surface integrins via an RGD motif, thereby mediating substrate adhesion, at least in vitro.[36] Ek-Rylander et al.[31] found that osteopontin is a substrate for the phosphoprotein phosphatase activity of osteoclast TRAP in vitro, resulting in dephosphorylation of serine residues. Other phosphatase enzymes, including alkaline phosphatase and prostatic acid phosphatase, lacked this enzymic activity. Following dephosphorylation of osteopontin by TRAP, it was no longer able to support osteoclast-substrate binding,[31] suggesting that TRAP could regulate osteoclast adhesion to the bone surface. This effect would not necessarily inhibit resorption; detachment could permit osteoclasts to migrate across the bone surface to new resorptive sites. Osteopontin is itself an osteoclast product secreted into the resorption space[7,26,60,83,118] and is also a potent inhibitor of de novo hydroxyapatite nucleation and crystal growth. Dephosphorylation of osteopontin also abolishes this inhibitory effect,[13,55] although the consequences of this activity for resorption are unclear. The major matrix protein, osteonectin, is also a TRAP substrate,[4] so the major function of TRAP in resorption may simply be the catabolic degradation of bone matrix phosphoproteins, thereby aiding access by specific proteinases. This is similar to the function proposed for TRAP in the degradation of red cell membrane and cytoskeletal phosphoproteins during erythrophagocytosis by macrophages.[107]

Other Possible Functions of TRAP Enzymes

An alternative, but not mutually exclusive, model for TRAP function in bone resorption is based on the observation that the enzyme is able to catalyze the generation of reactive oxygen species (ROS). Because TRAP in its native reduced form has an Fe(III)-Fe(II) active site, the ferrous ion is able to act as an electron donor. The relevant reactions involved in the generation of ROS, known as Haber–Weiss–Fenton chemistry, are shown as follows:

Hydroxyl radical formation:

$$\text{TRAP-Fe(III)-Fe(II)} + H_2O_2 \text{ ' TRAP-Fe(III)-Fe(III)} + OH^{\cdot} + OH^{-}$$

TRAP regeneration:

$$\text{TRAP-Fe(III)-Fe(III)} + O_2^{-} \text{ ' TRAP-Fe(III)-Fe(II)} + O_2$$

Osteoclasts possess the same NADPH-oxidase-dependent superoxide-generating system utilized by macrophages and granulocytes to generate free radicals for microbial killing.[113,114] There is clear evidence that oxygen free radicals are produced at the resorptive interface with bone and are required for active resorption.[23,40,65,97] The potential activity of TRAP as a Fenton catalyst was recognized in studies of the porcine enzyme.[111] Hayman et al.[51] demonstrated that recombinant human TRAP is able to catalyze iron-dependent peroxidation of luminol, which can be monitored as chemiluminescence, independent of phosphatase activity. Similarly, Halleen et al.[46] reported that RAW264 macrophages overexpressing TRAP produced reactive oxygen metabolites. More recently, this group reported that ROS can be targeted to destroy collagen and other proteins in resorbing osteoclasts. TRAP was found in transcytotic vesicles transporting matrix degradation products through the cell.[47] This suggests that TRAP-generated ROS can participate in bone matrix degradation not only in resorption lacunae but also intracellularly in transcytotic vesicles. This mechanism could be important in tissue remodeling and as an adjunct defense mechanism in TRAP-expressing phagocytic cells.[47]

As noted earlier, the purified porcine enzyme is irreversibly inhibited when treated with hydrogen peroxide in the presence of ascorbate. The Fe(III)-Zn(II) derivative of the enzyme was stable under the same conditions. The results are consistent with the generation of hydroxyl radicals by the enzyme, and subsequent inactivation as a result of reaction of the hydroxyl radicals with active-site residues.[9] The presence of the inactive form of TRAP in the circulation (see earlier) could be indirect evidence that a strong oxidative environment occurs during bone resorption in vivo and may also suggest a simple mechanism of feedback regulation of TRAP enzyme activity by product inhibition.

Among its range of established substrates, TRAP has long been known to be capable of hydrolyzing pyrophosphate.[37] Pyrophosphate is a known inhibitor of bone resorption,[108] so pyrophosphate activity of TRAP per se could lead to an increase in bone resorption by osteoclasts. The bisphosphonates are a class of antiosteoporotic drugs with structures analogous to pyrophosphate; the P-O-P structure is replaced by a nonhydrolyzable P-C-P. In principle, the bisphosphonates could act as substrate analog TRAP inhibitors, but etidronate, a commonly used bisphosphonate, is a relatively poor inhibitor of TRAP.[2] We also found no correlation between biological activity of bisphosphonates and TRAP-inhibitory activity (unpublished observations). Bisphosphonates act more globally on osteoclast viability. Nitrogen-containing bisphosphonates, such as alendronate, iban-

dronate, and risedronate, inhibit posttranslational prenylation of Ras in osteoclasts, which leads to apoptosis.[78] In the case of alendronate at least this results from the inhibition of an unidentified enzyme involved in the conversion of mevalonate to geranylgeranyldiphosphate, the substrate for prenylation of most GTP binding proteins.[33]

Directions for Future Research

Mammalian TRAP is clearly expressed at very high levels in osteoclasts via the use of a separate conserved promoter. Teleologically, it is difficult to believe that such specific expression lacks a purpose. Transgenic mice, both knockouts and overexpressed, indicate that TRAP can be limiting for resorption. The full extent of this role is probably masked by the ability of the bone synthetic pathways to compensate for deficient resorption, an observation that is hardly surprising given the tight homeostatic control of serum calcium. Greater insight may come from subjecting the animals to stresses such as ovariectomy or calcium loading, or by crossing them to other stains that have altered bone metabolism (e.g., op/op) or lower intrinsic bone mineral density (e.g., C57BL/6). Ongoing studies of the active site of plant and mammalian TRAP enzymes, and identification of high-affinity substrates and inhibitors, suggest that active-site antagonists are possible and might be candidate antiosteoporotic drugs.

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