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Tartrate-resistant acid phosphatase from human osteoclastomas is translated as a single polypeptide

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Tartrate-resistant acid phosphatases have been isolated from a number of sources. These enzymes consist of one subunit (M_r 30000–40000) or two dissimilar subunits (M_r 15000–20000). Previously we isolated the enzyme from human osteoclastomas, as a two-subunit protein. By Northern blotting and hybridization with radiolabelled oligonucleotides corresponding to the *N*-terminal sequences of the two subunits, we demonstrate here that the enzyme is transcribed as one mRNA which is translated *in vitro* to produce a single polypeptide of approx. M_r 33000. Transcription as a single mRNA species is also the case in other tissues. These results suggest that the osteoclastoma enzyme undergoes post-translational modification in the form of cleavage of a single peptide bond to give a disulphide-bonded two-subunit protein.

INTRODUCTION

Type 5 tartrate-resistant acid phosphatase is an orthophosphoric monoester phosphohydrolase (EC 3.1.3.2) active at acid pH. It is an iron-containing protein of M_r 30000–40000. It is one of the acid hydrolases secreted by osteoclasts during bone resorption, and elevated levels have been observed in the serum of patients suffering from osteoporosis [1]. Increased levels have also been observed in the spleens and serum of patients with Gaucher's disease [2], and in the spleens and circulating white cells of patients with leukaemic reticuloendotheliosis [3]. The enzyme has been suggested to play a role in iron transport in the pig uterus [4], and also in the retrieval of iron released by erythrophagocytosis [5]. However, the function of the enzyme in bone is unclear.

Tartrate-resistant acid phosphatases have been isolated from a variety of tissues, including bone [6–11], spleen [12–15], uterus [16] and placenta [17]. Some of these enzymes were isolated as two-subunit proteins, whereas others were isolated as a single polypeptide. The cDNA sequence of tartrate-resistant acid phosphatase obtained from human placental cDNA libraries contained the complete coding region for the enzyme as a single mRNA species [18,19]. A two-subunit protein might arise by a number of mechanisms, including alternative splicing of the initial transcript, the use of alternative translation initiation sites, or post-translational proteolytic processing. It has been suggested that tartrate-resistant acid phosphatases may be a family of enzymes which share the same *N*-terminal sequences but have differences throughout the remainder of the molecule [20]. This paper investigates whether the osteoclastoma enzyme is transcribed as one or two messages.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP, [^{35}S]DNA size markers, [^{35}S]methionine, Autofluor, nitrocellulose membranes, Hyperfilm MP and *in vitro* translation kits were obtained from Amersham. The mRNA isolation kit was from Stratagene. Antibodies to pig uteroferrin were obtained from R. Michael Roberts, University of Missouri, Columbia, MO, U.S.A. Protein A–Sepharose was from Pharmacia. RNA markers were obtained from Gibco–BRL and protein M_r markers were purchased from Sigma. All chemicals were of analytical grade and were obtained from either BDH or Sigma. Osteoclastomas were collected immediately after surgery and were stored at −70 °C.

Labelling of oligonucleotides

Oligonucleotides were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase [21].

Isolation of RNA

Total RNA was isolated from tissues by the acid guanidinium thiocyanate/phenol/chloroform method [22]. mRNA was produced from total RNA using a kit from Stratagene.

Northern blotting

Total RNA (20 μg samples) was subjected to electrophoresis through 1% agarose gels containing formaldehyde [23]. Gels were rinsed (3 × 10 min) with water and vacuum-blotted on to nitrocellulose membranes (Amersham) in 10 × SSC (SSC = 0.3 M-NaCl and 0.03 M-sodium citrate, pH 7.0). After blotting, the membranes were heated at 80 °C for 2 h in a vacuum oven. The blots were then wetted in 6 × SSC and prehybridized for 2 h in 5 × SSC, 50 mM-sodium phosphate (pH 6.5), 2 mg of Ficoll/ml, 3 mg of polyvinylpyrrolidone/ml, 3 mg of bovine serum albumin/ml and 250 μg of calf thymus DNA/ml at 37 °C. Radiolabelled oligonucleotides were added to the prehybridization solution and the incubation was continued at 37 °C overnight. Membranes were washed in 6 × SSC/0.05% SDS for 1 h at 20 °C, followed by 1 h at 42 °C, before being exposed to X-ray film for 1–3 days at −70 °C.

In vitro translation

Osteoclastoma mRNA (5 μg) was translated using a reticulocyte lysate translation kit in the presence of [^{35}S]methionine (100 μCi). The translation product was divided into three portions and treated as follows. The first portion was used to determine total translation, while the second and third portions were incubated overnight with 10 μl of antibodies raised to the tartrate-resistant acid phosphatase from pig uterus (known as uteroferrin) and normal rabbit serum respectively at 4 °C, followed by 2 h at 20 °C in the presence of Protein A–Sepharose

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Table 1. Oligonucleotide sequences

Oligonucleotides were synthesized corresponding to the *N*-termini of the two osteoclastoma tartrate-resistant acid phosphatase subunits [6]. The third codon for each amino acid was taken from the human placental cDNA sequence [18].

A subunit (M_r 15000)										
Amino acid sequence	A	A	A	R	E	D	Y	V	L	
cDNA sequence	5′-GCG	GCG	GCC	AGG	GAG	GAC	TAC	GTG	CTG-3′	
Oligonucleotide	3′-CGC	CGC	CGG	TCC	CTC	CTG	ATG	CAC	GAC-5′	
B subunit (M_r 17500)										
Amino acid sequence	A	T	P	A	L	R	F	V	A	V
cDNA sequence	5′-GCC	ACC	CCT	GCC	CTG	CGC	TTT	GTA	GCC	GTG-3′
Oligonucleotide	3′-CGG	TGG	GGA	CGG	GAC	GCG	AAA	CAT	CGG	CAC-5′

(50 μl). The Sepharose beads were washed three times with 500 μl of phosphate-buffered saline (142 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.2) and the bound proteins were eluted by incubation in a boiling water bath for 5 min in the presence of 100 μl of gel sample buffer (2% SDS, 10% sucrose, 5% 2-mercaptoethanol, 0.001% Bromophenol Blue, 60 mM-Tris/HCl, pH 6.8). After pelleting, the supernatants were analysed by SDS/polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue, destained, treated with Autofluor for 30 min, dried and exposed to X-ray film at −70 °C for 1 week [24].

RESULTS

Northern blotting

Oligonucleotides of 27 and 30 nucleotides were synthesized, corresponding to the 9 and 10 amino acids at, or close to, the *N*-terminal ends of each of the two subunits of tartrate-resistant acid phosphatase. The sequences used are taken from the partial sequences of the osteoclastoma enzyme that we have previously determined [6]. The amino acid sequence of the osteoclastoma enzyme shows some differences from that of the placental enzyme [18]. However, the amino acid sequences corresponding to the two oligonucleotide probes are identical in the two enzymes. These sequences are shown in Table 1; the third base for each amino acid was selected from the cDNA sequence of the enzyme from human placenta [18]. The B subunit of the osteoclastoma enzyme corresponds to the *N*-terminus of the placental enzyme (residues 3–12), whereas the osteoclastoma A subunit sequence corresponds to an internal sequence (residues 172–180) of the placental enzyme [18].

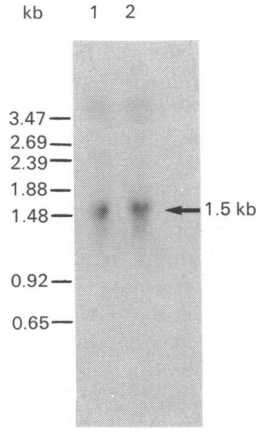

Fig. 1. Autoradiograph of a Northern blot of osteoclastoma total RNA hybridized with the two radiolabelled oligonucleotides

Lane 1, oligonucleotide corresponding to the A subunit; lane 2, oligonucleotide corresponding to the B subunit. The positions of [^{35}S]DNA size markers are given.

Northern blots of total RNA from osteoclastomas when hybridized with the two radiolabelled oligonucleotides revealed a single band of 1.5 kb with each of the two probes (Fig. 1). Bands of a similar size were also observed when a mixture of the two oligonucleotides was used to hybridize Northern blots of total RNA from spleen, lung, uterus, liver and kidney (Fig. 2).

In vitro translation

Osteoclastoma mRNA was translated *in vitro* using a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Immunoprecipitation of the reaction products using antibodies to uteroferrin, followed by SDS/PAGE under reducing conditions and autoradiography, demonstrated a major translation product with an M_r of 33000 (Fig. 3).

DISCUSSION

The tartrate-resistant acid phosphatase originally isolated as a two-subunit protein from human osteoclastomas is coded for by a single mRNA species. This was also true of the enzyme from spleen, lung, uterus, liver and kidney. The size of the transcript in all of these tissues was approx. 1.5 kb. This is in keeping with the size of the cDNA sequences obtained from human placenta [18,19].

Although the enzyme originally isolated from osteoclastomas [6] was purified as a two-subunit protein, the osteoclastoma transcript was translated *in vitro* to produce a single polypeptide of approx. M_r 33000. Several other isolations of tartrate-resistant acid phosphatases from tissues have resulted in a two-subunit protein [11–13,15,25].

A bone tartrate-resistant ATPase also appears to consist of two subunits [26]. A more recent isolation of tartrate-resistant acid phosphatase from osteoclastomas [20] reported it to exist as a single polypeptide. In this latter purification, chromatography under reducing conditions on Superose-12 f.p.l.c. resulted in a major peak (M_r 30000) and a smaller second peak (M_r 16000). The *N*-terminal amino acid sequence of the major peak was identical with the sequence of the M_r-17500 subunit given in Fig. 1. However, the sequence of the smaller peak was not reported. We believe that this enzyme is similar to our previously reported enzyme [6]. In our first report, reverse-phase h.p.l.c. of the reduced and alkylated enzyme yielded two peaks in the ratio 1:2 which were designated A and B, corresponding to the M_r-15000 and -17500 subunits respectively. We now suggest that peak A is solely the M_r-15000 subunit and peak B, which gives the appearance of a doublet, is actually a mixture of the intact M_r-30000 enzyme and the B subunit. The results of the more recent enzyme purification from osteoclastomas [20] could also be

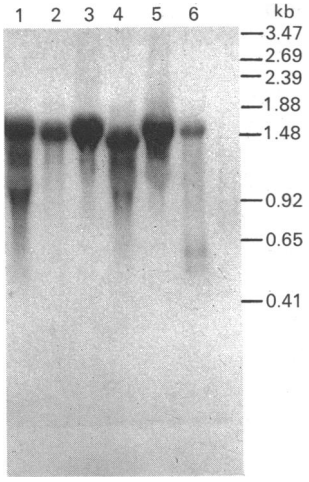

Fig. 2. Autoradiograph of a Northern blot of total RNA from several tissues hybridized with a mixture of the two radiolabelled oligonucleotides

Lanes 1–6 contain total RNA from osteoclastoma, spleen, lung, uterus, liver and kidney respectively. Tissues were from the rat, with the exception of osteoclastoma, which was human. The positions of [^{35}S]DNA size markers are given.

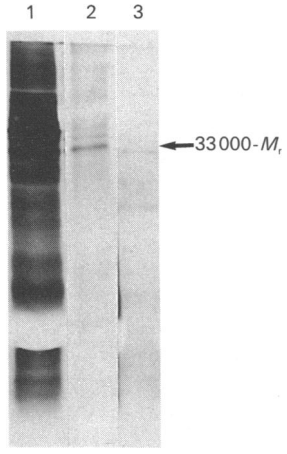

Fig. 3. *In vitro* translation of osteoclastoma tartrate-resistant acid phosphatase

Osteoclastoma mRNA was translated using a reticulocyte lysate translation kit in the presence of [^{35}S]methionine. The resulting proteins were immunoprecipitated with an antibody to the tartrate-resistant acid phosphatase from the pig uterus. These were separated by SDS/PAGE and subjected to fluorography. Lane 1 represents total translation, lane 2 contains anti-uteroferrin antibody-precipitated proteins, and lane 3 contains translated proteins treated with normal rabbit serum.

interpreted in the same way. The major peak resulting after chromatography on Superose-12 f.p.l.c. may well correspond to the M_r-30000 enzyme. However, the smaller second peak (M_r 16000) could be a mixture of the M_r-17500 (B) and -15000 (A) subunits. Although subunits of such similar size would not be separated under these conditions, the peak appears to be a merged doublet. We can only speculate here, since the composition of the peak was not proven by sequencing. It was suggested that the M_r-16000 peak from the Superose-12 f.p.l.c. [20] is an artifactual product of the enzyme due to storage or reducing conditions. The specificity of the cleavage makes this unlikely.

It was reported that the *N*-terminal amino acid sequences of the osteoclastoma and two hairy-cell leukaemia spleen tartrate-resistant acid phosphatases were identical, and also of the same M_r, but different in their amino acid composition [20]. The sequences were similar to that of human placental type-5 acid phosphatase and to those of the enzymes from the pig uterus and bovine spleen. Due to differences in amino acid composition, it was suggested that these enzymes belong to a large family of multigene proteins. Using a PCR probe corresponding to a common sequence in human placenta, pig uterus and bovine spleen tartrate-resistant acid phosphatase, Southern blots indicated the presence of a single-copy gene, making the existence of a multigene family unlikely [19]. It therefore seems likely that all the tartrate-resistant acid phosphatases sequenced so far are products of the same gene.

We therefore conclude that the tartrate-resistant acid phosphatase from osteoclastomas is transcribed as a single mRNA species and translated as a single polypeptide. This then undergoes partial processing in the form of cleavage of a single peptide bond to give a disulphide-bonded two-subunit protein. The fact that the enzyme occurs in some tissues as a single polypeptide and in others as a two-subunit protein suggests that there is cell specificity in the post-translational modification of the enzyme. Expression of tartrate-resistant acid phosphatase in model cell systems would be required to answer this question.

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REFERENCES

1. Lau, K. H. W., Onishi, T., Wergedal, J. E., Singer, F. R. & Baylink, D. J. (1987) Clin. Chem. **33**, 458–462
2. Robinson, D. B. & Glew, R. H. (1980) Clin. Chem. **26**, 371–382
3. Yam, L. T., Li, C. Y. & Finkel, H. E. (1972) Arch. Int. Med. **130**, 248–256
4. Buhi, W. C., Ducsay, C. A., Bazer, F. W. & Roberts, R. M. (1982) J. Biol. Chem. **257**, 1712–1723
5. Schindelmeiser, J., Munstermann, D. & Witzel, H. (1987) Histochemistry **87**, 13–19
6. Hayman, A. R., Warburton, M. J., Pringle, J. A. S., Coles, B. & Chambers, T. J. (1989) Biochem. J. **261**, 601–609
7. Lau, K. H. W., Freeman, T. K. & Baylink, D. J. (1987) J. Biol. Chem. **262**, 1389–1397
8. Kato, T., Hara, A., Nakayama, T., Sawada, H., Hamatake, M. & Matsumoto, Y. (1986) Comp. Biochem. Physiol. **83B**, 813–817
9. Anderson, T. R. & Toverud, S. U. (1986) Arch. Biochem. Biophys. **247**, 131–139
10. Allen, S. H., Nuttleman, P. R., Ketcham, C. M. & Roberts, R. M. (1989) J. Bone Miner. Res. **4**, 47–55
11. Andersson, G., Ek-Rylander, B. & Hammarström, L. (1984) Arch. Biochem. Biophys. **228**, 431–438
12. Robinson, D. B. & Glew, R. H. (1980) J. Biol. Chem. **255**, 5864–5870
13. Ketcham, C. M., Baumbach, G. A., Bazer, F. W. & Roberts, R. M. (1985) J. Biol. Chem. **260**, 5768–5776
14. Stepan, J. J., Lau, K. H. W., Mohan, S., Kraenzlin, M. & Baylink, D. J. (1989) Biochem. Biophys. Res. Commun. **165**, 1027–1034
15. Davis, J. C., Lin, S. S. & Averill, B. A. (1981) Biochemistry **20**, 4062–4067
16. Schlosnagle, D. C., Bazer, F. W., Tsibris, J. C. M. & Roberts, R. M. (1974) J. Biol. Chem. **249**, 7574–7579
17. Waheed, A., Laidler, P. M., Wo, Y. P. & Van Etten, R. L. (1988) Biochemistry **27**, 4265–4273
18. Ketcham, C. M., Roberts, R. M., Simmen, R. C. M. & Nick, H. S. (1989) J. Biol. Chem. **264**, 557–563

19. Lord, D. K., Cross, N. C. P., Bevilacqua, M. A., Rider, S. H., Gorman, P. A., Graves, A. V., Moss, D. W., Sheer, D. & Cox, T. M. (1990) Eur. J. Biochem. **189**, 287–293
20. Stepan, J. J., Lau, K. H. W., Mohan, S., Singer, F. R. & Baylink, D. J. (1990) Biochem. Biophys. Res. Commun. **168**, 792–800
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. (eds.) (1989) Molecular Cloning: A Laboratory Manual, Ch. 11, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (eds.) (1989) Molecular Cloning: A Laboratory Manual, Ch. 7, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Darbre, A. (1987) Practical Protein Chemistry, Ch. 8, John Wiley & Sons, New York
25. Anderson, R. E., Woodbury, D. M. & Jee, W. S. S. (1986) Calcif. Tissue Int. **39**, 252–258
26. Reinholt, F. P., Widholm, S. M., Ek-Rylander, B. & Andersson, G. (1990) J. Bone Miner. Res. **5**, 1055–1061

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