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Tyrosine-rich acidic matrix protein (TRAMP) is a tyrosine-sulphated and widely distributed protein of the extracellular matrix

Euan G. Forbes, Andrew D. Cronshaw, Jonathan R.E. MacBeath**, David J.S. Hulmes*

Department of Biochemistry, University of Edinburgh, Hugh Robson Buiding, George Square, Edinburgh, EH8 9XD, UK

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Abstract Tyrosine-rich acidic matrix protein (TRAMP; 22 kDa extracellular matrix protein; dermatopontin) is a protein that co-purifies with lysyl oxidase and with dermatan sulphate proteoglycans, with possible functions in cell–matrix interactions and matrix assembly. Using a rabbit polyclonal antiserum raised against porcine TRAMP, which cross-reacts with both the human and murine forms of the protein, we show by immunoblotting that TRAMP has a widespread tissue distribution, including skin, skeletal muscle, heart, lung, kidney, cartilage and bone. In cultures of human skin fibroblasts, TRAMP incorporates both [^{35}S]sulphate and [^{3}H]tyrosine and is secreted into the medium, as shown by immunoprecipitation. Amino acid analysis of immunoprecipitated TRAMP demonstrates that many of the tyrosine residues in TRAMP are sulphated.

Key words: Extracellular matrix; Tyrosine sulphation; TRAMP

1. Introduction

The extracellular matrix plays fundamental roles in morphogenesis, cell differentiation, cell migration, metastasis, connective tissue disease and repair [1–3]. In vertebrates, the matrix consists of collagens [4,5], proteoglycans [6], and other glycosylated and non-glycosylated proteins [1]. A common feature of many matrix macromolecules is their ability to interact with other components of the matrix during the formation of various supra-molecular aggregates, e.g. collagen fibrils. In the last few years, several non-collagenous matrix components have been shown to control the assembly of fibril-forming collagens into fibrils [7]. These include the small, leucine-rich proteoglycans decorin, fibromodulin and lumican [5], which delay fibril formation and control fibril diameter [8–12].

Collagen fibrils are stabilised by inter- and intra-molecular covalent cross-links, which form spontaneously after oxidative de-amination of specific lysine (or hydroxylysine) residues by the enzyme lysyl oxidase [13]. During the course of purifying lysyl oxidase (32 kDa) from porcine skin, we recently characterised a protein (22 kDa) that co-purifies with the enzyme [14]. This new protein is acidic and rich in tyrosine, and we refer to it as TRAMP (tyrosine-rich acidic matrix protein). TRAMP is the same as a protein previously isolated from bovine skin, which co-purifies with dermatan sulphate proteoglycans [15]. The bovine form of TRAMP has cell adhesion activity, which is blocked by dermatan sulphate proteoglycans [16], hence the protein is thought to be involved in cell–matrix interactions in vivo. The human equivalent of TRAMP, called dermatopontin, has recently been identified at the cDNA level [17]. Comparison of the cDNA derived sequence with the complete protein sequence of the mature protein ([15]; Cronshaw et al., unpublished data) indicates that TRAMP is synthesised with an 18 residue, N-terminal signal sequence, but without a propeptide region. Recently we have found an additional possible function of TRAMP, namely to accelerate the assembly of collagen into fibrils [7]. TRAMP appears then to be a multi-functional protein of the extracellular matrix. Here, using a polyclonal antiserum raised against porcine TRAMP, we show that TRAMP has a widespread tissue distribution. Furthermore, TRAMP is synthesised and secreted by human fibroblasts in culture, and the protein is tyrosine sulphated.

2. Materials and methods

2.1. Materials

Reagents for cell culture were obtained from Gibco, Paisley, Strathclyde, UK. L-[3,5-^{3}H]Tyrosine (specific activity 1.81 TBq/mmol) and [^{35}S]sulphate (carrier free) were purchased from Amersham International plc, Amersham, Bucks., UK, and [^{14}C]formaldehyde (specific activity 1.5 GBq/mmol) was from ICN Biomedicals Ltd., Thame, Oxfordshire, UK. Killed *Staphylococcus A.* bacteria (Pansorbin) were obtained from Calbiochem Novabiochem Ltd., Nottingham, UK. Donkey anti-rabbit IgG was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, UK. Other reagents were from BDH/Merck, Poole, Dorset, UK.

2.2. Preparation of polyclonal antiserum

TRAMP was purified from the skins of stillborn piglets, by DEAE ion-exchange chromatography and selective interaction with Sephacryl S-400, followed by preparative reverse-phase chromatography on a Pharmacia ProRPC HR5/2 column, as described [14]. Polyclonal antiserum was raised against TRAMP by subcutaneous injection of New Zealand White rabbits with an initial dose of 200 μg TRAMP emulsified in a 1:1 mixture of complete Freund's adjuvant, followed by a 100 μg boost (in incomplete Freund's adjuvant) and then four further boosts injected every 6 weeks.

2.3. Tissue extraction and Western blotting

Tissues were dissected (in phosphate-buffered saline containing 1 mM phenylmethyl-sulphonylfluoride (PMSF)) from stillborn piglets and from 3- to 4-week-old c57 black mice. Human foreskin (Department of Dermatology) and chicken skin (commercial broiler) were also analysed. Tissues were homogenised in glass homogenisers (most tissues) or pulverised, after freezing in liquid N_2, in a stainless-steel bomb (bone, cartilage, tendon). Extraction was at 4°C, with continuous rotation for 24–72 h, in 6 M urea, 10 mM sodium phosphate, pH 7.8, con-taining 1 mM PMSF, using approximately 2 ml buffer per g of tissue (wet weight). Homogenates were centrifuged in a Beckman TL-100 centrifuge (66,000 × *g*, 10 min) and supernatants were collected for determination of protein concentration by absorbance at 280 nm or

*Corresponding author. Fax: (44) (31) 650 3711.

***Present address:* School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester, M13 9PT, UK.

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using the bicinchoninic acid assay (Pierce) at 60°C. Approximately 100 μg of each extract was then analysed, after reduction with 2-mercaptoethanol, by discontinuous SDS-PAGE [18] with 12% acrylamide, 0.32% bis-acrylamide in the separating gel.

Gels were electro-blotted on to cellulose nitrate (Schleicher and Schuell) using a Bio-Rad blotting tank [19]. After identification of protein bands by staining with Ponceau S [20], the nitrocellulose was blocked with 5% (w/v) dried milk in TBS-T (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.2% Tween-20) then incubated in the rabbit, polyclonal antiserum (or pre-immune serum) diluted 1,000- to 10,000-fold. After repeated washing to remove unbound proteins, bound antibodies were detected by enhanced chemiluminescence (Amersham) using horseradish peroxidase-coupled donkey anti-rabbit IgG, diluted 1:5,000, and either Kodak X-Omat AR or Amersham Hyperfilm MP film.

2.4. Cell culture

Human foreskin fibroblasts (passage 7) were a gift from Dr. G.W. Priestley, Department of Dermatology, University of Edinburgh. The cells were cultured in 75 cm^2 flasks in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 37°C in an atmosphere of 5% CO_2. At confluency, the medium was replaced, without washing, by serum-free minimum essential medium (MEM; 10 ml per 75 cm^2 flask) prepared (from a Gibco Select-Amine kit) either without tyrosine, or without sulphate ($MgSO_4$ and $CaSO_4$ replaced by $MgCl_2$ and $CaCl_2$), and supplemented with the corresponding radiolabel, either L-[3,5-^{3}H]tyrosine (50 μCi/ml) or [^{35}S]sulphate (100 μCi/ml). Cells were incubated in the presence of radiolabel for 20–24 h.

Following incubation, the culture medium was collected and placed on ice, proteinase inhibitors were added (from a 10× stock in 0.5 M Tris-HCl, pH 7.5) to final concentrations of 20 mM EDTA, 10 mM *N*-ethylmaleimide (NEM), 1 mM phenylmethylsulphonylfluoride (PMSF), and the medium was centrifuged at 3,000 × *g* for 10 min, then stored at −20°C. The cell layers were lysed in 1.5 ml (per flask) of lysis buffer (1% Nonidet NP-40, 0.15 M NaCl, 20 mM EDTA, 10 mM NEM, 1 mM PMSF, 50 mM Tris-HCl, pH 7.5) at 0°C for 30 min. Lysates were collected using a cell scraper, centrifuged (10,000 × *g* for 10 min) and the supernatants collected and stored.

2.5. Immunoprecipitation

Proteins were immunoprecipitated from culture medium or from cell lysates using killed *Staphylococcus A.* bacteria that were pre-loaded [20] either with pre-immune serum (PIS) or with anti-TRAMP antiserum (ATS). Briefly, the *Staph. A.* bacteria were washed and resuspended in buffer A (9 parts MEM : 1 part 500 mM Tris-HCl, 200 mM EDTA, pH 7.5) to a final concentration of 10% (w/v). For pre-loading, *Staph. A.* bacteria were incubated for 30 min at 4°C with a 1/10th vol. of ATS or PIS, followed by repeated centrifugation and resuspension in buffer A, to a final concentration of 20% (w/v). To the cell culture medium and cell lysates, bovine serum albumin was added to a final concentration of 100 μg/ml. The PIS-loaded *Staph. A.* bacteria were then added to the medium (volume ratio 1:20), or to the lysate (1:2), followed by incubation with gentle agitation for 2 h at 4°C. Proteins bound non-specifically to the PIS-loaded *Staph. A.* bacteria were then removed by centrifugation (10,000 × *g*, 1 min) and the supernatant was retained for immunoprecipitation with ATS-loaded *Staph. A.* bacteria. Cell pellets (PIS and ATS precipitates) were extracted in SDS-PAGE sample buffer (2% (w/v) SDS, 0.125 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 3% (v/v) 2-mercaptoethanol, 0.005% (w/v) Bromophenol blue) for 3 min at 100°C, followed by centrifugation (10,000 × *g*, 1 min) and analysis of supernatants by SDS-PAGE [18].

2.6. Identification of sulphotyrosine

Sulphotyrosine was synthesised by reaction of L-tyrosine with concentrated sulphuric acid [21]. Briefly, 5 g tyrosine and 10 ml concentrated sulphuric acid (both pre-cooled to −20°C) were mixed and stirred rapidly on ice for 10 min, then neutralised with $Ba(OH)_2$, and the sulphotyrosine separated from unreacted tyrosine on a Bio-Rad AG 50W-X8 column [22]. The elution position of the phenylthiocarbamyl (PTC) derivative of sulphotyrosine was determined on an Applied Biosystems 420A amino acid analyser [14] after derivatisation with phenylisothiocyanate. PTC-sulphotyrosine eluted between PTC-proline and phenylthiourea.

To identify radiolabelled tyrosine and sulphotyrosine, excess unlabelled porcine TRAMP was added to the immunoprecipitates and, after SDS-PAGE, proteins were electro-blotted (in 10 mM CAPS, 10% methanol, pH 11) on to ProBlott membrane [23] using a semi-dry blotting unit. TRAMP bands were identified by Coomassie blue staining (0.1% (w/v) in 50% methanol), cut out, de-stained (10% acetic acid, 50% methanol) and dried. As the ester bond of sulphotyrosine is acid labile but resistant to alkaline hydrolysis [22], radiolabelled TRAMP bands on ProBlott membrane were hydrolysed in 0.2 M $Ba(OH)_2$ at 110°C for 22 h, followed by neutralisation and precipitation of $Ba(SO_4)_2$ with 1 M H_2SO_4. After removal of the precipitate by centrifugation (15,600 × *g*, 5 min) supernatants were applied to the amino acid analyser.

2.7. Other procedures

Purified porcine TRAMP and molecular weight standards (Pharmacia) were radiolabelled by reductive alkylation with [^{14}C]formaldehyde and cyanoborohydride [24] using Amicon C-10 microconcentrators for buffer exchange and concentration. Radiolabelled proteins separated by SDS-PAGE were detected by fluorography [25] using Kodak X-Omat AR film.

3. Results

3.1. Tissue distribution of TRAMP

By Western blotting, the rabbit antiserum raised against porcine TRAMP recognised a single band at the migration position of purified TRAMP in urea extracts of porcine, murine and human skin (not shown). No cross-reactivity was seen against extracts of chicken skin, and the pre-immune serum control was negative (not shown).

Extracts from a number of porcine and murine tissues were examined for the presence of TRAMP using the polyclonal antiserum. The results for porcine tissues (Fig. 1) showed TRAMP to be present in extracts of several tissues, including skin, skeletal muscle, heart, lung, articular cartilage, long bone, calvaria, with smaller amounts in kidney, and essentially none in brain, liver and spleen. The distribution of TRAMP in extracts of murine tissues was similar, and no reaction was seen with pre-immune serum (not shown).

3.2. Biosynthesis and tyrosine sulphation of TRAMP

To examine the biosynthesis of TRAMP in cell culture,

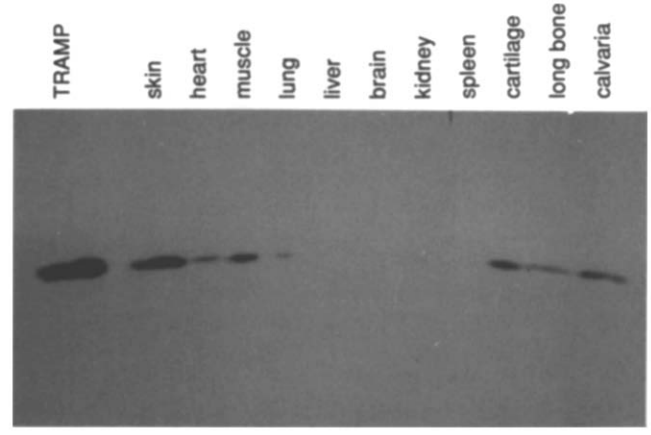

Fig. 1. Tissue distribution of TRAMP. Purified porcine TRAMP and extracts of porcine tissues in 6 M urea, 10 mM sodium phosphate, pH 7.8, 1 mM PMSF were analysed by discontinuous SDS-PAGE (12% acrylamide, 0.32% bisacrylamide in the separating gel), electro-blotted to nitrocellulose and probed with a 1:1,000 dilution of the polyclonal antiserum. Bound antibodies were detected by enhanced chemiluminescence using peroxidase-coupled donkey anti-rabbit IgG. Lane 1, purified porcine TRAMP (0.5 μg); lanes 2–12, 100 μg protein per lane.

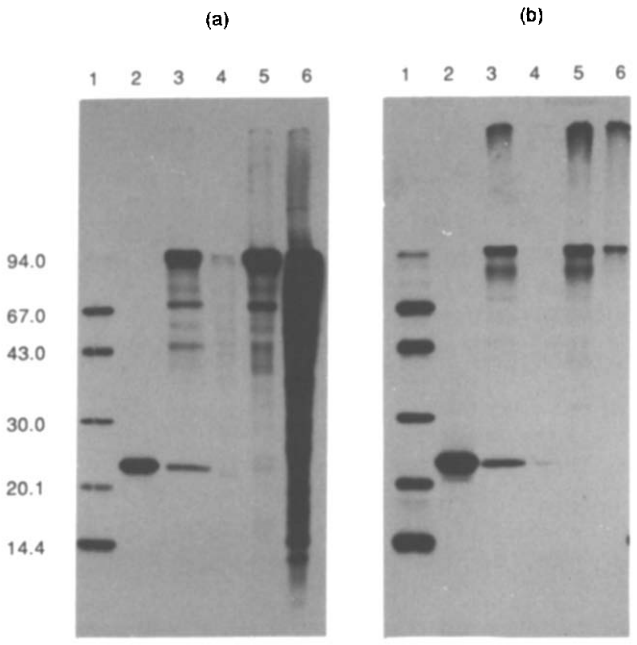

Fig. 2. Immunoprecipitation of (a) [^{3}H]tyrosine labelled TRAMP and (b) [^{35}S]sulphate-labelled TRAMP from human fibroblast cultures. Lane 1, ^{14}C-labelled molecular mass markers; lane 2, ^{14}C-labelled TRAMP; lane 3, immunoprecipitate from culture medium, anti-TRAMP antiserum; lane 4, immunoprecipitate from cell lysate, anti-TRAMP antiserum; lane 5, pre-cleared precipitate from culture medium, pre-immune serum; lane 6, pre-cleared precipitate from cell lysate, pre-immune serum.

human fibroblasts were incubated in the presence of L-[3,5-^{3}H]tyrosine, and proteins in both medium and cell layer were immunoprecipitated with the polyclonal antiserum. Analysis by SDS-PAGE and fluorography (Fig. 2a) showed that the [^{3}H]tyrosine was incorporated into a protein that was specifically precipitated by the anti-TRAMP antiserum. The protein migrated at the same position as ^{14}C-labelled, purified porcine TRAMP, and was present mainly in the culture medium. Higher molecular mass proteins were also precipitated from the culture medium, but these were bound non-specifically, as shown by the pre-immune serum control. From the cell lysates, a protein of apparent molecular mass somewhat less than the [^{14}C]TRAMP standard was immunoprecipitated.

Fibroblasts were also cultured in the presence of [^{35}S]sulphate. As with the [^{3}H]tyrosine label, a ^{35}S-labelled protein that co-migrated with [^{14}C]TRAMP was specifically precipitated with the anti-TRAMP antiserum (Fig. 2b) and this protein was present mainly in the culture medium. Higher molecular mass bands were also present but these were precipitated non-specifically. The ^{35}S-labelled band that co-migrated with [^{14}C]TRAMP was also clearly visible after SDS-PAGE and fluorography of crude, concentrated culture medium (not shown).

Previous work has indicated that TRAMP is not a glycoprotein [15], and the effect of sulphatase treatment or mild acid hydrolysis on Alcian blue staining indicated that TRAMP contained sulphotyrosine [14]. To examine this possibility, immunoprecipitated TRAMP from the human fibroblast culture medium was separated by SDS-PAGE, then transferred to ProBlott and subjected to alkaline hydrolysis. When separated by reverse-phase chromatography on the amino acid analyser, the major peak in hydrolysates of the ^{35}S-labelled TRAMP was at the elution position of the PTC-sulphotyrosine standard (Fig. 3). Hydrolysates of the [^{3}H]tyrosine-labelled TRAMP gave two peaks, at the elution positions of PTC-tyrosine and PTC-sulphotyrosine, in approximately equal proportions.

4. Discussion

The results obtained by Western blotting show that TRAMP has a widespread tissue distribution in both porcine and murine tissues. The protein is most abundant in tissues that are relatively rich in extracellular matrix, such as skin, lung, cartilage and bone, though significant amounts are present in skeletal muscle, heart and kidney. The results on the protein distribution of TRAMP are in good agreement with the Northern blot analysis [17] on the human form of TRAMP, dermatopontin.

The cell culture work confirms that TRAMP is a secreted

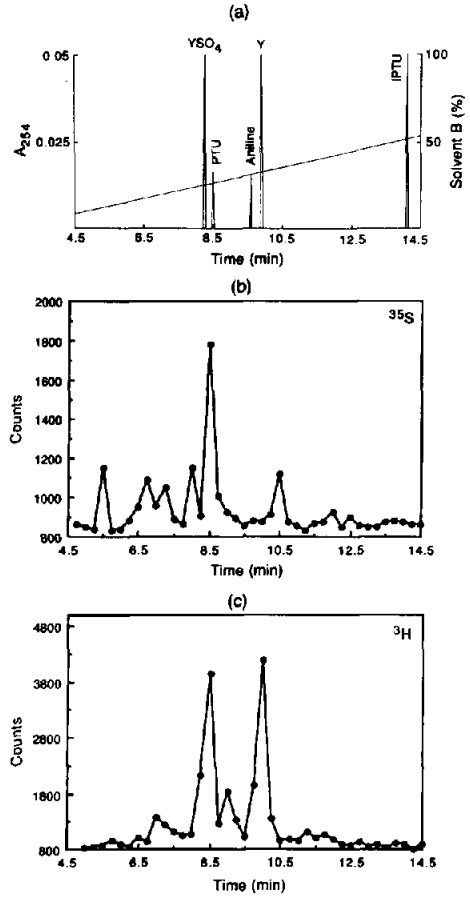

Fig. 3. Amino acid analysis of radiolabelled TRAMP in alkaline hyrolysates. (a) Separation of PTC-sulphotyrosine and PTC-tyrosine standards by reverse-phase chromatography on the PTC C-18 column of the amino acid analyser. (b, c) Separation of radiolabelled amino acids after alkaline hydrolysis of immunoprecipitated TRAMP after transfer to ProBlott membranes. Counts were for 40 min with (b) [^{35}S]sulphate-labelled TRAMP and (c) [^{3}H]tyrosine-labelled TRAMP. The column (5 μm particle size; 2.1 × 220 mm) was pre-equilibrated with 3% (v/v) acetonitrile, 50 mM sodium acetate, pH 5.4, and eluted with a linear gradient from 2% to 64% buffer B (70% acetonitrile, 32 mM sodium acetate, pH 6.1) over 18.8 min.

protein. Unlike the observations of Superti-Furga et al. [17], we found that TRAMP was labelled with [^{35}S]sulphate during biosynthesis. Northern blot analysis of dermatopontin [17] demonstrated the presence of two mRNA species, of 1.0 and 2.2 kb, with the possible intrepretation that there may be different, alternatively spliced forms of TRAMP. We found no evidence for any additional forms of TRAMP that could be labelled with [^{3}H]tyrosine or [^{35}S]sulphate and immunoprecipitated with the polyclonal antiserum. With the [^{3}H]tyrosine-labelled cultures, immunoprecipitates from the cell lysates revealed a protein that migrated somewhat faster than the [^{14}C]TRAMP standard or the [^{3}H]TRAMP from the culture medium. It is likely that this is an intracellular form of TRAMP, prior to tyrosine sulphation.

Studies by Lewandowska et al. [16] indicate that the bovine form of TRAMP interacts with dermatan sulphate proteoglycans. We found no evidence in the immunoprecipitates for a complex between TRAMP and sulphated proteoglycans, as the high molecular mass ^{35}S-labelled material in the stacking gel and also at the top of the separating gel, in the region expected for decorin, biglycan and other proteoglycans [17], was also present in the pre-immune serum controls (Fig. 2).

We have established that TRAMP contains sulphotyrosine residues, and therefore TRAMP can be included in the list of other extracellular matrix proteins (e.g. procollagen III [26], procollagen V [27], fibromodulin [28], fibronectin [29], vitronectin [30], bone sialoprotein [31]) that are known to be post-translationally modified in this way. Tyrosine sulphation has been shown to be important in the interaction properties of several secreted proteins [29,32–35]. Further studies are required to determine if the known interactions of TRAMP, with collagen in fibril assembly [7] and with a putative cell surface receptor in cell adhesion [16], are also influenced by tyrosine sulphation.

The widespread tissue distribution of TRAMP suggests that it may have a general function in the extracellular matrix. We note that bovine [15], human [17] and porcine (Cronshaw et al., unpublished observations) TRAMP contain the sequence Asn-Tyr-Asp, which conforms to the proposed consensus sequence (Asn-Tyr-Asp/Glu) for the formation of topaquinone in copper-dependent amine oxidases [36–38]. This suggests the possibility that TRAMP may itself have amine oxidase activity, in addition to that of lysyl oxidase. Experiments to test this hypothesis are in progress.

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