Characterization and expression of human bifunctional 3′-phosphoadenosine 5′-phosphosulphate synthase isoforms

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Sulphonation, a fundamental process essential for normal growth and development, requires the sulphonate donor molecule 3′-phosphoadenosine 5′-phosphosulphate (PAPS), which is produced from ATP and inorganic sulphate by the bifunctional enzyme PAPS synthase. In humans, two genes encode isoenzymes that are 77 % identical at the amino acid level, and alternative splicing creates two subtypes of PAPS synthase 2. The question as to whether distinctions in amino acid composition are reflected in differences in activity has been examined. The specific activity of the PAPS synthase 2 subtypes is 10- to 15-fold higher than that for PAPS synthase 1. The greater catalytic efficiency of the PAPS synthase 2 subtypes is demonstrated further by the 3- to 6-fold higher k_{cat}/K_m ratios for ATP and inorganic sulphate as compared with the ratios for PAPS synthase 1. In humans, PAPS synthase 1 is expressed ubiquitously, and is the dominant isoform in most tissues, whereas expression of the PAPS synthase 2 subtypes is variable and tissue-specific. It is noteworthy that, similar to other human tissues, PAPS synthase 1 also appears to be the dominant isoform expressed in cartilage. The latter finding initially created a conundrum, since there is a specific human dwarfing disorder that is known to be caused by a mutation in the PAPS synthase 2 gene. This apparent enigma would seem to be resolved by examination of cartilage from guinea-pigs as an animal model. Similar to humans, cartilage from mature animals predominantly expresses PAPS synthase 1. In contrast, expression of PAPS synthase 1 is relatively low in the cartilage of immature guinea-pigs, including the growth plate of long bones, whereas PAPS synthase 2 is the highly expressed isoenzyme.

Key words: cartilage, sulphate activation, sulphoconjugation, sulphonate donor, sulphonation.

INTRODUCTION

Sulphonation is essential for normal growth and development, as well as maintenance of the internal milieu. The transfer of a sulphonate group (SO_3^{1-}) from a donor to an acceptor molecule is a fundamental process in the biotransformation of a host of endogenous compounds, as well as drugs and xenobiotics [1]. In order for sulphonation to occur, however, inorganic sulphate must be activated to a high-energy form prior to being transferred to an acceptor molecule [2,3]. The activated form of sulphate and universal sulphonate donor molecule is 3′-phosphoadenosine 5′-phosphosulphate (PAPS). There are two catalytic reactions involved in the formation of PAPS: the first reaction carried out by ATP-sulphurylase results in the formation of adenosine 5′-phosphosulphate (APS); the second reaction carried out by APS kinase results in the formation of PAPS. Although the two catalytic reactions are located on separate polypeptide chains in bacteria, fungi, yeast and plants, in animal species gene fusion has occurred and the two reactions are contained in a single protein (PAPS synthase). The APS kinase catalytic domain is located in the N-terminal portion of the protein, whereas the ATP-sulphurylase catalytic domain is in the C-terminal region [4].

Bifunctional PAPS synthase was originally cloned from the marine worm [5] and the mouse [6]. Cloning of human PAPS synthase [4,7,8] revealed that it is 98 % and 95 % identical at the amino acid level with the mouse [6] and guinea-pig (unpublished work) PAPS synthases respectively, indicating that the protein is highly conserved. Following the initial cloning of PAPS synthase, a second isoform (PAPS synthase 2) was reported for human [9] and mouse [9,10] species. The mouse and human PAPS synthase 2 isoenzymes were discovered as a result of investigating cartilage-specific developmental abnormalities, i.e. brachymorphism in mice and a form of spondyloepimetaphyseal dysplasia in humans. The gene for human PAPS synthase 1 is located on chromosome 4 (unpublished work), whereas the gene for human PAPS synthase 2 is located on chromosome 10 [9]. An interesting feature regarding human and mouse PAPS synthase 2 is the occurrence of alternative splicing, resulting in the formation of two subtypes distinguished by the presence or absence of a 5-amino-acid segment in the ATP-sulphurylase domain of the protein [11]. It is not clear why there are two genes regulating PAPS formation; furthermore, the biochemical and physiological relationships of the PAPS synthase isoforms are not understood. One aspect of these uncertainties that we have examined relates to distinctions in their amino acid composition and whether such variance might be associated with functional differences. Furthermore, mRNA expression was examined in a variety of human tissues, with particular attention to cartilage because of the specific dwarfing disorder occurring in humans that is caused by a defect in the PAPS synthase 2 gene. The expression studies were performed as ancillary investigations to ongoing studies involving transcriptional regulation of the two human PAPS synthase genes [12,13].

EXPERIMENTAL

Construction of human PAPS synthase 1, 2a and 2b expression vectors

Because of the presence of polymorphisms in reported human PAPS synthase 1 and 2, the amino acid sequences used in these studies were the consensus sequences derived from multiple

Abbreviations used: APS, adenosine 5′-phosphosulphate; GST, glutathione S-transferase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; RT, reverse transcriptase.

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PAPS synthase 1 and 2 clones. A MacVector 7.0 Clustal W alignment program from Genetics Computer Group, Inc. (Madison, WI, U.S.A.) was used to analyse four human PAPS synthase 1 clones (GenBank® accession nos. U53447, Y10387, AF033026 and AF105227) and six PAPS synthase 2 clones (accession nos. AF091242, AF150754, AF074331, AF160503, AF173365 and AF313907).

Full-length cDNAs of PAPS synthase 1 and 2a were generated using the ThermoScript reverse transcriptase (RT)-PCR system from Invitrogen (Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Briefly, 1 μg of brain total RNA for PAPS synthase 1, and 5 μg of adrenal gland total RNA for PAPS synthase 2a, were used. First-strand cDNAs were generated using the gene-specific antisense primer (5′-AGGAAGCATGCCAG-ACAGACACC-3′) for PAPS synthase 1, and random hexamer primers for PAPS synthase 2a. Reverse transcriptions were performed at 60 °C for 60 min for PAPS synthase 1, and 50 °C for 50 min for PAPS synthase 2a. PCR primers used for PAPS synthase 1 were 5′-TAAGGATCCATGGAGATCCCCGGGA-3′ (sense) and 5′-AGGTCGACCTAAGCTTTCTCCAAGGAT-TTG-3′ (antisense) (*Bam*HI and *Sal*I restriction sites, respectively, are underlined). PAPS synthase 2a PCR primers were 5′-ACGCGTCGACATGTCGGGGATCAAGAAGCAAA-AG-3′ (sense) and 5′-ACGCGTCGACTTAGTTCTTCTCCA-GGGACCTG-3′ (antisense) (a *Sal*I restriction site is underlined in each primer). PCR conditions for PAPS synthase 1 were: pre-heat denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min; the number of cycles was 30. PCR conditions for PAPS synthase 2a were: pre-heat denaturation at 94 °C for 1 min, denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 3 min; the number of cycles was 35. After digestion with *Sal*I and/or *Bam*HI, PCR products were gel-purified and ligated to *Sal*I- or *Sal*I/*Bam*HI-digested pGEX-6P-3 glutathione S-transferase (GST)-fusion protein expression vector from Amersham Biosciences (Piscataway, NJ, U.S.A.) using DNA ligation kit version 2 from Panvera Corp. (Madison, WI, U.S.A.). Selection of proper clones was confirmed by restriction-enzyme digestion analysis, and inserts were verified by sequencing both strands using the dideoxy chain termination method Sequenase version 2 (Amersham Biosciences). To create vectors containing nucleotide sequences that express the consensus amino acid sequences, the conversion of two amino acid residues for PAPS synthase 1 and one residue for PAPS synthase 2a was necessary; this was accomplished using the QuikChange XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA, U.S.A.). The consensus amino acid sequences of PAPS synthases 1 and 2a were confirmed by sequencing.

The PAPS synthase 2b subtype, which contains a 5-amino-acid Gly-Met-Ala-Leu-Pro (GMALP) segment missing from PAPS synthase 2a, was generated by PCR using an Excite PCR-Based Site-Directed Mutagenesis kit (Stratagene) to add the appropriate 15 nt sequence to cloned PAPS synthase 2a. The primers used were 5′-GCATGGCCCTTCCTGATGGCGTGA-TCAACATGAGCATC-3′ (sense) and 5′-CATCTAGCAGGG-TGTCAAAGTG-3′ (antisense). The 5′-end of these primers was phosphorylated, and the linear PCR products were ligated by T4 DNA ligase. Selection of proper clones was confirmed by sequencing.

Purification of fusion proteins

Recombinant PAPS synthase proteins were purified using the GST Gene Fusion System (Amersham Biosciences). Briefly, after transformation of *Escherichia coli* strain BL-21 Gold (DE3) pLysS (Stratagene) with PAPS synthase expression vectors, bacteria were incubated in 100 ml of Luria–Bertani medium containing ampicillin at 37 °C for 3 h, and then overnight at 26 °C to minimize expression of proteins as inclusion bodies. Overnight cultures were added to 1 litre of Luria–Bertani medium containing ampicillin, and incubated until a D_{595} of 0.6 was attained, at which time isopropyl β-D-thiogalactoside was added (to a final concentration of 50 μM). After overnight incubation at 26 °C, bacteria were collected by centrifugation, and the bacterial pellets were frozen at −80 °C for 1 h. Pellets were homogenized by sonication in 25 ml of ice-cold PBS containing one protease-inhibitor tablet from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) and 1 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO, U.S.A.). Sonication was performed for 1 min, and repeated four times. The bacterial sonicated material was centrifuged at 204000 ***g*** for 1 h at 4 °C. Supernatants were collected, mixed with 1 ml of glutathione–Sepharose 4B resin (Amersham Biosciences) and incubated for 1–3 h at 4 °C. The resin mixture was transferred to a plastic column, and washed with 25 column-vols. of cleavage buffer [50 mM Tris/HCl (pH 7.0)/150 mM NaCl/1 mM EDTA/1 mM dithiothreitol]. PreScission protease [from Amersham Biosciences; 40 μl (80 units)] in 920 μl of cleavage buffer was applied to the column, and incubated overnight at 4 °C. The eluate was collected and analysed by SDS/PAGE, using GELCODE Blue Stain reagent from Pierce (Rockford, IL, U.S.A.) to reveal the cleaved proteins. Protein concentrations were determined using the bicinchoninic protein assay kit (Pierce) and BSA as a standard.

PAPS synthase assay

Catalytic activity was determined in a total volume of 10 μl consisting of 3 μl of reaction buffer [150 mM Tris/HCl (pH 8.0)/50 mM KCl/15 mM $MgCl_2$/3 mM EDTA/45 mM dithiothreitol], 1 μl of enzyme preparation (1 μg and 0.5 μg per tube for PAPS synthases 1 and 2 respectively), 1 μl of 50 mM ATP and 5 μl of 20 mM $Na_2{}^{35}SO_4$ (6.6×10^7 c.p.m.) obtained from NEN Life Science Products, Inc. (Boston, MA, U.S.A.). Reactions were performed at 37 °C for 5 min (PAPS synthase 1) and 1 min (PAPS synthase 2a and 2b), and then stopped by placing the reaction tubes in boiling water for 5 min. After centrifugation at 16000 ***g*** for 5 min, 1 μl aliquots were transferred to PEI-TLC plates obtained from Merck (Darmstadt, Germany) and developed using 0.9 M LiCl as the solvent system. Following chromatography, the PEI-TLC plates were dried and exposed overnight to X-OMAT films (Eastman Kodak Co., Rochester, NY, U.S.A.). The PAPS 'spots' were excised, and the radioactivity was determined by liquid-scintillation spectrometry. Statistical analyses were performed using StatView SE software from Abacus Concepts, Inc. (Cary, NC, U.S.A.).

Cartilage RNA isolation

Human cartilage RNA

Frozen cartilage (from a 60-year-old Caucasian male) was obtained from the National Disease Research Interchange (Philadelphia, PA, U.S.A.) and 0.6 g was pulverized under liquid nitrogen using a mortar and a pestle. Following removal of the liquid nitrogen, the powdered material was added to a TRIzol solution (Invitrogen), homogenized with a Polytron® and incubated at room temperature for 5 min. After centrifugation at 16000 ***g*** for 10 min, the supernatant was decanted, extracted with chloroform at room temperature and precipitated with

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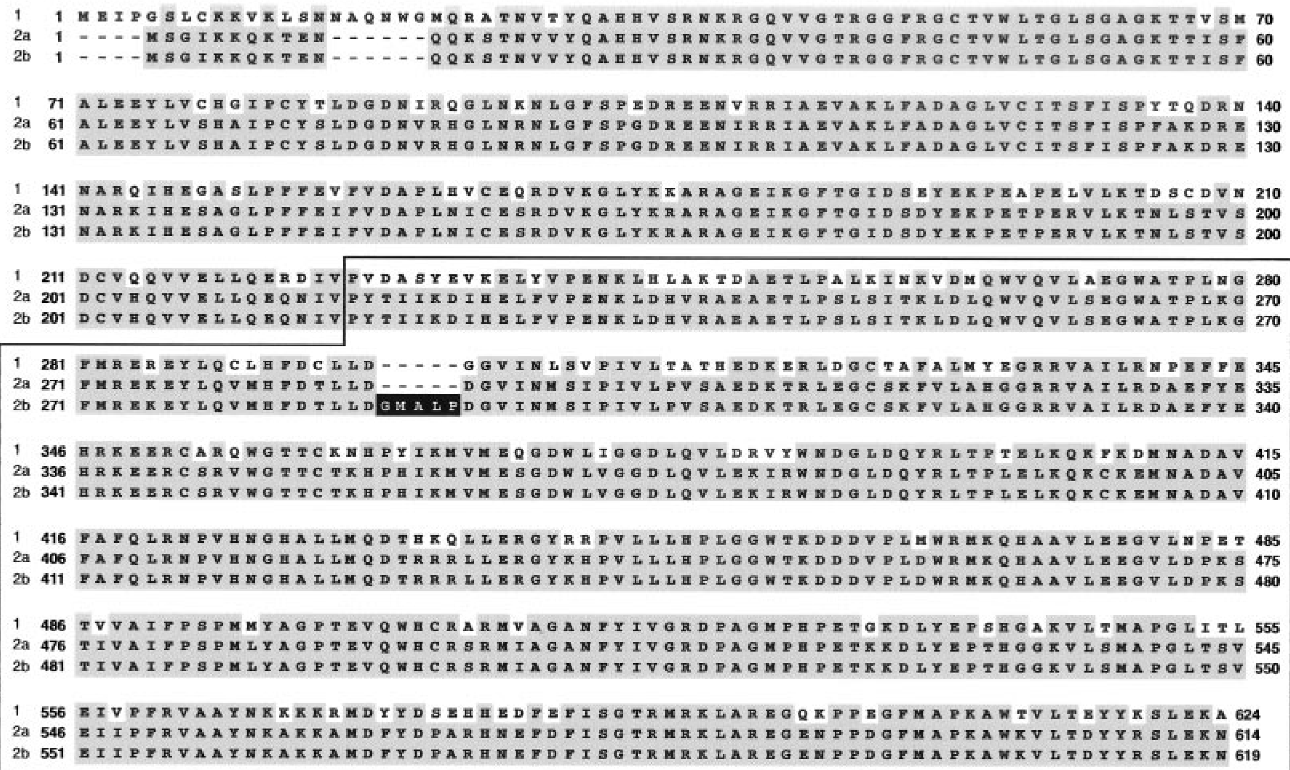

Figure 1 Amino acid sequence alignment of human PAPS synthase isoforms

Human PAPS synthase 1, PAPS synthase 2a and PAPS synthase 2b amino acid sequence alignment, showing amino acid identities on a shaded grey background and the GMALP segment unique to PAPS synthase 2b highlighted in reveresd-out lettering on a black background. The boxed area signifies the ATP-sulphurylase domain, whereas the unboxed region represents the APS kinase domain of these bifunctional enzymes.

isopropyl alcohol. Samples were treated with DNase using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene).

Guinea-pig cartilage RNA

Pregnant and non-pregnant female and male out-bred Hartley guinea-pigs were purchased from Charles River (Wilmington, MA, U.S.A.). Animals were anaesthetized with CO_2, and decapitated using a guillotine. Distal femurs and proximal tibiae were removed, along with costal cartilage of false ribs 7, 8 and 9 from fetuses and 3-week-old animals, and the xiphisternum cartilage from animals at 7–8 weeks and 1 year of age. The long bones were cut in half longitudinally, and the growth plates were identified by their whitish colour and scraped from the underlying bone. Cartilage obtained from long-bone growth plates, false ribs and xiphisterna was incubated in 0.05 % (w/v) trypsin and 0.53 mM EDTA at 37 °C for 30 min to digest connective and fibrous tissue. After centrifugation at 600 ***g*** for 10 min, pellets were incubated in 0.4 % (w/v) collagenase in Dulbecco's modified Eagle's medium at 37 °C for 4 h, filtered through two layers of gauze, and centrifuged at 600 ***g***. Chondrocytes were recovered and added to 0.5 ml of lysis solution [4 M guanidine thiocyanate/25 mM sodium citrate/0.1 M 2-mercaptoethanol] and 1.5 ml of proteinase K solution [0.72 g/l proteinase K/ 20 mM Tris/HCl (pH 7.5)]. Following incubation at room temperature for 20 min, the solution was extracted with phenol/ chloroform/isopentanol solution (25:24:1, by vol.) and precipitated with isopropyl alcohol. Samples were treated with DNase as described above.

RT-PCR analysis

Human

Reverse transcription was performed using the ThermoScript RT-PCR system, according to the provided instructions (Invitrogen). Briefly, 3 μg of total RNA extracted from human tissues obtained from Clontech (Palo Alto, CA, U.S.A.) and Stratagene were used as templates. First-strand cDNAs were made using 10 pmol each of PAPS synthase 1 and 2 gene-specific antisense primers in one reaction tube (5′-GTAGAGTCCTT-TGACATCCCTCTGTTCAC-3′ for PAPS synthase 1, and 5′-AGAGGTGAGGCCAGGGGCCATGCTC-3′ for PAPS synthase 2). Reverse transcription was performed at 50 °C for 60 min. After heat-inactivation, PCR was performed following addition of oligonucleotide primers to one reaction tube: 5′-TCATGGAGATCCCCGGGAGCTTGTGCAAG-3′ sense and 5′-TGCCTTGCATTGTTGCG-ATCCTGAGTG-3′ antisense, for PAPS synthase 1; 5′-CACTCCCCTCAAAGGTTTC-3′ sense and 5′-CAGCGTCTCGTAAGATAGC-3′ antisense, for PAPS synthase 2. PCR conditions were: pre-heat denaturation at 94 °C for 2 min, denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 30 s; the number of

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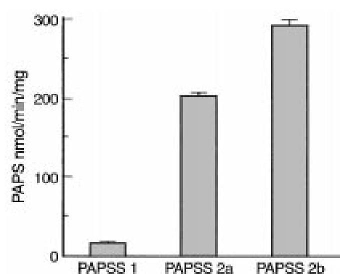

Figure 2 Catalytic activity of human PAPS synthase isoforms

Overexpressed and purified human PAPS synthase (PAPSS)1 (1 μg/tube), PAPSS 2a (0.5 μg/tube) and PAPSS 2b (0.5 μg/tube) were incubated with 10 mM radiolabelled inorganic sulphate and 5 mM ATP, and assayed for PAPS formation as described in the Experimental section. Each preparation was assayed using six replicates. Error bars represent one standard deviation; all P values are < 0.001.

cycles was 30. PCR products were analysed by electrophoresis using 3% (w/v) agarose gels.

Human and guinea-pig cartilage

Briefly, 0.6 and 1 μg of total RNA from human and guinea-pig cartilage respectively was used as a template. First-strand cDNAs were made using 25 pmol of oligo(dT)$_{20}$ and 25 ng of random primers (Invitrogen) in a reaction volume of 20 μl. Following heat denaturation at 65 °C for 5 min, reverse transcription was performed at 25 °C for 10 min, and then 60 °C for 50 min. Human primer sequences were: 5′-GCACAGTTTGGCTAACAGGCTTG-3′ (sense) and 5′-CGGGCTTTTTGTAGAGTCCTTTG-3′ (antisense) for PAPS synthase 1; 5′-CCAGCAGAAATCCACCAATGTAGTC-3′ (sense) and 5′-GACCAGACCAGCATCAGCAAAC-3′ (antisense) for PAPS synthase 2; and 5′-CTGGCACCACACCTTCTACAATG-3′ (sense) and 5′-AATGTCACGCACGATTTCCC-3′ (antisense) for β-actin (GenBank® accession no. E00829). Primers for guinea-pig were generated on the basis of the sequence of guinea-pig PAPS synthase 1 (GenBank® accession no. AF004875) and PAPS synthase 2 (GenBank® accession no. AF251798). Primer sequences were: 5′-CTCTGCACGTTTGCGAACAG-3′ (sense) and 5′-GCATGTCCACCTTGTTAATCTG-3′ (antisense) for PAPS synthase 1; 5′-CTCTTCCTCACTGTCAATTAC-3′ (sense) and 5′-CAGCCGTGCCTTGTCATCTG-3′ (antisense) for PAPS synthase 2; and 5′-TGGTATCGTGGAAGGACTCATG (sense) and 5′-GTCACAGGACACAACCTGGTC-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GenBank® accession no. U51572). PCR conditions were: preheat denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s; the number of cycles was 30. PCR products were analysed by agarose-gel electrophoresis.

RESULTS

Amino acid alignment of human PAPS synthase isoforms

Amino acid sequence alignment analysis using the MacVector 7.0 Clustal W program revealed that human PAPS synthases 1 and 2 are 77% identical at the amino acid level (Figure 1). Furthermore, the PAPS synthase 2 subtypes are almost identical, except for the presence (synthase 2b) or absence (synthase 2a) of a 5-amino acid GMALP sequence (highlighted in Figure 1) located in the ATP-sulphurylase domain of the

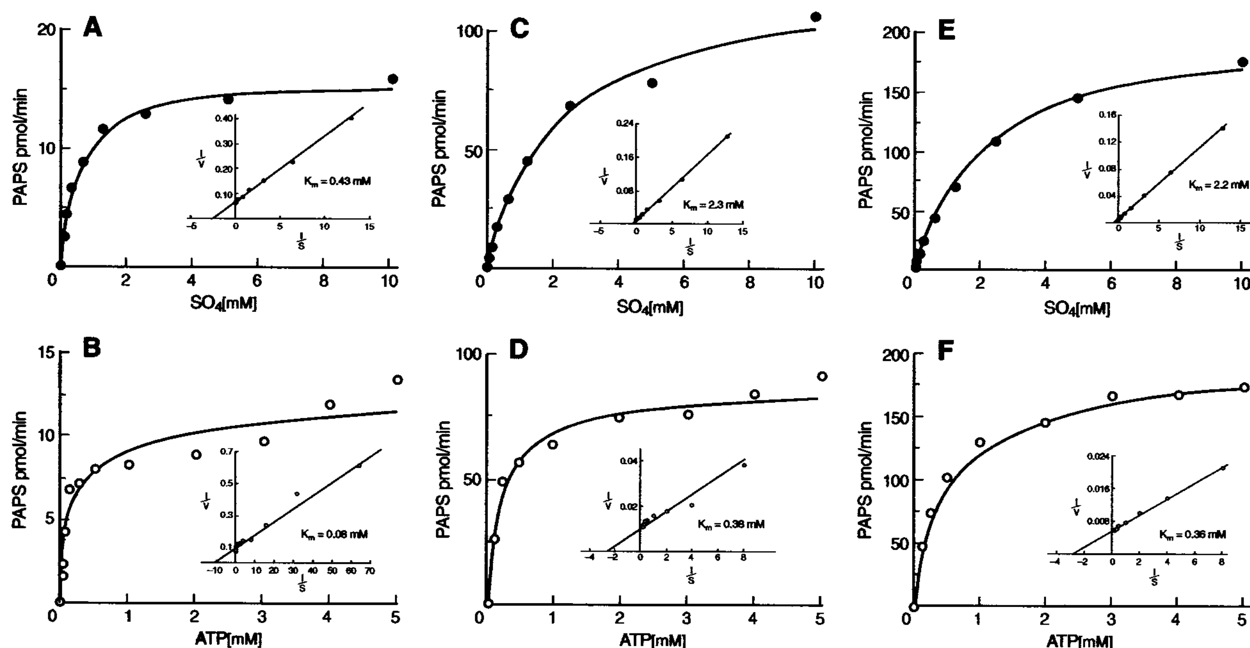

Figure 3 Steady-state kinetics of human PAPS synthase isoforms

Overexpressed and purified human PAPS synthase 1 (**A** and **B**), PAPS synthase 2a (**C** and **D**) and PAPS synthase 2b (**E** and **F**) were incubated with radiolabelled inorganic sulphate and ATP and assayed for PAPS formation as described in the Experimental section. Each datum point represents the average of duplicate determinations; a Lineweaver–Burk plot is presented as an inset in each panel.

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Table 1 Human PAPS synthase isoform kinetic data

Data are derived from the results presented in Figure 3. PAPSS, PAPS synthase.

	SO_4^{2-}			ATP		
	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
PAPSS 1	1.9×10^{-2}	4.3×10^{-4}	45	1.7×10^{-2}	8×10^{-5}	210
PAPSS 2a	3.3×10^{-1}	2.3×10^{-3}	150	2.3×10^{-1}	3.8×10^{-4}	610
PAPSS 2b	5.0×10^{-1}	2.2×10^{-3}	230	4.4×10^{-1}	3.6×10^{-4}	1200

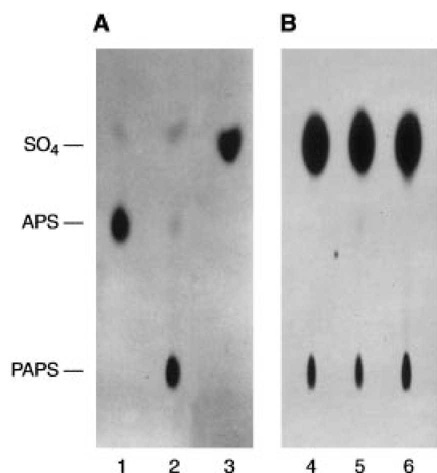

Figure 4 PEI-TLC chromatogram of radioactive components

(**A**) ^{35}S-labelled standards were applied to a PEI-TLC plate and developed using 0.9 M LiCl as the solvent system. Following chromatography, the PEI-TLC plate was dried and exposed overnight: APS (lane 1), PAPS (lane 2) and inorganic SO_4 (lane 3). (**B**) Overexpressed and purified human PAPS synthase isoforms were incubated with radiolabelled inorganic sulphate and ATP and assayed for PAPS formation, as described in the Experimental section. An aliquot (1 μl) of each enzyme incubation was transferred to a PEI-TLC plate, which was similarly developed and processed as noted above: PAPS synthase 1 (lane 4), PAPS synthase 2a (lane 5) and PAPS synthase 2b (lane 6).

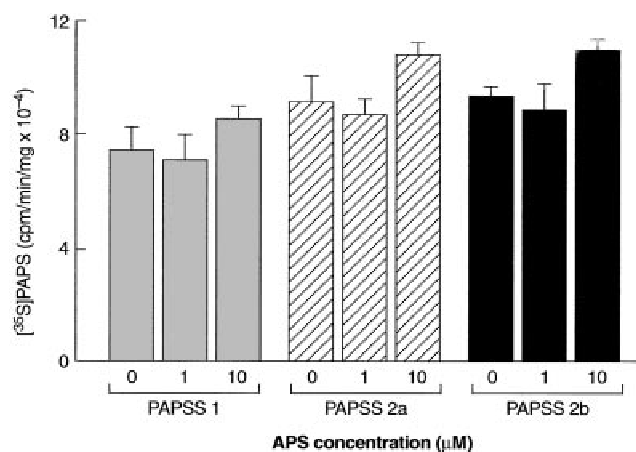

Figure 5 Assay of human PAPS synthase isoforms in the presence of added APS

Overexpressed and purified human PAPS synthase (PAPSS) isoforms were incubated with 0.6 μM radiolabelled inorganic sulphate and 5 mM ATP, and assayed for PAPS formation as described in the Experimental section in the presence or absence of exogenously added non-radioactive APS, as indicated.

protein (boxed region in Figure 1), a difference resulting from alternative splicing of the PAPS synthase 2 transcript [11].

Catalytic activity and kinetic analysis of human PAPS synthase isoforms

On the basis of SDS/PAGE analysis, the enzyme preparations used in these experiments were 91–95% pure (as determined from densitometry); furthermore, they demonstrated activity that was linear over time and with protein concentration (results not shown). The specific activities of PAPS synthase 2a and 2b were 10- to 15-fold higher than the specific activity for PAPS synthase 1, as shown in Figure 2. Steady-state kinetic analyses of overexpressed and purified recombinant proteins are depicted in Figure 3. PAPS synthase 1 (Figures 3A and 3B), PAPS synthase 2a (Figures 3C and 3D) and PAPS synthase 2b (Figures 3E and 3F) essentially exhibited saturation kinetics with respect to both ATP and inorganic sulphate substrates. Double reciprocal transformations revealed linear plots for both substrates, as indicated in the insets of each panel in Figures 3(A)–3(F) . The K_m values for ATP were 0.08, 0.38 and 0.36 mM for PAPS synthases 1, 2a and 2b respectively; K_m values for inorganic sulphate were 0.43, 2.3 and 2.2 mM for PAPS synthases 1, 2a and 2b respectively. The greater catalytic efficiency of the PAPS synthase 2 subtypes as compared with PAPS synthase 1 was reflected in 3- to 6-fold higher k_{cat}/K_m ratios for ATP and inorganic sulphate (Table 1).

Bifunctional PAPS synthase directly transfers or channels the intermediate APS from the ATP-sulphurylase active site to the APS kinase site [14]. Thus radioactive APS should not accumulate in the bulk medium during the PAPS synthase reaction, and this was indeed the case, as shown in Figure 4. Furthermore, just as radioactive APS does not egress from the protein during the PAPS synthase reaction, exogenous non-radioactive APS should not be able to enter the protein and co-mix with the endogenous radioactive APS. This also appears to be the case, since the addition of non-radioactive APS to the incubation medium up to a concentration of 10 μM did not reduce the specific radioactivity of the PAPS product, as shown in Figure 5.

Comparative mRNA expression of human PAPS synthase isoforms

RT-PCR, which was used to perform a comparative analysis, as shown in Figure 6, revealed that PAPS synthase 1 is expressed robustly and appears to be the dominant PAPS synthase isoform in all tissues examined, with the exception of the liver. On the other hand, as also depicted in Figure 6, expression of the PAPS synthase 2a and 2b subtypes is highly variable and tissue-specific, i.e. certain tissues express both subtypes, some express one or the

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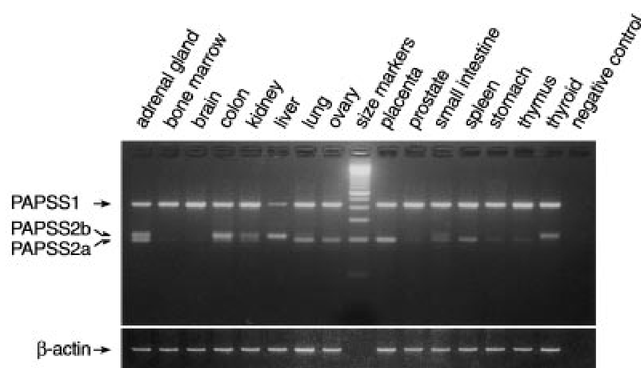

Figure 6 Expression of mRNA for human PAPS synthase isoforms

RT-PCR was performed as described in the Experimental section. Briefly, 3 μg of total RNA from each human tissue was used as a template. First-strand cDNAs were made using 10 pmol of gene-specific antisense primers, and PCR was performed using gene-specific sense and antisense primers added to one reaction tube; the number of cycles was 30. PAPS synthase 1 (PAPSS1), PAPS synthase 2a (PAPSS2a), PAPS synthase 2b (PAPSS2b) and β-actin PCR products were analysed by electrophoresis using 3% (w/v) agarose gels. Size markers (100 bp) are indicated in the centre lane.

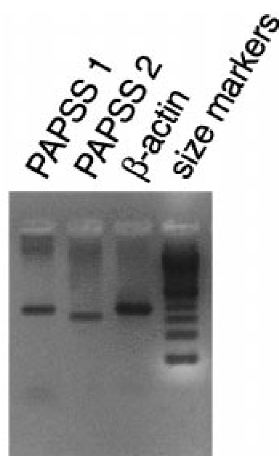

Figure 7 Expression of mRNA for PAPS synthase isoforms in human cartilage

RT-PCR was performed as described in the Experimental section. Briefly, 0.6 μg of total RNA was used as a template. First-strand cDNAs were made using oligo(dT)$_{20}$ and random primers, and PCR was performed using gene-specific sense and antisense primers; number of cycles was 30. PAPS synthase 1 (PAPSS1), PAPS synthase 2 (PAPSS2) and β-actin PCR products were analysed by electrophoresis using 3% agarose gels. Size markers (100 bp) are indicated.

other subtype, and for some tissues expression is barely, if at all, detectable. For example, the adrenal gland expresses prominently both 2a and 2b subtypes, the liver expresses only the 2b subtype, and the ovary expresses only the 2a subtype, whereas neither subtype can be detected definitively in the brain.

Comparative mRNA expression of human and guinea-pig PAPS synthase isoforms in cartilage

RT-PCR analysis revealed that in adult human (Figure 7) and guinea-pig (Figure 8) cartilage PAPS synthase 1 is the predominant isoform present. In contrast, however, expression of PAPS synthase 1 is relatively low in cartilage obtained from immature guinea-pigs, including the growth plate of long bones, whereas PAPS synthase 2 appears to be expressed strongly in this tissue (Figure 8).

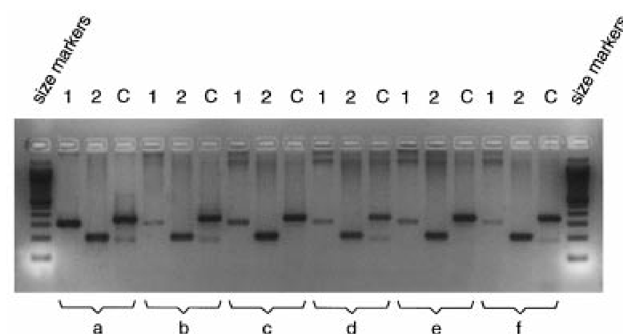

Figure 8 Expression of mRNA for PAPS synthase isoforms in guinea-pig cartilage

RT-PCR was performed as described in the Experimental section. Briefly, 1 μg of total RNA was used as a template. First-strand cDNAs were made using oligo(dT)$_{20}$ and random primers, and PCR was performed using gene-specific sense and antisense primers; the number of cycles was 30. PCR products were analysed by electrophoresis using 3% (w/v) agarose gels. The numbers 1 and 2 and the upper-case C at the top of the gel refer to PAPS synthase 1, PAPS synthase 2 and the glyceraldehyde-3-phosphate dehydrogenase control respectively. The lower case letters at the bottom of the gel refer to: (a) xiphisternum at 1 year of age; (b) xiphisternum at 7–8 weeks; (c) growth plate at 3 weeks; (d) costal cartilage of false ribs at 3 weeks; (e) fetal growth plate; and (f) fetal costal cartilage of false ribs. Size markers (100 bp) are indicated on each side of the gel.

DISCUSSION

While examination of the individual catalytic reactions involved in the formation of PAPS, i.e. the ATP-sulphurylase and APS kinase steps, would be of interest, we chose to limit our investigation to analysing the 'overall' physiological reaction. Our aim was to determine how the three PAPS synthase isoforms prepared similarly would perform during formation of PAPS when presented with the required ATP and inorganic sulphate substrates under the same conditions *in vitro*. Thus it was of interest that a clear distinction in the ability of the isoforms to produce PAPS was demonstrable, with the PAPS synthase 2a and 2b subtypes being 15–20 times more active than PAPS synthase 1.

The finding that ATP-sulphurylase and APS kinase are contained within a single bifunctional protein led to the discovery that translocation of the intermediate, APS, involves a direct transfer from the sulphurylase catalytic centre to the kinase active site without entering the bulk medium [14]. This channelling process has an efficiency of approx. 96% [15]. It is thus noteworthy that, with all three PAPS synthase preparations, no radioactive APS intermediate was found to accumulate during the course of the incubations, suggesting an efficient transfer of APS from the first to the second active site. Furthermore, the addition of non-radioactive APS to the reaction media did not lead to any dilution of the radioactive PAPS product, indicating further a proficient coupling of the two catalytic reactions.

The three enzyme preparations basically exhibited Michaelis–Menten kinetics. Steady-state kinetic analysis of the overall reaction, which had not been reported previously, revealed that the K_m values of PAPS synthase 1 for ATP and inorganic sulphate were 5-fold lower than those of the PAPS synthase 2 subtypes. Nevertheless, the k_{cat}/K_m ratios of ATP and inorganic sulphate were, respectively, 3- and 6-fold higher for PAPS

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synthase 2a and 2b than those for PAPS synthase 1, indicating a greater catalytic efficiency for the PAPS synthase 2 subtypes. Additionally, our data revealed that deletion of the GMALP segment from PAPS synthase 2a yielded only a modest effect on catalytic efficiency, i.e. the k_{cat}/K_m ratios for ATP and inorganic sulphate of PAPS synthase 2a were one-half the ratios found for PAPS synthase 2b. The GMALP segment that is normally spliced out of the PAPS synthase 2 transcript to create the 2a subtype is located in the ATP-sulphurylase domain of the protein [4], and the physiological significance of this splice variant is not appreciated. In a recent publication reporting the kinetic properties of only the PAPS synthase 2a subtype, an S_{50} value for ATP of 0.70 mM and a K_m for SO_4^{2-} of 0.87 mM was noted [16], whereas our data for PAPS synthase 2a revealed K_m values for ATP and SO_4^{2-} of 0.38 mM and 2.3 mM respectively. The difficulty in comparing these results is that the methods employed are so different. For instance, we used PAPS synthase isoforms that were bacterially overexpressed as fusion proteins followed by cleavage and column purification. On the other hand, Xu et al. [16] transfected COS-1 cells with PAPS synthase 2a and then used cytosolic extracts for enzyme analysis. Furthermore, we assayed PAPS formation directly, whereas Xu et al. [16] used an 'indirect' or coupled radiochemical assay.

The RT-PCR data are novel in that mRNA expression of the PAPS synthase 2 subtypes could be distinguished; something that is not possible with Northern blot analysis [17]. Those tissues expressing both the 2a and 2b subtypes usually favoured expression of one or the other, with the exception of the adrenal gland, which was the one tissue in our study that appeared to express both subtypes similarly; we have not examined the adrenal cortex and medulla separately (a phaeochromocytoma was found to contain only the 2a subtype). The colon and, to a lesser extent, the thyroid largely express 2b, whereas 2a is weakly expressed; the reverse is the case for the lung, where 2a is abundantly expressed and 2b weakly so. Other tissues, such as the kidney and the small intestine, express the two subtypes only weakly. On the other hand, the ovary, placenta and spleen express only the 2a subtype (possibly the stomach as well, but very weakly), whereas the liver clearly expresses only the 2b subtype. Several tissues, such as the brain, bone marrow and prostate, did not unequivocally express either PAPS synthase 2 subtype. Expression of the PAPS synthase 2 subtypes is in contrast with the expression of PAPS synthase 1, which is robustly expressed and is the dominant PAPS synthase isoform in all tissues except for the liver, where it appears to be less vigorously expressed. In fact, of the various tissues examined, the liver is unique, i.e. it is the one tissue to solely express PAPS synthase 2b, which forms the dominant PAPS synthase isoform in that tissue. The relatively low expression of PAPS synthase 1 in the liver is consistent with previous reports based on Northern blot analysis [7,17]. The physiological significance of the differential expression of the 2a and 2b subtypes is not understood.

The biological import of having two genes encoding for related proteins that carry out an identical function, i.e. synthesis of the essential sulphonate donor molecule PAPS, is unclear. It is difficult to imagine that this represents a 'backup system', given that PAPS synthase 2 appears to be expressed in a tissue-specific manner, in contrast with PAPS synthase 1, which is expressed ubiquitously. What then is the relationship of PAPS synthase 1 to PAPS synthase 2? It was recently reported that PAPS synthase 1 is located in the nucleus of mammalian cells [18]. It was noted further that PAPS synthase 2 is located in the nucleus, but only when co-expressed with PAPS synthase 1, suggesting that PAPS synthase 1 regulates the distribution of PAPS synthase 2 [18]. These authors concluded that PAPS synthase 1 localizes to the nucleus in most cells, whereas PAPS synthase 2 localizes to the cytoplasm only in tissues where PAPS synthase 1 levels are low, such as the liver. Notwithstanding that this hypothesis is interesting, it is difficult to understand its physiological significance. Although some sulphonation may necessarily occur in the nucleus, perhaps involving soluble sulphotransferases such as oestrogen sulphotransferase [19], the major site of sulphonation involving secretory and membrane proteins, glycosaminoglycans and proteoglycans, which involves membrane-associated sulphotransferases, occurs in the *trans*-Golgi complex [20,21]. Furthermore, the sulphonation of galactoglycerolipids, sphingolipids and a myriad of low-molecular-mass compounds, such as hormones and neurotransmitters as well as drugs and xenobiotics, probably occurs in the cytoplasm.

Human PAPS synthase 2 was discovered during a search for the genetic basis of a developmental abnormality causing a form of spondyloepimetaphyseal dysplasia that presents with a skeletal phenotype involving the spine and long bones. It was discovered that this recessive dwarfing disorder is caused by a nonsense mutation located in the ATP-sulphurylase domain of PAPS synthase 2 [9]. Interestingly, there is a comparable dwarfing abnormality occurring in mice, termed 'brachymorphism', which is also due to a mutation in the gene for PAPS synthase 2; in this case, a missense mutation in the APS kinase domain of the protein [10]. Initially, it was baffling that, in the genetic disorder involving human PAPS synthase 2, which produces the osteochondrodysplasia phenotype, the cartilage-specific defect occurs in spite of the co-expression of PAPS synthase 1 in cartilaginous tissue [9]. In fact, as we were able to demonstrate, PAPS synthase 1 is comparatively more robustly expressed than PAPS synthase 2 in cartilage from human adults; similar results were obtained with the human chondrosarcoma-derived SW1353 cell line (results not shown). The pathology in human spondyloepimetaphyseal dysplasia is considered to be similar to that found in the brachymorphic mouse, i.e. undersulphation of extracellular matrix proteoglycan [22]. We believe that this apparent enigma has now been resolved by the demonstration that, in the cartilaginous growth plate of guinea-pig long bones, PAPS synthase 2 is, indeed, the predominant isoform being expressed, whereas PAPS synthase 1 is poorly expressed. This appears to be a general phenomenon in immature animals, i.e. cartilage obtained from non-growth plate sites (e.g. sternum and rib cage) also more robustly expresses PAPS synthase 2, in contrast with the situation in mature animals, where PAPS synthase 1 is more prominently expressed in cartilage. It is quite likely that this finding involving immature guinea-pigs as an animal model will also hold true for human development where tissues are not readily available for examination.

Only a limited amount of information is available regarding regulation of the expression of the PAPS synthase genes, and no information is available regarding their differential expression during growth and development. We have embarked on the study of the transcriptional regulation of these genes, and have identified their proximal promoters. Both genes lack TATAAA and CAATT motifs, and both genes are regulated, at least in part, by the Sp1 family of transcription factors [12,13]. These studies are, of course, in their infancy.

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