ヒト硫酸転移酵素遺伝子ファミリーの網羅的機能解析に関する研究

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総 括 研 究 報 告 書

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ヒト硫酸転移酵素遺伝子ファミリーの網羅的機能解析に関する研究

主任研究者　　榊原　陽一　　宮崎大学・助教授

研究要旨

本研究は、生体内において非常に多様な機能に関与するヒト硫酸転移酵素に関して網羅的機能解析を行い、トキシコゲノミクス分野における硫酸転移酵素の機能解明と研究成果の医薬品の効率的な開発およびテーラーメイド医療への応用の可能性を検討するものである。現在までの研究で、硫酸転移酵素はシトクロム P-450 酵素群と同様に大きな遺伝子ファミリーを形成し、非常に多様な分子種により構成されていることが明らかとなっている。現在、ゲノム情報をもとにヒトゲノム上に異なる機能を持った硫酸転移酵素遺伝子が少なくとも１２種類存在し、スプライスバリアントを含めるとそれ以上に多様な種類の酵素タンパク質として存在すると考えられている。我々は、平成１５年度から１６年度に新規硫酸転移酵素遺伝子のクローニングを行った。その結果、ヒト SULT1C1 をクローニングし、本酵素がC端のエクソンを使い分けることで２種類のスプライスバリアントが存在する可能性を示した。現在、これらスプライスの違いに由来する２種の ORF を発現し、活性の確認を行っている。これらの研究結果より、ヒト SULT1C1 はスプライシングにより酵素活性を変化し、機能を多様にしている可能性が考えられた。さらに SULT6 という新規ファミリーに属すると考えられる硫酸転移酵素 SULT6A1 をヒトおよびマウスにおいてクローニングした。平成１６年度は、１２種類の薬物成分（p-Acetoamidophenol, Amoxicillin, Allopurinol 、 Ethinylestradiol, (R)-(-)-Phenylephrine hydrochloride, Potassium Guaiacol-4-sulfate hemihydrat, Sodium salicylate, Salbutamol sulfate, Tetracycline hydrochroride, Methotrexate, Folic acid）に対する基質特異性を検討した。さらに、内分泌かく乱物質（Octylphenol, Nonylphenol, Bisphenol A, Diethylstilbestrol）による阻害作用を検討した。

平成１６年度は以上のように、新規硫酸転移酵素のクローニングおよび硫酸転移酵素による薬物代謝に関する研究が進展した。さらに、硫酸転移酵素の遺伝子多型に関する研究にも取り組み、興味深い結果が得られている。将来的には、これらの結果を活用し、トキシコゲノミクス分野における硫酸転移酵素の機能解明とこれらの研究成果の医薬品の効率的な開発およびテーラーメイド医療への応用を目指す。

A．研究目的

生体内において非常に多様な機能に関与するヒト硫酸転移酵素に関して網羅的機能解析を行い、トキシコゲノミクス分野における硫酸転移酵素の機能解明と研究成果のテーラーメイド医療への応用の可能性を検討する。現在までに硫酸転移酵素は非常に多様な分子種からなり、シトクロム P-450 酵素群と同様に大きな遺伝子ファミリーを形成していることが明らかとなっている。しかしながら、現時点ではゲノム上に何種類の異なる機能を持った硫酸転移酵素が存在しているのかといったことも正確には把握されていない。そこで、本研究計画において、全ての硫酸転移酵素遺伝子(SULT)ファミリーのクローニングとリコンビナント酵素の調製を行い、ヒト硫酸転移酵素の網羅的機能解析を行う。

生体内における硫酸化は、生体外異物や薬物の解毒代謝機構、ステロイドホルモンや神経伝達物質の生体内濃度調節機構、食品機能性成分の作用機構への関与などが知られている。このような観点から、硫酸転移酵素はテーラーメイド医療やテーラーメイド栄養指導のための指標として注目を集めつつある。今後、トキシコゲノミクス分野においてヒト硫酸転移酵素を網羅的に機能解析し、生体外異物（食品添加物、環境ホルモン、環境変異原物質など）や薬物にたいする解毒代謝機構としての硫酸化に関して生化学的に諸性質を検討する必要がある。そこで、

平成１６年度は、１１種類の薬物成分（ *p*-Acetoamidophenol, Amoxicillin, Allopurinol 、 Ethinylestradiol, (R)-(-)-Phenylephrine hydrochloride, Potassium Guaiacol-4-sulfate hemihydrat, Sodium salicylate, Salbutamol sulfate, Tetracycline hydrochroride, Methotrexate, Folic acid）に対する硫酸転移酵素の基質特異性を検討した。さらに、内分泌かく乱物質（Octylphenol, Nonylphenol, Bisphenol A, Diethylstilbestrol）による硫酸転移酵素の阻害作用についても検討した。

平成１６年度は以上のように、新規硫酸転移酵素のクローニングおよび硫酸転移酵素による薬物代謝に関する研究が進展した。さらに、硫酸転移酵素の遺伝子多型に関する研究にも取り組み、興味深い結果が得られている。将来的には、これらの結果を活用し、トキシコゲノミクス分野における硫酸転移酵素の機能解明とこれらの研究成果の医薬品の効率的な開発およびテーラーメイド医療への応用を目指す。

B．研究方法

新規ヒト硫酸転移酵素 SULT6A1 はゲノムデータベースの解析により発見し、PCR により ORF の増幅を行いそのアミノ酸配列を決定した。さらに、SULT1C1 同様に

pGEX ベクターにサブクローニングし、リコンビナント酵素を GST との融合タンパク質として発現した。菌体をフレンチプレスにより破砕後グルタチオンセファロースによる精製を行った。

ヒト硫酸転移酵素 SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1E1, SULT2A1, SULT1B1b の８種は、大腸菌で GST 融合タンパク質として発現するベクターpGEX-2TK にサブクローニングし、発現および酵素活性を確認した物を使用した。精製酵素は、１１種類の薬物成分（ *p*-Acetoamidophenol, Amoxicillin, Allopurinol 、 Ethinylestradiol, (R)-(-)-Phenylephrine hydrochloride, Potassium Guaiacol-4-sulfate hemihydrat, Sodium salicylate, Salbutamol sulfate, Tetracycline hydrochroride, Methotrexate, Folic acid）に対する基質基質特異性を検討した。

さらに、同じく８種の硫酸転移酵素を用いて４種の内分泌かく乱物質（Octylphenol, Nonylphenol, Bisphenol A, Diethylstilbestrol）が硫酸転移酵素を阻害し、内因性のホルモン濃度調節機構としての硫酸化に与える影響を検討した。

硫酸転移酵素の活性測定は[^{35}S]-放射活性硫酸でラベルされた硫酸供与体 3'-Phosphoadenosine 5'-Phosphosulfate (PAPS) を酵素的に合成し PAPS から基質への放射活性の転移を測定することで行った。PAPS の合成にはリコンビナント PAPS 合成酵素を使用し、ATP および[^{35}S]-放射活性無機硫酸より合成した。この活性硫酸 PAPS は硫酸転移酵素の研究に不可欠であり、我々は非常に効率のよい合成方法を開発して研究に使用している。

さらに、ヒト硫酸転移酵素遺伝子ファミリーの全体を明らかにするために、ヒト以外にマウスおよびゼブラフィッシュの硫酸転移酵素のクローニングを行った。これらの生物種を比較することで未発見の新規ヒト硫酸転移酵素をより効率よく発見できると考えている。現在、マウスに関しては宮崎大学で行い、ゼブラフィッシュに関しては共同研究者であるテキサス大学の Dr. Ming-Cheh Liu によって精力的に行われている。

倫理面への配慮

新規ヒト硫酸転移酵素のクローニングにおいて、その試料提供者への倫理面への配慮が必要と考えられる。しかしこれらに関しては市販の RNA やライブラリーを用いることにより対処し、倫理的な問題が発生しないよう配慮した。さらにヒト硫酸転移酵素の多型に関する情報が考えられるが、本研究においては遺伝子の多型に関する情報はデータベース上で公開されているもののみを使用することで対応することで倫理面への配慮を十分に行った。

C. 研究結果

ヒトSULT6A1はヒト由来トータルRNAの混合物を鋳型にRT-PCRにより行った。使用したPCRプライマーはゲノムデータベースの解析から判明したN端の開始コドンからC端の終止コドンを含むプライマーをデザインした。その結果、912bpのORFを完全に含むPCR産物が増幅し、303アミノ酸をコードしていることが判明した。SULT6A1のORFはpGEX-4T1ベクターのBamHIサイトにそれぞれサブクローニングし、酵素とGSTとの融合タンパク質として大腸菌で発現し、それぞれのリコンビナント酵素を調製した。酵素活性の確認は、硫酸供与体として[^{35}S]放射活性硫酸ラベルした3'-Phoshoadenosine 5'-Phosphosulfate（活性硫酸PAPS）を用いて、基質の硫酸化反応を行った。反応後はTLCにより酵素反応によって[^{35}S]放射活性硫酸ラベルされた基質の硫酸体を分離し、イメージアナライザーFLA3000により放射活性を酵素活性として測定した。SULT6A1はマウスにおいてヒドロキシステロイド類を硫酸化する結果がごく最近得られた。よって、ヒトに関しても同様な基質を硫酸化することが考えられる（表1）。マウスSULT6A1がPregnenoloneやDHEAに活性を示したことから、現在ヒトSULT6A1のリコンビナント酵素を調製し、同様な活性測定を行っている。

さらに、８種類のヒト硫酸転移酵素SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1E1, SULT2A1, SULT1B1bを使用して、下記の１１種類の薬物成分（*p*-Acetoamidophenol, Amoxicillin, Allopurinol、Ethinylestradiol, (R)-(-)-Phenylephrine hydrochloride, Potassium Guaiacol-4-sulfate hemihydrat, Sodium salicylate, Salbutamol sulfate, Tetracycline hydrochroride, Methotrexate, Folic acid）（構造式は図１参照）に対する基質基質特異性を検討した結果を表２にまとめた。

さらに、同じく８種類のヒト硫酸転移酵素SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1E1, SULT2A1, SULT1B1bを使用して、４種類の内分泌かく乱物質（Octylphenol, Nonylphenol, Bisphenol A, Diethylstilbestrol）が硫酸転移

表１．リコンビナントマウスSULT6A1の基質特異性

基質	v(pmol/min/mg)
Pregnenolone	1.36±0.17
DHEA	2.75±0.09
β-Estradiol	0. 16±0.01
Cholesterol	N.D.
Bisphenol A	N.D.
o-Bromophenol	N.D.
p-Nitrophenol	N.D.
Dopamine	N.D.
Naphtylamine	N.D.
3,3',5-L-Triiodothyronine	N.D.

基質濃度＝10μM

N.D.＝not detected

酵素活性を阻害し、ホルモンや神経伝達物質の濃度調節機構としての硫酸化を阻害するか検討した。その結果として、阻害剤を作用しない条件をコントロール100%とし、相対活性で表３にまとめた。

D. 考察

上記の結果より、SULT1A1 が Amoxicillin, Ethinylestradiol, (R)-(-)-Phenylephrine に対し硫酸化を触媒し、SULT1A3 が Allopurinol、Ethinylestradiol, (R)-(-)-Phenylephrine, Salbutamol sulfate に対し硫酸化を触媒することが判明した。ここで得られた結果より、ヒトにおいては SULT1A1 および SULT1A3 が薬物成分の解毒代謝機構において中心的な役割を果たしていると考えられた。

さらに、内分泌かく乱物質による硫酸転移酵素阻害作用を検討した結果、基質が異なると阻害作用が異なること及び酵素の種類によっても異なることが判明した。特に、SULT1A1 が Dopamine の硫酸化に対する今回試験した内分泌かく乱物質の阻害作用は著しく、全く活性は検出されなくなった。

E. 結論

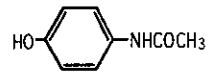
平成１６年度は、新規硫酸転移酵素のクローニングとして平成１５年度から引き続きヒトおよびマウス SULT6A1 のクローニングと大腸菌におけるリコンビナント酵素の発現を行った。クローニングの結果、ヒトやマウスといった哺乳動物では、硫酸転移酵素は少なくとも SULT1 から SULT6 までの６種のファミリーから構成されることが判明した。

種々の薬物成分の硫酸化に関する研究より、ヒトにおいては SULT1A1 および SULT1A3 が薬物成分の解毒代謝機構において中心的な役割を果たしていると考えられた。しかしながら、今後より多くの医薬品及び医薬品候補成分に関する研究の必要性が強く考えられた。そして、生体外異物が硫酸転移酵素によって代謝されるとともに内因性のホルモンや神経伝達物質の濃度調節機構としての硫酸化を阻害することが内分泌かく乱物質を用いた研究で明らかになった。今後、より多くの医薬品及び医薬品候補成分に関して硫酸化を検討するのみならず、硫酸転移酵素活性を阻害する作用も同時に検討する必要が考えられた。

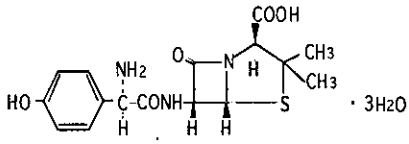
F. 健康危険情報

平成１６年度の研究においては、特に健康危険情報として早急に報告すべき研究結果や事例は見られなかった。

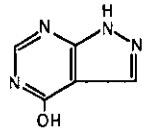
p-Acetamidophenol
解熱、頭痛・関節痛の緩和



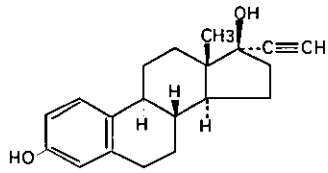
Amoxicillin
グラム陽性菌を中心に殺菌



Allopurinol
尿酸合成阻害剤（痛風治療）

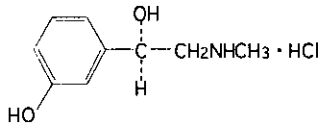


Ethinylestradiol
卵胞ホルモン剤(前立腺ガンの育増殖抑制)



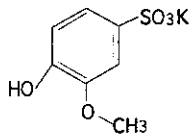
(R)-(-)-Phenylephrine

気管支拡張、診断及び治療を目的とする散瞳と調節麻痺



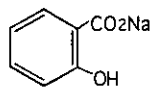
Potassium Guaiacol-4-Sulfate Hemihydrat

鎮咳去痰剤



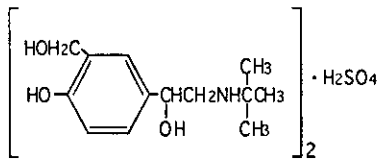
Sodium Salicylate

鎮痛消炎剤

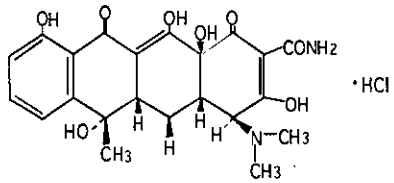


Salbutamol Sulfate

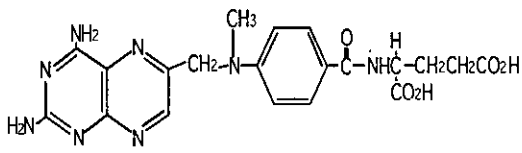
気管支拡張剤



Tetracycline Hydrochoride
抗菌作用(グラム陽性・陰性菌，レプトスピラ，リケッアクラミジアに強く作用)



Methorexate
関節の腫れや痛みを緩和(慢性関節リウマチ治療薬)



Folic Acid
貧血治療

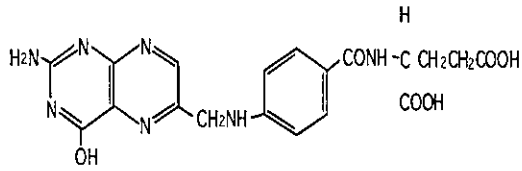


図１．使用した薬物成分及びその構造

表２．ヒト硫酸転移酵素による薬物成分の硫酸化

Substrates (10 μM)	Specific activity (*pmol/min/mg*)							
	SULT1A1	SULT1A2	SULT1A3	SULT1B1	SULT1C2	SULT1E1	SULT2A1	SULT2B1b
p-Nitrophenol	1434.65±58.40	830.80±30.57	61.74±2.30	346.17±12.38	5.40±0.21	1411.96±64.87	ND	ND
Cholesterol	ND	ND	ND	ND	ND	ND	ND	16.98±0.76
Pregnenolone	ND	ND	ND	ND	ND	263.93±12.12	363.74±16.26	273.32±13.50
o-Bromophenol	259.51±14.08	160.50±8.47	2452.30±102.52	414.34±18.27	7.82±0.45	996.25±44.25	ND	ND
p-Acetamidophenol	ND	ND	56.85±2.44	ND	ND	ND	ND	ND
Amoxicillin	81.64±4.06	ND	ND	ND	ND	ND	ND	ND
Allopurinol	ND	ND	58.73857±1.38	ND	ND	ND	ND	ND
Ethynylestradiol	517.40±22.93	ND	51.15044±1.10	ND	ND	357.13±13.17	88.20±4.43	ND
(R)-(-)-Phenylephrine hydrochloride	4.58±0.26	ND	2577.99±134.41	ND	ND	ND	ND	ND
Potassium Guaiacol-4-sulfate Hemihydrate	ND	ND	ND	ND	ND	ND	ND	ND
Sodium Salicylate	ND	ND	ND	ND	ND	ND	ND	ND
Salbutamol Sulfate	ND	ND	2446.67±86.93	ND	ND	ND	ND	ND
Tetracycline Hydrochoride	ND	ND	ND	ND	ND	ND	ND	ND
Methotrexate	ND	ND	ND	ND	ND	ND	ND	ND
Folic acid	ND	ND	ND	ND	ND	ND	ND	ND

ND; not detected

表３．内分泌かく乱物質による硫酸転移酵素阻害活性

Substrate (100 mM)	DHEA			17β-Estradiol					Dopamine		Pregnenolone		
SULTs	1E1	2A1	2B1b	1A1	1A2	1A3	1E1	2A1	1A1	1A3	1E1	2A1	2B1b
Inhibitor (100 mM)	*Relative Activity (%)												
Octylphenol	0	57.1	64.5	0	0	36.1	26.9	0	0	18.2	0	65.0	181.3
Nonylphenol	25.7	77.1	172.7	6.5	0	91.2	48.6	74.3	0	48.4	40.9	110	232
Diethylstilbestrol (DES)	55.6	88.2	142.6	28.6	97.2	90.3	134.6	80.6	0	48.4	0	117.4	129.2
Bisphenol A (BPA)	52.9	92.5	152.3	77.1	22.2	67.1	100.0	76.7	0	35.3	3.2	130.4	69.2

* Results were expressed using activity obtained without inhibitors as 100%.

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Zebrafish tyrosylprotein sulfotransferase: molecular cloning, expression, and functional characterization

Emi Mishiro, Ming-Yih Liu, Yoichi Sakakibara, Masahito Suiko, and Ming-Cheh Liu

Abstract: By employing the reverse transcriptase – polymerase chain reaction technique in conjunction with 3′ rapid amplification of cDNA ends, a full-length cDNA encoding a zebrafish tyrosylprotein sulfotransferase (TPST) was cloned and sequenced. Sequence analysis revealed that this zebrafish TPST is, at the amino acid sequence level, 66% and 60% identical to, respectively, the human and mouse TPST-1 and TPST-2. The recombinant form of the zebrafish TPST, expressed in COS-7 cells, exhibited a pH optimum at 5.75. Manganese appeared to exert a stimulatory effect on the zebrafish TPST. The activity of the enzyme determined in the presence of 20 mM $MnCl_2$ was more than 2.5 times that determined in the absence of $MnCl_2$. Of the other nine divalent metal cations tested at a 10 mM concentration, Co^{2+} also showed a considerable stimulatory effect, while Ca^{2+}, Pb^{2+}, and Cd^{2+} exerted some inhibitory effects. The other four divalent cations, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}, inhibited completely the sulfating activity of the zebrafish TPST. Using the wild-type and mutated P-selectin glycoprotein ligand-1 N-terminal peptides as substrates, the zebrafish TPST was shown to exhibit a high degree of substrate specificity for the tyrosine residue on the C-terminal side of the peptide. These results constitute a first study on the cloning, expression, and characterization of a zebrafish cytosolic TPST.

Key words: XXXXXXXX.

Résumé : À l'aide de la technique d'amplification en chaîne par polymérase après transcription inverse (RT-PCR) et de l'amplification rapide des extrémités 3′ d'ADNc (3′-RACE), un ADNc pleine longueur codant une tyrosylprotéine sulfotransférase (TPST) du poisson-zèbre a été cloné et séquencé. L'analyse de la séquence des acides aminés montre que cette TPST du poisson-zèbre est, respectivement, 66% et 60% identique aux TPST-1 et TPST-2 de la souris et humaines. La TPST recombinante du poisson-zèbre, exprimée dans des cellules COS-7, a un pH optimum de 5,75. Le manganèse semble avoir un effet stimulant sur la TPST du poisson-zèbre. L'activité de l'enzyme en présence de $MnCl_2$ 20 mM est 2,5 fois plus élevé qu'en absence de $MnCl_2$. Des neuf autres cations divalents métalliques évalués à une concentration de 10 mM, le Co^{2+} a également un effet stimulant considérable, alors que le Ca^{2+}, le Pb^{2+} et le Cd^{2+} ont un petit effet inhibiteur. Les quatre autres cations divalents, le Fe^{2+}, le Cu^{2+}, le Zn^{2+} et le Hg^{2+}, inhibent complètement l'activité de sulfatation de la TPST du poisson-zèbre. En utilisant les peptides N-terminaux du ligand glycoprotéique 1 de la P-sélectine (PSGL-1) de type sauvage ou muté comme substrats, nous montrons que la TPST du poisson-zèbre a une très grande spécificité de substrat envers le résidu tyrosine du côté C-terminal du peptide. Ceci constitue la première étude sur le clonage, l'expression et la caractérisation d'une TPST cytosolique du poisson-zèbre.

Mots clés :(non transmis).

[Traduit par la Rédaction]

Introduction

The structure of a mature protein is dependent on not only its amino acid sequence but also on various covalent modifications that take place after ribosomal protein synthesis. These posttranslational protein modifications, including glycosylation, proteolytic cleavage, phosphorylation, acylation, adenylation, farnesylation, ubiquitination, and sulfation, abound among proteins in eukaryotic cells (Tuboi et al. 1992). First discovered in a peptide (fibrinopeptide B) derived from fibrinogen (Bettelheim 1954), protein tyrosine sulfation has emerged as a widespread post-translational modification in multicellular

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eukaryotes (Huttner 1982). It has been shown to be involved in the alteration of biological activity of proteins (Jensen et al. 1981; Brand et al. 1984), proteolytic processing of bioactive peptides (Rosa et al. 1985), change in half-life of proteins in circulation (Pauwels et al. 1987), intracellular transport of secretory proteins (Hille et al. 1984), and modulation of extracellular protein–protein interactions (Kehoe and Bertozzi 2000). More recently, tyrosine sulfation has been demonstrated to be a key modulator of protein–protein interactions that mediate inflammatory leukocyte adhesion (Pouyani and Seed 1995; Sako et al. 1995; Wilkins et al. 1995). The recent discovery of tyrosine-sulfated chemokine receptors (Farzan et al. 1999) suggests an even broader role in the inflammatory response.

Tyrosylprotein sulfotransferase (TPST), the enzyme responsible for protein tyrosine sulfation, catalyzes the transfer of a sulfonyl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the "active" sulfate, to specific tyrosine residues of proteins (Lee and Huttner 1983). This enzyme, following its first description in rat pheochromocytoma 12 cells (Lee and Huttner 1983), has been found in numerous cell lines and tissues and has been characterized from the bovine adrenal medulla (Lee and Huttner 1985) and heart (Suiko et al. 1997), rat brain (Vargas et al. 1985), liver (Rens-Domiano and Roth 1989; Rens-Domiano et al. 1989; Ramaprasad and Kashinathan 1998), gastric mucosa (Kashinathan et al. 1992), submandibular salivary glands (Sundaram et al. 1992; William et al. 1997), human liver (Lin and Roth 1990; Young 1990), and platelets (Sane and Baker 1993). Comparison of the properties reported for TPSTs from the mammalian tissues mentioned above reveals some common properties including acidic pH optima, stimulation by Mn^{2+}, and the use of PAPS as the sulfate donor. Biochemical evidence indicates that TPST is an integral membrane protein with a lumenally oriented active site localized in the trans-Golgi network. Tyrosine sulfation was demonstrated to take place in the same compartment as galactosylation and sialylation (i.e., the trans-Golgi), occurring shortly after these terminal glycosylation reactions (Baeuerle and Huttner 1987). A major advance in the field came with the molecular cloning of two distinct TPSTs, designated TPST-1 and TPST-2, from both human and mouse (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998). Both enzymes were shown to be membrane-bound, *N*-glycosylated Golgi enzymes with a lumenal catalytic domain, a single-span transmembrane domain, and a short cytosolic tail. The two human TPSTs share 67% sequence identity. Northern analysis indicated that both human TPSTs are expressed in many tissues (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998). The existence of more than one TPST might explain the diversity of sequences that are tyrosine sulfated. The two enzymes might have differential substrate specificity and act upon different subsets of target proteins. A recent gene-knockout study revealed that mice deficient in TPST-1 had reduced body mass and increased postimplantation fetal death (Ouyang et al. 2002).

Zebrafish has in recent years emerged as a popular animal model for a wide range of studies (Briggs 2002; Ward and Lieschke 2002). Its advantages, compared with mouse, rat, or other vertebrate animal models, include the small size (3–4 cm length for adult fish), the availability of relatively large number of eggs (>200 eggs per week per mature female), rapid development externally of virtually transparent embryo (in 2–4 days), and short generation time (~3 months). These unique characteristics of the zebrafish make it an excellent model for a systematic investigation on the ontogeny of the expression of various proteins and their tissue- and cell-type-specific distribution as well as physiological relevance.

We report here the molecular cloning of a full-length zebrafish TPST cDNA. Using COS-7 cells as the host, the zebrafish TPST was expressed in a recombinant form. The enzymatic properties of the recombinant zebrafish TPST were analyzed using as substrates wild-type and a series of mutated P-selectin glycoprotein ligand-1 (PSGL-1) N-terminal peptides.

Experimental procedures

Materials

Adenosine 5′-triphosphate (ATP), sodium dodecyl sulfate, *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid, Trizma base, and dithiothreitol were products of the Sigma Chemical Company (St. Louis, Mo.). A zebrafish cDNA clone, ID 2644043 (GenBank accession No. AW344308), encoding the N-terminal region of a TPST was obtained from Genome Systems, Inc. Ampli*Taq* DNA polymerase was a product of Perkin Elmer. Takara *Ex Taq* DNA polymerase and the 3′-Full RACE core set were purchased from PanVera Corporation. T_4 DNA ligase and all restriction endonucleases were from New England Biolabs. XL1-Blue MRF′ *Escherichia coli* host strain was purchased from Stratagene (La Jolla, Calif.). Eukaryotic TOPO TA cloning kit and Lipofectamine 2000 were products of Invitrogen (Carlsbad, Calif.). TNT coupled reticulocyte lysate system was from Promega (Madison, Wis.). Oligonucleotide primers were synthesized by MWG Biotech. Recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase was prepared as described previously (Yanagisawa et al. 1998). COS-7 SV40 transformed African green monkey kidney cells (ATCC CRL1651) were from the American Type Culture Collection. TRI reagent was from Molecular Research Center, Inc. Total RNA from zebrafish was prepared using the TRI reagent according to the manufacturer's instructions. Sep-Pak Plus C18 cartridges were from Waters. Cellulose thin-layer chromatography plates were products of EM Science. Carrier-free sodium [^{35}S]sulfate and [^{35}S]methionine were from ICN Biomedicals. All other reagents were of the highest grades commercially available.

Molecular cloning of the zebrafish TPST

By searching the expressed sequence tag database, a zebrafish cDNA clone (GenBank accession No. AW344308) encoding the N-terminal region of a TPST was identified. This zebrafish TPST cDNA was purified and subjected to nucleotide sequencing based on the cycle sequencing method using, respectively, M13 forward and M13 reverse as primers. The nucleotide sequences, as well as the deduced amino acid sequences, of the cDNA were analyzed using BLAST search for sequence homology to known TPSTs.

To obtain the 3′-coding region and the untranslated sequence further downstream, 3′ rapid amplification of cDNA

ends (3'-RACE) was performed using the Takara 3'-Full RACE core kit. First-strand cDNA was synthesized using AMV reverse transcriptase with zebrafish total RNA as the template in conjunction with an oligo dT-3 sites adaptor primer. Afterwards, a polymerase chain reaction (PCR) was carried out using an oligonucleotide (5'-CGTAGTGGCACCACGCTA-ATGC-3') designed based on the sequence determined for the above-mentioned zebrafish TPST cDNA and a three sites adaptor primer as the primer pair with the first-strand cDNA as the template. Amplification conditions were 25 cycles of 30 s at 94 °C, 30 s at 59 °C, and 1 min at 72 °C. The reaction mixture was analyzed by agarose electrophoresis. A discrete PCR product detected was isolated and subcloned into pSTBlue-1 cloning vector and subjected to nucleotide sequencing (Sanger et al. 1977). The nucleotide sequences, as well as the deduced amino acid sequences, of the cDNA were analyzed using BLAST search for sequence homology to known TPSTs.

To amplify the full-length zebrafish TPST cDNA for subcloning into the pcDNA3.1 eukaryotic expression vector, a sense primer (5'-CCCTGACTTTTGCCCCCACCTGC-3') corresponding to a region 65 residues upstream from the initiation codon of the open reading frame (see Fig. 1) and an antisense primer (5'-GGTAGTTGTGACTCCG-3') corresponding to a region 116 residues downstream from the stop codon were synthesized. With these two oligonucleotides as primers, PCR in a 50-μL reaction mixture was carried out under the action of *Ex Taq* DNA polymerase using zebrafish first-strand cDNA as the template. Amplification conditions were 25 cycles of 40 s at 94 °C, 45 s at 47.5 °C, and 1 min and 20 s at 72 °C. The final reaction mixture was applied onto a 1.2% agarose gel and separated by electrophoresis. The discrete PCR product band, visualized upon ethidium bromide staining, was excised from the gel and the DNA fragment therein was purified by spin filtration. The purified zebrafish TPST cDNA, containing 3' A-overhangs, was subcloned into the 3' T-overhangs-containing pcDNA3.1 eukaryotic expression vector at a cloning site located between *Bst*X I and *Eco*RV restriction sites. To verify its authenticity and direction of cloning, the cDNA insert was subjected to nucleotide sequencing (Sanger et al. 1977).

Transient expression of the zebrafish TPST in COS-7 cells

COS-7 cells, routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, were used as the host cells for the expression of recombinant zebrafish TPST protein. Dishes (100 mm) of COS-7 cells were individually transfected with 10 μg of pcDNA3.1 vector only or pcDNA3.1 harboring the zebrafish TPST cDNA using the Lipofectamine 2000 mediated procedure. Transfection was for 18 h at 37 °C. Afterwards, the transfected cells were incubated at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. At the end of a 48-h incubation, the cells were rinsed twice with phosphate-buffered saline and lysed in an ice-cold lysis buffer containing 20 mM Hepes–NaOH (pH 7.0), 1% Triton X-100, and 10 μg aprotinin/mL. The lysate was subjected to centrifugation and the supernatant was used in the TPST assays described below. Lysates of untransfected COS-7 cells or COS-7 cells transfected with pcDNA 3.1 alone were prepared as controls.

Preparation of wild-type and mutated PSGL-1 N-terminal peptides as substrates for the zebrafish TPST

For the preparation of cDNA encoding the N-terminal region (ATEYEYLDYDFL) of PSGL-1, two complementary 42-mer oligonucleotides (sense strand 5'-GATCCGCCACCGAAT-ATGAGTACCTAGATTATGATTTCCTGG-3' and antisense strand 5'-AATTCCAGGAAATCATAATCTAGGTACTCA-TATTCGGTGGCG-3') were synthesized with, respectively, *Bam*HI and *Eco*RI restriction sites incorporated at the ends. The annealed oligonucleotides were subcloned into the *Bam*HI/*Eco*RI site of pGEX-4T-1 and transformed into *E. coli* XL1-Blue MRF'. For the preparation of cDNA encoding mutated PSGL-1 N-terminal peptides, sense and antisense mutagenic primers with the TAT or TAC codon changed to TTT or TTC were synthesized and similarly processed and subcloned into pGEX-4T-1 and transformed into *E. coli* XL1-Blue MRF'. Figure 2 shows the amino acid sequences of the wild-type and mutated PSGL-1 N-terminal peptides. Competent *E. coli* BL21 cells transformed with pGEX-4T-1 harboring cDNA encoding the wild-type or mutated PSGL-1 N-terminal sequence were grown to A_{600nm} = 0.5 in 100 mL of LB medium supplemented with 100 μg ampicillin/mL. Upon induction with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 25 °C, the cells were collected by centrifugation at 1000*g* for 10 min at 4 °C and homogenized in 15 mL of an ice-cold lysis buffer (containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA) using an Aminco French press. The crude homogenate thus prepared was subjected to centrifugation at 10 000*g* for 20 min at 4 °C. The collected supernatant that includes the fusion protein was fractionated using 0.5 mL of glutathione–Sepharose for 1 h, and the Sepharose was washed three times with lysis buffer and twice with thrombin digestion buffer (containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 2.5 mM $CaCl_2$). Afterwards, the fusion protein bound to the Sepharose was treated with 1 mL of a thrombin digestion buffer containing 5 units thrombin/mL. After a 2-h incubation at room temperature, the preparation was subjected to centrifugation. The recombinant peptide released into the supernatant was purified using a Waters Sep-Pak Plus C18 cartridge according to the manufacturer's instructions. Purified peptide was used as substrate in the enzymatic assay.

Enzymatic assay

The standard assay for TPST activity was carried out in a reaction mixture (25 μL final volume) consisting of 50 mM MES (pH 5.75), 50 mM NaF, 20 mM $MnCl_2$, 0.1% Triton X-100, 40 μM peptide substrate, and 15 μM PAP[^{35}S]. The reaction was started by the addition of the enzyme, allowed to proceed for 30 min at 28 °C, and terminated by heating at 100 °C for 3 min. The precipitates formed were cleared by centrifugation, and the supernatants collected were analyzed for [^{35}S]sulfated product using a previously developed thin-layer chromatography procedure (Liu and Lipmann 1984) with *n*-butanol – isopropanol – 88% formic acid – water (3:1:1:1 by volume) as the solvent system. To examine the pH dependence, different buffers (50 mM MES at pH 5.5, 5.75, or 6.0; MOPS at pH 6.25, 6.5, or 7.0) instead of 50 mM MES (pH 5.75) were used in the reactions. For the kinetic studies on the sulfation of peptide substrates, varying

Fig. 1. Nucleotide and deduced amino acid sequences of the zebrafish TPST cDNA. Nucleotides are numbered in the 5′ to 3′ direction. The translation stop codon is indicated by an asterisk. The putative transmembrane segment and the residues involved in the binding of the 5′-phosphosulfate group and the 3′-phosphate group of PAPS are underlined.

```
   1  CTGTTGGCCTACTGGAGCGATTATTTACATACAGACAGAATTCACGAGAAATGTGCTCGC
  61  GGTGTCAGATTCTGAGTCTCCTGAGAGGTTTATAGCTCCATTTACACGATCTGTTCGTCT
 121  GCATTTCATCACGCGATTCTGGAGAATGTAGAATTCGAGGAGTTGTGCTTTCTGTACGGC
 181  ACAATGGTGCTGACAGAGAATATATGGCTTGATTTACCATGTCTGAGTTTATTGAAGCCT
 241  CAAGCAAAGCCCAGAAGACCTGCTGACTGAGGAAAAAAATCTCCCATCCCTTCCCTCCCC
 301  CCAAAAAAACTTTTACACTTCGCCTTTTACATTCCTTCACCCTGACGCTTTTTGGAAACA
 361  TTCACTCCGTCTTTCCCCTTCCCTGACTTTTGCCCCCACCTGCAAAATCTGTTTAACTCA
 421  TTTGTTATTTTTCCCCCTACAAGCAAAGTACTTCCCTAACTGTAAGTCATGAGAAAACAA
   1                                                  M  R  K  Q
 481  ACGTGCAATGTTCTCCTAGTCTGTGGGGTCATCAGCTCCATCACAGTATTTTATCTTGGC
   5  T  C  N  V  L  L  V  C  G  V  I  S  S  I  T  V  F  Y  L  G
 541  CTCAGTACGATAGAGTGTCCGAATGCCCGTTCTCGTGCATCACAGCATGGGTGGGTGGTA
  25  L  S  T  I  E  C  P  N  A  R  S  R  A  S  Q  H  G  W  V  V
 601  AACCTACACGCTGGCAGAAACCTTAGTGACCCATTACAGCTCCCTGAGGAGTACAATGAG
  45  N  L  H  A  G  R  N  L  S  D  P  L  Q  L  P  E  E  Y  N  E
 661  GAAACTCCTCTCATTTTTGTTGGCGGAGTCCCTCGTAGTGGCACCACGCTAATGCGGGCT
  65  E  T  P  L  I  F  V  G  G  V  P  R  S  G  T  T  L  M  R  A
 721  ATGTTAGATGCTCACCCTATCGTGCGGTGCGGAGAAGAGACCCGGGTCATCCCTCGGTTG
  85  M  L  D  A  H  P  I  V  R  C  G  E  E  T  R  V  I  P  R  L
 781  TTAGCCATGCAGGCAACCTGGAGTCACTCGGCACGAGAGCGGGTCCGTCTGGATGAGGCT
 105  L  A  M  Q  A  T  W  S  H  S  A  R  E  R  V  R  L  D  E  A
 841  GGTGTCACTGATGATGTTTTGGACTCTGCTGTACGTGCGTTTCTTTTGGAGATCATAGTA
 125  G  V  T  D  D  V  L  D  S  A  V  R  A  F  L  L  E  I  I  V
 901  GGGCATGGGGAGCCAGCGCCGAGGCTCTGCAACAAGGACCCATTTGCTCTGAAGTCCATG
 145  G  H  G  E  P  A  P  R  L  C  N  K  D  P  F  A  L  K  S  M
 961  TCCTACCTCTCAAAACTCTTCCCAAAGGCGAAGTTTATTCTCATGCTTCGTGATGGCAGG
 165  S  Y  L  S  K  L  F  P  K  A  K  F  I  L  M  L  R  D  G  R
1021  GCCACCGTTCACTCGATGATCTCCCGCAAGGTTACTATTACTGGGTTTGACTTGACAAGT
 185  A  T  V  H  S  M  I  S  R  K  V  T  I  T  G  F  D  L  T  S
1081  TACCGGGATTGTTTGGTAAAGTGGAACCGGGCGGTGGAAGTGATGTACGACCAGTGCCTG
 205  Y  R  D  C  L  V  K  W  N  R  A  V  E  V  M  Y  D  Q  C  L
1141  GCTGCAGTGGATGGCAACTGTCTGCCTGTCCATTATGAGCAGCTTGTGCTGCATCCTGAG
 225  A  A  V  D  G  N  C  L  P  V  H  Y  E  Q  L  V  L  H  P  E
1201  CGGGTGATGCGCAGGCTCCTTCAGTTCTTGGATCTGCCATGGGACACTGCTGTGCTGCAT
 245  R  V  M  R  R  L  L  Q  F  L  D  L  P  W  D  T  A  V  L  H
1261  CATGAACAGCTAATTGGGAAAGTCGGAGGAGTTTCGCTGTCAAAGGTGGAACTGTCAACA
 265  H  E  Q  L  I  G  K  V  G  G  V  S  L  S  K  V  E  L  S  T
1321  GATCAAGTAGTGAAGCCAGTGAATACAGAGGCTCTGTCTAAATGGGTGGGCAAGATTCCT
 285  D  Q  V  V  K  P  V  N  T  E  A  L  S  K  W  V  G  K  I  P
1381  GCTGATGTAGTGAAGGACATGCCCAGCCTTGCCCCCATGCTGAGTCGCTTGGGTTATGAC
 305  A  D  V  V  K  D  M  P  S  L  A  P  M  L  S  R  L  G  Y  D
1441  CCTCTGGCCAACCCACCAAACTACAACAAGCCTGATCTCTTATATCTGAACAACACAAAA
 325  P  L  A  N  P  P  N  Y  N  K  P  D  L  L  Y  L  N  N  T  K
1501  ATAGTAAGGCCGATGTAAAACTGAAAGGTCATCTCTGTGGATACAACCTGTGAATATTTG
 345  I  V  R  P  M  *
1561  GAAGCAATAAACAATGTGCATTTTTTGTAGCACGCAAAGTCTCTACTTGAATTAAGAACA
1621  TGTATTGACAAAACGGAGTCACAACTACCATTGTTTCATACAAAGTGAGTTCTAGTTTTA
1681  ATGTTGTATTTTAAGTCATTTCTCAATTAAGTGCATTTCAGATGTTTTGCTGTTTGTACC
1741  GATGTTATGATGTTGTTATTAATGTCAGTCCGATGACTATAAGGTGGCTTTTGTCTTGTG
1801  GTTCTATTTGTATGCATGCATGTCACAGTAAATGTGTCCTAAAGAGTGATTATCACACAG
1861  GAAACTATGTTTGTTGAAAGAGGAAGTCAAAGTTGCACAGAAATATCACTATTTTTCTCT
1921  GACCGCAGGAGAACTAAGTAAGTTCCATCTAC
```

concentrations of the substrate compounds and 50 mM MES at pH 5.75 were used.

Miscellaneous methods

PAP[^{35}S] (15 Ci/mmol (1 Ci = 37 GBq)) was synthesized from ATP and [^{35}S]sulfate using recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase as described previously (Yanagisawa et al. 1998). In vitro transcription/translation of the zebrafish TPST was performed using the TNT coupled reticulocyte lysate system according to the manufacturer's instructions. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–

Fig. 2. Amino acid sequences of the wild-type (wt) and mutated PSGL-1 N-terminal peptides. The three tyrosine residues and the mutated phenylalanine residues are boldfaced.

PSGL-1 wt	ATEYEYLDYDFL
PSGL-1 1,2 Y	ATEYEYLDFDFL
PSGL-1 1,3 Y	ATEYEFLDYDFL
PSGL-1 2,3 Y	ATEFEYLDYDFL
PSGL-1 1Y	ATEYEFLDFDFL
PSGL-1 2Y	ATEFEYLDFDFL
PSGL-1 3Y	ATEFEFLDYDFL
PSGL-1 F	ATEFEFLDFDFL

PAGE) was performed on 12% polyacrylamide gels using the method of Laemmli (1970). Protein determination was based on the method of Bradford (1976) with bovine serum albumin as the standard.

Results and discussion

Although considerable progress has been made in recent years in the study of the TPST enzymes, some fundamental questions concerning their ontogeny, regulation, and physiological involvement remain unanswered. The present study was prompted by an attempt to develop a zebrafish model to address these important issues. As a first step toward achieving this goal, we have decided to clone, express, and characterize the TPST enzymes present in zebrafish.

Molecular cloning of the zebrafish TPST

The zebrafish TPST cDNA cloned by reverse transcription – PCR in conjunction with 3′-RACE was subjected to nucleotide sequencing, and the nucleotide sequence obtained was deposited at the GenBank database under accession No. AY263386. Figure 1 shows the nucleotide and deduced amino acid sequences of this zebrafish enzyme. The open reading frame encompasses 1047 nucleotides and encodes a 349 amino acid polypeptide with a calculated molecular mass of 39 030 Da (without considering posttranslational modifications). Sequence analysis based on BLAST search revealed that the deduced amino acid sequence of the zebrafish TPST displayed, respectively, 66% and 60% identity to those of human and mouse TPST-1 (Ouyang et al. 1998) and TPST-2 (Beisswanger et al. 1998; Ouyang and Moore 1998). It should be pointed out that although the cloned zebrafish TPST showed higher percent homology to human or mouse TPST-1, it may actually correspond to TPST-2. We have recently cloned and sequenced another zebrafish TPST (Liu et al., unpublished data), and sequence analysis revealed it to resemble more closely human or mouse TPST-1. Moreover, it is worthwhile mentioning that TPSTs have also been found to be present in lower animals such as *Caenorhabditis elegans* and *Drosophila melanogaster* but not in yeast (Moore 2003). The zebrafish TPST cloned in the present study, while displaying higher percent identity in amino acid sequence to human and mouse TPSTs, also exhibited significant homology to TPSTs from these latter animals. Hydropathy analysis revealed that, similar to human or mouse TPSTs, the zebrafish TPST cloned in the present study contains a putative transmembrane segment (as underlined) located near the N terminus. The zebrafish TPST therefore appears to be a type II transmembrane protein with a short N-terminal cytoplasmic tail with the bulk of this putative Golgi protein being present in the Golgi lumen. Previous studies employing X-ray crystallography in conjunction with sequence alignment have revealed that all cytosolic and Golgi sulfotransferases contain two conserved sequence elements (Negishi et al. 2001). These two sequence elements, designated the 5′-phosphosulfate binding motif and the 3′-phosphate binding motif, are responsible for binding to, respectively, the 5′-phosphosulfate group and the 3′-phosphate group of PAPS, a cosubstrate for sulfotransferase-catalyzed sulfation reactions (Lipmann 1958). Examination of the amino acid sequence of the zebrafish TPST also revealed residues 76–80 (RSGTT, underlined) that correspond to the 5′-phosphosulfate binding motif. And, similar to human and mouse TPSTs previously reported, the zebrafish TPST also contains two conserved residues (Arg^{181} and Ser^{189}, boldfaced and underlined) proposed to be involved in binding the 3′-phosphate group of PAPS (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998).

Expression of recombinant zebrafish TPST in COS-7 cells

The full-length zebrafish TPST cDNA packaged in pcDNA3.1, a eukaryotic expression vector, was used. To ensure that the cDNA can be used as a template for transcription followed by translation for the synthesis of TPST protein, an in vitro transcription/translation experiment was first carried out. As shown in Fig. 3, a 39-kDa protein band was detected in the reaction mixture upon SDS–PAGE, indicating the production of the TPST protein. pcDNA3.1 harboring the zebrafish TPST cDNA was then used to transfect COS-7 cells for the expression of the recombinant enzyme. Lysates of untransfected cells and cells transfected with pcDNA3.1 vector alone or pcDNA3.1 harboring the zebrafish TPST cDNA were then assayed for TPST activity using the wild-type PSGL-1 N-terminal peptide as substrate. As shown in Table 1, lysates of untransfected cells and cells transfected with pcDNA3.1 alone showed approximately the same level of TPST activity. This basal TPST activity is due to the endogenous TPST of COS-7 cells. In contrast, there was a six-fold increase in TPST activity in lysate of COS-7 cells transfected with pcDNA3.1 harboring the zebrafish TPST cDNA, indicating clearly the production of functionally active zebrafish TPST.

Characterization of the recombinant zebrafish TPST

The wild-type and a series of mutated PSGL-1 N-terminal peptides (see Fig. 2) were prepared for the characterization of the recombinant zebrafish TPST.

Temperature dependence, pH optimum, and divalent cation requirement

We first examined the temperature dependence of the activity of the zebrafish TPST using the wild-type PSGL-1 N-terminal peptide as substrate. As shown in Fig. 4, the zebrafish TPST exhibited approximately the same level of activity at 28 and 37 °C. At 21 °C, there was a 50% decrease in the sulfating activity of the enzyme, and only a very low level of activity

Fig. 3. Production of the zebrafish TPST protein by in vitro transcription/translation. The figure shows the autoradiograph taken from the dried polyacrylamide gel used for the SDS–PAGE of the reaction mixtures. Samples analyzed: lane 1, in vitro transcription/translation using pcDNA3.1 vector only as the template; lane 2, in vitro transcription/translation using pcDNA3.1 harboring the zebrafish TPST cDNA as the template.

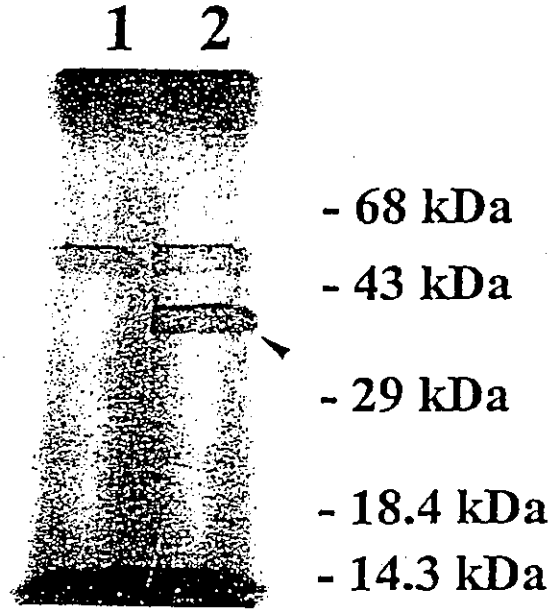


Table 1. Expression of recombinant zebrafish TPST in COS-7 cells.

	Specific activity (pmol·min^{-1}·mg^{-1})	Relative activity (fold)
None	2.03±0.67	1.0
pcDNA3.1	2.07±0.38	1.0
pcDNA3.1-TPST-2 cDNA	11.7±0.20	5.8

Note: Values are means ± SD derived from three determinations.

was detected at 44 °C. As indicated in Table 1, the untransfected COS-7 cell lysate contained a low level of TPST activity due to the endogenous enzyme. It is likely that this endogenous TPST activity may account for the bulk of the activities detected at 37 and 44 °C. It is worthwhile mentioning that although zebrafish are subjected to fluctuation in body temperature in their natural habitat, they have been shown to be best maintained in aquaria heated to 28 °C (Westerfield 2000). Taking into consideration their optimal temperature of growth and the results from the temperature dependence study, we therefore decided to characterize the other enzymatic properties of the zebrafish TPST at 28 °C. Another important property is with regard to the pH optimum of the enzyme. In a pH dependence experiment, the zebrafish TPST displayed a pH optimum of 5.75 (Fig. 5). Previous studies have revealed TPSTs as Golgi enzymes with the catalytic domain being located in the Golgi lumen (Ouyang et al. 1998; Beisswanger et al. 1998; Ouyang and Moore 1998). The acidic pH optimum determined for the zebrafish TPST, therefore, is compatible with the acidic environment in the

Fig. 4. Temperature dependence of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out at designated temperatures under standard assay conditions as described in Materials and methods. The data represent calculated mean values derived from three experiments.

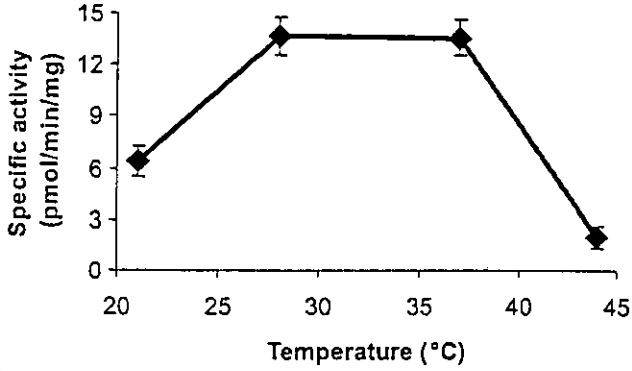
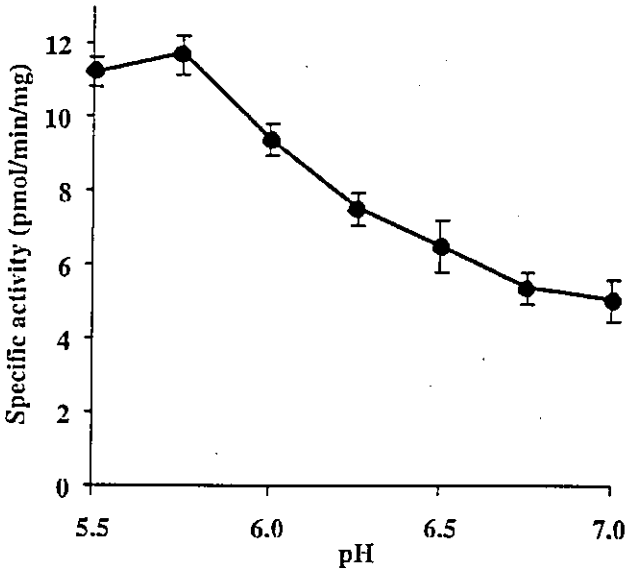
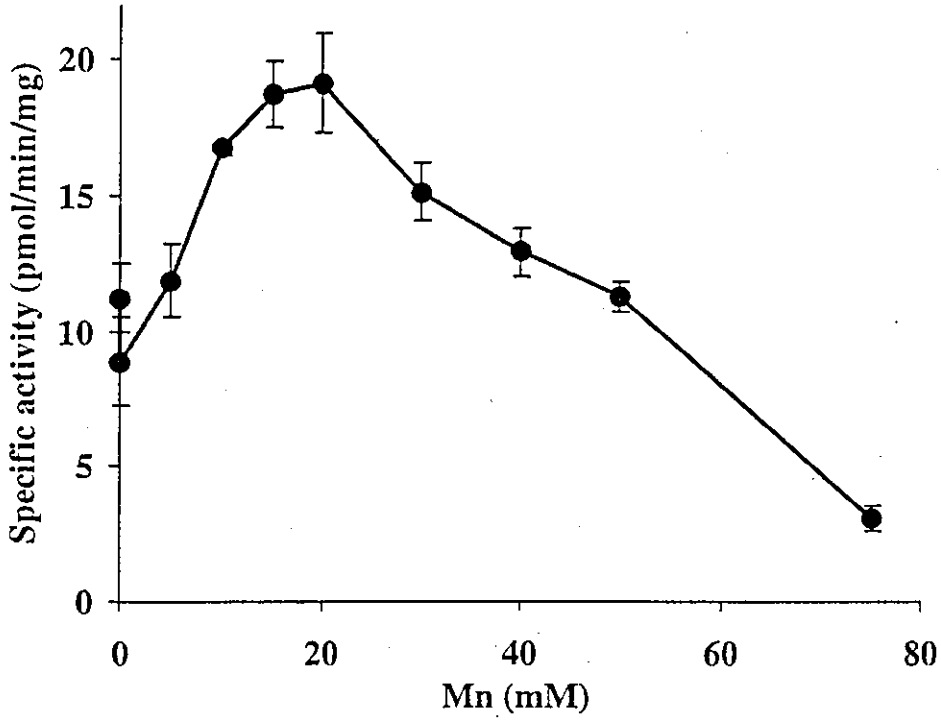


Fig. 5. pH dependency of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out under standard assay conditions, as described in Materials and methods, using different buffer systems. The data represent calculated mean values derived from three experiments.



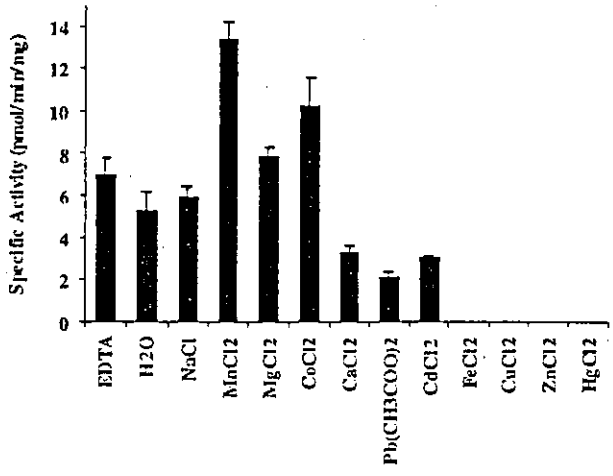
Golgi lumen (Mellman et al. 1986). Previous studies have revealed stimulatory effects of manganese on the activity of some mammalian TPSTs (Lin et al. 1994). We were interested in examining whether manganese is also capable of stimulating the activity of the zebrafish TPST. In a concentration dependence experiment (Fig. 6), it was found that in the presence of 20 mM $MnCl_2$, the sulfating activity of the zebrafish TPST was more than 2.5 times that determined in the absence of $MnCl_2$. At concentrations higher than 50 mM,

Fig. 6. Manganese dependence of the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The enzymatic assays were carried out in the presence of different concentrations of $MnCl_2$ under standard assay conditions as described in Materials and methods. The data represent calculated mean values derived from three experiments.



however, manganese became inhibitory. The mechanism underlying the stimulatory effects of manganese remains to be clarified. We further tested nine other divalent cations for their stimulatory/inhibitory effects on the activity of the zebrafish TPST. As shown in Fig. 7, at a 10 mM concentration, Co^{2+} showed a significant stimulatory effect, while Ca^{2+}, Pb^{2+}, and Cd^{2+} exerted some inhibitory effects. In contrast, the other four divalent cations, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+}, inhibited completely the sulfating activity of the zebrafish TPST.

Fig. 7. Effects of divalent metal cations on the sulfating activity of the zebrafish TPST with the wild-type PSGL-1 N-terminal peptide as substrate. The sulfating activity of the zebrafish TPST was assayed in the presence of different divalent metal cations or NaCl (as a control for the counter ion Cl^-) under standard conditions as described in Materials and methods. The concentration of the divalent metal cations tested was 10 mM, and the concentration of NaCl tested was 20 mM.



Substrate specificity

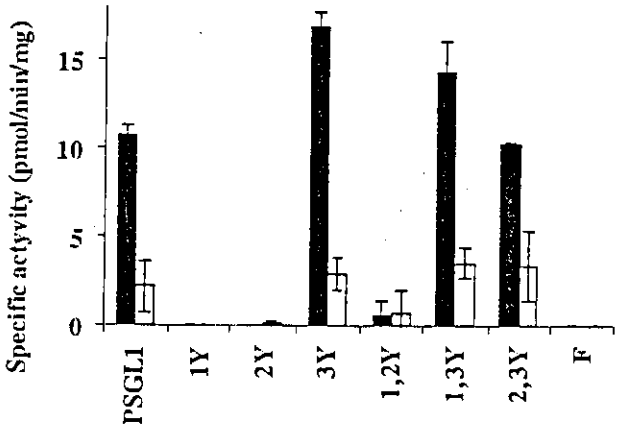
Previous studies have led to a consensus that a key feature of the tyrosine sulfation site is the presence of acidic residues on the N-terminal side of the sulfatable tyrosine (Huttner 1984; Hortin et al. 1986). As shown in Fig. 2, the wild-type PSGL-1 N-terminal peptide contains three tyrosine residues and all of them fulfill such a requirement for sulfation. We were interested in finding out whether the three tyrosine residues of the wild-type PSGL-1 N-terminal peptide are equally or differentially sulfated by the zebrafish TPST. Mutated peptides with one or two of the three tyrosine residues being replaced by phenylalanine were prepared and used as substrates. As shown in Fig. 8, the zebrafish TPST appeared to be most active toward mutated peptides that retained the C-terminal tyrosine residue. In contrast, mutated peptides where this C-terminal tyrosine residue was mutated served as very poor substrates for the zebrafish enzyme even when the N-terminal and (or) middle tyrosine residue(s) were intact. These results indicated clearly the specificity of the zebrafish TPST for the C-terminal tyrosine residue in the wild-type PSGL-1 N-terminal peptide. Kinetic constants for the wild-type and the three mutated PSGL-1 N-terminal peptide that have the C-terminal tyrosine residue intact were determined. Table 2 shows the differential K_m

Table 2. Kinetic constants of zebrafish TPST with wild-type and mutant PSGL1 N-terminal peptides as substrates.

Substrate	K_m (μM)	V_{max} (pmol·min^{-1}·mg^{-1})	V_{max}/K_m (pmol·min^{-1}·mg^{-1}/μM)
PSGL-1 (wild type)	35.9	4.1	0.11
3Y	31.5	42.2	1.34
1,3Y	5.2	17.5	3.37
2,3Y	8.5	10.0	1.18

Note: Values are means ± SD derived from three determinations.

Fig. 8. Substrate specificity of the zebrafish TPST with the wild-type and mutated PSGL-1 N-terminal peptides as substrates. The enzymatic assays were carried out under standard assay conditions as described in Experimental procedures. The data represent calculated mean values derived from three experiments. The solid bars correspond to the activities detected using the zebrafish TPST-expressing COS-7 cell lysate, and the open bars correspond to the activities detected using the control COS-7 cell lysate.



and V_{max} values of these peptide substrates. Interestingly, in the absence of both the N-terminal and the middle tyrosine residues, the C-terminal tyrosine residue was sulfated 10 times faster than in the presence of those two tyrosine residues.

To summarize, we have cloned a zebrafish TPST and expressed and characterized the recombinant enzyme. The recombinant zebrafish TPST exhibited some properties, including acidic pH optimum and stimulation by manganese, that are similar to those previously determined for mammalian TPSTs. Further studies concerning the ontogeny, regulation, and physiological involvement of the zebrafish TPST are now in progress.

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