A common African polymorphism abolishes tyrosine sulfation of human anionic trypsinogen (PRSS2)

Zsolt Rónai[1], **Heiko Witt**[2,§], **Olga Rickards**[3], **Giovanni Destro-Bisol**[4], **Andrew R. M. Bradbury**[5], and **Miklós Sahin-Tóth**[1,*]

[1]*Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, MA, 02118*

[2]*Department of Hepatology and Gastroenterology, Charité, 13353 Berlin, Germany*

[3]*Department of Biology, University of Rome Tor Vergata, 00133 Rome, Italy*

[4]*Istituto Italiano di Antropologia, 00185, Rome, Italy and Department of Human and Animal Biology, University of Rome "La Sapienza", 00185 Rome, Italy*

[5]*Biosciences Division, TA-43, HRL-1, MS M888, Los Alamos National Laboratory, Los Alamos, NM 87545*

Abstract

Human pancreatic trypsinogens undergo post-translational sulfation on Tyr154, catalyzed by the Golgi-resident enzyme tyrosylprotein sulfotransferase 2. Sequence alignments suggest that sulfation of Tyr154 is facilitated by a unique sequence context characteristically found in primate trypsinogens. In search for genetic variants that might alter this sulfation motif, we identified a single nucleotide polymorphism (c.457G>C) in the human anionic trypsinogen gene (*PRSS2*), which changed Asp153 to His (p.D153H). The p.D153H variant is common in subjects of African origin, with a minor allele frequency of 9.2%, whereas it is absent in subjects of European descent. We demonstrate that Asp153 is the main determinant of tyrosine sulfation in anionic trypsinogen, as both the natural p.D153H variation and the p.D153N mutation result in complete loss of trypsinogen sulfation. In contrast, mutation of Asp156 and Glu157 only slightly decrease tyrosine sulfation, whereas mutation of Gly151 and Pro155 are without consequence. With respect to the biological relevance of the p.D153H variant, we found that tyrosine sulfation had no significant effect on the activation of anionic trypsinogen or the catalytic activity and inhibitor sensitivity of anionic trypsin. Taken together with previous studies, the observations suggest that the primary role of trypsinogen sulfation in humans is to stimulate autoactivation of cationic trypsinogen (PRSS1), whereas sulfation of anionic trypsinogen is unimportant for normal digestive physiology. As a result, the p.D153H polymorphism which eliminates this modification could become widespread in a healthy population.

Keywords

tyrosine sulfation; tyrosylprotein sulfotransferase; sulfation motif; trypsinogen mutation; trypsinogen autoactivation

*Correspondence to Miklós Sahin-Tóth, 72 East Concord Street, Evans-433; Boston, MA 02118;, Tel: (617) 414-1070; Fax: (617) 414-1041; E-mail: E-mail: miklos@bu.edu.
§Present address: Department of Pediatrics and Else Kröner-Fresenius-Zentrum (EKFZ), Technische Universität München (TUM), 80804 Munich, Germany

INTRODCUTION

Trypsinogen is the most abundant digestive proenzyme produced by the pancreas. Trypsin, the active form of trypsinogen, plays a central role in digestive physiology as the universal activator of digestive proenzymes. Trypsin also facilitates digestion of dietary proteins by cleaving arginyl and lysyl peptide bonds. In the human pancreas trypsinogen is expressed as three isoforms, which exhibit close to 90% identity in their primary sequence. The three isoenzymes are encoded by separate genes, the serine protease 1 (*PRSS1*) and serine protease 2 (*PRSS2*) genes on chromosome 7 and the serine protease 3 (*PRSS3*) gene on chromosome 9. The biochemical literature traditionally denotes PRSS1 as cationic trypsinogen, PRSS2 as anionic trypsinogen and PRSS3 as mesotrypsinogen. These common names reflect the relative isoelectric points of the zymogens. Cationic and anionic trypsinogen represent >90–97% of trypsinogen in the pancreatic juice [1].

The two major human trypsinogen isoforms undergo post-translational sulfation on Tyr154 [2]. This modification is catalyzed by the Golgi-resident enzyme tyrosylprotein sulfotransferase 2 (TPST2), which is highly expressed in the human pancreas [3–5]. Trypsinogen sulfation seems to be facilitated by the unique sequence context of Tyr154, characteristically found in primate trypsinogens [2]. Mesotrypsinogen is also likely to be sulfated but experimental evidence is lacking. Trypsinogen sulfation was first described by Scheele et al. (1981) who incubated human pancreatic slices with $[^{35}S]Na_2SO_4$ and demonstrated $[^{35}S]$ incorporation into trypsinogens by two-dimensional gel electrophoresis [6]. Subsequently, the crystal structure of native human cationic trypsin indicated the presence of a modification on Tyr154, which was erroneously described as phosphorylation [7]. Conclusive evidence that human trypsinogens are sulfated on Tyr154 came from our recent study; in which we isolated the modified tyrosine amino acid from hydrolyzed pancreatic trypsinogens and showed that it was tyrosine sulfate and not tryrosine phosphate [2]. Furthermore, we demonstrated incorporation of $[^{35}S]$ from $[^{35}S]Na_2SO_4$ into cationic trypsinogen expressed by transfected human embryonic kidney (HEK) 293T cells, and labeling was abolished by mutation of Tyr154 to Phe. We also found that tyrosine sulfation stimulated autoactivation of human cationic trypsinogen. More recent studies using mass spectrometry confirmed tyrosine sulfation of pancreatic trypsinogens and also showed that trypsinogen expressed by tumors is not sulfated [8]. It is interesting to note that pancreatic trypsinogens appear completely sulfated, as judged by two-dimensional gel electrophoresis, native gel electrophoresis or mass spectrometry [2,6,7,8].

In the present study we characterized the sequence determinants that promote sulfation of Tyr154 on human trypsinogens. Furthermore, we identified the c.457G>C (p.D153H) alteration in *PRSS2* as a common polymorphic variant in subjects of African origin. We demonstrated that Asp153 is the main determinant of tyrosine sulfation in anionic trypsinogen, and the p.D153H variation causes loss of trypsinogen sulfation without any appreciable effect on anionic trypsinogen function.

METHODS

Study subjects

The study was approved by the medical ethical review committee of the Charité University Hospital. All patients gave informed consent for genetic analysis. The study population included 1,305 healthy German subjects and 198 German patients suffering from chronic pancreatitis with either idiopathic or hereditary etiology. The clinical diagnosis of chronic pancreatitis was based on criteria published previously [9]. To determine the frequency of p.D153H in non-Europeans, we studied 201 Native Americans from Ecuador and 946 unrelated individuals of African descent originating from Benin (n=161, 52 females, 106 males, 3 with

unknown gender, age 3–80 yrs, unknown 88, median 31 yrs; mean 31 yrs); Cameroon (n=410, 303 females, 107 males, age 10–79 yrs, unknown 1, median 28 yrs, mean 32 yrs), Ethiopia (n=155, 80 females, 75 males, age unknown) and Ecuador (n=220, 107 females, 113 males; age 6–80 yrs, unknown 2, median 31 yrs, mean 34 yrs). All non-European subjects were recruited for genetic population studies between 1986 and 1990 (subjects originating from Cameroon) and 1990–1998 (subjects originating from Benin, Ethiopia and Ecuador).

Genotyping

We performed melting curve analysis to detect the p.D153H variant using a pair of fluorescence resonance energy transfer (FRET) probes and the LightCycler (Roche Diagnostics). Primers and PCR conditions for exon 4 of *PRSS2* were published previously [9]. The p.D153H sensor probe was 5'-LC-TCT GGG TAG TGG GCT GTG AGG AT-ph (LC, LightCycler Red 705 attached to 5' terminus; ph, 3' phosphate) and the anchor probe was 5'-CAG CAC AGG AGC ATC CAG GCA CTG CAG CTC-FL (FL, 5,6-carboxyfluorescein attached to 3'-O-ribose). Primers and probes were designed and synthesized by TIB MOLBIOL (Berlin, Germany).

Plasmid construction and mutagenesis

The *PRSS2* coding sequence (GenBank NM_002770.2) was PCR amplified from GeneStorm clone H-M27602M (Invitrogen) using the Hu2-XbaI sense primer (5' TTT TTT TCT AGA CAC ACT CTA CCA CCA TGA ATC TAC TTC TGA TCC T 3') and the Hu2-BamHI antisense primer (5' TTT TTT GGA TCC GGA CCA GGG GCT TTA GCT GTT GGC AGC TAT G 3'). The PCR product was digested with XbaI and BamHI and ligated into the pcDNA 3.1(−) expression vector. DNA sequencing revealed that compared to the GenBank reference entry our *PRSS2* sequence contained a known polymorphic variant in Ala90 (c.270A>G) and an unexpected mutation in codon 91 (c.272C>T; p.A91V), which was also present in the GeneStorm clone used as template for the PCR amplification. This mistake was subsequently fixed by overlap extension PCR mutagenesis. Missense mutations in the sulfation motif of PRSS2 were generated by overlap extension PCR and were cloned into the pcDNA 3.1(−) expression plasmid.

Construction of the pcDNA3.1(−) expression plasmid carrying the *PRSS1* gene was described previously [10].

The protein-coding portion of the TPST2 cDNA (GenBank NM_003595.3) was PCR amplified from IMAGE clone #4857366 (GenBank BC017509) using the TPST2 XhoI sense primer (5'-CCC TGC CTC GAG GCC ACC ATG GGC CTG TCG GTG CGG AGG -3') and the TPST2 BamHI antisense primer (5'- GAG ATC GGA TCC TCA CGA GCT TCC TAA GTG GGA GGA GGT -3'). The sense primer changes the 5' upstream sequence (CCCAGC>**G**CCA**CC**) and codon 2 (CGC>**G**GC; Arg2>Gly) to create an optimal Kozak sequence. The PCR product was digested with XhoI and BamHI and cloned into the pcDNA3.1(−) plasmid.

The coding region of the tyrosylprotein sulfotransferase 1 (TPST1) cDNA (GenBank NM_003596.2) was PCR amplified from an Incyte full length cDNA clone (clone ID LIFESEQ3598404) using the TPST1 XhoI sense primer (5'-CAA GAT CTC GAG GCC ACC ATG GTT GGA AAG CTG AAG CAG AAC TTA CTA-3') and the TPST1 BamHI antisense primer (5'-CTC CTG GGA TCC CTA CTC CAC TTG CTC AGT CTG TGG-3'). The sense primer changes the 5' upstream sequence to an optimal Kozak sequence (ATCAAG>**GC**CA**CC**). The PCR product was digested with XhoI and BamHI and cloned into the pcDNA3.1(−) plasmid.

Cell culture and transfection

To study trypsinogen sulfation, we have used HEK 293T cells, because this cell line does not express endogenous trypsinogens. HEK 293T cells were cultured in 6-well tissue culture plates (10^6 cells per well) in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin / streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were carried out in a total volume of 2 mL DMEM using 10 µL Lipofectamine 2000 (Invitrogen) and 2 µg wild type or mutant PRSS2 plasmid and 0.5 µg TPST1 or TPST2 plasmid, unless indicated otherwise. After overnight incubation at 37 °C, the transfection medium was replaced with 2 mL OptiMEM containing 1 mM benzamidine to prevent autoactivation of secreted trypsinogen.

Expression and purification of anionic trypsinogen

For larger scale expression of sulfated and non-sulfated anionic trypsinogen, HEK 293T cells were cultured in 75 cm^2 flasks as described above and transfections were performed in 20 mL DMEM using 75 µL Lipofectamine 2000 with 24 µg PRSS2 plasmid with or without 6 µg TPST2 plasmid. Cells were incubated overnight at 37 °C, and the transfection medium was then replaced with 20 mL OptiMEM containing 1 mM benzamidine. Conditioned media were collected after 32 h, supplemented with Tris-HCl (pH 8.0) to 0.1 M final concentration and anionic trypsinogen was purified by ecotin affinity chromatography as described earlier [11].

Expression and purification of cationic trypsinogen

The procedure to express and purify sulfated and non-sulfated human cationic trypsinogen was essentially the same as described above for anionic trypsinogen with the following modifications. We found that HEK 293T cells express wild type cationic trypsinogen to 5–10-fold lower levels than anionic trypsinogen. To increase expression, we used the p.K237D/ p.N241D cationic trypsinogen variant, which is expressed almost 3-fold better than wild type (E. Kereszturi, M. S.-T., unpublished observation). In contrast to anionic trypsinogen, cationic trypsinogen was partly sulfated by HEK 293T cells, therefore, we included 50 mM Na-chlorate in the growth medium when our aim was to purify non-sulfated cationic trypsinogen. Because cationic trypsinogen autoactivated in the growth medium even in the presence of 1 mM benzamidine, we collected the conditioned medium every 8 h and replaced it with fresh OptiMEM medium.

Trypsin activity assay

Aliquots (10 µL) of conditioned media were mixed with 1 µL 1 M Tris-HCl (pH 8.0), 1 µL 10 mM $CaCl_2$ and 1 µL human enteropeptidase (1.4 µg/mL; R&D Systems, Minneapolis, MN) and incubated at 37 °C for 1 h. Trypsin activity was then measured using the synthetic chromogenic substrate, *N*-benzyloxycarbonyl(CBZ)-Gly-Pro-Arg-*p*-nitroanilide at 0.14 mM final concentration. One-minute time courses of *p*-nitroaniline release were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0) and 1 mM $CaCl_2$ at room temperature in a Spectramax Plus 384 microplate reader (Molecular Devices).

Native gel electrophoresis

To analyze trypsinogens by polyacrylamide gel electrophoresis under non-denaturing conditions, 400 µL conditioned media were precipitated with 10% trichloroacetic acid. Samples were centrifuged at 13,200 rpm for 10 min in an Eppendorf microcentrifuge and pellets were resuspended in native loading buffer devoid of SDS or reducing agents. Samples were then electrophoresed on 13% Tris–glycine gels. The gel apparatus was placed in ice and gels were run for 2–2.5 h at 35 mA constant current. Proteins were visualized by Coomassie Blue staining.

SDS-PAGE and Western blotting

Conditioned media were precipitated with 10% trichloroacetic acid (final concentration), resuspended in Laemmli sample buffer with 100 mM dithiothreitol, heat-denatured at 95 °C for 5 min and electrophoresed on 15% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue R. For immunodetection of sulfated trypsinogens, 30 µL conditioned media were precipitated and electrophoresed on 15% SDS-polyacrylamide gels. The proteins were then transferred onto an Immobilon-P membrane (Millipore, Billerica, MA) at 300 mA for 1.5 h. The membrane was blocked overnight at 4 °C with 5% milk powder dissolved in PBS with 0.1% Tween 20 (PBST) and then incubated in the same solution with anti-tyrosine-sulfate IgG [12,13] added at a dilution of 1:2,000 for 1 h at room temperature. After three 10 min washes with PBST, the membrane was blocked again for 1 h at room temperature and the secondary antibody (horse-radish peroxidase (HRP) conjugated rabbit polyclonal antibody against human IgG; Abcam, catalog# ab6759) was applied at a dilution of 1:10,000 for 1 h. After three 10 min PBST washes, HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

The p.D153H variant alters the sulfation motif in human anionic trypsinogen

Recognition of the target Tyr amino acid by sulfotransferases is aided by the sequence context, commonly referred to as the sulfation motif. Although there is no consensus sequence that describes this motif, certain amino acids are strongly preferred or excluded, as summarized in Fig 1 [14–17]. Thus, an acidic amino acid (Asp or Glu) is frequently found adjacent to the sulfated Tyr, and at least three acidic residues are usually present within the five amino acids flanking the Tyr on either side. Turn-forming amino acids Pro and Gly are often seen in sulfation motifs. Cysteine and basic amino acids are generally excluded, and hydrophobic amino acids are rare. With the exception of the Cys at position +6, the trypsinogen sulfation sequence conforms to these requirements. In search of the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) for genetic variants that might alter the trypsinogen sulfation motif, we identified the c.457G>C (rs1804564a) single nucleotide polymorphism (SNP) in the human anionic trypsinogen gene (*PRSS2*), which changes Asp153 to His (p.D153H). To determine the prevalence of this variant, we screened 1,305 healthy German subjects and 198 German patients with chronic pancreatitis. Surprisingly, we found the p.D153H variant only in a single healthy individual whose mother was German and the father was of African descent. Next, we analyzed 946 subjects of African origin from four different countries and 201 Native Americans from Ecuador and found the p.D153H variant with an average minor allele frequency of 9.2% in subjects of African origin, but not in Native Americans (Table 1). Thus, p.D153H is a common African polymorphism. The p.D153H variant changes the Asp residue closest to Tyr154 within the putative sulfation motif, and thus it is expected to have a significant impact on tyrosine sulfation of anionic trypsinogen.

Tyrosine sulfation of human anionic trypsinogen by TPST2 in HEK 293T cells

To study tyrosine sulfation of anionic trypsinogen by TPST2 in a quantitative manner, we established a cellular system in which trypsinogen and sulfotransferase are co-expressed from separate plasmids, thus allowing independent manipulation of either protein. HEK 293T cells were co-transfected with 2 µg PRSS2 plasmid and increasing amounts (0, 2.5, 8, 25, 80, and 250 ng) of TPST2 plasmid. Conditioned media containing secreted anionic trypsinogen was analyzed by native polyacrylamide gel electrophoresis. Because tyrosine sulfation introduces an extra negative charge, sulfated anionic trypsinogen migrates faster on native gels than its nonsulfated counterpart. As Fig 2A demonstrates, with increasing amounts of TPST2 plasmid, the slower mobility non-sulfated anionic trypsinogen form is gradually converted to the higher mobility sulfated form. Complete sulfation was observed with 250 ng TPST2 plasmid used for

co-transfection. The experiment also demonstrates that endogenous tyrosylprotein sulfotransferase activity in HEK 293T cells is low, as essentially no tyrosine sulfation occurs in the absence of added TPST2 plasmid. To confirm these findings with an independent method, western blot analysis was carried out using an antibody that specifically recognizes tyrosine sulfate [12,13]. Fig 2B demonstrates that in the absence of TPST2 plasmid no immunosignal was detected, whereas inclusion of increasing amounts of TPST2 plasmid resulted in increasing levels of immunoreactivity, which perfectly correlated with the amount of sulfated anionic trypsinogen observed on native gels. Although data are not shown, co-transfection with higher amounts ($\geq 1\,\mu g$) of TPST2 plasmid resulted in a marked suppression of the secretion of anionic trypsinogen. This phenomenon was unrelated to the sulfation of anionic trypsinogen, as production of β-galactosidase was also suppressed by co-transfection with TPST2 under similar conditions. On the basis of these results, in our subsequent experiments we chose to use $0.5\,\mu g$ TPST2 plasmid which provided complete sulfation without affecting protein secretion.

Asp153 is the critical determinant of tyrosine sulfation in anionic trypsinogen

To investigate the significance of the p.D153H variant and the amino acids neighboring Tyr154 in tyrosine sulfation, we subjected this region to mutational analysis. We mutated Tyr154 to Phe (p.Y154F), Asp153 to His (p.D153H) and Asn (p.D153N), Asp156 to Asn (p.D156N) and Glu157 to Leu (p.E157L), because in the majority of mammalian trypsinogens Leu is found at this position. Furthermore, Gly151 and Pro155 were mutated to Ala (p.G151A and p.P155A). HEK 293T cells were transfected with wild type and mutant PRSS2 plasmids and co-transfected with TPST2 plasmid. Conditioned media were analyzed by reducing SDS-PAGE, trypsin activity assays and western blotting. The Coomassie Blue stained gel in Fig 3A demonstrates that wild type and mutant trypsinogens were secreted to similar levels. Note that on SDS-PAGE the sulfated and non-sulfated forms are not separated due to the denaturing conditions. Trypsin activity of wild type and mutant trypsinogens was also comparable when measured after activation with enteropeptidase, indicating that the mutations have no detrimental effect on the catalytic properties of the enzyme (Fig 3B). On the other hand, western blot analysis revealed that the trypsinogen mutants were sulfated to an extent that varied considerably among the mutants (Fig 3C). As expected, removal of the phenolic hydroxyl from Tyr154 by the p.Y154F mutation abolished sulfation. A striking loss of sulfation was also observed when the negative charge on the adjacent Asp153 residue was eliminated by mutations p.D153N or p.D153H. Mutants p.D156N and p.E157L exhibited decreased sulfation, indicating that although Asp156 and Glu157 are not essential, the two acidic residues increase the efficiency of this modification. On the other hand, sulfation of mutants p.G151A and p.P155A was unchanged relative to wild type anionic trypsinogen.

To confirm that the changes detected in immunoreactivity reflect different sulfation levels, we analyzed tyrosine sulfation of the trypsinogen mutants on native gels. In this experiment, PRSS2 mutants were expressed in HEK 293T cells with or without co-transfection with TPST2 plasmid. Conditioned media were then electrophoresed under non-denaturing conditions on Tris-glycine gels. As shown in Fig 3D, the results were in perfect agreement with the western blots shown in Fig 3C. Thus, wild type trypsinogen and mutants p.G151A and p.P155A were completely sulfated; mutants p.D156N and p.E157L were partially sulfated, whereas no sulfation was detectable on mutants p.D153N, p.D153H and p.Y154F.

Although TPST2 is the sulfotransferase isoform responsible for trypsinogen sufation in the pancreas [3,5], we wondered how well TPST1 would catalyze sulfation of the trypsinogen mutants. As judged by western blot analysis, co-transfection with TPST1 plasmid resulted in sulfation of wild type anionic trypsinogen, which was abolished by mutations p.D153N, p.D153H and p.Y154F, and moderately decreased by mutations p.D156N and p.E157L

(Supplementary Figure S1). The results indicate that determinants of the sulfation motif on anionic trypsinogen are recognized by TPST1 and TPST2 in a similar manner, suggesting that natural selection of TPST2 overexpression in the human pancreas was not driven by substrate specificity.

Biochemical characteristics of sulfated and non-sulfated PRSS2

We previously demonstrated that native, sulfated cationic trypsinogen purified from human pancreatic juice exhibited faster autoactivation than its non-sulfated recombinant counterpart purified from *Escherichia coli* [2]. These observations suggested that tyrosine sulfation can facilitate trypsinogen activation. To confirm and extend these experiments, we purified sulfated and non-sulfated anionic and cationic trypsinogen from conditioned media of HEK 293T cells transfected with PRSS1 and PRSS2 plasmids with or without TPST2 plasmid. Autoactivation experiments were carried out at pH 8.0, in the presence of 1 mM and 10 mM $CaCl_2$ (Fig 4). As reported previously, autoactivation of cationic trypsinogen was stimulated by tyrosine sulfation, particularly in the high calcium milieu. In contrast, autoactivation of anionic trypsinogen was slightly inhibited by tyrosine sulfation.

We also compared the activation, catalytic activity, inhibitor sensitivity and degradation of sulfated and non-sulfated anionic trypsin(ogen) in a variety of biochemical assays, which are detailed and illustrated in the online Supplementary Material, Supplementary Table S1 and Supplementary Figures S1–S6. Small differences were observed in inhibitor sensitivity and degradation by chymotrypsin C; however, none of these appeared biologically significant.

DISCUSSION

Tyrosine-O-sulfation is a ubiquitous post-translational modification of secretory and membrane proteins [18,19]. It is catalyzed by two isoforms of tyrosylprotein sulfotransferase, TPST1 and TPST2, which are localized in the trans-Golgi system [3,4,20]. The amino acid sequences of the two TPST enzymes are 64% identical and their substrate specificity overlaps, although affinity to some synthetic substrates is slightly different [21]. Both are expressed in all tissues at varying levels, in humans the highest TPST1 mRNA expression was measured in the testis, whereas the TPST2 transcript is most abundant in the pancreas [3,5,20,21]. To address the biological role of tyrosine sulfation, mouse strains deficient in TPST1, TPST2 or both isoenzymes were engineered [22–24]. Selective disruption of the TPST1 gene resulted in reduced body weight and increased post-implantation fetal death, whereas TPST2 knock-out mice were infertile, hypothyroid, and exhibited delayed growth. Double knock-outs died in the early postnatal period with signs of cardiopulmonary insufficiency. The undersulfated target proteins responsible for the phenotypic changes in the knock-out mice have not been identified yet. Hypothyroidism was also observed in a dwarf mouse strain which carried the natural p.H266Q mutation in the *TPST2* gene [25]. The authors proposed that defective sulfation of the thyroid-stimulating hormone receptor was the direct cause of the phenotype.

Biochemical studies on a handful of tyrosine-sulfated proteins led to the current consensus that tyrosine sulfation modulates extracellular protein–protein interactions. For example, sulfation of P-selectin glycoprotein ligand-1 present on leukocytes is necessary for binding to P-selectin on the endothelial cells during adhesion [26]. Similarly, tyrosine sulfation of endoglycan on endothelial cells is required for binding to L-selectin on lymphocytes during homing [27]. The sulfation of the three tyrosine residues on platelet glycoprotein Ibα promotes its interaction with von Willebrand factor, which is essential for the initiation of hemostasis [28]. Sulfation of Tyr1680 in coagulation factor VIII is important for binding to von Willebrand factor, and the p.Y1680F missense mutation (Y1699F when counted from the initiator methionine) causes a mild form of hemophilia A [29,30]. Finally, sulfation of tyrosine residues in the N terminus

of CC chemokine receptor 5 promotes chemokine binding and facilitates HIV-1 entry [31, 32].

In the present study we characterized the amino acid requirements for tyrosine sulfation of human trypsinogens. On the basis of sequence alignments, we previously suggested that human trypsinogens are sulfated because of a special sequence motif around Tyr154 [2]. Mutational analysis of this sulfation motif confirmed that Asp153 is the critical determinant, but Asp156 and Glu157 are also important for efficient trypsinogen sulfation. In contrast, the turn-forming residues Gly151 and Pro155 within the sulfation motif are dispensable. Our findings are in agreement with published studies demonstrating that acidic residues, particularly in the immediate vicinity of the target Tyr, are important for tyrosine sulfation (see Fig 1) [14–17]. Since Asp153 is absent in most vertebrate trypsinogens, our results confirm that trypsinogen sulfation is largely primate specific. Interestingly, trypsinogen genes in the recently sequenced genome of the laboratory opossum (*Monodelphis domestica*) contain amino acids that correspond to Asp153, Tyr154, and Asp156, although Glu157 is missing. Furthermore, a newly sequenced platypus (*Ornithorhynchus anatinus*) trypsinogen gene also contains amino acids corresponding to Asp153 and Tyr154, which can be viewed as the minimal requirement for trypsinogen sulfation. In all likelihood, the presence of the sulfation motif in the trypsinogen genes of monotremes, marsupials and primates is the result of convergent evolution. Alternatively, trypsinogen sulfation may have begun to evolve in their common ancestor and was subsequently lost in all placental mammals with the exception of primates.

We identified a common polymorphism in the *PRSS2* gene that altered Asp153 and abolished sulfation of anionic trypsinogen. The p.D153H variant was found in subjects of African origin with an average minor allele frequency of 9.2%, whereas it was absent in subjects of European descent or in Native Americans originating from Ecuador. Previously, variants of the *PRSS1* gene were shown to cause hereditary chronic pancreatitis and a variant of the *PRSS2* gene was shown to afford protection against chronic pancreatitis [9,33]. Thus, variants in human trypsinogen genes can play important pathological roles in defining the risk for chronic pancreatitis. Due to the unavailability of African subjects with chronic pancreatitis, we could not study potential disease association of the p.D153H variant, but the high frequency of the alteration suggests that p.D153H cannot be a significant risk factor for chronic pancreatitis, which is a relatively rare disease with ~0.01–0.1% prevalence.

The functional significance of post-translational sulfation in human trypsinogens was first addressed in our previous study where we showed that autoactivation of human cationic trypsinogen was stimulated by tyrosine sulfation [2]. These observations have been confirmed in the present study. Surprisingly, however, the stimulatory effect of sulfation on autoactivation proved to be isoform specific as we found that autoactivation of anionic trypsinogen was slightly inhibited by this modification. Furthermore, catalytic activity and inhibitor sensitivity of anionic trypsin were also unaffected by tyrosine sulfation. Taken together, we conclude that the functional role of trypsinogen sulfation in humans is to stimulate autoactivation of cationic trypsinogen, whereas sulfation of anionic trypsinogen appears to be inconsequential, at least with respect to autoactivation and other enzymatic properties studied here. The prevalence of the p.D153H variant in subjects of African origin also seems to support the contention that tyrosine sulfation of anionic trypsinogen is redundant; therefore, a polymorphism which abolishes this modification could become widespread in a healthy population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium

HEK, human embryonic kidney

mAU, milli-absorbance units

OptiMEM, reduced serum medium

PAGE, polyacrylamide gel electrophoresis

PBST, phosphate buffered saline with 0.1% Tween 20

PRSS1, serine protease 1, human cationic trypsinogen

PRSS2, serine protease 2, human anionic trypsinogen

PRSS3, serine protease 3, human mesotrypsinogen

SDS, sodium dodecyl sulfate

TPST1, tyrosylprotein sulfotransferase 1

TPST2, tyrosylprotein sulfotransferase 2.

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Amino acids in sulfation motifs			L −6	S −5	S −4	G −3	A −2	D −1	Y 0	P +1	D +2	E +3	L +4	Q +5	C +6
Acidic	≥ 1	−2 to +2						**+**			**+**				
Acidic	≥ 3	−5 to +5						**+**			**+**	**+**			
Basic	≤ 1	−5 to +5													
Hydrophobic	≤ 3	−5 to +5											**+**		
Cysteine	= 0	−7 to +7													⊖
Turn forming	≥ 1	−7 to +7				**+**				**+**					

Figure 1.
The sulfation motif in human anionic trypsinogen. The *PRSS2* sequence between amino acids 148 and 160 is shown; the sulfated Tyr154 is denoted as position 0. The number and position of amino acids characteristic of sulfation motifs are shown according to [14–17]. Positive signs indicate amino acids in anionic trypsinogen that conform to the typical sulfation motifs, whereas the ⊖ sign indicates a cysteine residue that is in conflict.
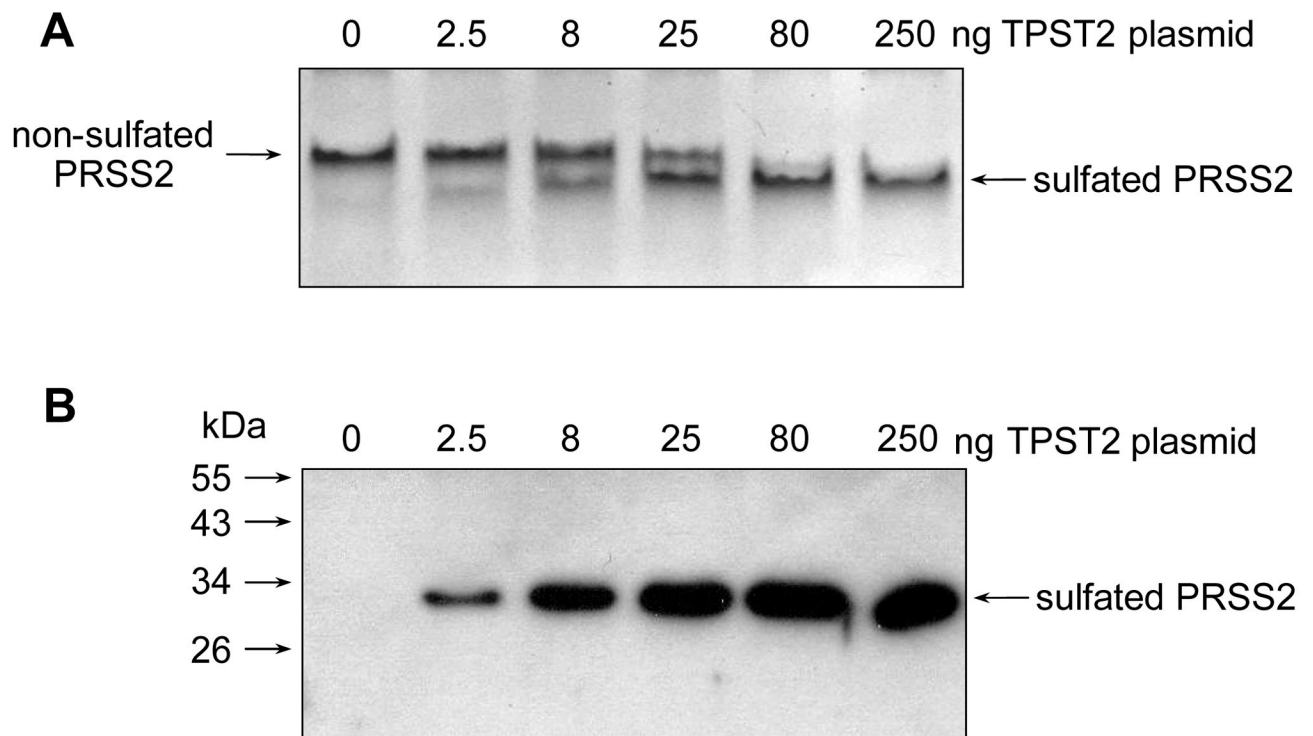
A

B

Figure 2.
Tyrosine sulfation of human anionic trypsinogen in HEK 293T cells by TPST2. Cells were co-transfected with 2 µg PRSS2 plasmid and the indicated amount of TPST2 plasmid. Conditioned media were collected 48 h after transfection. **A.** Aliquots (400 µL) of conditioned media were precipitated with 10% trichloroacetic acid, resuspended in native sample buffer, electrophoresed on a 13% native polyacrylamide gel, and stained with Coomassie Blue. **B.** Aliquots (30 µL) of conditioned media were analyzed by western blotting using an antibody against tyrosine sulfate, as described in *Methods*.
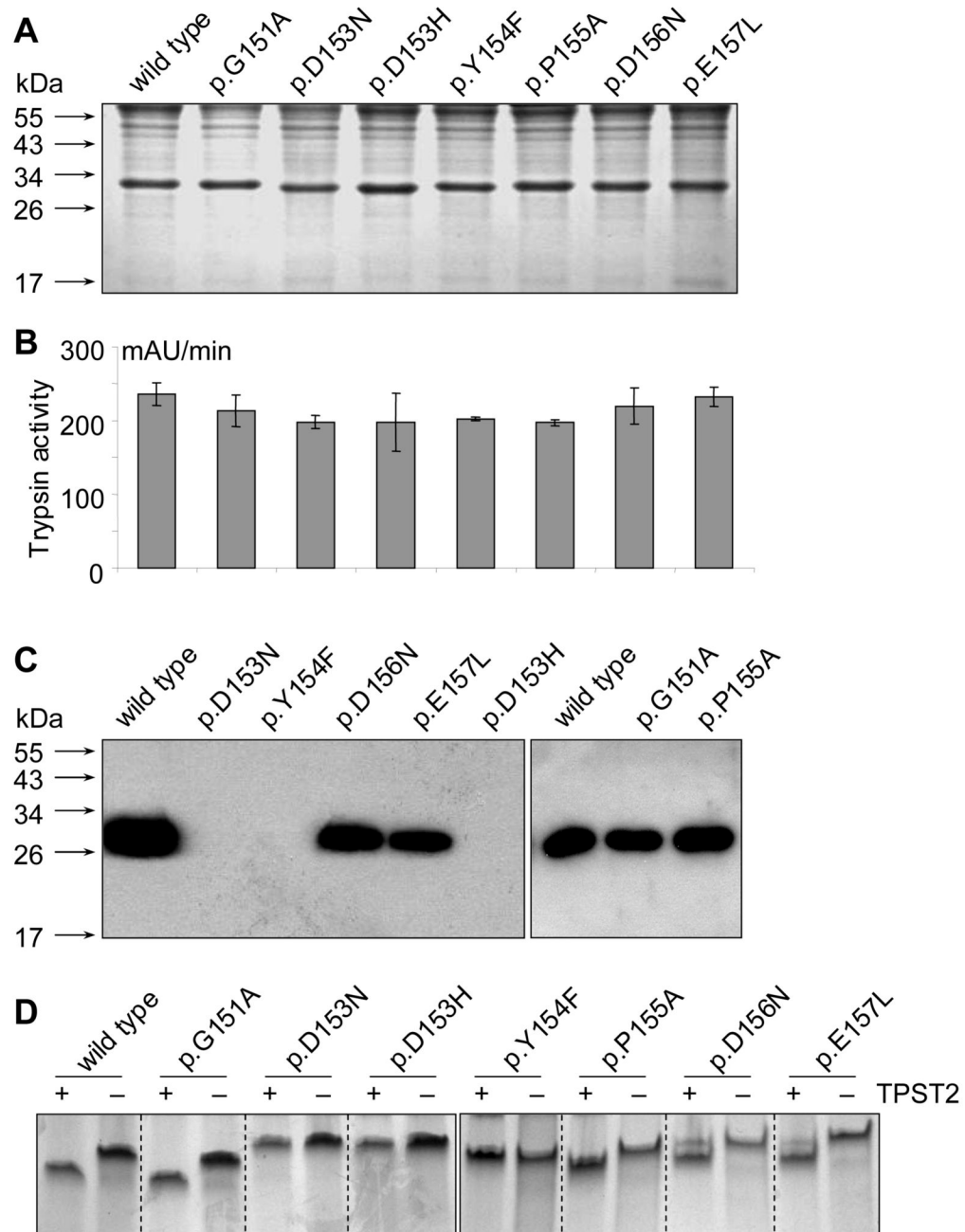
Figure 3.
Mutational analysis of the sulfation motif in human anionic trypsinogen. HEK 293T cells were transfected with 2 µg PRSS2 plasmid carrying the indicated mutation and 0.5 µg TPST2 plasmid. Conditioned media were collected 48 h after transfection. **A.** Aliquots (200 µL) of conditioned media were precipitated with 10% trichloroacetic acid and analyzed by reducing SDS-PAGE and Comassie Blue staining, as described in *Methods*. **B.** Aliquots (10 µL) of conditioned media were activated by enteropeptidase, and trypsin activities were determined as described in *Methods*. The average (± SEM) of three measurements is shown. **C.** Aliquots (30 µL) of conditioned media were analyzed by western blotting using an antibody against tyrosine sulfate, as described in *Methods*. **D.** Aliquots (400 µL) of conditioned media were

precipitated with 10% trichloroacetic acid, resuspended in native sample buffer, electrophoresed on a 13% native polyacrylamide gels, and stained with Coomassie Blue.
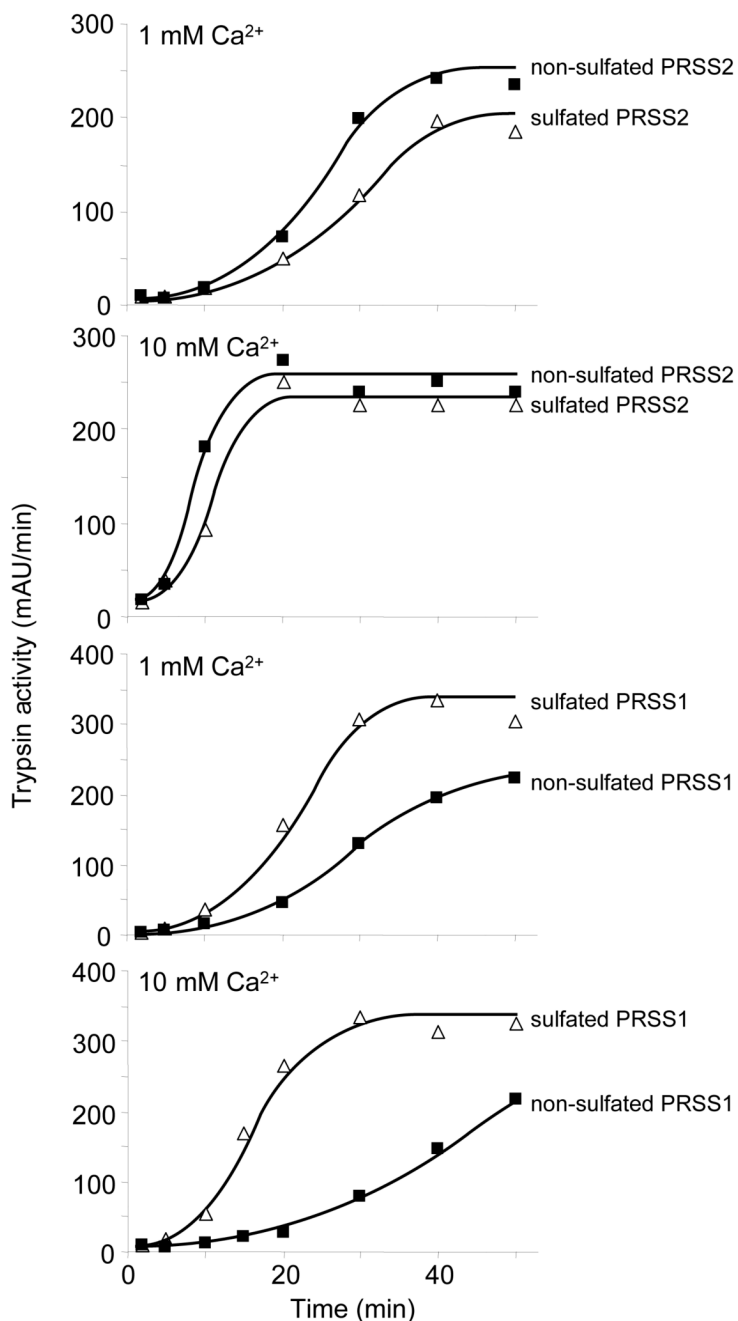
Figure 4.
Autoactivation of sulfated and non-sulfated forms of human anionic and cationic trypsinogens. Trypsinogens were incubated at 1.5 µM concentration in 100 mM Tris-HCl (pH 8.0), with 1 mM or 10 mM CaCl$_2$, in 100 µL final volume, at 37 °C. Autoactivation reactions were initiated by addition of 10 nM trypsin. Trypsin activity was measured from 2 µL aliquots with 0.14 mM *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide substrate (final concentration) as described in *Methods*. Trypsin activity was expressed as the slope of the linear portion of the absorbance increase in mAU/min. The figure shows representative results of three independent experiments using two different trypsinogen preparations. The solid lines through the data points were drawn by polynomial interpolation.

Table 1

Genotype distribution of the p.D153H polymorphism in subjects of African origin from four different countries. *N*, number of individuals in each group. The allele frequency of p.D153H is 9.2%.

	N	Homozygote wild type	Heterozygote	Homozygote p.D153H
Benin	161	71.4%	25.5%	3.1%
Cameroon	410	84.1%	14.6%	1.2%
Ecuador	220	82.3%	16.8%	0.9%
Ethiopia	155	92.3%	7.7%	0.0%
Total	**946**	**82.9%**	**15.9%**	**1.3%**