New Consensus Features for Tyrosine O-Sulfation Determined by Mutational Analysis*

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Tyrosine sulfation is an ubiquitous modification of proteins synthesized along the secretory pathway. It enhances protein-protein interactions and may be necessary for the bioactivity of secreted proteins and peptides. To predict tyrosine sulfation, a consensus has been proposed based on sequence comparisons of known substrates and on in vitro studies using synthetic peptides. This consensus predicts the presence of acidic residues on the amino-terminal side of the target tyrosine as the key feature. Using site-directed mutagenesis, we have examined the role of residues neighboring the sulfation site of an intact protein, human progastrin, in vivo. The results show that the charge of the residue in the amino-terminal position (−1) of the tyrosine is critical and can be neutral or acidic, whereas a basic residue abolishes sulfation. In addition, the degree of sulfation is influenced by the residues in positions −2 and −3. Hence, surprisingly a basic residue in position −2 enhances sulfation. Our data suggest a considerably broader range of substrates for the tyrosylprotein sulfotransferase (TPST), a transmembrane enzyme, than hitherto assumed and that the tyrosylprotein sulfotransferase is cell-specifically expressed.

Tyrosine sulfation plays a decisive role in receptor binding of the peptide hormone cholecystokinin (CCK) (6), interactions between hirudin and thrombin (7, 8), von Willebrand factor and Factor VIII (9), and P-selectin with the P-selectin glycoprotein ligand-1 (10, 11). The incomplete sulfation has been ascribed to the lack of an acidic residue in position −1, which contains an alanine. Five glutamic acid residues amino-terminally hereof, however, are thought to direct sulfation (see Table I for sulfation site structure). In support of this hypothesis, we have shown that substitution of alanine in position −1 with aspartate completes sulfation (19).

The aim of our study was to identify additional features determining the sulfation pattern for progastrin. We have used site-directed mutagenesis of the human gastrin gene followed by transient expression in an endocrine cell line known to sulfate gastrin-17 and gastrin-34 partially after transfection (27). The results show that the neighboring residues contribute only moderately to sulfation and that a high degree of sulfation is obtained despite dramatic changes in the charge distribution of the neighboring residues. One critical position appears to be −1, which should be a neutral or acidic residue. Surprisingly, we found that not only are basic residues allowed around sulfation sites, but they even enhance sulfation. Hence, our data suggest that secondary structures may play an important role as determinants of tyrosine sulfation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The hamster insulinoma cell line HIT (28) was cultured at 10% CO₂ and 37 °C in Dulbecco’s modified Eagle’s medium (with Glutamax, 5 mM glucose) supplemented with 10% FBS. This cell line stably expresses high concentrations of the human progastrin precursor (29) and thus allows for the evaluation of sulfation of this protein. Progastrin is synthesized mainly in G-cells of the antral mucosa. Progastrin matures to gastrin-17 and gastrin-34, the predominant bioactive forms, which are carboxy-amidated and partially tyrosine sulfated (Fig. 1). Receptor binding of gastrin and its homologue, CCK, requires carboxy-amidation, but contrary to CCK, sulfation of gastrin is not necessary for binding to the gastrin (CCK-B) receptor. Sulfation has, however, been shown to affect both intracellular processing of progastrin (19) and peptide degradation (20–22). Mammalian gastrins display a complex expression pattern with tissue-specific proteolytic processing and sulfation. However, since plasma gastrin originates mainly from antral G-cells where sulfation is incomplete, circulating gastrin is only partially sulfated (20, 23–26). The incomplete sulfation has been ascribed to the lack of an acidic residue in position −1, which contains an alanine. Five glutamic acid residues amino-terminally hereof, however, are thought to direct sulfation (see Table I for sulfation site structure). In support of this hypothesis, we have shown that substitution of alanine in position −1 with aspartate completes sulfation (19).

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The abbreviations used are: TPST, tyrosylprotein sulfotransferase; CCK, cholecystokinin; ab, antibody.
fetal calf serum and 1% penicillin-streptomycin, all purchased from Life Technologies. Cells were split 1 day before transfection and transiently transfected using the modified calcium phosphate method as described (29, 30).

Expression Vector Constructions and Site-directed Mutagenesis—The wild type gastrin expression vector has previously been described (27). Site-directed mutagenesis was performed in a PCR with primers carrying the mutations using the Pwo polymerase (Boehringer Mannheim) and cloned directly into this expression vector except for the KAY-gastrin mutant which was constructed using the method of Kunkel (31), both methods as described previously (27). DNA cloning procedures were as described (30); enzymes were obtained from Boehringer Mannheim or Promega and used according to the manufacturers’ instructions. The identity of all mutants was confirmed by DNA sequencing both during cloning and after propagation for cell culture transfection. Sequencing was performed using either the Sequenase version 2.0 kit (Life Science) or the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). DNA for transfections was prepared using the Qiagen plasmid Mega Prep kit (Qiagen). The sequences of mutant primers are shown in Table II.

Preparation of Cell Extracts and Ion-Exchange Chromatography—Two days after transfection, culture medium was recovered, and cells were washed with phosphate-buffered saline with the addition of 2 g/liter EDTA. Cells were counted and stored at −20 °C as pellets. Pellets were resuspended in 1 ml of boiling water and boiled for 30 min. Cell debris was removed by centrifugation, and extracts were stored at −20 °C. Anion-exchange chromatography was performed using a MonoQ column in a fast protein liquid chromatography system (Pharmacia). Buffer A was Tris-Cl, pH 8.2 (Sigma), with 10% acetonitrile (Rathburn Chemicals Ltd.). Buffer B was equivalent to buffer A with 1 M NaCl (Sigma). Peptides were eluted at a flow of 1 ml/min for 60 min in a linear gradient of NaCl, the gradient depending on the number of negative charges in the expressed constructs. The gradient used were from 0–50% buffer B up to 10–70% buffer B. Fractions of 1 ml were collected and, after dilution, analyzed directly in radioimmunoassays.

Radioimmunoassays—Radioimmunoassays were performed using a library of monospecific antibodies, most specific for gastrin but some cross-reacting with the related peptide, CCK. However, endogenous CCK expression in HIT cells is much lower than gastrin expression after transfection and result in negligible background (27). Ab.2609 recognizes both sulfated and nonsulfated carboxy-amidated gastrin (32), whereas ab.2605 is specific for the nonsulfated, amidated forms (24). Similarly, ab.7270 recognizes both sulfated and nonsulfated glycine-extended gastrin in contrast to ab.3284, which is specific for the corresponding nonsulfated forms (33). Ab.8017 and ab.2145 are specific for the amino terminus of human gastrin-17 and gastrin-34, respectively, regardless of carboxyl-terminal processing (34, 35). The radioimmunoassays were performed as described in the references.

Sulfation Ratio Calculations—Data from radioimmunoassays of the chromatograms were plotted using GraphPad Prism 2.0 software (GraphPad Software Inc.). The sulfation ratio was calculated from area determination under the curve generated by the program. Differences in cross-reactivity of the various antibodies with different gastrin forms were taken into account during calculations. Hence, ab.2609 cross-reacts 131% with sulfated gastrin-17 but only 63% with gastrin-34. Likewise, ab.7270 cross-reacts 133% with sulfated gastrin-17-Gly and 78% with gastrin-34-Gly (data not shown).

RESULTS
To determine the requirements for tyrosine sulfation of gastrin, a number of mutations were introduced around the sulfation site (Table I). Wild type gastrin and the mutants were then expressed transiently in the β-cell line, HIT. The expression levels of the individual constructs varied <50% compared

![Image](57x400 to 298x568)

**FIG. 1. Schematic diagram of the structures of preprogastrin and its major processing products.** Glycine-extended gastrin-17 and gastrin-34 is obtained by cleavage at dibasic sites (RR and KK). The antibody epitopes are also indicated. Antibodies 5284 and 2605 are specific for the nonsulfated and gastrin-34 is obtained by cleavage at dibasic sites (RR and KK). Related peptide structures are shown in bold italic, sulfated tyrosines are underlined.

| Structural Requirements for Tyrosine Sulfation | 21701 |
|---|---|
| **TABLE I** | **List of human gastrin mutants expressed in this study and selected related peptide structures** |
| Gastroin structures presented are the sequences of the completely processed, carboxyamidated gastrin-17 forms. Altered residues are shown in bold italics, sulfated tyrosines are underlined. | |
| Related peptide structures | |
| Horse gastrin | Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 0 |
| Turtle gastrin | -Ala-Glu-Leu-Arg-Pro-Leu-His-Asp-His-Asp-Tyr-Pro-Gly-Trp-Met-Asp-NH₂ | 0 |
| Conin | -Glu-His-Met-Gln-Arg-Met-Glu-ApGpGly-Trp-Met-Asp-Ape-NH₂ | 100 |
| hGH | -Asp-Phe-Thr-Tyr-Glu-Glu-Glu-Glu-Ala-Aryle-Pro-Are-Glu-Gly | n.d. |
| % sulfation | |
| Wild type gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 71.8 ± 7.2* |
| YE-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | 59.9 ± 1.5 |
| YA-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 90.2 ± 1.7 |
| EDY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | 100.0 ± 0.0 |
| ERY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 0.0 ± 0.0 |
| KAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 86.8 ± 3.9 |
| AAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 91.1 ± 3.1 |
| AKE-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 61.1 ± 1.7 |
| AKEEAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 70.8 ± 1.4 |
| AAKAAAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 70.7 ± 6.2 |
| HDHYY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 100.0 ± 0.0 |
| HDHAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | 89.6 ± 1.1 |
| KEEAAAY-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | 87.3 ± 2.4 |
| IGEG-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | >50 |
| AE-gastrin | pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH₂ | >50 |
| % sulfation | 50 |
| Related peptide structures | |
| Horse gastrin | Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Ape-NH₂ | 0 |
| Turtle gastrin | -Ala-Glu-Leu-Arg-Pro-Leu-His-Asp-His-Asp-Tyr-Pro-Gly-Trp-Met-Asp-NH₂ | 0 |
| Conin | -Gly-His-Met-Gln-Arg-Met-Glu-Glu-Glu-Glu-Ala-Tyr-Ile-Pro-Are-Glu-Gly | 100 |
| hGH | -Asp-Phe-Thr-Tyr-Glu-Glu-Glu-Glu-Ala-Aryle-Pro-Are-Glu-Gly | n.d. |

* Mean ± S.D. (n = 3, except wild type gastrin, n = 7).

a n.d., not determined.
Structural Requirements for Tyrosine Sulfation

Table II
Sequences of oligonucleotides used for site-directed mutagenesis

| 5′-specific primer                                      | 3′-specific primer                                      |
|---------------------------------------------------------|---------------------------------------------------------|
| ERY-gastrin                                             | KAY-gastrin                                             |
| EEEEEAY-gastrin                                         | YA-gastrin                                              |
| EEEAAEY-gastrin                                         | IGEG-gastrin                                            |
| EEEAEEY-gastrin                                         | HDHIDY-gastrin                                          |
| EEEAAY-gastrin                                         | HDHIDY-gastrin                                          |
| EAAE-gastrin                                             | KEAAAY-gastrin                                          |
| EIGEG-gastrin                                           | KAAAY-gastrin                                           |

Fig. 2. Analysis of expression pattern, using anion-exchange chromatography, of the gastrin mutant, KAY-gastrin, heterologously expressed in HIT cells and monitored by sequence-specific radioimmunoassays for human progastrin and its products. The elution profile of the various forms are indicated with arrows, where s denotes the sulfated and ns the nonsulfated forms, respectively. Cell extracts were applied on a MonoQ column and eluted using a linear NaCl gradient. Fractions were analyzed for carboxyamidated gastrins (A) and glycine-extended gastrins (B). Monospecific antibodies for the N terminus of gastrin-17 and gastrin-34 as well as antibodies specific for the nonsulfated forms of carboxyamidated and glycine-extended gastrins were used for further characterization of the peaks. The sulfation ratio was determined by determination of the area under the curves. All determinations were based on at least three independent transfections and chromatograms.

expressing the KAY-gastrin mutant analyzed with antisera specific for amidated or glycine-extended gastrins.

Role of Basic Residues Flanking the Tyrosine Sulfation Site—Basic residues constitute the most important consensus feature of tyrosine sulfation, and in human gastrin a stretch of 5 glutamates are present in positions −2 to −6. To determine the individual contributions to sulfation of these residues, we sequentially replaced each glutamate with alanine (Fig. 3 and Table I). Surprisingly, we found that substitution of glutamate in position −2 increased sulfation from 72% to 91%, whereas a moderate decrease in sulfation to 61% was observed by substitution of position −3. In contrast, substitutions of positions −4, −5, or −6 gave no effect. Moreover, when three of the glutamates are removed by a 9-base pair deletion or substituted with uncharged residues (ΔE-gastrin and IGEG-gastrin, respectively), gastrin is still at least 50% sulfated. Because the various forms of these two mutants were not completely separated by ion-exchange chromatography, their sulfation ratios can only be estimated from analysis using several different radioimmunoassays. To examine whether acidic residues are required at all, we substituted all 5 glutamates with alanines. However, the expression level of this construct was extremely low compared with normal expression levels, indicating that the highly charged region of the peptide is necessary for correct expression. Taken together our data imply a less strict requirement of acidic residues than suggested by the previous accepted sulfation consensus. Moreover, the data indicate that charged or hydrophilic residues within the gastrin-17 fragment are necessary for expression of progastrin.

The effect of an acidic residue on the carboxyl-terminal side of the tyrosine was examined by substitution of the glycine in position +1 with a glutamate (YE-gastrin). This substitution decreases sulfation to 60%, a decrease possibly inflicted by the removal of a turn-inducing residue. Turn-inducing residues have been proposed to affect tyrosine sulfation, and in progastrin the only turn-inducing residue, a glycine, is found in the +1 position. Substituting this residue with a glutamate (YE-gastrin) results in a minor decrease in sulfation, but it also alters the elution profile on an anion-exchange column (27). The difference could be due to changes in secondary structures masking the additional negative charge. To examine this possibility, we substituted the glycine with an alanine (YA-gastrin). This substitution increased sulfation from 72 to 90% (see Fig. 4A) and resulted in an elution profile similar to that of wild type gastrin in both anion-exchange chromatography and gel chromatography (data not shown). Thus, neither the glycyl residue nor an acidic residue in position +1 enhance sulfation.

Role of Basic Residues Flanking the Sulfation Site—Basic residues are generally thought to be excluded around sulfation sites. We have analyzed the effect of basic residues in the proximal amino-terminal region of the sulfation site. We have previously shown that substituting the alanine in the −1 posi-
tion of wild type gastrin with an aspartate leads to complete sulfation (19). When alanine is substituted with an arginine, sulfation is completely abolished, see Fig. 4B. However, when the proximal glutamate in position −2 is substituted with a lysine (KAY-gastrin), sulfation increases to 87%, similar to that of the substitution of an alanine (AAY-gastrin) (Fig. 4C). Hence, both a neutral and a basic residue in position −2 is preferable to an acidic residue.

Gastrin has been isolated from a number of vertebrate species that display individual sulfation patterns (36). For instance, neither turtle nor horse antral G-cells sulfate gastrin. Although turtle gastrin is structurally different from human gastrin (37), horse gastrin is similar2 (Table I). However, turtle gastrin has two histidines in positions −2 and −4 which could account for the lack of sulfation. When a similar structure was introduced into human gastrin (HDHDY-gastrin), the resulting peptide was found to be completely sulfated (Fig. 4D). Even when aspartate in position −1 was changed to an alanine (HDHAY-gastrin), sulfation was more complete than in wild type gastrin. This again suggests that basic residues residing elsewhere than position −1 do not inhibit sulfation. When a structure is introduced that is identical with that of horse gastrin-17, peptides expressed in HIT cells are 89% sulfated, i.e. to an even higher level than wild type gastrin. Thus, it appears that peptide structures resembling human gastrin that are not sulfated on eutopic expression are substrates for TPST when they are expressed in HIT cells.

DISCUSSION

Gastrin is an excellent model system for studies of tyrosine sulfation for several reasons. First, sulfation of the peptide varies in a tissue specific manner, and since gastrin is partially sulfated it should be possible to identify both positive and negative factors influencing sulfation. Second, libraries of monospecific antibodies against various molecular forms and sequences of gastrin, including sulfation-specific antisera, are available. Third, we have established a transient expression system for mutational studies of progastrin biosynthesis that facilitates expression of a large number of mutants. Using this system, we have introduced mutations in the residues neighboring the sulfated tyrosine and examined the effect on sulfation. We find that tyrosylprotein sulfotransferase has a consider-

Fig. 3. Alanine scanning of the positional effects of the 5 acidic residues amino-terminally of the sulfated tyrosine in human gastrin. Glutamyl acid residues were sequentially substituted with alanine and analyzed for effects on sulfation. Data are presented as percentage of sulfation ± S.D. (n = 7 for wild type gastrin and n = 3 in all mutants).

Fig. 4. Sulfation ratios of human gastrin mutants altered in position +1 (A), position −1 (B), and position −2, respectively (C), and analysis of the effect of basic residues in a turtle gastrin-like structure (D). Data are presented as percentage of sulfation ± S.D. (n = 7 for wild type gastrin and n = 3 in all mutants).

erably broader substrate specificity than determined by previous studies using synthetic peptides or structural homologies between known sulfation sites (1, 12, 17).

In contrast to most known TPST substrates, progastrin lacks an acidic residue in the amino-terminal position (−1) of the tyrosine. Wild type gastrin is ~72% sulfated when expressed in HIT cells, and we have previously shown that substitution of the neutral residue in position −1 with an aspartate led to complete sulfation (19). Thus, in agreement with the sulfation consensus, an acidic residue in this position is important. To compensate for the neutral residue in position −1, 5 acidic residues present on the amino-terminal side hereof are thought to determine the partial sulfation pattern of the peptide. We have substituted these residues sequentially with alanines and examined the effect on sulfation. Surprisingly, we find that an alanine in position −2 of the tyrosine enhances sulfation, whereas alanine in position −3 decreases sulfation. No effect was observed by substitutions of positions −4 to −6. These findings are surprising in relation to the previously published consensus sequence for sulfation. In comparison, Niehrs et al. (14) found that adjacent acidic residues are more important than distal ones, which agrees with our findings that the adjacent positions are the most important. However, Lin et al. (15) suggested that acidic residues in the −5 to +5 region contribute quantitatively and independently to the overall affinity between peptide and TPST in a position-dependent manner. Combined with our study, this would imply that positions −1 and possibly −3 are the only positions where acidic residues enhance sulfation, but that they are not required to obtain a

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partially sulfated. To examined whether acidic residues are required for sulfation at all, we substituted all 5 glutamates in position -2 to -6 with alanines, but expression resulted in very low levels of expression, indicating that the acidic stretch is necessary for expression of progastrin.

We then addressed the effect of a basic residue in position -1 by substituting alanine with arginine. The mutant was not sulfated at all, demonstrating that a basic residue in position -1 prevents sulfation. This is in conflict with a previous report that leuenkephalin in the brain is sulfated on a tyrosine preceed by a dibasic motif (38). However, the existence of sulfated enkephalin has not been confirmed and might be an artifact. In contrast to previous assumptions, we have found that basic residues in positions other than -1 do not reduce sulfation. On the contrary, they may even enhance sulfation. Thus, a basic residue in position -2 grossly enhances sulfation, and substitutions of both acidic residues in positions -2 and -4 with histidines (HDDAY-gastrin) also increases sulfation.

Turn-inducing residues are frequently found in tyrosine sulfation sites and have been suggested to affect sulfation (1). However, little influence was found on sulfation of synthetic peptides (14), and we did not see any correlation between sulfation and the only turn-inducing residue in the sulfation site of progastrin, i.e. the glycine in position +1. In contrast, sulfation increased when glycine was substituted with an alanine. Hence, turn-inducing residues may be necessary only for sulfation of proteins, in which their absence otherwise would impose secondary structures that inhibit sulfation.

On the basis of this work we can then ask: What is the true determinant of tyrosine sulfation of human progastrin and is it possible to predict the occurrence? Possibly sulfation is directed by a combination of charge distribution around the sulfation site (especially in position -1) and the accessibility of the site to the TPST, the latter criteria being the most important. Human growth hormone contains the structural motif (-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-) which judged from the present data includes a putative sulfation site. However, there have been no reports of tyrosine sulfation of growth hormone. In contrast to human gastrin, the crystal structure of growth hormone is known (39) and shows that the potential sulfation site is surface exposed and situated immediately after an α-helix (ending on Glu-Glu-). Thus, the presence of the α-helix may mask the sulfation site, indicating that secondary structures may act as determinants of sulfation sites. This would also explain the frequent presence of turn-inducing residues in sulfation sites. Thus, information of linear sequences should be combined with structural data for exact prediction of sulfation. Another sulfated protein that deviates from the sulfation consensus is the neuropeptide precursor procinion. Cionin is related to gastrin and shares the bioactive carboxyl-terminal pentapeptide sequence but is otherwise structurally distinct from gastrin (40, 41). In cionin, the most proximal acidic residue to the two neighboring tyrosines comprising the sulfation site, is located in position -3 and there is a basic and a neutral residue located in the -2 and -1 positions, respectively (Table 1). When cionin is expressed in HIT cells, the peptide is doubly sulfated, whereas substitution of either of the tyrosines with phenylalanine results in partial sulfation, as observed with gastrin in our study. Hence, our data on requirements of sulfation can substantiate predictions of the sulfation pattern observed in procinion.

Tyrosine sulfation is a widespread modification and is believed to occur in all animal tissues and in many cell lines (reviewed in Refs. 1 and 17). Our transfection studies indicate that relatively profound changes in charge distribution and structure around the sulfation site do not affect sulfation dramatically. Nevertheless, eutopically expressed gastrin is completely nonsulfated in pituitary corticotrophs (42, 43) and in ileal mucosa cells (44). Moreover, antral gastrin is not sulfated either in the horse or in the structurally more remote turtle gastrin (37), although both proteins are readily sulfated in HIT cells. Considering the broad substrate specificity of TPST, we have described here in HIT cells, it seems unlikely that structural differences are the reason for the lack of sulfation. It appears most likely that certain cell types do not sulfate potential substrates although all tissues examined display TPST activity. It has been proposed that more than one TPST may exist, and it is possible that HIT cells express several forms with different substrate specificities. Our data strongly suggest that TPST or its isoforms are expressed in a cell-specific manner.

In conclusion, we have shown that TPST activity in HIT cells has a broad substrate specificity and that basal sulfation levels may be determined by a combination of linear amino acid sequences and secondary structures. Considering the overlapping substrate specificity between TPST and tyrosine kinases (14) and the fact that the tyrosine sulfation site in gastrin can be phosphorylated in vitro (45), it would be interesting to investigate whether the same recognition pattern is valid for certain tyrosine kinases. Finally, experimental data suggest that certain cell types are incapable of sulfating gastrin forms that are sulfation targets in HIT cells, indicating a cell-specific expression pattern of TPST activity.

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REFERENCES

1. Huttner, W. B., and Baeuerle, P. A. (1988) in Modern Cell Biology (Satir, B., ed), pp. 97–140, Alan R. Liss, Inc., New York
2. Hille, A., Braulke, T., von Figura, K., and Huttner, W. B. (1990) Eur. J. Biochem. 188, 577–586
3. Hille, A., and Huttner, W. B. (1990) Eur. J. Biochem. 188, 587–596
4. Baeuerle, P. A., and Huttner, W. B. (1985) J. Biol. Chem. 260, 6434–6439
5. Niehrs, C., and Huttner, W. B. (1990) EMBO J. 9, 35–42
6. Murti, V. (1980) in Gastrointestinal Hormones (Glass, G. B. J., ed), pp. 169–221, Raven Press, New York
7. Stone, S. R., and Hofstænæ, J. (1986) Biochemistry 25, 4622–4628
8. Hofstænæ, J., Stone, S. R., Donella-Deana, A., and Pinna, L. A. (1990) Eur. J. Biochem. 188, 55–59
9. Leyte, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verbeet, M. P., Mertens, K., and van Mourik, J. A. (1991) J. Biol. Chem. 266, 740–746
10. Pouyani, T., and Seed, B. (1985) Cell 30, 333–347
11. Wilkie, P. P., Moore, K. L., McRuer, R. F., and Cumming, R. D. (1995) J. Biol. Chem. 270, 22677–22680
12. Hultin, G. L., Felt, R., Gordon, J. I., and Strauss, A. W. (1986) Biochem. Biophys. Res. Commun. 141, 326–333
13. Rens-Domiano, S., Hultin, G. L., and Roth, J. A. (1989) Mol. Pharmacol. 36, 647–655
14. Niehrs, C., Kraft, M., Lee, R. W. H., and Huttner, W. B. (1990) J. Biol. Chem. 265, 8525–8532
15. Lin, W., Larsen, K., Huttner, G. L., and Roth, J. A. (1992) J. Biol. Chem. 267, 2677–2679
16. Rosenquist, G. L., and Nicholas, H. B., Jr. (1993) Protein Sci. 2, 215–222
17. Niehrs, C., Beisswanger, R., and Huttner, W. B. (1994) Chem.-BioL Interact. 92, 257–271
18. Walsh, J. H. (1987) in Physiology of the Gastrointestinal Tract (Johnson, L. R., Christiansen, J., Jackson, M. J., Jacobson, R. D., and Walsh, J. H., eds), pp. 181–253, Raven Press, New York
19. Bundgaard, J. R., Vuust, J., and Rehfeld, J. F. (1995) EMBO J. 14, 3073–3079
20. Pawels, S., Deckrey, G. J., and Walker, R. (1987) Gastroenterology 92, 1229–1225
21. Pawels, S., Najdovski, T., Dimaline, R., Lee, C. M., and Deschott-Lanceman, M. (1989) Biochim. Biophys. Acta 996, 82–88
22. Rehfeld, J. F., Hansen, C. P., and Johnsen, A. H. (1995) EMBO J. 14, 389–396
23. Gregory, R. A., Tracy, H. J., and Grossman, M. I. (1966) Nature 209, 583
24. Andersen, B. N., De Magistris, L., and Rehfeld, J. F. (1993) Clin. Chim. Acta 209, 25–39
25. Andersen, B. N., Abramovich, D., Brand, S. J., Petersen, B., and Rehfeld, J. F. (1995) Regul. Pept. 10, 329–338
26. Bardram, L., Hilsted, L., and Rehfeld, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 298–302
27. Bundgaard, J. R., Cowland, J. B., Vuust, J., and Rehfeld, J. F. (1996) DNA Cell Biol. 15, 147–157

8J. U. Thorup, J. R. Bundgaard, and J. F. Rehfeld, manuscript in preparation.
28. Santerre, R. F., Cook, R. A., Crisel, R. M. D., Sharp, J. D., Schmidt, R. J., Williams, D. C., and Wilson, C. P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4339–4343
29. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) Current Protocols in Molecular Biology, Wiley Interscience, New York
31. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
32. Rehfeld, J. F. (1978) J. Biol. Chem. 253, 4016–4021
33. Hilsted, L., and Rehfeld, J. F. (1986) Anal. Biochem. 152, 119–126
34. Bardram, L., and Rehfeld, J. F. (1989) Scand. J. Clin. Lab. Invest. 49, 173–182
35. van Solinge, W. W., and Rehfeld, J. F. (1990) Clin. Chim. Acta 192, 35–46
36. Andersen, B. N. (1985) Gen. Comp. Endocrinol. 58, 44–50
37. Johnsen, A. H., and Rehfeld, J. F. (1992) Eur. J. Biochem. 207, 419–428
38. Unsworth, C. D., Hughes, J., and Morley, J. S. (1982) Nature 295, 519–522
39. Abdel-Meguid, S. S., Shieh, H.-S., Smith, W. W., Dayringer, H. E., Vial, B. N., and Bentle, L. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6434–6437
40. Johnsen, A. H., and Rehfeld, J. F. (1990) J. Biol. Chem. 265, 3054–3058
41. Monstein, H.-J., Thorup, J. U., Folkesson, R., Johnsen, A. H., and Rehfeld, J. F. (1993) FEBS Lett. 331, 60–64
42. Rehfeld, J. F. (1978) Nature 271, 771–773
43. Rehfeld, J. F., and Larssen, L. T. (1981) J. Biol. Chem. 256, 10426–10429
44. Friis-Hansen, L., and Rehfeld, J. F. (1994) FEBS Lett. 343, 115–119
45. Baldwin, G. S., Knesel, J., and Monckton, J. M. (1983) Nature 301, 435–437