

# Tyrosine O-sulfation promotes proteolytic processing of progastrin

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**Tyrosine O-sulfation is a common post-translational modification of secretory and membrane proteins. The biological function of sulfation is known in only a few proteins, where it appears to enhance protein–protein interactions. Based on known sequences around sulfated tyrosines, a consensus sequence for prediction of target tyrosines has been proposed. However, some proteins are tyrosine sulfated at sites that deviate from the proposed consensus. Among these is progastrin. It is possible that the deviation explains the incomplete sulfation characteristic for bioactive gastrin peptides. In order to test this hypothesis, we have performed site-directed mutagenesis of the gastrin gene followed by heterologous expression in an endocrine cell line. The results show that substitution of the alanyl residue immediately N-terminal to the sulfated tyrosine with an acidic amino acid promotes the sulfation of gastrin peptides. Hence, the study supports the proposed consensus sequence for tyrosine sulfation. Importantly, however, the results also reveal that complete sulfation increases the endoproteolytic maturation of progastrin. Thus, our study suggests an additional function for tyrosine sulfation of possible general significance.**

**Key words:** gastrin biosynthesis/prohormone processing/proteolytic cleavage/tyrosine sulfation

## Introduction

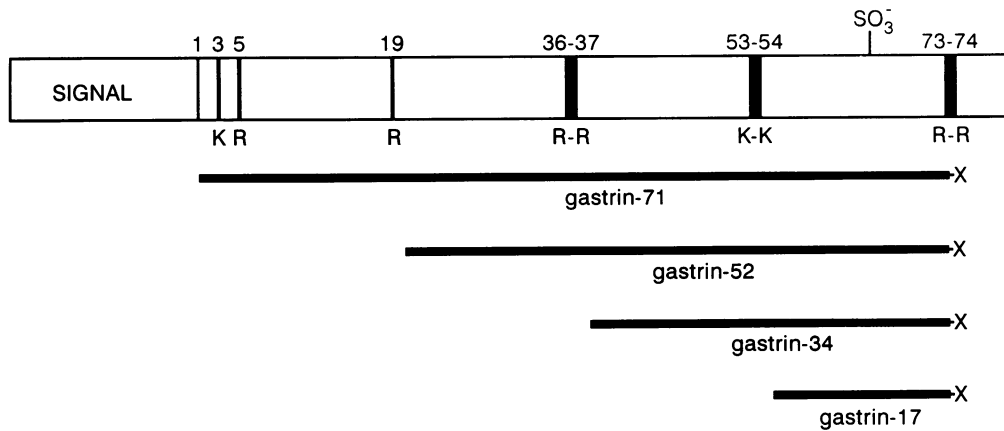
In the last decade tyrosine sulfation has been recognized as a common post-translational modification (Huttner, 1982; Huttner and Baeuerle, 1988). The sulfation is catalysed by tyrosylprotein sulfotransferase (TPST), an integral membrane protein located in the *trans* Golgi network (Huttner and Baeuerle, 1988; Niehrs and Huttner, 1990). Accordingly the modified proteins belong to the classes of secretory, membrane and lysosomal proteins (Hille and Huttner, 1990; Hille *et al.*, 1990). A large number of tyrosine sulfated proteins with considerable diversity of biological activities have now been identified (reviewed by Huttner and Baeuerle, 1988; Niehrs *et al.*, 1994). However, the biological significance of tyrosine sulfation is known for only a few proteins, in which sulfation appears to improve protein–protein interactions.

The sequences around sulfated tyrosines are known for

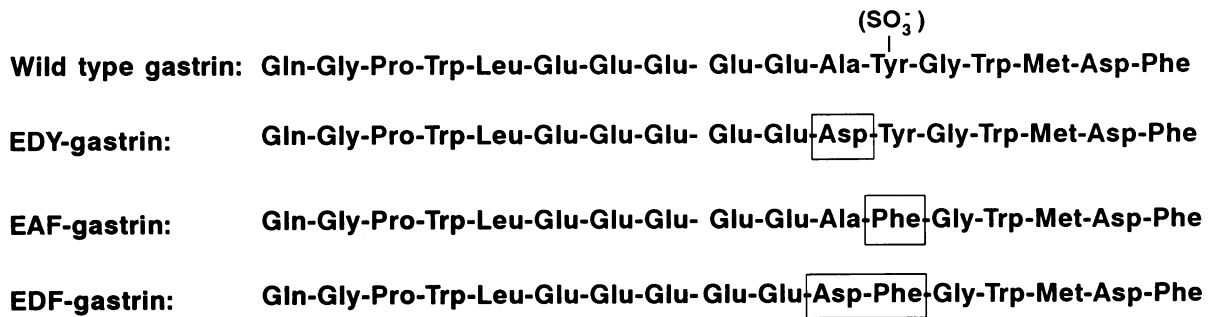
a number of proteins, and based on these a consensus sequence of sulfation sites has been proposed (Hortin *et al.*, 1986; Huttner and Baeuerle, 1988). Hence, a tyrosine sulfation site requires acidic amino acids around the tyrosine, in particular in the immediate N-terminal position –1. The significance of this position has been substantiated by *in vitro* sulfation of synthetic peptides using cell lysates enriched in TPST activity, although peptides with other structures could also act as TPST substrates (Rens-Domiano *et al.*, 1989; Niehrs *et al.*, 1990; Lin *et al.*, 1992). Niehrs *et al.* (1992) have demonstrated that a heptapeptide motif from chromogranin B, which has an ideal consensus site for sulfation, was sufficient to obtain complete stoichiometric sulfation of an artificial protein, sulfophilin, in a transfected cell culture. However, no studies have yet examined consensus demands of tyrosine sulfation of intact proteins.

Gastrin is an important gastrointestinal hormone, which regulates gastric acid secretion and growth of the gastrointestinal mucosa (Walsh, 1987). It belongs to a family of tyrosine sulfated peptides sharing the C-terminal structure (Trp-Met-Asp-Phe-NH<sub>2</sub>), but with varying structures around the sulfated tyrosine and different patterns of sulfation and proteolytic processing. In mammals, only half of the progastrin is sulfated (Gregory *et al.*, 1964; Hilsted and Rehfeld, 1987) in contrast to the homologous hormone cholecystokinin (CCK), which is completely sulfated (Mutt and Jorpes, 1968). The difference could be due to the structural variations or to different sulfation potentials of cells expressing the hormone genes. In order to examine these hypotheses, we have now expressed wild-type gastrin and gastrin mutants in the  $\beta$ -cell line, HIT, which is well equipped with TPST activity.

Substantial knowledge about progastrin processing in normal antral G-cells has already accumulated. Hence, in the endoplasmic reticulum, a 21-amino acid signal sequence of human preprogastrin is removed to release the 80-amino acid progastrin (Figure 1). To obtain biological activity progastrin, however, requires processing at the C-terminus by cleavage at Arg73–Arg74. After removal of the basic residues, Gly72-extended gastrin is the target of the amidating enzyme complex, PAM, which uses Gly72 as donor for  $\alpha$ -amidation of Phe71 (Suchanek and Kreil, 1977; Bradbury *et al.*, 1982). The amidated gastrins are bioactive, i.e. their C-terminus is Trp-Met-Asp-Phe-NH<sub>2</sub>. Apart from C-terminal processing, progastrin is also processed at various mono- or dibasic sites. Thus, cleavage at Arg36–Arg37 or Lys53–Lys54 gives rise to gastrin-34 or gastrin-17 (or the corresponding glycine-extended forms), the major forms secreted by G-cells. Besides proteolytic processing, progastrin is partially sulfated at Tyr66, and partially phosphorylated at Ser75 (Varro *et al.*, 1988).



**Fig. 1.** Schematic diagram of preprogastrin and progastrin processing products. Amino acid numbering denotes the position in progastrin and basic residues involved in proteolytic cleavage are shown. Some major processing products are shown. The C-terminus marked -X denotes -NH<sub>2</sub> for amidated gastrins, and -Gly72 for glycine extended gastrins. The position of the partially sulfated Tyr66 is also shown.



**Fig. 2.** Primary sequence of wild-type progastrin surrounding the sulfated tyrosine and the corresponding sequences of the mutants, EDY-gastrin, EAF-gastrin and EDF-gastrin. The partially sulfated tyrosyl residue in wild-type gastrin is marked, and the altered amino acids are boxed. The sequences presented are the sequences of gastrin-17 or mutated equivalents. See Introduction for nomenclature of progastrin processing.

## Results

### Expression vector constructions

An expression vector based on fusion of the human ubiquitin (*UbB*) promoter region with the human gastrin gene was constructed. Since gastrin displays a tissue-specific tyrosine sulfation pattern, we investigated the role of the primary structure surrounding the sulfation site using heterologous expression of mutated human gastrin genes, altered by site-directed mutagenesis. The original and altered structures of the peptides expressed are shown in Figure 2. Introduction of an acidic amino acid immediately N-terminal to the tyrosine resulted in a gastrin mutant (EDY-gastrin), that has a perfect consensus sequence for tyrosine sulfation (Hortin *et al.*, 1986; Huttner and Baeuerle, 1988).

### Expression of wild-type gastrin

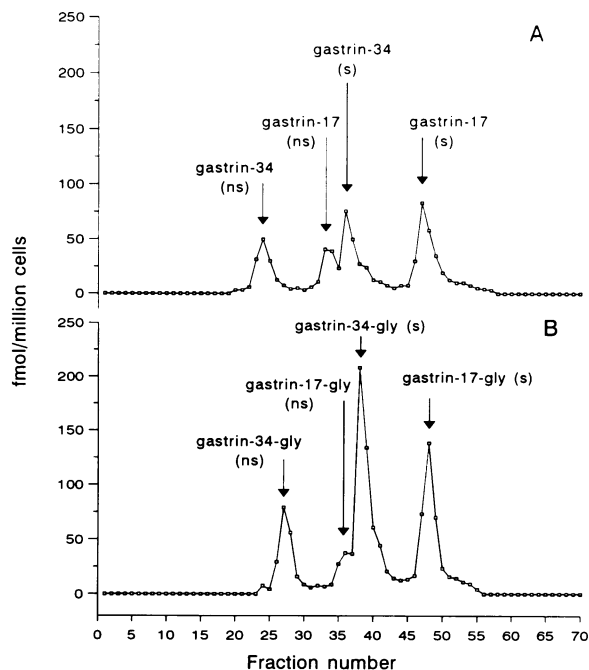
The  $\beta$ -cell line, HIT, was transfected transiently in independent series with the plasmid expressing wild-type gastrin leading to expression at high levels. Radioimmunoassay of non-transfected HIT cell extracts using gastrin-specific antibodies demonstrated that gastrin is not expressed endogenously. The processing and sulfation patterns were analysed using ion-exchange chromatography. The elution pattern of extracts from transfected cells expressing wild-type gastrin is shown in Figure 3. This demonstrates that HIT cells process human progastrin to the same molecular forms which are predominantly

expressed in normal antral G-cells, i.e. gastrin-17 and gastrin-34. Moreover, sulfated and non-sulfated gastrins are entirely separated in this chromatographic system, which shows the sulfation of all forms to be identical (60%). The degree of sulfation was verified with measurements using sulfation-specific antibodies (data not shown). Glycine-extended gastrins corresponding to the amidated forms were also found. However, the ratio of glycine-extended gastrin-34 to glycine-extended gastrin-17 is much higher than the ratio for the corresponding amidated forms, a relationship described earlier and which is characteristic of gastrin expression (Hilsted *et al.*, 1986; Hilsted and Rehfeld, 1987).

### Expression of EDY-gastrin

The expression in HIT cells transfected with the EDY-gastrin construct was also measured after ion-exchange chromatography (Figure 4). In contrast to wild-type gastrin, non-sulfated EDY-gastrins were not detectable. Radioimmunoassays specific for the N-terminus of human gastrin-34 and gastrin-17 were used to identify EDY-gastrins. As expected from the introduction of an additional acidic residue, all EDY-gastrins eluted chromatographically at higher salt concentrations than wild-type gastrin. The results demonstrates the importance of an acidic amino acid located N-terminally to the tyrosyl residue for complete sulfation.

The proteolytic processing pattern of EDY-progastrin



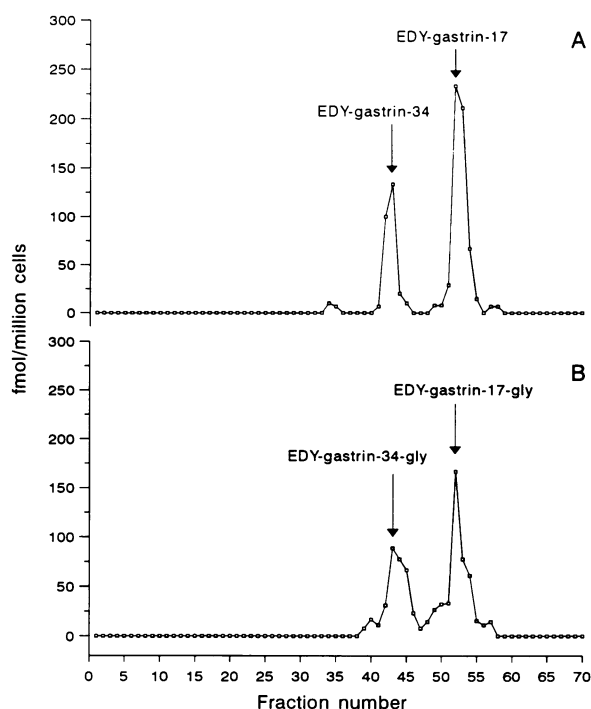
**Fig. 3.** Characterization of gastrin processing pattern using anion exchange chromatography of extracts of HIT cells transfected with the wild-type gastrin expression vector. Fractions were collected and analysed by radioimmunoassay for amidated (A) and glycine-extended gastrin (B). Elution positions of the different gastrin forms, determined using specific antisera, are shown. The (s) denotes tyrosine-sulfated gastrins, whereas (ns) denotes the non-sulfated forms. The chromatogram shown is representative of three independent experiments.

differed from that of wild-type progastrin. Hence, the ratios between amidated and glycine-extended forms did not differ for EDY-gastrins as is observed for wild-type gastrins. On the contrary, the EDY-gastrin-34 to EDY-gastrin-17 ratios were identical, regardless of the C-terminal structure, i.e. carboxamidated or glycine-extended. Moreover, the concentrations of carboxamidated EDY-gastrins were higher than those of wild-type gastrin. Identical relationships were found in culture media, and the findings were also reproducible in all transfection series after gel chromatography of cell extracts (data not shown). Hence, the endoproteolytic processing of EDY-gastrin is more efficient than that of wild-type gastrin.

#### Expression of EAF- and EDF-gastrin

To identify whether the increased processing described above was due to increased sulfation of tyrosine, to the introduction of an acidic amino acid or both, we constructed two additional gastrin mutants (Figure 2). In EAF-gastrin the sulfation target, Tyr66, was substituted with a phenylalanine. Moreover, if the aspartate (which substitutes alanine in the EDY-gastrin mutant) in itself affects the processing of the mutant, the corresponding substitution in EDF-gastrin should also alter the processing pattern.

HIT cells were transfected with expression vectors encoding the EAF- and EDF-gastrin mutants. Figure 5A and B show the elution patterns of amidated and glycine-extended EAF-gastrins after ion-exchange chromatography. The cells synthesized more carboxamidated EAF-gastrin-17 than EAF-gastrin-34, whereas glycine-extended



**Fig. 4.** Characterization of EDY-gastrin processing pattern using anion exchange chromatography of extracts of HIT cells transfected with the EDY-gastrin expressing construct. Fractions were analysed by radioimmunoassay for amidated (A) and glycine-extended EDY-gastrin (B).

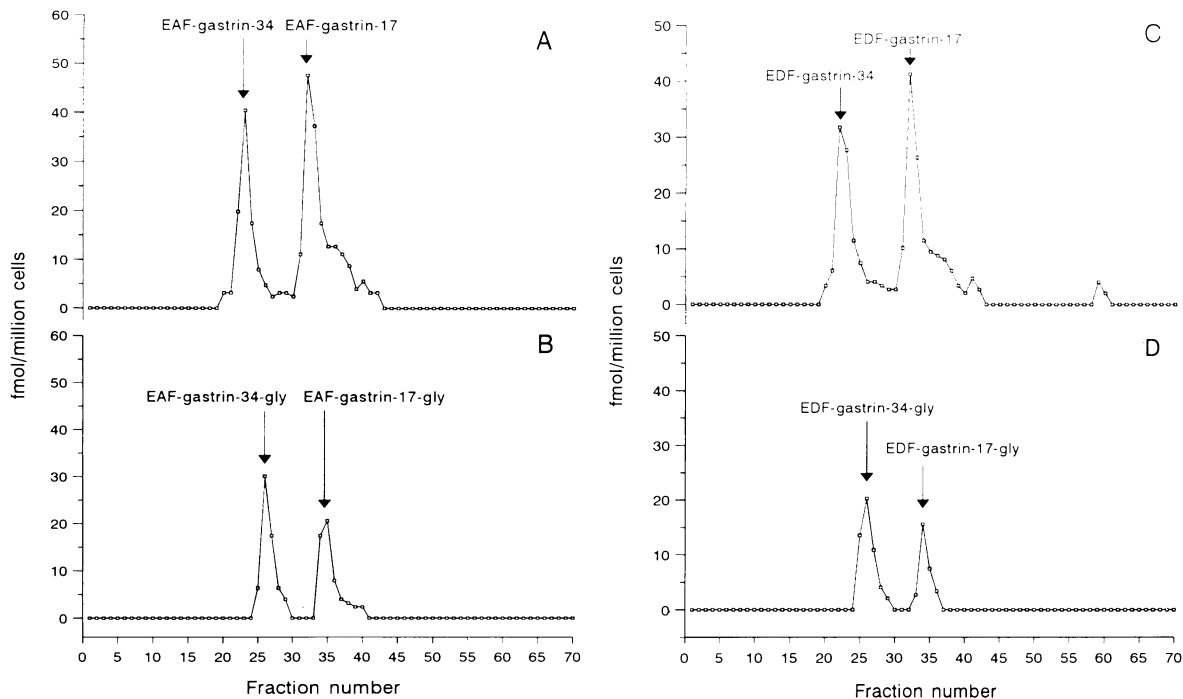
EAF-gastrin-34 was the most abundant glycine-extended form. This pattern of expression is similar to that of wild-type gastrin. Moreover, the expression pattern of EDF-gastrin was similar to that of EAF-gastrin (Figure 5C and D). Hence, substitution of alanine with an aspartate as such does not increase the endoproteolytic maturation, suggesting that the altered processing of EDY-gastrin is due solely to the increased tyrosine O-sulfation.

#### Effect of sulfation on proteolytic processing at Lys53–Lys54

In order to quantitate the effect on proteolytic cleavage, six independent transfections with all four constructs were performed. The ratio of gastrin-17 versus gastrin-34 regardless of the extent of C-terminal processing, was determined in cell extracts from each transfection using antisera specific for these two forms. The results demonstrate that the processing at Lys53–Lys54 is unaltered in EDF-gastrin compared with wild-type gastrin (Figure 6). In contrast, substitution of Tyr66 with a phenylalanine leads to a small, but significant ( $P < 0.04$ ) decrease in gastrin-17 production. Conversely, substitution of Ala55 with an aspartate resulted in a significant ( $P < 0.0001$ ) increase in gastrin-17 versus gastrin-34 ratio. No significant difference in processing ratio could be demonstrated between EAF-gastrin and EDF-gastrin.

#### Effects of mutations on C-terminal processing

The chromatograms indicate, that the overall ratios of amidated versus glycine-extended gastrin mutants are higher than in wild-type gastrin. Indeed, when independently transfected extracts were analysed using the specific antisera against these forms, a 2- to 3-fold increase is



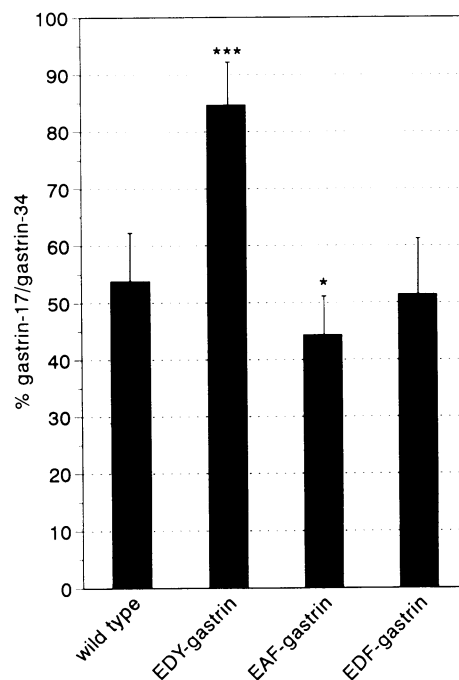
**Fig. 5.** Characterization of EAF- and EDF-gastrin processing patterns using anion exchange chromatography of extracts of HIT cells transfected with EAF-gastrin (A and B) or EDF-gastrin expression vectors (C and D). Fractions were analysed by radioimmunoassay for amidated (A and C) or glycine-extended, mutated gastrins (B and D).

found (data not shown). However, this increase may be a result of a changed reactivity of the antibodies with the mutants. The antisera have higher affinities for sulfated than for non-sulfated gastrin and may therefore be sensitive to structural changes around the tyrosine. Thus, in order to investigate the effect on C-terminal processing we will need a more precise determination of antibody reactivity with the mutants. However, since all mutants exhibited increased amidation ratio, it cannot be mediated by tyrosine sulfation.

**Discussion**

This study has shown, that the charge of the amino acid residue immediately N-terminal to the tyrosine to be sulfated is an essential determinant of the degree of sulfation. Moreover, the results show that the degree of sulfation governs the degree of proteolytic processing. Hence, tyrosine sulfation becomes an important regulator of phenotypic gene expression.

O-Sulfation is the most common modification of tyrosyl residues. It has been estimated that 1% of the tyrosines in a cell may be sulfated (Baeuerle and Huttner, 1985). A number of proteins are known to be tyrosine-sulfated, but the structure of the sulfation site is known in only a few proteins. Based on the primary structures of these sites, consensus features have been proposed (Table I) (Hortin *et al.*, 1986; Huttner and Baeuerle, 1988). Evaluated by *in vitro* sulfation of synthetic peptides, the most important feature is the presence of acidic residues, particularly in the -1 position (Rens-Domiano *et al.*, 1989; Niehrs *et al.*, 1990; Lin *et al.*, 1992). However, in some peptides the structures of the sulfation sites are different. Cionin, for example, is completely sulfated in two neighbouring tyrosines, despite considerable deviations from the pro-



**Fig. 6.** Ratios of gastrin-17/gastrin-34 in the four different constructs. Cell extracts from six independent transfections of each construct were analysed with antisera specific for gastrin-17 (ab.8017) and gastrin-34 (ab.2145). Data are presented as mean  $\pm$  S.D. ( $n = 6$ ) and compared using an unpaired, two-tailed Student's *t* test. \*,  $P < 0.04$ ; \*\*\*,  $P < 0.0001$ .

posed consensus sequence (Johnsen and Rehfeld, 1990; Monstein *et al.*, 1993). Human gastrin contains a neutral residue in the -1 position, and in some mammalian species an additional neutral residue is found in the -2 position. Immediately N-terminal to this, however, gastrin has a

**Table I.** Consensus demands for prediction of tyrosine sulfation sites based on structures of known sulfation targets

- (i) Presence of at least three acidic amino acids (Asp, Glu) in the -5 to +5 amino acid stretch with one placed in the -1 position. Alternatively, four acidic amino acids are found from -5 to +5 without an acidic amino acid in -1.
- (ii) Presence of turn-inducing amino acids (Pro, Gly) from -7 to +7.
- (iii) Absence of Cys- and N-glycosylation sites.
- (iv) C-terminal location of the target tyrosine.

(Hortin *et al.*, 1986; Huttner and Baeuerle, 1988; Niehrs *et al.*, 1994). The numbering refers to the position relative to the sulfated tyrosine.

stretch of 4–5 acidic amino acids. The sulfation pattern of gastrin is complex, showing both species and tissue variations. The sulfation of antral gastrins ranges from 24 to 80% in mammals (Andersen, 1985). Complete lack of sulfation is found in the adenohypophysis (Rehfeld and Larsson, 1981) and in the ileum (Friis-Hansen and Rehfeld, 1994). In contrast, gastrin is completely sulfated in the fetal pancreas (Brand *et al.*, 1984). These variations could be due either to deviations from ideal sulfation consensus and/or to different sulfation potentials of cells expressing gastrin.

We have addressed this question using transient expression of human progastrin in endocrine cells. The transient nature of the system is expedient for studies of prohormone biosynthesis using site-directed mutagenesis. We constructed a mutant, EDY-gastrin, which fulfils the sulfation consensus by the substitution of alanine in the -1 position with aspartate. After expression in HIT cells, EDY-gastrin was completely sulfated, in contrast to wild-type gastrin which was only 60% sulfated. Thus, the acidic amino acid in the -1 position is important for stoichiometric sulfation. Our data therefore support the proposed consensus requirements in agreement with *in vitro* experiments using synthetic peptides and membrane fractions enriched in TPST activity (Rens-Domiano *et al.*, 1989; Niehrs *et al.*, 1990; Lin *et al.*, 1992).

The biological significance of tyrosine sulfation is known in only a few cases. It has been demonstrated that sulfation is important for the binding of von Willebrand factor to Factor VIII (Leyte *et al.*, 1991) and for hirudin to thrombin (Stone and Hofsteenge, 1986; Hofsteenge *et al.*, 1990). Sulfation also increases the activity of the fourth component of complement (C4) (Hortin *et al.*, 1989). Moreover, binding to the CCK<sub>A</sub> receptor requires tyrosine sulfation of the ligand (Mutt, 1980), whereas tyrosine sulfation has only limited effect on the binding to the CCK<sub>B</sub>/gastrin receptor (Huang *et al.*, 1989). Recently we described another role of tyrosine sulfation. Upon maturation of progastrin in antral G-cells small bioactive peptides are produced which are protected from aminopeptidase degradation by the sulfated tyrosine (Rehfeld *et al.*, 1995). Additional functions in progastrin maturation have been suggested earlier. Thus, examinations of plasma and tissue samples from hypergastrinaemic patients showed a direct correlation between the degree of sulfation and the maturation of progastrin to gastrin-17 (Andersen and Stadil, 1983; Jensen *et al.*, 1989). Moreover, adeno-physeal and ileal gastrin is not sulfated and processed only to gastrin-34 (Rehfeld and Larsson, 1981; Friis-Hansen and Rehfeld, 1994).

In the present study we have shown, that HIT cells processes wild-type progastrin mainly to gastrin-34 and gastrin-17. Notably, HIT cells do not cleave gastrin-17 to small carboxyamidated gastrins as shown for antral G-cells (Rehfeld *et al.*, 1995). Less than half of the C-terminally processed gastrin was amidated in HIT cells, and the ratio of gastrin-34 versus gastrin-17 was much larger for glycine-extended gastrins than for the amidated forms (Figure 3). In contrast, by expression of EDY-gastrin, the ratios between gastrin-34 and gastrin-17 were equal regardless of C-terminal structure (Figure 4), suggesting that the altered structure and/or the change in degree of sulfation affects the processing of gastrin-34 to gastrin-17. Evidence that the effect is mediated by tyrosine sulfation is presented by EAF-gastrin and EDF-gastrin. They show that introduction of an aspartate in itself has little effect on processing, whereas both EDY-gastrin and EAF-gastrin display an altered processing pattern. These findings may have relevance for other proteins. In C4, tyrosine sulfation increases the rate of cleavage by C1s, and this could be directed by sulfation (Hortin *et al.*, 1989). Moreover, inhibition of tyrosine sulfation by chlorate treatment of a MTC cell line reduced the processing of proCCK to CCK-8 (Beinfeld, 1994). Although both expression and secretion of CCK were also affected, the observation is consistent with a correlation between tyrosine sulfation and proteolytic cleavage.

It remains to be shown how proteolytic cleavage is affected by tyrosine sulfation. Since tyrosine sulfation is often involved in protein-protein interactions, it is possible that sulfation enhances the interaction between prohormone convertases and propeptides. Accordingly, in CCK a mono-basic processing site is close to the sulfated tyrosine, whereas in gastrin, eleven amino acids separate the tyrosine from a dibasic cleavage site. Alternatively, sulfation could alter the kinetics of secretion of gastrin and thereby influence propeptide processing.

## Materials and methods

### Construction of a human gastrin expression vector

A gastrin-expressing vector, pJB72 was constructed (J.R. Bundgaard, J.B. Lowland, J. Vuust and J.F. Rehfeld, unpublished results). This vector consisted of a human ubiquitin three-repeat (*UbB*) gene promoter (Baker and Board, 1980) in fusion with the entire human gastrin gene, including part of the 3' flanking region (Wiborg *et al.*, 1984).

### Site-directed mutagenesis and construction of mutant vectors

The EDY-gastrin mutation was prepared as follows: the 3'-end of the human gastrin gene was recovered by *StuI* and *NheI* digestion of pG8E, a plasmid carrying an *EcoRI* fragment with the entire human gastrin gene including the 3'- and 5'-flanking regions (Wiborg *et al.*, 1984). The *StuI-NheI* fragment was cloned in the *HincII* and *XbaI* sites of pBluescript II SK(+) (Stratagene). Using helperphage R408 (Stratagene), single-stranded phagemid DNA was made as described (Dente *et al.*, 1985). Site-directed mutagenesis was performed according to the method of Kunkel (1985), by incorporation of uracil into single-stranded phagemid DNA. The mutant oligonucleotide sequence was 5'-AAG AAG AAG ACT ATG GAT GG-3'. Recovered clones were sequenced using the Sequenase Kit (USB), and a 391 bp *HindIII* fragment carrying the mutation was subcloned into the gastrin expression vector, pJB72. The mutants EAF-gastrin and EDF-gastrin were generated with PCR. Mismatches were incorporated in the upstream PCR primer surrounding the *NcoI* site of the human gastrin gene. The sequence of the mutation primers were: EAF-gastrin: 5'-GGG ACC ATG GCT GGA GGA AGA AGA AGA AGC CTT TGG ATG GAT GG-3' and EDF-gastrin: 5'-

GGG ACC ATG GCT GGA GGA AGA AGA AGA CTT TGG ATG GAT GG-3'. The downstream primer, 5'-GGC ACT CAG ATC TTC TCC CT-3', is located in position 6905 [numbered as in Kariya *et al.* (1986)] in the 3' flanking region of the gastrin gene. In PCR, pG8E was used as the DNA template, and a 156 bp *NcoI*-*SpeI* fragment of the product was cloned directly into the equivalent sites in the gastrin expression vector, pJB72, and sequenced. DNA modifying enzymes and Taq polymerase were obtained from Boehringer Mannheim or Gibco-BRL and were used as recommended by the manufacturers.

DNA was purified on CsCl gradients (Ausubel *et al.*, 1992), and preparations used for transfection were sequenced before use.

### Cell culture and transfection

The hamster  $\beta$ -cell line, HIT (Santerre *et al.*, 1981), was cultured in Dulbecco's Modified Eagle's Medium (DMEM 1965) supplemented with 1% glutamine and 1% penicillin/streptomycin (all purchased from Gibco-BRL). Cells were incubated in 10% CO<sub>2</sub> at 37°C. Cells were split 1 day before transfection. Transfections were performed using the method of Chen and Okayama (1987) as described in (Ausubel *et al.*, 1992).

### Preparation of cell extracts

Two days after transfection, culture media were recovered and cells harvested using PBS with 2 mg/ml EDTA, counted, and kept at -20°C as cell pellets until peptide extraction. Culture media were extracted by 30 min boiling and dried in a Speed-Vac centrifuge. Media were resuspended in 0.02 M veronal buffer, pH 8.4, with 0.1% albumin. Cell pellets were resuspended in 1 ml redistilled water, boiled for 30 min, centrifuged for removal of cell debris, and stored at -20°C for later analysis.

### Ion-exchange chromatography

Ion-exchange chromatography was performed on a FPLC system, using a MonoQ column (Pharmacia). Buffer A was Tris-HCl, pH 8.2 (Sigma) with 10% acetonitrile. Buffer B was equivalent to buffer A with 1 M NaCl. Elution was performed by a flow of 1 ml/min in a linear gradient of NaCl, from 10 to 70% buffer B in 60 min. Fractions of 1 ml were collected and dried in a Speed-Vac. Fractions were re-dissolved in 0.02 M veronal buffer, pH 8.4, with 0.1% albumin, and analysed by radioimmunoassay.

### Radioimmunoassay

Radioimmunoassays were performed using the antibodies ab.2609, ab.7270, ab.8017 and ab.2145. Ab.2609 is specific for the common amidated C-terminus of gastrin and cholecystokinin (Rehfeld, 1978). Ab.7270 recognizes the corresponding glycine-extended forms (Hilsted and Rehfeld, 1986). Ab.8017 is specific for the N-terminus of human gastrin-17 (Bardram and Rehfeld, 1989), and ab.2145 is specific for the N-terminus of human gastrin-34 (van Solinge and Rehfeld, 1990).

Radioimmunoassays were performed as previously described (Rehfeld, 1978; Hilsted and Rehfeld, 1986; Bardram and Rehfeld, 1989; van Solinge and Rehfeld, 1990). Determination of sulfation ratios were made with corrections for the different affinities of the antibodies towards the different peptide products. Hence, ab.7270 recognizes sulfated gastrin ~133% compared with the non-sulfated form (L.Hilsted, personal communication). Similarly, ab.2609 exhibits 131% cross-reactivity with sulfated gastrin (Rehfeld *et al.*, 1981), whereas ab.8017 cross-reacts 108% (Bardram and Rehfeld, 1989). In contrast, the antibodies bind gastrin-34 with decreased affinity. Using ab.2609 the cross-reactivity of gastrin-34 is 63%, and for ab.7270 is 78% (L.Hilsted, personal communication).

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### References

Andersen,B.N. (1985) Species variation in the tyrosine sulfation of mammalian gastrins. *Gen. Comp. Endocrinol.*, **58**, 44-50.  
Andersen,B.N. and Stadil,F. (1983) Sulfation of gastrin in Zollinger-

Ellison sera: evidence for association between sulfation and proteolytic processing. *Regul. Peptides*, **6**, 231-239.  
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.  
Baeuerle,P.A. and Huttner,W.B. (1985) Tyrosine sulfation of yolk protein 1, 2, and 3 in *Drosophila melanogaster*. *J. Biol. Chem.*, **260**, 6434-6439.  
Baker,R.T. and Board,P.G. (1980) The human ubiquitin gene family: Structure of a gene and pseudogenes from the UbB subfamily. *Nucleic Acids Res.*, **15**, 443-463.  
Bardram,L. and Rehfeld,J.F. (1989) Production and evaluation of monospecific antibodies for a processing-independent sequence of human progastrin. *Scand. J. Clin. Lab. Invest.*, **49**, 173-182.  
Beinfeld,M.C. (1994) Inhibition of pro-cholecystokinin (CCK) sulfation by treatment with sodium chlorate alters its processing and decreases cellular content and secretion of CCK 8. *Neuropeptides*, **26**, 195-200.  
Bradbury,A.F., Finnie,M.D.A. and Smyth,D.G. (1982) Mechanism of C-terminal amide formation by pituitary enzymes. *Nature*, **298**, 686-688.  
Brand,S.J., Andersen,B.N. and Rehfeld,J.F. (1984) Complete tyrosine-O-sulphation of gastrin in neonatal rat pancreas. *Nature*, **309**, 456-458.  
Chen,C. and Okayama,H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745-2752.  
Dente,L., Sollazzo,M., Baldari,C., Cesareni,G. and Cortese,R. (1985) In Glover,D.M. (ed.), *DNA cloning - The pEMBL family of single-stranded vectors*. IRL Press, Oxford, Vol. I, pp. 101-107.  
Friis-Hansen,L. and Rehfeld,J.F. (1994) Ileal expression of gastrin and cholecystokinin. *FEBS Lett.*, **343**, 115-119.  
Gregory,H., Hardy,P.M., Jones,D.S., Kenner,G.W. and Sheppard,R.C. (1964) The antral hormone gastrin. *Nature*, **204**, 931-933.  
Hille,A. and Huttner,W.B. (1990) Occurrence of tyrosine sulfate in proteins - a balance sheet. 2. Membrane proteins. *Eur. J. Biochem.*, **188**, 587-596.  
Hille,A., Braulke,T., Figura,K.v. and Huttner,W.B. (1990) Occurrence of tyrosine sulfate in proteins - a balance sheet. 1. Secretory and lysosomal proteins. *Eur. J. Biochem.*, **188**, 577-586.  
Hilsted,L. and Rehfeld,J.F. (1986) Measurement of precursors for  $\alpha$ -amidated hormones by radioimmunoassay of glycine-extended peptides after trypsin-carboxypeptidase B cleavage. *Anal. Biochem.*, **152**, 119-126.  
Hilsted,L. and Rehfeld,J.F. (1987)  $\alpha$ -carboxyamidation of antral progastrin. *J. Biol. Chem.*, **262**, 16953-16957.  
Hilsted,L., Rehfeld,J.F. and Schwartz,T.W. (1986), Impaired  $\alpha$ -carboxyamidation of gastrin in vitamin C-deficient guinea pigs, *FEBS Lett.*, **196**, 151-154.  
Hofsteenge,J., Stone,S.R., Donella-Deana,A. and Pinna,L.A. (1990) The effect of substitution phosphotyrosine for sulphotyrosine on the activity of hirudin. *Eur. J. Biochem.*, **188**, 55-59.  
Hortin,G.L., Folz,R., Gordon,J.I. and Strauss,A.W. (1986) Characterization of sites of tyrosine sulfation in proteins and criteria for predicting their occurrence. *Biochem. Biophys. Res. Commun.*, **141**, 326-333.  
Hortin,G.L., Farries,T.C., Graham,J.P. and Atkinson,J.P. (1989) Sulfation of tyrosine residues increases activity of the fourth component of complement. *Proc. Natl Acad. Sci. USA*, **86**, 1338-1342.  
Huang,S.C., Yu,D.-H., Wank,S.A., Mantey,S., Gardner,J.D. and Jensen,R.T. (1989) Importance of sulfation of gastrin or cholecystokinin (CCK) on affinity for gastrin and CCK receptors. *Peptides*, **10**, 785-789.  
Huttner,W.B. (1982) Sulphation of tyrosine residues- a widespread modification of proteins. *Nature*, **299**, 273-276.  
Huttner,W.B. and Baeuerle,P.A. (1988) Protein sulfation on tyrosine. *Mod. Cell Biol.*, **6**, 97-140.  
Jensen,S., Borch,K., Hilsted,L. and Rehfeld,J.F. (1989) Progastrin processing during antral G-cell hypersecretion in humans. *Gastroenterology*, **96**, 1063-1070.  
Johnsen,A.H. and Rehfeld,J.F. (1990) Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. *J. Biol. Chem.*, **265**, 3054-3058.  
Kariya,Y., Kato,K., Hayashizaki,Y., Himeno,S., Tarui,S. and Matsubara,K. (1986) Expression of human gastrin gene in normal and gastrinoma tissue. *Gene*, **50**, 345-352.  
Kunkel,T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488-492.  
Leyte,A., van Schijndel,H.B., Niehrs,C., Huttner,W.B., Verbeet,M.P., Mertens,K. and van Mourik,J.A. (1991) Sulfation of tyr1680 of human

- blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J. Biol. Chem.*, **266**, 740–746.
- Lin, W.-H., Larsen, K., Hortin, G.L. and Roth, J.A. (1992) Recognition of substrates by tyrosylprotein sulfotransferase. *J. Biol. Chem.*, **267**, 2876–2879.
- Monstein, H.-J., Thorup, J.U., Folkesson, R., Johnsen, A.H. and Rehfeld, J.F. (1993) cDNA deduced proclonin. Structure and expression in protochordates resemble that of procholecystokinin in mammals. *FEBS Lett.*, **331**, 60–64.
- Mutt, V. (1980) In Glass, G.B.J. (ed.), *Gastrointestinal hormones. Cholecystokinin: Isolation, Structure, and Functions*. Raven Press, New York, pp. 169–221.
- Mutt, V. and Jorpes, J.E. (1968) Structure of porcine cholecystokinin-pancreozymin. *Eur. J. Biochem.*, **6**, 156–162.
- Niehrs, C. and Huttner, W.B. (1990) Purification and characterization of tyrosylprotein sulfotransferase. *EMBO J.*, **9**, 35–42.
- Niehrs, C., Kraft, M., Lee, R.W.H. and Huttner, W.B. (1990) Analysis of the substrate specificity of tyrosylprotein sulfotransferase using synthetic peptides. *J. Biol. Chem.*, **265**, 8525–8532.
- Niehrs, C., Huttner, W.B. and Rütger, U. (1992) *In vivo* expression and stoichiometric sulfation of the artificial protein sulfophilin, a polymer of tyrosine sulfation sites. *J. Biol. Chem.*, **267**, 15938–15942.
- Niehrs, C., Beisswanger, R. and Huttner, W.B. (1994) Protein tyrosine sulfation, 1993 – an update. *Chem.-Biol. Interact.*, **92**, 257–271.
- Rehfeld, J.F. (1978) Immunochemical studies on cholecystokinin. *J. Biol. Chem.*, **253**, 4016–4021.
- Rehfeld, J.F. and Larsson, L.-I. (1981) Pituitary gastrins: different processing in corticotrophs and melanotrophs. *J. Biol. Chem.*, **256**, 10426–10429.
- Rehfeld, J.F., De Magistris, L. and Andersen, B.N. (1981) Sulfation of gastrin: effect on immunoreactivity. *Regul. Peptides*, **2**, 333–342.
- Rehfeld, J.F., Hansen, C.P. and Johnsen, A.H. (1995) Post-poly(Glu) cleavage and degradation modified by O-sulfated tyrosine: a novel post-translational processing mechanism. *EMBO J.*, **14**, 389–396.
- Rens-Domiano, S., Hortin, G.L. and Roth, J.A. (1989) Sulfation of tert-butoxycarbonylcholecystokinin and other peptides by rat liver tyrosylprotein sulfotransferase. *Mol. Pharmacol.*, **36**, 647–653.
- Santerre, R.F., Cook, R.A., Crisel, R.M.D., Sharp, J.D., Schmidt, R.J., Williams, D.C. and Wilson, C.P. (1981) Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic  $\beta$  cells. *Proc. Natl Acad. Sci. USA*, **78**, 4339–4343.
- Stone, S.R. and Hofsteenge, J. (1986) Kinetics of the inhibition of thrombin by hirudin. *Biochemistry*, **25**, 4622–4628.
- Suchanek, G. and Kreil, G. (1977) Translation of melittin messenger RNA *in vitro* yields a product terminating with glutaminyglycine rather than with glutaminamide. *Proc. Natl Acad. Sci. USA*, **74**, 975–978.
- van Solinge, W.W. and Rehfeld, J.F. (1990) Radioimmunoassay for sequence 38–54 of human progastrin: increased diagnostic specificity of gastrin-cell diseases. *Clin. Chim. Acta*, **192**, 35–46.
- Varro, A., Desmond, H., Pauwels, S., Gregory, H., Young, J. and Dockray, G.J. (1988) The human gastrin precursor. *Biochem. J.*, **256**, 951–957.
- Walsh, J.H. (1987) In Johnson, L.R., Christensen, J., Jackson, M.J., Jacobson, E.D. and Walsh, J.H. (eds), *Physiology of the Gastrointestinal Tract. Gastrointestinal Hormones*. Raven Press, New York, pp. 181–253.
- Wiborg, O., Berglund, L., Boel, E., Norris, F., Norris, K., Rehfeld, J.F., Marcker, K.A. and Vuust, J. (1984) Structure of a human gastrin gene. *Proc. Natl Acad. Sci. USA*, **81**, 1067–1069.

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