Both N-terminal and C-terminal regions of steroid sulfatase are important for enzyme activity

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Abstract

Steroid sulfatase (STS) is localized in the endoplasmic reticulum and catalyzes desulfation of 3 β -hydroxysteroid sulfates. X-linked ichthyosis (XLI) is an inherited skin disorder caused by deficiency of STS enzyme activity. We previously reported a case in which XLI with a one-base change in the STS gene and variation in amino acid Q560P developed. In this study, we performed molecular analysis to determine the importance of terminal regions of STS and the effect of mutant STS on STS enzyme activity. To examine the effect of terminal truncated STS on the enzyme activity, N- and C-terminal truncated STS expression vectors were transfected into COS-1 cells. The activity of truncated STS lacking the N-terminal regions declined, and the activity of C-terminal-truncated STS declined with extension of the truncated C-terminal

region. Although the results of pulse-chase experiments showed that a one-base mutant STS (Q560P) and C-terminal-truncated STS (Δ C2 (1–559)) had no effects on protein synthesis and degradation, the mutant STS and C-terminal-truncated STS have dominant negative effect on STS enzyme activity when the STS mutant or truncated STS protein and a wild-type STS protein coexist in cells. Results of coprecipitation of the truncated STS with an STS-FLAG fusion protein showed that STS formed a dimer conformation in cells. In this study, we have shown that both the N-terminal region and C-terminal region are important for STS enzyme activity. The C-terminal mutant has a dominant negative effect on wild-type STS.

Journal of Endocrinology (2006) **188**, 365–374

Introduction

Steroid sulfatase (STS) (EC 3.1.6.2) is present in various mammalian tissues, such as the liver, ovary and testis, and is especially abundant in the placenta (Warren & French 1965). This enzyme is a membrane-bound protein that is localized in the endoplasmic reticulum (ER) and catalyzes desulfation of cholesterol sulfate and 3 β -hydroxysteroid sulfates, such as progesterone sulfate, dehydroepiandrosterone sulfate and estrone sulfate (Willemssen *et al.* 1988, Stein *et al.* 1989). The sulfated forms of steroid hormones, which are inactive because of their failure to bind to their receptors, become active after desulfation by STS (Warren & French 1965). STS has been purified from microsomal fractions of tissues with detergents (Gauthier *et al.* 1978). Although the molecular mass of STS determined by SDS-PAGE is 63 kDa, STS seems to be a 126 kDa protein from its active appearance determined by gel-filtration analysis (Noel *et al.* 1983). Digestion of STS by endoglycosidase H has shown that STS has two N-glycosylation sites (Stein *et al.* 1989).

X-linked ichthyosis (XLI) is an inherited skin disorder caused by a deficiency of STS enzyme activity. Lack of

STS enzyme activity results in an excessive amount of epidermal cholesterol sulfate in the epidermis and alterations in the physical properties of corneocytes (Elias *et al.* 1984, Zettersten *et al.* 1998). XLI is characterized by a generalized scaling of the skin, with large, polygonal, dark-brown scales. XLI has been diagnosed by assaying STS activity in the placenta of the patient's mother or in the lymphocytes of the patient after birth (Traupe & Ropers 1982). The STS gene has been cloned, and it has been found that most patients with XLI have large deletions of the STS gene (Yen *et al.* 1987, Shapiro *et al.* 1989, Stein *et al.* 1989). However, point mutations in the STS gene have been reported in some XLI patients with complete STS deficiency (Alperin & Shapiro 1997). We previously reported two cases with a one-base change in the STS gene, and variations in amino acids Q560P and H444R were found in those cases (Sugawara *et al.* 2000). The finding of impairment of STS enzyme activity in a Q560P mutant in the C-terminal region of STS indicates that the C-terminal region of the STS enzyme is important for STS enzymatic function (Sugawara *et al.* 2000). In this study, we performed molecular analysis of both

Figure 1 Artificial truncated STS mutant proteins. ΔC2 lacks the C-terminal region of the STS protein including glutamine of codon 560. ΔN1 lacks the first N-terminal 24 amino-acid residues encoding a signal peptide from wild-type STS. ΔN2 lacks the first 52 N-terminal amino acids that include an N-glycosylation site, the residue of which is asparagine codon 47. Wild-type STS has 583 amino acids. STS cDNA and mutant STS cDNA were inserted into a pSV-SPORT-1 expression vector to express the truncated STS protein.

terminal regions of the STS protein and clarified the effect of a mutant STS protein on wild-type STS protein.

Materials and Methods

Plasmid constructs

Human STS cDNA (2.0 kb) encoding a full-length STS protein expression vector (pSTS) was prepared by inserting an EcoRI fragment from human STS cDNA into pSV-SPORT-1 (Sugawara *et al.* 2001). We previously reported a case of XLI with a one-base change in the STS gene and variation in amino acid Q560P, a mutant lacking STS enzyme activity (Sugawara *et al.* 2000). Q560P mutation was produced with a Transformer Site Directed Mutagenesis kit (Clontech) to yield the plasmid Q560P, as described previously (Sugawara *et al.* 2001). STS has N-terminal signal peptides of 23 amino acids, which start with a methionine and an N-glycosylation site at asparagine residue 47 in the N-terminal region. Various truncated STS expression constructs were prepared by PCR using human STS cDNA as a template and were cloned into pSV-SPORT1 at EcoRI restriction enzyme sites. The truncated STS proteins are illustrated in Fig. 1. ATG was added to the N-terminal region of the truncated STS mutant so that the mutant translates from methionine, and the terminal codon TAG was also added to the C-terminal region of the truncated STS mutant. STS (1–583), which lacks the 5′- and 3′-untranslated regions of the STS gene, encodes the full length of STS protein. ΔC1 (1–572) lacks 11 C-terminal amino acids, and

ΔC2 (1–559) lacks 24 C-terminal amino acids, including glutamine 560. ΔN1 (24–583) lacks the first 24 N-terminal amino acids that encode a signal peptide to microsomes, and ΔN2 (52–583) lacks the first 52 N-terminal amino acids that include an N-glycosylation site, codon 47 (Asn). ΔN1C2 (24–559) lacks the N-terminal 24 amino acids and the C-terminal 24 amino acids, and ΔN2C2 (52–559) lacks the N-terminal 52 amino acids and the C-terminal 24 amino acids. To produce an STS and C-terminal FLAG fusion protein, which is called an STS–FLAG fusion protein expression vector, an EcoRI fragment of the human STS cDNA was amplified by PCR from pSTS and cloned into the p3x FLAG vector (Sigma).

Cell culture and transfection

Monkey kidney COS-1 cells were obtained from RIKEN Bank (Tsukuba, Japan). COS-1 cells were grown in 35 mm plastic dishes and cultured in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 50 µg/ml gentamycin. COS-1 cells were transfected with wild-type STS cDNA and truncated mutant STS cDNA expression vectors, using FuGENE 6 (Roche), as described previously (Sugawara *et al.* 2001). Some cultures were cotransfected with wild-type STS cDNA and truncated mutant STS. The cells were allowed to incubate for 48 h. At the end of the culture, extracts were collected for evaluation of STS enzyme activity and for Western blotting. The cells were washed twice with PBS, and

cell extracts were prepared in 200 µl STS lysis buffer (10 mM Tris-HCl, 1% (v/v) Triton-X, 1 × proteinase inhibitor (Roche)). Protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Each experiment was performed on at least three separate occasions with three different cell preparations to confirm the generality of the findings.

STS enzyme activity

STS enzyme activity was assayed by the previously described method (Sugawara *et al.* 1994). The reaction mixture, which contained 20 mM Tris-HCl (pH 8.0), 50 000 d.p.m. of 7-[³H]dehydroepiandrosterone sulfate (DHEAS) (16 Ci/mmol; New England Nuclear, Boston, MA, USA), 100 pmol DHEAS, 1% (v/v) Triton-X-100 and 25 µl cell extracts, was incubated at 37 °C for 1 h. At the end of incubation, desulfated DHEA was extracted by the addition of benzene and was assayed by a liquid scintillation counter after adding 3 ml scintillation mixture. One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 pmol DHEAS per h/mg.

Western blot analysis

Cultures of subconfluent COS-1 cells were plated so that 35 mm tissue culture dishes received equal numbers of cells. COS-1 cell extracts were harvested for Western blot analysis after transfection. An amount of 10 µg cell extract was then subjected to SDS-PAGE. After electrophoresis, the gels were transferred to nitrocellulose membranes for immunodetection with rabbit anti-STS serum, which was prepared by immunization of rabbits with human STS, as described previously (Sugawara *et al.* 1994). Rabbit polyclonal anti-STS serum was used as a primary antibody at a dilution of 1:1000. After incubation for 1 h at 25 °C, the blot was further incubated for 60 min at 25 °C with horseradish peroxidase-conjugated affinity pure goat anti-rabbit antibody (Roche) at a dilution of 1:10 000. The signal was detected by chemiluminescence with ECL Western blotting detection reagents (Amersham Pharmacia Biotech). The membrane was then exposed to RX-U film (Fuji, Tokyo, Japan).

Northern blot analysis and preparation of a cDNA probe

COS-1 cells were cultured for 1 day after being subcultured in 100 mm plastic dishes and were then transfected with various kinds of pSTS and truncated mutant STS cDNA expression vectors using FuGENE 6. The protocols for the preparation, culture and isolation of total RNA from COS-1 cells have been described in detail previously (Sugawara & Fujimoto 2004). For Northern blot analysis, 30 µg total RNA from each dish were separated by electrophoresis and transferred to a

nylon membrane (Biodyne, ICN, Glen Cove, NY, USA). Detection of the signal was performed in accordance with the standard protocol for a nucleic acid detection kit (Roche). Northern blots were probed with human STS cDNA. The human STS cDNA in pSPORT-1 was linearized with SalI. Digoxigenin-labeled human STS probes were produced by *in vitro* transcription with T7 RNA polymerase and an RNA labeling kit (Roche).

In vitro translation

STS proteins were synthesized *in vitro* with an Sp6 RNA polymerase-based TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's manual. An amount of 500 ng pSTS or truncated expression vector was mixed with *in vitro* translation reaction mixtures. Translation reactions were performed with SP6 TNT RNA polymerase for 90 min at 30 °C. A volume of 10 µl 2 × SDS sample buffer was added to 10 µl *in vitro* translated products, heated for 5 min and subjected to 12% (w/v) SDS-PAGE and then to Western blot analysis.

Pulse-chase experiments

COS-1 cells were transfected with pSTS (2.0 µg) or mutant STS expression vectors 1 day before metabolic labeling. COS-1 cells were incubated with methionine-free DMEM for 15 min and then labeled with ³⁵S-methionine (0.4 mCi/ml) for 30 min. After labeling, the radioactive medium was replaced with DMEM containing 4 mM methionine, and the cells were incubated for the indicated time. Then the cells were washed with PBS and scraped into 400 µl RIPA buffer (50 mM Tris-HCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) deoxycholate, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 1 × proteinase inhibitor, Compete Mini (Roche)). Equal aliquots of protein from cell extracts were precleared with 20 µl protein-G Sepharose (Amersham Pharmacia) for 30 min at 4 °C. After centrifugation, supernatants were incubated with 1 µl anti-STS serum for 3 h at 4 °C and then overnight on a rocking platform with 20 µl protein-G Sepharose at 4 °C. Immunocomplexes were washed four times by resuspension in 500 µl RIPA buffer and collected by centrifugation. Pellets were resuspended in 10 µl 2 × SDS sample buffer and then subjected to SDS-PAGE. Gels were dried and exposed to Fuji RX-U film.

Coimmunoprecipitation with STS-FLAG fusion protein

COS-1 cells were cultured in 100 mm plastic dishes and were transfected with the STS mutant and STS-FLAG fusion expression vectors with FuGENE 6. For coprecipitation, whole-cell extracts (1 mg) from COS-1 cells in 400 µl RIPA buffer were incubated with anti-FLAG IgG (Sigma) for 1 h at 4 °C in 500 µl IP buffer

(50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1.5 mM EGTA, 0.1% (v/v) Triton X-100, 1 mM DTT, 1 mM PMSF and 1 × protease inhibitor). Then 20 µl protein A Sepharose-FF (Amersham Pharmacia) were added, and the mixture was incubated overnight at 4 °C. The bound complex was then washed three times with IP buffer. The precipitated proteins were eluted with 20 µl 2 × SDS sample buffer containing 2% (v/v) 2-mercaptoethanol (2-ME) and resolved on a 10% (w/v) SDS polyacrylamide gel. Western blot analysis was performed with a rabbit polyclonal anti-STC serum at 1:1000 dilution. Some precipitated proteins were eluted with 100 µl of 100 mM glycine-HCl buffer (pH 3.0) and then neutralized with 100 µl of 1 M Tris-HCl (pH 8.0), and STC enzyme activity was assayed.

Data analysis

Values are presented as means ± S.E. One-way ANOVA was used to test differences in repeated measures across experiments. Significant results from ANOVA were further analyzed by Tukey's post-hoc test. $P < 0.05$ was considered significant.

Results

Effect of terminal truncated STC

To examine the effect of truncated STC on the enzyme activity, truncated STC expression vectors were transfected into COS-1 cells, which do not express endogenous STC enzymes, and STC enzyme activities were assayed (Fig. 2). The enzyme activity level of STC in the mock transfection was low. The activity level of STC in COS-1 cells transfected with truncated STC lacking the N-terminal regions (Δ N1 and Δ N2) or the C-terminal regions (Δ C1 and Δ C2) was significantly lower ($P < 0.01$) than that in COS-1 cells transfected with wild-type STC. The activity level of STC in COS-1 cells decreased greatly, depending on the number of missing amino acids in the truncated C-terminal region. The STC enzyme activity in COS-1 cells transfected with the Δ C2 truncated expression vector that lacks C-52 amino acids was only 5.1% of that in COS-1 cells transfected with wild-type STC and did not differ significantly from that in the mock transfection. When the truncated STC expression vectors that lack both the N-terminal and C-terminal regions (Δ N1C2 and Δ N2C2) were transfected into COS-1 cells, STC enzyme activity was completely impaired.

To examine the protein expression of truncated STC in COS-1 cells, we performed Western blot analysis of extracts from COS-1 cells into which various kinds of truncated STC expression vectors had been transfected (Fig. 3A). The protein levels of the C-terminal truncated STC (Δ C1 and Δ C2) were almost the same as the levels of wild-type STC protein in COS-1 cells. When the

Figure 2 STC enzyme activity of truncated STC. pSV-SPORT-1 (Mock), STC (wild-type), STC (1-583), Δ C1 (1-572), Δ C2 (1-559), and Δ N1, Δ N2, Δ N1C2 and Δ N2C2 plasmids were transfected into COS-1 cells. STC activities in transfected cell extracts in STC lysis buffer (10 mM Tris-HCl, 1% Triton-X, 1 × protease inhibitor) were assayed. Values presented are means ± S.E. of STC enzyme activities from four separate experiments. Different superscript letters indicate significant differences ($P < 0.05$), as determined by ANOVA followed by Tukey's post-hoc test.

truncated STC lacking the C-terminal region was transfected into COS-1 cells, the protein translation was not impaired. On the other hand, the protein enzyme levels of truncated STC lacking the N-terminal region (Δ N1, Δ N2, Δ N1C2 and Δ N2C2) decreased. The molecular sizes of truncated proteins detected by antihuman STC serum were smaller and the amounts of protein were less than those of wild-type STC. Several minor protein bands were also detected in COS-1 cells transfected with the N-terminal truncated STC expression vectors. To examine the expression of truncated STC, we performed Northern blot analysis, using total RNA from COS-1 cells that had been transfected with truncated STC expression plasmids (Fig. 3B). STC mRNA levels were unaltered in COS-1 cells that had been transfected with truncated expression vectors. These observations suggest that truncated STC transcription has the same effect as that of wild-type STC.

To determine the effect of N-terminal truncated STC (Δ N1, Δ N2, Δ N1C2 and Δ N2C2) on protein synthesis processes, we examined the efficiency of plasmid expression, using *in vitro* transcription/translation (Fig. 4). Translation products were subjected to SDS-PAGE and then to Western blot analysis. The amounts of STC translation products in *in vitro* transcription-translation reaction mixtures were determined. Although several

Figure 3 Truncated STS protein and STS gene expression. Truncated STS expression vectors were transfected into COS-1 cells with FuGENE 6. (A) A volume of 10 µg cell extracts was subjected to Western blot analysis with anti-human STS serum. The membrane was stripped according to the supplier's protocol, and immunodetection was carried out with anti-human GAPDH IgG. (B) RNA was extracted from COS-1 cells after 24-h cotransfection treatment. Total RNA (30 µg) was subjected to Northern blot analysis with wild-type human STS RNA probes.

minor bands were detected, the truncated STS lacking the N-terminal signal peptides of STS (ΔN1 and ΔN1C2) had no apparent effect on protein synthesis, indicating that the N-terminal regions of STS have only a small effect on translational processes in a cell-free system.

We performed pulse-chase experiments to determine whether a C-terminal one-base mutant STS (Q560P) and a C-terminal truncated STS (ΔC2) have effects on the synthesis and degradation of STS protein (Fig. 5). COS-1 cells were transfected with wild-type STS, ΔC2 and Q560P expression plasmids and then metabolically labeled with ³⁵S-methionine. During a pulse period up to 24 h, the labeled protein levels of wild-type STS and the ΔC2 and Q560P mutants decreased with progress of time. The results of pulse-chase experiments on COS-1 cells that had been transfected with the one-base mutant Q560P and N-terminal region-truncated STS were compared with

Figure 4 Truncated STS protein in an *in vitro* translation system. Translated proteins were synthesized in an *in vitro* system. A volume of 10 µl of *in vitro* translated products was subjected to Western blot analysis with anti-human STS serum.

the results of pulse-chase experiments on COS-1 cells that had been transfected with a wild-type STS expression vector. The results showed that both the C-terminal one-base mutant and truncated mutant protein disappeared from COS-1 cells as fast as did wild-type STS. Thus, the degradation of STS protein is not affected by a C-terminal mutation of STS.

Dominant negative effect of C-terminal STS mutant

The STS enzyme activity in COS-1 cells that had been transfected with ΔC2 or that in cells that had been transfected with Q560P was significantly ($P < 0.05$) lower than that in cells that had been transfected with wild-type STS alone (Table 1). The enzyme activity levels in COS-1 cells into which Q560P had been introduced and in COS-1 cells into which ΔC2 had been introduced were 2.8% and 3.5% respectively of the level in cells into which the wild-type STS alone had been transfected. The enzyme activity level in COS-1 cells into which both 1 µg wild-type STS and 1 µg ΔC2 truncated expression vector had been introduced was significantly ($P < 0.05$) decreased compared with that in cells transfected with the wild-type STS alone. The enzyme activity level in cells into which both wild-type STS and ΔC2 truncated had been introduced did not differ from that in cells transfected with both wild-type STS and Q560P. The enzyme activity levels in COS-1 cells that had been cotransfected with wild-type STS and ΔC2 and in COS-1 cells that had been cotransfected with wild-type STS and Q560P were 37.9% and 40.8% respectively of the level in COS-1 cells that had been transfected with only wild-type STS.

Figure 5 Results of pulse-chase experiments on transfection with wild-type STS (pSTS), mutant STS (pQ560P) and truncated STS (Δ C2) expression plasmids. COS-1 cells were transfected with pSTS, pQ560P and pSTSM1A6 (2 μ g/plate), using FuGENE 6. After transfection, COS-1 cells were pulse-labeled with 35 S-methionine for 30 min and then chased with an excess of cold methionine followed by immunoprecipitation with anti-human STS serum of the proteins and SDS-PAGE. Autoradiograms with wild-type STS, mutant STS (Q560P) and truncated STS (Δ C2) are shown. Each experiment was performed on two separate occasions.

To determine the dose-dependent effect of Δ C2 on the production of wild-type STS, 0.01–3 μ g Δ C2 were coinroduced with 1 μ g wild-type STS expression vector

Table 1 C-terminal mutated STS reduced enzyme activity of wild-type STS

Plasmids	STS enzyme activity (pmol/h.mg)
Mock	1.5 $\pm$ 0.5 ^c
STS (Wild-type)	31.4 $\pm$ 4.5 ^a
Q560P	0.9 $\pm$ 0.5 ^c
Δ C2	1.1 $\pm$ 0.4 ^c
STS+Q560P	12.8 $\pm$ 5.7 ^b
STS+ Δ C2	11.9 $\pm$ 1.8 ^b

Values presented are means $\pm$ S.E. of STS enzyme activities from three separate experiments. Values with a different superscript letter are significantly different ($P < 0.05$) from others.

into COS-1 cells, and the rates of decrease in STS enzyme activity level were assayed (Fig. 6A). The levels of STS enzyme activity in COS-1 cells that had been cotransfected with 0.01, 0.05, 0.1 and 0.5 μ g Δ C2 and 1 μ g wild-type STS decreased by 9%, 13%, 29% and 50% respectively. Truncated STS has a dominant negative effect on wild-type STS when both truncated STS and wild-type STS proteins exist in cells. To examine the effect of truncated STS on STS protein levels, we performed Western blot analysis of extracts from cells into which truncated STS and wild-type STS plasmids had been transfected (Fig. 6B). The levels of STS, including truncated STS, in COS-1 cells that had been transfected with wild-type STS and truncated STS were not affected by the amount of truncated STS protein.

To determine whether the C-terminal STS mutant has a dominant negative effect on wild-type STS, we coinroduced Q560P mutant expression vectors with wild-type STS expression vectors into COS-1 cells (Fig. 7). The levels of STS enzyme activity in COS-1 cells

Figure 6 STS enzyme activity of truncated STS and wild-type STS in cells. (A) COS-1 cells were cotransfected with 1 μg pSTS and increasing amounts of truncated STS expression vectors. Values presented are the means ± S.E. of STS enzyme activities, expressed as a percentage of the wild-type STS activity, from four separate experiments. Different superscript letters indicate significant differences ($P < 0.01$), as determined by ANOVA followed by Tukey's post-hoc test. (B) Western blot analysis was performed with COS-1 cell extracts, using anti-STS.

that had been cotransfected with 0.01, 0.05, 0.1 and 0.5 μg Q560P and 1 μg wild-type STS decreased by 7%, 17%, 24% and 51% respectively. Q506P has a dose-dependent effect on STS enzyme activity in COS-1 cells transfected with wild-type STS. Q560P has a dominant negative effect on wild-type STS when both Q560P mutant STS and wild-type STS protein exist in cells.

Dimer conformation of STS

To investigate the dominant negative effect on wild-type STS, we performed immunoprecipitation with an STS-FLAG fusion protein. Whole-cell extracts from COS-1 cells cotransfected with ΔC2 and pSTS-FLAG were incubated with anti-FLAG IgG. The protein complex was precipitated with protein A Sepharose-FF. The protein complexes were subjected to 10% SDS-PAGE by running the gel under reducing conditions and were then analyzed by Western blotting with an STS antibody. As shown in Fig. 8A, FLAG antibody detected STS-FLAG fusion protein of mass 66 kDa in cell extracts from COS-1 cells. As shown in Fig. 8B, STS antibody detected both STS-FLAG and ΔC2 mutant protein in cell extracts.

Figure 7 STS enzyme activity of Q560P STS mutant and wild-type STS in cells. COS-1 cells were cotransfected with 1 μg pSTS and increasing amounts of pQ560P STS mutant expression vectors. Values presented are the means ± S.E. of STS enzyme activities, expressed as a percentage of the wild-type STS activity, from three separate experiments. Different superscript letters indicate significant differences ($P < 0.01$), as determined by ANOVA followed by Tukey's post-hoc test.

ΔC2 mutant protein was precipitated with STS-FLAG fusion protein (Fig. 8C). The coprecipitation results showed that STS-FLAG interacted with ΔC2 mutant protein in cells. The results showed the STS forms a dimer conformation in cells.

To determine the dominant negative effect of Q560P, various amounts of pQ560P were transfected into COS-1 cells with pSTS-FLAG (0.5 μg/plate) and pFLAG. Whole-cell extracts from COS-1 cells cotransfected with expression plasmids were incubated with anti-FLAG IgG. The protein complex was precipitated with protein A Sepharose-FF. Precipitated proteins were eluted with 100 mM glycine-HCl buffer (pH 3.0), and STS enzyme activity was assayed. The STS activity of COS-1 cells that had been transfected with pQ560P and the STS activity of COS-1 cells not transfected with pQ560P were compared (Fig. 9). The STS activity levels of COS-1 cells decreased by 50%, 71% and 78% when the cells were transfected with 0.12, 0.25 and 0.5 μg pQ560P and 0.5 μg pSTS-FLAG respectively.

Discussion

STS is synthesized in the ER and is transported to the lumen side of the ER by translocation hydrophobic signal peptides on the N-terminal region of STS. N-terminal signal sequences are cleaved by signal peptidases that recognize Ser21-His22 or Ala23-Ala24 equally at the *trans* side of the membrane (von Heijne 1986). Lower levels of protein were detected when N-terminal region-deleted STS protein expression vectors (ΔN1, ΔN2, ΔN1C2 and ΔN2C2) were transfected into COS-1 cells.

Figure 8 Interaction between STS-FLAG fusion protein and M1A6 STS mutant in cells. Whole-cell extracts were prepared from COS-1 cells that had been transfected with 0.12–1.0 μg $\Delta C2$ together with 0.5 μg STS-FLAG fusion protein expression vector or an empty FLAG vector, so that the total amount of plasmids transfected in COS-1 cells was 1.5 μg . Preimmunoprecipitated cell extracts were subjected to 10% SDS-PAGE, and then Western blot analysis was performed with anti-FLAG (A) and anti-STS antibodies (B). Cell extracts were incubated with anti-FLAG IgG and immunoprecipitated by protein A Sepharose-FF. Washed beads were boiled in 2 $\times$ SDS sample buffer containing 2% 2-mercaptoethanol, and precipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting with an anti-STS antibody (C).

The enzyme activity level of truncated proteins decreased in proportion to the amount of protein. The steroidogenic associated enzyme P450c21 with an N-terminal region deletion produces small amounts of protein, and the N-terminal hydrophobic domain has been shown to be important for protein stability (Hsu *et al.* 1993). N-terminal signal peptide-deleted STS protein may not move to an appropriate intracellular position and may not fold properly in cells. Correct folding of a protein is important for its stability, since denatured proteins are known to be degraded more rapidly than are native forms (Pakula & Sauer 1989). Proteins acquire their proper tertiary and quaternary structures by glycosylation in the lumen of the ER in mammals. The importance of N-linked oligosaccharides in the folding of glycoproteins is widely recognized (Hurtley & Helenius 1989). Two sites (Asn47 and Asn259) of the four potential N-glycosylation sites of STS are used for glycosylation (Stein *et al.* 1989). Several minor protein bands that were detected in Western blots were thought to be degraded

STS proteins rather than nonspecific protein bands. Although *in vitro* translation reactions of both $\Delta N1C2$ and $\Delta N1$ proceeded properly and the N-terminal signal seems to have no influence on the translation, N-terminal region-deletion mutants, $\Delta N2$ and $\Delta N2C2$, which lack the N-glycosylation site, may fail to fold properly and may degrade rapidly in cells. Several minor protein bands were thought to result in initiation of translation at internal downstream methionines.

Since the level of $\Delta N2C2$ expression in an *in vitro* translation system is lower than that of $\Delta N2$, a lack of C-terminal regions also appears to have some effects on STS protein levels. Western blot analysis of C-terminal region-truncated STS proteins ($\Delta C1$ and $\Delta C2$) has shown that the C-terminal region does not affect protein expression, and the results of a pulse-chase experiment have shown that the C-terminal region of STS ($\Delta C2$) has no effect on protein degradation. The results of x-ray crystallography of STS have recently been reported (Hernandez-Guzman *et al.* 2003). The STS structure

Figure 9 Dominant negative effects of STS enzyme activity of STS mutant. COS-1 cells were cotransfected with 0.5 μg pSTS-FLAG and increasing amounts of pQ560P STS. Cell extracts were incubated with anti-FLAG IgG and immunoprecipitated by protein A Sepharose-FF. Precipitated proteins were eluted with sodium acetate buffer, and STS enzyme activity was assayed. Values presented are the means $\pm$ S.E. of STS enzyme activities, expressed as a percentage of the STS-FLAG activity, from three separate experiments. Different superscript letters indicate significant differences ($P < 0.01$), as determined by ANOVA followed by Tukey's post-hoc test.

consists of antiparallel β -helices that traverse the membranes and anchor the functional domain on the membrane surface facing the ER lumen. Therefore, the polar catalytic domain rests on the lumen side of the lipid bilayer. Residues from the membrane-associated region line the groove leading to the active site. Residues 468–500 and 548–568 constitute the entrance to the active site. These two residues are thought to be an important region for enzyme activity, and Q560P and truncated Δ C2 mutant proteins lack STS enzyme activity in cells.

Dominant-negative mutations in membrane receptors or DNA-binding proteins, including oncogenes, have been reported (Herskowitz 1987). The binding ability of these proteins depends on the cooperation of two or more motifs belonging to different subunits. Although a dominant negative mutation is not usually expected for diseases involving defects of enzymes, since enzymes are so active that there is no loss of function unless their levels have decreased to less than 10–15% of normal levels, several dominantly inherited metabolic diseases have been reported (Xiao *et al.* 1995, Chamberlin *et al.* 1997). In a heterozygous state, these mutants antagonize the activity of the remaining wild-type allele and give a phenotype approaching a null. STS proteins form homodimer complexes (Iwamori *et al.* 1976, Gniot-Szulzycka & Januszewska 1986). Mutant Q560P protein may not only fail to have the proper conformation like wild-type STS but also bind wild-type STS, alter catalytic domains and

abrogate the enzyme activities. The mutant binds wild-type STS, and the stability of the whole heteroallelic complexes may be decreased. The sequence identity of STS was 32% of arylsulfatase A sequence. According to sequence alignment, codon Q560 is conserved in codon 486 of arylsulfatase A, and the shape, size and structure of that domain resemble those of the soluble form of human sulfatase (Lukatela *et al.* 1998). A nonsense mutation (Q486X) in the arylsulfatase A gene has been reported in a patient with metachromatic leukodystrophy (Harvey *et al.* 1994). This site is important for both sulfatase enzyme activity and structure of protein constructs because the C-terminal STS mutant has shown a dominant negative effect.

In this study, we have shown that both the N-terminal regions and C-terminal region are important for STS enzyme activity. C-terminal region mutants have a dominant negative effect on the activity of wild-type STS. Since estrone sulfate is an important source of estrogens in postmenopausal women, development of inhibitors of estrone sulfatase activity may be of value in the treatment of hormone-dependent breast cancers. Plasmids that have a dominant negative effect on STS activity might be useful in treatment of hormone-dependent cancer. Future studies are needed to determine the application of this to cancer therapy.

Acknowledgements

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 7 November 2005

Accepted 15 November 2005

Made available online as an Accepted Preprint

22 November 2005