Analysis of the thyrotropin-releasing hormone-degrading ectoenzyme by site-directed mutagenesis of cysteine residues

Cys68 is involved in disulfide-linked dimerization

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Thyrotropin-releasing hormone (TRH) acts both as a hypothalamic hypophysiotropic hormone (reviewed in [1,2]) and a neuropeptide or neurotransmitter that elicits a wide range of pharmacological and behavioral effects in extra- and hypothalamic brain areas (reviewed in [3–5]). These functions are mediated by high-affinity TRH-receptors [6–8] and imply the existence of an efficient inactivation system.

Inactivation was found to be initiated by cleavage of the l-pyroglutamyl-histidinyl bond by a particular ectoenzyme (reviewed in [9,10]) that, similar to the TRH-degrading serum enzyme, exhibits a high degree of substrate specificity and does not cleave any of the other known pyroGlu-containing neuropeptides [11–14]. Thus, this enzyme has been termed thyroliberinase, TRH-specific aminopeptidase, and TRH-degrading ectoenzyme (TRH-DE; EC 3.4.19.6). It is also known as pyroglutamyl-peptidase II or pyroglutamyl-aminopeptidase II (PAP II) to distinguish it from the unspecific, cytosolic pyroglutamyl-aminopeptidase I (PAP I). It is found in synaptosomal [12] and adenohypophyseal [15] membrane fractions. In cell cultures from murine brain it is found on the surface of neuronal cells but not on glial cells [16,17], and is thus adequately located to terminate neurotropic TRH signals. This notion is strengthened by the analysis of the region-specific expression of TRH-DE in the rat nervous system [18].

Following purification of the enzyme [19] and cloning of the cDNA of the rat TRH-DE [20], the cDNA of the human TRH-DE has also been cloned recently [21]. Rat and human TRH-DE reveal an unusually high degree of conservation. Overall, there are 96% identical residues around the Zn-binding consensus sequence. In fact, there is a stretch of 224 amino acids without any substitution. Based on biochemical studies and cDNA cloning data, the rat and human TRH-DE were identified as a glycosylated, type II metalloproteinase [19,20,22]. The extracellular domain contains the HEXXH + E motif that is characteristic of Zn-dependent aminopeptidases (reviewed in [23]). A comparison of the complete amino-acid sequence of rat TRH-DE and those in the translated GenBank revealed significant homology with rat aminopeptidase N [20,24] (34%) and mouse aminopeptidase A (32%) [25]. While the intracellular parts and the transmembrane-spanning regions of these three enzymes are completely different, some sequences in the extracellular domain display a high degree of homology. Notably, an
alignment of the C-terminal domains of TRH-DE and aminopeptidase N revealed that in these enzymes the last four cysteine residues are conserved (Fig. 1). However, in the sequence of aminopeptidase A this homology does not exist. Moreover, TRH-DE consists of nine cysteines all included in the extracellular domain. TRH-DE is unique in the Zn-dependent aminopeptidase family in that it is the only peptidase that contains a cysteine residue within the zinc-binding motif. We were therefore interested in studying whether this cysteine residue is of functional significance, especially with respect to the unusually high degree of substrate specificity. Moreover, we were interested in exploring the potential role of all cysteine residues of TRH-DE as chemical modification studies previously established that cysteine residues are not directly involved in enzyme catalysis [26] and the odd number of cysteines thus indicated that the enzyme may exist as a disulfide-linked homodimer.

By site-directed mutagenesis we therefore replaced each cysteine individually by a serine residue, expressed the mutant cDNA in BHK-cells and investigated the enzymatic and structural properties of the mutated enzyme.

**EXPERIMENTAL PROCEDURES**

**Site-directed mutagenesis**

Site-directed mutagenesis was performed according to the one-step overlap extension PCR-method of Urban et al. [27]. For mutagenesis, the target DNA-fragments were cloned into pBluescript II KS and SK (Stratagene). These fragments served as templates in a single PCR reaction in the presence of the universal primer T7 (5'-GTAATACGACTCACTATAGGGC-3') and two mutagenic primers which contained an overlapping region of 19 nucleotides. The PCR products were loaded onto a 1% (w/v) agarose gel and purified using the JetSorb Purification Kit (Genomed). The presence of the mutation and the absence of nonspecific mutations was confirmed by DNA sequencing by an Applied Biosystems 371 automated sequencer using fluorescent dNTPs (ABI). Sequence analysis was performed using the program macmolly tetra (SoftGene GmbH). Each mutant was then subcloned back to the wild-type plasmid pBs II KS/rat TRH-DE.

Mutants C68S and C174S were restricted by XhoI and SmalI, mutant C279S by BspMI and BglII. The C338S PCR fragment was cleaved by SmaI and BglII and the C444S fragment by BglII and BsmI. SacI was used for subcloning the C823S, C830S, C859S and C895S mutants and the region around the mutation was sequenced.

**Construction of expression plasmids**

The full-length rat TRH-DE cDNA for wild-type and the mutants was restricted by NotI and XbaI and subcloned into the expression vectors pcDNA3.1/His A (Invitrogen) and pFLAG-CMV1 (Kodak).

For subcloning the N-terminal fragment (350 nucleotides) of wild-type rat TRH-DE and the C68S mutant into the pEGFP-N1 Vector (Clontech) a PCR-reaction was performed. The

**Fig. 1.** Schematic representation of the primary structure of rat TRH-DE, rat APN and mouse APA. Cysteine residues and their individual positions are indicated by vertical lines. The open box indicates the N-terminal intracellular domain and the black box indicates the transmembrane domain. The C-terminal extracellular domain, with the zinc-binding site (HEXXH-motif) is represented by the hatched box. aa, Amino acids.

**Fig. 2.** Western blot analysis of wild-type and mutated rat TRH-DEs expressed in BHK-cells. The top diagram shows the schematic representation of the primary structure of rat TRH-DE in pcDNA3.1. The complete open-reading frame was fused in-frame C-terminal to the polyhistidine tag. The nine cysteine residues of the wild-type transcript are indicated as vertical lines on the top of the diagram. The closed box indicates the transmembrane spanning domain and the hatched box indicates the zinc-binding consensus motif. Following transient transfection of the BHK-cells with pcDNA3.1, pcDNA3.1/lacZ, wild-type and mutants, the membrane fractions containing the ectoenzyme were subjected to SDS/PAGE as described under Experimental procedures. The His-tagged proteins were detected using the SuperSignal HisProbe Western Blotting Kit (Pierce). The positions of the molecular mass markers (in kDa) are indicated.
forward primer 5'-ATATCCAGCGAATTCGCGGCCGCCAT-GG-3' used included the Start ATG, the NotI restriction site and an EcoRI site. As reverse primer 5'-GTTGCGCGGATCC-GATCCTGGGTAGCTG-3' was selected to introduce a BamHI site for the continuous in frame cloning with GFP. The amplified-fragment was digested by EcoRI and BamHI and subcloned into the corresponding site of the pEGFP-N1-vector. Conditions for the PCR reaction were the following: initial 2 min at 95 °C, 30 s at 50 °C, 1 min at 72 °C followed by 2 cycles of 1 min at 95 °C, 30 s at 55 °C, 1 min at 72 °C followed by 30 cycles of 20 s at 95 °C, 20 s at 60 °C, 1 min at 72 °C, with an additional extension at 72 °C for 3 min in the last cycle.

**Tissue culture and tranfection of BHK-cells**

BHK-cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum in a 37 °C incubator with 10% CO₂. For transient expression, BHK-cells were plated onto six-well plates at 2 × 10⁵ cells per well or on 150-cm² Petri dishes at 2 × 10⁶ cells. After 18 h of growth, the cells were washed twice with DMEM and transfected (2 μg of DNA per well or 10 μg of DNA per dish) by using LipofectAmine-PLUS (Life Technologies) as cationic lipid (1 μg DNA per 6 μg lipid). The cells were incubated for 4 h, and DMEM containing 10% fetal bovine serum was added. At 48 h after transfection, the cells were collected, washed with NaCl/Pi, and homogenized in ice-cold 20 mM potassium phosphate, pH 7.4. After centrifugation (20 000 g for 30 min at 4 °C) the cell pellet was washed in the same buffer and then resuspended in ice-cold 20 mM potassium phosphate, 500 mM NaCl, pH 7.4. After 30 min on ice the membranes were collected by centrifugation (20 000 g for 30 min at 4 °C). This step was repeated once. The final membrane pellet was homogenized in 20 mM potassium phosphate, pH 7.4 and stored at −80 °C until used for Western blot analysis and enzyme assays. The protein was determined by a modification of the Lowry method as described by Peterson [28].

**Confocal fluorescence microscopy**

BHK-cells expressing recombinant enhanced green fluorescence protein (EGFP)-tagged rat TRH-DE were harvested 24 h after transfection and cultured on glass coverslips for 48 h, washed in NaCl/Pi, fixed with 3% formaldehyde in NaCl/Pi for 20 min at room temperature and washed in NaCl/Pi again. Cells were viewed under a Leica confocal fluorescence microscope equipped with the appropriate filter set and photographed.

**SDS/PAGE and Western blot analysis**

Polyacrylamide gel electrophoresis was carried out according to Laemmli [29] using a 4% stacking and a 7.5% separating gel. After blotting [30] onto nitrocellulose membranes (BAS83, Schleicher and Schuell) by use of a tank blot (Biorad Trans Blot Cell) the proteins were detected either by the chemical luminescence technique (SuperSignal HisProbe Western Blotting Kit, Pierce) or by an immunochemical method using either anti-FLAG monoclonal antibody M2 (Kodak) as primary
antibody and alkaline-phosphatase conjugated goat anti-(mouse IgG) Ig (Biorad) as secondary antibody or the living colors peptide antibody-AP conjugate (Clontech). The protein expression levels were estimated by densitometric scanning (Duoscan T1200, Agfa) and calculated using the program NIHIMAGE (NIMH).

Enzyme assay

The activity of the TRH-DE was measured by the radio-chemical assay in the presence of the inhibitors of the soluble TRH-degrading enzymes as described in [16] using [pyroGlu-3H]TRH as substrate. The kinetic parameters ($K_m$ and $V_{max}$) were determined from Lineweaver–Burk plots.

RESULTS

Characterization of cysteine mutants of rat TRH-DE expressed in BHK-cells

Each of the nine cysteine residues in rat TRH-DE was mutated individually to serine. The mutant cDNAs were expressed in BHK-cells. Western blot analysis of the membrane fractions prepared from these cells showed that each mutant enzyme had the same apparent molecular mass (130 kDa) as the recombinant wild-type enzyme, and thus appeared to be similarly glycosylated (Fig. 2).

The activity of each mutant enzyme was determined in the membrane fractions by radiometabolic assay and expressed relative to the amount of the wild-type transcript and the purified brain ectoenzyme (trypsin-fragment), as quantified by Western blot analysis (densitometric scanning of bands, Duoscan T1200, Agfa/NIMH-Image, NIMH). Using the same amount of recombinant enzyme we detected specific enzymatic activities similar to that of the wild-type enzyme only for the C68S mutant (Fig. 3). The $K_m$ and $V_{max}$ values were also not significantly different (Table 1). The C-terminal mutants C823S, C830S and C895S were completely inactive, indicating that even the elements remote from the active site are important for the overall structure of the enzyme. The specific activities of all the other mutants were markedly decreased. The specific activities of the C279S and the C338S mutants were 10-fold and fivefold lower and correspondingly a decrease in the $V_{max}$ values could be demonstrated, while the $K_m$ values were not significantly different.

Fig. 5. Heat inactivation of wild-type and C68S mutant. Membrane preparations of recombinant wild-type (■) or the C68S mutant (○) enzyme were preincubated for 40 min and then assayed at the temperatures indicated. [pyroGlu-3H]TRH was used as substrate.

Fig. 6. Subcellular distribution of the truncated GFP-fusion proteins with either cysteine or serine at position 68, as analyzed by confocal laser fluorescence microscopy. BHK cells were transfected either with the empty pEGFP-N1 vector (A), the EGFP-tagged, truncated wild-type cDNA of TRH-DE (B) or the corresponding C68S mutant (C) and processed as described in Experimental procedures.
In the Zn-binding HEICH domain, substitution of the cysteine by serine resulted in a 60-fold decrease in the specific enzymatic activity of the mutant enzyme, suggesting that this cysteine is an important structural element that is not limited to restraining only the substrate specificity of the enzyme.

### Cys68 is involved in disulfide-linked dimerization

For the proteolytically truncated enzyme a molecular mass of 230 kDa has been estimated by gel-filtration chromatography, whereas a single band with an apparent molecular mass of 116 kDa could be detected after SDS/PAGE under both reducing and nonreducing conditions [19]. From the alignment of the amino-acid sequences we speculated that Cys68, the only Cys residue of the N-terminal peptide fragment that could be left in the membrane after trypsin cleavage, might be involved in disulfide-linked dimerization. Therefore, the wild-type and the C68S mutant were fused in-frame C-terminal of the FLAG-epitope of pFLAG-CMV1 (see Experimental procedures) and expressed in BHK-cells. Membrane fractions from BHK-cells expressing the wild-type or the mutant enzyme were subjected to SDS/PAGE under reducing and nonreducing conditions and analyzed by immunoblotting with the anti-FLAG M2 monoclonal antibody (Kodak). Under reducing conditions (1.5% dithiothreitol), a band of immunoreactive protein with a molecular mass of 130 kDa could be detected for both proteins, the wild-type and the C68S mutant (Fig. 4A, lanes 2 and 3). Under nonreducing conditions, the same band was also visualized for the C68S mutant (Fig. 4A, lane 6) but not for the wild-type protein (Fig. 4A, lane 5), which migrated as a high molecular mass protein. These data are compatible with the interpretation that the mutant enzyme may exist as a monomeric fusion protein, while the wild-type protein represents a disulfide-linked homodimer. This hypothesis is further supported by the analysis of the proteolytically truncated, catalytically fully active ectoenzyme from rat brain. Under both reducing and nonreducing conditions, this protein migrates on SDS/PAGE as a single band with an apparent molecular mass of 116 kDa (Fig. 4B) indicating that other cysteine residues are not involved in dimer formation. To analyze whether the homodimer formation via the Cys68 S-S bridges might contribute to the stability of TRH-DE, we studied the temperature-dependant inactivation of the wild-type and the C68S mutant but found no difference (Fig. 5).

To study whether the interchain bridge at Cys68 might be important for the proper intracellular trafficking and insertion

### Table 1. Kinetic parameters for the hydrolysis of [pyroGlu-3H]-TRH by wild-type and mutant rat TRH-DE.

| Enzyme | Specific activity (nmol·min⁻¹·mg⁻¹) | \(K_m\) (μM) | \(V_{max}\) (nmol·min⁻¹·mg⁻¹) | \(V_{max}/K_m\) (mL·min⁻¹·mg⁻¹) |
|--------|---------------------------------|--------------|----------------------------|----------------------------|
| Wild-type | 250 ± 44 | 32.5 ± 1 | 3727 ± 104 | 114.67 |
| C68S | 210 ± 36 | 29.3 ± 0.4 | 2900 ± 489 | 98.97 |
| C174S | 6.25 ± 0.2 | – | – | – |
| C279S | 24.56 ± 4.15 | 27 ± 4 | 238 ± 51 | 10.48 |
| C338S | 46.38 ± 5.11 | 33 ± 3 | 716 ± 28.27 | 21.69 |
| C444S | 4.37 ± 2 | – | – | – |
| C823S | ND | – | – | – |
| C830S | ND | – | – | – |
| C859S | 9.37 ± 1.37 | – | – | – |
| C895S | ND | – | – | – |

with the anti-FLAG M2 monoclonal antibody (Kodak). Under reducing conditions (1.5% dithiothreitol), a band of immunoreactive protein with a molecular mass of 130 kDa could be detected for both proteins, the wild-type and the C68S mutant (Fig. 4A, lanes 2 and 3). Under nonreducing conditions, the same band was also visualized for the C68S mutant (Fig. 4A, lane 6) but not for the wild-type protein (Fig. 4A, lane 5), which migrated as a high molecular mass protein. These data are compatible with the interpretation that the mutant enzyme may exist as a monomeric fusion protein, while the wild-type protein represents a disulfide-linked homodimer. This hypothesis is further supported by the analysis of the proteolytically truncated, catalytically fully active ectoenzyme from rat brain. Under both reducing and nonreducing conditions, this protein migrates on SDS/PAGE as a single band with an apparent molecular mass of 116 kDa (Fig. 4B) indicating that other cysteine residues are not involved in dimer formation. To analyze whether the homodimer formation via the Cys68 S-S bridges might contribute to the stability of TRH-DE, we studied the temperature-dependant inactivation of the wild-type and the C68S mutant but found no difference (Fig. 5).

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### Fig. 7. Expression of wild-type and C68S mutant as truncated GFP-fusion proteins in BHK-cells.

The large extracellular domain of TRH-DE with the eight cysteine residues was replaced by EGFP. BHK-cells were transfected either with the empty pEGFP-N1 vector (lanes 1 and 4), the EGFP-tagged, truncated wild-type cDNA of TRH-DE (lanes 2 and 5) or the corresponding C68S mutant (lanes 3 and 6). The membrane fractions were subjected to SDS/PAGE (12% gel) in the presence (+) or absence (−) of dithiothreitol and analyzed by immunoblotting using the living colors peptide antibody-AP conjugate (Clontech). The positions of the molecular mass markers (in kDa) are indicated.
into the plasma membrane, fusion proteins were constructed in which the large extracellular domain of TRH-DE with the eight cysteine residues were deleted and replaced by EGFP. The truncated proteins thus contained at position 68 either the only Cys in the sequence or alternatively a Ser residue. As expected, cells transfected with the vector alone exhibited bright fluorescence distributed throughout the whole cell (Fig. 6A), whereas the truncated fusion protein with either cysteine or serine at position 68 was found to be localized on the plasma membrane (Fig. 6B,C).

On SDS/PAGE, the truncated mutant protein exhibited the same mobility irrespective of whether diithiothreitol was present. The same pattern was observed when the truncated wild-type protein was subjected to SDS/PAGE under reducing conditions, whereas in the absence of diithiothreitol a band of about twice the molecular mass could be visualized (Fig. 7).

**DISCUSSION**

Site-directed mutagenesis is an important and well established technique in protein chemistry to study the functional role of defined amino-acid residues. We used this method to investigate the structural and functional importance of the nine cysteine residues in TRH-DE by replacing individually each cysteine by a serine residue, and thus identified this enzyme as a new member of mammalian cell-surface peptidases that exist as disulfide-linked dimers. Previously, aminopeptidase A [31,32], meprin [33,34], membrane dipeptidase [35] and endothelin-converting enzyme-1 [36] have been the only established examples.

In agreement with previous biochemical data [19], the results of the present study clearly demonstrate that the eight cysteine residues within the large extracellular domain all contribute to the intrachain S-S-bridging of the subunits, the conformation of the catalytic domain and the enzymatic activity. The specific activities of all these mutant enzymes were either considerably reduced or completely abolished.

Within the M1 family of Zn-dependent aminopeptidases, TRH-DE and aminopeptidase N share some sequence similarity in the C-terminal region, especially with regard to the cluster of four cysteine residues [20,24] (see Fig. 1). Although remote from the active site, these cysteine residues are clearly of major importance to the structural integrity of the core domain, whereas in aminopeptidase A the differently structured C-terminal end is separated from the catalytic core domain by a protease susceptible region [32]. Although the functional significance of the C-terminal domain of TRH-DE and sequence-related enzymes awaits determination by other methods (e.g. crystallographic analysis), it is interesting to note that the porcine coronavirus transmissible gastroenteritis virus binds to the C-terminal domain of aminopeptidase N between residues 717 and 813 [37].

Among the large family of metalloproteases, TRH-DE is unique in that it contains a cysteine residue within the HEXXH zinc-binding domain. Mutation of this cysteine (Cys444) to serine resulted in a 60-fold decrease of the enzymatic activity. As part of an intrachain S-S bridge, this cysteine apparently contributes to establishing the tertiary structure of the active pocket. On the basis of the high substrate specificity of TRH-DE, we assumed that replacement of Cys444 by serine might ‘open’ the active pocket and thus might result in an enzyme that could hydrolyze different substrates other than TRH. However, with pyroglutamyl-β-naphthylamide, a low affinity substrate that is slowly hydrolyzed by TRH-DE, release of β-naphthylamine by the mutant enzyme could not be detected (data not shown). Furthermore, LH-RH, a decapeptideamide with the sequence pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly is also not hydrolyzed by the mutant enzyme (data not shown). LH-RH was tested because this peptide is not hydrolyzed by TRH-DE, yet it is apparently recognized by the enzyme as the degradation of TRH by TRH-DE is effectively inhibited by this peptide [13].

In contrast to the cysteines constituting the catalytic domain, Cys68, which resides very close to the membrane surface (just four residues away from the hydrophobic membrane-spanning region), could be substituted by serine without affecting significantly the specific activity of the mutant enzyme. This cysteine is positioned within the presumed stalk region, a stretch of about 40 amino-acid residues that is a general feature of ectoenzymes and is thought to be important for surface exposition [38]. The hypothesis that this stretch forms a separate domain is supported by the fact that in TRH-DE [21], as in other enzymes such as in aminopeptidase N (reviewed in [39]), this region is considerably less conserved than the catalytic domain. As in aminopeptidase A [31,32], this cysteine is obviously responsible for intersubunit S-S bridging and thus for the formation of the homodimeric wild-type TRH-DE. Surprisingly, however, covalent dimerization seems not to be essential for the formation and expression of the catalytically active enzyme. Moreover, as also shown with the truncated mutant protein in which the catalytic domain was replaced by GFP, the intermolecular disulfide link via Cys68 appeared not to be essential for trafficking and transport of the protein to the cell surface. In agreement with these results are the data of previous biochemical studies on the dimeric assembly of enterocyte brush border enzymes (e.g. aminopeptidase N and A, dipeptidyl peptidase IV) which demonstrated that homodimerization is not an absolute requirement for transport of these enzymes to, and through, the Golgi complex [40] although dimeric assembly may increase the rate of intracellular transport. For the mouse meprin peptidase, site-directed mutagenesis studies also demonstrated that the covalent dimerization of subunits is not essential for efficient biosynthesis, trafficking or post-translational processing of the secreted protease [33]. Moreover, for membrane dipeptidase, another enzyme with a single disulfide bond close to the membrane surface, mutation of the critical cysteine residue to glycine neither influenced the expression of the mutant enzyme to the cell surface nor the enzymatic properties of the peptidase [35].

Taking these data together, it might be reasonable to assume that the noncovalent hydrophobic domain–domain interactions of the catalytic subunits may represent the major elements that are critical for the structure and function of TRH-DE and related dimeric proteins.

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