Glycoconj J (2010) 27:359–366
DOI 10.1007/s10719-010-9283-4

Involvement of a cysteine protease in the secretion process of human xylosyltransferase I

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Received: 14 December 2009 / Revised: 10 February 2010 / Accepted: 12 February 2010 / Published online: 3 March 2010

Abstract Xylosylation of core proteins takes place in the Golgi-apparatus as the transfer of xylose from UDP-xylose to specific serine residues in proteoglycan core proteins. This initial and rate-limiting step in glycosaminoglycan biosynthesis is catalyzed by human xylosyltransferase I (XT-I). XT-I is proteolytically cleaved from the Golgi surface and shed in its active form into the extracellular space. The secreted, circulating glycosyltransferase represents a serum biomarker for various diseases with an altered proteoglycan metabolism, whereas a physiological function of secreted XT-I is still unknown. To shed light on the secretion process of XT-I and on its biological function, the cleavage site was examined and the group of proteases involved in the cleavage was identified in this study. The peptide mass fingerprint from partly purified secreted XT-I revealed the cleavage site to be localized in the amino-terminal 231 amino acids. The addition of a cysteine protease inhibitor cocktail to cells recombinantly expressing XT-I led to a concentration-dependent shift of enzyme activity towards the cell lysates attended by consistent total intracellular and extracellular XT-I activities. In conclusion, our findings provide a first insight into the XT-I secretion process regulated by a cysteine protease and may contribute to understanding the biological and pathological role of this process.

Keywords Extracellular matrix · Glycosaminoglycans · Proteoglycans · Secretion process · Xylosyltransferase

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Abbreviations

AAA	Abdominal aortic aneurysm
CRTL	Control
EC	Extracellular
IC	Intracellular
XT	Xylosyltransferase
XT-I	Xylosyltransferase I

Introduction

Proteoglycans form a group of polyanionic glycoproteins present in virtually every animal cell on the cell surface and in the extracellular (EC) matrix [1]. Serving a wide range of functions in distinct biological processes, they are involved in viral and bacterial infections, cell-cell interactions and tumor cell growth. The hallmarks of proteoglycans are varying numbers and types of glycosaminoglycan chains which are covalently attached to a core protein [2]. The glycosaminoglycans chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin are bound to the proteoglycan core via a common tetrasaccharide linker, consisting of one glucuronic acid, two galactoses and one xylose [2]. The initial transfer of D-xylose from UDP-D-xylose to specific serine residues of the core protein is, amongst others, catalyzed by xylosyltransferase I (XT-I, EC 2.4.2.26), which has been shown to be the rate-limiting enzyme in the glycosaminoglycan biosynthesis [3, 4]. XT-I is a Golgi localized glycosyltransferase featuring a type-II integral membrane protein structure, containing a short N-terminal cytoplasmic tail, a single hydrophobic transmembrane domain, a luminal stem region, and the large C-terminal catalytic domain situated in the lumen of the Golgi apparatus [5–7]. For its complete retention in the Golgi

compartment, XT-I requires the N-terminal 214 amino acids [7]. Such as other glycosyltransferases [8], in addition to its Golgi localization XT-I is described as appearing in its active form in the EC space [9, 10]. To clarify the appearance of XT-I activity in the EC space, human chondrocytes were previously subjected to colchicine treatment. The toxin is an inhibitor of microtubule-mediated vesicular transport and has been shown to inhibit the release of glycosaminoglycans into the EC matrix [11] without influencing protein synthesis [12, 13]. As XT secretion was blocked under colchicine treatment, the enzyme is known to be present in the EC space due to an active secretion process after cleavage of the membrane-bound Golgi-enzyme.

Secreted, circulating XT is described as a potential serum biomarker of an altered proteoglycan metabolism in various diseases [9]: its elevated serum activity is accompanied by an increased proteoglycan synthesis in systemic sclerosis [14, 15] and in pseudoxanthoma elasticum [16]. Accordingly, multiple tissues of diabetic patients show a reduced proteoglycan biosynthesis in correlation with a decreased serum XT activity in patients with diabetes mellitus [17]. However, its usage as a biomarker for several diseases does not explain the biological function of EC XT-I. Especially as donor UDP-xylose is known to be absent in significant amounts outside the Golgi compartment [18], proteoglycan biosynthesis cannot represent the main physiological role of secreted XT-I.

To clarify the biological function of secreted XT-I, identification of the protease involved in the cleavage process of the enzyme might be helpful. Therefore, in the present study, we investigated the XT-I cleavage site in CHO cells stably transfected with XT-I cDNA and questioned which protease was responsible for its cleavage. Based on the results of inhibitor assays with various protease inhibitors, a cysteine protease was identified as a plausible protease responsible for the secretion of XT-I. This is the first report on the involvement of a cysteine protease in the secretion process of human XT-I.

Experimental procedures

Site-directed mutagenesis

The vector coding for the C-terminal His-tagged form of XT-I was generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the plasmid pSS1 [7] as a template. The insertion of the 6xHis-tag was performed with the primers 5′-GATGGCCGGCTCAGG **CACCATCACCATCACCATTAGTGA**TCTAGAATGGC TAGCAAAGGAGAAGAA-3′ and 5′-TTCTTCTCC TTTGCTAGCCATTCTAGA**TCACTAATGGTGATGGT GATGGTG**CCTGAGCCGGCCATC-3′ (inserted nucleotides are in bold typeface and underlined). Mutagenesis was performed according to the manufacturer's instructions apart from the following: Polymerase chain reaction annealing was performed at 60°C for 2 min, the plasmids were *Dpn* I digested for 3 h and transformed into TOP10 competent cells (Invitrogen, Groningen, Netherlands). The plasmids were isolated and purified using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). The obtained plasmid pSS1_6His was proved by DNA sequencing using an ABI 310 DNA sequencing system.

Cell culture

Xylosyltransferase-deficient pgsA-745 CHO cells [19] were grown in Ham's F12 medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Biowest, Nuaille, France), 300 µg/mL L-glutamine (PAA Laboratories GmbH, Pasching, Österreich), and antibiotic-antimycotic (Biowest). The cells were cultured at 37°C in an atmosphere of 5% CO_2 in air at 100% relative humidity.

Stable transfection and overexpression of r6His_XT1

Xylosyltransferase-deficient pgsA-745 CHO cells were transfected with the plasmid pSS1_6His using FuGENE 6 transfection reagent (Roche Applied Science, Mannheim, Germany) as recommended by the manufacturer. Stable transfectants were selected with 400 µg/mL Geneticin and the single clone pgsA-6HisXT1-K4, expressing the His-tagged XT-I (r6His_XT1), was isolated. pgsA-6HisXT1-K4 cells releasing r6His_XT1 in the cell culture supernatant were cultivated as a monolayer in 175-cm^2 T-flasks with 25 mL of supplemented Ham's F12 medium containing 400 µg/mL Geneticin. When confluence was reached, the adherent cells of 2 flasks (about 2×10^7 cells in total) were removed and transferred to a 10 cm culture dish containing 10 mL of serum-free ProCHO5 medium (Cambrex, Verviers, Belgium) supplemented with 300 µg/mL L-glutamine and 400 µg/mL Geneticin. After 4 d the cell suspension was centrifuged at 1,100 g for 5 min, the cell culture supernatant was harvested for the purification of r6His_XT1, and the cells were discarded.

Partial purification and identification of r6His_XT1

Fifty-five milliliter ProCHO5 cell culture supernatant containing r6His_XT1 were concentrated using a 50,000 MWCO ultrafiltration cell with a polyethersulfone membrane (Sartorius Stedim Biotech S.A., Aubagne Cedex, France). Partial purification of the concentrate was performed by nickel chelate chromatography via a HisTrap Ni-Chelate column (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The r6His_XT1 containing

eluate fraction was concentrated and desalted using a Microcon 10 column with a MWCO of 10,000 and a regenerated cellulose membrane (Millipore, Billerica MA). Thirty-two microgram purified recombinant protein was subjected to denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which was carried out on a 4–12% Bis-Tris polyacrylamide gel with MOPS running buffer (Invitrogen). Subsequently, Western blotting was performed using a semi-dry electro-blotting apparatus. After protein transfer, nonspecific binding sites were blocked with 5% milk powder in DPBS (Invitrogen) over night at 4°C. Following several washing steps with 0.05% Tween 20 (Sigma, Taufkirchen, Germany) in DPBS, the membrane was incubated with monoclonal anti-polyhistidine peroxidise conjugated antibody (Sigma), diluted 1:2,000 in 1% BSA and 0.05% Tween 20 in DPBS, for 2 h. Unbound antibody was washed from the membrane and detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Following the Western blot, gel resident protein bands were detected with Coomassie brilliant blue. The supposed recombinant XT-I band, possessing a molecular weight of about 65 kDa, was excised and characterized by MALDI mass spectrometry after tryptic digestion.

Inhibitor treatment

After 3×10^5 pgsA-6HisXT1-K4 cells were cultured in supplemented Ham's F12 medium containing 400 µg/mL Geneticin in a 6-well plate for 24 h, they were washed with DPBS. Two milliliter of serum-free medium containing the solvent with / without the particular protease inhibitor in various concentrations were added to each well and the cells were cultivated for an additional 40 h. For the determination of intracellular (IC) and EC XT-I activity both fractions were obtained as described below. The following protease inhibitors were tested under the conditions described here: (a) ß-Secretase Inhibitor II (Merck KGaA, Darmstadt, Germany), solvent DMSO, solvent concentration in medium 0.1%; (b) γ-Secretase Inhibitor DFK-167 (MP Biomedicals, Aurora, OH), solvent DMSO, solvent concentration in medium 1%; (c) Serine Protease Inhibitor Cocktail Set I (Merck KGaA, Darmstadt, Germany), solvent H_2O, solvent concentration in medium 1%; (d) Protease Inhibitor Cocktail Set VIII (Merck KGaA, Darmstadt, Germany), solvent DMSO, solvent concentration in medium 1%.

Production of IC and EC fractions

One milliliter of the conditioned medium was centrifuged at 1,100 g for 5 min to remove any cell debris. The supernatant was used as EC fraction. To obtain the IC fraction, cell lysates were prepared with Nonidet P-40 cell lysis buffer (50 m*M* Tris-HCl, 150 m*M* NaCl, 1% Nonidet P-40, pH 7.8). Therefore, the adherent cells were washed with DPBS and incubated for 10 min at 4°C with 300 µL Nonidet P-40 buffer. After centrifugation at 10,000 g and 4°C for 10 min the supernatant was used as IC fraction.

XT-I enzyme activity assay

The method for determining the XT-I activity is based on the incorporation of radioactive [^{14}C]D-xylose into silk fibroin as an acceptor according to a previously described method [20, 21].

Measurement of total protein concentration

Protein concentration was determined by the Bicinchoninic Acid Protein Assay kit (Sigma) using BSA as a standard.

Determination of specific XT-I activity

The specific XT-I activity was calculated using the quotient of XT-I activity to total protein concentration.

Statistics

All statistical tests were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). The Mann-Whitney test was used for single comparisons between two groups. *P* values of <0.05 were considered statistically significant.

Results

Secretion of r6His_XT1 and analysis of its cleavage site

To shed light on the secretion process of the generally low expressed XT-I, the generation of a stable cell line overexpressing the enzyme was essential. The generated single cell clone pgsA-6HisXT1-K4 expressed r6His_XT1 and secreted it in its active form into the culture medium (XT-I activity (IC), 0.677 ± 0.040 µU/1×10^6 cells; XT-I activity (EC), 4.945 ± 0.636 µU/1×10^6 cells; XT-I activity (ratio EC/IC), 7.305 ± 0.851). To analyze the cleavage site of XT-I, the secreted enzyme was partly purified from conditioned ProCHO5 medium of pgsA-6HisXT1-K4 cells and subjected to SDS-PAGE. Protein bands were visualized by Coomassie brilliant blue staining. The band with a molecular mass of about 65 kDa (Fig. 1a), corresponding to the positive band detected in the Western blot of r6His_XT1 (Fig. 1b), was analyzed by MALDI mass spectrometry after tryptic digestion. The analyzed protein was confirmed to be XT-I, as 37% of the amino acid

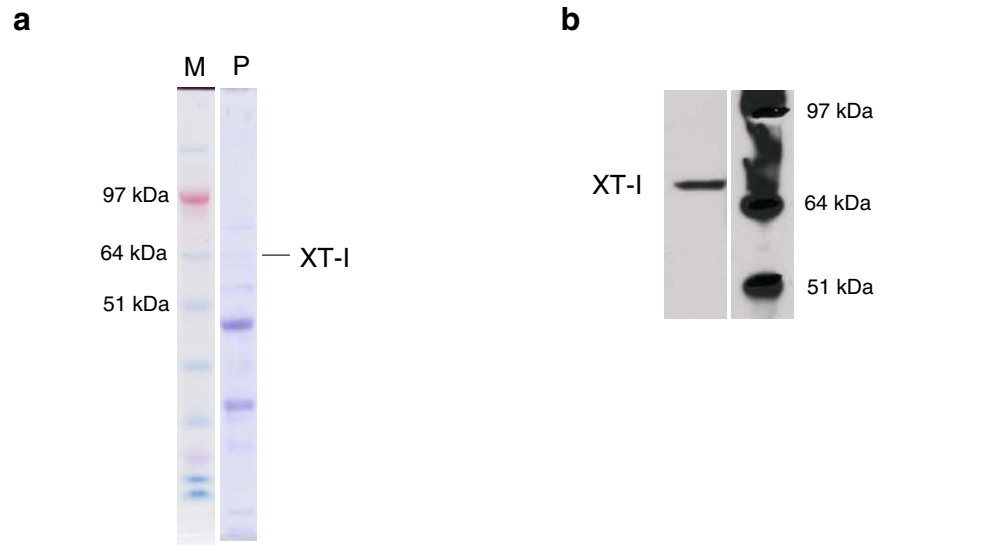

Fig. 1 Characterization of r6His_XT1. **a** SDS-PAGE after partial purification of secreted r6His_XT1. *Lane M*, Marker; *Lane P*, partly purified secreted r6His_XT1, whereby both lanes derive from different SDS-gels. The marked band in lane P was shown to be XT-I by MALDI mass spectrometry after tryptic digestion. **b** Western blot analysis after partial purification of secreted r6His_XT1. **c** XT-I amino acid sequence with XT-I peptides identified shown in red. Identification of XT-I was performed via mass spectrometry after excision and tryptic digestion of the marked protein band shown in Fig. 1a

c

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1    MVAAPCARRL  ARRSHSALLA  ALTVLLLQTL  VVWNFSSLDS  GAGERRGGAA
51   VGGGEQPPPA  PAPRRERRDL  PAEPAAARGG  GGGGGGGGGG  RGPQARARGG
101  GPGEPRGQQP  ASRGALPARA  LDPHPSPLIT  LETQDGYFSH  RPKEKVRTDS
151  NNENSVPKDF  ENVDNSNFAP  RTQKQKHQPE  LAKKPPSRQK  ELLKRKLEQQ
201  EKGKGHTFPG  KGPGEVLPPG  DRAAANSSHG  KDVSRPPHAR  KTGGSSPETK
251  YDQPPKCDIS  GKEAISALSR  AKSKHCRQEI  GETYCRHKLG  LLMPEKVTRF
301  CPLEGKANKN  VQWDEDSVEY  MPANPVRIAF  VLVVHGRASR  QLQRMFKAIY
351  HKDHFYYIHV  DKRSNYLHRQ  VLQVSRQYSN  VRVTPWRMAT  IWGGASLLST
401  YLQSMRDLLE  MTDWPWDFFI  NLSAADYPIR  TNDQLVAFLS  RYRDMNFLKS
451  HGRDNARFIR  KQGLDRLFLE  CDAHMWRLGD  RRIPEGIAVD  GGSDWFLLNR
501  RFVEYVTFST  DDLVTKMKQF  YSYTLLPAES  FFHTVLENSP  HCDTMVDNNL
551  RITNWNRKLG  CKCQYKHIVD  WCGCSPNDFK  PQDFHRFQQT  ARPTFFARKF
601  EAVVNQEIIG  QLDYYLYGNY  PAGTPGLRSY  WENVYDEPDG  IHSLSDVTLT
651  LYHSFARLGL  RRAETSLHTD  GENSCRYYPM  GHPASVHLYF  LADRFQGFLI
701  KHHATNLAVS  KLETLETWVM  PKKVFKIASP  PSDFGRLQFS  EVGTDWDAKE
751  RLFRNFGGLL  GPMDEPVGMQ  KWGKGPNVTV  TVIWVDPVNV  IAATYDILIE
801  STAEFTHYKP  PLNLPLRPGV  WTVKILHHWV  PVAETKFLVA  PLTFSNRQPI
851  KPEEALKLHN  GPLRNAYMEQ  SFQSLNPVLS  LPINPAQVEQ  ARRNAASTGT
901  ALEGWLDSLV  GGMWTAMDIC  ATGPTACPVM  QTCSQTAWSS  FSPDPKSELG
951  AVKPDGRLR
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sequence of XT-I was detected (Fig. 1c). The matched peptides were spread all over the enzyme, not even lacking the enzyme's C-terminus as only the last two amino acids were not identified by MALDI mass spectrometry. Interestingly, no XT-I matching peptides were found within the first 231 amino acids. These results indicate that secreted XT-I, in contrast to the Golgi-anchored enzyme, lacks no more than the first 231 amino acids. Therefore, the cleavage site is localized in the aminoterminal 231 amino acids.

Inhibition of XT-I cleavage and secretion by cysteine protease inhibitors

For the identification of the protease involved in XT-I secretion, ß- and γ-secretases, as well as serine and cysteine proteases, were investigated in pgsA-6HisXT1-K4 cells. Neither the quotients of specific EC to specific IC XT-I activity nor the total XT-I activities in inhibitor-treated cells compared to solvent-only treated (CRTL) cells were significantly changed regarding the inhibition of ß-secretase (Fig. 2a and b), γ-secretase (Fig. 2c and d) and serine proteases (Fig. 2e and f). However, γ-secretase inhibition led to inconsistent ratios of specific EC to specific IC XT-I activity. Interestingly, the addition of a protease inhibitor cocktail consisting of three protease inhibitors with specificity for cysteine proteases led to mentionable results: As shown in Fig. 3a, with increasing inhibitor concentrations the EC specific XT-I activity (significantly) decreased in comparison to DMSO-only treated (CRTL) cells (CRTL, 1.00±0.04; dilution of 10,000, 0.96±0.16; dilution of 5,000, 0.94±0.04, P=0.01; dilution of 1,000, 0.88±0.11, P=0.03). Concurrently, the IC-specific XT-I activity was

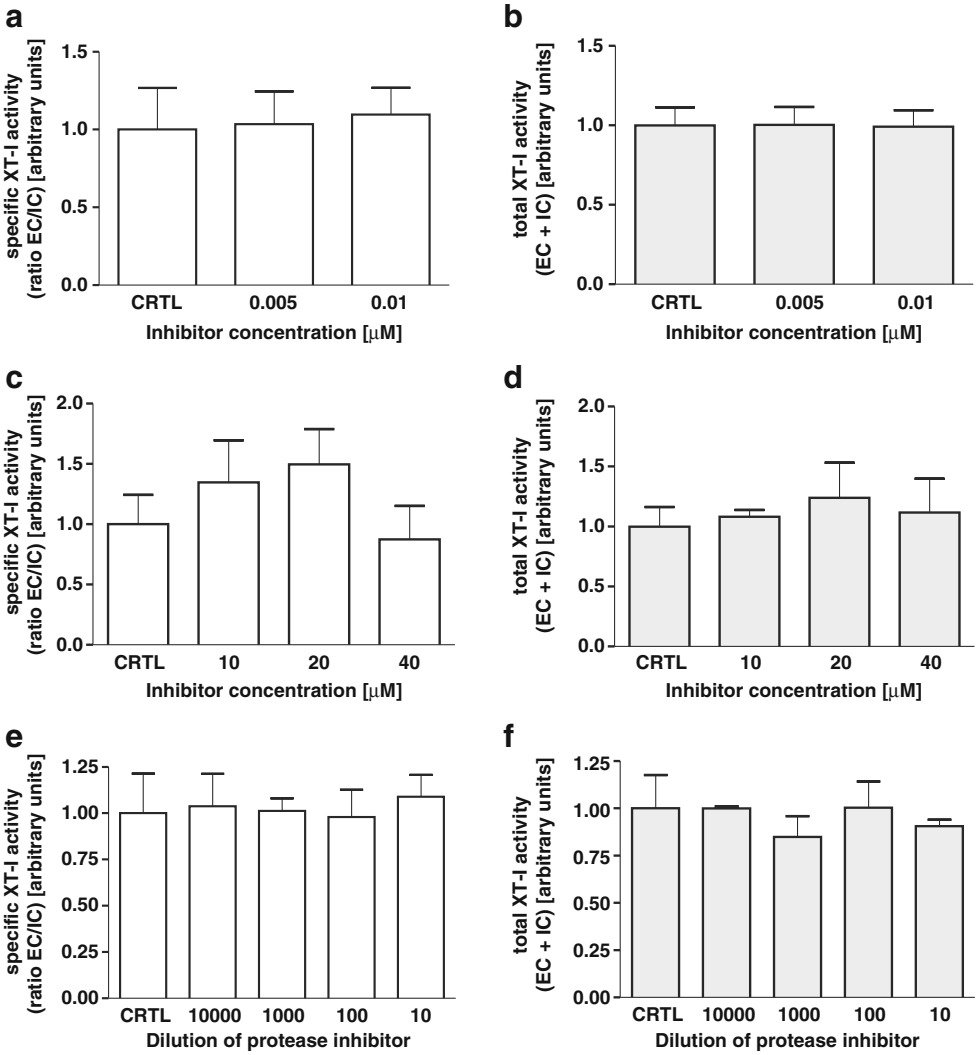

Fig. 2 Influence of different protease inhibitors on XT-I secretion and total XT-I activity in pgsA-6HisXT1-K4 cells. Cells were treated with the given protease inhibitor concentrations / dilutions for a period of 40 h. Quotients of EC and IC specific XT-I activity (**a**, **c**, **e**) and total XT-I activity (**b**, **d**, **f**) depending on the protease inhibitor concentration / dilution are shown for the addition of (**a**, **b**) ß-Secretase Inhibitor II, (**c**, **d**) γ-Secretase Inhibitor DFK-167 and (**e**, **f**) Serine Protease Inhibitor Cocktail Set I. Values are normalized to 1.0 and displayed as the mean with the corresponding standard deviation. The data of two independent experiments with two replicates each are shown in (**a**) to (**d**); (**e**) and (**f**) represent the data of one experiment with two replicates

(significantly) increased in comparison to DMSO-only treated (CRTL) cells (CRTL, 1.00±0.10; dilution of 10,000, 0.99±0.09; dilution of 5,000, 1.22±0.17, P=0.01; dilution of 1,000, 1.30±0.22, P=0.002) (Fig. 3b). Therefore, the quotients of specific EC to specific IC XT-I activity in inhibitor-treated cells compared to DMSO-only treated (CRTL) cells were (significantly) reduced (CRTL, 1.00±0.10; dilution of 10,000, 0.96±0.13; dilution of 5,000, 0.78±0.10, P=0.002; dilution of 1,000, 0.68±0.10, P=0.0003) (Fig. 3c). Thus, using a cocktail of cysteine protease inhibitors, the XT-I secretion was inhibited in a dose-dependent manner compared with DMSO treatment only in recombinant pgsA-6HisXT1-K4 cells. To exclude the possibility of the protease inhibitors influencing XT-I activity, total XT-I activities of EC and IC fractions were calculated. As shown in Fig. 4, total XT-I activities are nearly identical in the control and in the cells treated with the different protease inhibitor concentrations. Therefore, the observed effect of the cysteine protease inhibitor cocktail does not result from different enzyme activities due to distinct inhibitor concentrations. Further, unchanged cell viability under the inhibitor treatment was demonstrated for the concentrations used by the cell proliferation reagent WST-1 (Roche, Mannheim, Germany) (data not shown). All values given in the text are indicated as mean±standard deviation, whereby arbitrary units are used.

Discussion

Xylosylation of core proteins takes place in the Golgi apparatus. Nevertheless, only a small part of total XT activity is located membrane-bound in the cells within this compartment. The majority of XT activity is accumulated in a time-dependent manner in the cell culture supernatant of cultured cells [14, 15]. As XT activity has been observed in culture supernatants of all cell lines investigated, XT secretion seems to be independent of cell type and organ system [14, 22]. The secreted, circulating glycosyltransferase represents a confirmed biochemical marker, as elevated serum XT

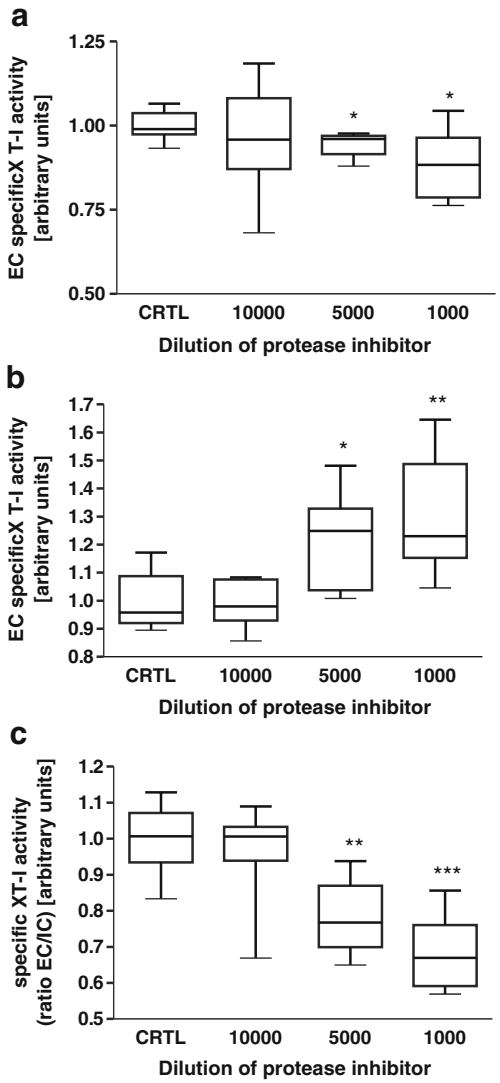

Fig. 3 Influence of the cysteine protease inhibitor cocktail on XT-I secretion in pgsA-6HisXT1-K4 cells. Cells were treated with the given protease inhibitor dilutions for a period of 40 h. (**a**) EC specific XT-I activity depending on the protease inhibitor dilution. (**b**) IC specific XT-I activity depending on the protease inhibitor dilution. (**c**) Resulting quotients of EC and IC specific XT-I activity depending on the protease inhibitor dilution. Each *box* represents the values from the lower to the upper quartile (25 to 75 percentile), and the *middle horizontal line* in each *box* represents the median. The *vertical line* extends from the minimum to the maximum value. The data of four independent experiments with two replicates, each, are shown. ***$p<0.001$, **$p<0.01$, *$p<0.05$ *versus* non-inhibitor treated cells

activity is accompanied by an increased proteoglycan synthesis in systemic sclerosis [14, 15] and in pseudoxanthoma elasticum [16], whereas reduced serum XT activity in correlation with a decreased proteoglycan biosynthesis is observed in multiple tissues of diabetic patients [17]. However, a physiological function of secreted XT-I is still unknown.

To provide a first insight into the XT-I secretion process, the goal of the present study was analysis of the XT-I cleavage site and identification of the protease involved in the cleavage and secretion of XT-I. Because of the marginal physiological expression of the glycosyltransferase, a stable cell line overexpressing XT-I was generated. The generated single-cell clone pgsA-6HisXT1-K4 secreted about 90% of the total XT-I into the culture medium. As EC XT fractions from cell lines physiologically secreting the enzyme are similar [9], problems of overexpression systems, such as mislocalization or increased shedding, can be excluded. Therefore, the generated overexpression system was proved to represent an adequate tool to assess the XT-I secretion.

As shown by peptide mass fingerprinting (Fig. 1c), no peptides were found from the first 231 amino acids of XT-I, whereas detected XT-I consistent peptides are spread all over the remaining protein part, beginning with amino acid number 232. These results indicate that the cleavage site is localized among the first 231 amino acids, whereby a cleavage in between the stem domain, beginning with amino acid number 36, seems presumable. Interestingly, as complete Golgi retention of XT-I requires the first 214 N-terminal amino acids of the enzyme [7], Golgi localization and cleavage from the Golgi surface might be dependent on the same amino acid region. However, the exact localization of the cleavage site remains to be elucidated in further investigations.

To identify the protease involved in XT-I secretion, ß- and γ-secretases, as well as serine and cysteine proteases, were inhibited in CHO cells recombinantly expressing the glycosyltransferase. The involvement of ß- and γ-secretases and of a serine protease could be excluded, as the quotients of specific EC to specific IC XT-I activity in inhibitor-treated cells compared to solvent-only treated (CRTL) cells were not significantly changed (Fig. 2). Due to their non-significance and their inconsistency, the observed varying ratios of specific EC to specific IC XT-I activity following γ-secretases inhibition (Fig. 2c) do not indicate an

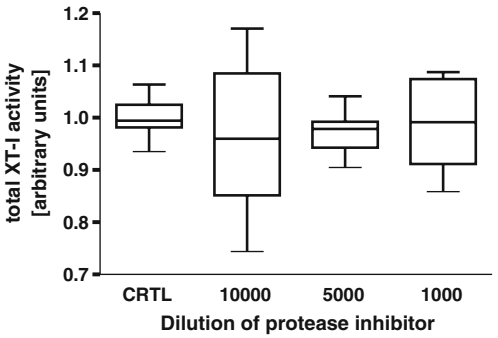

Fig. 4 Influence of the cysteine protease inhibitor cocktail on the total XT-I activity in pgsA-6HisXT1-K4 cells. Cells were treated with the given protease inhibitor dilutions for a period of 40 h. The total EC and IC XT-I activities are shown. Plot representation is analogous to the description in Fig. 3. The data of four independent experiments with two replicates, each, are shown

involvement of γ-secretase in XT-I secretion. Remarkably, as shown in Fig. 3, the inhibition of cysteine proteases led to a significantly reduced XT-I secretion in a dose-dependent manner compared with DMSO treatment only in pgsA-6HisXT1-K4 cells. Although it cannot be completely excluded that the inhibitor treatment might also have an additional effect on the enzyme distribution, the possibility of different XT-I activities arising from distinct inhibitor concentrations was excluded, as total enzyme activities were similar in the control and in the cells treated with different protease inhibitor concentrations (Fig. 4). Furthermore, cell viabilities were not influenced by inhibitor treatment in different concentrations. Therefore, reduced XT-I secretion in cysteine protease inhibitor treated cells points to an involvement of a cysteine protease in the XT-I secretion process. The used Protease Inhibitor Cocktail Set consists of three protease inhibitors in the given concentrations (ALLN, 1.56 m*M*; Cathepsin Inhibitor I, 0.5 m*M*; E-64 Protease Inhibitor, 1.5 m*M*) with specificity for cysteine proteases, including cathepsins, proteasomes and papain.

Proteases, known to catalyze the irreversible hydrolysis of amide bonds, are divided into four major groups—cysteine, serine, aspartate and metallo-proteases—based on their distinct reaction mechanisms [23]. Of these, the cysteine proteases are widely distributed among living organisms, including mammals, as well as plants, viruses and bacteria [24, 25]. They can be subdivided into several families, including the family of enzymes related to the interleukin-1ß-converting enzyme, the calpain family and the papain family [26, 27]. The most abundant cysteine proteases are those of the latter family, which consists of papain, related plant proteases and the cathepsins [26, 28]. Although well known as lysosomal enzymes, cysteine proteases have been recognized as multi-function molecules, involved in inflammation, apoptosis, membrane-bound protein cleavage, as well as in EC matrix turnover and in processes of tissue remodeling [23, 25].

In particular, the cathepsins—through their contribution to EC matrix remodeling and inflammation—may play an important role in aneurysm formation, which is characterized by a thinning of the vessel wall due to EC matrix degradation [23]. Increased protein levels and activities of distinct cathepsins, among them the cathepsins B, L and S, have been observed in aneurysm walls in comparison to normal arteries [23]. Assuming an involvement of cathepsins in the XT-I cleavage and secretion, this process might be enhanced in aneurysm patients due to the higher cathepsin activity. The resulting lower content of IC XT-I may lead to a reduced biosynthesis of proteoglycans and thereby influence the vessel wall structure in aneurysm patients.

A further indication of the involvement of a cathepsin in the XT-I cleavage might be the presence of a potential cleavage site for one of the cathepsins. Although they have a relatively broad substrate specificity [29], it is known that the cathepsins B, L and S prefer arginine and lysine at the P1 position, strictly hydrophobic amino acids at the P2 position and broader specificities at the P3 and P4 positions [30]. Interestingly, within the stem region of XT-I two potential cleavage sites, possessing these characteristics, have been found. Thus, potential cathepsin cleavage sites might occur between Arg^{147} and Thr^{148} and between Lys^{194} and Arg^{195}. However, these interesting considerations require further examination by mutagenesis experiments. Additionally, as the cysteine protease inhibitor cocktail used contained inhibitors with specificity for the cathepsins B, L and S, as well as for proteasomes and papain, investigations to determine the exact cysteine protease involved in XT-I secretion will be necessary. However, this is the first report on the involvement of a cysteine protease in glycosyltransferase cleavage and secretion.

In summary, our data provide new insights into the secretion process of human XT-I, which at least in part seems to be regulated by a cysteine protease, maybe by one of the cathepsins B, L or S. Future experiments will focus on identification of the exact cysteine protease involved in XT-I secretion and on its physiological and pathological implication. The cleavage process could play an important role in the regulation of glycosaminoglycan attachment to core proteins and in the production of soluble XT-I, related to an altered proteoglycan metabolism in various diseases. Therefore, a selective cysteine protease inhibitor for the XT-I secretion may represent a possible tool for the inhibition of XT-I related proteoglycan alterations in some diseases. Furthermore, the exact localization of the XT-I cleavage site has to be identified.

Acknowledgements The authors thank Xiaoping Xu and Gudrun Bokermann for their excellent technical assistance. In addition, we thank Sarah L. Kirkby for her linguistic advice.

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