Cloning and Recombinant Expression of Active Full-length Xylosyltransferase I (XT-I) and Characterization of Subcellular Localization of XT-I and XT-II*

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Xylosyltransferase I (XT-I) catalyzes the transfer of xylose from UDP-xylose to serine residues in proteoglycan core proteins. This is the first and apparently rate-limiting step in the biosynthesis of the tetrasaccharide linkage region in glycosaminoglycan-containing proteoglycans. The XYLTI-II gene codes for a highly homologous protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known. Here we present for the first time the construction of a vector encoding the full-length protein, but its physiological function is not yet known.
are responsible for intracellular localization. The function of the stem region is supported by the cytoplasmic and the transmembrane domains. The catalytic domain of XT-I is not implicated in their localization, and the region responsible for Golgi targeting is not associated with the activity of the enzyme. This is the first report on gene cloning of the full-length human XT-I and the application of GFP-tagged XT-I and XT-II variants to identify the subcellular localization and the signals required for this.

**MATERIALS AND METHODS**

**Cell Culture**—HEK-293 cells (human, embryonic kidney) (ICN Biomedicals, Meckenheim) were grown in Dulbecco’s modified Eagle’s medium (PAA Laboratories, Pasching, Austria) and SAOS-2 cells (human, osteosarcoma) (ATCC, Manassas, VA) in RPMI 1640 (Cambrex, Verviers, Belgium). Both media were supplemented with 10% (v/v) fetal calf serum (Biowest), L-glutamine (PAA Laboratories), and antibiotics-antimycotic (Biowest). The cells were cultured at 37 °C under an atmosphere of 5% CO2 in air at 100% relative humidity.

**Generation and Cloning of Full-length GFP-tagged Human XTLYT-I cDNA**—The IMAGE clone 2116905 (GenBank accession number AL1524006) contains the N-terminal part (nucleotides 85 to 724) of the human XTLYT-I cDNA, lacking two nucleotides (CG, between positions 40 and 41). These nucleotides were inserted using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the following oligonucleotides: 5’-CGCCGCTGCTCGCCGAGCGCATCTTCGGAGCGCGGCC-3’ and 5’-GAGCAGCGCGAGGTGACATTTTCGAGCGCGGCCGACG-3’ (inserted nucleotides are in boldface type and underlined). The obtained plasmid pSS0 was proved by double-stranded DNA sequencing using an ABI 310 DNA sequencing system with the ABI Prism Big Dye Terminator cycle sequencing kit and the ABI Prism 310 genetic analyzer from PerkinElmer Life Sciences. We used the pCG255-1 vector for the C-terminal XTLYT-I region. This vector included nucleotides 444–2877 in the pMIB/V5 vector (Invitrogen) and encodes for the catalytic active region of the enzyme. This is the first report on gene cloning of the full-length human XT-I and the application of GFP-tagged XT-I and XT-II variants to identify the subcellular localization and the signals required for this.

**Expression and Localization of XT-I and XT-II**

**Assay for XT-I Enzyme Activity**—Cell lysates were prepared with Nonidet P-40 (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8) to analyze intracellular XT-I activity. The method for determination of XT-I activity is based on the incorporation of [14C]D-xylose into silk fibroin as an acceptor (19). The standard reaction mixture (100 µl) for the assay contained 50 µl of cell culture supernatant or templates and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) for amplification. The obtained products were cloned into the pcDNA3.1/CT-GFP-TOPO vector and transformed into chemically competent E. coli TOP10 cells using the GFP Fusion TOPO TA expression kit (Invitrogen) (Fig. 1). The deletion mutants were generated from suitable vectors with the QuikChange™ site-directed mutagenesis Kit. Proceeding with pSS4 (Fig. 1) using the primers 5’-CGCCGCTGCTCGCCGAGCGCATCTTCGGAGCGCGGCC-3’ and 5’-GCCGAGCTCCGACTCGGCCGTCTC-3’ and 5’-GACGAGCGCGAGGTGACATTTTCGAGCGCGGCCGACG-3’ we constructed the XT-I T variant. The XT-II S mutant was generated from pSS12 with the oligonucleotides 5’-GGCGAGAAGATGTTGAGCTTCAGCG-3’ and 5’-CGCTGAAGCTCTCATCCTTTGAGCGCG-3’. The entire XT-II variant with only the T region was built from pSS13 with 5’-GGCGAAGAAGATGTTGAGCTTCAGCG-3’. We used the pCG255-1 vector for the C-terminal XT-I parts. Multiple clones were sequenced to confirm the deletions and to ensure that no additional changes were introduced. Schematic of all mutants are shown in Fig. 1.

**Transfection**—Plasmids were purified from bacterial cultures using the GenElute (HP) Plasmid Midiprep kit (Sigma). HEK-293 and SAOS-2 cells were seeded onto 6-well plates containing sterile coverslips. After 24 h, when the cells reached 30–40% confluence, the cells were transiently transfected using Fugene6 transfection reagent (Roche Applied Science) as recommended by the manufacturer. Cells were then incubated for 48–72 h before immunostaining. At least two vectors encoding each XTLYT variant and three independent transfection experiments were performed to ensure reproducibility of the results.

**Immunostaining**—Cells on coverslips were washed with phosphate-buffered saline (PBS) and fixed in −20 °C chilled methanol for 15 min and then rinsed again in PBS. Fixed cells were permeabilized with PBS containing 0.1% Triton X-100 (Sigma) for 15 min at room temperature. The cells were washed with 0.1% bovine serum albumin in PBS. The cells were incubated for 1 h with the rabbit polyclonal to mannosidase II-Golgi marker (Abcam, Cambridge, UK) diluted 1:200 in 1% bovine serum albumin in PBS. In a second approach, untransfected cells were incubated for 1 h with the polyclonal rabbit antiserum, which was raised against a synthetic peptide homologous to the human XT-I (33). To remove unbound primary antibody, the cells were washed several times for 30 min with PBS containing 0.1% bovine serum albumin. The samples were then incubated for 1 h with TRITC- or FITC-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) diluted 1:1000 or 1:100 in PBS with 1% bovine serum albumin. After several washes with PBS, nuclear staining was performed using DAPI (1:1000 in PBS; Chemicon, Hampshire, UK) for 30 min. The cells were washed and mounted with Fluoromount-G (Southern Biotech, Birmingham, UK). The fluorescence pattern was analyzed using a Nikon Eclipse TE2000-S microscope (Nikon, Düsseldorf, Germany).

**Inhibitor Treatment**—Cells were incubated with 100 µg/ml cycloheximide (Sigma) either for 120 min in culture medium at 37 °C or for 90 min and then treated with 5 µg/ml brefeldin A (BFA; Sigma) for 30 min in the presence of cycloheximide in the culture medium. Fixation and staining were performed as described above.

**Assay for XT-I Enzyme Activity**—Cell lyses were prepared with Nonidet P-40 cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8) to analyze intracellular XT-I activity. The method for determination of XT-I activity is based on the incorporation of [14C]D-xylose into silk fibroin as an acceptor (19). The standard reaction mixture (100 µl) for the assay contained 50 µl of cell culture supernatant or
diluted cell lysate, 25 mM 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM MgCl₂, 5 mM MnCl₂, 1 μM UDP-[¹⁴C]D-xylose (9.88 kBq/nmol; DuPont), and 30 μl of solubilized silk fibroin (10 mg/ml). After incubation at 37 °C for 90 min, the reaction mixtures were placed on nitrocellulose discs (Sartorius, Göttingen, Germany). The dried discs were washed with 10% trichloroacetic acid for 10 min and three times with 1% trichloroacetic acid. Incorporated radioactivity was measured after the addition of 3.5 ml of scintillation mixture using a LS5000TD liquid scintillation counter (Beckman Coulter, Fullerton, CA). The enzyme activity was expressed in units (1 unit = 1 μmol of incorporated xylose/min).

RESULTS

Construction and Recombinant Expression of Active Full-length GFP-tagged XT-I—The separately existing N- and C-terminal parts of human XT-I cDNA were connected using PCR, restrictions, ligation, and site-directed mutagenesis. The obtained plasmid pSS1 contained 79 nucleotides of the 5'-untranslated region, including the native translation initiation sequence and the coding region (nucleotides 1–2877) of XT-I in frame with cycle 3 GFP. HEK-293 and SAOS-2 cells were transfected with pSS1 and analyzed by fluorescence microscopy, and the enzymatic activity was determined. The appearance of fluorescence 24 h post-transfection confirmed that GFP was in frame with XT-I. The activity of recombinant full-length GFP-tagged human XT-I was analyzed in the culture medium and in the cell lysates 72 h after transfection in comparison with the native XT-I activity in cells transfected with the control plasmid. HEK-293 cells show a 50-fold increase, and SAOS-2 cells show a 17-fold increase in XT-I activity in the supernatant and the lysates in comparison with the controls (Fig. 2). The XT-I activity in the supernatant is higher than in the cell lysates: 3-fold in HEK-293 and 4-fold in SAOS-2 cells. However, the ratio of secreted and retained XT-I activity is not altered in pSS1-transfected cells and the controls.

Intracellular Localization of the Native XT-I—We investigated the subcellular localization of native XT-I in untransfected HEK-293 and
SAOS-2 cells. Fluorescence microscopy was used after immunostaining with the XT-I or the Golgi antibody. The observed perinuclear staining pattern of the FITC-marked native XT-I is typical for localization in the Golgi compartment. Comparison with nuclear DAPI staining and the anti-mannosidase antibody confirmed this localization (Fig. 3A). The staining patterns were always similar to that of α-mannosidase II, which is a known resident enzyme of the medial Golgi complex. Cells were further stained with the XT-I or the Golgi marker after treatment with cycloheximide alone or cycloheximide and BFA. Cycloheximide blocks the synthesis of proteins, and the fungal metabolite BFA has been used to distinguish between proteins residing within the cis-/medial/trans-Golgi cisternae and the trans-Golgi network. BFA causes the collapse of the contents of the cis-, medial, and trans-Golgi back to the ER, whereas proteins residing within the trans-Golgi network traffic to the microtubule organizing center (34, 35). This redistribution allows the straightforward assignment of localization either in the Golgi cisternae or trans-Golgi network. The incubation with cycloheximide did not result in any altered XT-I or mannosidase staining patterns (figures not shown), indicating that the observed fluorescence reflects the true location of the mature proteins and is not the result of nascent protein still in the process of trafficking within the Golgi compartment or of protein being merely secreted in the extracellular space. After cycloheximide and BFA treatment and staining with the anti XT-I or the anti-mannosidase antibody, the perinuclear staining pattern was altered to diffuse cytoplasmic staining indicative of a localization in the endoplasmic reticulum (36) (Fig. 3A). All fluorescence patterns were identical for both cell lines used in our study. Together, these results support the conclusion that native XT-I is a Golgi-resident enzyme that is localized in the Golgi-cisternae and not in the trans-Golgi network.

Intracellular Localization of Recombinant Full-length GFP-tagged XT-I—The subcellular localization of the recombinant full-length GFP-tagged XT-I was investigated in HEK-293 and SAOS-2 cells after transfection with pSS1. A perinuclear staining pattern of the GFP-tagged recombinant XT-I was observed, indicating a Golgi localization of the enzyme; co-localization with the anti-mannosidase antibody confirmed this Golgi localization (Fig. 3B). Furthermore, an analysis of the subcellular distribution of XT-I-GFP and the Golgi markers after treatment with cycloheximide alone and a combined cycloheximide and BFA treatment was performed. Again, cycloheximide treatment induced no alterations of the fluorescence pattern, whereas cycloheximide and BFA treatment resulted in a diffuse cytoplasmic staining typical of the ER (Fig. 3B). The merged images reveal that the localization of XT-I-GFP and mannosidase is still identical after Golgi disruption by cyclohexi-
mide and BFA treatment. All stainings were identical for all cell lines used, and no differences were observed between the localization of recombinant full-length XT-I-GFP or the native XT-I. In conclusion, these findings confirm that the recombinant full-length XT-I-GFP is also retained in the cisternae of the Golgi apparatus.

Role of C, T, and S domains in XYLT-I—In order to determine which domains of XT-I are responsible for targeting the enzyme to the Golgi apparatus, cDNA constructs encoding GFP-tagged C, T, CT, S, or CTS domains with various lengths of S were made (Fig. 1A). We used predictions for the lengths of the putative C and T region based on hydropathy plot analysis. The NH2-terminal C-tail comprises the amino acids 1–13, and the T domain is located at positions 14–35. The length of the S region is not known, but we could recently show that the N-terminal truncated XT-I (H9004 1–148) is active in High Five insect cells (30–32). The PCR products of the C, CT, and CTS variants were cloned at the C-terminal end in frame with GFP, and the deletion mutants T and S were made from CT and CTS using site-directed mutagenesis. All constructs were transfected into HEK-293 and SAOS-2 cells, and 2–3 days post-transfection, immunostaining with the Golgi marker was performed with or without cycloheximide and BFA treatment. The different staining patterns were studied by fluorescence microscopy. In all experiments, no differences between HEK-293 and SAOS-2 cells were observed. The GFP fusion protein consisting of the regions CTS (amino acids 1–214; pSS2) is the only truncated variant reaching full Golgi retention as indicated by the identical punctuated staining pattern, the co-localization with the Golgi marker, and the diffuse ER-like pattern after BFA treatment as shown for the full-length XT-I (figures not shown, but patterns were identical to pSS1-transfected cells). The deletion of 56 amino acids (pSS3) from the S region changed the microscopic picture, and we could observe only a partial Golgi retention indicated by the higher density of GFP fluorescence in the perinuclear region. After BFA treatment, the fluorescence patterns changed, since the GFP fusion proteins that were formerly located in the Golgi cisternae were now redistributed into the ER (Fig. 4A). A complete loss of Golgi localization was reached when the S region was truncated to 69 amino acids (pSS6). The fluorescence was irregularly distributed over the whole cell, leaving out the nucleus (Fig. 4B). Comparison with the Golgi marker showed no concordance with the localization of the GFP-XT-I mutant, and in addition, BFA treatment did not result in any redistribution of the GFP fusion protein (figures not shown). We also observed identical staining patterns when we expressed the CT- and T-variants in pSS8- and pSS10-transfected cells (figures not shown). Expression of the C- and the S-constructs in pSS9- and pSS11-transfected cells resulted in GFP fluorescence being homogeneously distributed all over the cell identical to the pattern of the control vector, which codes for the cycle 3 GFP without any signal sequence (figure not shown).

Intracellular Localization of XT-II and Function of C, T, and S Domains—Analogous to XT-I, we designed five GFP fusion proteins consisting of the CTS, CT, C, T, and S region for XT-II to analyze their subcellular localization and the signal sequence responsible for targeting this glycoyltransferase (Fig. 1B). The hydropathy analysis of XT-II revealed that the cytoplasmic tail is built by amino acids 1–15 and that the potential membrane-spanning region consists of 16 hydrophobic amino acid residues at positions 16–32 (24). All GFP fusion proteins consisting of the regions CTS (amino acids 1–214; pSS2) is the only truncated variant reaching full Golgi retention as indicated by the identical punctuated staining pattern, the co-localization with the Golgi marker, and the diffuse ER-like pattern after BFA treatment as shown for the full-length XT-I (figures not shown, but patterns were identical to pSS1-transfected cells). The deletion of 56 amino acids (pSS3) from the S region changed the microscopic picture, and we could observe only a partial Golgi retention

FIGURE 4. Localization of representative truncated GFP-tagged XT-I variants in HEK-293 cells. A, cells transfected with pSS3 show a small region of intense perinuclear staining, which is primarily co-localized with the Golgi marker (top). This fluorescence pattern is disrupted in the presence of cycloheximide and BFA (bottom). B, the GFP fluorescence of the CTS-XT-I-variant encoded by pSS6 appeared constant over the whole cell except the nucleus. No co-localization with the Golgi marker α-mannosidase II was observed. C, expression of a GFP-tagged XT-I-variant that only contains the stem domain and is encoded by pSS11 results in a cytoplasmic fluorescence evenly distributed over the whole cell.
including amino acids 1–45, in frame with GFP (pSS12) resulted in the Golgi-typical punctuated staining pattern (Fig. 5A). Furthermore, this GFP fusion protein shows a perfect co-localization with the Golgi marker. The incubation with cycloheximide did not result in any altered fluorescence patterns (figures not shown), demonstrating that the observed fluorescence reflects the true location of the protein and is not the result of nascent protein still trafficking within the Golgi apparatus. When the cells were incubated with cycloheximide and BFA, the punctuated staining pattern disappeared, and the fluorescence was redistributed to the ER (Fig. 5A). These results confirm that XT-II is also located within the Golgi cisternae. The GFP fluorescence of the XT-II mutant containing the CT region (pSS13) was located in perinuclear regions (Fig. 5B) identical to the fluorescence patterns observed for the variant with the isolated T domain (pSS15; figures not shown). Cycloheximide and cycloheximide/BFA treatment did not result in any different staining patterns in the T- and CT-XT-II variants. The XT-II variants coding for the C and S region did not localize GFP to the Golgi, but fluorescence was distributed throughout the whole cytoplasm (Fig. 5C). Therefore, we suggest that the short S-part encoded by exon 1, together with the C and T domains, is sufficient for the Golgi retention.

**DISCUSSION**

Primary sequence and hydrophobicity analyses predicted that the xylosyltransferases XT-I and XT-II are, like the majority of eukaryotic glycosyltransferases, type-II transmembrane proteins with a short NH2-terminal cytoplasmic tail, a single transmembrane domain, a stem region of variable length, and a large COOH-terminal globular catalytic domain (2, 24). Several investigations suggested that soluble glycosyltransferases were formed from membrane-bound enzymes by proteolytic cleavage between the catalytic and the transmembrane domain (37, 38). We have shown that XT-I is secreted into the extracellular space together with proteoglycans (22, 39), and that serum XT-I activity is a confirmed biochemical marker for the determination of fibrotic activity in systemic sclerosis (22, 24).

Here we report for the first time the construction and recombinant expression of the full-length GFP-tagged XT-I in mammalian cells. The XT-I activity in the cell culture supernatant and the cell lysates is 20–50-fold increased in comparison with cells transfected with the control plasmid. About two-thirds of the enzyme is secreted into the extracellular space and is accumulated in the cell culture supernatant. The ratio of intra- and extracellular XT-I activity was not altered after transfection. These results confirmed that the GFP-tagged recombinant XT-I was properly folded, proteolytically cleaved by intracellular proteases, and secreted into the extracellular space independent of the transfected cell line. Furthermore, these findings demonstrate that expression of the recombinant GFP-XT-I fusion protein under control of the strong cytomelovirus promoter does not result in any artificial subcellular localization or increased shedding of this enzyme. This is also supported by the identical fluorescence patterns obtained for the native XT-I and the recombinant GFP-tagged XT-I in our study. Until now, only a soluble recombinant form of the human XT-I lacking amino acids 1–148 was expressed in High Five insect cells and CHO-K1 cells (30–32). Cloning and expression of the active full-length XT-I is an important prerequisite for studies investigating the intracellular localization of the enzyme, the shedding mechanism, or the molecular effects of disease-associated genetic variations.

The analysis of the subcellular localization of native and recombinant XT-I revealed that this enzyme is Golgi-resident. This was shown by the punctuated staining pattern of the FITC-marked native XT-I in untransfected cells and GFP-tagged XT-I in transfected cells. The exact
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co-localization with the Golgi marker and the equal sensitivity to BFA verified these results. BFA causes the collapse of the contents of the cis-, medial, and trans-cisternae back to the ER, whereas proteins residing within the trans-Golgi network traffic to the microtubule organizing center. This redistribution allows the straightforward assignment of localization either in the cisterna of the Golgi compartment or the trans-Golgi network. In our study, BFA treatment resulted in a redistribution of both α-mannosidase II, which is a well known component of the medial Golgi cisternae, and XT-I into the ER. These results strongly support a localization of human XT-I in the early Golgi cisternae. Furthermore, the comparison of transfected and untransfected cells indicates that the Golgi localization is not caused by an overexpression of the recombinant enzyme under the strong cytomegalovirus promoter or is an artifact induced by the carboxyl-terminal GFP tag. Moreover, all human cell lines investigated in our study exhibit an identical Golgi localization of the xylosyltransferases. Consequently, this is the first report of determination of the subcellular localization using a tagged form of the protein. Previous studies performed in recent decades using techniques like density gradient centrifugation for the isolation of subcellular organelles could not exactly elucidate the intracellular localization of XT-I. There were some data suggesting a Golgi localization and some suggesting an endoplasmic reticulum localization (40).

Information about the characteristics that localize glycosyltransferases in intracellular compartments is needed in order to gain a better understanding of the underlying mechanisms that regulate glycan biosynthesis. With the intention of defining the primary sequence responsible for Golgi retention of XT-I, cDNAs encoding truncated enzymes were fused to a GFP reporter protein and were transiently expressed in mammalian cells. The localization was investigated by immunofluorescence microscopy after immunostaining with Golgi markers and BFA treatment.

A XT-I variant with a total length of 214 amino acids, consisting of the CTS region with 179 amino acids in the S domain, showed an efficient Golgi localization. Truncation to a total of 158 to 119 amino acids (123 to 84 amino acids of the S region) resulted in impaired retention, and the tagged protein was only partially detected in the Golgi. However, the fact that some of the recombinant proteins still localized in the Golgi apparatus emphasizes the importance of the stem region rather than the transmembrane domain in targeting these molecules to the Golgi. As a limitation of our study, it should be noted that a steric influence of the carboxyl-terminal GFP domain in the CTS variants on the Golgi retention cannot be completely excluded. However, this is rather unlikely, since the pSS12-encoded CTS-XT-II-GFP variant that contains the shortest “spacer” between the transmembrane region and the GFP domain is also completely retained in the Golgi cisternae. To finally overcome this limitation, the GFP domain of all fusion proteins would have to be replaced by different peptide tags. The deletion of the C and T region from the 134 amino acids including the CTS variant (pSS4; 99 amino acids of the S region) changed the formerly incomplete Golgi retention of the protein to that of a soluble protein exhibiting diffuse cytoplasmic staining. This result indicates that the stem region of XT-I is not the sole determinant for its Golgi localization. GFP-tagged proteins expressed by CTS variants with a stem region of 69 or fewer amino acids and mutants only consisting of the CT or T domain lost their Golgi retention, and fluorescence was detected irregularly over the whole cell with the exception of the nucleus. This staining pattern differs from those with the isolated S or C region, indicating that the transmembrane domain plays a major role not only in specific localization but also in directing the proteins into intracellular membrane systems. These findings confirm the results from the initial hydrophobicity plot analysis that the predicted amino acid sequence at positions 14–35 is the T region.

XT-II is a potential glycosyltransferase highly homologous to XT-I. Since it shares the same predicted topology, we also analyzed five variants of the N-terminal CTS regions of XT-II to compare the retention mechanisms of those proteins. The CTS variant with 13 amino acids of the S region is able to ensure full Golgi retention of XT-II. The staining patterns of the C, CT, S, and T mutants were equivalent to those for XT-I. BFA treatment resulted in a redistribution of the medial Golgi marker α-mannosidase II and XT-II into the ER, indicating that human XT-II is also localized in the early Golgi cisternae. The observed localization of both XT-I and XT-II in the cis-Golgi or medial Golgi compartments gives support to the hitherto unproven hypothesis that XT-II is also a xylosyltransferase with different and narrow substrate specificity.

The results obtained from these in vitro targeting experiments clearly demonstrate that the transmembrane domain and parts of the flanking stem regions of XT-I and XT-II are sufficient to target the normally cytoplasmic protein GFP to the Golgi compartment. Interestingly, the required length of the S region differs between the two xylosyltransferases. No more than the 13 amino acids of the stem region of XT-II encoded from exon 1 are sufficient for Golgi retention. In contrast, for complete Golgi retention, XT-I requires a maximum number of 179 amino acids from the stem region encoded by exons 1 and 2 and parts of exon 3. The way in which our XT-I and XT-II data can be reconciled with the kin recognition theory should be investigated in further studies. The present investigations reported that for some glycoyltransferases, oligomerization of the stem region was responsible for their Golgi retention (41), whereas others still showed Golgi retention after the disruption of the protein-protein interactions (42, 43). We could only hypothesize that the truncated stem region of XT-I leads to decreased formation of aggregates and therefore to impaired Golgi retention. We propose that the transmembrane domain acts as a general anchor or retention sequence holding proteins in the membranes. The comparison of the Golgi retention sequences revealed no homologies between the two xylosyltransferases. This result confirms the observation that no amino acid consensus sequence for localization exists (44). In this regard, a homologous sequence or a required length of the stem regions in the human xylosyltransferases does not exist and would not be necessary for Golgi retention.

The N-terminal 214 amino acids that are responsible for Golgi localization are not required for the enzymatic function of XT-I, because we could show that the deletion of amino acids 1–266 of XT-I did not influence the enzymatic activity (45). Similar results have been reported for GnT-V, a Golgi-resident protein whose stem region is not required for its enzymatic activity but is responsible for its intracellular localization (41). However, it is not yet known whether this is also valid for XT-II, and we will have to investigate this after identification of the enzymatic function of this protein.

In conclusion, we have constructed a vector encoding the GFP-tagged enzymatic active full-length XT-I, which is an important prerequisite for the analyses of the shedding mechanism and the localization of the secreted XT-I in the extracellular matrix. Furthermore, we have shown that XT-I and XT-II are Golgi-resident enzymes. Our results indicate that for XT-I 214 amino acids and for XT-II 45 amino acids from the CTS region are sufficient to target to and retain the GFP reporter in the Golgi apparatus.

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