Mapping the Interaction of the STT3 Subunit of the Oligosaccharyl Transferase Complex with Nascent Polypeptide Chains*

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Many secretory and membrane proteins are N-glycosylated by the oligosaccharyl transferase complex during their translocation across the endoplasmic reticulum membrane. Several experimental observations suggest that the highly conserved STT3 subunit contains the active site of the oligosaccharyl transferase. Here, we report a detailed study of the interaction between the active site of the STT3 protein and nascent polypeptide chains using an in vitro photocrosslinking technique. Our results show that the addition of a glycan moiety in a stretch of ~15 residues surrounding a QXK*T cross-linking site impairs the interaction between the nascent chain and STT3.

N-Linked glycosylation is one of the most common types of protein modification in eukaryotic cells. The attachment of carbohydrates to asparagines in secretory and membrane proteins takes place during cotranslational translocation across the endoplasmic reticulum membrane. The transfer of high mannose oligosaccharides from a dolichol-carrier to Asn-Xaa-Thr/Ser acceptor sites (with Xaa being any amino acid except proline) is catalyzed by the oligosaccharyl transferase (OST) enzyme (1), and the transfer efficiency depends on many factors such as the translation rate, the levels of OST, and availability of the dolichol-bound oligosaccharides. As a result of this complex cellular balance, not all potential glycosylation sites are modified, the most important being the consensus sequence Asn-Xaa-Thr/Ser.

Previously, we have shown that a photoreactive probe incorporated into a nascent polypeptide chain carrying a cryptic glycosylation site (Gln-Lys*-Thr, where Lys* carries the photodeactivatable group) can be cross-linked to the mammalian STT3 in dog pancreas microsomes (11). No significant photocrosslinking to other OST subunits was detected, and therefore our results strongly indicated that the active site of the OST complex is located in STT3.

To further characterize the co-translational interaction between STT3 and nascent polypeptide chains, we have now studied the effect of nascent chain cross-linking of introducing bona fide Asn-Xaa-Thr glycosylation sites close to the Gln-Lys*-Thr cross-linking site. We find that cross-linking to STT3 is greatly reduced when a glycosylated Asn-Xaa-Thr site is present between positions ~9 (N-terminal) and +4 (C-terminal) relative to the Gln-Lys*-Thr site. Additionally, we find that two closely spaced Asn-Xaa-Thr sites are less efficiently glycosylated than when they are placed further apart.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all enzymes, plasmid pGEM1, and rabbit reticulocyte lysate were from Promega or New England BioLabs. [14C]Met, [15N]HCl, [13C]-methylated marker proteins, ribonucleotides, and the cap analogs m7G(5′)ppp(5′) G and G(5′)ppp(5′)G were from GE Healthcare. Ex Taq polymerase was from TaKaRa Biomedicals (Shiga, Japan). Protein A-Sepharose was from Sigma. The antibody to STT3A was raised against the C terminus (residues 693–705) of human STT3A (7). Dog pancreas column-washed rough microsomes (CRMs), SRP, and wheat germ extract were prepared as described (12, 13). eANB-[14C]Lys-tRNA(1) was prepared as detailed previously (14, 15). BigDye Terminator v1.1 Cycle Sequencing Kit was from AB Applied Biosystems (Washington, WA), and oligonucleotides were from CyberGene AB (Stockholm, Sweden).

DNA Plasmids—Bovine preprolactin (pPL) glycosylation constructs were made from the previously described preprolactin construct (pPL-sk) and were cloned into the pVV1 plasmid behind the SP6 promoter (16). The pPL-sk polypeptide lacks residues 2–9 of natural pPL and therefore does not have any lysines in its sequence (15). In all nascent chains the lysine codons at position 91, 128, 146, 164, and 181 in pPL-sk...
were altered to a non-lysine codon, and this Lys-less construct was used as a basis for the following mutagenesis (11). Two main series of pPL derivatives were constructed in the present work by site-specific mutagenesis. The first series pPL-sK(QK*T) contains a Q66KT cross-linking site (11) and an NST glycosylation site in different positions (one in each construct) with the asparagine (N) in positions 32, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 69, 70, 71, 72, 78, 90, or 95. The second series pPL-sK(NKT) contains a glycosylation site N66KT and a second glycosylation site NST in the same positions as above. To examine the closest approach of two glycosylation sites, the NKT sequence in pPL-sK(NKT) was replaced by the amino acid sequence ..NST.., ..QNST.., ..NTS.., ..QNTS.., ..NNTT.., ..QNTT.., ..NNTNNT.., or ..NSTNST... Leader peptidase (Lep) constructs were made from the pGEM1 plasmid, cloned, and expressed behind the SP6 promoter as previously described (17). The sequence of the endogenous glycosylation site, ..NET.. at position 215–217, was replaced by the amino acid sequence ..NNTT.. or ..NSTNST... to make Lep constructs with neighboring glycosylation sites.

Site-specific mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit from Stratagene. All mutants were confirmed by the sequencing of plasmid DNA at BM labbet AB (Furulund, Sweden). All cloning steps were done according to standard procedures using restriction enzymes from Promega.

Transcription in Vitro—Transcription of full-length pPL-sK mRNAs from the preprolactin plasmid and full-length Lep mRNAs from the pGEM1 plasmid using SP6 RNA polymerase was performed as described previously (18). The DNA template for in vitro transcription of truncated pPL-sK mRNAs was prepared using PCR to amplify a fragment from the plasmid. The 5′-primer was situated 100 bases upstream of the translation start, and the amplified fragment thus contained the SP6 transcriptional promoter. The 3′-primer was chosen to produce a truncated fragment ending at the desired codon (156), and no stop codon was included. Truncated mRNAs were transcribed from the amplified DNA fragments as described (13), except that the GTP concentration was raised to 0.5 mM after 90 min to ensure completion of all transcripts.

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Leader peptidase (Lep) constructs were made from the pGEM1 plasmid, cloned, and expressed behind the SP6 promoter as previously described (17). The sequence of the endogenous glycosylation site, ..NET.., at position 215–217, was replaced by the amino acid sequence ..NNTT.. or ..NSTNST.. to make Lep constructs with neighboring glycosylation sites.

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Interaction of Nascent Chains with STT3

Translation in Vitro and Photocrosslinking—In vitro translations (usually 25-μl total volume, but 50 μl for immunoprecipitations) of mRNA in wheat germ cell-free extract were incubated at 22 °C for 30 min in the presence of 40 nM canine SRP, [35S]Met (usually 0.7 μCi/ml, but 2 μCi/ml for immunoprecipitations), 8 eq of CRMs, and 15 pmol of eANB-[14C]Lys-tRNA^lys/25 μl of incubation (13, 19). Samples were photolyzed on ice for 15 min using a 500-W mercury arc lamp and filters that only transmitted light with a wavelength greater than 300 nm. After photolysis, membranes were sedimented through a 0.5 M sucrose cushion in a Beckman TLA ultracentrifuge at 4 °C for 4 min at 100,000 rpm in a TLA100 rotor. For immunoprecipitation using STT3-A-specific antibodies, microsome pellets were resuspended in buffer A (50 mM Tris-HCl (pH 7.6), 95 mM NaCl, 3 mM EDTA) containing 2% (w/v) SDS and placed at 55 °C for a minimum of 30 min. The samples were then diluted 10 times with buffer B (50 mM Tris-HCl (pH 7.6), 95 mM NaCl, 3 mM EDTA, 1.25% (v/v) Triton X-100, 0.2% (w/v) SDS) and precleared by rocking with protein A-Sepharose at room temperature for 60 min. The Sepharose beads were removed by sedimentation. STT3-A-specific antiserum was added to a precleared sample, and the samples were rocked overnight at 4 °C. Protein A-Sepharose was then added to each sample and incubated for a minimum of 2 h at 4 °C. The immunoprecipitates were recovered by sedimentation, washed twice with buffer B, and then washed a final time with the same buffer containing no detergent. The immunoprecipitated proteins were analyzed by SDS-PAGE and visualized in a Bio-Rad FX Molecular Imager using the Quantity One Quantification software.

Translations of full-length pPL-sK mRNAs and full-length Lep mRNAs were performed at 30 °C for 60 min as described (20) in a total volume of 14 μl containing nuclease treated reticulocyte lysate, 40 units of RNase inhibitor, [35S]Met (0.7 μCi/μl), 70 μM concentration of each amino acid except Met, and 4 eq of CRMs. Samples were then analyzed by SDS-PAGE, and proteins were visualized in a Fuji FLA-3000 PhosphorImager using the Image Reader V1.8J/Image Gauge V 3.45 software.

RESULTS

Using photocrosslinking from an engineered Gln-Lys*-Thr (QK*T) site in a nascent polypeptide chain, we have shown previously (11) that nascent secretory proteins are adjacent to the OST component STT3 during their translocation across the endoplasmic reticulum membrane. We also showed that the efficiency of photocrosslinking to STT3 reached a maximum when the QK*T site was positioned 70–100 amino acids from the P-site in the ribosome, in agreement with the observation that an Asn-Ser-Thr (NST) glycosylation acceptor site must be located at least 65–75 residues from the P-site for efficient glycosylation (21).

To probe the interaction between STT3 and nascent chains in more detail, we sought to block photocrosslinking to the QK*T site by introducing a nearby bona fide Asn-Xaa-Thr (NKT) glycosylation acceptor site. In parallel, we also determined the inhibitory effect on glycosylation when two NXT sites are placed close to each other. In a first set of constructs, a potential glycosylation acceptor site (NST) was engineered at various positions either N- or C-terminal to the QK*T cross-linking site in the model secretory protein pPL, and the efficiency of photocrosslinking to STT3 was determined for each construct after in vitro translation of truncated mRNAs in the presence of dog pancreas rough microsomes (11). In a second set, an NST site was placed either N- or C-terminal to an authentic glycosylation site in the model secretory protein pPL, and the efficiency of photocrosslinking to STT3 was determined for each construct after in vitro translation of full-length pPL in the presence of RM.

Glycosylation Inhibits Cross-linking to STT3—For the first set of constructs, translocation intermediates for the photocrosslinking experiments were prepared by in vitro translation in the presence of SRP, endoplasmic reticulum microsomes, and the photoreactive eANB-Lys-tRNA. In these experiments the mRNAs were truncated within the
interaction of nascent chains with STT3

In vitro translation in the absence (-) or presence (+) of rough microsomes (CRM) of full-length pPL-sk (A and C) and Lep (B) constructs carrying two closely spaced glycosylation sites. Doubly glycosylated and singly glycosylated products are indicated by two open circles and one open circle, respectively (A and B). Singly glycosylated and non-glycosylated products that were cleaved by signal peptidase are indicated by an open and closed circle, respectively, whereas unprocessed singly glycosylated and non-glycosylated products are indicated by an open and closed square, respectively (C). The percentage of doubly glycosylated pPL-sk (A) and Lep (B) molecules is indicated by DG for each construct (A and B), and the percentage of singly glycosylated molecules is indicated by SG in C. All values are an average of two independent experiments, except for NET (B) and NST (C), which are from single experiments.

Only weak STT3 photocrosslinks (11) were detected for constructs where the asparagine in the NST sequence was placed between residues 57 and 71 (positions −9 to +5 relative to the Gln in the QK*T sequence) compared with constructs where the NST site was placed further away, Fig. 1. We conclude that the introduction of a bulky glycan moiety in a stretch of −15 residues surrounding the QK*T cross-linking site impairs the interaction between the nascent chain and STT3.

Modification of Neighboring Glycosylation Sites—Because a glycosylated NST sequence placed near the QK*T sequence inhibits cross-linking to STT3, we considered the possibility that there may be a similar interference between two closely spaced bona fide NST glycosylation acceptor sites.

We therefore made a second set of constructs where a NST glycosylation site was located at the same distances from an NKT acceptor site as from the QK*T cross-linking site in the first set of constructs. Indeed, a decrease in the fraction of chains carrying two glycans was seen when the NST site was placed in positions −10 to +4 relative to the NST site, Fig. 2. The inhibitory effect on glycosylation was less pronounced than the inhibition of STT3 photocrosslinking, but the sensitive region was approximately the same size and had the same asymmetry as the region identified by photocrosslinking.

Only one of the two overlapping acceptor sites in the sequence NNTT was modified, Fig. 3A, and, for very closely spaced sites, glycosylation efficiency was found to depend to some extent on sequence context. Thus, the extent of double glycosylation of chains was smaller for two NNTNNT sites than for two NSTNST sites. Comparing the sequences NSTRNKT and NKTNST, 45% of the NSTRNKT-containing chains had two glycans, whereas 73% of NKTNST had two glycans (Fig. 3B). Clearly, when glycosylation efficiency is reduced, sequence context becomes important, as has been seen earlier for single, non-optimal glycosylation sites (22, 23). The sequence context surrounding two glycosylation sites seems in some cases more important than the distance between them; e.g., the glycosylation efficiency of the sequences NSTRNKT (18%) is lower than for the more closely spaced sequences NSTNST (45%) (Fig. 2B) (positions of NST site are −4 and −3, respectively).

The result for overlapping sites was confirmed using another well-characterized model protein, Lep, which is normally located in the inner membrane of Escherichia coli. Lep is efficiently glycosylated on N215ET when expressed as a full-length protein in the presence of microsomes (17). As shown in Fig. 3B, only one site in the sequence NNTT in Lep was efficiently glycosylated, whereas both sites in constructs with NST-NST sequence were glycosylated.

The finding that a “cryptic” QK*T-site can be cross-linked to OST (11) suggests that QXS/T sequences might affect the glycosylation efficiency of a nearby NXS acceptor site. To test this, we made a third set of pPL constructs with overlapping QXT/S and NXT/S sites, Fig. 3C. Indeed, a QKT sequence overlapping with the less than optimal NXS acceptor site (22, 23) reduced glycosylation of the latter (compare constructs NTS and QNTS) but had no effect on the glycosylation efficiency of the optimal NXS sequence (construct QNTT and QNST).

DISCUSSION

Here we have carried out a detailed study of how the interaction between a nascent chain and the STT3 subunit of the OST is affected by the presence of a nearby glycosylated residue. Two different assays of nascent chain interaction with STT3—photocrosslinking from a nearby QK*T site and glycosylation of a nearby NKT site yield similar results, a glycan located between 9 residues N-terminal and 4 residues C-terminal of the QK*T or the NKT target site markedly affects the nascent chain.
interaction with STT3. The simplest interpretation of these data is that the OST complex contacts roughly 15 residues of the nascent chain. Our data for constructs carrying two closely spaced bona fide glycosylation sites are in agreement with previous work where it has been noted that the smallest separation between neighboring glycosylated sites found so far in a natural protein (24) is three residues (a NHSENAT sequence in haptoglobin-1 (25, 26)). It may be that steric hindrance prevents more closely spaced sites from being glycosylated in the same nascent chain, as we found for the sequence NNNT in yeast invertase (28). Mutagenesis of the overlapping acceptor sites to NNST in yeast invertase resulted in glycosylation of the second NNTS in yeast invertase (28). Mutagenesis of the overlapping acceptor sites to NNST in yeast invertase resulted in glycosylation of the second Asn residue (29). Both the efficiency of transfer and the selection of a preferred site within closely spaced or overlapping glycosylation sites (29) therefore seem to be influenced by the preference of OST for Asn-Xaa-Thr sites relative to Asn-Xaa-Ser sites (22, 23) and the local sequence context.

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