The Amino Acid Following an Asn-X-Ser/Thr Sequon Is an Important Determinant of N-Linked Core Glycosylation Efficiency

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ABSTRACT: Many eukaryotic proteins are modified by Asn-linked (N-linked) glycosylation. The number and position of oligosaccharides added to a protein by the enzyme oligosaccharyltransferase can influence its expression and function. N-Linked glycosylation usually occurs at Asn residues in Asn-X-Ser/Thr sequons where X ≠ Pro. However, many Asn-X-Ser/Thr sequons are not glycosylated or are glycosylated inefficiently. Inefficient glycosylation at one or more Asn-X-Ser/Thr sequons in a protein results in the production of heterogeneous glycoprotein products. These glycoforms may differ from one another in their level of expression, stability, antigenicity, or function. The signals which control the efficiency of N-linked glycosylation at individual Asn residues have not been fully defined. In this report, we use a site-directed mutagenesis approach to investigate the influence of the amino acid at the position following a sequon (the Y position, Asn-X-Ser/Thr-Y). Variants of rabies virus glycoprotein containing a single Asn-X-Ser/Thr sequon at Asn^{37} were generated. Variants were designed with each of the twenty common amino acids at the Y position, with either Ser or Thr at the hydroxy (Ser/Thr) position. The core glycosylation efficiency of each variant was quantified using a cell-free translation/glycosylation system. These studies reveal that the amino acid at the Y position is an important determinant of core glycosylation efficiency.

N-Linked glycosylation begins with the enzymatic transfer of a pre-synthesized core oligosaccharide, $Glc_3Man_9GlcNAc_2$, from a dolichol phosphate oligosaccharide donor to an Asn residue *(1, 2)*. This reaction usually occurs cotranslationally, as nascent proteins enter the endoplasmic reticulum *(3−6)*, and is catalyzed by oligosaccharyltransferase, a large heteromeric membrane protein complex *(7)*. Core glycosylation usually occurs at Asn residues in the sequon Asn-X-Ser/Thr, where X is any amino acid except Pro *(8−10)*. However, many Asn-X-Ser/Thr sequons are not glycosylated or are glycosylated at a low level *(11)*. Inefficient glycosylation results in the formation of glycoprotein variants which differ from one another in the number and position of N-linked oligosaccharides. These variants may differ from one another in terms of their expression, stability, or biological function *(12, 13)*. The heterogeneity of glycoproteins which results from inefficient core glycosylation can complicate the production of recombinant proteins for research or clinical applications.

Studies by Imperiali and colleagues support a mechanistic model for core glycosylation in which the hydroxy (Ser/Thr) residue in a sequon participates in the oligosaccharide transfer reaction. In this model, glycosylation requires the formation of an Asx turn at the sequon to facilitate critical hydrogen bonding reactions between the Asn residue, the hydroxy residue, and the peptide backbone *(14)*. Amino acids near a sequon could potentially modulate core glycosylation efficiency by influencing either the conformation of the sequon or the potential of functional groups in the sequon to participate in hydrogen bonding reactions. The importance of amino acids in the sequon itself (i.e., at the X and hydroxy positions) has been confirmed using peptides as oligosaccharide acceptors, comparing amino acids in glycosylated and nonglycosylated sequons, and using site-directed mutagenesis to modify the sequons in recombinant proteins *(11)*. Similar studies suggest that core glycosylation efficiency is also influenced by amino acids at other positions outside the sequon but near the Asn residue. While Asn-X-Ser/Thr tripeptides can be glycosylated if the N- and C-termini are blocked *(15)*, the glycosylation rate of sequon-containing peptides increases with increasing chain length. This suggests that amino acids on either side of a sequon contribute to the formation of an active conformation *(16, 17)*. Information about how specific amino acids near Asn-X-Ser/Thr sequons affect core glycosylation efficiency is limited. The presence of Pro at the position following a sequon usually blocks glycosylation *(9, 18, 19)*. Cys residues near sequons can also inhibit glycosylation, possibly by introducing conformational constraints in proteins as a result of disulfide bonding *(17, 20−22)*.

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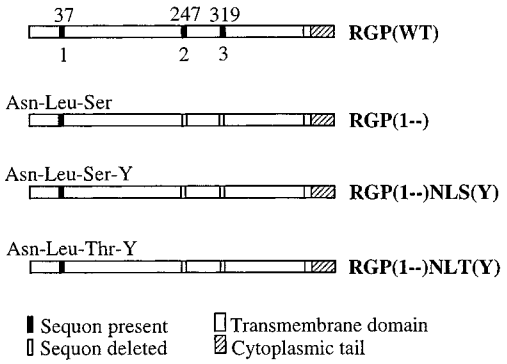

FIGURE 1: Structure of RGP and RGP variants. The extracellular domain of wild-type RGP contains three sequons, at Asn^{37} (sequon 1), Asn^{247} (sequon 2), and Asn^{319} (sequon 3) (RGP(WT)). RGP(1- -) contains a single sequon with the wild-type sequence Asn^{37}-$Leu^{38}Ser^{39}$ (sequon 1). In RGP(1- -), sequons 2 and 3 were deleted by site-directed mutagenesis, replacing the Thr in each sequon with Ala. RGP(1- -)NLS(Y) and RGP(1- -)NLT(Y) variants were derived from RGP(1- -) by oligonucleotide cassette mutagenesis. RGP(1- -)NLS(Y) variants contain a single Asn-X-Ser sequon, whereas RGP(1- -)NLT(Y) variants contain a single Asn-X-Thr sequon. Amino acid substitutions were introduced into the variants at the Y position following the sequon.

In this report, we use rabies[1] virus glycoprotein (RGP) as a model protein to examine systematically the impact of the Y amino acid in the sequence Asn-X-Ser/Thr-Y on core glycosylation efficiency. Wild-type RGP contains three sequons for N-linked glycosylation at Asn^{37}, Asn^{247}, and Asn^{319} (Figure 1) *(23)*. A variant of RGP with a single sequon at Asn^{37}, RGP(1- -), was previously generated to examine core glycosylation at that site *(24)*. This sequon, $Asn^{37}Leu^{38}Ser^{39}$, is inefficiently glycosylated when RGP(1- -) is expressed in a cell-free system or in Chinese hamster ovary cells *(24)*. Remarkably, core glycosylation at Asn^{37} is dramatically improved by replacing Leu^{38} with more favorable X amino acids *(25)* or by replacing Ser^{39} with Thr *(26, 27)*. This indicates that the sequon is fully accessible to oligosaccharyltransferase for glycosylation. In this report, amino acid substitutions at the Y position of RGP(1- -) (i.e., at amino acid 40) were introduced by site-directed mutagenesis. The core glycosylation efficiency of the sequon in each of these RGP variants was quantified using a cell-free translation/glycosylation system. These studies reveal that the amino acid at the Y position is an important determinant of core glycosylation efficiency, particularly when Ser is the hydroxy amino acid.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Encoding RGP Variants with Amino Acid Substitutions at the Y Position following Sequon 1. RGP variants with amino acid substitutions at the Y position following sequon 1 were generated using a cassette mutagenesis approach described previously *(25)*. The degenerate oligonucleotides used to generate RGP(1- -)NLS(Y) variants were 5′-GAAGGATGCACCAACCTGTCAXXXTTCTCCTACATGGAGCT-3′ (sense) and 5′-CCATGTAGGAGAAXXXTGACAGGTTGGTGCATCCTTC-3′ (antisense); X indicates that A, C, G, or T may be present. Degenerate oligonucleotides used to generate RGP(1- -)NLT(Y) variants were 5′-GAAGGATGCACCAACCTGACCXXXTTCTCCTACATGGAGCT-3′ (sense) and 5′-CCATGTAGGAGAAXXXGGTCAGGTTGGTGCATCCTTC-3′ (antisense). The positions of bases corresponding to Asn-X-Ser/Thr sequons are underlined. Successful mutagenesis was confirmed in each variant by DNA sequencing.

Expression of RGP Variants in a Cell-Free System. Methods for expression of RGP variants in a cell-free system were described previously *(24, 25)*. Briefly, RNA encoding RGP variants was generated by in vitro transcription and translated using a rabbit reticulocyte lysate system supplemented with [^{35}S]methionine and canine pancreas microsomes. Following translation, the reaction mixtures were treated with proteinase K and analyzed by SDS−PAGE and autoradiography *(25)*. Densitometric analysis was performed using gel autoradiographs exposed in the linear range.

RESULTS

Construction of Plasmids Encoding RGP Variants with Amino Acid Substitutions at the Y Position. We previously generated a variant of RGP(WT) which contains only one glycosylation sequon, $Asn^{37}Leu^{38}Ser^{39}$ (Figure 1, RGP(1- -)) *(24)*. This sequon was selected for further analysis using the cell-free translation/glycosylation system described below for three reasons. First, because this sequon is inefficiently glycosylated, it is possible to detect the influence of neighboring amino acids which either increase or decrease core glycosylation efficiency. Second, previous studies using this cell-free system demonstrate that this sequon can be fully glycosylated if more favorable amino acids are substituted at the X or hydroxy position *(25−27)*. This indicates that this sequon is accessible to the glycosylation apparatus and that the cell-free system has a high level of enzymatic activity, capable of fully glycosylating favorable sequons. Third, the glycosylation efficiencies of RGP variants in this system are qualitatively similar to those obtained when the same variants are expressed in transfected cells *(24, 26)*. Furthermore, sequons at Asn^{37} in RGP(1- -) variants that are identified as poor oligosaccharide acceptors in this system are inefficiently glycosylated in peptides and are less likely to be glycosylated in other proteins synthesized in cells and in vivo *(27)*. This suggests that findings obtained using RGP(1- -) as a model protein in this cell-free system are of general relevance.

In the present study, 40 variants of RGP(1- -) were generated to examine systematically the effect of the amino acid at the Y position. Each variant contained a single sequon at Asn^{37} with Leu at the X position. The variants were generated with either Ser or Thr at the hydroxy position (Figure 1, RGP(1- -)NLS(Y) and RGP(1- -)NLT(Y), respectively), to allow comparison of the influence of the Y amino acid in Asn-X-Ser and Asn-X-Thr sequons. RGP(1- -)NLS(Y) variants and RGP(1- -)NLT(Y) variants were generated with each of the 20 common amino acids at the Y position using a cassette mutagenesis approach. The amino acid sequence of the variant encoded by each plasmid was confirmed by DNA sequencing.

Expression of RGP Variants in a Cell-Free System. We compared the core glycosylation efficiency of RGP(1- -)NLS(Y) variants and RGP(1- -)NLT(Y) variants in a rabbit

[1] Abbreviations: Rabies virus glycoprotein (RGP); wild type (WT); sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

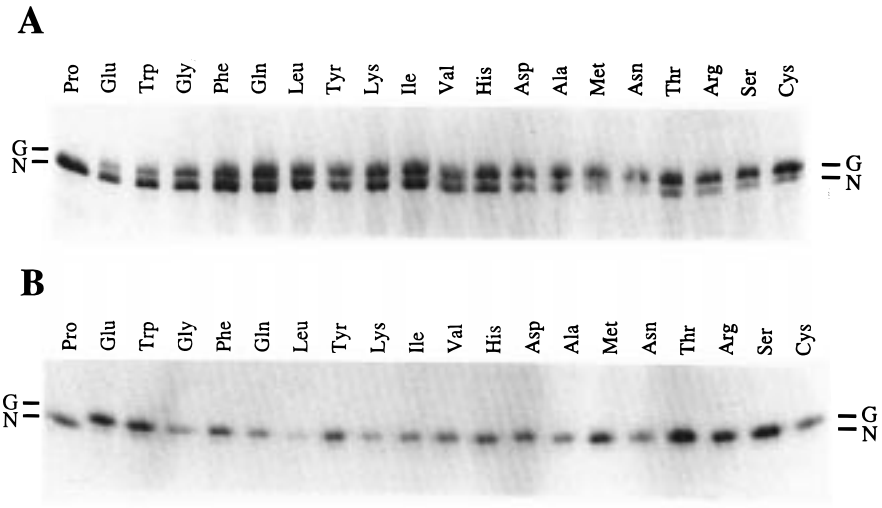

FIGURE 2: Core glycosylation of RGP variants. RNA encoding each RGP variant was generated by in vitro transcription and expressed in a cell-free translation system in the presence of canine pancreas microsomes and [^{35}S]methionine. Translation products were treated with proteinase K and analyzed by SDS−PAGE and autoradiography. The amino acid at the Y position in each variant is indicated above each lane. The migration positions of the nonglycosylated protein (N) and the protein glycosylated with a single core oligosaccharide (G) are indicated. (A) Translation of RGP(1- -)NLS(Y) variants with Asn-X-Ser sequons. (B) Translation of RGP(1- -)NLT(Y) variants with Asn-X-Thr sequons.

reticulocyte cell-free translation system supplemented with [^{35}S]methionine and canine pancreas microsomes *(28, 29)*. These microsomes support the cotranslational addition of core oligosaccharides to Asn-X-Ser/Thr sequons. The reaction conditions used in this study were optimized for efficient translation and glycosylation of RGP variants. Under these conditions over 90% of RGP synthesized is translocated into microsomes *(25)*. Our previous studies confirm that RGP variants with favorable sequons at Asn37 are fully glycosylated in this system *(25, 27)*. Processing of core oligosaccharides is limited in this system, simplifying analysis of core glycosylation. Prior to glycosylation analysis, the full set of variants was translated in parallel in the absence of microsomes (without glycosylation) to confirm that none of the amino acid substitutions introduced at position 40 altered the electrophoretic mobility of the nonglycosylated protein (data not shown).

To compare the core glycosylation efficiency of the RGP variants, all 20 RGP(1- -)NLS(Y) variants and all 20 RGP-(1- -)NLT(Y) variants were translated in parallel in the presence of microsomes. For each experiment, the translation reagents and microsomes were premixed and then aliquoted to tubes containing RNA. This ensured that each variant was translated and glycosylated under essentially identical conditions in which the activity of oligosaccharyltransferase and the amounts of other components of the glycosylation system (e.g., the dolichol oligosaccharide donor) were constant. Following incubation, samples were treated with proteinase K to remove proteins which were not targeted to microsomes *(30)*. This ensured that only those proteins which entered the microsomal lumen were included in the determination of core glycosylation efficiency. Digestion with proteinase K also removes the nonglycosylated cytoplasmic tail of RGP; this results in a shift in electrophoretic mobility of the protein, providing a control for the completeness of proteinase K digestion *(25)*.

Each RGP variant analyzed contains only one glycosylation sequon. Translation of each variant can therefore yield two types of protein products: the RGP protein with a single core oligosaccharide at Asn^{37} and the nonglycosylated protein. The proportion of glycosylated and nonglycosylated proteins produced directly reflects the efficiency of core glycosylation at Asn^{37} by oligosaccharyltransferase. Glycosylated and nonglycosylated proteins can be separated by SDS−PAGE and analyzed by autoradiography. Autoradiographs showing the translation products of RGP(1- -)NLS-(Y) variants and RGP(1- -)NLT(Y) variants are shown (Figure 2, panels A and B, respectively). The proportions of glycosylated (G) and nonglycosylated (N) protein produced for each variant were quantified by densitometric scanning of gel autoradiographs. Core glycosylation efficiency was calculated as [G]/[G + N] × 100%, as described *(25)*. In this system, the core glycosylation efficiency of the sequon normally present at Asn^{37} in RGP (i.e., NLSG) is essentially unchanged over a 50-fold range of input RNA concentration *(27)*. The core glycosylation efficiency at this sequon is also similar following 30, 60, or 120 min of incubation in the cell-free system (data not shown). The full set of RGP(1- -)NLS(Y) and RGP(1- -)NLT(Y) variants was translated and analyzed in three independent experiments (Figure 3, panels A and B, respectively).

As expected, the presence of Pro at the Y position had a strong inhibitory effect on the core glycosylation of both NLS and NLT sequons. In general, the core glycosylation efficiencies of RGP(1- -)NLT(Y) variants were high with Y amino acids other than Pro (Figure 3B), tending to minimize the influence of the Y amino acid; mean core glycosylation efficiencies of these variants ranged from 81% to 93%. In contrast, the amino acid at the Y position had a pronounced effect on core glycosylation of RGP(1- -)NLS(Y) variants (Figure 3A). The core glycosylation efficiencies of these variants ranged from 31% to 78%. The Y amino acid normally present in RGP(1- -) is Gly. The core glycosylation efficiency of the RGP variant with this sequon (i.e., NLSG) is approximately 40% (Figure 3A), which is consistent with results obtained in our previous studies *(25, 27, 34)*. Only two Y amino acids, Glu and Trp, lowered the core glycosylation efficiency of the sequon relative to its wild-type

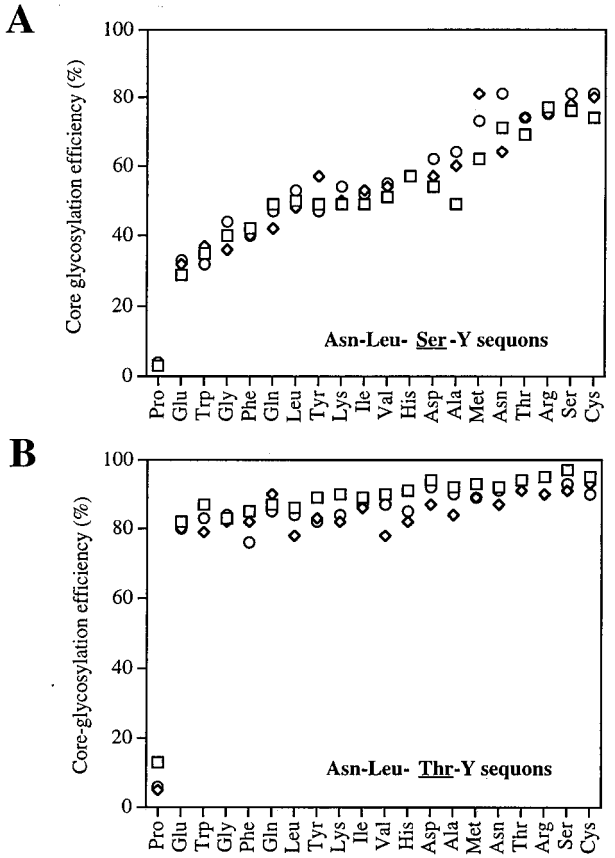

FIGURE 3: Influence of the Y amino acid on the core glycosylation efficiency of RGP variants. RGP variants were analyzed in the cell-free system as described in Figure 2 in three independent experiments (circles, squares, triangles). Gel autoradiographs from each experiment were exposed in the linear range and analyzed by densitometric scanning. The core glycosylation efficiency of each variant was calculated as described in the text for each experiment. (A) Data for RGP(1- -)NLS(Y) variants with Asn-X-Ser sequons. (B) Data for RGP(1- -)NLT(Y) variants with Asn-X-Thr sequons.

sequence in RGP(1- -). Other Y amino acids were more favorable. Although the effect of the Y amino acid was minimal for the RGP(1- -)NLT(Y) variants compared to the RGP(1- -)NLS(Y) variants, a similar pattern of inhibition with different Y amino acids was observed. The core glycosylation efficiencies of sequons in selected RGP variants were examined after different periods of incubation in the cell-free system. Translation products were first detected after 15 min of incubation. The core glycosylation efficiencies of the variants were similar after 15, 30, and 60 min of incubation (37%, 38%, and 39% for RGP(1- -)NLSE; 41%, 51%, and 47% for RGP(1- -)NLSK; and 61%, 69%, and 72% for RGP(1- -)NLSS), respectively). These findings demonstrate for the first time that amino acids other than Pro at the Y position can influence core glycosylation efficiency. This effect is marked when Ser is present at the hydroxy position.

DISCUSSION

Inefficient core glycosylation at one or more sequons in a glycoprotein results in the synthesis of heterogeneous glycoforms. This may offer an advantage in some cases, since individual glycoforms may have unique physical or biological properties *(31)*. However, for recombinant protein production, heterogeneity of glycoprotein products may be undesirable *(32)*. Previous studies have demonstrated that the glycosylation efficiency of sequons can be modulated by site-directed mutagenesis of the X or hydroxy amino acids *(25−27, 33)*. Further characterization of the protein signals which influence core glycosylation will facilitate the design of recombinant proteins with optimized properties.

Previous studies have suggested that amino acids near sequons can influence core glycosylation efficiency. For example, Pro, which blocks core glycosylation when it is present at the X position, also inhibits glycosylation when it is present at the Y position *(9, 18, 19)*. Cys residues near sequons can also impair glycosylation when there is potential for local disulfide bonding *(17, 20−22)*. This report is the first to examine the impact of amino acids other than Pro at the Y position and to identify the Y amino acid as an important determinant of core glycosylation efficiency.

Our studies reveal that Trp inhibits core glycosylation when it is present at either the X or the Y position *(25, 27)*. The large side group on Trp may block accessibility of the sequon to oligosaccharyltransferase or the dolichol oligosaccharide donor, or it may induce an unfavorable local protein conformation. The impact of charged amino acids at the X and Y positions is more complex. Glu inhibits core glycosylation at either the X or the Y position, whereas Asp, which is also negatively charged, only exerts a marked inhibitory effect at the X position. Also, positively charged X amino acids are generally favorable, whereas Arg appears to be more favorable than either His or Lys at the Y position. Interestingly, Thr, Ser, and Cys are among the most favorable amino acids at both the X and Y positions *(25)*. This suggests that the hydroxy or sulfhydryl side groups on these amino acids may facilitate catalytic reactions involving the amino acids at the Asn and Ser/Thr positions in the sequon. Further studies are needed to clarify the role of specific amino acids near Asn residues in oligosaccharide addition.

RGP has proved to be a useful model for studies of the regulation of core glycosylation *(24−27, 34)*. However, additional studies are needed to confirm the general relevance of these results to other proteins. One must also consider that core glycosylation is influenced by a variety of factors other than the local amino acid sequence *(1, 11)*. Even an optimal sequon may be inefficiently glycosylated if it is not fully accessible to oligosaccharyltransferase during protein synthesis. Sequon accessibility may be hindered if sequons are very close to one another, of if a sequon is close to a membrane-spanning segment, a disulfide-bonded Cys residue, or a signal sequence *(11)*. The rate of protein translocation into the ER or the interaction of nascent proteins with ER lumenal proteins may also influence sequon accessibility *(11)*. In addition, conditions used for glycoprotein expression may affect glycosylation efficiency *(35−37)*, as can the levels of oligosaccharyltransferase, dolichol, and dolichol-P−P-oligosaccharide *(1,7)*. For these reasons, the task of predicting the level of glycosylation of individual sequons in a glycoprotein remains a difficult one. Further characterization of oligosaccharyltransferase, and of the local protein structures that influence oligosaccharide addition, will provide further insight into the mechanism of core glycosylation. This information will facilitate the design of recombinant glycoproteins.

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