Glycosylation efficiency of Asn-Xaa-Thr sequons is independent of distance from the C-terminus in membrane dipeptidase

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In vitro transcription/translation studies with model proteins have shown that glycosylation of Asn-Xaa-Thr sequons is reduced when the sequon is within 60 residues of the C-terminus of the protein. We have previously shown that in living cells N-glycosylation of the prion protein (PrP) is also abolished when its Asn-Ile-Thr and Asn-Phe-Thr sequons are less than 60 residues from the C-terminus (Walmsley and Hooper [2003] Biochemical Journal, 370, 351–355). To investigate whether sequon distance to the C-terminus is a general determinant of N-glycosylation in living cells, Asn-Ile/Phe-Thr sequons were introduced into another glycosylphosphatidylinositol (GPI) anchored protein, membrane dipeptidase (MDP), at similar distances from the C-terminus as those in PrP. When expressed in the human neuroblastoma SH-SY5Y cell line, the introduced sequons were fully N-glycosylated even when they were less than 60 residues from the C-terminus in both GPI-anchored and secreted forms of MDP. These data demonstrate that the utilization of sequons in some proteins is independent of their distance from the C-terminus.

Key words: glycosyl-phosphatidylinositol anchor/membrane dipeptidase/N-glycosylation/oligosaccharyltransferase/prion protein

Introduction

The N-glycosylation of proteins involves the transfer of a preassembled oligosaccharide, Glc3Man9GlcNAc2, from a lipid precursor to the side chain of an Asn residue in the sequon Asn-X-Thr/Ser (X ≠ Pro) and is catalyzed within the lumen of the endoplasmic reticulum (ER) by the translocon-associated multisubunit enzyme complex oligosaccharyltransferase (OST) (Garlich et al., 1992; Silberstein and Gilmore, 1996). N-linked oligosaccharides perform a broad range of functions in glycoproteins, ranging from stabilization of the native conformation to intracellular targeting (Helenius and Aebi, 2001). However, N-glycosylation of Asn-X-Thr/Ser sequons is not obligatory; sequons may be fully or variably utilized or may remain unglycosylated (Gavel and von Heijne, 1990). For example, the prion protein (PrP), the causative agent of the neurodegenerative transmissible spongiform encephalopathies (TSEs), contains two sequons (Asn180-Ile-Thr and Asn196-Phe-Thr) that are each variably N-glycosylated to generate un-, mono-, and diglycosylated species (Rudd et al., 2002). In the case of the pathogenic form of PrP, the relative proportion of these species is a characteristic and, in many cases, transmittable molecular marker for TSE strains (Jackson and Collinge, 2001).

Apart from the sequence in and around the sequon, the efficiency of N-glycosylation is greatly influenced by the accessibility of the sequon to the lumenally orientated active site of OST. The presence of disulfide bonds and the conformation of the nascent polypeptide chain in the vicinity of the sequons can influence N-glycosylation (Helenius and Aebi, 2001; Imperiali and Rickert, 1995). For example, in tissue-type plasminogen activator (tPA), disulfide bond formation during the folding of the nascent polypeptide inhibits N-glycosylation, most likely by sterically hindering the accessibility of the sequons to OST (Allen et al., 1995). Additionally, studies utilizing in vitro transcription/translocation systems have shown that the physical parameters of the translocon–OST complex also restrict sequon utilization. In such studies N-glycosylation efficiency was seen to rapidly decrease when the sequon was fewer than 60 residues from the C-terminus (Nilsson and von Heijne, 2000; Whitley et al., 1996). Because the distance between the ribosome P-site and OST active site is estimated to be around 65 residues, this inefficient N-glycosylation is believed to be due to the sequon failing to reach the active site of OST prior to the dissociation of the polypeptide from the ribosome (Nilsson and von Heijne, 2000; Whitley et al., 1996).

Recently, we have shown that the N-glycosylation of a nonanchored form of PrP in neuronal cells is abolished when the sequons are fewer than 60 residues from the C-terminus (Walmsley and Hooper, 2003; Walmsley et al., 2001). These observations led us to conclude that sequon utilization in PrP is a cotranslational process. In the present study, we have investigated whether sequon distance from the C-terminus is a general determinant of N-glycosylation in living cells by introducing the sequons of PrP into another glycosylphosphatidylinositol (GPI)-anchored protein, membrane dipeptidase (MDP), and expressing the protein in the same neuronal cells as used to study the glycosylation of PrP. In the same cell type the introduced sequons in MDP were fully utilized even when they were fewer than 60 residues from the C-terminus, indicating...
that in two different proteins the same sequons in similar positions near the C-terminus can be differentially utilized by OST.

Results
To determine whether sequon distance to the C-terminus is a general determinant of N-glycosylation in living cells, we introduced each of the sequons of PrP, Asn-Ile-Thr, and Asn-Phe-Thr into another GPI-anchored protein, porcine MDP, at distances from the C-terminus similar to those in PrP (Figure 1). To examine the N-glycosylation of the introduced sequons, the two naturally occurring N-glycosylation sequons of MDP were inactivated by substitution of their acceptor Asn residues for Gln residues (Figure 1). The MDP constructs were stably expressed in the human neuroblastoma cell line SH-SY5Y and the lysates and conditioned medium subjected to western blot analysis with an anti-MDP antibody (Figure 2A). MDP\textsuperscript{D\textsubscript{N}}, MDP\textsubscript{N332}, and MDP\textsubscript{N358} were detected solely in the cell lysate and not in the medium, as expected for GPI-anchored proteins (Figure 2A). MDP\textsuperscript{D\textsubscript{N}} migrated with an apparent molecular weight of 45 kDa, consistent with the size of the deglycosylated porcine kidney protein (Littlewood et al., 1989).

The presence of a GPI anchor on the MDP constructs was assessed by digestion with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (Figure 2B). MDP (Littlewood et al., 1989), like many other GPI-anchored proteins, such as variant surface glycoprotein (Cardoso de Almeida and Turner, 1983), the prion protein (Stahl et al., 1987; Walmsley et al., 2001), and the nogo-66 receptor (Pignot et al., 2003), undergoes a characteristic shift in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), migrating with a higher molecular weight when the GPI anchor has been cleaved by PI-PLC. Accordingly, the molecular weights of MDP\textsuperscript{AN}, MDP\textsubscript{N322}, and MDP\textsubscript{N358} were increased following treatment with PI-PLC (Figure 2B), confirming the presence of the GPI anchor. To assess the glycosylation status of the MDP constructs, cell lysates were digested with peptide N-glycosidase F (PNGase F), an enzyme that cleaves the N-glycans from glycoproteins resulting in a reduction in their molecular weight (Figure 2C). Treatment with PNGase F failed to alter the mobility of MDP\textsuperscript{AN} (Figure 2C), confirming that the two naturally occurring Asn-Leu-Ser sequons of MDP had been inactivated by mutation of their acceptor Asn residues to Gln residues. The reduction in the molecular weight of MDP\textsubscript{N312} and MDP\textsubscript{N358} on PNGase F treatment to that of MDP\textsuperscript{D\textsubscript{N}} demonstrated that each of the Asn-Ile-Thr and Asn-Phe-Thr sequons of PrP were N-glycosylated when introduced into GPI-anchored MDP.

To assess whether the N-glycosylation of the introduced Asn-Ile/Phe-Thr sequons was dependent on their distance to the C-terminus, the GPI signal sequence of the MDP constructs was incubated for 3 h in the presence or absence of bacterial PI-PLC prior to SDS-PAGE and western blot analysis with antibody RP209. (B) Cell lysates from SH-SY5Y cells expressing the MDP constructs were incubated for 3 h in the presence or absence of bacterial PI-PLC prior to SDS-PAGE and western blot analysis with antibody RP209. (C) Cell lysates from SH-SY5Y cells expressing the MDP constructs were incubated for 16 h in the absence or presence of PNGase F and the MDP constructs in the digests detected by western blot analysis with antibody RP209.
In the present study, we have examined whether sequon glycosylation in living cells by introducing Asn-Ile/Phe-Thr sequons into the GPI-anchored protein, porcine MDP. In MDP, at positions similar to those in PrP and examining its glycosylation status in the same mammalian cell line. Porcine MDP, like PrP, contains two N-glycosylation sequons, Asn-Ile-Thr and Asn-Phe-Thr sequons into the PrP is critically dependent on their distance to the C-terminus, with N-glycosylation only occurring when their distance from the C-terminus was greater than ~60 residues (Walmsley and Hooper, 2003). Because the active site of OST has been estimated to be ~65 residues from the C-terminus of the nascent polypeptide chain (Nilsson and von Heijne, 2000; Whitley et al., 1996), we concluded that the sequons of PrP must reach the OST active site prior to the termination of translation for N-glycosylation to occur (Walmsley and Hooper, 2003). In the present study, we have examined whether sequon distance to the C-terminus is a general determinant of N-glycosylation in living cells by introducing Asn-Ile/Phe-Thr sequons into the GPI-anchored protein, porcine MDP, at positions similar to those in PrP and examining its glycosylation status in the same mammalian cell line. Porcine MDP, like PrP, contains two N-glycosylation sequons, Asn57-Leu-Ser and Asn279-Leu-Ser, both of which are mutated in the mature glycoprotein (Brewis et al., 1995; Rached et al., 1990). These two sequons were inactivated by mutation of the Asn residues to Gln, and sequons identical to those in PrP were introduced so that any observed differences could not be attributed to differences in the second and third residues of the sequons (Kaplan et al., 1987; Shakin-Eshleman et al., 1996). These new sequons were introduced into porcine MDP at positions homologous to the two additional N-glycosylation sites present in human MDP (Hooper et al., 1990) to minimize the possibility that their N-glycosylation was governed by factors, such as conformation, other than their distance from the C-terminus.

Unlike in PrP, the introduced sequons in both the GPI-anchored and nonanchored forms of MDP were efficiently N-glycosylated even when they were fewer than 60 residues from the C-terminus. For example, even though it would be inside the ribosome on chain termination (Matlack and Walter, 1995), the Asn-Phe-Thr sequon 26 residues from the C-terminus of MDP was efficiently utilized. These findings suggest that the Asn-Xaa-Thr sequons near the

**Discussion**

We have recently shown that the utilization of the Asn-Ile/Phe-Thr sequons in PrP is critically dependent on their distance to the C-terminus, with N-glycosylation only occurring when their distance from the C-terminus was greater than ~60 residues (Walmsley and Hooper, 2003). Because the active site of OST has been estimated to be ~65 residues from the C-terminus of the nascent polypeptide chain (Nilsson and von Heijne, 2000; Whitley et al., 1996), we concluded that the sequons of PrP must reach the OST active site prior to the termination of translation for N-glycosylation to occur (Walmsley and Hooper, 2003). In the present study, we have examined whether sequon distance to the C-terminus is a general determinant of N-glycosylation in living cells by introducing Asn-Ile/Phe-Thr sequons into the GPI-anchored protein, porcine MDP, at positions similar to those in PrP and examining its glycosylation status in the same mammalian cell line. Porcine MDP, like PrP, contains two N-glycosylation sequons, Asn57-Leu-Ser and Asn279-Leu-Ser, both of which are mutated in the mature glycoprotein (Brewis et al., 1995; Rached et al., 1990). These two sequons were inactivated by mutation of the Asn residues to Gln, and sequons identical to those in PrP were introduced so that any observed differences could not be attributed to differences in the second and third residues of the sequons (Kaplan et al., 1987; Shakin-Eshleman et al., 1996). These new sequons were introduced into porcine MDP at positions homologous to the additional N-glycosylation sites present in human MDP (Hooper et al., 1990) to minimize the possibility that their N-glycosylation was governed by factors, such as conformation, other than their distance from the C-terminus.
C-terminus of MDP, in contrast to those in PrP, can be N-glycosylated following detachment of the polypeptide chain from the ribosome. Such posttranslational glycosylation of proteins has been reported in only a limited number of cases (Hessa et al., 2003; Kolhekar et al., 1998; Olivares et al., 2003).

The lack of N-glycosylation of PrP may be due to the sequons becoming inaccessible to the active site of OST during the folding of the nascent polypeptide, as appears to be the case for tPA (Allen et al., 1995). In PrP the two sequons (Asn180 and Asn196) lie between the two cysteine residues (Cys178 and Cys213) that form the only intrachain disulfide bond in the protein. Interestingly, this bond can only form posttranslationally as Cys213, being only 41 residues from the C-terminus of the nascent polypeptide chain, would not be accessible to the lumen of the ER during translation (Whitley et al., 1996). The formation of the intrachain disulfide bond following chain termination may compromise the accessibility of the Asn180-Ile-Thr and Asn196-Phe-Thr sequons to the OST active site and thus prevent their N-glycosylation. In agreement with this is the finding that treatment of neuronal cells with the thiol reductant dithiothreitol resulted in an increase in the thiol content of living cells but also highlights the difficulty involved in predicting N-glycosylation sequon occupancy in vitro (Gavel and von Heijne, 1990).

Materials and methods

Materials
All materials were obtained from Sigma (St. Louis, MO) unless otherwise stated. The RP209 polyclonal antibody was raised in rabbits against affinity-purified porcine kidney MDP (Littlewood et al., 1989).

Generation of constructs
MDP in pClneo has been previously described (Pang et al., 2001). All mutants were generated using the Quickchange site-directed mutagenesis kit (Stratagene, LaJolla, CA) according to the manufacturer’s instructions and the mutations confirmed by sequencing on both strands (DNA sequencing service, University of Durham, U.K.). MDPAGPI was generated from MDP in pClneo using the following mutagenic primers: sense primer 5'-ACGGCTACTCATGACCCCCAGCCT-3' and antisense primer 5'-AGGCTGAGGAGGCTAGTACGGCTG-3'. MDPAN and MDPANAGPI were generated from MDP and MDPAGPI, respectively, by two rounds of site-directed mutagenesis using the following primers: sense primer 5'-ACCCGGGGGCCCCAAGCTTCCAGC-3' and antisense primer 5'-AGGCTCGAGAGTTGGCCGCCC-3' for mutation of Asn41 to Gln; sense primer 5'-CGGCCAGGCCCAATTGTTGCCAAG-3' and antisense primer 5'-ACTCTGAGAATTGGCCCTTTGCA-3' for mutation of Asn263 to Gln. MDPN332 and MDPAGPI332 were generated from MDPAN and MDPANAGPI, respectively, using the following mutagenic primers: sense primer 5'-GTCAGAGGAAGACTACGAGGCGG-3' and antisense primer 5'-CGGCCCTCCGATGTCTCTCCGAGC-3'. MDPN358 and MDPAGPI358 were generated from MDPAN and MDPANAGPI, respectively, using the following mutagenic primers: sense primer 5'-GGCCAGCAATTTCACTCAG TTCCAG-3' and antisense primer 5'-CTGGAACCTGA GTGAAATTGCTGGCC-3'.

Cell culture, transfection, and lysis
SH-SY5Y cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium with Glutamax-I, sodium pyruvate, 4.5 mg/ml glucose, and pyridoxine supplemented with 10% (v/v) fetal calf serum (Gibco, Paisley, Scotland) in a humidified atmosphere of 5% CO2/95% air. Cells were electroporated with linearized DNA and selected with G418 as described elsewhere (Walmseley et al., 2001). Briefly, cells at midconfluence were harvested with trypsin and resuspended in complete medium at a concentration of ~1×10⁶ cells/ml. An 800-μl aliquot of cell suspension was placed in a 4-mm electroporation cuvette and incubated for 1 min with 30 μg linearized DNA. Cells were pulsed at 1650 μF/250 V using the Easy-Ject electroporator (Flowgen, Leicestershire, UK) and immediately transferred to fresh complete medium.

Selection for antibiotic resistance was imposed 48 h after electroporation by incubating the cells with complete medium containing 1 mg/ml G418. Because pClneo is a bicistronic vector, all G418-resistant cells have a higher level of target protein expression, and thus cloning is not required (Rees et al., 1996). Each stably expressing cell line represented a pooled population of G418-resistant colonies (<50 colonies/cell line) that expressed their target protein at levels sufficient for detection by western blot analysis.

Cells at confluence were rinsed twice with phosphate buffered saline (PBS; 1.5 mM KH₂PO₄, 2.7 mM Na₂HPO₄, 150 mM NaCl, pH 7.4), scraped into the same buffer, and harvested by centrifugation for 3 min at 500 × g. The cells were resuspended in lysis buffer (10 mM Tris–HCl, pH 7.8, 0.5% [w/v] sodium deoxycholate, 0.5% [v/v] Nonidet P40, 100 mM NaCl, 10 mM ethylenediamine tetra-acetic acid [EDTA], 0.1 mM phenylmethanesulfonyl fluoride) and incubated for 30 min at room temperature. The lysates were clarified by centrifugation for 1 min at 13,000 × g. Medium samples were concentrated by the addition of five volumes of methanol and incubated for 30 min at −20°C. The precipitate was harvested by centrifugation for 5 min at 13,000 × g, dried, and resuspended in lysis buffer.

SDS–PAGE and western blot analysis
Samples containing ~10 μg of total protein were mixed with an equal volume of SDS dissociation buffer (125 mM
Tris–HCl, pH 6.8, 2% [w/v] SDS, 20% [v/v] glycerol, 100 mM dithiothreitol, bromophenol blue) and boiled for 5 min. Immunoprecipitates were resuspended in SDS dissociation buffer and boiled. Proteins were resolved by electrophoresis through 15% polyacrylamide gels. For western blot analysis, resolved proteins were transferred to a Hybond-P poly(vinylidenefluoride) membrane (Amersham, Little Chalfont, U.K.). The membrane was blocked by incubation for 1 h with PBS containing 0.1% (v/v) Tween 20 and bovine serum albumin. Samples were boiled for 5 min, diluted fivefold with 1.25% Triton X-100, 2% SDS, 100 mM Tris–HCl, pH 6.8, 20% glycerol, and boiled. Proteins were resolved by electrophoresis through 15% polyacrylamide gels. For western blot analysis, resolved proteins were transferred to a Hybond-P poly(vinylidenefluoride) membrane (Amersham, Little Chalfont, U.K.). The membrane was blocked by incubation for 1 h with PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) dried milk powder. Incubations with primary and peroxidase-conjugated secondary antibodies were performed for 1 h in the same buffer. MDP was detected with the polyclonal antibody RP209 (Littlewood et al., 1989). Bound peroxidase conjugates were visualized using an enhanced chemiluminescence detection system (Amersham).

PI-PLC and PNGase F digestion
For cleavage of the GPI anchor with PI-PLC, cell lysates or concentrated media samples at a total protein concentration of ~100 μg/ml were incubated for 3 h at 37°C with 1 U/ml Bacillus thuringiensis PI-PLC prepared as described (Low, 1992). Enzymatic deglycosylation was performed with PNGase F according to the supplier’s instructions (Glyko, Novato, CA). Briefly, samples of cell lysate or concentrated media were made 20 mM with respect to sodium phosphate, pH 7.6, 50 mM with respect to EDTA, 5% (w/v) with respect to SDS, and 5% (v/v) with respect to β-mercaptoethanol. Samples were boiled for 5 min, diluted fivefold with 1.25% (v/v) Triton X-100, and incubated for 16 h at 37°C with 1 U peptide N-glycosidase F/100 μg total protein.

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Abbreviations
EDTA, ethylenediamine tetra-acetic acid; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; MDP, membrane dipeptidase; OST, oligosaccharyltransferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide N-glycosidase F; PrP, prion protein; SDS, sodium dodecyl sulfate; tPA, tissue-type plasminogen activator; TSEs, transmissible spongiform encephalopathies.

References


