The role of protein glycosylation in the control of cellular N-sialyltransferase activity

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Abstract  Protein glycosylation, which is a key post-translational event, is catalysed by the glycosyltransferase family of enzymes. There is an increasing body of evidence to suggest that these enzymes may themselves be glycosylated, possibly as an autocatalytic event. Using a novel in vitro system, we have investigated the role of enzyme glycosylation in sialyltransferase catalytic activity. The enzyme activity is glycosylation dependent, with the penultimate galactose residue on complex N-linked oligosaccharides playing a pivotal role. These results serve to underline the complexity of the glycosylation process. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Sialyltransferase; Autoglycosylation; Sialoglycoproteins; Lectin

1. Introduction

Glycosylation is a key post-translational event, primarily associated with membrane-bound and secreted proteins [1]. Proteins are glycosylated in a specific manner that is determined by their route through the endoplasmic reticulum and Golgi. Furthermore, with few exceptions, this is an irreversible process and is viewed as a mechanism by which glycoprotein function and processing can be controlled.

The negatively charged sialic acid residues, which exert a particular influence on the 3-dimensional structure of their protein backbone, are located at the non-reducing terminal end of N-linked complex and hybrid oligosaccharides, primarily in α2,6 and α2,3 linkages to a penultimate galactose residue. The transfer of these residues is catalysed by the ST3N and ST6N members of the sialyltransferase (STN) group of enzymes [2] whose activity is modified by a number of factors including oncogene expression [3], second messenger systems [4], steroid hormones [5] and heavy metals [6]. Indeed, it has been proposed that the STN enzymes themselves may be glycosylated, possibly by an autocatalytic mechanism, thus providing another mechanism for the control of enzyme activity.

Sialic acid can also be attached to a complex N-linked oligosaccharide chain in an α2,8 linkage. The transfer of the terminal sialic acid residue and the subsequent elongation of the chain, which is expressed almost exclusively on the neural cell adhesion molecule, NCAM, is catalysed by the PST and STX polysialyltransferase enzymes [2]. These enzymes have also been demonstrated to be capable of autopolysialylation both in vitro and in vivo [7,8]. Initial studies suggested that while polysialylation of the enzyme enhances the polysialic acid (PSA) content of NCAM, it is not an absolute prerequisite [9]. More detailed studies have demonstrated that PST/STX enzymes lacking sialic acid and galactose are, in fact, inactive in the catalysis of the polysialylation of either the enzyme or the NCAM protein [10]. Other glycosyltransferase enzymes can also be glycosylated and this has been proposed to play a role in enzyme stability, their subcellular distribution within the cell [11-15] and the modification of their catalytic activity [16,17].

Determining the role of individual sugar residues of oligosaccharide chains in protein function and processing can be particularly difficult as there are relatively few sufficiently selective tools with which to modify the activities of individual glycosyltransferase or glycosidase enzymes. While the generation of stably transfected cell lines is one approach that can be taken [18], the over-expression of certain glycosyltransferase enzymes may actually disrupt the cellular glycosylation machinery [19]. However, a number of mutant Chinese hamster ovary (CHO) cell lines have previously been generated, each of which exhibits a specific glycosylation defect [20]. These provide an excellent tool with which to study the role of individual sugar residues in protein processing and in this study, we have demonstrated that both terminal sialic acid and galactose residues on N-linked oligosaccharides play a pivotal role in determining STN catalytic activity using these cell lines.

2. Materials and methods

2.1. Cell culture

Control (Pro 5) and mutant (Lec 1, 2 and 8) CHO cell lines [20] were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, penicillin (2 U/ml) and streptomycin (0.25 mg/ml) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. They were routinely trypsinised upon reaching confluence and seeded at a density of 2.2 x 10⁶ cells per 75 cm² tissue culture flask. The cells were harvested by scraping into a phosphate-buffered saline solution and pelleted by centrifugation at 15 000 rpm for 3 min at 4°C. The cell pellets were stored at −20°C until use.

2.2. STN assay

Total cellular STN activity was determined using cytidine-5-monophosphate-4,5,6,7,8,9,14C-N-acetyllactosaminic acid (CMP-14C-Neu5Ac, Radiochemical Centre, Amersham; specific activity 293 mCi/μmol) as the sialic acid donor and asialofetuin (Sigma) as an exogenous acceptor [21].

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2.3. Lectin blot analysis

Although they have been previously published, the glycosylation potential of the individual cell lines was confirmed for the present study by lectin blot analysis using digoxigenin-labelled lectins (Boehringer Mannheim) as previously described [18]. The terminal sialic acid (in an α2,6 linkage) was identified using the Sambucus Nigra (SNA) lectin; the penultimate Gal-GlcNAc disaccharide group was identified by the Phaseolus Vulgaris lectin; the GlcNAc-Man disaccharide identified by the Datura Stramonium lectin and the (Manα2-Manβ branch trisaccharide by the Galanthus Rivalis lectin. Samples were treated with 5 U N-glycosidase F for 2 h at 37°C (Boehringer Mannheim).

2.4. Polyacrylamide gel electrophoresis

The protein content of the samples was determined colorimetrically [22]. The individual polypeptide components of the samples were separated by discontinuous SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore) by electroblotting as previously described [18]. All lanes were loaded equally with 50 µg protein unless otherwise stated. The ST6N protein was visualised by Western blot analysis using a polyclonal antibody (kind gift of Prof. Eric Berger, University of Zurich) [18].

3. Results

The cell lines have the title 'Lec' to indicate that they are resistant to the binding of certain plant lectins that label specific oligosaccharide units. Initially, their glycosylation status was confirmed using appropriate lectins. All four cell lines exhibited a lectin-binding spectrum that was consistent with their mutations (Fig. 1A). The Pro 5 cell lysates (control) were labelled by all four lectins used in the study confirming that they express a complex N-linked sugar chain. However, the staining intensity of the GNA lectin blot was somewhat weaker than in the other cell lines with this staining probably being masked by the expression of the negatively charged terminal sialic acid residue. The Lec 2 cell line has a dysfunctional CMP-sialic acid transporter, resulting in an inability of the activated sugar to enter the Golgi cisternae with the resultant abolition of SNA lectin labelling of terminal sialic acid residues. The Lec 8 cells are lacking the UDP-galactose transporter, thus preventing the transfer of the penultimate galactose residue (and by implication a terminal sialic acid residue) onto the complex oligosaccharide chain. This resulted in the lack of binding of both the SNA and PHA-L lectins. The Lec 8 cell line has a dysfunctional GlcNAc T1 transferase enzyme resulting in N-linked sugars blocked at the Manα2-GlcNAc2-Asn stage and abolishing its ability to bind the DSA lectin. The cellular glycoproteins are, however, labelled by the DSA lectin that recognises oligosaccharide chains containing the trimannosyl core sugar. Lectin blots label a large number of proteins rather than individual bands, hence, the non-resolved...
nature of the blots. However, some individual bands, corresponding to proteins expressing large levels of a specific oligosaccharide epitope, can be identified. These are labelled accordingly.

Total cellular STN activity was similar in the Pro 5 and Lec 2 cell lines, indicating that the removal of the terminal sialic acid residue had minimal effect on enzyme activity (Fig. 2). However, there was a 60% decrease in the catalytic activity in both the Lec 8 and Lec 1 cell lines when compared with the Pro 5 cells, suggesting that the presence of a penultimate galactose residue is a critical component of the oligosaccharide chain that is required for optimal enzyme activity. The subsequent removal of the GlcNAc residue had no obvious influence on enzyme activity.

In the control cells, the ST6N enzyme appeared as a doublet of 44 and 48 kDa. A similar pattern was observed in the Lec 2 cells. However, in the Lec 8 and Lec 1 cells, only the single lower molecular weight band could be detected (Fig. 3A). Treatment of the ST6N doublet in the Pro 5 cells with N-glycosidase F resulted in the abolition of the higher band, suggesting that it represents a glycosylated form of the enzyme (Fig. 3B).

4. Discussion

The concept of kinase phosphorylation (including autophosphorylation) has been well characterised and it plays a key role in modifying enzyme activity [23]. The concept of glycosylation of glycosyltransferase enzymes is, however, still relatively poorly understood [13]. Early studies reported that STN exists as a glycoprotein [24,25] although the significance of this concept remained unclear. More recent studies have shown that although glycosylation is not required for catalytic activity of the ST6Gal I enzyme in vitro, the deglycosylated form of the enzyme has no catalytic activity in vivo, possibly because of altered enzyme protein oligomerisation and aggregation within the Golgi [12]. The PST enzymes (PST/STX) have also been demonstrated to be autosialylated and contain the PSA oligosaccharide chain [7,8]. There appears, however, to be some disagreement as to whether, for example, PST/STX autopolyglycosylation is an absolute requirement for enzyme activity or if it purely modifies activity [9]. The most recent evidence suggests that the former is indeed the case [10].

One primary problem in the field of glycobiology is the lack of sensitive tools with which to investigate the effects of altered glycosylation on protein structure, function and processing. There are inherent problems with over-expressing (or knocking out) glycosyltransferase enzymes in cell lines by transfection as this has been reported to upset the subcellular distribution or functional roles of other Golgi enzymes [19]. While the generation of proteins with mutated glycosylation sites (usually Asn residues) or treatment with agents such as tunicamycin both act to completely block the glycosylation process, they are not sufficiently sensitive to provide any information on the role of individual sugar residues. Deoxymannojirimycin, which acts to block mannosidase II, is somewhat more sensitive as it allows for the differentiation between high mannose and complex oligosaccharides [26,27]. The CHO mutant cell lines generated by Stanley in 1975 [28] have therefore proven to be an ideal tool with which to investigate the functional role of protein glycosylation.

In these cells, the ST6N enzyme exists as a doublet of 44 and 48 kDa. While the presence of sialic acid on complex and hybrid N-linked oligosaccharide chains does not appear to be a prerequisite for STN activity, the lack of both sialic acid and galactose residues results in a significant decrease in catalytic activity. This is in broad agreement with previous studies that demonstrate that ST6N sialylation is not required optimal enzyme activity [29].

An ST6N dimer has been previously reported with the two bands differing according to their glycosylation states [29,30]. Altered cellular sialylation potential in cells transfected with ST6N also resulted in a glycosylation-associated modification of the protein molecular size. The higher molecular weight 48 kDa form in the Pro 5 cells corresponds to the glycosylated form while the lower 44 kDa species is likely to be deglycosylated. This was confirmed by the removal of N-linked oligosaccharides by treatment with N-glycosidase F which resulted in a concomitant shift to the lower molecular weight band. The differential expression of these two forms of the enzyme mirrors well its catalytic activity, with the cells expressing the 48 kDa form exhibiting the highest enzymatic activity.

It was of interest to note, however, that the decrease in catalytic activity associated with sequential sugar removal was not a step-wise process and only two glycoforms could be detected. Indeed, the higher molecular weight band remained the primary form of the enzyme even in the absence of a charged sialic acid residue in the Lec 2 cells. This suggests that the enzyme may only have a very limited sialic acid content and therefore, its expression in a cell line incapable of

Fig. 2. Total STN activity in the individual cell lines (n= 3). *P< 0.05 (ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 3. A: Expression of the ST6N enzyme protein in the individual cell lines as determined by Western blot analysis. The approximate molecular weight of the protein bands is indicated. B: Treatment of the cell homogenate with glycosidase F for 18 h at 37°C prior to analysis.
Sialylation has little effect on the enzyme glycosylation state or activity. In the Lec 8 cells, which lack the subsequent galactose residue, the significant decrease in activity, with the loss of the higher 48 kDa band suggests that the penultimate galactose is a key residue. This was confirmed in the Lec 1 cells whereby the absence of a subsequent N-acetylgalactosamine residue had no effect. Indeed, the removal of all sugars by endoglycosidase F did not generate a protein band that was significantly different to that observed in the Lec 8 cells.

Protein glycosylation plays a specific role in the catalytic activity of other glycosyltransferase enzymes although it is likely that this varies significantly between individual enzymes. These include α1,3-fucosyltransferase [13,16], β1,4-N-acetylgalactosaminyltransferase and GD3 synthase [14,17]. While we have demonstrated that glycosylation plays a role in, but is not an absolute requirement for, the catalytic activity of the ST6N enzyme, its overall significance remains to be determined. Future detailed studies on enzyme glycosylation in vivo should assist in increasing our knowledge on this topic.

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References