The efficiency of N-linked glycosylation of bovine DNase I depends on the Asn-Xaa-Ser/Thr sequence and the tissue of origin

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Bovine DNase I contains two potential N-linked glycosylation sites with the sequences Asn¹⁸-Ala-Thr and Asn¹⁰⁶-Asp-Ser. A previous report established that pancreatic DNase I has only one sugar chain at Asn¹⁸ [Liao, Salnikow, Moore and Stein (1973) J. Biol. Chem. **248**, 1489–1495]. We found, however, that bovine DNase I expressed in COS-1 cells was glycosylated about 70 % at Asn¹⁰⁶ in addition to being completely glycosylated at Asn¹⁸. Glycosylation of Asn¹⁰⁶ increased to 97 % when Asp¹⁰⁷ was mutated to Glu or when Ser¹⁰⁸ was mutated to Thr. Mutation of Asp¹⁰⁷ to Trp had no effect, whereas a substitution with Pro at

this position abolished glycosylation of Asn¹⁰⁶. Analysis of the state of glycosylation of DNase I purified from a variety of bovine tissues revealed that DNase I from spleen, submaxillary gland, lung and adrenal had two sugar chains, whereas enzyme from pancreas and kidney had only one sugar chain. These findings demonstrate a major difference in the ability of various tissues to utilize N-linked glycosylation signals that contain suboptimal residues in the second and third positions.

Key words: N-linked oligosaccharide, oligosaccharyltransferase.

INTRODUCTION

Asparagine-linked glycosylation occurs in the endoplasmic reticulum by the transfer of a glucosylated high-mannose oligosaccharide from a lipid-linked precursor to selected asparagine residues on nascent proteins by oligosaccharyltransferase [1,2]. The consensus sequence for this reaction is Asn-Xaa-Ser/Thr with Asn-Xaa-Cys also functioning in rare instances [3–10]. However, only about 30 % of potential N-glycosylation sites appear to be used in all polypeptides bearing this consensus sequence, although this percentage can vary significantly among specific glycoproteins [11]. In addition, the extent of glycosylation at particular sites may be incomplete. With the exception of proline, every amino acid in the Xaa position of the consensus sequence allows glycosylation to occur [5,6,9,10]. Using an *in vitro* glycosylation assay, Shakin-Eshleman et al. [12] showed that the efficiency of glycosylation was very dependent on the particular amino acid in the Xaa position.

Bovine DNase I, which is secreted by the pancreas, has been characterized extensively with respect to its enzymic activity, stability [13], amino acid sequence [14,15], crystal structure [16] and glycosylation [17]. Whereas there are two potential N-glycosylation sites on bovine DNase I, one located at Asn¹⁸ and the other at Asn¹⁰⁶, it has been found that DNase I purified from bovine pancreas contains a single N-linked sugar chain at position 18 [14]. However, when we transfected COS-1 cells with an expression vector encoding bovine DNase I, most of the expressed DNase I contained two N-linked sugar chains. In this study we have examined the basis for this difference. First, we show that the consensus sequence for N-linked glycosylation at the Asn¹⁰⁶ site is not as favourable as the sequence at the Asn¹⁸ site. But beyond this, there is a strong influence of the tissue source on the efficiency of glycosylation at the 106 site.

EXPERIMENTAL

Materials

COS-1 cells were obtained from ATCC. Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs. Pro-mix

[³⁵S]Met/Cys labelling mixture, anti-rabbit antibody horseradish peroxidase conjugate and the ECL[®] kit were purchased from Amersham Pharmacia Biotech. Lipofectamine, calf serum, Opti-MEM and other media were from Gibco-BRL. Other reagents were from Sigma and Katayama Chemical Industries.

Plasmids

The bovine DNase I expression vector with the cDNA of bovine DNase I subcloned into the pSVK3 vector was constructed according to our previous report [18]. The expression vectors containing the various mutations in the bovine DNase I cDNA were constructed using the sequential PCR procedure [18]. All plasmids used for transfection were purified with the Qiagen plasmid kit.

COS-1 cell transfections

COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % calf serum. The cells were transfected with purified plasmid DNA using the Lipofectamine method as follows: on the day before transfection approx. 2×10^5 cells were plated into a 60 mm culture dish and incubated at 37 °C for 16 h in a CO₂ incubator. Plasmid DNA (3 µg) was dissolved in 100 µl of Opti-MEM. Lipofectamine solution was prepared by mixing 5 µg of Lipofectamine and 100 µl of Opti-MEM. The DNA and Lipofectamine solutions were mixed gently and incubated at room temperature for 15 min. The cells were washed with 2 ml of PBS followed by 2 ml of Opti-MEM. Then 0.8 ml of Opti-MEM and the Lipofectamine–DNA complex were added to the plate. After 24 h of incubation, the transfection reagents were replaced with 3 ml of culture medium. Following an additional 24 h incubation, the cells were washed twice with PBS and incubated with 1.0 ml of Dulbecco's modified Eagle's medium without methionine/cysteine containing 10 % dialysed bovine serum, 10 mM NH₄Cl and 0.2 mCi of [³⁵S]Met/Cys labelling mixture for 4 h at 37 °C, and then the culture medium was harvested.

Abbreviation used: PNGase F; peptide N-glycosidase F.

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Immunoprecipitation and glycosylation analysis

Rabbit anti-bovine DNase I serum was prepared by immunizing a rabbit with bovine DNase I that had been purchased from Sigma and purified by an additional step on a concanavalin A-Sepharose column. Anti-bovine DNase I-specific antibody was purified from the serum on a bovine DNase I affinity column.

The harvested COS-1 cell-culture media were incubated with 2 μ l of antisera overnight with rotation at 4 °C. Then 100 μ l of a 50 % Protein A-agarose bead suspension was added. After 1 h of additional rotation, the bead suspension was transferred to a fresh tube. The beads were washed three times with 100 mM Tris/HCl, pH 8.0/150 mM NaCl/1 % Triton X-100/5 mM EDTA/10 trypsin-inhibitory units/ml of aprotinin. The immunoprecipitated protein was eluted from the beads in 50 μ l of 0.1 M Tris/HCl, pH 7.0/2 % SDS/10 % 2-mercaptoethanol/20 % glycerol by boiling for 5 min. The eluted samples were subjected to SDS/PAGE using 13 % acrylamide gel. Following electrophoresis, the gels were rinsed with 7 % acetic acid/10 % methanol for 15 min, soaked in Amplify solution (Amersham Pharmacia Biotech) for 15 min, and dried. Autoradiography was then carried out, and the intensity of the bands corresponding to the amount of DNase I was quantified by a scanning densitometer (using Quantity One software, PDI, Huntington Station, NY, U.S.A.).

To analyse the glycosylation of the bovine DNase I isolated from different tissues, the tissues were homogenized with 4 vol. of 10 mM Tris/HCl buffer, pH 7.4, containing 0.1 mM PMSF, 0.1 mM benzamidine and 0.25 M sucrose using a Polytron homogenizer. The homogenates were subjected to centrifugation at 8000 *g* for 10 min to remove mitochondria, nuclei and intact cells. The supernatant fraction was collected and the DNase I immunoprecipitated using affinity-purified anti-bovine DNase I antibody. After SDS/PAGE, the protein was transferred to a Hybond-P membrane and ECL Western blotting was carried out according to the manufacturer's method.

PNGase F digestion

Immunoprecipitated DNase I was released from the beads by boiling for 10 min in 0.5 % SDS/1.0 % 2-mercaptoethanol solution. After centrifugation, the concentrated reaction mixtures were adjusted to contain 50 mM sodium phosphate, pH 7.5, and 1.0 % Nonidet P-40 according to the manufacturer's instructions (New England Biolabs). PNGase F (0.15 units) was added and the reactions were incubated at 37 °C for various intervals. Following incubation, the samples were subjected to SDS/PAGE.

RESULTS

Glycosylation of bovine DNase I expressed in COS-1 cells

Bovine DNase I contains two potential N-linked glycosylation sites, Asn¹⁸-Ala-Thr and Asn¹⁰⁶-Asp-Ser [14,15]. Previously we found that when a plasmid carrying a cDNA for bovine DNase I is transfected into COS-1 cells, both sites are glycosylated on the expressed protein [18]. However, the extent of glycosylation was not determined. To measure the efficiency of glycosylation at each site, mutants of DNase I were constructed in which the glycosylation sites were deleted (Figure 1a). When these constructs were expressed in COS-1 cells and analysed for the extent of glycosylation by SDS/PAGE, it became apparent that

Figure 1 Structure of bovine DNase I and DNase I variants

(a) and (b) show the structure of mutated bovine DNase I. 1Δ, Asn¹⁸ was mutated to Gln, so that no sugar chain could be transferred; 2Δ, Asn¹⁰⁶ was mutated to Gln; 1·2Δ, both Asn¹⁸ and Asn¹⁰⁶ were mutated to Gln. 1Δ+D107X mutant, Asp¹⁰⁷ of the 1Δ mutant was changed to either Pro, Glu, Trp or Ser; 1Δ+S108T, Ser¹⁰⁸ of the 1Δ mutant was changed to Thr. * denotes a potential glycosylation site.

Figure 2 Analysis of the number of sugar chains on DNase I expressed in COS cells

The DNase I constructs expressed by COS cells were subjected to SDS/PAGE. Lane 1, wild-type DNase I; lane 2, 2Δ; lane 3, 1Δ; lane 4, 1·2Δ. The amino acid sequences of the wild type, 1Δ, 2Δ and 1·2Δ are shown in Figure 1. The glycosylation efficiency (%) was calculated as described in the text for each experiment, and each mean value from the three independent experiments was determined and is shown under the gel for DNase I bearing 2, 1 or 0 sugar chains. All expressed mutants had enzymic activity measured according to our previous report [30] (and results not shown). The migration positions of the marker proteins are indicated: (a) bovine pancreatic DNase I (purchased from Sigma); (b) carbonic anhydrase (30 kDa).

glycosylation at Asn¹⁸ was nearly 100 % (the 2Δ construct), whereas glycosylation at Asn¹⁰⁶ was only 70 % (the 1Δ construct; Figure 2). As expected, the wild-type DNase I with both glycosylation sites exhibited the average of these two, with 81 % of the molecules having both sites glycosylated, 18 % only one site glycosylated and 1 % lacking glycosylation. From these results we conclude that the reason why wild-type DNase I expressed in COS-1 cells is a mixture of mono- and di-glycosylated DNase I depends on the glycosylation efficiency at Asn¹⁰⁶.

The effect of the amino acid at the Xaa and terminal positions on glycosylation

To pursue the basis for the incomplete glycosylation at Asn¹⁰⁶, Asp¹⁰⁷ was changed to four other amino acids and Ser¹⁰⁸ was changed to Thr, as shown in Figure 1(b). These constructs were then expressed in COS-1 cells and the extent of glycosylation

Figure 3 Analysis of the extent of glycosylation of mutant DNase I

The migration positions of the unglycosylated DNase I (0) and monoglycosylated DNase I (1) are indicated. Lane 1, 1 Δ +D107E; lane 2, 1 Δ +D107S; lane 3, 1 Δ +D107P; lane 4, 1 Δ +D107W; lane 5, 1 Δ +S108T; lane 6, 1 Δ . The percentages of the monoglycosylated (1) and unglycosylated (0) DNase I are given below the gel. These values are means that were calculated from the three independent experiments.

Figure 4 Analysis of the number of sugar chains on DNase I from various bovine tissues

Bovine DNase I was immunoprecipitated from several tissues and subjected to SDS/PAGE followed by Western-blot analysis as described in the Experimental section. DNase I was tested from the following tissues: lane 1, ovary; lane 2, liver; lane 3, kidney; lane 4, spleen; lane 5, parotid gland; lane 6, lung; lane 7, adrenal; lane 8, pancreas; lane 9, submaxillary gland. Bars indicate the tentative position of di- (2) and mono-glycosylated (1) DNase I. The percentages of the diglycosylated and monoglycosylated DNase I are given under the gel.

determined (Figure 3). The construct with the D107S substitution was highly glycosylated (97%), whereas the construct with the D107W mutation was glycosylated to the same extent as the wild-type construct (73% versus 70%, respectively), and the D107E construct was glycosylated less well (52%). The construct with the D107P substitution was not glycosylated at all. These results show that the nature of the amino acid at the Xaa position of the Asn-Xaa-Ser sequon has a profound effect on glycosylation.

The DNase I encoded by the construct with the S108T mutation was 97% glycosylated, consistent with prior reports that a Ser in the last position of the glycosylation sequence is less effective than a Thr in that position [8–10,19,20].

The number of sugar chains on native bovine DNase I varies with the tissue of origin

Liao et al. [14] showed that there was only one sugar chain on bovine pancreatic DNase I. Since our results demonstrate that the majority of bovine DNase I produced by COS-1 cells contains two sugar chains, we investigated the impact of the tissue of origin on the efficiency of glycosylation *in vivo* of endogenous DNase I. Previous studies have reported that DNase I is present in many tissues other than the pancreas, including the kidney, liver, testis and parotid gland [21–23]. As shown in Figure 4, there is a significant difference in the number of sugar chains on DNase I obtained from various tissues. Most of the DNase I isolated from spleen, submaxillary gland, parotid gland, lung

Figure 5 PNGaseF digestion of bovine DNase I

DNase I purified from submaxillary gland (lanes 1–4) and kidney (lanes 5–7) was treated with PNGase F for various times up to 15 min. After digestion, the samples were subjected to SDS/PAGE and Western-blot analysis. Intact submaxillary-gland DNase I is shown in lane 1 and intact kidney DNase I in lane 5. The bars indicate the position of di- (2), mono- (1) and un-glycosylated (0) DNase I.

and adrenal has two sugar chains. The DNase I in the ovary and liver consists of a mixture of molecules with one or two sugar chains, whereas the DNase I in the kidney and pancreas has only one sugar.

To confirm that the differences in migration on SDS/PAGE are due to variability in the number of N-linked oligosaccharides and not the result of some other modification, the DNase I purified from submaxillary gland and kidney were incubated with PNGase F for 2, 5 and 15 min and then aliquots of the reaction mixture were applied to SDS/polyacrylamide gels. Western-blot analysis showed that the timed digestion of the submaxillary-gland DNase I gave rise to an intermediate form that co-migrated with the intact kidney DNase I, whereas the 15 min digest caused both materials to migrate in the position of aglyco-DNase I (Figure 5). This result supports the conclusions that submaxillary-gland DNase I contains two N-linked sugar chains whereas kidney DNase I has only one.

DISCUSSION

N-linked glycosylation plays a critical role in the synthesis and functioning of many cell-surface and secreted proteins [24]. Consequently it is important whether or not a protein acquires an N-linked sugar chain during its biosynthesis. The N-linked sugar chains are transferred from a dolichol-linked precursor oligosaccharide to asparagine residues in the consensus tripeptide sequon Asn-Xaa-Ser/Thr on the target protein by the oligosaccharyltransferase complex. However, the presence of this sequon in the protein does not always lead to glycosylation. One factor known to influence the efficiency of glycosylation is whether the third amino acid of the sequon is Thr or Ser. Previous studies have shown that the exchange of Ser to Thr increases the glycosylation efficiency 2–40 times [8–10,19,20]. Similarly, when Ser¹⁰⁸ in the second glycosylation site of DNase I was changed to Thr, the extent of glycosylation at this site increased from 70% to almost 100% in the COS-1 cell-expression system. Therefore it is likely that the presence of a Thr in the third position of the Asn¹⁰⁶ site makes this site more subject to the influence of tissue-specific factors that affect the efficiency of glycosylation.

The nature of the amino acid at the Xaa position is also known to influence the N-linked glycosylation reaction [9,10]. The most extensive study of this phenomenon was carried out by Shakin-Eshleman et al. [12], who used a rabbit reticulocyte lysate cell-free system supplemented with canine pancreas microsomes. They found that Ala in the Xaa position was much more favourable than Asp, helping to explain further why the Asn¹⁸-Ala-Thr sequon is glycosylated better than the Asn¹⁰⁶-Asp-Ser

sequon. One difference in their results compared with ours is that they found that Trp functioned very poorly in the Xaa position, whereas it worked reasonably well in our experiments. In both studies the presence of Ser in the Xaa position resulted in optimal glycosylation.

The most significant finding in our study was the striking differences in the extent of glycosylation of DNase I among the various bovine tissues. This ranged from the synthesis of molecules with only a single sugar chain at Asn¹⁸ (pancreas, kidney) to the formation of molecules with two sugar chains (spleen, lung, parotid gland, adrenal, submaxillary gland). Abe and Liao [21] suggested previously that parotid-gland DNase I had two sugar chains. The important conclusion is that the ability of the N-linked glycosylation system to utilize Asn-Xaa-Ser sequons with suboptimal amino acids in the Xaa position varies greatly between tissues. The basis for this variation is unclear at this time. One possibility is that the level of lipid-linked oligosaccharide precursor varies among the tissues. In this regard Carson et al. [25] reported that supplementation of oviduct tissue slices with dolichylphosphate enhanced N-linked glycosylation of RNase. These investigators speculated that dolichylphosphate availability may be a limiting factor in the glycosylation *in vivo* of proteins in these systems. Another factor that could potentially influence the efficiency of glycosylation of DNase I is the nature of the oligosaccharyltransferase itself. It is now known that this enzyme is a multisubunit complex, but the role of the individual subunits is still under investigation. One speculative possibility is that the subunit content might vary in different tissues, with the consequence that a given N-linked sequon might be recognized with greater or lesser efficiency depending upon the composition of the oligosaccharyltransferase in that tissue.

Whereas prior work from a number of laboratories [26–29] has documented a tissue-specific effect in the processing of N-linked glycans at the same position on a protein, these studies did not evaluate the efficiency of glycosylation at these sites. The importance of our observations with DNase I is that it shows that a protein may undergo different extents of glycosylation depending on the tissue in which it is synthesized. This, in turn, could have a significant effect on the properties of the underlying protein.

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