The identification of threonine-95 as the major site of glycosylation in normal human myelin basic protein

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The enzymic transfer of *N*-acetylgalactosamine to myelin basic protein and that to peptides derived from basic protein were compared. Basic protein treated with pepsin and trypsin before glycosylation resulted in decreases of 7% and 23% in the amount of *N*-acetylgalactosamine transferred to the peptides respectively. However, digestion of basic protein had little effect on the sites glycosylated. It was found that Thr-95 was the major site for glycosylation in both the intact human basic protein and in the tryptic peptides.

Basic protein is a major component of human central-nervous-system myelin. Although it is not found glycosylated in mature myelin, it does serve as an acceptor for *N*-acetylgalactosamine (Hagopian *et al.*, 1971; Young *et al.*, 1979; Briand *et al.*, 1981; Suguira *et al.*, 1982; Cruz & Moscarello, 1983). Most of the *N*-acetylgalactosamine is *O*-glycosidically linked to the two tryptic-digest peptides 92–105 and 98–105 (Hagopian *et al.*, 1971; Cruz & Moscarello, 1983). Hagopian *et al.* (1971) identified Thr-98 as the major acceptor site for *N*-acetylgalactosamine in peptide 98–105 of the bovine protein. However, their study did not determine whether the *N*-acetylgalactosamine residue was bound to the Thr-95 or -98 in peptide 92–105. In addition to these major sites, two minor glycosylation sites were identified as tryptic-digest peptides 143–152 and 66–75 (Cruz & Moscarello, 1983).

Young *et al.* (1979) found that synthetic peptides containing sequences similar to the region of the Thr-95 and -98 were equally as good acceptors as the intact basic protein. Hence we determined the effect of digestion of basic protein on the amount and specificity of the *N*-acetylgalactosamine transferred to the peptides. In addition, it was necessary to determine which of the threonine residues in peptide 92–105 was glycosylated. Thr-95 was found to be the major site for glycosylation both in

the intact basic protein and in the tryptic-digest peptides.

Experimental

Basic protein was isolated from myelin of human central-nervous-system white matter by the method of Lowden *et al.* (1966). Non-radioactive UDP-*N*-acetyl-D-galactosamine was prepared from galactosamine 1-phosphate essentially as described by Carlson *et al.* (1964). Tritiated UDP-*N*-acetyl-D-galactosamine was purchased from New England Nuclear. The Triton X-100 extract of UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetyl-D-galactosaminyltransferase (EC 2.4.1.41) was prepared from pig submaxillary glands by a slight modification of the procedure of Schwyzer & Hill (1977). Basic protein was cleaved at the tryptophan residue by using BNPS-skatole, and the peptides were separated by the method of Boggs *et al.* (1981).

Tryptic-digest peptides of glycosylated basic protein were prepared by dissolving basic protein (200 µg) in a mixture of 150 µl of water, 150 µl of 2% (w/v) NH₄HCO₃ and 7 µl of 0.1 M-NaCl containing 5 µg of trypsin. The samples were incubated for 24 h at 37°C and then centrifuged at 10000 *g* for 10 min, and the peptides were separated by h.p.l.c. as described previously (Cruz & Moscarello, 1983). Tryptic-digest peptides of basic protein used for the glycosylation reaction were prepared in the same way, except that 1.5 mg of basic protein and 30 µg of trypsin were used. Peptic-digest peptides of basic protein were pre-

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; BNPS-skatole, 3'-bromo-3-methyl-2-(2-nitrobenzenesulphenyl)indolenine.

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pared by dissolving 1.5 mg of basic protein in a mixture of 150 μ l of water and 150 μ l of 1.0M-sodium formate buffer, pH 6.0, containing 30 μ g of pepsin and incubated at 37°C for 24 h. The tryptic and peptic digests were boiled for 10 min and then divided into fractions containing 200 μ g of protein for the glycosylation reaction.

Basic protein and the peptides were glycosylated as described previously (Cruz & Moscarello, 1983). Briefly, reaction mixtures containing 200 μ g of basic protein or peptides, 50 nmol of UDP-*N*-acetyl-D-galactosamine (100 000 c.p.m.), 5 μ mol of MnCl₂, 5–10 μ g of Triton X-100, 10 μ mol of Mes (4-morpholine-ethanesulphonic acid) buffer, pH 7.6, and 50 μ l of enzyme (total volume, 100 μ l) were incubated for 6 h at 37°C. In order to determine the amount of *N*-acetylgalactosamine transferred to intact basic protein, the reaction mixtures were spotted on paper, and the protein was precipitated with 20% (w/v) trichloroacetic acid, washed with 10% (w/v) trichloroacetic acid and its radioactivity counted (Cruz & Moscarello, 1983). The extent of glycosylation of the peptides was determined by two methods: high-voltage paper electrophoresis and h.p.l.c. For electrophoresis, the reaction mixture was spotted on Whatman 3MM paper and run for 1.5 h in 0.05M-ammonium formate buffer, pH 4.0, at 3 kV. The paper was dried and scanned for radioactivity. The peptides remained near the origin, whereas the free UDP-*N*-acetylgalactosamine migrated 15–20 cm towards the anode. The region containing the peptides was cut out and its radioactivity determined.

For the h.p.l.c. separations, the reaction mixture was loaded on a C₁₈ μ Bondapak column and run for 5 min with 0.05% trifluoroacetic acid and then 10 min with linear gradient of 0–60% (v/v) acetonitrile in 0.5% trifluoroacetic acid. The peptides were eluted with the latter solution and their radioactivities determined.

The major glycosylated peptides I and II of the trypsin-digested glycosylated protein were collected after h.p.l.c. separation and freeze-dried (Cruz

& Moscarello, 1983). After glycosylation of the tryptic digest of basic protein, peptide III_T was isolated in the same way. The peptides were resuspended in a mixture of 55 μ l of water, 30 μ l of 40 mM-NH₄HCO₃, 8 μ l of 20 mM-dithiothreitol and 8 μ l of clostripain (2 mg/ml) and incubated at 37°C for 24 h in order to digest the Arg-Thr bond that was resistant to trypsin. The digests were centrifuged at 10 000 *g* for 10 min and loaded on the C₁₈ μ Bondapak column. The peptides were separated by running a 1 h linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid. Samples were collected at 30 s intervals and their radioactivities determined.

Results and discussion

Effect of proteolytic and chemical cleavage of myelin basic protein on glycosylation

Intact basic protein and peptides derived from the BNPS-skatole cleavage, the peptic digest and the tryptic digest were compared as acceptors for *N*-acetylgalactosamine. The results are shown in Table 1. No significant difference in the amount of *N*-acetylgalactosamine transferred to the mixture of BNPS-skatole-cleavage peptides was observed when compared with intact protein. However, digestion of basic protein with pepsin and trypsin before glycosylation resulted in decreases of 7% and 23% in the amount of *N*-acetylgalactosamine transferred to the peptides respectively. In this respect, it may be noted that tryptic digestion of basic protein was much more extensive than that with pepsin. Peptic digestion of basic protein produced 10–12 peptides, whereas trypsin treatment produced 25–30 peptides (h.p.l.c. analyses, results not shown).

Effect of tryptic digestion on the glycosylation of the major sites in myelin basic protein

Tryptic-digest peptides prepared from glycosylated basic protein and peptides glycosylated after tryptic digestion of basic protein were compared by h.p.l.c. analysis. Each of the peaks detected at 210 nm was collected and the radioactivity determined. The distribution of radioactivity associated with the tryptic-digest peptides is shown in Fig. 1. Most of the radioactivity when the intact protein was glycosylated was associated with three tryptic peptides, I, II and III, which contained 16, 69 and 6% of the total radioactivity respectively (Fig. 1a). On the other hand, glycosylation of the tryptic-digest peptides resulted in the transfer of *N*-acetylgalactosamine to five peptides, termed I_T–V_T for convenience. Peptides I_T, II_T, III_T, IV_T and V_T contained 9, 7, 61, 5 and 10% of the total radioactivity respectively (Fig. 1b). The retention times of peptides I and III were identical with those of peptides I_T and IV_T respectively.

Table 1. *Effects of proteolytic and chemical cleavage on the glycosylation of peptides from myelin basic protein*
Samples of peptides or basic protein (200 μ g) were incubated for 6 h at 37°C under optimum conditions for glycosylation. The transfer of *N*-acetylgalactosamine to the peptides was determined by using high-voltage paper electrophoresis and h.p.l.c. analysis as described in the Experimental section.

Treatment	Glycosylation (% of control)
None	100 $\pm$ 3
BNPS-skatole	104 $\pm$ 4
Pepsin	93 $\pm$ 3
Trypsin	77 $\pm$ 6

Fig. 1. Tryptic peptides of glycosylated myelin basic protein
The tryptic peptides (200 μ g) were separated by h.p.l.c., by running a 1 h linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid. All of the peptide peaks detected at 210 nm were collected and their radioactivities determined. The results represent the major radioactive peptides; those peptides containing less than 2% of the total radioactivity are not shown. The Roman numerals I, II and III refer to tryptic peptides 98–105, 92–105 and 66–75 respectively. The subscript T (e.g. in I_T – V_T) refers to tryptic-digest peptides subsequently glycosylated. (a) Intact basic protein was glycosylated and then digested with trypsin; (b) basic protein was digested with trypsin before glycosylation; (c) a mixture of (a) and (b) containing equal amounts of protein.

However, the major peptide (II) isolated from the glycosylated protein migrated differently on the h.p.l.c. from the major radioactive peptide (III_T) glycosylated after tryptic digestion. This difference in elution from h.p.l.c. was shown to reflect differences in primary sequence rather than variation in the elution time (Fig. 1c).

Identification of the major glycosylated site in myelin basic protein

Our previous study identified the major sites on the glycosylated protein (Cruz & Moscarello,

Fig. 2. Comparison of the major glycosylated tryptic-digest peptides

The major glycosylated peptides I, II and III_T were collected after h.p.l.c. separation and freeze-dried. Approx. 3600 c.p.m. of each peptide was loaded on the C_{18} μ Bondapak column and run for 1 h with a linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid. Samples were collected at 30 s intervals and their radioactivities determined. (a) Peptide II; (b) peptide III_T ; (c) peptide II treated with clostripain; (d) peptide I.

1983). From the amino acid analysis of the tryptic-digest peptides, I and II were identified with peptides 98–105 and 92–105 respectively. The major peptide glycosylated after trypsin digestion, III_T , was identified with peptide 92–97. This result suggested that Thr-95 was the preferred site for glycosylation after tryptic digestion of basic protein. The relatively smaller amount of *N*-acetylglactosamine bound to peptide 98–105 may be partially due to the free *N*-terminal amino group on Thr-98. Similar peptides have been shown to be better substrates for *N*-acetylglactosaminyl transferase (Briand *et al.*, 1981) when the *N*-terminal amino group was blocked.

Since the major glycosylated peptide from the intact protein (peptide 92–105) contained two threonine residues (95 and 98), both of which could serve as acceptors of *N*-acetylglactosamine, we determined which of these residues was preferred. Peptide II from glycosylated basic protein contained an Arg–Thr bond that was resistant to trypsin cleavage, whereas this bond was readily hydrolysed in the non-glycosylated peptide. How-