Evidence for a Novel O-Linked Sialylated Trisaccharide on Ser-248 of Human Plasminogen 2*

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Human plasminogen, the inactive precursor of plasmin, exists in two major glycoforms. Plasminogen 1 contains an N-linked oligosaccharide at Asn-289 and an O-linked oligosaccharide at Thr-345. Plasminogen 2 is known to contain only an O-linked oligosaccharide at Thr-345. However, plasminogen 2 displays a further well documented microheterogeneity dependent on the N-acetylenuraminic acid content, which has functional consequences with regard to activation of plasminogen. The proposed structure and number of known oligosaccharide linkages in plasminogen 2 is insufficient to account for this microheterogeneity. In the present study, a combination of trypsin digestion, lectin affinity chromatography, Edman degradation amino acid sequence analysis, carbohydrate composition analysis, and mass spectrometry revealed the existence of a novel site for O-linked glycosylation on plasminogen 2 at Ser-248. Direct evidence for the structure of the carbohydrate was obtained from a combination of lectin affinity chromatography, desialylation experiments, and mass spectrometry analysis. These findings provide a structural basis for some of the observed microheterogeneity, and have implications with regard to the known functional consequences of the extent of sialylation of plasminogen.

Plasminogen (Pg)³ is the inactive precursor of plasmin, quantitatively the most important proteinase involved in fibrinolysis. Pg exists in two major glycoforms, Pg 1, which possesses an N-linked high mannose-type carbohydrate chain located at Asp-289 and an O-linked carbohydrate chain linked at Thr-345, and Pg 2, which contains only the carbohydrate chain present at Thr-345 (1, 2). The sialylated O-linked carbohydrate chain at Thr-345 contains N-acetylenuraminic acid (NeuNac), galactose (Gal), and N-acetylgalactosamine (GalNac) and has the structure NeuNacα2–3Galβ1–3GalNac. In 1–5% of Pg molecules, there is a further NeuNac linked directly to the GalNac (1, 3). It has long been known that Pg 2 can be resolved by isoelectric focusing techniques into at least six glycoforms that differ only in their N-acetylenuraminic acid (NeuNac) content (4, 5). Recently, we have separated the glycoforms of human Pg 2 by employing a combination of lectin affinity chromatography and chromatofocusing (6). Our data showed that the NeuNac content of Pg 2 glycoforms varied from 1.3 mols/mol of protein to 13.65 mols/mol (6). Furthermore, the individual Pg 2 glycoforms display markedly different kinetic behavior when activated with tissue-type plasminogen activator (tPA), urinary-type plasminogen activator (uPA), and streptokinase (6, 7). Activation of Pg 2 by tPA was most dependent on NeuNac content with a steady decrease in catalytic efficiency with increased sialylation, whereas catalytic efficiencies of activation by uPA appeared to be unaffected up to a threshold of NeuNac content. The most highly sialylated glycoform of Pg 2 was essentially resistant to activation by both tPA and uPA (7). Interestingly, streptokinase activation of human Pg was also regulated by NeuNac content, with the most highly sialylated glycoform activated 20-fold less efficiently than the least sialylated glycoform. Although carbohydrate did not stop streptokinase forming an initial activator complex with human Pg, as demonstrated by gel filtration experiments, the carbohydrate was hypothesized to interfere with the stability of the Michaelis complex (7). In contrast to the effect on Pg activation, NeuNac content did not interfere with the inhibition of generated plasmin glycoforms by a₂-antiplasmin (6).

Further evidence that the glycosylation of Pg modulates the functional activity of the protein has been provided by Mori et al. (8), who demonstrated differential activation of Pg 1 and 2 by tPA 1 and II, respectively. Davidson and Castellino (9) have shown that differently glycosylated forms of Pg exhibit different kinetic parameters for activation by uPA. In addition, neonatal Pg 2, which has 18 times more NeuNac than adult Pg 2, is activated 6-fold less efficiently by tPA (10). Pg 2 also binds to cell surfaces with greater affinity than the more glycosylated Pg 1 (11). Unglycosylated Pg expressed in Escherichia coli, was resistant to activation by tPA and uPA and was cleared significantly faster than glycosylated Pg molecules (5). Together, these data suggest an important role for carbohydrate, in general, and NeuNac, in particular, in regulating the function of Pg.

Examination of the proposed structure of the carbohydrate chain on Thr-345 of Pg 2 (3) predicts the possibility of only two glycoforms based on the NeuNac content; however, the exist-

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The abbreviations and trivial names used are: Pg, plasminogen(s); NeuNac, N-acetylenuraminic acid; GalNac, N-acetylgalactosamine; NANAse, neuraminidase; tPA, tissue-type plasminogen activator; uPA, urinary-type plasminogen activator; FACE, fluorophore-assisted carbohydrate analysis; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; ES-MS, electrospray ionization-mass spectrometry.

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ence of at least six glycoforms is well documented (5, 6). To address this apparent discrepancy, we have reassessed the glycosylation of Pg2.

In this study, we present data derived from amino-terminal sequence analysis of tryptic peptides, mass spectrometry, and fluorophore-assisted carbohydrate analysis (FACE), demonstrating that Pg 2 contains a novel O-linked carbohydrate chain linked to Ser-248.

EXPERIMENTAL PROCEDURES

**Proteins**—Pg 2 was purified from fresh frozen plasma (American Red Cross, Durham, NC) as described previously using a combination of lysine-Sepharose and concanavalin A-Sepharose affinity chromatography (6). Each batch of Pg 2 was purified from 4–8 units of fresh frozen plasma. At least three separate batches of prepared Pg 2 were used in these studies. Trypsin was obtained from Sigma. Jacalin-agarose (4 mg/ml) was obtained from Vector Labs Inc. (Burlingame, CA). Neuraminidase (NANase III) was a kind gift from GLYKO (Novato, CA). Proteinase SV8 was obtained from Boehringer Mannheim.

**Chemicals**—Dithiothreitol, iodoacetamide, α-methylacytylamino-side, and all buffer chemicals were purchased from Sigma. All other reagents were of reagent grade quality.

**Peptides**—Peptides were produced by limited proteolysis essentially as described previously (12). Briefly, Pg 2 (2–5 mg/ml) was incubated for 2 h at 37 °C in 6 μl guanidine hydrochloride, 0.1 mM Tris-HCl, pH 8.2, 10 mM dithiothreitol. The solution was adjusted to 30 mM iodoacetamide and incubated in the dark at room temperature for 30 min. After dialysis against 0.1 mM Tris-HCl, pH 8.2, overnight, the Pg 2 solution (including precipitated material) was transferred to a 50-ml conical tube, and trypsin was added such that the final molar ratio of Pg to trypsin was 100:1. The reaction was allowed to proceed for 4–16 h at 37 °C, after which the solution was adjusted to pH 7.0 with HCl. Peptides containing an O-linked carbohydrate chain were purified by applying the peptide mixture to a jacalin-agarose column (16 × 100 mm) equilibrated in 100 mM Tris-HCl, pH 7.0. Jacalin is a lectin with affinity for the disaccharide 1-β-galactopyranosyl-3-(α-D-mannopyranosyl-2-deoxygalactopyranoside), the core disaccharide of mucin-type carbohydrate chains (13). The column was washed in 100 mM Tris-HCl buffer (3 column volumes), and bound peptides were eluted using 100 mM Tris-HCl, 20 mM α-methylacytylamino-side. Glycopeptides were further purified using HPLC. Separations were performed on an octadecylsilane (C18) column (4.6 × 250 mm, 5 μm particle size) using a linear gradient of 0.1% trifluoroacetic acid (TFA) in acetonitrile (50%) containing 1% formic acid. Spectra were acquired in the multi-channel acquisition mode from mass/charge (m/z) 600–1600 with a scan time of 10 s. For some experiments, the reversed phase fractions were resubjected to reverse phase-HPLC (Deltabond, ODS 150 × 1 mm, Keystone Scientific, Bellefonte, PA) using an Isco (Lincoln, NA) microbore system. The effluent was monitored at an absorbance of 216 nm and split evenly into the two streams. One stream was fed directly to the ion source of the mass spectrometer. Spectra were acquired in continuum mode from mass/charge (m/z) ratio of 600–1600 with a scan time of 5 s.

**RESULTS**

**HPLC Separation of Glycopeptides**—The glycopeptides purified by jacalin-agarose affinity chromatography resolved into three peptides, eluting at 20, 27, and 27.5% acetonitrile (Fig. 1). Although the peptides eluting at 27 (pep2) and 27.5% (pep3) acetonitrile have been previously described (12), the peptide eluting at 20% (pep1) acetonitrile is novel. Edman degradation amino acid sequence analysis of the first 20 amino acid residues of peptides 2 and 3 confirmed that both of these peptides were derived from Pg 2 and consisted of a fragment commencing at Ile-329 (Table I). Confirmation of the sequence of the last 18 residues was obtained by further digesting peptides 2 and 3 with SV8 as described under "Experimental Procedures." The identity of pep1 was obtained by amino acid sequencing of the entire tryptic peptide. The sequences (Table I) differed slightly from the Pg sequence previously published. No phenylthiohydantoin amino acid derivative was detected in the cycle during Edman degradation at position Thr-345 in peptides 2 and 3, consistent with a modification of the threonine by the known O-linked carbohydrate chain present at this residue (3). Ser-338 in peptide 2 was also modified and both peptides 2 and 3...
had Gln-341 instead of the expected Glu-341, as reported previously (12). Peptides 2 and 3 always eluted from the C18 column in equimolar concentrations as indicated by peak area (data not shown).

Amino acid sequence analysis of peptide 1 (Table I) indicated that it was a Pg 2 derived fragment, commencing at Cys-242 and terminating at Lys-257. There was a blank cycle at position Ser-248 in this peptide tide, indicating a modification of the serine. The amount of peptide obtained from HPLC was consistently 13–15% of the amounts of peptides 2 or 3, suggesting that not all Pg 2 molecules have this modification.

**Mass Spectrometry Analysis**—To determine the nature of the modification inferred by the blank cycles found during sequence analysis, we performed ES-MS analysis. ES-MS analysis of peptide 1 (Table II) revealed a mass of 2449.9 Da. The expected mass of the unmodified carboxyamidomethylated peptide is 1796.0 Da. As the predicted mass of a mucin carbohydrate trisaccharide chain attached to the side chain of serine or threonine is 656.6 Da, this indicates that Ser-248 may possess an O-linked mucin carbohydrate chain.

Similar analysis of peptide 2 revealed a mass of 4896.6 ± 1 Da (Table II). The expected mass of the carboxyamidomethylated peptide is 4162.5. Mass spectrometry analysis of peptide 3 consistently revealed a peptide with mass value of 4816.2 ± 0.6 Da (Table II). Thus, peptides 1, 2, and 3 all have a greater mass than would be expected from a simple analysis of the primary sequence, indicating that all peptides isolated from jacalin-agarose are modified by the addition of at least one trisaccharide moiety on Ser or Thr. To confirm the composition and structure of the carbohydrate on these glycopeptides we performed FACE analysis.

**FACE Analysis**—Peptides 1, 2, and 3 were analyzed by FACE to determine carbohydrate composition. A representative analysis is shown in Fig. 2. The only monosaccharides detected in peptides 1, 2, and 3 were galactose, N-acetylgalactosamine, and NeuNAc. The ratios of N-acetylgalactosamine to protein (Table III) are essentially equimolar for peptides 2 and 3, indicating that the additional modification of Ser-338 noted in peptide 2 is unlikely to be a carbohydrate modification.

**Desialylation of Glycopeptides**—Glycopeptides 1–3 were treated with NANase III, a neuraminidase with specificity for α(2–3), α(2–6), and α(2–8) linkages for 16 h at 37°C. The peptides were then analyzed by ES-MS. The mass differences before and after NANase III treatment are consistent with the loss of the NeuNAc (Table IV), indicating that each of the glycopeptides purified only has one NeuNAc residue.

**DISCUSSION**

In this study, we provide evidence that Pg 2 molecules contain an additional O-linked carbohydrate chain and have localized the site of attachment of this second carbohydrate chain to Ser-248. We have isolated three jacalin-reactive peptides from Pg 2, of which one (peptide 1) is a novel glycopeptide. The other two peptides, designated peptides 2 and 3, have been described previously (12). The mass spectrometry and carbohydrate composition analysis of jacalin-purified peptide 1 indicates that the carbohydrate attached to Ser-248 of Pg 2 has the structure

### Table I

| Peptide  | Sequence |
|---------|----------|
| Peptide 1 | $242^{C}CTPDPXSGPTYQCLK^{257}$ |
| Peptide 2 | $323^{P}IPCSDSPVXTQELAP^{336}$ |
| Peptide 3 | $323^{P}IPCSDSPVSTQEFLAP^{336}$ |

### Table II

| Peptide | ES-MS | Expected mass | Difference |
|---------|-------|---------------|------------|
| Peptide 1 | 2449.9 ± 0.1 | 1796.01 | 653.9 |
| Peptide 2 | 4896.6 ± 1.0 | 4162.5 | 734.1 |
| Peptide 3 | 4816.2 ± 0.6 | 4162.5 | 653.7 |

### Table III

| Glycopeptide | pmol Peptide | pmol N-acetylgalactosamine |
|--------------|-------------|---------------------------|
| Peptide 2    | 150 ± 6.2   |                            |
| Peptide 3    | 122 ± 11.0  |                            |

* Determined by amino acid analysis. Values are mean ± S.E., n = 4.

**NeuNACo2–3Galβ1–3GalNAc, identical to the known structure of the carbohydrate chain on Thr-345 of Pg 2.**

Pg contains five kringle domains that mediate binding to substrate surfaces, such as fibrin and fibronectin and cell receptors, and that regulate the activation of Pg as well as plasmin activity. The site of attachment for the novel sialylated trisaccharide chain described here is located between kringle 2 and 3. We and others (6, 8) have demonstrated the importance of carbohydrate in the regulation of Pg activation by tPA. We have also previously demonstrated that a decrease in the catalytic efficiency of Pg activation by tPA correlates with increasing Neu-
Data are derived from ES-MS analysis. Desialylation was performed as described under “Experimental Procedures.” The predicted loss of mass due to desialylation is 291.3 Da. The technique has an expected error of \( \pm 1 \) Da per 1 kDa.

| Glycopeptide | Peptide 1 | Peptide 2 | Peptide 3 |
|--------------|-----------|-----------|-----------|
| Mass before desialylation (Da) | 2449.9 | 4896.6 | 4816.2 |
| Mass after desialylation (Da) | 2141.3 | 4604.1 | 4526.0 |
| Difference (Da) | 308.6 | 292.5 | 290.2 |

TABLE V

| Isoform | NeuNAc content\textsuperscript{a} | Activator | Catalytic efficiency of activation\textsuperscript{a} mol/mol protein M\textsuperscript{-1} min\textsuperscript{-1} |
|---------|----------------------------------|-----------|------------------------------------------------|
| Pg2α    | 1.3 ± 0.317                      | tPA       | \( 3.84 \times 10^6 \) |
| Pg2β    | 2.2 ± 0.201                      | tPA       | \( 1.78 \times 10^6 \) |
| Pg2γ    | 2.95 ± 0.035                     | tPA       | \( 2.72 \times 10^6 \) |
| Pg2δ    | 5.77 ± 2.50                      | tPA       | \( 2.85 \times 10^6 \) |
| Pg2ε    | 5.34 ± 0.68                      | tPA       | \( 1.71 \times 10^6 \) |
| Pg2κ    | 13.65 ± 4.07                     | tPA       | ND |

\textsuperscript{a} Data are derived from Ref. 6.
\textsuperscript{b} Data are derived from Ref. 7.
\textsuperscript{c} ND, not detectable.

Since there are at least six glycoforms of Pg 2 (4, 5, 6), the presence of a second sialylated trisaccharide on Ser-248 does not fully account for all the known microheterogeneity of this protein. There are other types of O-linked saccharide chains that can contain NeuNAc, notably oligosaccharide chains attached via a fucos residue (16) that are found in a variety of proteins involved in coagulation (17). The presence of such carbohydrate chains on plasminogen or the possibility of polysialic acid (6) must also be considered. Our data also demonstrate a modification of Ser-338 of plasminogen 2. Hortin and Yu (18) have presented evidence indicating that plasminogen 2 is phosphorylated at Ser-338. The mass difference of 80 Da we report here between peptides 2 and 3 provides further evidence in support of this hypothesis.

In conclusion, we provide evidence for a novel O-linked carbohydrate chain on Pg 2. This chain, attached at Ser-248, is a trisaccharide terminated with NeuNAc. The presence of this trisaccharide between kringles 2 and 3, coupled with the previously reported trisaccharide between kringles 3 and 4 (3), provides a structural basis for the observed correlation of NeuNAc content with structural and functional microheterogeneity.