O-Linked L-Fucose Is Present in Desmodus rotundus Salivary Plasminogen Activator

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DSPα1 (Desmodus rotundus salivary plasminogen activator), a plasminogen activator from the saliva of the vampire bat Desmodus rotundus, is an effective thrombolytic agent. An unusual type of posttranslational modification, in which L-fucose is O-glycosidically linked to threonine 61 in the epidermal growth factor domain was found for natural DSPα1 and its recombinant form isolated from Chinese hamster ovary cells.

In the present study a combination of carbohydrate and amino acid composition analysis, amino acid sequencing, and mass spectrometry revealed that the L-fucose is bound to residues 56–68 of DSPα1. The amino acid sequence of this glycosylation site agreed with the suggested consensus sequence Cys-Xaa-Xaa-Gly-Gly-Ser/Thr-Cys described for other proteins. A new strategy for the identification of the modified amino acid was established. Direct evidence for the occurrence of fucosyl-threonine was obtained by mass spectrometry after digestion of the glycopeptide with a mixture of proteases. On the basis of these results, DSPα1 is a suitable model for studying the influence of O-fucosylation on clearance rates, particularly in comparative studies with the identically fucosylated and structurally related tissue plasminogen activator.

Plasminogen activators (PAs) are involved in many physiological processes and play a key role in fibrinolysis. They are serine proteases that catalyze the conversion of plasminogen to plasmin by a specific proteolytic cleavage (5–8). Plasmin, which is also a serine protease, efficiently degrades the fibrin network. Due to their thrombolytic properties PAs are used as therapeutic agents in acute myocardial infarction.

DSPα1 (Desmodus rotundus salivary plasminogen activator) is a plasminogen activator from the sālīva of the vampire bat Desmodus rotundus (9, 10). In contrast to other plasminogen activators DSPα1 displays a remarkable selectivity toward fibrin-bound plasminogen (1) and is therefore potentially a safer and more efficacious thrombolytic agent than other PAs. For further characterization the glycoprotein was cloned and expressed in baby hamster kidney cells (11, 12) as well as Chinese hamster ovary (CHO) cells (13). Like human tissue plasminogen activator (t-PA), DSPα1 is a multidomain protein consisting of a finger domain, a serine protease, a kringle domain, and an epidermal growth factor region. This type of multidomain structure is found in several proteins that are active in coagulation or fibrinolysis (14). Comparison of the amino acid sequence of t-PA and DSPα1 reveals a homology of 72% for the corresponding domains. In contrast to t-PA, which contains two kringle segments, only one kringle region is present in DSPα1. In t-PA the cleavage sequence of Arg92-Phe111, which is responsible for the conversion of the single chain form to the higher active two-chain form, is located in the kringle 2 domain. rDSPα1 lacks this domain and is the first example of a naturally occurring PA acting as a single chain enzyme. DSPα1 contains the consensus sequence of two N-glycosylation sites, which corresponds to t-PA minus the kringle 2 region.

Epidermal growth factor domains are found in many proteins involved in coagulation or fibrinolysis (14). In recent years three different types of posttranslational modifications have been identified within this region (15). β-Hydroxylation of Asp and Asn residues in the amino acid sequence Cys-Xaa-Asp/Asn-Xaa-Xaa-Xaa-Tyr/Phe-Xaa-Cys/Xaa-Cys is characteristic for many proteins, e.g. protein C, factor IX, factor X, protein S, protein Z, and others (16–21). In addition to β-hydroxylation, two other types of posttranslational modification have been described, both involving glycosylation. Thus the disaccharide Xyl-Glc and the trisaccharide Xyl2-Glc2 are present in bovine factor VII, factor IX, protein Z, and thrombospordin (22–24) and in human factor VII and factor IX (25); secondly O-linked L-fucose was first discovered in the EGF domain by Kentzer (26) and confirmed by Buko (27) for human pro-urokinase. Human factor VII (28), human factor XII (29), and t-PA derived from different cell lines (30) all contain O-linked L-fucose. Factor IX contains a tetrasaccharide bound via fucose to serine (31). In all published accounts, L-fucose is attached to a conserved consensus sequence Cys-Xaa-Xaa-Gly-Gly-Thr/Ser/Cys and is found only in proteins of human origin, with the exception of bovine factor VII (32).

Since this consensus sequence is present in DSPα1, the possible O-fucosylation of natural and CHO cell-derived rDSPα1 was investigated, and its occurrence is described in this report.

EXPERIMENTAL PROCEDURES

Materials—rDSPα1 from CHO cells was obtained from Schering AG (Berlin, Germany). Mexican DSPα1 (80 μg) was purified from 30
ml of saliva of the vampire bat D. rotundus according to Schleuning et al. (1). Trypsin (c~-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was purchased from Serva (Heidelberg, Germany). Chymotrypsin (1-chloro-3-tosylamido-7-aminotriheptanone-treated) and trifluoroacetic acid were obtained from Sigma. Pronase E was purchased from Merck (Germany). a-L-Fucosidase from chicken liver was obtained from Oxford GlycoSystems (Abingdon, United Kingdom), and a-L-fucosidase from Charonia lampas was from Sekagaku (Japan).

Peptide Mapping—DSPAn1 was reduced and carboxymethylated in a buffer containing 6 M guanidine hydrochloride, pH 8.0, according to Wadxl (2) with some modifications. Large scale tryptic digestion of rDSPAn1 (3 mg) was performed overnight at 37 °C after exchange of the buffer into 0.1% NH4HCO3, pH 8.0, by the addition of two portions of trypsin (1/100, w/w) at 0 and 8 h. Small scale tryptic digestion of natural or rDSPAn1 (60 µg) was carried out overnight at 37 °C in 0.8 M guanidine hydrochloride, pH 8.0, by the addition of two portions of trypsin (1/50, w/w) at 0 and 8 h.

Peptides were separated by a chromatography workstation (Bio-Rad) equipped with a Europleer C-100 column (4.6 × 250-mm, Knauer) at room temperature with a flow rate of 1 ml/min. The applied gradient consisted of solvent A (H2O, 0.1% trifluoroacetic acid) and solvent B (CH3CN/H2O(70/30), 0.1% trifluoroacetic acid). The two-stage gradient started 3 min after injection and went from 0 to 12% solvent B in 10 min and then up to 50% B in 110 min. Absorbance of the effluent was monitored at 215 nm.

Chymotryptic digestion (approximately 0.25 µg/mmol of peptide) of the HPLC-purified tryptic peptide was performed in 1% NH4HCO3 overnight. Peptides were separated as described above.

Pronase E (1 mg/ml) was preincubated at 60 °C in 1% NH4HCO3, pH 7.0, for 30 min. Digestion was performed for 48 h at 37 °C in 1% NH4HCO3, pH 7.0, containing 10 mM CaCl2, the Pronase E (approximately 100 µg/mmol of peptide) being added in two portions, one at 0 h and the other at 24 h. Proteins were removed by centrifugation using a Centricon 3 microconcentrator at 2000 × g.

Peptide Analysis—N-terminal sequence analysis was carried out by automated Edman degradation using a 477A system from Applied Biosystems. Phenylthiohydantoin-derivates were identified by an Applied Biosystems A 120 analyzer.

Peptide hydrolysis was performed with 6 M HCl at 110 °C for 24 and 48 h. Amino acids were derivatized with p-phenylaldehyde and 3-mercaptotrianionpic acid. Effluent was monitored by fluorescence at an emission wavelength of 330 nm with excitation at 450 nm.

Sugar Component Analysis—Monosaccharides were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (3) and gas chromatography coupled to mass spectrometry (GC-MS). For analysis with HPAEC-PAD the peptides were hydrolyzed with 2 M trifluoroacetic acid for 3.5 h at 100 °C. After evaporation under vacuum and the addition of an internal standard (2-deoxyribose), the samples were injected into a Biosystems LC from Dionex equipped with a Carapac PA 1 column. Monosaccharides were separated by isocratic elution with 15 mM NaOH. Postcolumn addition of 0.5 M NaOH enabled an amperometric detection (PAD) at 300 nA. Identification of the monosaccharides was improved by calculating the relative retention times. Sugar analyses with GC-MS were performed as described previously (4).

Mass Spectrometry—Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was carried out on a VG TOF Spec (Fisons, Manchester, United Kingdom) or, more recently, using a Bruker Biflex (Bruker, Bremen, Germany). Ionization was accomplished with a 337-nm beam from a nitrogen laser. Mass spectra were recorded in the positive and negative ion mode using the reflector for better resolution. 2,5-Dihydroxybenzoic acid (10 mg/ml) in 40% acetonitrile and 0.1% trifluoroacetic acid was used as matrix. Typically, an aqueous solution of the peptide (2–20 pmol/µl) was mixed (1/1, v/v) with the matrix, and 0.1–1 µl was placed on the target.

Defucosylation—Digestion with a-L-fucosidase from chicken liver was performed with 0.2 units/~3 nmol of peptide in a buffer containing 100 mM sodium citrate-phosphate, pH 6.0, for 18 h at 37 °C according to the protocol of the manufacturer.

Digestion with a-L-fucosidase from C. lampas was carried out with 0.05 units/~3 nmol of peptide in a buffer containing 200 mM sodium citrate-phosphate, pH 4.1, for 18 h at 37 °C according to the manufacturer’s protocol.

RESULTS

To elucidate the covalent attachment of fucose to the EGF domain, rDSPAn1 was chemically reduced, carboxymethylated, and enzymatically digested with trypsin. The resulting peptides were separated by reversed-phase HPLC (RP-HPLC) (Fig. 1), and their carbohydrate content was analyzed by monosaccharide composition analysis. Besides two theoretical N-glycosylation sites, we found a peptide (P1) that carried only one type of monosaccharide. Glycan composition analysis by HPAEC-PAD yielded a signal corresponding to the retention time of fucose (Fig. 2A). To verify this result, monosaccharide analysis by GC-MS was used. Attachment of fucose was confirmed by calculating the retention time relative to an internal standard and by analysis of the mass spectrometric fragmentation pattern (Fig. 2B). The molecular mass of the fucosylated peptide (P1) was determined by MALDI-TOF-MS. In the positive ion mode, a signal at m/z = 1781.3 was detected (Fig. 3A). By increasing the laser intensity a second signal consistent with the loss of fucose was observed. Measurements in the negative ion mode yielded a signal at m/z = 1778.9, indicating an intact glycopeptide (Fig. 3B). The expected mass for the carboxymethylated tryptic peptide comprising residues 56–82 (numbering from the N terminus) was 3186.5 (M + H) + or 3349.6 (M + H) + with one fucose residue attached. The observed mass of 1781.3 was consistent with a sodium adduct of a peptide containing residues 56–68 plus one fucose residue (Table I). Amino acid composition analysis and sequence determination of the first amino acids confirmed the suggested peptide, located in the EGF domain.

To determine the anomeric configuration of the glycosidic linkage, the tryptic glycopeptide was digested with a-L-fucosidase from chicken liver and from C. lampas. Three peptides were isolated by RP-HPLC after treatment with a-L-fucosidase from chicken liver; these contained presumably no phenylalanine (amino acid residue 68) and had lost some fucose, as indicated by mass spectrometry. Incubation of peptide P1 with a-L-fucosidase from C. lampas also generated some new peptides, which were not further analyzed. Peptidase activity is presumably due to impurities in the enzyme preparation. To circumvent the problem of degradation, the peptide was treated with chymotrypsin. Chymotryptic peptides were separated by RP-HPLC (Fig. 4A) and subjected to monosaccharide composition analysis. Fucose was found to be attached to two
peptides, which showed small differences in retention times. Mass determination of the major peak P2 resulted in signals at m/z = 1172.1 and m/z = 1148.1, using the positive or negative ion mode, respectively. This agreed with the calculated mass for a sodium adduct of the peptide comprising residues 56–63 plus one fucose residue and its deprotonated form, respectively (Table I).

It is known that the Asn-Gly sequence can be converted to Asp-Gly by deamidation in alkaline solution (30, 33). The minor peak P3 was identified by MALDI-TOF-MS as the deamidation product of peptide P2. Incubation of peptide P2 with a-L-fucosidase from chicken liver produced two peptides, which were separated by RP-HPLC (Fig. 4B). Mass spectrometric analysis revealed that peptide P2* corresponded to the fucosylated peptide P2, whereas peptide P4 represented the defucosylated form of P2. Comparison of the peak areas for P2* and P4 indicated nearly 60% removal of L-fucose.

To determine whether rDSPa1 was partially or completely modified by O-linked L-fucose, tryptic peptides were analyzed by MALDI-TOF-MS. Starting from the elution time of the fucosylated peptide P1, the subsequent 10 ml of the RP-HPLC run were fractionated and subjected to mass determination. No signal for the defucosylated peptide was observed. Furthermore, we compared the elution profile of the defucosylated peptide, obtained by treatment of the tryptic peptides with a-L-fucosidase from C. lampas, with the profile of untreated peptides. A very weak signal from the untreated peptides corresponded to the retention time of the defucosylated peptide, but mass spectrometry showed that this signal did not represent the defucosylated peptide.

Comparative Study of Natural and Recombinant DSPa1—To determine if O-fucosylation was restricted to DSPa1 when overexpressed in CHO cells, we studied natural DSPa1 derived from the Mexican vampire bat D. rotundus. The small scale tryptic maps for natural and rDSPa1

| Table I | Determination of peptides by mass spectrometric analysis |
|---------|----------------------------------------------------------|
| HPLC peak | Ion | Mass of peptide | Observed mass | Peptide |
| P1 | (M + Na)⁺ | 1634.8 | 1780.9 | 1781.3 | CFNGGTCWQAVYF + fucose |
| (M + Na – 2H)⁻ | 1632.8 | 1778.9 | 1778.9 | CFNGGTCWQAVYF + fucose |
| P2 | (M + Na)⁺ | 1026.1 | 1172.2 | 1172.1 | CFNGGTCW + fucose |
| (M – H)⁻ | 1002.1 | 1148.2 | 1148.1 | CFNGGTCW + fucose |
showed a very similar pattern when developed under identical conditions (Fig. 6). Mass spectrometry of peptides R1 and R2 of rDSPα1 yielded ions at m/z = 1781.0 and m/z = 1781.7, indicating the presence of the fucosylated peptide and the deamidated fucosylated form containing amino acid residues 56–68. All peptides of natural DSPα1 that eluted between 85 and 110 min were subjected to mass spectrometric analysis. The observed masses of 1780.9 Da for peptide N1 and 1782.0 Da for peptide N2 correspond to the sodium adduct of the fucosylated peptide and the deamidated form characterized from rDSPα1. The covalent attachment of fucose to the peptides R1, R2, N1, and N2 was confirmed by monosaccharide analysis. Mass spectrometric data, monosaccharide analysis, and the nearly identical retention times on RP-HPLC for the peptides R1/N1 and R2/N2 demonstrated that natural and recombinant DSPα1 were O-fucosylated in the same peptide domain.

In the present study we established that natural DSPα1 and CHO cell-derived rDSPα1 are modified by the presence of a single L-fucose residue, which is O-glycosidically linked to threonine 61 in the epidermal growth factor domain. The fucosylated tryptic peptide was isolated by RP-HPLC and identified by a combination of amino acid and carbohydrate analysis as well as mass spectrometry. Monosaccharide composition analysis by HPAEC-PAD and GC-MS revealed the presence of the deoxyhexose L-fucose. The C terminus of the tryptic peptide was phenylalanine, a typical cleavage point for chymotrypsin but less favorable for trypsin. Cleaving at the C-terminal side of phenylalanine, tryptophan, tyrosine, leucine, or methionine is frequently observed, even when chymotryptic activity has been chemically inhibited (34, 35). Cleavage of bonds adjacent to aromatic residues may therefore be due to small amounts of ψ-trypsin rather than to chymotrypsin (36).

The α-anomeric configuration of the glycosidic linkage and the presence of fucose in the L-enantiomeric form were demonstrated by susceptibility to α-L-fucosidase from chicken liver.
and from C. lampas. Comparison of RP-HPLC profiles of peptides treated and untreated with \( \text{fucosidase} \), followed by mass spectrometric analysis, indicated that threonine 61 is always fucosylated.

Different techniques of mass spectrometry have been previously reported for the identification of modified peptides (27, 31, 32). In this study mass determination was performed by MALDI-TOF-MS. The quality of spectra recorded in the positive and the negative ion modes were comparable, with no basic differences in sensitivity (the same number of ions was formed) or undesired fragmentation. Loss of fucose during mass spectrometry (29) can be minimized by attenuating the laser intensity and working at the ionization threshold.

Characterization of the fucose binding size is complicated by its lability under alkaline or acidic conditions. \( \beta \)-Elimination applied to the O-glycosylated amino acid residues, followed by comparative amino acid analysis, has been reported (30, 32). In the present study an alternative approach was developed for the identification of the modified amino acid. The fucosylated peptide was exhaustively digested with Pronase E to cleave all peptide bonds, followed by mass spectrometric analysis. Attachment of \( \text{fucoside} \) to threonine was demonstrated directly by the detection of fucosyl-threonine with MALDI-TOF-MS. This new strategy may represent the most favorable method for the identification of this type of glycosylation site.

Attachment of \( \text{O-linked fucose} \) has been described previously for human proteins. This modification was also observed in bovine factor VII but not described in detail (32). The proteins examined were either obtained from human plasma or expressed in different cell lines. In the present study we demonstrated that the vampire bat protein DSPA\(_{a1}\) and its recombinant form rDSPA\(_{a1}\), expressed in CHO cells, are posttranslationally \( \text{O}-\)fucosylated. In all reports so far, the attachment of \( \text{fucose} \) is restricted to the suggested consensus sequence -Cys-Xaa-Xaa-Gly-Thr/Ser-Cys- located in EGF domains (Table II). This motif is also valid for DSPA\(_{a1}\), since fucosylated threonine 61 is bound to a corresponding sequence in the EGF domain. The result shows that \( \text{O-linked fucose} \) is not limited to proteins of human origin but may be more widespread than earlier suspected. Stults and Comming (37) suggested that many glycoproteins carry \( \text{O-linked fucose} \), based on investigations using a CHO cell line deficient in N-acetylglucosaminyl-transferase I (Lec1). In this study Lec1 cells were metabolically radiolabeled with \([6-\text{H}]\text{fucose}\). \( \beta \)-Elimination of the radiolabeled proteins resulted in the release of \([6-\text{H}]\text{fucitol}\). However, the fucose attachment site was not assigned to a specific sequence or domain. Apart from this report, O-fucosylation has been observed only in EGF domains, and all of the modified proteins are involved in blood coagulation or fibrinolysis.

Presumably O-linked \( \text{fucose} \) has some physiological relevance, but no specific function has so far been discovered. It is noteworthy that \( \text{fucose} \) is an important constituent of many carbohydrate antigenic epitopes such as the ABO system and Lewis X structures, recognized by selectins (38, 39). It remains to be determined whether O-linked \( \text{fucose} \) also represents a lectin recognition signal. Preliminary data indicate that this modification may influence the clearance rate of t-PA. Hajjar and Reynolds (40) reported binding of t-PA to suspended HepG2 cells, followed by a rapid internalization and degradation of t-PA. Inhibition studies and treatment with \( \alpha \)-fucosidase suggested the involvement of O-linked \( \text{fucose} \). It is not yet known whether binding of t-PA to the low density lipoprotein receptor-related protein/\( \alpha \)-2-macroglobulin receptor (LPR receptor) (41) depends on O-fucosylation. Thus O-linked \( \text{fucose} \)-mediated clearance may be a new mechanism or an essential part of a described clearance pathway. Comparative studies by Witt et al. (42) in dogs showed that the clearance of rDSPA\(_{a1}\) is 4 times less than that of t-PA. In both proteins, O-linked \( \text{fucose} \) may contribute to clearance, but it cannot account for the significantly different clearance rates. The half-lives of these proteins therefore seem to be regulated by different mechanisms. The role of O-linked \( \text{fucose} \) as a recognition signal and its contribution to clearance rates remain to be established.

![Fig. 6. Detail of the RP-HPLC tryptic maps obtained by small scale tryptic digestion of recombinant (A) and natural (B) DSPA\(_{a1}\).](image)

**Table II**

Partial amino acid sequences of EGF-like domains modified by O-linked \( \text{fucose} \)

Amino acids characterizing the suggested consensus sequence are printed in boldface type. The fucosylation sites are underlined.

| Protein  | Origin | Partial Amino Acid Sequence | Residues | Reference |
|----------|--------|-----------------------------|----------|-----------|
| DSPA\(_{a1}\) | Bat    | CSEPRCRCFNFGGCGCWQAVERYFSDFVCC | 51-73    | 13        |
| t-PA     | Human  | CSEPRCRCFNFGGCGCWQAVERYFSDFVCC | 51-73    | 44        |
| Urokinase| Human  | PSNCPCLNCCVSNKYSNIHWC | 8-30     | 45        |
| Factor VII| Human  | CASSPCNCNQCSSCKDQLQSYICFC | 50-72    | 46        |
| Factor VII| Bovine| CASSPCNCNQCSSCKDQLQSYICFC | 50-72    | 47        |
| Factor IX | Human  | CSNPCNLNGCSSCKDINYESCWC | 51-73    | 48        |
| Factor XII| Human  | CSHKSPCQKGGRCCVNMPGSPHCCL | 79-102   | 49        |
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