Tissue Plasminogen Activator Has an O-Linked Fucose Attached to Threonine-61 in the Epidermal Growth Factor Domain

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ABSTRACT: An unusual type of glycosylation has been observed for tissue plasminogen activator (t-PA). The monosaccharide fucose is glycosidically linked to threonine-61 in the epidermal growth factor region of t-PA. The presence of O-linked fucose was demonstrated by carbohydrate analysis and mass spectrometry of tryptic and chymotryptic peptides that contain this site. The susceptibility of the fucose residue to α-fucosidase indicated that it was in the α-anomeric configuration. Fucosylation of threonine-61 was observed in t-PA isolated from the Bowes melanoma cell line and from recombinant expression systems using Chinese hamster ovary or human embryonic kidney cells. Fucosylation of the homologous residue in prourokinase has also been reported recently. Our results indicate that this novel type of glycosylation may be common to the epidermal growth factor domains found in coagulation and fibrinolytic proteins and, therefore, suggest that the modification may have functional significance.

Tissue-type plasminogen activator (t-PA)[1] is a glycoprotein that converts plasminogen to plasmin in the presence of a fibrin clot; the plasmin thus formed then degrades the clot (Hoylaerts et al., 1982, Rijken et al., 1982). Recombinant t-PA is used clinically as a thrombolytic agent for the treatment of myocardial thrombosis. Purified t-PA has been isolated from several sources, including the Bowes melanoma cell line (Rijken & Collen, 1981, Wallen et al., 1983) and transfected Chinese hamster ovary (Vehar et al., 1984) and human embryonic kidney (293) cells (Higgins et al., 1990). The amino acid sequence of t-PA was originally deduced by cDNA cloning (Pennica et al., 1983) and later confirmed by both genomic cloning (Ny et al., 1984) and peptide mapping experiments (Pohl et al., 1984). The t-PA molecule comprises several domains that are commonly found in coagulation and fibrinolytic proteins: a type I finger domain, an epidermal growth factor like region, two kringle domains, and a serine protease (Pennica et al., 1983; Banyai et al., 1983).

The Asn-linked carbohydrate moieties of melanoma-derived and CHO-cell-expressed recombinant t-PA have been extensively characterized (Pohl et al., 1987; Spellman et al., 1989; Parekh et al., 1989a,b); these were found to have attached high mannose oligosaccharides at Asn-117, with mainly fucosylated complex-type oligosaccharides attached at Asn-184 and Asn-448. The Asn-184 site is glycosylated in approximately 50% of the t-PA molecules from either source.

No O-linked carbohydrates have yet been reported for t-PA. The fucose content of rt-PA has been reported as 2.5 mol/mol of protein (Vehar et al., 1986), but only 1.5 mol of fucose can be accounted for from the fucosylated N-linked oligosaccharides. This led us to examine the possibility that there is a fucose residue directly O-linked to a threonine or serine residue of t-PA in a fashion similar to that recently reported for prourokinase (Kentzer et al., 1990). Here we report that the t-PA molecules from the Bowes melanoma and two recombinant cell lines (Chinese hamster ovary and human embryonic kidney) have fucose attached via a glycosidic linkage of the α-anomeric configuration to Thr-61 in the epidermal growth factor domain.

Materials and Methods

Melanoma-derived t-PA was obtained from Dr. Desire Collen (University of Leuven, Belgium), while the CHO-cell expressed rt-PA studied was that produced by Genentech (Activase). Human embryonic kidney (293) cell-derived rt-PA was prepared as described (Higgins et al., 1990). Chymotrypsin and TPCK-treated trypsin were purchased from Worthington (Freehold, NJ). TFA was from Pierce (Rockford, IL). Chicken liver α-fucosidase was purchased from Oxford GlycoSystems (Rosedale, NY).

Peptide Mapping. All of the t-PA samples were S-carboxymethylated as described (Chloupek et al., 1989), then exchanged into a buffer containing 10 mM Tris, 100 mM NaOAc, and 1 mM $CaCl_2$ at pH 8.0, and treated with TPCK-trypsin for 8 h at 37 °C with aliquots (1/100 w/w) of the enzyme added at 0 and 4 h. Peptide separations were performed with a Hewlett-Packard 1090M HPLC system equipped with a Vydac C18 column (4.6 × 250 mm) operating at 30 °C with a flow rate of 1.0 mL/min. After equilibration with 0.1% TFA (solvent A), elution of peptides was carried out by using a two-stage linear gradient starting 3 min after sample injection that went from 0% to 25% solvent B (0.1% TFA in acetonitrile) in 50 min and then from 25% to 60% solvent B in 35 min.

Further digestion of the HPLC-purified T8 peptide was achieved by incubation with chymotrypsin (0.125 μg/nmol of peptide) for 24 h at room temperature in 0.1 M NH_4HCO_3. Separations of the chymotryptic fragments of the T8 peptide were performed with the HPLC system described above operating at 0.25 mL/min with a Vydac C18 column (2.1 × 250 mm) and a linear gradient from 0% to 60% solvent B in 60 min starting 5 min after sample injection.

[1] Abbreviations: t-PA, tissue-type plasminogen activator; mt-PA, t-PA isolated from Bowes melanoma cells; rt-PA, t-PA isolated from Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells transfected with the gene for human t-PA; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; RCM, reduced and S-carboxymethylated; PTH, phenylthiohydantoin; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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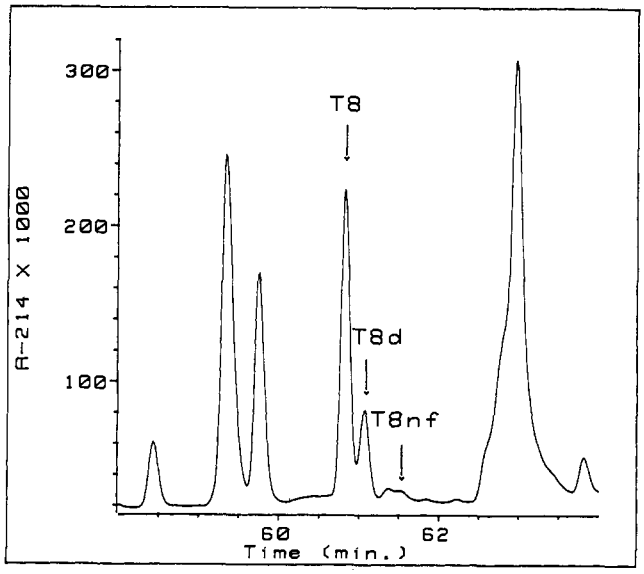

FIGURE 1: Detail of the RP-HPLC tryptic map of 2 nmol of RCM rt-PA. The elution positions of the T8 peptide (residues 56–82) and its deamidated (T8d) and nonfucosylated (T8nf) forms are indicated. Chromatography conditions are described under Materials and Methods.

Peptide Analyses. Samples for amino acid analysis were subjected to hydrolysis for 24 h in 6 N HCl at 110 °C in vacuo. Amino acid analyses were performed on a Beckman Model 6300 analyzer. N-Terminal sequence analyses were performed with an ABI Model 477A/120A system. Carbohydrate compositions were determined by high-pH anion-exchange chromatography with pulsed amperometric detection using a Dionex BioLC system (Hardy et al., 1988) on samples that had been subjected to hydrolysis at 125 °C for 1 h with 4 N TFA. Mass spectrometry was performed with a SCIEX API III triple quadropole instrument operating in the positive ion mode by atmospheric pressure chemical ionization (Covey et al., 1988). For alkaline β-elimination experiments, peptide samples were incubated in 0.1 N NaOH for 16 h at 55 °C (Anderson et al., 1964) and then neutralized with acetic acid. Incubation of the T8 peptide (5 nmol) with 0.2 unit of α-fucosidase in a buffer containing 100 mM sodium citrate–phosphate at pH 6 for 18 h at 37 °C was used to determine whether the glycosidic linkage was the α or β configuration.

Results

Numbering sequentially from the N-terminus, the eighth of the expected tryptic fragments (comprising residues 56–82 of t-PA) is referred to as T8. The region of the RP-HPLC profile of trypsin-digested RCM rt-PA where T8 elutes is given in Figure 1. The main peak fraction containing the T8 peptide was identified by amino acid analysis. Monosaccharide composition analysis of hydrolysates of the T8 peptide revealed the presence of 0.8 mol of fucose/mol of peptide. No other monosaccharides were detected in the T8 hydrolysates.

Covalent attachment of the fucose was demonstrated by mass spectrometric analysis using atmospheric pressure chemical ionization. This type of ionization generates multiply charged forms of the peptide (Covey et al., 1988). The expected mass of the S-carboxymethylated T8 peptide is 3153.3 without fucose or 3299.5 with one fucose residue attached. The results (Figure 2) clearly show doubly, triply, and quadruply charged forms that correspond to the latter (a calculated average mass of 3299.8).

The T8 peptide contains an Asn-Gly sequence that is highly susceptible to deamidation during incubation at high pH (Bornstein & Balian, 1977) during the S-carboxymethylation procedure. The deamidated form of T8 eluted 0.25 min later than unmodified T8 in the tryptic map (Figure 1). A minor nonfucosylated form of T8 (observed mass of 3154.2) eluting 0.4 min after the deamidated form was also observed.

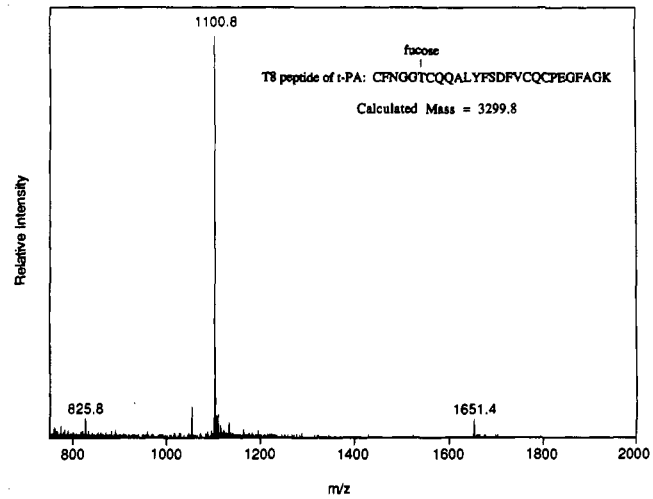

FIGURE 2: Positive ion mass spectrum of T8 from RCM rt-PA. Indicated are the doubly (1651.4), triply (1100.8), and quadruply (825.8) charged forms of fucosylated T8. The peptide mass (M) for each of these forms is derived from the formula $M = [(m/z)n - n]$, where n is the net charge. The calculated mass is the mean of these values (Covey et al., 1988).

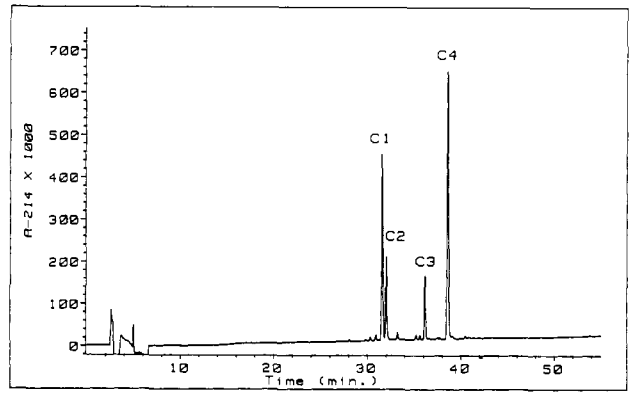

FIGURE 3: RP-HPLC purification of fragments generated by chymotryptic digestion of the T8 peptide. Conditions are described under Materials and Methods. The peptide identities of the labeled peaks are given in Table I.

Table I: Identification of the Chymotryptic Fragments of the T8 Peptide by Mass Spectrometry[a]

HPLC fraction	calcd mass +fucose	calcd mass −fucose	obsd mass	peptide identity
C1	1566.6	1420.4	1566.4	CFNGGTCQQALY + fucose
C2	1567.6	1421.4	1566.8	CFDGGTCQQALY + fucose
C3	1897.1	1750.9	1750.4	FSDFVCQCPEGFAGK
C4	1640.8	1494.6	1493.9	FSDFVCQCPEGF

[a] The average molecular masses are given.

We further digested the fucosylated T8 peptide from rt-PA with chymotrypsin in order to generate fragments that would contain only one of the possible O-glycosylation sites (Thr-61 or Ser-69). These fragments were purified by RP-HPLC (Figure 3) and identified by mass spectrometry (Table I). Peak C1 contains a fragment (residues 56–67) that includes the Thr-61 site. The observed mass (1566.4) is consistent with the attachment of one fucose residue within this fragment; peak C2 is the deamidated form of the same peptide. The C3 (residues 68–82) and C4 (residues 68–79) peaks contain portions of the T8 peptide that include Ser-69 but not Thr-61;

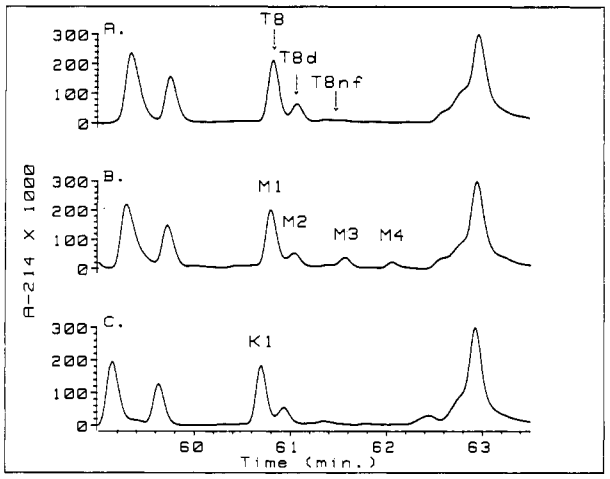

FIGURE 4: Detail showing the RP-HPLC tryptic maps of CHO-cell rt-PA (A), mt-PA (B), and HEK-cell rt-PA (C). The peaks labeled T8d and T8nf are the deamidated and nonfucosylated forms of T8, respectively. The peaks numbered M1–M4 and K1 in the lower profiles were collected for identification.

these peptides were both found to be unmodified.

The localization and nature of the linkage between fucose and the peptide were further examined under conditions intended to promote β-elimination of O-linked carbohydrates (Anderson et al., 1964). Alkaline treatment would be expected to result in elimination of fucose with concomitant loss of the attachment threonine as a consequence of its conversion to α-aminocrotonic acid, which is destroyed during acid hydrolysis. After incubation at high pH, only 0.1 mol of threonine was recovered by amino acid analysis from the C1 peptide due to β-elimination of the fucose. As a control, the chymotryptic fragment containing the same residues (56–67) from the nonfucosylated T8 peptide was also subjected to incubation at high pH prior to acid hydrolysis; this fragment showed 0.5 mol of threonine. The combined results show that Thr-61 is the site of the fucose attachment.

The anomeric configuration of the glycosidic linkage between fucose and threonine was examined by digestion with α-fucosidase. Treatment with α-fucosidase shifted the RP-HPLC elution of the T8 peptide to the position of the nonfucosylated form of the peptide. Removal of fucose was confirmed by mass spectrometry (an observed mass of 3153.5).

Isoaspartyl bonds at the expected position of the Asn–Gly bond (residues 58–59) in the deamidated form of T8 (residues 56–82) and the chymotryptic fragment C2 (residues 56–67) were demonstrated by Edman degradation (data not shown). The deamidated form of T8 represents approximately 22% (by area) of total T8 in each of the S-carboxymethylated t-PA preparations that we studied. This consistency suggests that deamidation is an artifact of sample preparation. Similarly, the nonfucosylated T8 fraction consistently appears at a level of 4–5%. Given the base lability of the fucose residue, it is also likely that this minor species results from sample preparation.

To address the question of whether the attachment of O-linked fucose to Thr-61 was unique to the CHO-cell expression system, t-PA preparations from two other cell lines were also examined. The tryptic map profiles for mt-PA and HEK-cell-expressed rt-PA were nearly identical with CHO-cell-expressed rt-PA (Figure 4). The peaks corresponding to T8 (peaks M1 and K1 of Figure 4) were subjected to mass spectrometric analysis. The masses observed for the T8 fractions from mt-PA and HEK rt-PA were 3299.6 and 3299.9, respectively, corresponding to the mass of fucosylated T8. The identical RP-HPLC retention times and peptide masses for the T8 peptide from each source indicate that the same type of fucosylation that was found for T8 from CHO-cell-expressed rt-PA is also found on T8 from mt-PA and HEK rt-PA.

The peak that elutes just after T8 in the mt-PA tryptic profile (M2 of Figure 4) is the deamidated fucosylated form of T8. Two new peaks in the mt-PA profile were also identified by N-terminal sequence analysis. Peak M3 is a tryptic fragment of a contaminating protein (concanavalin A) introduced during the purification procedure (Rijken & Collen, 1981), while peak M4 contains the 280–296 fragment of t-PA.

Discussion

We have demonstrated that the monosaccharide fucose is glycosidically linked to Thr-61 in the growth factor domain of t-PA. The linkage was found to have the α-anomeric configuration. This glycosylation does not appear to be cell-type specific, as it has been found on t-PA isolated from the Bowes melanoma cell line as well as on rt-PA from transfected CHO and HEK cells. Physiological t-PA is synthesized in endothelial cells, but the paucity of such material in plasma (1–5 ng/mL) precludes the examination of endothelial cell t-PA. Fucosylation of the N-linked oligosaccharides of CHO-cell-expressed rt-PA can account for approximately 1.5 mol of fucose/mol of protein (Spellman et al., 1989). The observation of an additional mole of directly O-linked fucose brings the expected total to 2.5, which matches the value observed for the intact protein (Vehar et al., 1986). Therefore, we do not expect that any other sites contain fucose in a fashion similar to Thr-61.

Prior primary structure characterization studies on t-PA (Pohl et al., 1984; Chloupek et al., 1988) overlooked this structure as it is extremely acid-labile; consequently, it is not readily apparent during amino acid analysis or N-terminal sequence analysis. Close examination of the PTH-amino acid analysis profile obtained at the Thr-61 position during N-terminal sequence analysis of the T8 peptide revealed that, in addition the PTH-Thr and its β-elimination products, a minor peak that elutes just before PTH-Ser is present. Experiments are underway to determine whether this corresponds to the PTH derivative of fucosylthreonine.

Pohl et al. (1984) found heterogeneity in the elution position of the T8 peptide in the RP-HPLC tryptic map of mt-PA. They speculated that this elution heterogeneity might be due to O-glycosylation because they recovered hexosamines (presumably galactosamine) in one of their T8 HPLC fractions. Carbohydrate analysis did not reveal any hexosamines in our mt-PA T8 fractions. The T8 elution heterogeneity that we have observed results from deamidation at the Asn–Gly bond (residues 58–59 of t-PA) and the presence of a minor nonfucosylated form.

A recent, preliminary report for prourokinase is the only other observation of this type of protein modification (Kentzer et al., 1990). However, these investigators did not demonstrate conclusively the attachment residue or the nature of the linkage between fucose and the polypeptide. They also left open the question of whether the modification was a manifestation of the particular cell line used or whether it was a more general phenomenon.

Prourokinase is a fibrinolytic protein similar to t-PA, comprising a growth factor domain, one kringle, and a serine protease. As fucosylation of prourokinase and t-PA occurs at the homologous threonine residue within their respective growth factor domains, it is likely that this type of modification will be found in other related proteins. Many human coagu-

lation and fibrinolytic proteins contain growth factor domains (Davie et al., 1986; Doolittle, 1989). An examination of the Genentech protein sequence databank showed that factor XII, protein C, and transforming growth factor α all have threonine in the homologous position as was found fucosylated in t-PA, while factor VII, factor IX, and protein S have serine at this position. Growth factor domains are also found in factor X, thrombomodulin, the low-density lipoprotein receptor, and epidermal growth factor, but these do not have threonine or serine in the appropriate position. The threonine that is fucosylated in human t-PA is also conserved in rat and mouse t-PA.

O-Linked fucose is a novel carbohydrate structure. The most common ("mucin-type") mammalian O-linked structures have *N*-acetylgalactosamine attached to threonine or serine (Kornfeld & Kornfeld, 1976). Hart et al. (1989) have observed O-linked *N*-acetylglucosamine as a constituent residue of some nuclear pore, chromatin, and cytoskeletal proteins. The O-linked fucose described here is unusual because it appears on a secreted serum glycoprotein. Because the glycosidic linkage is both base- and acid-labile, it is possible that this modification has previously been overlooked because it was lost during protein purification.

The complete fucosylation at Thr-61 in t-PA suggests that this modification may have a functional role. The functional significance of the epidermal growth factor domains found in coagulation/fibrinolysis proteins is not known. The O-linked fucose may influence the interaction between such domains and their ligand(s). A reexamination of the posttranslational modifications of the other O-fucosylation candidate proteins should help to clarify the role of this modification. The identification of the fucosyl transferase and its subcellular localization and substrate specificity also merit investigation.

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Registry No. t-PA, 105913-11-9; Thr, 72-19-5; fucose, 2438-80-4.

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