THE GLYCOPROTEIN NATURE OF HUMAN PLASMINOGEN ACTIVATORS

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1. Introduction

Protein glycosylation may have a function in protein secretion [1], orientation of membrane proteins [2,3], protein conformation [4], or in protection of proteins from degradation [5]. Because of our interest in the biochemistry of human plasminogen activators, we have utilized several different techniques to determine whether plasminogen activators as they are secreted by many human cell lines in vitro [6–8] might be glycoproteins. Gas chromatography results described here demonstrate that acid hydrolysates of purified human urokinase (a urinary PA) contain monosaccharides characteristic of other glycoproteins, with mannose, galactose, *N*-acetylglucosamine and *N*-acetylneuraminic acid in approximate molar ratios of 3.3:2.3:4.2:3.0, respectively. Further, urinary urokinase, and both immunologic types of PA secreted by Detroit 562, a human pharyngeal carcinoma cell line [8], bind to lentil lectin Sepharose, and are specifically eluted with α-methyl-mannoside. To confirm the glycoprotein nature of the urokinase-like PA [8] secreted by Detroit 562 cells, the protein was metabolically labeled with [^{3}H]glucosamine and isolated by immunoadsorption chromatography [9]. As expected, the isolated PA contained [^{3}H]glucosamine. Taken together, these results indicate that human PA are glycoproteins.

2. Materials and methods

2.1. *Urokinase purification and carbohydrate analysis*

Urokinase (5×10^5 CTA units 'Winkinase', Sterling Winthrop; $6–7 \times 10^4$ CTA units/mg protein) was purified further by *p*-aminobenzamidine–Sepharose affinity chromatography [10]. Following elution with 0.5 M arginine, 0.4 M NaCl, 0.1 M phosphate buffer (pH 7.5), the urokinase was concentrated ~20-fold by dialysis against dry Biogel P-150. Urokinase was then purified further by gel filtration on a 0.9×60 cm column of Biogel P-150 equilibrated and eluted with 0.1 M NH_4HCO_3 (pH 7.5). The NH_4HCO_3 was removed by 2 cycles of lyophylization, and the sample was dissolved in 0.3 ml distilled water and stored at −20°C. Electrophoresis on an SDS–10–15% polyacrylamide gradient slab gel of an unreduced aliquot of this sample revealed 3 protein bands with app. mol. wt 59–64, 35 and 19.5×10^3; these values are similar to those published for high-molecular-weight urokinase and its component polypeptide chains [11,12]. Urokinase samples were prepared for carbohydrate analysis by methanolysis and re-*N*-acetylation of amino sugars [13–15]. Trimethylsilyl ethers of the methylglycosides were separated on a 10 ft × 1/8 in. column of OV 17 on Chromasorb (20–100 mesh) (Pierce Chemical Co.) with the temperature programmed at 5°C/min from 125–260°C, followed by a 5 min isothermal period. A Packard model 420 Becker gas chromatograph with a flame ionization detector was used. Arabitol was used as an internal standard and sugars were identified by comparison with a standard mixture of sugars and those derived by methanolysis of fetuin.

2.2. *[^{3}H]Glucosamine labeling and immunoadsorption*

A detailed description of the technique used for immunoadsorption using a specific anti-urokinase Ig column can be found in [9]. For labeling secreted proteins, confluent cultures of pharyngeal carcinoma-derived Detroit 562 cells [8] in 2 plastic roller bottles were each incubated in 40 ml serumless RPMI1640 medium containing 10% of the normal amount of

Abbreviations: PA, plasminogen activator(s); SDS, sodium dodecyl sulfate; I; LcH, *Lens culinaris* hemagglutinin

glucose, 100 U/ml penicillin and 100 μg/ml streptomycin, and 1 mCi [^{3}H]glucosamine (38 Ci/mmol, Amersham) for 24 h. After immunoadsorption the eluted proteins were dialyzed immediately at 4°C against 0.1% SDS (British Drug Houses, special pure), lyophylized and analyzed by SDS gel electrophoresis.

2.3. *Other methods*

SDS–10–15% polyacrylamide slab gels were prepared as in [16] and the separated proteins were stained with Coomassie blue or the gels were sliced and assayed for plasminogen activator activity in ^{125}I-labeled fibrin-coated wells [8]. PA activity in *Lens culinaris* hemagglutinin–Sepharose column eluates was measured in ^{125}I-labeled fibrin-containing wells as in [17], except that the assays were incubated at 37°C for 15 min. Radioactivity in gel slices was measured by solubilizing the gel slice in 0.1 ml 30% H_2O_2 followed by scintillation counting in 10 ml Hydrofluor (National Diagnostics). LcH (Miles-Yeda) was coupled to CNBr–Sepharose as in [18]. Sugars were obtained from Pfanstiehl Lab.

3. Results and discussion

Over 90% of the PA activity from Detroit 562 harvest fluids is bound by LcH–Sepharose columns, but only 68% of the PA activity in urokinase containing media is bound (table 1). Binding was preferentially inhibited by 0.1 M α-methylmannoside as compared to other sugars. SDS gel analysis of Detroit 562 PA α-methylmannoside eluants (data not shown) demonstrated that they contain both the 73 × 10^3 mol. wt and the 55–60 × 10^3 mol. wt (urokinase-like) PA expected for Detroit 562 [8]. Thus, the lectin binding behavior of urinary urokinase, and both kinds of PA secreted by Detroit 562 cells suggests that these PA are glycoproteins with accessible carbohydrates in the α-mannosyl configuration [19]. The partial (68%) binding of urinary urokinase to LcH–Sepharose may be due to carbohydrate heterogeneity, incomplete glycosylation during synthesis, or degradation of the carbohydrate chain in urine or during enzyme isolation.

Since human urokinase has already been purified to homogeneity [11], and is available in large amounts, the carbohydrate composition of highly purified urokinase was confirmed by gas chromatography. The quantities and molar ratios of the carbohydrates observed are listed in table 2. When precautions were taken in sample preparation to avoid leaching of carbohydrates from Sephadex or dialysis tubing, <0.2 μg glucose/100 μg protein was observed. The molar ratios

Table 1
Binding of PA activity to LcH–Sepharose

A.	Percent of PA activity bound	
	Detroit 562 culture medium	Urinary urokinase
no sugar	95	68
0.01 M α-methylmannoside	63	32
0.1 M α-methylmannoside	11	5
0.1 M D-glucose	85	62
0.1 M D-galactose	92	60
0.1 M D-*N*-acetylglucosamine	79	62
B.	Initial activity recovered	
0.1 M α-methylmannoside	75%	56%

Samples of enzyme (0.15 ml) in PBS (Gibco, K-13) buffer, 0.1 mg/ml bovine serum albumin and the respective sugar were applied to a 0.3 ml column of LcH–Sepharose, and eluted with 5 column vol. PBS buffer containing BSA bovine serum albumin and the respective sugar. The samples were diluted appropriately and assayed. In (B) samples were adsorbed in the absence of sugar and eluted from the column with 5 column vol. PBS buffer containing 0.1 M α-methylmannoside

Table 2
Carbohydrate composition of urokinase

	μg/100 μg protein[a,b]	moles/55 000 daltons
Mannose	1.07 ± 0.10	3.3
Galactose	0.76 ± 0.16	2.3
N-Acetylglucosamine	1.76 ± 0.25	4.2
N-Acetylneuraminic acid	1.66 ± 0.27	3.0
Fucose	⩽0.28	⩽0.9
N-Acetylgalactosamine	⩽0.36	⩽0.9

[a] Protein was assayed as in [25], using bovine serum albumin as the standard

[b] Values are mean ± standard deviation of triplicate gas chromatographic determinations on 2 separate protein hydrolysates

of mannose, galactose, *N*-acetylglucosamine and *N*-acetylneuraminic acid in table 2 are comparable to those of asparagine-linked complex oligosaccharides described for Ig [20] and vesicular stomatitis virus glycoprotein [21]. The presence of small amounts of *N*-acetylgalactosamine suggests the possible presence of an oligosaccharide containing an *O*-glycosidic linkage [20].

At present, insufficient urokinase-like Detroit 562 PA has been isolated to permit chemical determination of its carbohydrate composition by gas chromatography, however its glycoprotein nature was confirmed by metabolic labeling of the 55–60 × 10^3 mol. wt protein with [^{3}H]glucosamine, and subsequent isolation of the ^{3}H-labeled PA using a specific anti-urokinase Ig–Sepharose immunoadsorption technique [9]. We have shown [9] that both a 55 × 10^3 and a 60 × 10^3 mol. wt urokinase-like PA are secreted by Detroit 562 cells, and that purified urinary urokinase preparations contain both of these species (fig.1C). The glycoprotein nature of the urokinase-like Detroit 562 protein is illustrated by comparison of fig.1A and B. Fig.1A shows that the [^{3}H]glucosamine-labeled anti-urokinase Ig–Sepharose column eluants from Detroit 562 contain a large ^{3}H-labeled peak in the 55–60 × 10^3 dalton region (fig.1A) which comigrated with urokinase-like PA enzyme activity (fig.1B). This PA activity was almost totally inhibitable by anti-urokinase IgG (data not shown). A smaller peak of ^{3}H radioactivity was observed with 33 × 10^3 mol. wt the size of a previously described low molecular weight form of urokinase [22]. Some ^{3}H-labeled peaks in fig.1A were observed >68 × 10^3 mol. wt, and may

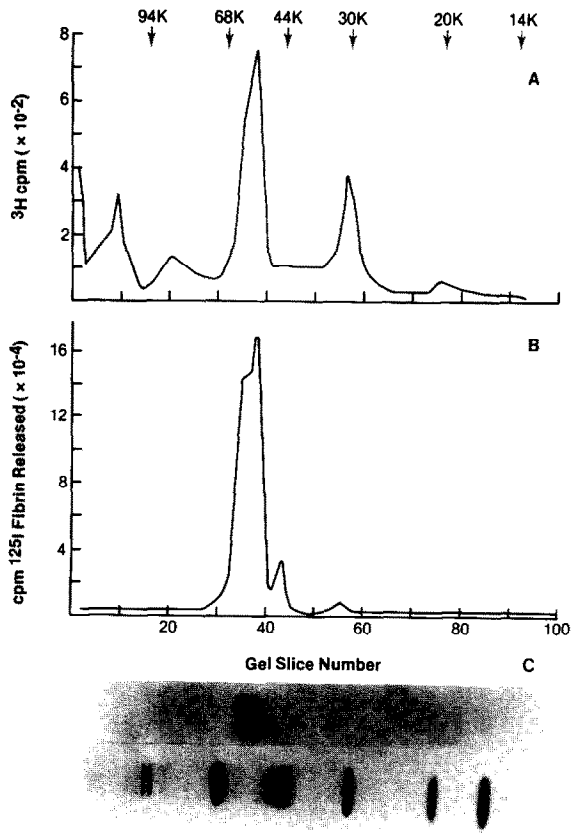

Fig.1. SDS–10–15% polyacrylamide gel electrophoresis of proteins eluted from specific anti-urokinase IgG–Sepharose column. (A) ^{3}H cpm from components of Detroit 562 cell conditioned medium labeled with [^{3}H]glucosamine. (B) PA activity of components from Detroit 562 cell conditioned medium. (C) Coomassie blue-stained protein bands from Detroit 562 conditioned medium (top), and molecular weight markers of (× 10^3) 94, 68, 44, 30, 20, 14 (bottom).

be aggregates of the 55–60 × 10^3 and 33 × 10^3 mol. wt components. To establish that the ^{3}H radioactivity in the Detroit 562 proteins was still associated with carbohydrate residues, we hydrolyzed an aliquot of the material shown in fig.1A, added small amounts of unlabeled glucosamine and galactosamine, and separated the products on an amino acid analyzer. Of the recovered ^{3}H radioactivity, 48% and 30% co-chromatographed with the glucosamine and galactosamine, respectively (data not shown). Thus, our results indicate that the urokinase-like 55–60 × 10^3 mol. wt PA from Detroit 562 cells is a glycoprotein containing glucosamine and some galactosamine. Our demonstration that urokinase is a glycoprotein containing sialic acid is compatible with the observed heterogeneity of

this enzyme upon isoelectric focusing [10]. Also, by analogy to our results, chick cell PA may be a glycoprotein, and thus the stimulatory effect of retinoic acid on secretion of chick cell PA activity [23] may be the result of the stimulatory effect of retinoic acid on carbohydrate incorporation into glycoproteins [24].

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