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Apolipoprotein D is the major protein component in cyst fluid from women with human breast gross cystic disease

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GCDFP(gross-cystic-disease-fluid protein)-24, a progesterone-binding protein present in large amounts in cyst fluid from human breast gross cystic disease, was purified in a one-step procedure by size-exclusion h.p.l.c. Peptide fragments obtained by trypsin digestion of the intact protein were purified by reverse-phase h.p.l.c. and analysed for their amino acid composition and subjected to automated Edman degradation. A search of the National Biomedical Research Foundation Data Bank revealed that all the sequenced tryptic peptides from protein GCDFP-24 matched perfectly with regions present in the amino acid sequence determined for human apolipoprotein D. Additional data on *N*-terminal sequence of the unblocked proteins, carbohydrate-attachment sites, amino acid composition and molecular-mass estimations supported the identity between both molecules. On the basis of this identity a possible role of apolipoprotein D in progesterone transport is proposed.

INTRODUCTION

Gross cystic breast disease is very common in premenopausal women, affecting about 7% of this population [1]. Although the cysts themselves are only rarely precancerous, several studies over the last two decades [2–6] have indicated that patients with breast cysts are at a 2–4-fold greater risk of developing breast cancer than the normal female population.

The biochemical composition of cyst fluid has been studied in order to understand the mechanisms controlling cyst formation as well as to define their possible role in carcinogenesis. Data are available for the ionic composition [7], enzyme levels [8], hormone content [9] and protein components [10–13]. These analyses have revealed the presence of several unusual proteins that seem to be specific secretory products of epithelial cells surrounding the cysts [14]. These proteins have been named GCDFP-70, -44, -24 and -15 (indicating gross-cystic-disease-fluid proteins), with the numbers being in accordance to their respective molecular masses GCDFP-70 is immunologically identical with plasma albumin, although it is present in cyst fluid at approx. 100-fold lower concentration than in plasma. GCDFP-44 is Zn-α_2-glycoprotein found at 10 times its concentration in plasma. GCDFP-15 is present in small amounts in the plasma of normal women, but its amino acid sequence does not reveal any significant similarity to that of other known human proteins [14]. Finally, and in spite of the fact that GCDFP-24 accounts for over half the total protein present in cyst fluid, very little is known about its source, biological function and biochemical characteristics. GCDFP-24 has been described as a glycoprotein with progesterone-binding activity [15] and is immunologically identical with a component of human plasma. However, antisera against 30 components of normal plasma failed to identify GCDFP-24 as one of these plasma proteins [14]. Some authors have suggested that this protein may represent a previously unidentified component of human plasma probably presenting proteinase activity [14,16]. To investigate these possibilities, studies were undertaken to perform a detailed molecular characterization of this major protein component of cyst fluid. According to the amino-acid-sequence analysis of the purified protein, we report herein that GCDFP-24 is apolipoprotein D, a component of high-density lipoprotein in human plasma involved in cholesterol transport [17]. The possible implications of apolipoprotein D in the binding and transport of progesterone are also discussed.

EXPERIMENTAL

Materials

Cyst fluid was obtained, with informed consent, by needle aspiration from patients under treatment for gross cystic disease. It was centrifuged at 35000 *g* for 1 h at 4 °C and stored at −20 °C until used. Trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] was obtained from Merck. Pyroglutamate aminopeptidase was from Boehringer-Mannheim. H.p.l.c. reagents were from Carlo Erba, Milan, Italy. Triethylamine and phenyl isothiocyanate were from Pierce. Reagents for amino acid sequencing were from Applied Biosystems. Other chemicals used were of the highest reagent grade available.

Protein purification

Cyst fluid containing about 2 mg of protein was applied to a TSK-3000 SWG column (2.15 cm × 30 cm) equilibrated and eluted at a constant flow rate of 0.4 ml/min with 0.5 M-ammonium acetate, pH 6.5. The runs were carried out at room temperature using a Waters (Milford, MA, U.S.A.) h.p.l.c. apparatus equipped with a u.v. detector (model 481), an automated gradient controller (model 680) and two solvent-delivery systems (model 510). Measurements were made at 280 nm in the sensitivity range of 2.0. Fractions (1.0 ml) were collected and analysed by SDS/PAGE by the method of Laemmli [18].

Trypsin digestion

Purified protein GCDFP-24 (about 250 μg) was dissolved in 200 μl of 97% (v/v) formic acid, to which 300 μl of chilled performic acid were added. After incubation for 2 h in an ice/water bath, the mixture was diluted with cold distilled water and freeze-dried. Oxidized protein was dissolved in 0.1 M-ammonium bicarbonate, pH 8.5, and digested with trypsin for 16 h at 37 °C at an enzyme/substrate ratio of 1:50 (w/w).

Abbreviation used: GCDFP, gross-cystic-disease-fluid protein.

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Purification of tryptic peptides

Tryptic peptides from protein GCDFP-24 were fractionated by reverse-phase h.p.l.c. on a C_{18} Nova-Pak column (3.9 mm × 150 mm) equilibrated with 0.1% trifluoroacetic acid. The runs were carried out in the above-described chromatograph, at room temperature and at a flow rate of 0.8 ml/min. Peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid as indicated in Fig. 3 (below). The absorbance was monitored at 214 nm. Peaks were collected manually. The peptide-containing fractions were freeze-dried and subjected to amino acid analysis and sequence determination.

Amino acid analysis

Purified tryptic peptides were hydrolysed with 80 μl of 5.7 M-HCl containing 0.05% (v/v) 2-mercaptoethanol in evacuated and sealed tubes at 110 °C for 20 h. After reaction with phenyl isothiocyanate, the hydrolysates were analysed by reverse-phase h.p.l.c. as previously described [19].

Digestion with pyroglutamate aminopeptidase

Oxidized protein GCDFP-24 was dissolved in 0.1 M-sodium phosphate buffer, pH 8.0, containing 10 mM-EDTA, 5% (v/v) glycerol and 5 mM-dithiothreitol, and treated with pyroglutamate aminopeptidase for 4 h at 37 °C at an enzyme/substrate ratio of 1:40 (w/w).

Amino-acid-sequence analysis

Samples containing about 0.5 nmol of peptide were subjected to *N*-terminal sequence analysis by sequential degradation on an Applied Biosystems 477A sequenator in the presence of Polybrene [20]. The amino acid anilinothiazolinones were converted into phenylthiohydantoin derivatives in the automatic conversion flask of the sequenator and identified and quantified with an on-line phenylthiohydantoin analyser (model 120A) [21]. Computer analysis of protein sequences were performed by using the FASTA program of Pearson & Lipman [22] and the Protein Sequence Database of the Protein Identification Resource.

RESULTS

Purification of protein GCDFP-24 from cyst fluid

The different protein components in cyst fluid from human breast gross cystic disease were separated by size-exclusion h.p.l.c. and the results obtained are shown in Fig. 1. Analysis by SDS/PAGE showed that fractions corresponding to an elution volume of 65–66 ml contained GCDFP-24, the major protein component in the cyst fluid (Fig. 2). It is remarkable that, although fractions 100–110 in the chromatographic eluate show the highest absorbance, analysis by SDS/PAGE revealed the absence of protein components in these fractions. This high absorbance is probably due to the presence of small sized molecules that give to the cyst fluid a characteristic yellow–brown colour. Calibration of the column under the same chromatographic conditions (results not shown) revealed that GCDFP-24 is eluted in a volume corresponding to a molecular mass of about 100 kDa. Considering that SDS/PAGE indicates that the monomer size of the protein is 24 kDa, it is presumed that the protein is a tetramer in its native state; however, an abnormal chromatographic mobility of the protein due to its high carbohydrate content cannot be ruled out. The purified protein was subjected to automated Edman degradation in a gas–liquid-phase sequenator. However, no phenylthiohydantoin amino acid derivatives could be identified in two separate runs, suggesting that the protein GCDFP-24 was blocked at its *N*-terminal end.

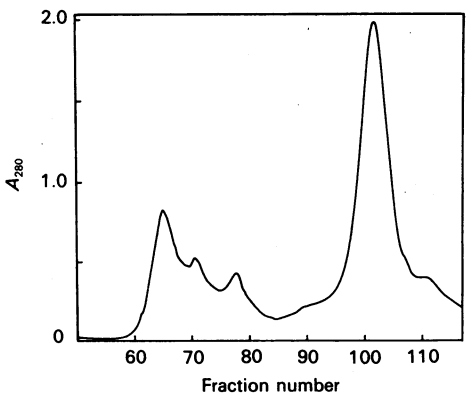

Fig. 1. Fractionation of GCDFP species by size-exclusion h.p.l.c.

Cyst fluid was applied to a TSK-3000 SWG column (2.15 cm × 30 cm) in 0.5 M-ammonium acetate, pH 6.5. The flow rate was 0.4 ml/min, and 1.0 ml fractions were collected.

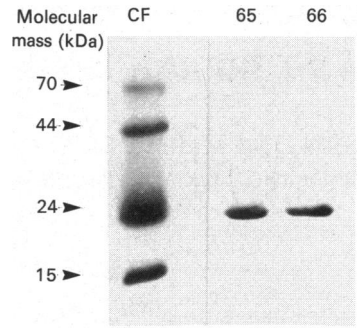

Fig. 2. PAGE of total proteins from cyst fluid and purified protein GCDFP-24

Aliquots of cyst fluid (CF, 1 μl) and indicated column fractions (65 and 66, 10 μl) were freeze-dried, treated with reducing SDS sample buffer and run on 10%-polyacrylamide/SDS Minigels. The gel was stained with Coomassie Blue.

Isolation and sequencing of tryptic peptides from protein GCDFP-24

Assuming that the *N*-terminal of protein GCDFP-24 was blocked, an alternative strategy involving proteolytic digestion of the purified protein was carried out to obtain information regarding internal amino acid sequences. Preliminary attempts showed that this protein was very resistant to proteolytic cleavage. Thus purified protein was previously oxidized with performic acid and then extensively treated with trypsin to generate multiple fragments. The tryptic peptides were fractionated by reverse-phase h.p.l.c. and the result obtained is shown in Fig. 3. The amino acid composition of the purified tryptic peptides, corresponding to the numbered peaks in Fig. 3, was determined and is shown in Table 1. The amino acid composition of the intact purified protein GCDFP-24 is also shown in Table 1.

Most of the peptides isolated by h.p.l.c. and further characterized by their amino acid compositions were directly subjected to automatic Edman degradation. Typically, 300–500 pmol of peptide was applied per run, and the amino acid sequences obtained are shown in Table 2. The repetitive yields of representative runs ranged from 92 to 95%. A search of the National Biomedical Research Foundation Data Bank revealed that all the sequenced tryptic peptides from protein GCDFP-24 could be matched with regions present in the amino acid sequence

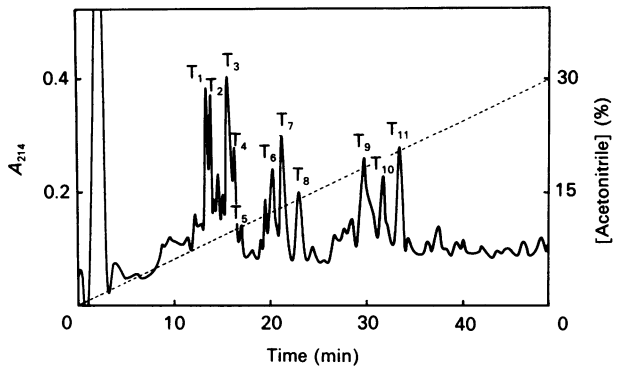

Fig. 3. Fractionation of the tryptic peptides from oxidized protein GCDFP-24 by reverse-phase h.p.l.c.

The tryptic digest was dissolved in 0.1% (v/v) trifluoroacetic acid and applied to a Nova-Pak column (3.9 mm × 150 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid. The elution of peptides was performed by means of a 0–30% gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min.

Table 2. Amino acid sequences of tryptic peptides from GCDFP-24 and comparison with sequence deduced from human apolipoprotein D(ApoD) cDNA

GCDFP-24 peptide	Amino acid sequence	Amino acid position in Apo D sequence
T_1	MTVTDQVNCPK	157–167
T_2	KMTVTDQVNCPK	156–167
T_3	CIQAXYSLMENGK	41–53
T_4	YLGR	22–25
T_5	XYEIEK	26–31
T_6	VLNQELR	56–62
T_7	CPNPPVQENFDVNK	8–21
T_8	IPTTFENGR	32–40
T_9	ADGTVNQIEGEATPVXLTEPAK	63–84
T_{10}	NPNLPPETVDSLK	132–144
T_{11}	NILTSNNIDVK	145–155
T_{12}	FSXFMPSAPYXILATDYENYALVYS CTCIIQLFXVDFAXILA	89–130

predicted from the cDNA sequence determined for human apolipoprotein D [17] (Table 2).

A large part of the sequence reported for apolipoprotein D was overlapped with the fragments isolated and sequenced from GCDFP-24 during the present study. The di- and tetra-peptides corresponding to positions 54–55, 168–169 and 85–88 were missed in h.p.l.c., probably owing to their small size. The largest tryptic peptide present in apolipoprotein D, corresponding to positions 89–131, was also missed. One possible explanation for the absence of this fragment could be that, owing to its large size, it was retained in the C_{18} column under the chromatographic conditions selected for the purification of tryptic peptides from GCDFP-24 (gradients up to 30% acetonitrile). To test this hypothesis, we made several attempts to isolate the above-mentioned peptide by mean of extensions of the acetonitrile gradient used for the elution. Finally we were able to isolate and identify an additional peptide (designated 'T_{12}'), which was eluted at about 56% acetonitrile. Its amino acid composition (Table 1) shows some discrepancies with that deduced from the corresponding apolipoprotein D peptide. However, these discrepancies could be mainly due to values not being corrected for destruction during hydrolysis or incomplete hydrolysis of this large fragment, since its amino acid sequence (Table 2) was identical with the corresponding region reported for apolipoprotein D.

Table 1. Amino acid composition of human GCDFP-24 and its isolated tryptic peptides*

Amino acid	Composition (molar ratio)													
	GCDFP-24	Peptide...	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}
Asp†	21.6 (23)		1.7 (2)	1.6 (2)	1.5 (2)			0.8 (1)	3.5 (4)	1.0 (1)	2.6 (3)	2.7 (3)	3.5 (4)	2.8 (3)
Thr	10.5 (11)		1.5 (2)	1.6 (2)						1.8 (2)	2.8 (3)	0.8 (1)	0.7 (1)	2.0 (2)
Ser	10.7 (7)				0.7 (1)							0.7 (1)	0.9 (1)	2.9 (3)
Glu†	20.5 (19)		1.2 (1)	1.1 (1)	1.8 (2)		2.1 (2)	1.8 (2)	2.1 (2)	1.1 (1)	3.5 (4)	1.1 (1)		2.8 (2)
Pro	12.0 (12)		0.8 (1)	0.8 (1)					3.5 (3)	1.2 (1)	1.6 (2)	2.8 (3)		2.6 (2)
Gly	7.8 (6)				1.2 (1)	1.3 (1)				1.3 (1)	2.3 (2)			
Ala	10.1 (10)				1.2 (1)						3.3 (3)			3.3 (5)
Cys‡	n.d. (5)		0.8 (1)	0.9 (1)	1.0 (1)				0.8 (1)					1.1 (2)
Val	12.3 (12)		1.9 (2)	2.1 (2)				0.9 (1)	1.6 (2)		1.9 (2)	1.1 (1)	1.0 (1)	2.4 (2)
Met§	3.7 (3)		0.7 (1)	0.6 (1)	0.5 (1)									0.9 (1)
Ile	9.6 (11)				0.8 (1)		1.0 (1)			1.0 (1)	1.0 (1)		2.1 (2)	2.4 (4)
Leu	15.8 (15)				1.2 (1)	1.2 (1)		2.1 (2)			1.2 (1)	2.3 (2)	1.3 (1)	3.8 (4)
Tyr	5.6 (7)				0.6 (1)	0.6 (1)	0.7 (1)							1.9 (4)
Phe	7.2 (7)								0.8 (1)	0.7 (1)				2.0 (4)
His	1.7 (2)													1.1 (1)
Lys	8.5 (11)		1.0 (1)	1.6 (2)	1.0 (1)		1.0 (1)		1.0 (1)		1.0 (1)	1.0 (1)	1.0 (1)	
Arg	4.6 (4)					1.0 (1)		1.0 (1)		1.0 (1)				1.0 (1)
Trp	n.d.‖ (4)						— (1)							— (3)

* The values in the Table are molar ratios without correction for destruction during hydrolysis or incomplete hydrolysis. Within parentheses are given the number of residues calculated from the sequence reported for apolipoprotein D.

† Asp and Glu determinations are Asp + Asn and Glu + Gln respectively.

‡ Determined in peptides as cysteic acid.

§ Determined in peptides as methionine sulphone.

‖ n.d.: not determined.

The asparagine residues corresponding to positions 5 and 16 from peptides T-3 and T-9 respectively were obtained in very low yields in the sequential degradations. The amino acid composition of the corresponding tryptic peptides is compatible with the presence of aspartic acid or asparagine residues at these positions (Table 1). In addition, both residues are present in *N*-glycosylation consensus sequences Asn-Xaa-(Ser/Thr). These data strongly suggest that these two asparagine residues from GCDFP-24 contain *N*-linked carbohydrate groups. Further comparison with the sequence reported for apolipoprotein D supported these results, since glycosylation has been found to occur at the same positions [17].

N-Terminal sequence analysis of protein GCDFP-24

The availability of the sequence data reported for apolipoprotein D suggested an additional experiment to provide further confirmation on the fact that purified protein GCDFP-24 from cyst fluid is intact apolipoprotein D. According to these data [17] the mature apolipoprotein D showed cyclized glutamine as *N*-terminal residue, which was released by treatment with pyroglutamate aminopeptidase. In the present work we used the same strategy in order to unblock the *N*-terminal of purified protein GCDFP-24. Oxidized protein was treated with pyroglutamate aminopeptidase and then subjected to automated Edman degradation. The resulting amino acid sequence (AFHLGKCPN) was identical with the one reported for mature apolipoprotein D.

DISCUSSION

It has been suggested that human gross cystic breast disease may be closely linked with an abnormality in the production and in the physiological function of the proteins present in the fluid filling the cysts. However, it is striking that protein GCDFP-24, which accounts for most of the protein content in cyst fluid [14], has not yet been characterized at the molecular level. In the present paper, and on the basis of amino-acid-sequence analysis of the purified protein, we present evidence that this major protein component of cyst fluid is apolipoprotein D. To our knowledge this is the first report in which high levels of apolipoprotein D are found in a fluid associated with a pathological process.

Preliminary attempts to obtain the sequence information on GCDFP-24 were not successful, because the α-amino group of the polypeptide chain was blocked. The alternative approach involving a trypsin digestion of the purified protein allowed one to obtain reliable amino acid sequences required to demonstrate that GCDFP-24 is apolipoprotein D. Additional data on the molecular characteristics of both proteins provided further evidence supporting the identity between them. They are glycoproteins with the α-*N*-terminal blocked, which is released after pyroglutamate aminopeptidase treatment. The subsequent amino acid sequence determined for GCDFP-24 is identical with the one reported for apolipoprotein D [17]. Furthermore, the amino acid composition obtained for protein GCDFP-24 conforms very well to the one deduced for the mature apolipoprotein D from the cDNA nucleotide sequence [17]. The molecular mass reported for apolipoprotein D varies from 22.1 kDa [23] to 33 kDa [17], 19.3 kDa being the calculated size from the cDNA sequence [17]. All these values are in the range of the molecular mass determined for monomeric GCDFP-24 (24 kDa), with slight variations in the electrophoretic mobility, probably due to the extensive glycosylation reported for both molecules [24]. In relation to this, it is noteworthy that, during the amino acid sequence determinations of GCDFP-24, we detected the presence of carbohydrate groups bound at the same two asparagine residues reported to be glycosylated in apolipoprotein D [17].

The identification of apolipoprotein D as the major protein component in cyst fluid of gross cystic breast disease may also provide some additional information on the biological function of this lipoprotein. Apolipoprotein D has been classified as a member of a new family of proteins designated 'lipocalins', which are similar in amino acid sequence, disulphide-bond arrangement, three-dimensional structure and gene structure organization [25–31]. The common function of the proteins of this family seems to be the ability to bind and transport small hydrophobic molecules in serum, cholesterol esters being the specific ligands described for apolipoprotein D [17]. However, considering that the previously called GDCFP-24 protein has been characterized as a progesterone-binding protein [14,15], it seems reasonable to assume that apolipoprotein D may also be involved in the binding and transport of progesterone. This fact would indicate that a given protein belonging to the lipocalin family might bind a wide variety of ligands instead of being highly selective for a specific molecule. Additional evidence comes from previous observations indicating that several lipocalins, such as protein HC [32,33] or odorant-binding protein [28], are also associated with more than one lipophilic ligand.

It will now be worthwhile to investigate the possible role of apolipoprotein D in the induction of the pathological process characteristic of breast cystic disease as well as its hypothetical implication in the development of human breast cancer. Elucidation of these questions will require the use of large amounts of purified apolipoprotein D. It is therefore of interest to consider that the levels of apolipoprotein D in the cyst fluid are in the range of 10–50 mg/ml [16], which is at least 500-fold higher than the values reported for the concentration of this protein in normal plasma [34]. Taking into account the easy accessibility of the fluid and the relatively high frequency of the disease, the single-step procedure described herein to isolate this protein will be helpful for future studies on both normal and pathological functions of apolipoprotein D.

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