Hucolin, a new corticosteroid-binding protein from human plasma with structural similarities to ficolins, transforming growth factor-β1-binding proteins

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Abstract In order to study the cortisol-binding factors in blood, human plasma was applied to a 11β-hydroxy-3-oxo-4-androstene-17β-carboxyaminoethylamine-(HACA) 1,4-butanediol diglycidyl ether-Sepharose column. Elution of the column with cortisol buffer produced two protein peaks, the minor peak yielded a protein complex of molecular weight ~200 kDa, subsequently termed hucolin. SDS-PAGE analysis under reducing and non-reducing conditions revealed hucolin was a disulphide-linked complex of 35-kDa and 75-kDa subunits. Twenty-five amino acid residues of the N-terminus of the 35-kDa subunit were determined and homology searches revealed an 88% sequence identity with the N-terminal region of β-ficolin, a transforming growth factor-β1 (TGF-β1)-binding protein purified from porcine uterus.

Key words: Elastin-binding protein; Corticosteroid-binding protein; Cortisol; Ficolin; Human plasma; Transforming growth factor-β1

1. Introduction

Cortisol, a naturally occurring glucocorticoid and its artificial analogues, are powerful therapeutic agents used in the treatment of a large group of inflammatory diseases which include rheumatoid arthritis [1,2]. Corticosteroid-binding globulin (CBG) binds >90% of cortisol in plasma and regulates the levels of free cortisol [3,4]. In order to study other cortisol-binding proteins in human plasma these proteins were isolated from plasma by HACA-affinity chromatography, which employs a derivative of corticosterone linked by a 13-atom spacer to Sepharose [5]. A novel corticosteroid-binding protein, subsequently termed hucolin, was isolated and found to share a high degree of sequence homology and structural similarity with porcine ficolins. Ficolins are recently identified porcine transforming growth factor-β (TGF-β1)-binding proteins, with fibrinogen and collagen like domains [6]. TGF-β1 is a member of a family of 25-kDa dimeric proteins, derived from platelets, and is a potent growth inhibitor of haemopoetic progenitor cells, lymphocytes, epithelial and endothelial cells. TGF-β1 is also involved in wound healing and scarring and has been implicated in the pathogenesis of glomerulonephritis, liver cirrhosis and pulmonary fibrosis [6,7,8].

2. Materials and methods

Plasma was obtained as out-of-date fresh frozen plasma. Ultrafiltration units were from Amicon, MA, USA. Pre-stained molecular weight markers were from New England Biolabs, MA, USA and chemicals were obtained from Sigma Chemicals, UK. Problott PVDF membrane was obtained from Applied Biosystems CA, USA.

Purification of hucolin was achieved, essentially by the method of Mickelson et al. [5], by the affinity chromatography of steroid-stripped plasma with 11β-hydroxy-3-oxo-4-androstene-17β-carboxyaminoethylamine-1,4-butanediol diglycidyl ether-Sepharose as the resin [9]. Plasma (1 litre) was passed through a column (2.5 × 15.5 cm, of the affinity resin) at a flow rate of 150 ml/h at 4°C. The column was washed with 0.5 M KCl, 50 mM sodium phosphate, pH 9.0 buffer, at 4°C, until the UV absorbance of the washings was negligible (<0.01). The column was then placed at room temperature and, after 1 h, eluted with 0.1 M NaCl, 50 mM sodium phosphate buffer, pH 7.4, containing 280 μg/ml cortisol. The second, minor, protein peak was collected and concentrated by ultrafiltration.

Polyacrylamide gel electrophoresis employing 4% stacking and 9% resolving gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Laemmli [10].

Two-dimensional PAGE was accomplished by applying protein to a well in the stacking gel of a 9% SDS-PAGE gel and carrying out electrophoresis till the dye front was 5 mm from the end of the plates. The track containing protein was excised and soaked in a 50% (v/v) solution of stacking gel buffer containing 2% (v/v) 2-mercaptoethanol and 0.33% (w/v) SDS for 45 min before washing the track, three times 15 min, in soaking solution without 2-mercaptoethanol. The strip of polyacrylamide was then placed between two glass plates and a 9% resolving SDS polyacrylamide gel poured below it and a 4% stacking gel poured around it. Electrophoresis was carried out as described above and proteins transferred to PVDF membrane by electroblotting in 10% (v/v) methanol, 96 mM glycine, 12.5 mM Tris, pH 8.5, buffer at 30 V constant for 1.5 h [11]. The membrane was stained with Coomassie brilliant blue R-250 and protein spots cut out for N-terminal amino acid sequence determination.

The PIR protein, SWISSPROT protein and EMBL DNA databases were searched for sequence homology using sequence strings generated by N-terminal sequence analysis and the BLAST search program available at the world wide web address 'http://www.genome.ad.jp/SIT/BLAST.html' on the Internet.

3. Results

Steroid-stripped human plasma was applied to the HACA-steroid Sepharose before washing extensively in high salt buffer to remove non-specifically bound plasma proteins. Specifically bound proteins were eluted by low salt buffer containing cortisol. The elution profile exhibited one major and one minor protein peak (Fig. 1). Protein from the major peak contained mainly corticosteroid binding globulin. The minor peak was concentrated and resolved into 5 protein bands of molecular weights between 25 and 200 kDa, when separated by SDS-PAGE under non-reducing conditions (Fig. 2a). Under reducing conditions the same sample resolved into four major bands and numerous minor bands, of molecular weights between 25 and 80 kDa, indicating that some of the steroid binding proteins

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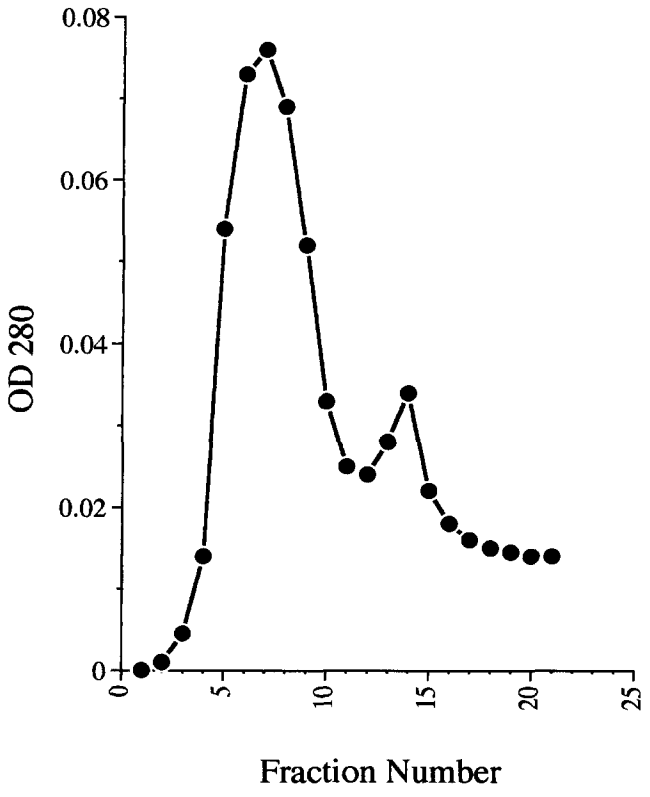

Fig. 1. Elution profile of bound plasma proteins from HACA-Sepharose. The column was eluted with 280 μg/ml cortisol in 50 mM sodium phosphate, O.1 M NaCl buffer pH 7.4, and fractions of 7.5 ml were collected. The minor protein peak, fractions 13–17, were pooled and concentrated.

were disulphide-linked structures (results not shown). Therefore it was decided to characterise the proteins, in the minor elution peak, by separation in two dimensions: SDS-PAGE non-reducing in the first and reducing in the second before transferring proteins to PVDF membrane to determine their N-terminal amino acid sequence (Fig. 2b).

Spot A2, MW 35 kDa (Fig. 2b), yielded the 25 amino acid sequence: LQAADTCPEVKMVGLEGSDKLTILR, and derived by reduction from band A, MW ~200 kDa (Fig. 2a). Spot D1, MW 35-kDa, yielded the identical but shorter sequence: LQAADTCPEVKMVGLEGSDK and derived by reduction from band D, MW 35-kDa (Fig. 2a). The 25 amino acid sequence shared 88% identity with domestic pig (*Scrofa domestica*) β-ficolin and 80% with α-ficolin, when protein databases were searched (Table 1). Spot A2 and D1 are, therefore likely to represent the same protein.

Spot A1, MW 75 kDa (Fig. 2b), although faint, was consistently noted in each experiment and also derived by reduction from band A at ~200 kDa (Fig. 2a). It yielded the six amino acid N-terminal sequence: AAFDDLQ. The initial N-terminal amino acid sequence yields for A1 and A2 were 6 and 68 pmol, respectively, which agree qualitatively with the relative staining intensities of the spots. Other spots were characterised as either, CBG, albumin or CBG-IgG complexes, all of which bind cortisol.

These results suggest the protein, subsequently termed hucolin (represented by band A; Fig. 2a), is a disulphide-linked multimeric protein which comprises predominately ficolin-like 35-kDa subunits and a minor dissimilar 75-kDa subunit. Hucolin specifically elutes from HACA-Sepharose indicating it has a steroid-binding site.

4. Discussion

Ichijo et al. [12] partially purified a previously unidentified transforming growth factor-β1 binding protein from porcine uterus that had a disulphide-linked multimeric structure, formed of subunits of MW 40-kDa. Upon cloning the gene they identified fibrinogen- and collagen-like domains from the DNA sequence, and consequently named it ficolin [12]. They later expressed the 40-kDa ficolin subunit and found it spontaneously formed multimers which were sensitive to reducing agents. The recombinant ficolin, however, did not bind TGF-β1 and they suggested an unidentified dissimilar subunit could be required for binding activity. In addition they found porcine ficolin cDNA probes hybridised with human mRNA and concluded that the genes for α- and β-ficolin are 'strongly conserved between pig and human' [12].

Hucolin is a candidate for the conserved 'human ficolin' gene product as it shares a highly conserved N-terminal sequence with the ficolins and a similar disulphide-linked multimeric structure comprising, predominately, a 35-kDa subunit. In addition, the 75-kDa subunit of hucolin could represent an equivalent of the dissimilar ficolin subunit postulated by Ichijo et al. [12]. However, we have no evidence that the dissimilar subunit of the hucolin complex is required for its cortisol-binding and further characterisation of this subunit was precluded by its low concentration.

The N-terminus of α- and β-ficolin was predicted by Ichijo et al. [12] from the cDNA gene sequence and on this basis hucolin would have a six amino acid N-terminal extension with respect to the porcine ficolins (Table 1). Conservation of the predicted pre-peptide cleavage site (threonine-cysteine) between the ficolins and hucolin and the absence of cleavage of hucolin at this site suggests the true N-terminus of the ficolins may be more proximal.

Another candidate for the human ficolin gene product has recently been identified [13]. Harumiya et al. [13] report the purification of EBP-37, a novel elastin-binding protein from human plasma and presented 45 residues of exclusively internal amino acid sequence that included collagen like domains. This protein exhibited greater than 72% direct amino acid sequence identity with porcine ficolins. The largest continuous sequence presented was a 15 amino acid lysyl endopeptidase cleavage fragment. EBP-37 also has a disulphide-linked multimeric

Table 1
Amino acid sequence homology between hucolin and porcine ficolins α and β

	* 5	10	* 15	20	* 25
hucolin	LQAAD	TCPEV	KMVGL	EGSDK	LTILR
β-ficolin	aqaad	tCPEV	KVVGL	EGSDK	LSILR
α-ficolin	apald	tCPEV	KVVGL	EGSDK	LSILR
Human S-gal			VVGS	PSAQD	EASPL

Lower case denotes the predicted ficolin signal sequence and asterisks mark residues of hucolin that are not shared by α-ficolin. The sequence from the spliced variant of human β-galactosidase (Human S-gal) [14] illustrates an elastin binding site and is aligned with the predicted homologous region of the ficolins [13].

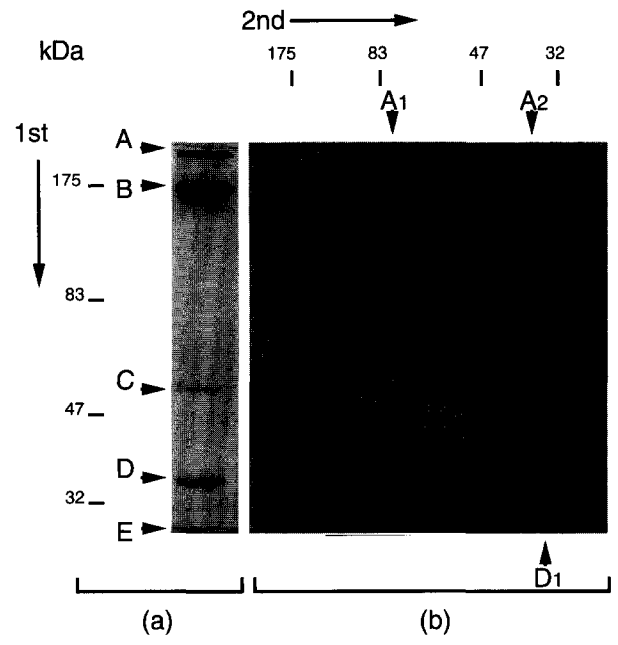

Fig. 2. Concentrated samples of the minor elution peak of the HACA-Sepharose column were separated by 9% SDS-PAGE (a) without reduction and (b) by two-dimensional 9% SDS-PAGE separation without reduction in the 1st dimension and with reduction (2% 2-mercaptoethanol for 45 min prior to separation) in the 2nd. Spots were cut out and subjected to N-terminal sequence analysis.

structure similar to both hucolin and the ficolins, with a similar subunit molecular weight of 37-kDa.

Harumiya et al. [13] suggest, by homology with elastin-binding proteins such as human β-galactosidase, for which the elastin binding site sequence has been determined [14] (Table 1), that the ficolins have an N-terminal elastin-binding region and predict that the ficolin N-terminal elastin-binding domain will be conserved in EBP-37, however, no N-terminal sequence data for EBP-37 was presented. The N-terminal data presented here not only demonstrates the high degree of conservation of the N-terminus between hucolin and the ficolins but supports the contention that hucolin has an elastin-binding site at the N-terminus and that hucolin and EBP-37 are identical or closely related proteins (Table 1).

Speculation as to the in vivo role of the ficolins centres on their fibrinogen- and collagen-like domains that may modify or localise their TGF-β1-binding activity [12]. It has been suggested, for EBP-37, that its elastin-binding function may serve to localise it to the extracellular matrix of blood vessels and that its collagen-like domains may bind to the C1q receptor [13]. When the roles of these proteins are being considered the part played by a steroid-binding site should be included as this may be a common feature or function of the hucolin/EBP-37/ficolin group. TGF-β_1 has been shown to antagonise glucocorticoid induced impairment of wound healing [15] and it may be that the human ficolin gene product has a role in modulating anti-inflammatory glucocorticoids and growth inhibitors such as TGF-β1 where elastin is exposed in the arterial vessels by vascular disease.

The N-terminal sequence information, provided by this investigation, should aid the design of oligonucleotide probes to clone the gene for hucolin/EBP-37 by reverse PCR screening of human cDNA libraries. In addition HACA-chromatography may provide a step in an improved purification procedure for ficolins or hucolin/EBP-37 should they prove to be as highly conserved as these results suggest.

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