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# Protein chemical characterization of Gc globulin (vitamin D-binding protein) isoforms; Gc-1f, Gc-1s and Gc-2

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**Abstract**

Gc globulin, also called vitamin D-binding protein, is a plasma protein involved in the extracellular actin-scavenger system, vitamin D transport and possibly also other biological activities. Low levels of Gc globulin have been found to correlate with multiple organ failure and non-survival of patients with fulminant hepatic failure and trauma. Here, we characterize the dominant isoforms of plasma-derived Gc globulin from Cohn fraction IV paste with respect to amino acid sequence and posttranslational modifications. Gc globulin was purified in large scale and the isoforms separated by ion exchange chromatography. The separated isoforms and several commercial preparations of individual isoforms were characterized by mass spectrometry. This revealed that the major isoforms were non-glycosylated. Compared to the Gc-1f isoform the other dominating isoforms represented an Asp/Glu substitution (Gc-1s) and a Thr/Lys substitution (Gc-2) in agreement with DNA sequencing studies. The commercial preparations were found to represent mainly one or two isoforms. An O-linked glycan with a mass of 656 Da and terminating with a sialic acid residue was detected on a minor proportion of Gc globulin molecules.


*Keywords:* Gc globulin; Vitamin D-binding protein; Isoforms; Glycosylation; Mass spectrometry

## 1. Introduction

Vitamin D-binding protein (DBP), also known as Gc (Group-specific component) globulin, is a plasma protein serving two major physiological functions: (1) binding and transport of vitamin D and its metabolites and (2) binding, sequestration and removal of monomeric actin [1–5].

Gc globulin is a member of the albumin family, which also comprises serum albumin, alpha-fetoprotein and afamin [6–12]. The genes for the proteins of the family are located on chromosome 4 in a multigene cluster [13]. Gc globulin is a single chain polypeptide of 458 amino acid residues and, like albumin, it consists of three domains [6–10]. Binding of vitamin D occurs with high affinity at a site located in domain I [14–16]. The binding site for actin is independent of the vitamin D binding site and has been located to a region covering residues 360–372 [15]. However, crystallographic studies have shown that the binding of actin occurs in a large cavity composed of all three domains [17,18]. Depolymerization of filamentous F actin by plasma gelsolin and sequestration of monomeric G actin by Gc globulin is crucial to prevent

*Abbreviations:* BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; CIE, crossed immunoelectrophoresis; DBP, vitamin D-binding protein; DTT, dithiothreitol; ESI, electro spray ionization; FPLC, fast protein liquid chromatography; Gc, group-specific component; HCCA, alpha-cyano-4-hydroxycinnamic acid; HILIC, hydrophilic interaction chromatography; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LC, liquid chromatography; MAF, macrophage-activating factor; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; RP-HPLC, reversed phase HPLC; TFA, trifluoroacetic acid; TNBP, tri-N-butylphosphate; TOF, time of flight; TTN, 50 mM Tris, 0.3 M NaCl, 1% Tween 20, pH 7.5

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circulatory disturbances in cases of severe cell necrosis or apoptosis, and exhaustion of the extracellular actin scavenger system may lead to deposition of actin, obstruction of blood flow and, eventually, multiple organ failure [4,19–39].

In addition to the two major functions described above, Gc globulin has also been reported to have a co-chemotactic activity for C5a [40,41] and to be a precursor for a potent macrophage-activating factor (MAF) [42–47], which also has anti-tumor and anti-angiogenic properties [46–52].

The conversion of Gc globulin to Gc-MAF is supposed to occur by the action of glycosidases on an O-linked trisaccharide resulting in successive removal of neuraminic acid (by neuraminidase) and galactose (by β-galactosidase) leaving an O-linked N-acetyl-galactosamine (GalNAc) [42–49]. However, except for one study [53] the location and the structure of the O-linked glycan has not been thoroughly characterized and its existence is mainly based on indirect evidence.

The concentration of Gc globulin in plasma is in the range of 200–500 mg/L [22,54–60] and it occurs in three major isoforms, which can be separated by isoelectric focusing (IEF) and are denoted Gc-1f, Gc-1s, Gc-2 [59,61–68]. The Gc-1f and Gc-1s isoforms have been reported to be sialylated [69], whereas Gc-2 was reported to be non-glycosylated [53]. However, DNA sequencing studies have associated the isoforms with amino acid substitutions at positions 416 and 420. Gc-1f has aspartic acid at position 416 and threonine at position 420, Gc-1s has glutamic acid and then threonine, and Gc-2 has aspartic acid and lysine at these positions [70,71].

In order to characterize the nature of the different isoforms, we have purified Gc globulin from plasma fraction IV [72] and characterized the three major isoforms by protein chemical methods, including mass spectrometry (MS) in combination with protease digestion.

## 2. Materials and methods

### 2.1. Large scale purification

Human plasma Gc globulin was purified using fraction IV paste as starting material [72]. Paste IV was precipitated during ethanol fractionation (40% ethanol at pH 5.9) of human plasma as described by Kistler and Nitschmann [73]. The fraction IV paste was dissolved in 20 mM Tris, pH 8.0, by overnight stirring at 4 °C, followed by serial depth and delipid filtration (50 LA, 90 LA, and delipid filters). The filtrate was added NaCl to 0.04 M, and applied onto a Q-Sepharose F.F. column. The column was washed using 20 mM Tris pH 8.0, 0.05 M NaCl, before elution with 20 mM Tris, 0.11 M NaCl, pH 8.0. The eluted material was ultra-filtered to half of the original volume, and dia-filtered against 20 mM phosphate buffer, pH 5.7. After ultra-dia-filtration the material was loaded onto a CM-Sepharose F.F. column, and the run-through fraction was collected, and treated by solvent-detergent (1% Tween 80, 0.3% tri-N-butylphosphate (TNBP)) overnight at room temperature, followed by a dilution with 20 mM phosphate buffer, 48 mM NaCl, pH 9.4 to a final pH of 7.8. The solution was then applied onto a Q-Sepharose F.F. column. Following washing with 20 mM phosphate buffer, pH 7.8, 0.04 M NaCl, the column was eluted with 20 mM phosphate buffer, pH 7.8, 0.1 M NaCl. The peak fraction was collected and ultra-filtered, followed by gel filtration on a Sephacryl S200 column equilibrated with 8 mM $Na_2HPO_4$, 1.4 mM $NaH_2PO_4$, 130 mM NaCl, pH 7.0,. The main peak was collected and subjected to nano-filtration through a 20 nm and a 15 nm filter connected in series, and finally stabilized by addition of 5% maltose before sterile filtration and filling.

### 2.2. Small scale ion exchange chromatography for separation of isoforms

10 mg of human Gc globulin purified from plasma fraction IV was dialyzed against 20 mM sodium acetate pH 4.5, followed by further separation by ion exchange chromatography on a MonoS column connected to a fast protein liquid chromatography (FPLC) system, using a flow rate of 1 mL/min and collecting fractions of 2.5 mL. The column was eluted with 20 mM sodium acetate, pH 4.5, 0.5 M NaCl according to the following setup: 30 min of 0% elution buffer, followed by a 10 min linear increase from 0 to 50%, and then a linear increase from 50 to 80%. Immediately after the chromatography, the collected fractions were dialyzed against 50 mM Tris, pH 7.5 before further analysis.

### 2.3. SDS-PAGE

SDS-PAGE was performed as described [74,75] using precast gels. The PAGE was performed using precast 4–20% Tris-glycine gels following the instructions of the manufacturer, using a variation of the recommended sample buffers. Samples were mixed with an equal volume of sample buffer (non-reduced SDS-PAGE: 50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.06% pyronin G; reduced SDS-PAGE: as non-reduced but added 0.1 M dithiothreitol (DTT)) before loading into the wells of the gel. After electrophoresis, gels were either stained with Coomassie Brilliant Blue (GelCode) according to the manufacturer's recommendation, or electroblotted to nitrocellulose membranes for subsequent immunoblotting.

### 2.4. Immunoblotting

SDS-PAGE gels were sandwiched with nitrocellulose membranes between 12 sheets of Whatman paper no. 1 soaked in 10 times diluted electrophoresis buffer, and subjected to semi-dry electroblotting overnight at 0.1 mA/cm$^2$. The membranes were blocked for 1 h in 50 mM Tris, 0.3 M NaCl, 1% Tween 20, pH 7.5 (TTN), incubated with monoclonal antibody against Gc globulin diluted 1:100 or rabbit antibodies against human serum proteins diluted 1:1000 in TTN for 1 h, followed by three washes using TTN. Next the membranes were incubated for 1 h with alkaline phosphatase-conjugated goat immunoglobulins against mouse or rabbit immunoglobulins diluted 1:1000 in TTN, followed by three washes in TTN. Finally, bound antibodies were visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate.

### 2.5. Isoelectric focusing

Isoelectric focusing was performed using precast pH 3–7 gels following the directions of the manufacturer. Samples were mixed with an equal volume of the recommended sample buffer before loading onto the gel. After electrophoresis and fixing, gels were stained with Coomassie Brilliant Blue (GelCode) according to the manufacturer's directions.

### 2.6. Glycosidase treatment of Gc globulin

Samples of approximately 100 μg Gc globulin were vacuum dried and redissolved in 100 μL 50 mM $NH_4HCO_3$, pH 6. The samples were treated individually, one with neuraminidase, alpha-mannosidase and beta-galactosidase, another with neuraminidase and O-glycosidase and a control sample was left untreated. 1 mU of each enzyme was utilized and samples were incubated over night at room temperature. In a separate experiment for ESI MS, selected fractions from the cation exchange chromatography and commercial preparations (approximately 100 pmol Gc globulin in 10 μL water) were treated with neuraminidase (3 μL reaction buffer and 0.3 μL sialidase A (GLYCO Prozyme, San Leandro, CA) for 1 h at 37 °C.

### 2.7. Matrix assisted laser desorption ionization (MALDI)-MS of intact protein

A sample of intact Gc globulin was micro-purified on custom made POROS $R1_{50}$ columns and loaded onto a PerSeptive steel-target in a matrix sandwich of