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Purification and characterization of a 200 kDa fructosyllysine-specific binding protein from cell membranes of U937 cells

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Amadori-modified proteins are bound by macrophages and monocytes via fructosyllysine-specific receptors. Detergent extracts from U937 cell membranes were used to purify the binding proteins by affinity purification on glycated polylysine coated magnetic beads followed by SDS-PAGE. Two proteins of 200 and 100 kDa were isolated. MS-analysis of the 200 kDa protein showed high homologies with cellular myosin heavy chain, type A. Both fructosyllysine specific binding proteins, cellular myosin heavy chain and nucleolin, are glycosylated.

Keywords: **glycation, fructosyllysine, receptor, cellular myosin heavy chain, nucleolin**

Abbreviations: **EDTA, ethylenediamine tetraacetic acid, sodium salt; IgG, immunoglobulin G; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; PBS, phosphate buffered saline; PMSF, phenylmethyl-sulfonyl fluoride; RP, reversed phase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.**

Introduction

The postranslational nonenzymatic modification of proteins with glucose (glycation) is likely to play a role in the development of the diabetic microangiopathy [1]. Unprotonated amino groups of proteins, especially the ε-amino groups of lysine residues, form a condensation product with the carbonyl function of the open chain form of glucose, which rearranges into a ketoamine (Amadori product). Fructosyllysine is the main Amadori adduct in extracellular proteins [2]. Binding proteins with molecular masses of 100, 130 to 150 and 200 kDa in U397 cell membranes have recently been described to interact specifically with glycated albumin [3,4]. Partial amino acid sequences of the purified 100 kDa protein showed homologies with the nuclear protein nucleolin [4]. We report here the purification and structural characterization of the 200 kDa binding protein as cellular myosin heavy chain, type A. We show for the first time that membrane-bound nucleolin and cellular myosin heavy chain are glycosylated.

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Materials and methods

Short-term glycated albumin was prepared as described [1] by incubating the protein (fraction V, Sigma, Deisenhofen, Germany) with 100 mM glucose in 0.01 M Tris/HCl – 0.14 M NaCl – 1 mM EDTA – 3 mM NaN_3, pH 7.5, for 3 days at 37°C and isolated by boronate affinity chromatography. Glycated albumin contained 1 mol fructoselysine/mol protein and was free from AGEs as described by [1,3].

20 mg uncoated dynabeads (dynabeads M-280 tosylactivated, Deutsche Dynal, Hamburg, Germany) were incubated with 200 mg poly-L-lysine (m.w. 5000 to 15 000, Sigma) in 4 ml 0.01 M $Na_2B_4O_7$ – 2 M NaCl (pH 9.5) for 16 h at 37°C. After repeated washings the polylysine coated beads were suspended in 4 ml of 200 mM glucose – 0.1 M Tris/HCl – 0.15 M NaCl – 1 mM EDTA – 3 mM NaN_3 (pH 8.0) for 3 days at 37°C. Thereafter, non-bound glucose and reagents were removed by repeated washings with 0.1 M Tris/HCl – 0.15 M NaCl – 1 mM EDTA – 3 mM NaN_3 (pH 7.5).

1×10^9 U937 cells were harvested by centrifugation, washed twice with 0.01 M Na_2PO_4 – 0.14 M NaCl (pH 7.4) (PBS), once with PBS containing 10 mM EDTA and resuspended in homogenization buffer 10 mM Tris/HCl – 300 mM sucrose – 0.1 mM EDTA (pH 7.4) containing an

excess of protease inhibitors (10 mM PMSF, 20 mM benzamidine, 10 µg/ml antipain, leupeptin, pepstatin A, bacitracin, 50 µg/ml aprotinin (Sigma)). After repeated freezing and thawing (three times) cells were homogenized using a Turrax stirrer and centrifuged at 800× *g* for 9 min. The supernatant was collected and recentrifuged at 20 000× *g* for 30 min. To eliminate membrane associated proteins pelleted membranes were washed once with a tenfold volume of sucrose free homogenization buffer and once with 10 mM Tris/HCl – 0.1 mM EDTA (pH 7.4). Finally pelleted cell membranes were solubilized in 57 mM ß-octylglucoside – 0.02 M Tris/HCl – 0.23 M NaCl – 10 mM EDTA (pH 7.4) and proteinase inhibitors as described above for 30 min at 4°C. The lysates were centrifuged for 1 h at 100 000× *g* and 4°C and incubated with 20 mg of prepared magnetic beads for 2 h at 4°C. After five stringent washings of the beads with 57 mM *β*-octylglucoside – 0.02 M Tris/HCl – 0.23 M NaCl – 10 mM EDTA (pH 7.4) bound proteins were eluted with 200 µl 57 mM *β*-octylglucoside – 10 mM Tris/HCl – 0.4 M N^{ε}-fructosyl amino caproic acid – 0.2 M N^{ε}-fructosyllysine (pH 7.4), both prepared according to [5]. Eluted proteins were separated by SDS-PAGE using 7.5% acrylamide gels as described [3]. After electrophoresis proteins were stained with Coomassie blue and used for amino acid sequence analysis [4]. The identity and homogeneity of the isolated proteins were checked using ligand blots with glycated albumin [3] in combination with immunochemical detection of bound albumin with suitable antibodies (Sigma) and 2 D-electrophoresis (pH gradient 3–10, 2% Bio-Lyte 3/10, 1% Bio-Lyte 4/6 (Bio-Rad, Germany), 4% acrylamide in the first dimension, 7.5% acrylamide, 0.1% SDS in the second dimension). Electrophoresis patterns were evaluated after silver staining [6]. For amino acid sequence analysis the purified 200 kDa protein was digested with trypsin (Roche, Mannheim, Germany), 2 µg/ml in 50 mM Tris/HCl – 1 mM EDTA (pH 8.0) within the gel overnight at 37°C. The peptide mixture was eluted from the gel with 10% formic acid and an aliquot (about 10%) was directly investigated by MALDI-MS. The remaining peptide mixture was desalted by RP material (C18 ZIP-T, size p10, Millipore, Eschborn, Germany) and used for MS/MS-analysis.

After SDS electrophoresis proteins were blotted on nitrocellulose membranes (Sigma) and characterized for carbohydrates by staining with a glycan detection kit (Roche). Western blots were performed with polyclonal antisera against cellular myosin heavy chain (generous gift from Prof. Murano, Berlin-Buch) and nucleolin (generous gift from Dr. Kübler, Heidelberg) as well as an alkaline phosphatase conjugated second anti-rabbit IgG antibody (Sigma). For ligand blotting with glycated albumin an anti-albumin polyclonal rabbit antibody (Sigma) was used. Antigen antibody reactions were vizualized with NBT/BCIP (Roche) as a substrate for alkaline phosphatase.

Results and discussion

As shown in Figure 1, two proteins of 200 and 100 kDa were obtained after affinity purification on glycated dynabeads. The proteins were identified by ligand blotting with glycated albumin as fructosyllysine-specific binding proteins [3]. They appeared homogenous with regard to molecular weight but not homogeneous with regard to charge in 2 D-electrophoresis (Figure 2). This heterogeneity is probably due to enzymatic glycosylation, because the fructosyllysine-specific binding proteins can be bound by Con A sepharose (not shown) and stain positively with a glycan detection kit (Figure 3).

The 200 kDa protein was subjected to MALDI-MS and MS/MS. Peptide mass finger print analysis in the data bank NCBI (release 061299) with the program "MS-fit" identified

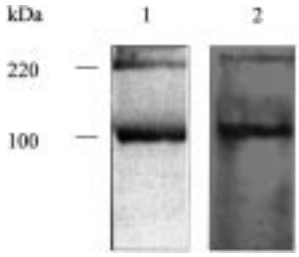

Figure 1. Lane 1: SDS-PAGE of U937 membrane proteins biospecifically eluted from glycated dynabeads, Coomassie blue stained; lane 2: ligand blot detection of isolated fructosyllysine-specific binding proteins after SDS-PAGE.

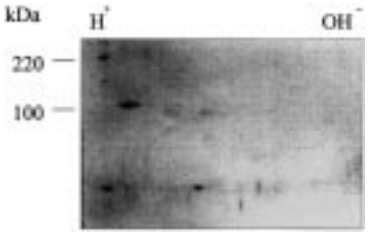

Figure 2. 2 D-electrophoresis of proteins eluted from glycated dynabeads. The 200 and 100 kDa proteins are homogenous with regard to molecular weight, but heterogenous with regard to charge.

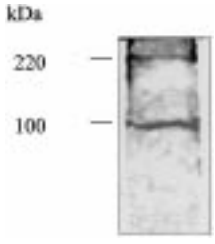

Figure 3. Evidence that the affinity purified fructosyllysine-specific binding proteins contain carbohydrates. The 100 and 200 kDa proteins (membrane-bound nucleolin and cellular myosin heavy chain A) were blotted on nitrocellulose and stained for carbohydrates using a glycan detection kit.

Table 1. MS/MS analysis of two tryptic peptides of the 200 kDa binding protein

Mass: m/z	*Charge*	*Mass: MH*	*Sequence tag*
653.36	2	1707.93	(644.39) ADFA (1048.57)
863.99	2	1725.98	(1059.58) NAQL (1485.9)

it as the cellular myosin heavy chain, type A. The matched peptides covered 20% (268/1337 amino acids) of the protein. From MS/MS spectra the following results were obtained (Table 1). Amino acid sequences are: peptide 1707.93 QLLQANPILEAFGNAK (positions 210–225), peptide 1725.98 EQADFAIEALAK (positions 408–419 of cellular myosin heavy chain) [7]. These sequences were not found in any of the AGE-receptors, from which sequence data are described up to now. In Western blots antisera against cellular myosin heavy chain stained the 200 kDa protein fraction, whereas antibodies against nucleolin reacted with the 100 kDa protein (Figure 4).

Cellular myosin heavy chain is a 224 kDa protein, which is also expressed in myeloid cell lines [7]. The molecular masses of 180 to 200 kDa of the fructosyllysine-specific binding protein indicate that a shortened product in comparison with the cytosolic protein may be inserted into the cell membrane. Cellular myosin has previously been shown also to be membrane-bound in neuronal plasma membranes [8]. Cellular myosin heavy chains anchored to plasma membranes induce cell motility. These associated cytosolic proteins are removed in the preparation of the membrane proteins by washing procedures [8] and are not cytosolic impurities in the affinity purified fructosyllysine-specific membrane receptor proteins. Furthermore, cytosolic and nuclear proteins did not bind glycated albumin [3,4].

Membrane-bound nucleolin and cellular myosin heavy chain are enzymatically glycosylated. Both proteins contain glycosylation sites in their amino acid sequence [7,9]. But the cytosolic forms of both proteins are free from carbohydrates.

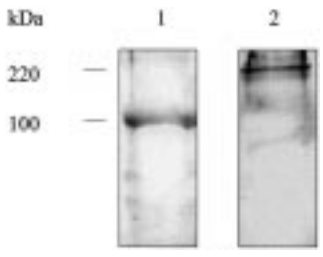

Figure 4. Immunochemical evidence that only the 200 kDa fructosyllysine-specific membrane protein reacts with an antibody for cellular myosin heavy chain. Lane 1: The 100 kDa protein was detected with an anti-nucleolin antibody as membrane-bound nucleolin. Lane 2: The 200 kDa protein reacted with an anti-cellular myosin heavy chain antibody.

Their glycosylation may be a signal directing them into the plasma membrane and a condition for their binding properties.

Amadori-modified proteins are also bound by macrophages, monocytes, endothelial cells and fibroblasts, as well as by the monocytic cell line MonoMac 6. Lymphocytes do not express fructoselysine-specific binding sites [1,3,4,10]. On monocytes and macrophages from rodents and humans a differing individual expression of these sites was found, which was positively associated with indices of diabetic microangiopathy and capillary basement membrane thickening [1,3]. Binding of glycated albumin by MonoMac 6 cells induced production of the cytokines ll-1 and TNF [3]. Therefore, binding of glycated proteins to fructoselysine-specific sites may participate in the pathogenesis of diabetic vascular complications.

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