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IDENTIFICATION OF A CELL CYCLE-DEPENDENT GENE PRODUCT AS A SIALIC ACID-BINDING PROTEIN

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Summary. A Ca^{2+}-dependent sialic acid-binding protein was purified on fetuin-Sepharose from various types of human tissue. The molecular mass was determined to be 10,315 Da by laser desorption mass spectrometry. Partial sequence analysis after cyanogen bromide cleavage that yielded one N-terminus accessible for Edman degradation revealed an identity to an internal stretch following the only methionine residue within a putative amino acid sequence (M_r 10,048), deduced from the cDNA of a cell cycle-specific gene. The reported biochemical identification is a prerequisite to infer the biological role of the so far undetected gene product. Initial glycohistochemical studies with sialic acid-(BSA-biotin) raised evidence for nuclear localization of sialic acid-binding sites that might reflect, at least in part, detection of this protein.

Besides the commonly accepted code system that is based solely on amino acid chains molecular recognition can well be mediated by the specific interplay of proteins and carbohydrate chains of cellular glycoconjugates (1). Due to their easy spatial accessibility and the presence of multiple forms with an array of modifications of a basic structure sialic acids deserve special attention among the carbohydrate moieties of glycoconjugates when considering the carbohydrate part of such a glycobiochemical interaction (2,3). Consequently, these reasons have prompted biochemical purifications of sialic acid-binding proteins from mammalian organisms (4-10). Herein, we describe purification of a further Ca^{2+}-dependent sialic acid-binding protein from diverse human tissue types and human Hodgkin tumors. Remarkably, the partial

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amino acid sequence of this protein is shown to be identical to the sequence of a cell cycle-specific gene, termed 2A9 or calcyclin, as deduced from its cDNA sequence (11). Since its implications in growth regulation have justified several studies on its expression (11-14), the reported identification of the gene product will aid in reaching a firm conclusion about the biological role of this protein in cell cycle progression.

MATERIALS AND METHODS

Purification of the sialic acid-binding protein. Processing of the tissue specimens started with homogenization in 5 - 6 vol of phosphate-buffered saline (75 mM Na_2HPO_4/75 mM KH_2PO_4, pH 7.2 and 575 mM NaCl) containing 4 mM β-mercaptoethanol and 2 mM ethylenediaminetetra-acetic acid (EDTA) in a Waring Blender at 4°C. The buffer was augmented by the addition of 0.01 mM benzylsulfonyl fluoride and 5 µg/ml leupeptin, antipapain and chymostatin, respectively, to minimize proteolytic activity. Moreover, 10 mM N-acetyl-neuraminic acid was included to optimize extractability. When the extract was run over pre-columns of other immobilized carbohydrates, namely lactose, D-xylose, L-fucose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, these sugars were also added to a final concentration of 50 mM each. Following homogenization and centrifugation at 20,000 g for 30 min, the resulting supernatant was dialyzed over 24 h with six changes against 50 vol of 20 mM Tris-HCl buffer, pH 7.8, containing 150 mM NaCl, 2 mM EDTA and 2 mM β-mercaptoethanol and any precipitating protein was discarded after centrifugation. Prior to affinity chromatography on the fetuin-Sepharose column (1.8 x 5 cm) the extract was adjusted to 20 mM $CaCl_2$ and passed over a set of pre-columns with different types of immobilized sugars, as given above, all affinity resins being prepared and checked for capacity as described elsewhere (8).The fetuin-containing column was extensively washed with 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM dithiothreitol, 20 mM $CaCl_2$ and first 150 mM NaCl, later 1 M NaCl to remove contaminants. The sialic acid-binding protein was eluted with this buffer, substituting 20 mM $CaCl_2$ with 4 mM EDTA. Further processing of the eluate and analytical procedures had been described in detail elsewhere (8,9). Standards for molecular weight designations (protein markers in the range of 97,400 - 14,300 as well as myoglobin fragments in the range of 2,510 - 16,950) were from Sigma (Munich, FRG). Further independent determination of the molecular mass was carried out by matrix-assisted ultraviolet laser desorption ionization mass spectrometry, as described in detail elsewhere (15). Specificity of the protein-carbohydrate interaction was further ascertained by a solid phase-binding assay (10), using sialic acid-(BSA-biotin), prepared and controlled for quality as described (16), and the isolated Ca^{2+}-dependent carbohydrate-binding protein, immobilized onto nitrocellulose.

Amino acid sequence analysis. The gas-phase sequencer model 470A in combination with the PTH-analyzer model 120A (Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404, USA) was used. The program was the normal 03RPTH run with gradient elution of the PTH-amino acids.

Cyanogen bromide cleavage in situ. At the end of a 03CPTH cycle the cartridge was removed from the holder, placed in a separate

glass vial equipped with a grinded lid. 30 µl of a solution containing 1 mg/ml cyanogen bromide were pipetted on the filter. 100 µl of this cyanogen bromide solution were placed in the vial in which the atmosphere was saturated by a solution of 70 % formic acid in water. After 8 h the cartridge was placed in a ventilated hood and the filter was dried by a stream of nitrogen. Drying was continued for another 10 min under argon in the sequencer. The following wash procedure was carried out before sequencing was restarted in the 03RPTH run: delivery of heptane (S1) until the filter is saturated with heptane, stop flow for 1 min and argon dry for 1 min. After this precipitation a cyclus of heptane- (1 min), butylchloride (1 min) and ethylacetate (0.5 min) washes interrupted by argon drying for 2 min was repeated three times.

Computer search. For identification of the determined amino acid sequence the Protein Identification Resource (PIR) from National Biomedical Research Foundation, 3900 Reservoir Road, N.W., Washington, DC 20007, USA with release 20.0 was used.

Glycohistochemical staining. Tissue specimens of human kidney were fixed in 4 % formaldehyde, embedded in paraffin, and 5 µm sections were glycohistochemically evaluated for the presence of sialic acid-binding sites using an optimized and standardized protocol with rigorous inherent specificity controls (9,16-18).

RESULTS

Affinity chromatography of extracts from human tissues on fetuin-Sepharose in the presence of Ca^{2+} resulted in isolation of a sialic acid-binding protein after elution with chelating agent, as shown for human muscle and kidney in Fig. 1. Gel electrophoretic analysis under denaturing and reductive conditions disclosed an apparent molecular weight of 7,500. Since this type of analysis is influenced by properties of the protein under investigation, we consequently employed another technique for independent molecular mass assessment. Laser desorption mass spectrometry revealed a molecular mass of 10,315 (Fig. 2). A similar value was obtained by extrapolation of a calibration curve with marker proteins from 97,400 to 14,300 in gel electrophoretic analysis (not shown). The yield of protein was 4.3 µg/g heart muscle, 1.2 µg/g skeletal muscle, 6.1 µg/g and 2.4 µg/g from kidney parenchyma and cortex and 12.4 µg/g from Hodgkin tumor cells, grown as tumors in nude mice. Since the complex carbohydrate chains of fetuin were employed as affinity ligands, any binding to other portions of these chains needed to be excluded by pre-columns, exposing different carbohydrate moieties that are present in fetuin as affinity ligands. Notably, chemically desialylated fetuin was also included as ligand of a pre-column to assure absence of any protein-protein interaction. This type of binding could otherwise mimic the supposed protein-carbohydrate binding and lead to erroneous conclu-

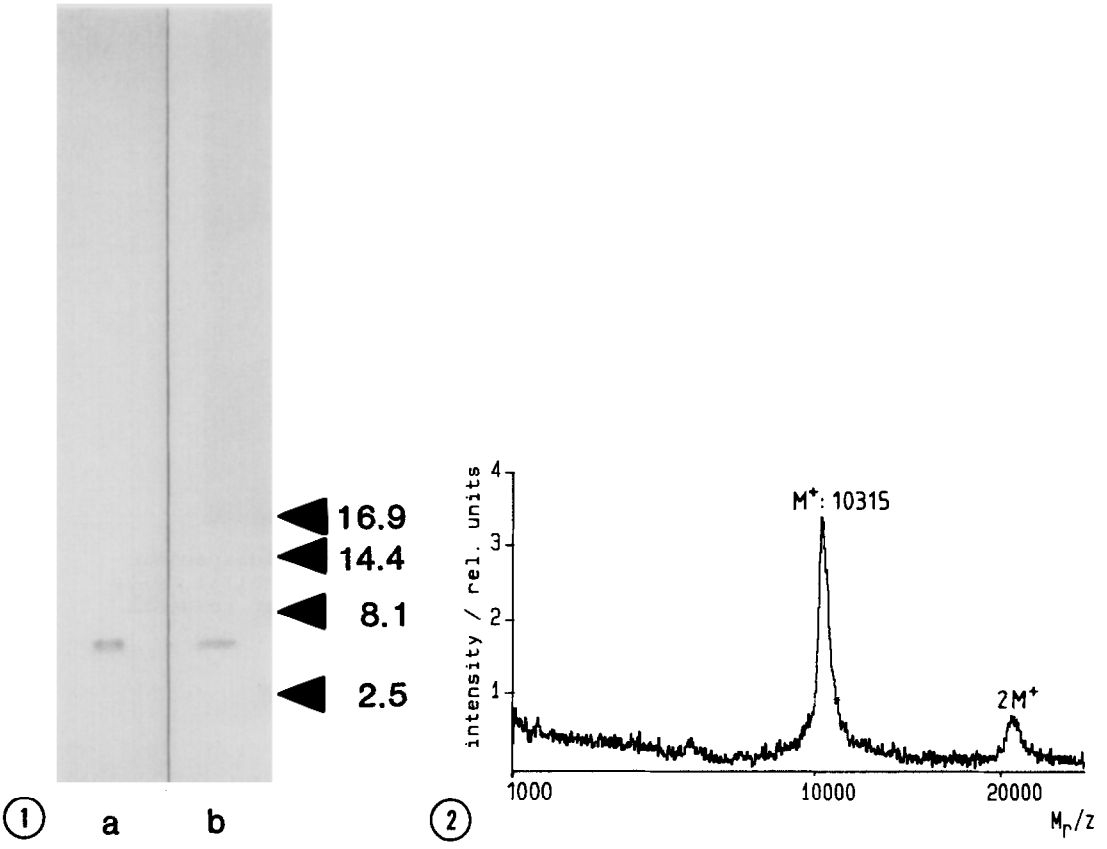

Fig. 1. Separation by SDS gel electrophoresis and silver staining of fractions of human muscle (a) and kidney (b) after elution with chelating agent from fetuin-Sepharose, using myoglobin fragments as standards for molecular weight designation.

Fig. 2. Matrix-assisted ultraviolet laser desorption ionization (UV-LDI) mass spectrum of the molecular ion range after 20 accumulated individual spectra.

sions. Moreover, the binding specificity in the presence of Ca^{2+} was independently ascertained by use of a labelled sialylated neoglycoprotein in a solid phase-assay (not shown).

The relatively low molecular weight of this protein prompted us to gain further structural information by N-terminal sequencing. However, these attempts were invariably not successful. A slightly increasing background within the first cycles of Edman degradation was rather indicative for a N-terminally blocked polypeptide, suggesting lack of proteolytic degradation during tissue processing. Consequently, cyanogen bromide cleavage was carried out on the glass-fibre disc with the material, already subjected to the conditions of Edman degradation. This cleavage apparently gained

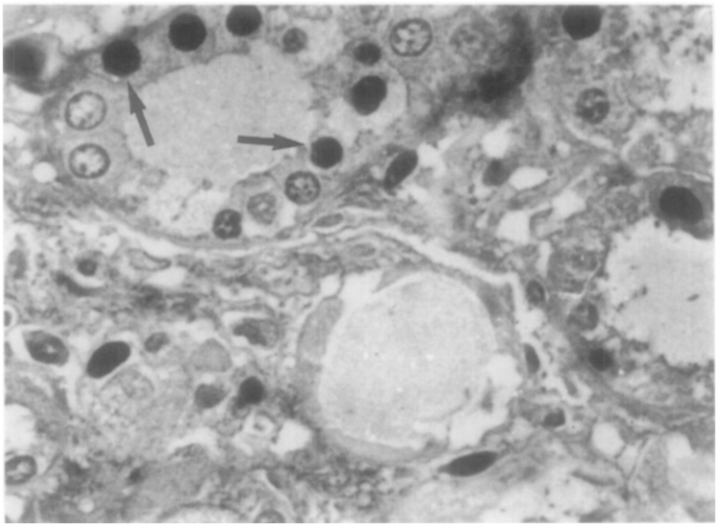

Fig. 3. Glycohistochemical localization of sialic acid-specific binding sites in the nuclei (arrows) of epithelial cells of distal convoluted kidney tubules, using ABC reagents for staining; original magnification x 800.

access to one N-terminus. The peaks from the resulting individual cycles allowed to unequivocally determine the following sequence: Glu-Asp-Leu-Asp-Arg-Asn-?-Asp-?-Glu-Val-Asn-Phe-?-Glu-Tyr-Val. This sequence was found to be identical to a sequence stretch, following the only internal methionine residue in position 56 of calcyclin. Calcyclin's sequence had so far only been deduced from a cloned cDNA of a cell cycle-specific gene (2A9), adding up to a calculated molecular weight of 10,048 with 89 amino acid residues (11).

Due to the present lack of its precise biological function, we attempted to infer the localization of sialic acid-binding sites in human kidney by glycohistochemistry with a sialylated neoglycoprotein. Since affinity chromatography on fetuin-Sepharose had resulted in isolation of one predominant sialic acid-binding protein, it is reasonable to assume that at present this approach may provide first substantiated evidence for its localization. Intense staining was detected in nuclei of epithelial cells of distal convoluted tubules of the kidney besides the basal membrane (Fig. 3).

DISCUSSION

Detection of cell cycle-dependent genes on the transcriptional level facilitates meaningful insights into the molecular events of

proliferation control. The precise assignment of the functional roles of the respective gene products can subsequently be substantiated on the protein level. Motivated in general by the conceptual framework of the importance of recognitive protein-carbohydrate interactions as well as particularly by the potentially eminent significance of sialic acid moieties among the different carbohydrate components of cellular glycoconjugates, we have described in this report the isolation of a Ca^{2+}-dependent sialic acid-binding protein. Concerning growth control regulatory influences can indeed be exerted by sialoglycopeptides, possibly by initiating intricate biosignaling via protein-sialic acid recognition (19,20). In this context, it is thus intriguing that we could demonstrate that in terms of partial sequence and molecular weight the isolated protein is identical to the putative amino acid sequence of the product of a cloned cell cycle-specific gene, termed calcyclin (11). The difference of 267 Da between the molecular mass, determined by laser desorption mass spectrometry, and the predicted molecular mass on the basis of the cDNA may be indicative for a post-translational modification that remains undefined at present. Gene expression for calcyclin on the mRNA level is inducible by growth factors and human acute myeloid leukemias reveal its overexpression and deregulation (12,13). Extended homologies to calcium-binding proteins have encouraged the proposal that calcyclin belongs to the family of calcium-modulated proteins (11). Interestingly, members of this family exhibit blocked N-terminal amino acids as calcyclin, among them N-acetylated alanine (21). As indicated in the term calcyclin, this assumed relationship could in principal link modulatory influences of calcyclin to changes in calcium levels, thereby causing changes in the status of cell proliferation. Taking the capacity for carbohydrate binding into account, further potential implications due to the presence of such a domain are reasonable. Alteration of crucial enzymatic activities by protein-carbohydrate interaction belongs to these proposals, where an appropriate precedent with an endogenous lectin has already been reported (22). Binding of two ligands, namely calcium and carbohydrate, can place calcyclin into the rapidly growing group of carbohydrate-binding proteins with at least one other type of functionally active domain (1,23,24). This notable capacity may perhaps account for its suggested relevance in growth control. Since a convenient method to purify calcyclin is now available, this attractive assumption can be readily tested experimentally.

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