Glycoconjugate Journal 16, 185–188 (1999)

SHORT COMMUNICATION

Megalin in normal tissues and carcinoma cells carries oligo/poly α2,8 deaminoneuraminic acid as a unique posttranslational modification

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In rat kidney, megalin, a member of the low density lipoprotein receptor gene family, is the sole glycoprotein which carries oligo/poly α2,8 deaminoneuraminic acid (KDN) as a posttranslational modification. We have investigated immunoprecipitated megalin from rat brain, lung and placenta, mouse yolk sac carcinoma and megalin synthesizing carcinoma cell lines, for presence of this unique glycan structure. Our immunoblot analysis revealed the presence of oligo/poly α2,8 KDN on megalin in all the studied normal tissues and carcinoma cells. Furthermore, it is demonstrated to be part of oligosaccharides *O*-glycosidically linked to megalin.

Keywords: **oligo/poly α2,8 deaminoneuraminic acid, megalin, lung, brain, placenta, F9 cells, L2 cells**

Introduction

In previous studies, we demonstrated the presence of oligo/poly α2,8 linked deaminoneuraminic acid (oligo/poly α2,8 KDN) in various embryonic, postnatal developing and adult mammalian tissues by immunohistochemistry and immunoblot analysis [1,2] with the use of the monoclonal antibody mAb.kdn8kdn [3]. The existence of KDN in mammalian tissues was furthermore confirmed by gas liquid chromatography analysis [1], and a sensitive fluorescent probe for HPLC [4] detection of KDN in tissues [5]. Western blot analysis of extracts of various rat tissues revealed the presence of a single reactive band in each tissue studied. The oligo/poly α2,8 KDN was found on a single 150 kDa glycoprotein except for a single >350 kDa glycoprotein in kidney [1,2].

Recently, we isolated and purified the oligo/poly α2,8 KDN bearing glycoprotein from rat kidney [6] and identified it as being megalin, a member of the low-density lipoprotein receptor gene family [7]. The presence of oligo/poly α2,8 KDN on kidney megalin was confirmed by combined immunoprecipitation / immunoblot analysis and on RAP-affinity purified megalin. Further supporting evidence was obtained by immunoelectron microscopy revealing an identical subcellular distribution of oligo/poly α2,8 KDN and megalin in rat kidney proximal tubules [6].

Megalin represents a major membrane protein of kidney proximal tubules but is additionally found in rat lung, choroid plexus and microvasculatur of brain, placenta, yolk sac epithelia, ciliary epithelium of the eye, parathyroid as well as inner ear [8]. Furthermore, by immunoblotting megalin was demonstrated in mouse F9 teratocarcinoma cells [9] and L2 rat yolk sac carcinoma cells [10].

Here, we report that megalin from various normal rat tissues, carcinoma tissue and carcinoma cell lines carries oligo/poly α2,8 KDN which is part of *O*-glycosidically linked oligosaccharides.

Materials and methods

Materials

For the detection of oligo/poly α2,8 KDN, purified IgM of the mouse monoclonal antibody mAb.kdn8kdn was used [3]. The hybridoma cell line 2*G*-5 [3] was kindly supplied by Dr. Ken Kitajima (University of Nagoya, Nagoya Japan). Polyclonal antibodies against purified rat kidney megalin were raised in rabbits as described previously [11] and IgG

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fractions prepared using Protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Alkaline phosphatase-conjugated donkey anti-mouse IgM (affinity-purified) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) and frozen rat tissues from Pel Freez Biologicals (Rogers, AR, USA). Mouse yolk sac tumor tissue and the L2 cell line were kindly supplied by Dr. Ulla Wewer (University Institute of Pathological Anatomy, Copenhagen, Denmark). Mouse F9 teratocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). Protease inhibitor cocktail tablets, recombinant N-Glycosidase F and digoxigenin-conjugated Concanavalin A were from Boehringer (Mannheim, Germany). Sephacryl S-400, XK 16/100 column were purchased from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade and purchased from Fluka (Buchs, Switzerland).

Cell cultures

L2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose and 10% fetal calf serum at 37°C in a humid atmosphere containing 5% CO_2. Mouse F9 teratocarcinoma cells were cultured in gelatin coated (0.1%) flasks in DMEM with high glucose and 15% fetal calf serum. To induce differentiation, F9 cells were incubated for 72 hours with 1 μM retinoic acid and 1 mM dibutyryl cyclic AMP.

Megalin immunoprecipitation

Protein A Sepharose CL-4B was incubated with PBS containing 1% BSA and 0.05% of Triton X-100 and Tween 20 for 1 h at 4°C, followed by incubation with anti-megalin antibodies for 18 h at 4°C. Rat kidney (0.7 g), lung (4.2 g), brain (25 g), placenta (4.2g), mouse yolk sac carcinoma tissue (4.2 g) and pellets of L2 and F9 cells (200 μg each) were homogenized in PBS containing protease inhibitors and Triton X-100 (final concentration 1%). After 60 min on ice, the homogenate was centrifuged at 100,000 × g for 60 min. The soluble extract was incubated with the anti-megalin / Protein A Sepharose CL-4B overnight at 4°C. Following two washes with PBS containing 0.1% Triton X-100, the Protein A Sepharose CL-4B was pelleted, placed in SDS-PAGE sample buffer and heated. The supernatant was analyzed by SDS-PAGE and Western blotting using anti-oligo/poly α2,8 KDN or anti-megalin antibodies as described previously [6].

Partial purification of megalin from mouse yolk sac carcinoma tissue

A mouse yolk sac carcinoma tissue homogenate was applied onto a XK 16/100 gel filtration column packed with Sephacryl S-400. Fractions, immunoreactive for oligo/poly α2,8 KDN were pooled and used for Western blot and lectin blot analysis.

β-elimination, N-Glycosidase F treatment and lectin blotting

For β-elimination, PVDF strips containing partially purified megalin from yolk sac carcinoma tissue were incubated with 0.1 N sodium hydroxide for 24 h at 37°C. As control, strips were incubated with PBS under the same conditions as described above. For N-Glycosidase F treatment, nitrocellulose strips with megalin were blocked with 1% BSA in 50 mM sodium acetate buffer (pH 5.5) followed by incubation with 5 U of N-Glycosidase F for 16 h at 37°C. For lectin blotting, strips were incubated with digoxigenin-conjugated Concanavalin A for 1 h followed by alkaline phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab′ fragments (150 U/ml). Color reaction was performed using nitroblue tetrazolium/BCIP-phosphate as substrates.

Results and discussion

In our previous studies [6] the >350 kDa oligo/poly α2,8 KDN bearing glycoprotein from rat kidney was identified as being megalin. Megalin is the most abundant membrane protein in proximal tubules of rat kidney but it is also expressed in other specialized epithelia [8] albeit at much lower levels. Immunoprecipitated megalin from rat lung, brain and placenta, from mouse yolk sac carcinoma tissue as well as L2 cells and from mouse F9 teratocarcinoma cells, induced to differentiate (data not shown) exhibited immunoreactivity for oligo/poly α2,8 KDN (Fig. 1). Thus, megalin present in these normal rat tissues and in carcinoma cells carries oligo/poly α2,8 KDN as a unique posttranslational modification. These findings on megalin are similar to those for a 150 kDa glycoprotein in lung which also carries oligo/poly α2,8 KDN both in normal human and rat embryonic and postnatal lung and in human lung carcinomas [2]. Furthermore, KDN has been detected in both normal human ovary and ovarian carcinoma cells but no data have been reported regarding its protein carrier [12].

β-elimination on stripes containing partially purified megalin from mouse yolk sac tissue resulted in the absence in immunoreactivity for oligo/poly α2,8 KDN. In contrast *N*-glycosidically linked oligosaccharides on megalin are not affected by this treatment as demonstrated by the reactivity with Conconavalin A (Fig. 2). Furthermore, N-Glycosidase F treatment increased the immunoreactivity for oligo/poly α2,8 KDN (Figure 2) indicating that oligo/poly α2,8 KDN is part of *O*-glycosidically linked oligosaccharides on megalin from mouse yolk sac carcinoma tissue. These data are in agreement with previously described results obtained from purified rat kidney megalin [6].

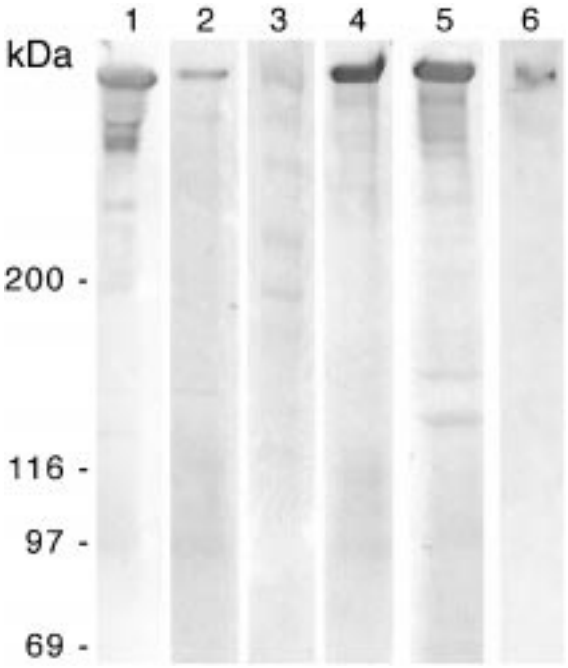

Figure 1. Immunoprecipitated megalin from rat kidney (lane 1), lung (lane 2) brain (lane 3) and placenta (lane 4), mouse yolk sac carcinoma (lane 5) and L2 cells (lane 6) exhibited immunoreactivity for oligo/poly α2,8 KDN. Molecular mass standards were as follows: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa).

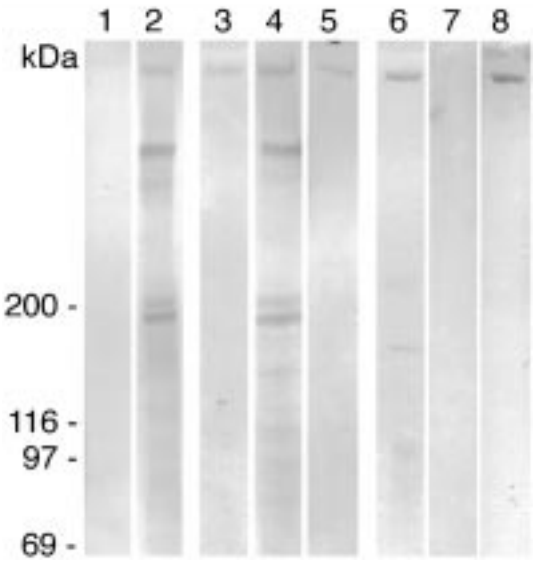

Figure 2. β-elimination and N-Glycosidase F treatment on partially purified megalin from yolk sac carcinoma tissue. β-elimination resulted in the absence of immunoreactivity for oligo/poly α2,8 KDN (lane 1). Lectin blot using Concanavalin A after β-elimination (lane 2). Immunoblot for oligo/poly α2,8 KDN (lane 3), Concanavalin A blot (lane 4) and immunoblot for megalin (lane 5) after incubation of the stripes with PBS at 37°C for 24 h. Increased immunoreactivity for oligo/poly α2,8 KDN after N-Glycosidase F treatment (lane 6) and absence of labeling for Concanavalin A (lane 7). Immunoblot for megalin after N-Glycosidase F treatment (lane 8).

Megalin has been found to bind a number of different ligands (for a review see [13]) *in vitro*. What may be a possible function of oligo/poly α2,8 KDN on megalin? Due to its localization in clathtrin-coated pits megalin was suggested to act as an endocytic receptor in kidney. Dependent on the organ localization of megalin and the composition of the surrounding fluids it can be anticipated that the nature of the ligands will vary from one organ to another. For example, in proximal tubules it was suggested that megalin is involved in the reabsorption of filtered proteins [14] and Ca^{2+}-ions [15]. In type II pneumocytes, megalin seems to be responsible for the clearance of protease / protease inhibitor complexes [16,17] from the alveolar space [18]. Charge interactions between negatively charged complement-type repeats and basic regions of the ligands seems to be important for receptor / ligand interaction. Furthermore, megalin is not only a Ca^{2+} binding protein but Ca^{2+}-ions are essential for ligand binding. *In vitro* oligo/poly α2,8 KDN binds Ca^{2+}-ions highly preferentially [19]. Therefore, we speculate that the polyanionic nature of oligo/poly α2,8 KDN together with the negatively charged complement-type repeats may be important for ligand binding to megalin and for the receptor activity in the various tissues. Studies are in progress to clarify this proposal.

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Received 4 November 1998, revised 19 February 1999, accepted 3 March 1999.