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Septin 7 reduces nonmuscle myosin IIA activity in the SNAP23 complex and hinders GLUT4 storage vesicle docking and fusion

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ABSTRACT

Glomerular epithelial cells, podocytes, are insulin responsive and can develop insulin resistance. Here, we demonstrate that the small GTPase septin 7 forms a complex with nonmuscle myosin heavy chain IIA (NMHC-IIA; encoded by *MYH9*), a component of the nonmuscle myosin IIA (NM-IIA) hexameric complex. We observed that knockdown of NMHC-IIA decreases insulin-stimulated glucose uptake into podocytes. Both septin 7 and NM-IIA associate with SNAP23, a SNARE protein involved in GLUT4 storage vesicle (GSV) docking and fusion with the plasma membrane. We observed that insulin decreases the level of septin 7 and increases the activity of NM-IIA in the SNAP23 complex, as visualized by increased phosphorylation of myosin regulatory light chain. Also knockdown of septin 7 increases the activity of NM-IIA in the complex. The activity of NM-IIA is increased in diabetic rat glomeruli and cultured human podocytes exposed to macroalbuminuric sera from patients with type 1 diabetes. Collectively, the data suggest that the activity of NM-IIA in the SNAP23 complex plays a key role in insulin-stimulated glucose uptake into podocytes. Furthermore, we observed that septin 7 reduces the activity of NM-IIA in the SNAP23 complex and thereby hinders GSV docking and fusion with the plasma membrane.

1. Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes and the most common cause of end-stage renal disease [1]. Much effort has therefore been devoted to understanding the mechanisms that promote glomerular damage in DN. Risk factors for the development of DN include, for example, hyperglycemia, hypertension, dyslipidemia and genetic factors [2]. Also insulin resistance is a risk factor for DN [2], and has been reported to be associated with microalbuminuria in patients with type 1 as well as type 2 diabetes [3,4].

At the cellular level the mechanisms leading to the development of insulin resistance include mutations in the insulin receptor itself [5] and impairments in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway which mediates the uptake of glucose into cells [6]. Glucose transporter 4 (GLUT4) is the major insulin-inducible glucose transporter. Insulin activates the translocation of GLUT4 storage vesicles (GSVs) from the intracellular storage site to the plasma membrane [7]. GSV trafficking consists of several steps, and disturbances in this trafficking process may also cause insulin resistance. The trafficking involves actin and microtubule networks, the exocyst complex proteins that help to tether the GSVs with the plasma membrane, and the soluble *N*-ethylmaleimide–sensitive fusion protein attachment protein receptor (SNARE) complex that facilitates the tethering, docking and fusion of GSVs with the plasma membrane.

Abbreviations: DN, diabetic nephropathy; GLUT4, glucose transporter 4; GSV, GLUT4 storage vesicle; NM-IIA, nonmuscle myosin IIA; NMHC-IIA, nonmuscle myosin heavy chain IIA; pp-RLC, phosphorylated myosin regulatory light chain; SNAP23, synaptosome-associated protein, 23 kDa; SNARE, N-ethylmaleimide–sensitive fusion protein attachment protein receptor; VAMP2, vesicle-associated membrane protein 2

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The SNARE complex includes a vesicle-SNARE on GSVs, such as vesicle-associated membrane protein 2 (VAMP2), and target-SNAREs on the plasma membrane, such as syntaxin 4 and synaptosomal-associated protein, 23 kDa (SNAP23) [7].

Interestingly, kidney glomerular epithelial cells, or podocytes, are insulin sensitive and able to rapidly transport glucose using the glucose transporters GLUT4 and GLUT1 [8]. Insulin signaling is necessary for normal kidney function, as deletion of insulin receptor specifically in podocytes induces a disease state reminiscent of DN in a normoglycemic environment [9]. Furthermore, podocytes isolated from diabetic db/db mice are unable to respond to insulin indicating that podocytes can develop insulin resistance [10]. Nephrin, an essential structural protein of the glomerular filtration barrier, aids in GSV docking by interacting with the v-SNARE VAMP2 [11]. We showed previously that the small GTPase septin 7 associates with nephrin and VAMP2, and negatively regulates glucose uptake in podocytes [12]. Our data further revealed that knockdown of septin 7 increased the complex formation of VAMP2 with nephrin and syntaxin 4 [12], indicating that depletion of septin 7 enhances the final stages of GSV exocytosis.

In this study we set out to define whether septin 7 plays a specific, regulatory role in the docking of the GSVs with the plasma membrane as suggested by its association with nephrin [12]. We found that nonmuscle myosin heavy chain IIA (NMHC-IIA), which regulates the docking and fusion of GSVs in adipocytes [13–15], is a novel interaction partner of septin 7. Specifically, we observed that septin 7 reduces the activity of nonmuscle myosin IIA (NM-IIA) in the SNAP23-containing SNARE complex at the plasma membrane. Our study thus proposes a novel mechanism by which septin 7, by reducing the activity of NM-IIA in the SNAP23 complex, hinders GSV docking and fusion with the plasma membrane and reduces glucose uptake into podocytes.

2. Materials and methods

2.1. Cell culture, stable overexpression of nephrin in podocytes and preparation of cell lysates

Conditionally immortalized human podocytes (AB 8/13) were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 1% ITS (Sigma-Aldrich, St. Louis, MO) at 33 °C and shifted to 37 °C for two weeks for differentiation [16]. Rat nephrin was previously subcloned into pLNCX2 retroviral vector [17]. HEK293T cells were co-transfected with pLNCX2-nephrin and packaging pKAT2 vectors using Lipofectamine 2000 (Invitrogen, Camarillo, CA, USA). Virus-containing medium was filtered through a 0.45 μm filter and used to infect mouse podocytes. Podocytes overexpressing nephrin were selected by culturing the cells in medium supplemented with 2,5 mg/ml of geneticin (G418) (Gibco, Life Technologies, Carlsbad, CA, USA) for 12 days. Mouse podocytes and mouse podocytes stably overexpressing nephrin were maintained in DMEM containing 4.5 g/L glucose, 10% FCS, penicillin and streptomycin (Sigma-Aldrich), supplemented with 10 U/ml INF-γ (Sigma-Aldrich) at 33 °C. Where indicated, podocytes were starved and stimulated with 20 nM insulin (NovoNordisk, Bagsværd, Denmark). HEK293T cells were maintained in DMEM supplemented with 10% FCS, 1% ultraglutamin, 1% streptomycin and 1% penicillin at 37 °C. Cells were lysed in 1% Nonidet P-40 (NP-40), 20 mM HEPES, pH 7.5, 150 mM NaCl, in 50 mM HEPES, pH 7.6, 0.5% Triton X-100, 0.5% CHAPS or in 0.5% NP-40, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA for co-immunoprecipitation as described [12]. All lysis buffers were supplemented with 50 mM NaF, 1 mM Na_3VO_4 and 1x Complete proteinase inhibitor cocktail (Roche, Basel, Switzerland).

2.2. Immunoprecipitation

Lysates were precleared with protein A-Sepharose (Invitrogen) or TrueBlot® anti-rabbit or anti-mouse Ig IP beads (eBiosciences, San Diego, CA, USA) and incubated with anti-septin 7, anti-SNAP23 or anti-nephrin antibodies and normal rabbit serum or rabbit/mouse IgGs (Zymed, South San Francisco, CA) as controls at 4 °C for 16 h. The immune complexes were bound to protein A-Sepharose or TrueBlot beads, washed with lysis buffer, stained with GelCode Blue (Pierce Chemical Co, Rockford, IL) or immunoblotted as described below.

2.3. Protein identification by LC-MS/MS

For mass spectrometry analysis the precipitated proteins were separated by SDS-PAGE and stained with GelCode Blue (Pierce Chemical Co). The > 200 kDa band obtained in the septin 7 immunoprecipitation was excised from gel, in-gel digested with trypsin and the resulting peptides were analyzed by LC-MS/MS using an Ultimate 3000 nano-LC (Dionex, Sunnyvale, CA) and a QSTAR Elite hybrid quadrupole TOF-MS (Applied Biosystems/MDS Sciex, Life Technologies, Carlsbad, CA) with nano-ESI ionization as described previously [18]. The LC-MS/MS data was searched with in-house Mascot through ProteinPilot 2.0 interface against the SwissProt database using criteria: Human-specific taxonomy, trypsin digestion with one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification.

2.4. Preparation of tissue lysates

Glomerular and tubular fractions were isolated from kidney cortices of male Sprague-Dawley and 40 weeks old, albuminuric obese or lean Zucker rats by graded sieving [19]. Zucker rats (Crl: ZUC-Leprfa) were purchased from Charles River Laboratories (Sulzfeld, Germany). Blood glucose, urinary albumin, and creatinine measurements have been described in [20]. The protocols were approved by the National Animal Experiment Board. Tissue lysates were prepared as above in NP-40 lysis buffer or in 50 mM HEPES, pH 7.6, 0.5% Triton X-100, 0.5% CHAPS.

2.5. Antibodies

Goat anti-septin 7 (N12) and rabbit anti-septin 7 (H120) IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and rabbit anti-septin 7 (C) IgG was from Immuno-Biological Laboratories Co., Ltd. (Gumma, Japan). Rabbit anti-nonmuscle myosin IIA heavy chain IgG was from Biomedical Technologies, Inc. (Stoughton, MA, USA), and rabbit anti-phospho-myosin light chain 2 (Thr18/Ser19) IgG from Cell Signaling (Danvers, MA, USA). Rabbit anti-nephrin IgG (#1034) is described in [21], and mouse anti-nephrin IgG (5-1-6) in [22]. Guinea pig anti-nephrin IgG was from PROGEN Biotechnik (Heidelberg, Germany), and rabbit anti-SNAP23 and mouse anti-VAMP2 IgGs were from Synaptic System (Goettingen, Germany). Mouse anti-SNAP23, mouse anti-tubulin and mouse anti-actin IgGs were from Sigma-Aldrich.

2.6. Immunoblotting

Glomerular lysates of 40 weeks old six individual obese Zucker (fa/fa) and six individual lean Zucker (fa/+) rats were used for analyzing NMHC-IIA, septin 7 and pp-RLC expression levels. Differentiated human podocytes were exposed to 10% patient sera in FCS-free culture medium for 48 h. The serum samples were from 4 normoalbuminuric and 5 macroalbuminuric patients with type 1 diabetes from the Finnish Diabetic Nephropathy Study (FinnDiane; www.finndiane.fi) (Supplemental Table S1). Immunoblotting was performed as in [17] and blots were quantified using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).