N-Acetylglucosamine Kinase is Localized to Dendritic Lipid Rafts and Caveolae of Rat Hippocampal Neurons

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A dynamic cycle of addition and removal of O-linked N-acetylglucosamine (O-GlcNAc) at serine and threonine residues is emerging as a key regulator of nuclear and cytoplasmic protein activity. In this work, immunocytochemistry was carried out to investigate the subcellular expression of GlcNAc kinase (NAGK, EC 2.7.1.59) that catalyzes the phosphorylation of GlcNAc to GlcNAc 6-phosphate. Immunostainings of cultured rat hippocampal neurons revealed patchy or punctate distribution of NAGK. When NAGK is doublestained with caveolin-1 or flotillin, markers for caveolae and lipid rafts, respectively, NAGK was co-localized with these markers. These results indicate that most, if not all, of the NAGK immunopunctae represent caveolae and lipid rafts, and suggest NAGK's role in these membrane microdomains.

Key words — N-Acetylglucosamine kinase, lipid raft, hippocampal neuron, immunocytochemistry

Introduction

N-acetylglucosamine (GlcNAc) kinase (NAGK, EC 2.7.1.59) catalyzes the phosphorylation of GlcNAc to GlcNAc 6-phosphate. Mammalian NAGK has been purified from man[8], pig[6] and rat[1]. The rat enzyme was characterized as a 39-kDa polypeptide which assembles as a homodimer. NAGK belongs to the group of N-acetylhexosamine kinases. The two other members of this group are N-acetylgalactosamine kinase[19] and N-acetylmannosamine kinase[9,24].

GlcNAc is a major component of complex carbohydrates. It is found in N-glycans[14,15] and O-glycans[10,29] as well as in glycolipids[13]. GlcNAc is also linked as a monosaccharide to serine and threonine residues of proteins[16]. O-linked N-acetylglucosamine (O-GlcNAc) proteome has revealed a diverse set of proteins engaged in numerous cellular functions. O-GlcNAc - modified proteins are usually phosphoproteins and are often components of macromolecular complexes such as transcription complexes or nuclear pores. Proteins engaged in functions ranging from carbohydrate metabolism, signaling, transcription and translation, and the stress response are well represented in the O-GlcNAc proteome (see a recent review[16]). A dynamic cycle of addition and removal of O-GlcNAc at ser-

ine and threonine residues is emerging as a key regulator of nuclear and cytoplasmic protein activity.

Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids that are involved in various cellular functions such as the trafficking and sorting of membrane proteins, secretory and endocytotic pathways, and signal transduction[3,23]. In mammalian CNS neurons, a multiprotein-signaling complex is assembled at the postsynaptic membrane of dendritic spines, some components of which have the lipid modifications typical for raft-associated proteins [e.g., postsynaptic density (PSD)-95 and glutamate receptor (GluR)-interacting protein (GRIP)][27,31]. Therefore, lipid rafts in neurons have been implicated in the mechanisms of cell polarity, cell migration, and motility of membrane protrusions such as growthcone and lamellipodia.

Eukaryotic peptide elongation factor-1A (eEF1A) is modified by GlcNAc[16]. Recently, my laboratory found that the domain 3 of the eEF1A (eEF1A-D$_{III}$) interacts with NAGK (manuscript in preparation). Immunocytochemistry using cultured rat hippocampal neurons showed that both NAGK and eEF1A punctae were distributed in somatodendritic domains. However, they did not overlap each other in mature neurons. The punctate, patchy distribution of NAGK was very similar to that of lipid rafts. In this work, immunocytochemistry using specific markers for lipid rafts was carried out. Here, evidence for the localization of NAGK in lipid rafts of cultured rat hippocampal neurons is presented.

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Materials and Methods

Neuronal culture

Embryonic day 18 (E18) rat hippocampal cells were dissociated by triturating trypsin-treated tissues, and were grown on poly-D-lysine-coated coverslips (1,000~1,500 cells/mm^2) in the Neurobasal medium supplemented with B27 and 0.25 mM glutamax I (Invitrogen, Carlsbad, CA), as previously described[2].

Immunocytochemistry

Coverslips (with cell-side up) were rinsed briefly in D-PBS at RT, and fixed with 4% (w/v) paraformaldehyde (PFA) for 10 min at RT. After rinsing in D-PBS briefly, coverslips were further fixed by methanol for 20 min at -20°C, and incubated in preblocking buffer (0.05% Triton X-100, 5% normal goat serum in h-PBS) overnight at 4°C. Primary antibodies were added in preblock buffer and incubated overnight at 4°C. Primary antibodies used are as follows; mouse monoclonal anti-caveolin-1 (1:250) and anti-flotillin-1 (1:100, BD Bioscience), chicken anti-PSD-95 (UCT-C1, 1:2,000; a gift from Dr. R. Walikonis, University of Connecticut), chicken anti-NAGK polyclonal IgY (GenWay Biotech, Inc., San Diego, CA). Coverslips were rinsed in preblock buffer (15 min x 3) and incubated with secondary antibodies in preblock buffer [goat anti-mouse IgG (Alexa 488, Molecular Probes, 1:1,000) and goat anti-chicken IgG (Alexa 647, Molecular Probes, 1:1,000)]. Coverslips were rinsed in preblock buffer (15 min x 1) followed by rinse in PBS (15 min x 2). Coverslips were mounted with 4% n-propylgallate in 90% glycerol, 10% sodium carbonate buffer (pH8.7) and viewed in a fluorescence laser-scanning confocal microscope (Leica TCS SP2, Wetzlar, Germany).

Results and Discussion

Extensive studies have demonstrated the existence of lateral membrane domains that contain a specific repertoire of lipids and proteins. Caveolae are small (50-100 nm) flask-shaped invaginations of the plasma membrane[17,18]. These membrane microdomains are thought to play a critical role in communication between cell surface membrane receptors and intracellular signaling protein cascades as well as other processes (see a recent review[22]). Caveolae have more recently become recognized as the prototypic lipid raft microdomain present in or near the plasma membrane. Along with lipid rafts in general, caveolae are rich in cholesterol and sphingolipids as well as glycosyl-phosphatidylinositol (GPI)-anchored proteins, such as the prion protein and GDNF family receptor (GFR) alpha1-4[21,26]. Following ligand activation of cellular receptors, intracellular signaling molecules are recruited to caveolae, which are thought to enhance signal transduction efficiency[22].

The presence of a caveolin protein family member is the proteinaceous hallmark of caveolae. This family contains at least three members, including caveolin-1 and caveolin-2, which have been reported to be expressed in many cell types, including neurons[5,7].

Caveolin-1 punctae co-localize with those of PSD-95

Caveolin-1 is expressed in hippocampal neurons maintained in culture, and is induced following glutamate stimulation, apparently through kainate and AMPA-type receptors [5]. In this work, antibody against caveolin-1 showed a punctate expression along the plasma membranes (Fig. 1A, caveolin, green). It is reported that PSD-95, GRIP, and AMPA receptors are present in lipid raft fractions isolated from rat brain[4,20,25]. In order to confirm
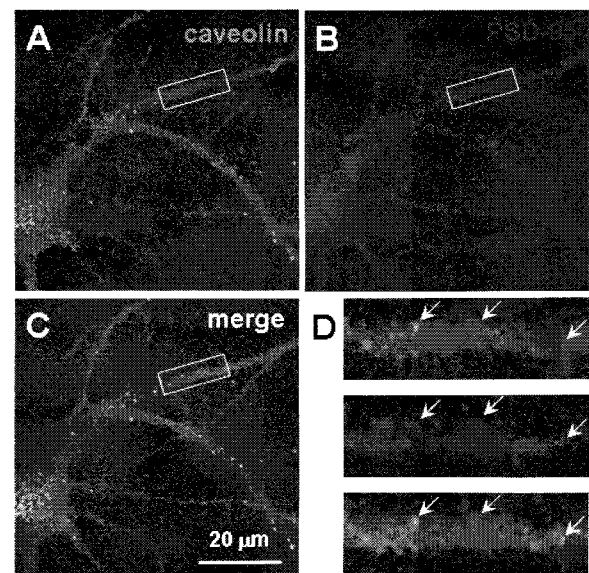

Fig. 1. Immunocytochemistry showing expression of caveolin-1 and PSD-95. Cultured rat hippocampal neurons were doublestained with antibodies against caveolin-1 and PSD-95. Primary antibodies were visualized using goat secondary antibodies conjugated for Alexa 488 (A; caveolin-1, green) or Alexa 647 (B; PSD-95, blue) fluorochromes. Two images were merged in C and the boxed areas are enlarged and shown in D. Scale bar; 20 μm.

the identity of caveolin-positive punctae as lipid raft, hippocampal neurons were doublestained with antibodies against caveolin-1 and PSD-95. The PSD-95 immunostain showed typical punctae along dendrites (Fig. 1B, PSD-95, blue). The merge of the two stainings (Fig. 1C, merge, and D) revealed many (~70%) punctae that are stained with both antibodies. This result supports that the caveolin-immunostained punctae represent lipid rafts.

NAGK is localized to lipid rafts

Immunocytochemistry of cultured rat hippocampal neurons showed patchy or punctate expression of NAGK in somatodendritic domains (Fig. 2B, NAGK, blue). The NAGK-positive punctate stainings were distributed throughout the shafts of dendrites but did not make protrusions. Therefore, the NAGK-positive immunopunctae is not likely to be spines. In order to find if these punctae are lipid rafts, hippocampal cultures were doublestained with antibodies against NAGK and caveolin-1. When the two images were merged, there appear many (~70%) punctae that are stained with both antibodies (Fig. 2C, merge and 2D). This result suggests that NAGK is localized to lipid rafts.

In order to confirm expression of NAGK in lipid rafts,

antibody against flotillin was used to doublestain hippocampal neurons with anti-NAGK antibody. Flotillin is another marker of rafts in the brain that co-localizes with saturated lipids[12]. As shown in Fig. 3A (flotillin, green), flotillin is highly expressed in hippocampal neuron. They are highly concentrated in the membrane in the punctate fashion. Merge of the flotillin image with NAGK one showed co-localization of the two proteins in most (>80%) punctae (Fig. 3C, merge and 3D). This result confirms that most, if not all, of the NAGK immunopunctae represent lipid rafts.

In this report I presented evidence for the expression of NAGK in lipid rafts in hippocampal neurons by showing co-localization of the protein with caveolin-1 and flotillin, two raft markers. Rafts are tissue-specific membrane microdomains composed of saturated phospholipids, sphingolipids, cholesterol and signaling proteins, and are defined as detergent-resistant with low buoyant density[28]. Neural tissues (glia and neurons) are enriched in rafts, and the protein flotillin is a marker of rafts in the brain that co-localizes with saturated lipids[12]. Caveolae are distinguished from rafts morphologically and by the presence of the cholesterol-binding protein caveolin-1; however, nei
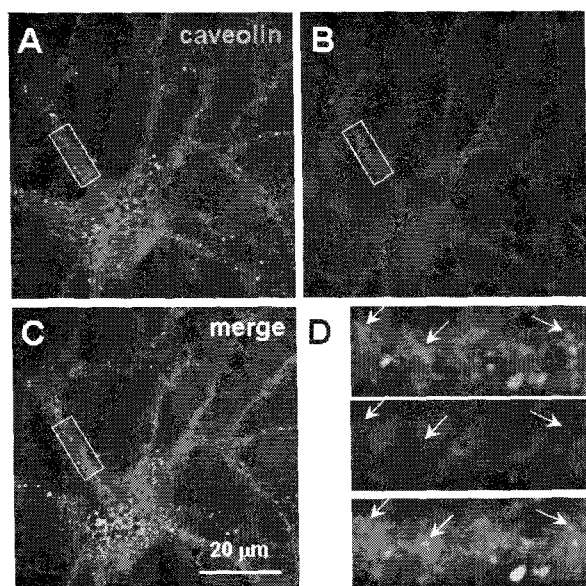

Fig. 2. Immunocytochemistry showing expression of caveolin-1 and NAGK. Cultured rat hippocampal neurons were doublestained with antibodies against caveolin-1 and NAGK. Primary antibodies were visualized using goat secondary antibodies conjugated for Alexa 488 (A; caveolin-1, green) or Alexa 647 (B; NAGK, blue) fluorochromes. Two images were merged in C and the boxed areas are enlarged and shown in D. Scale bar; 20 μm.
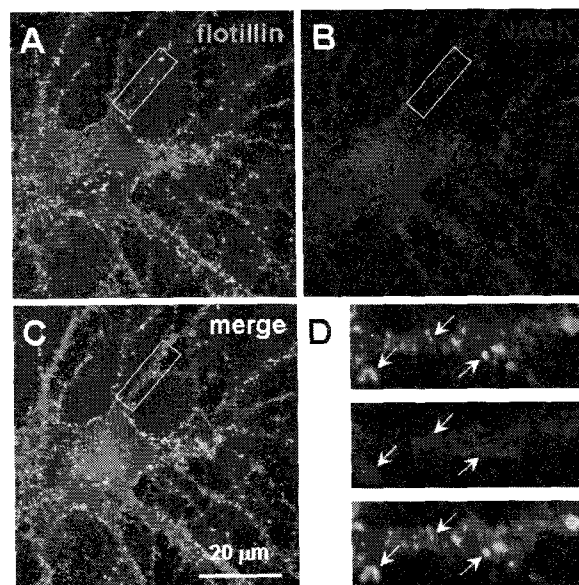

Fig. 3. Immunocytochemistry showing expression of flotillin and NAGK. Cultured rat hippocampal neurons were doublestained with antibodies against flotillin and NAGK. Primary antibodies were visualized using goat secondary antibodies conjugated for Alexa 488 (A; flotillin, green) or Alexa 647 (B; NGAK, blue) fluorochromes. Two images were merged in C and the boxed areas are enlarged and shown in D. Scale bar; 20 μm.

ther their specific shape nor caveolin expression appear to be exclusively characteristic for caveolae[30]. Apparently, both membrane microdomains, rafts and caveolae, may co-exist in the same cell membrane or represent different variants of a related structure.

A dynamic cycle of addition and removal of O-linked N-acetylglucosamine (O-GlcNAc) at serine and threonine residues is emerging as a key regulator of nuclear and cytoplasmic protein activity. Like phosphorylation, protein O-GlcNAcylation dramatically alters the posttranslational fate and function of target proteins. NAGK adds a phosphoryl group on the GlcNAc moiety, hence a second modification in addition to the primary GlcNAcetylation. To my best knowledge, the functions of NAGK in neurons are not reported at all. However, lipid rafts have been implicated in the mechanisms of cell polarity, cell migration, and motility of membrane protrusions. In this context, it is very interesting that NAGK is highly expressed in the protrusions such as lamelipodia and growth cones of early developmental neurons (manuscript in preparation). In mature neurons, as is in this work, NAGK is localized at lipid rafts suggesting its role in morphological changes in dendritic membranes. Experiments such as overexpression and RNA interference would shed light on its function.

Acknowledgment

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초록 : 흰쥐 해마신경세포 가지돌기의 lipid rafts 및 caveolae에서 N-acetylglucosamine kinase의 표현

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단백질의 serine 및 threonine 잔기에 O-linked N-acetylglucosamine (O-GlcNAc)의 수식은 핵단백질과 세포질 단백질의 주요 조절인자로 부각되고 있다. 본 연구에서는 GlcNAc를 인산화시켜 GlcNAc 6-phosphate로 만드는 GlcNAc kinase (NAGK, EC 2.7.1.59)의 세포내 표현을 면역화학적 방법으로 조사하였다. 배양한 해마신경세포에서 NAGK는 가지돌기를 따라 점박이(punctae)를 형성하였으며, 이 점박이들은 caveolin-1 혹은 flotillin 항체에도 염색이 되었다. 이들은 각각 caveolae와 lipid raft의 표지단백질이기 때문에 본 연구결과는 NAGK가 세포막의 이러한 특수 미세부분(microdomain)에 존재함을 의미하며, 이 미세부분에서 아직 알려지지 않은 어떤 기능을 할 것을 시사한다.