

Protrusion of N-acetylglucosamine Kinase Clusters into the Base of Excitatory Synapses

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N-Acetylglucosamine kinase (GlcNAc kinase or NAGK; EC 2.7.1.59) catalyzes the phosphorylation of GlcNAc to GlcNAc-6-phosphate (GlcNAc-6-P). Despite detailed characterization of the enzyme itself, there have been few studies on the expression of NAGK in mammalian tissues. In the rat hippocampal neuron in culture, NAGK-immunoreactivity (IR) formed clusters in somatodendritic domains. In this study we characterized the NAGK clusters that protrude out the long axis of dendritic shafts. By double-labeling of the neurons with antibodies against NAGK and various synaptic proteins, we show that NAGK is positioned at the base of spines, while there were no NAGK protrusions into inhibitory postsynaptic sites. Immunoblot analysis showed that NAGK was included in synaptosomes but not in PSD fractions. Our results indicate that the NAGK clusters at the dendritic periphery protrude into spines.

Key words : Dendrite, hippocampal culture, immunocytochemistry, microtubule, NAGK, spine

Introduction

N-Acetylglucosamine kinase (GlcNAc kinase or NAGK; EC 2.7.1.59) catalyzes the phosphorylation of GlcNAc to GlcNAc-6-phosphate (GlcNAc-6-P), which can enter a catabolic pathway, or it can enter an anabolic pathway leading to the formation of uridine diphosphate (UDP)-GlcNAc [7]. The rat and human NAGK in solution form homodimers of 37- and 39-kDa subunits, respectively (Hinderlich *et al.*, 1998). NAGK belongs to N-acetylhexosamine kinases, members of the sugar kinase/heat shock protein 70/actin superfamily [1,6]. Crystal structures of homodimeric human NAGK in complex with GlcNAc or with ADP and glucose revealed that the active site of NAGK is located in a deep cleft between the small and large domains of the V-shaped monomer [14]. The enzyme adopts a 'closed' configuration in the GlcNAc-bound complex by rotating the small domain 12 to 26° against the large domains from an 'open' configuration in the ADP/glucose-bound structure.

Despite detailed characterization of the enzyme itself, there have been few studies on the expression of NAGK in mammalian tissues. NAGK mRNA and protein are ubiqu-

itously expressed throughout various cell lines and tissues [6]. Our laboratory has been studying the expression of NAGK in nerve tissues. Immunohistochemistry (IHC) of rat brain sections showed that NAGK is highly expressed in neurons, while immunocytochemistry (IC) of dissociated cultures of rat hippocampal neurons showed that the strongest NAGK-immunoreactivity (IR) was associated with neuronal dendrites and negligible in axons (submitted). In dendrites of hippocampal neurons in culture, the NAGK-IR formed clusters along microtubule fibers. Interestingly, the NAGK clusters at the borders of dendrites protruded out of the microtubule shafts. In this study we characterized these NAGK protrusions in relation to spines. By double-labeling the hippocampal neurons with antibodies against NAGK and various spine markers, we show evidence that these protrusions correspond to the base of spines.

Materials and Methods

Antibodies

The following antibodies were used at the indicated dilutions: chicken polyclonal NAGK (α NAGK) (1:1,000; GenWay Biotech, Inc., San Diego, CA); MAb actin (α Actin, JLA20), MAb tubulin α -subunit (α Tubulin, 12g10) (1:2,000; Developmental Studies Hybridoma Bank, University of Iowa,

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Iowa City, IA); MAb gephyrin (α Geph; clone 7a, 1:1,000, Synaptic Systems, Göttingen, Germany); MAb PSD95 (α PSD95) (1:1,000; Upstate Biotechnology Inc., Lake Placid, NY); rabbit polyclonal NR2A (α NR2A), NR2B (α NR2B) (1:1,000; Upstate Biotechnology Inc.).

Primary culture of rat hippocampal neurons

Dissociated hippocampal cells from Sprague-Dawley rat at embryonic day 18 (E18) or E19 were plated onto 12-mm diameter polylysine/laminin-coated glass coverslips at a density of ~ 150 neurons/ mm^2 as described [2] and grown in astrocyte-conditioned Neurobasal media as described [5,13].

Immunocytochemistry (IC)

Cells were fixed by a sequential paraformaldehyde/methanol fixation procedure [incubation in 4% paraformaldehyde in phosphate buffered saline (20 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl) at room temperature (RT) for 10 min followed by incubation in methanol at -20°C for 20 min] [13]. IC was performed with indicated primary and secondary [Alexa Fluor 488-conjugated goat anti-mouse, Alexa Fluor 568-conjugated goat anti-rabbit and Alexa Fluor 647-conjugated goat anti-chicken IgG (each diluted 1:1,000 in blocking buffer; Invitrogen)] as described [13].

Subcellular fractionation and immunoblot

'One-Triton' PSD fractions were prepared from rat forebrain (FB) by washing synaptosomes (S) with 0.5% Triton X-100 for 15 min at 4°C , and immunoblotted as described previously [12]. In brief, synaptosomes were isolated from homogenates by sucrose step-gradient centrifugation using 0.85, 1.0 and 1.2 M sucrose layers, and then extracted with 0.5% Triton X-100 for 15 min at 4°C . The resulting 'One-Triton' PSD fraction was pelleted by centrifugation at $36,800\times g$ for 45 min. Forty micrograms of each fractions were subjected to immunoblotting, and the antigen-antibody complex was visualized with the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Rockford, IL).

Laser-scanning confocal microscopy.

Confocal images (1,024 \times 1,024 pixels) were acquired using a Leica TCS SP2 confocal system with laser lines at 488, 543, and 633 nm. Digital images were processed with the use of Adobe Systems Photoshop 5.0 software.

Results and Discussion

The NAGK clusters at the border of dendritic shafts often protrude out

Cultured hippocampal neurons were double-labelled with antibodies against NAGK and α Tubulin (Fig. 1). The NAGK-immunoreactivity (IR) was distributed throughout the cells with higher intensity in the dendrites (arrow) than in soma (asterisk). Apparently, NAGK-IR did not form a fiber even in the dendritic domains where it is denser than in soma. Instead, it made clusters (Fig. 1A, NAGK and inset). This feature was in contrast to that of tubulin-IR, which formed distinct fibers (Fig. 1A, tubulin and inset). The distribution of NAGK was very similar to that of tubulin. However, the merge image revealed differential localizations of the two IR's (Fig. 1B). In particular, many NAGK-IR clusters protrude out of the long axis at the periphery (Fig. 1B,

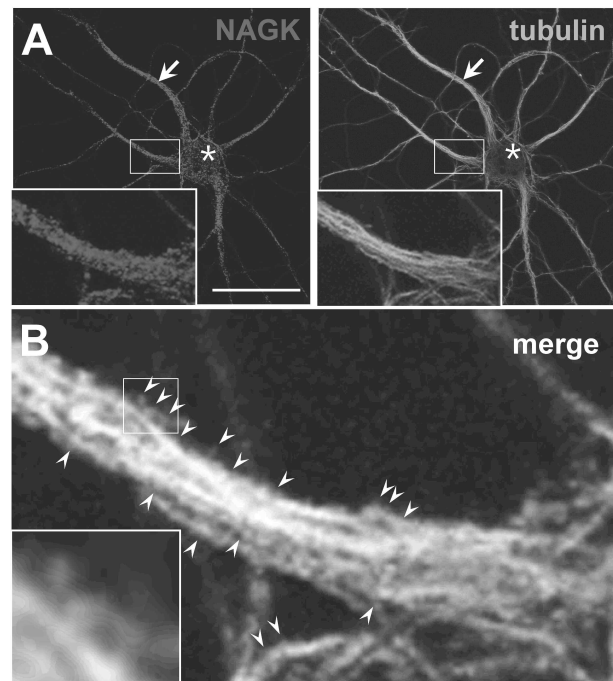


Fig. 1. The NAGK clusters at the border of dendritic shafts often protrude out. Cultured hippocampal neurons (21DIV) were double-labelled with antibodies against NAGK and α Tubulin, and confocal images were acquired using a 40X objective. *A*, A dendrite and a soma were marked with an arrow asterisk, respectively. The boxed area was shown enlarged in each insets. Scale bar, 30 μm . *B*, merge. Two images for the insets in *A* were merged, and some the NAGK protrusions were marked by arrowheads. The boxed area was further enlarged in inset. Note that NAGK clusters at the dendritic border protrude out of the shaft.

arrowheads and inset).

NAGK protrusions at the dendritic border are positioned at the base of spines

To characterize the NAGK protrusion at the periphery of the dendritic shafts, we double-labelled cultured hippocampal neurons with antibodies against NAGK and various synaptic markers. Actin is concentrated underneath cell membrane. Therefore, the distribution of actin would reveal the contour of a cell. To get insights on the position of NAGK protrusions in relation to dendritic contour, we double-labelled neurons with antibodies against NAGK and actin. As expected, confocal microscopic images of actin-IR revealed the contour of neurons. In addition, large actin-IR clusters appeared along the periphery of the dendritic shafts (Fig. 2A, actin). In neurons, F-actin is particularly enriched in dendritic spines [4,11]. In immunoelectron microscopic studies, bundles of F-actins are observed in the necks of dendritic spines, and lattices (network) of F-actins in the spine head [10]. Therefore, these large actin-IR clusters are thought to be spines. Interestingly, the actin clusters accompany

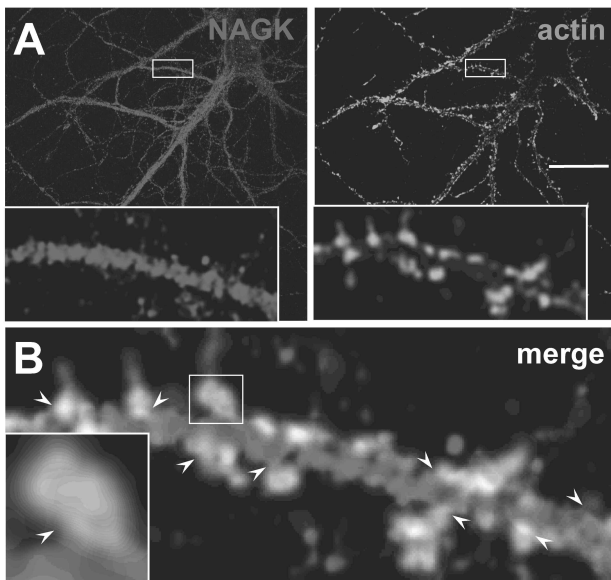


Fig. 2. NAGK protrusions are positioned at the base of spines. Cultured hippocampal neurons (21DIV) were double-labelled with antibodies against NAGK and actin, and confocal images were acquired using a 40X objective. *A*, single channel images for NAGK and actin, and enlarged images for the boxed areas were shown in each *insets*. Scale bar, 30 μ m. *B*, merge. The inset images of *A* were merged. The NAGK protrusions that abut against actin-IR clusters were marked by arrowheads. The boxed area was shown enlarged in *inset*. Note that the NAGK-IR clusters protrude into actin-IR clusters.

NAGK protrusions at the border of dendritic shafts (Fig. 2B, arrowheads). It can be observed that NAGK-IR protrude into actin clusters (Fig. 2B, arrowheads and inset). Since actin is highly enriched in spine head and necks, these results suggest that NAGK clusters at the border of dendritic shaft are positioned near spines.

NAGK protrusions at the dendritic border are positioned at the base of excitatory synapses

It is known that spine synapses are excitatory. To find the position of NAGK relative to excitatory synapses, we double-labelled neurons with antibodies against NAGK and *N*-methyl-*D*-aspartate receptor subunit 2B (NR2B), an excitatory postsynaptic marker [3,8,9,12]. NR2B clusters formed robust protrusions out of dendritic shafts (Fig. 3A, NR2B, arrows and inset). In contrast, NAGK protrusions were less robust and mostly confined within the shafts (Fig. 3A, NAGK, arrows and inset). The merge image showed that the NAGK-IR protrusions closely abut against those of NR2B which farther protrude out (Fig. 3B, insets). Since NR2B is enriched at the postsynaptic membrane, i.e., spine head, this

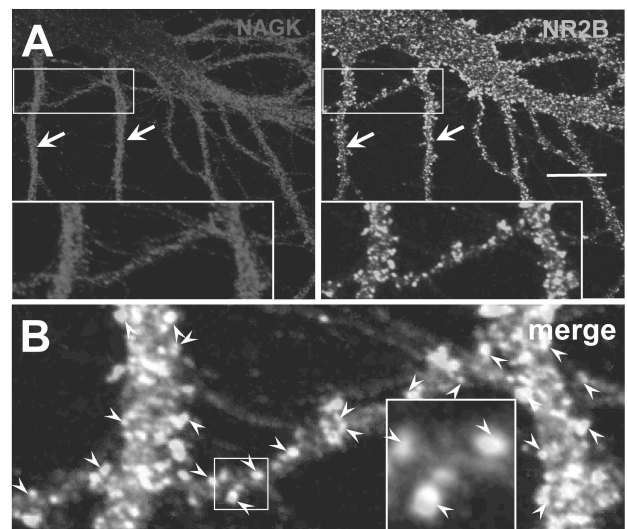


Fig. 3. NAGK protrusions are positioned at the base of excitatory synapses. Cultured hippocampal neurons (21DIV) were double-labelled with antibodies against NAGK and NR2B, an excitatory postsynaptic marker, and confocal images were acquired using an 100X objective. *A*, single channel images for NAGK and NR2B, and enlarged images for the boxed areas were shown in each *insets*. Scale bar, 15 μ m. *B*, merge. The inset images of *A* were merged. The NAGK protrusions that abut against NR2B-IR clusters were marked by arrowheads. The boxed area was shown enlarged in *inset*. Note that the NAGK-IR clusters protrude into NR2B-IR clusters.

feature can be interpreted that NAGK is located at the base or neck of spines.

To investigate the position of NAGK relative to inhibitory synapse, neurons were double-labelled with antibodies against NAGK and gephyrin, an inhibitory postsynaptic marker. The gephyrin-IR clusters were mostly formed at the periphery of dendritic shafts (Fig. 4A, *geph*). The merge image revealed that NAGK- and gephyrin-IR clusters did not overlap or abut each other (Fig. 4B). Furthermore, there were no NAGK protrusions where gephyrin clusters were formed (Fig. 4B, inset). Together, these results indicate that NAGK clusters protrude into excitatory synapses only.

NAGK is included in the synaptosome but not in the postsynaptic density fraction

If a protein is located at the base of spines, it is expected to be included in the synaptosome fraction. Immunoblots of rat brain forebrain synaptosome (S), postsynaptic density (PSD), total homogenates (H) showed that NAGK is present in BH and S fractions (Fig. 5A), but was absent from the PSD fraction. NAGK was not enriched in the synaptosome

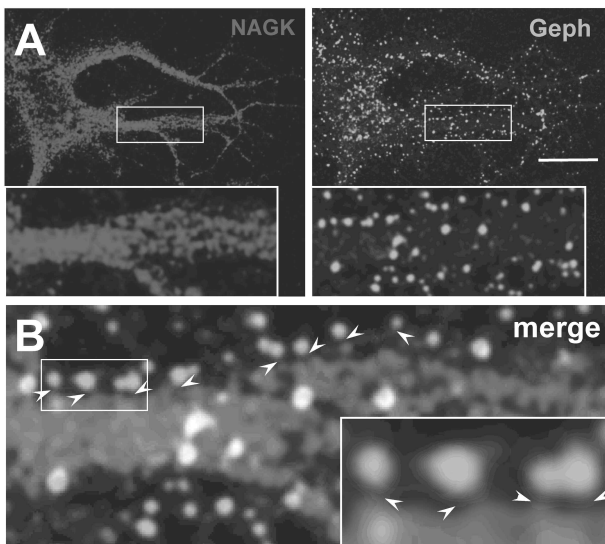


Fig. 4. NAGK-IR clusters do not protrude into inhibitory synapses. Cultured hippocampal neurons (21DIV) were double-labelled with antibodies against NAGK and gephyrin (Geph), an inhibitory postsynaptic marker, and confocal images were acquired using a 40X objective. *A*, single channel images for NAGK and gephyrin, and enlarged images for the boxed areas were shown in each *insets*. Scale bar, 30 μ m. *B*, merge. The inset images of *A* were merged. The NAGK-IR clusters near NR2B-IR clusters were marked by arrowheads. The boxed area was shown enlarged in *inset*. Note that the NAGK-IR clusters do not protrude into Geph-IR clusters.

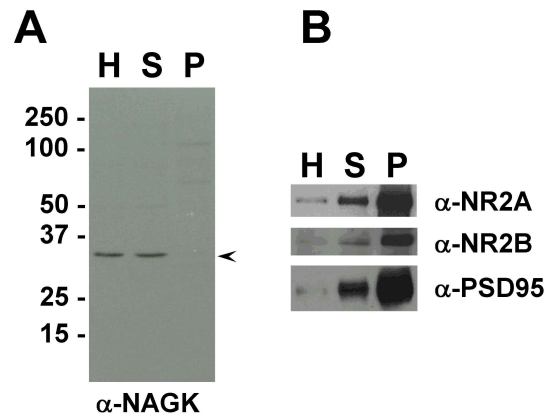


Fig. 5. NAGK is included in the synaptosome but not in the postsynaptic density fraction. Rat forebrain homogenate (BH), synaptosome (S) and postsynaptic density (PSD) fractions were immunoblotted with an antibody against NAGK (α -NAGK, *A*), and PSD markers (*B*). The position of NAGK is marked by an arrowhead. Note that NAGK is included in the synaptosome but not in the PSD fraction, while the known PSD proteins such as NR2A, NR2B and PSD-95 are highly enriched in the PSD fractions (*B*).

fraction, while PSD proteins such as NR2A, NR2B and PSD-95 [3,8,9] were highly enriched in both synaptosome and PSD fraction (Fig. 5B). These results supports that NAGK is not localized at the base of excitatory synapses.

In this study we have shown that NAGK is positioned at the base of spines. In hippocampal neurons in culture NAGK-IR formed clusters along microtubule fibers in the somatodendritic domain. The NAGK clusters were densely packed throughout dendritic shafts, while those at the periphery protrude out of the shaft. Double-labelling of the neurons with NAGK and various synaptic markers indicated that NAGK protrusions are positioned at the base of spines, while there were no NAGK protrusions into inhibitory postsynaptic sites. Positioning of NAGK at the base of spines was further supported by the inclusion in synaptosomes but not in PSD fractions. Since there are no studies on the function of NAGK other than the kinase activity, it is very difficult to understand the meaning of our results. However, selective protrusion into spines may indicate its role in spine formation.

Abbreviations

DIV, day *in vitro* GlcNAc kinase or NAGK, *N*-Acetylglucosamine kinase; IC, immunocytochemistry; IR, immunoreactivity; NR2B, *N*-methyl-*D*-aspartate receptor subunit 2B;

O-GlcNAc, O-linked N-acetylglucosamine; RT, room temperature; UDP-GlcNAc, uridine diphosphate N-acetylglucosamin

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초록 : 흥분성연접의 바닥으로 NAGK 클러스터의 돌출

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N-Acetylglucosamine kinase (GlcNAc kinase or NAGK; EC 2.7.1.59)는 GlcNAc를 인산화하여 GlcNAc-6-phosphate를 만드는 효소이다. 효소자체에 대한 자세한 연구에도 불구하고 포유류에서 NAGK의 표현에 대한 연구는 거의 없다. 배양한 흰쥐의 해마신경세포에서 NAGK은 세포체/가지돌기 영역에서 클러스터(cluster)를 형성한다. 본 연구에서는 가지돌기의 긴 축에서부터 가쪽으로 돌출되는 NAGK 클러스터에 대하여 연구하였다. 배양한 해마신경세포를 NAGK와 다양한 연접표지단백질에 대한 항체로 이중염색한 결과 NAGK 클러스터가 가지돌기의 바닥에는 있었지만 역제성 연접후부위에는 존재하지 않았다. 또한 흰쥐 전뇌(forebrain)의 균질액(homogenate, BH), 연접체(synaptosome, S), 연접후치밀질(postsynaptic density, PSD) 분획을 NAGK 항체로 면역염색한 결과 NAGK는 연접체에는 있었지만 PSD 분획에는 존재하지 않았다. 이러한 결과들은 NAGK가 가지돌기가시(spine)의 바닥쪽으로 돌출됨을 의미한다.