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Co-localization and interaction of DPYSL3 and GAP43 in primary cortical neurons

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Abstract

Dihydropyrimidinase-like 3 (DPYSL3) and GAP43 are both involved in neurite outgrowth, a crucial process for the differentiation of neurons. The present study shows for the first time that DPYSL3 co-localizes with GAP43 in primary cortical neurons. Further co-immunoprecipitation and overlay assay showed the ability of both recombinant and endogenous DPYSL3 to bind GAP43, indicating a specific interaction between DPYSL3 and GAP43 in primary cortical neurons.

Keywords: DPYSL3; DRP-3; CRMP-4; GAP43; Protein–protein interaction

Dihydropyrimidinase-like 3 (DPYSL3), a member of the TUC (TOAD-64/Ulip/CRMP) family, known also as CRMP-4 or TUC-4, is involved in neuronal plasticity and neurite outgrowth and extension [1–3]. The DPYSL3 interactions with structural proteins such as tubulin, actin, and also chondroitin sulfate proteoglycans, which are structurally and functionally important components of the extracellular matrix of the central nervous system, suggest that this protein may play a role in the regulation of cytoskeleton organization [4,5]. Regulation of the cytoskeleton is involved in basic cellular activities such as cell–cell interaction, exo- and endo-cytosis and cell adhesion and migration [6,7]. The precise synchronization of cytoskeleton organization is crucial for growth cone navigation, the process underlying the formation of neuronal connections.

The 43 kDa growth-associated protein (GAP43), also known as B50, neuromodulin and F1, plays an important role among the proteins involved in the regulation of neurite outgrowth, growth cone guidance and synaptic plasticity [8,9]. Its expression in neurons correlates with axonal outgrowth and the establishment of neuronal connections [10]. It is known, that within the cells, both GAP43 and CRMP family members (CRMP-1, -3, -5, and -4) accumulate in detergent soluble, cholesterol-enriched lipid rafts at the inner surface of the cell membrane [11,12].

An interesting observation was made by Zhao and Lu, who reported that Annexin-2 (ANX2) is co-distributed with both GAP43 and DPYSL3 in normal rat brain [13]. This finding, together with the fact that both DPYSL3 and GAP43 proteins are highly expressed in outgrowing neurites, raised a question as to whether the observed co-distribution is a result of specific interaction between DPYSL3 and GAP43. The present work provides direct evidence for such interaction in cortical neurons.

Materials and methods

Primary cortical cell culture. Rat primary cortical neurons (PCN) were cultured as previously described with slight modification [14,15]. Timed-pregnant Sprague–Dawley rats were purchased from Charles River

Abbreviations: CM, conditioned medium; CRMP-4, collapsin response mediated protein-4; DIV, days *in vitro*; DPYSL3, dihydropyrimidinase-like protein 3; FBS, fetal bovine serum; GAP43, 43-kDa growth-associated protein; MEM, minimal essential medium; PBS, phosphate buffered saline; PCN, primary cortical neurons; TOAD, turn-on after division, 64 kDa; TUC, TOAD/Ulip/CRMP.

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Canada (St. Constant, Quebec, Canada). The mother was sacrificed by cervical dislocation under halothane anesthesia and the fetuses were removed on embryonic day 18 (E18). The fetal brains were removed, placed in ice-cold PBS and cortices were dissected out. The cortical neurons were dispersed by triturating and the cells were centrifuged at 250*g* for 5 min at 4 °C. The cells were gently resuspended in plating medium consisting of 80% MEM, 10% heat-inactivated FBS, 10% heat-inactivated horse serum, 25 mM glucose, and 2 mM L-glutamine, and viable cells were determined by trypan blue exclusion. The cells were plated at 10^6 cells/cm^2 in poly-L-lysine-coated cell culture dishes in plating medium. The cultures were mixed populations containing both neurons and glial cells. To minimize glial growth, the cultures were treated with 15 μg/ml 5-fluoro-2′-deoxyuridine and 35 μg/ml uridine on day 4 *in vitro* (DIV). Seven days after plating, the cells were fed with growth medium consisting of 90% MEM, 10% horse serum, 25 mM glucose, and 2 mM L-glutamine. On 14–18 DIV primary cortical neurons were harvested in MSB buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1 mM sodium orthovanadate, 1 mM NaF, 1% NP-40) containing protease inhibitor cocktail (Sigma), and incubated for 30 min on ice, followed by centrifugation at 16,000*g* for 10 min. Protein concentration in the supernatant cell extract was estimated by Bradford method [16].

Immunocytochemistry. Cell monolayers were washed in PBS, fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 20 min at room temperature, and subsequently washed again with PBS. Non-specific sites were blocked using 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in PBS containing 0.1% Triton X, to permeabilize the cells, for 30 min at room temperature. Cells were incubated in primary antibody anti-DPYSL3 (1:500 v/v; BD PharMingen, Mississauga, Ontario, Canada) or anti-GAP43 1:500 v/v; Sigma, St. Louis, MO) in PBS containing 1% normal goat serum for 1 h at room temperature. After washing with PBS, the cells were incubated with Alexa568- and Alexa488-conjugated anti-IgG (1:400 v/v) (Molecular Probes, Eugene, Oregon) in PBS containing 1% normal goat serum for 30 min at room temperature. After washing with PBS, images were captured using a Zeiss Axiovert fluorescence microscope.

Cloning of DPYSL3. Total RNA from fetal rat brain RNA was isolated using TRIZOL reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription of 3 μg of RNA, using Superscript reverse transcriptase (Invitrogen) according to the manufacturer's protocol. DPYSL3 coding sequence was amplified using 1 μl of cDNA in total volume of 20 μl containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM each of the primers 5′-GCCAGAGCCACCCATATGTCCTACCAGGG-3′ (forward) and 5′-AAGCTTTTAACTCAGGGATGTGATGTTA-3′ (reverse) which introduced restriction sites for NdeI and HindIII, respectively (Alpha DNA, Montreal, Quebec, Canada), 0.25 mM of dNTP and 0.2 U of Platinum Taq Polymerase (Invitrogen, Carlsbad, CA). The primers for PCR were based on rat mRNA dihydropyrimidinase-like 3 (CRMP-4) sequence (NM_012934). Samples were amplified as follows: 5 min at 94 °C, 30 s/94 °C, 1 min/61 °C, 2 min/72 °C (30 cycles), and 10 min at 72 °C. The PCR product was 1670 bp. Both PCR product and pPROEX-1 plasmid were digested with NdeI and HindIII and 4 μl aliquots of PCR product were ligated with 1 μl of pPROEX (Gibco BRL, Gaithersburg, MD) for 20 min at 22 °C according to the manufacturer's instructions. Next, TOP10F' competent cells (Invitrogen, Carlsbad, CA) were transformed with pPROEX-1-DPYSL3 plasmid according to the manufacturer's protocol. The following day, transformants were selected and cultured. Plasmids were isolated using Mini Prep Kit (Qiagen) according to the manufacturer's protocol and later analyzed by sequencing using a ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

H6TV-DPYSL3 protein expression. Escherichia coli strain BL21(DE3) pLysS (Invitrogen, Carlsbad, CA) was transformed with an obtained plasmid according to the manufacturer's protocol. Expression of recombinant protein H6TV-DPYSL3 (rDPYSL3) was induced by the addition of IPTG to 1 mM final concentration and the culture was grown for 2 h at 37 °C. The culture was spun down and the cell pellet was resuspended in 30 ml of cold buffer D (50 mM phosphate buffer pH 6.0, 300 mM KCl) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Cells were sonicated for 4 times 1-min bursts with 1-min intervals, followed by centrifugation at 30,000*g* for 30 min. Supernatant was passed through a Ni–Resin (3ml) column pre-equilibrated with buffer D, washed with 20 ml of buffer D, 10 ml 25 mM imidazole in buffer D. Recombinant DPYSL3 protein was eluted with 100 mM imidazole in buffer D. Expression of recombinant DPYSL3 as a fusion protein with the H6TV peptide at the amino-terminus, using protocol described above produced about 12 mg of pure recombinant protein with a molecular weight of 64.84 kDa.

DPYSL-interacting proteins. To isolate DPYSL3-interacting proteins, 1 mg of total PCN extract was incubated with [DPYSL3(+)] or without [DPYSL3(−)] rDPYSL3 (20 μg) for 3 h at 4 °C. Next, 50 μl of Ni–Resin was added and samples were incubated overnight at 4 °C. Both samples were then centrifuged at 1000*g* for 5 min at 4 °C and the pellets were washed 3 times with buffer D. After washing, 40 μl of 200 mM imidazole in buffer D was added to each sample, followed by the addition of 5 times Laemmli's loading buffer [17]. Protein samples were resolved on 10% SDS–PAGE and transferred to PVDF membrane. Immunoblotting was performed with either mouse monoclonal anti-GAP43 (1:2000) or rabbit polyclonal anti-DPYSL3 (1:20,000) antibodies.

Overlay assay. Eight micrograms of rDPYSL3 protein and albumin (BSA, negative control) was separated on SDS–PAGE gel and transferred to PVDF membrane. The membrane was incubated with PCN lysate (200 μg/ml of total protein in TBS-T) to allow the interaction of PCN proteins with rDPYSL3 overnight at 4 °C. The membrane was washed 3 times with TBS-T and probed with mouse monoclonal anti-GAP43 antibody (1:2000).

Immunoprecipitation using GAP43 and actin antibodies. One milligram of total PCN lysate was incubated with either 3 μl of mouse monoclonal anti-GAP43 or mouse IgG2a or MSB buffer, for 1 h at 4° C. Next, 20 μl of Protein G Plus-Agarose was added and samples were incubated overnight. The pellet was collected and washed 3 times with MSB buffer. Fifty microliters of 1 times loading buffer was added to each sample and proteins were separated by SDS–PAGE and transferred to PVDF membrane. The membrane was probed with rabbit anti-DPYSL3 and mouse monoclonal anti-GAP43 antibodies.

Results

DPYSL3–GAP43 co-localization

Double immunofluorescent staining of primary cortical neurons with antibodies against DPYSL3 (red) and GAP43 (green) revealed that both these proteins are strongly expressed in these cells and they appear to be distributed in cytoplasm, membrane and neurite extensions. Areas of co-localization (overlay) were characterized by appearance of the yellow color, indicating a possible interaction between DPYSL3 and GAP43 proteins (Fig. 1).

Isolation of DPYSL3 interacting proteins

Since commercial antibodies against DPYSL3 were not suitable for immunoprecipitation experiments, recombinant DPYSL3 protein was used to study the DPYSL3–GAP43 interaction. As shown in Fig. 2A, DPYSL3 protein could be detected only in the sample spiked with rDPYSL3 (PCN lysate plus rDPYSL3 plus Ni–Resin), and not in the control sample (PCN lysate plus Ni–Resin). Under those conditions, the presence of GAP43 protein could be also detected in DPYSL3(+) but not in the control samples, suggesting co-sedimentation of GAP43 along with rDPYSL3.

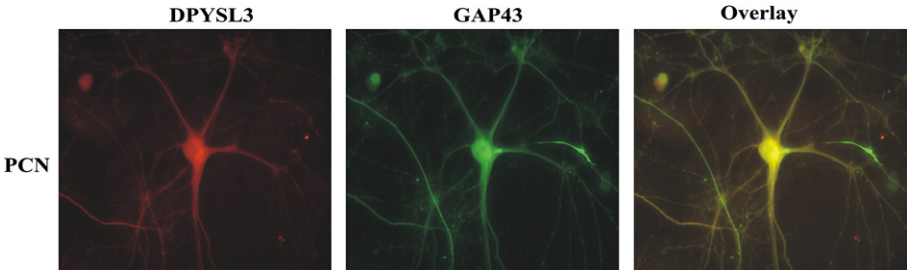

Fig. 1. DPYSL3 and GAP43 co-localization in primary cortical neurons. The fixed cells were labeled with antibody against DPYSL3 and GAP43, and the secondary antibodies conjugated with Alexa568 (red) and Alexa488 (green), respectively. Both DPYSL3 and GAP43 are expressed in cytoplasm and neurites. Areas of co-localization (overlay) of these two proteins are shown by the appearance of yellow color (green + red = yellow).

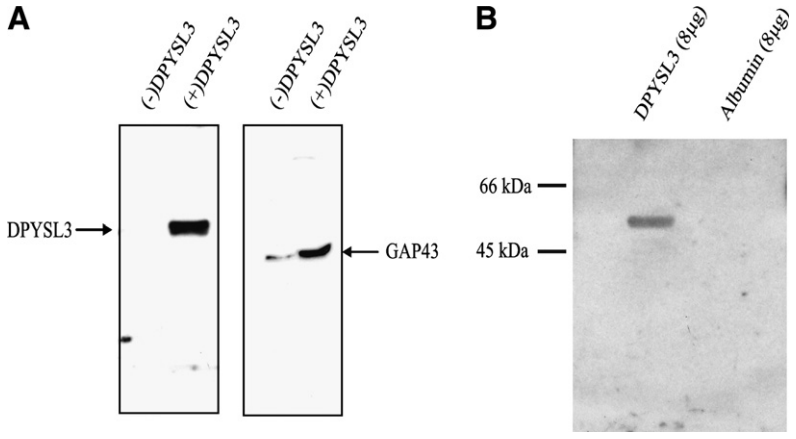

Fig. 2. Recombinant DPYSL3 binds endogenous GAP43. DPYSL3 interacting proteins were isolated as described in Materials and methods section. The presence of endogenous GAP43 is detected in samples enriched with recombinant DPYSL3(+), but not in control DPYSL(−) (2A) samples. Overlay assay with recombinant rDPYSL3 reveals binding of endogenous GAP43 to rDPYSL3 immobilized on the nitrocellulose membrane (2B).

Overlay assay

As shown in Fig. 2B, when an rDPYSL3 protein and albumin immunoblot was probed with an antibody against GAP43, a single band, matching the size of recombinant DPYSL3, was only observed in the DPYSL3 lane, and not in the albumin lane. These results indicate an interaction between the DPYSL3 and GAP43 present in the PCN extract. To ensure the specificity of the obtained signal, we have determined that the GAP43 antibody is specific and does not recognize endogenous or recombinant DPYSL3 (data not shown).

Immunoprecipitation experiments

Finally, immunoprecipitation experiments using monoclonal mouse anti-GAP43 antibody and PCN extract were performed to confirm DPYSL3–GAP43 interaction. As shown in Fig. 3, the presence of immunoprecipitated GAP43 was detected only in samples containing antibodies against GAP43, but not in the negative control or sample incubated with pre-immune serum. At the same time DPYSL3 protein could be detected in samples immunoprecipitated with GAP43 antibody, but not in samples immunoprecipitated with pre-immune serum.

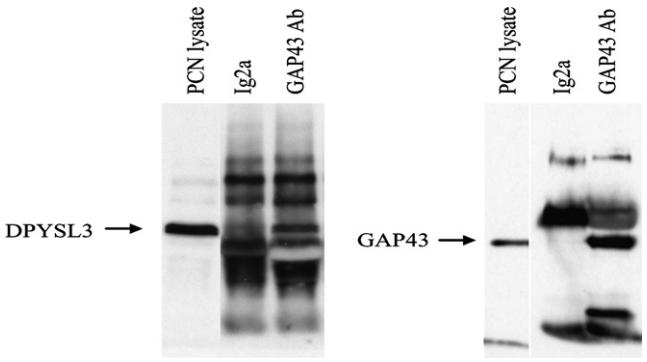

Fig. 3. Co-immunoprecipitation of endogenous proteins GAP43 and DPYSL3 from the PCN extracts. Immunoprecipitation assay was performed as describe in Materials and methods section. Western blot analysis of the protein complexes, immunoprecipitated with an antibody against GAP43, revealed the presence of both GAP43 and DPYSL3 proteins, indicating the interaction between those proteins.

Discussion

The present study shows for the first time the co-localization and interaction between synaptic proteins DPYSL3 and GAP43. The roles of both DPYSL3 and GAP43 have been associated with neurite outgrowth, a crucial process

for the differentiation of neurons; however, no reports on DPYSL3–GAP43 interaction could be found in the literature.

Our preliminary immunocytochemistry analysis revealed co-localization of DPYSL3 and GAP43, and supported our hypothesis about the possible interaction between those proteins. Since the commercially available anti-DPYSL3 antibody is not suitable for immunoprecipitation assays, recombinant DPYSL3 was used in our experiments. Both co-binding experiment and overlay assays showed that recombinant DPYSL3 was able to bind endogenous GAP43. At the same time, the reverse approach (immunoprecipitation using anti-GAP43 antibody) has proven that endogenous GAP43 was co-immunoprecipitating with endogenous DPYSL3, indicating a specific interaction between them.

Both DPYSL3 and GAP43 are involved in the regulation of F-actin structure which underlies the changes in the morphology of the growth cone. Rosslenbroich et al. have shown that DPYSL3 protein promotes F-actin bundling while the DPYSL3 (CRMP-4) knockout causes disorganization of actin filaments, indicating the role of DPYSL3 in the regulation of actin organization. The domain responsible for interaction with actin is localized within C-terminal 100 amino acids of CRMP-4. Once the C-terminus of CRMP-4 is removed, DPYSL3 is unable to promote F-actin bundling [5].

The GAP43 effect on the structure of actin filaments depends mostly on its phosphorylation status with the phosphorylated variant promoting the stabilization of long filaments [18,19]. The effect of GAP43 on the growth state of the presynaptic terminal is associated with its ability to interact with the membrane skeleton, the network of proteins on the inner face of the plasma membrane that governs its shape, motility and pathway guidance. Cytoskeletal proteins such as actin, α-actinin, talin, and spectrin can be found among GAP43-interacting proteins, [20,21]. For example, GAP43 together with MARCKS is known to affect local actin stability and bundling in vitro [10].

In light of the observations mentioned above, it is likely that DPYSL3–GAP43 interaction may play a role in actin stabilization and in the process of cytoskeleton regulation, which is essential for correct growth cone navigation leading to neuronal connections. Clearly, further studies are required to ascertain the key roles these protein–protein interactions play in the dynamics of cytoskeletal stability and growth cone navigation and neurite extension.

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