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p250GAP, a neural RhoGAP protein, is associated with and phosphorylated by Fyn

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Abstract

Fyn is a member of the Src-family protein tyrosine kinases and plays important roles in both neurons and oligodendrocytes. Here we report association of Fyn with p250GAP, a RhoGAP protein that is expressed predominantly in brain. p250GAP interacts with Fyn both in vitro and in vivo. p250GAP is tyrosine phosphorylated by Fyn when co-expressed in HEK293T cells. This phosphorylation appears to enhance the interaction between p250GAP and Fyn. Furthermore, the level of tyrosine phosphorylation of p250GAP increases upon differentiation of the oligodendrocyte cell line CG4. Given that Fyn activity is up-regulated during oligodendrocyte maturation, the results argue that p250GAP is phosphorylated by Fyn in oligodendrocytes. Tyrosine phosphorylation of p250GAP by Fyn would regulate its RhoGAP activity, subcellular localization, or interactions with other proteins, leading to morphological and phenotypic changes of oligodendrocytes.

Keywords: p250GAP; Fyn; Tyrosine phosphorylation; Oligodendrocyte; RhoGAP

Src-family nonreceptor protein tyrosine kinases (PTKs) play important roles in the nervous system. Among them, physiological importance of Fyn has been suggested by analyses of *fyn* knockout mice. These mice show defective long-term potentiation (LTP), impaired spatial memory, uncoordinated hippocampal structure, increased fearfulness, altered ethanol sensitivity, and impaired myelination [1–6]. Especially, hypomyelination is one of the most prominent phenotypes of *fyn* knockout mice. There is no significant reduction of myelin content in mutant mice lacking *src, yes*, or *lyn* [7,8]. Maturation of oligodendrocytes, which form myelin sheaths, requires the increase in the expression and activity of Fyn, rather than the other members of Src-family PTKs [9,10]. However, molecular mechanisms by which Fyn is involved in oligodendrocyte maturation are poorly understood.

p250GAP (also called as p200RhoGAP/Grit/RICS) is a recently identified brain-enriched RhoGAP (GTPase-activating protein) [11–14]. RhoGAP is a major class of regulators for Rho family GTPases and is implicated in various neural functions. p250GAP contains a RhoGAP domain at the N-terminal region and several proline-rich sequences at the C-terminal region. We previously showed that p250GAP is concentrated in the postsynaptic densities and is co-localized with the GluRε2 subunit of NMDA receptors. p250GAP is tyrosine phosphorylated in the brain and NMDA receptor stimulation leads to its dephosphorylation, which may result in redistribution of p250GAP in hippocampal slices [11]. Moreover, p250GAP is recruited to activated receptor tyrosine kinases and is tyrosine phosphorylated following ligand stimulation [13]. It is also known that the C-terminal portion of p250GAP is tyrosine phosphorylated by Src [12]. These findings suggest that the p250GAP activity is regulated by tyrosine phosphorylation.

In this paper, we show that p250GAP interacts with and becomes phosphorylated by Fyn, which may be

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relevant to oligodendrocyte differentiation as well as in neuronal functions.

Materials and methods

DNA constructs. For preparation of glutathione *S*-transferase (GST)-fusion proteins in bacteria, cDNA fragment encoding amino acids 1336–1738 of p250GAP was cloned in-frame into pGEX plasmid (Amersham Biosciences). The construct was verified by DNA sequencing. The expression plasmids pME-FynY531F, pME-FynK299M, and pME-Flag p250GAP were previously described [11,15].

Antibodies. Rabbit polyclonal antibodies against p250GAP were previously described [11]. Anti-Fyn polyclonal antibodies (Fyn3) were purchased from Santa Cruz Biotechnology. Anti-Flag epitope mAb (M2) was from Sigma. Anti-phosphotyrosine (pTyr) mAb (RC20) was from Transduction Laboratories. Anti-Fyn mAb (Fyn301) was from Wako Pure Chemicals.

Yeast two-hybrid screening. A cDNA encoding brain-type Fyn was cloned into pGBT9 vector (Clontech Laboratories) as a bait plasmid. The reporter yeast strain YJ69-2A harboring the bait plasmid was transformed with a human fetal brain cDNA library (Clontech Laboratories). Transformants were selected on synthetic media lacking leucine, tryptophan, adenine, and histidine. DNA sequences of the insert in the selected transformants were analyzed by the BLAST database.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in 5% CO_2. HEK293T cells were transfected with combinations of expression plasmids by the standard calcium phosphate method. The oligodendrocyte cell line CG4 was maintained and differentiated as described previously [16].

Preparation of lysates, immunoprecipitation, and immunoblotting. Transfected cells were lysed in TNE buffer (50 mM Tris–HCl, 1% Nonidet P-40, 5 mM EDTA, and 100 mM NaCl) containing 200 μM Na_3VO_4, 10 mM NaF. For immunoprecipitation, precleared lysates were incubated sequentially with the appropriate antibodies and protein G–Sepharose (Amersham Biosciences) for 2 h. Immunoprecipitates were washed five times with TNE buffer. Immunoprecipitates and lysates were resolved by SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membrane was then blocked with TBS-T solution containing 5% bovine serum albumin and immunoblotted with primary antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies and Renaissance Plus reagent (NEN Life Science Products) or CDP-Star (TROPIX) were used to visualize the immunoreactive proteins.

Results and discussion

Interaction between Fyn and p250GAP

To identify molecules that interact with Fyn, we performed yeast two-hybrid screening using full-length Fyn as a bait. We screened approximately 8.6×10^6 yeast transformants and obtained 80 candidate clones. Among them, two clones contained cDNA sequences coding for the C-terminal portion of p250GAP (Fig. 1A; TH1 and TH2). Direct interaction between Fyn and the C-terminal portion of p250GAP (p250GAPTH2; amino acids 1336–1738) was confirmed by the GST-pull down assay. As shown in Fig. 1B, the GST-p250GAPTH2 fusion protein specifically precipitated a constitutively active form of Fyn (FynY531F), indicating that p250GAP interacts directly with Fyn in vitro. As p250GAPTH2 contains the proline-rich sequence that may serve as SH3 domain binding sites [17], the interaction between p250GAP and Fyn in vitro may be mediated via the proline-rich sequence of p250GAP and the SH3 domain of Fyn.

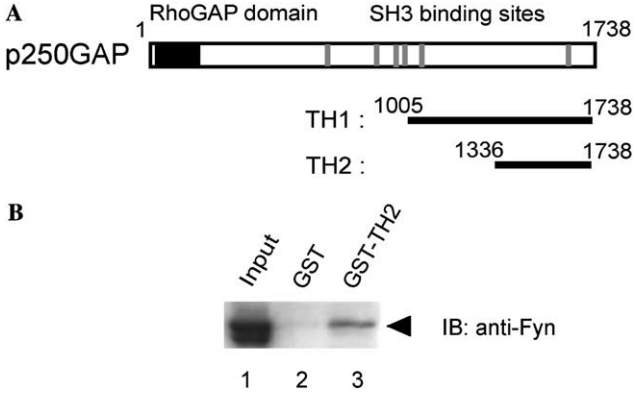

Fig. 1. Identification of p250GAP as a Fyn-interacting protein. (A) Schematic diagrams of p250GAP and the clones (TH1 and TH2) isolated by yeast two-hybrid screening. Filled box: the RhoGAP domain, gray bars: proline-rich sequences. (B) Interaction between GST-p250GAPTH2 and Fyn in vitro. HEK293T cells were transfected with Fyn (Y531F) expression plasmid. Lysates were incubated with GST alone or GST-p250GAPTH2 fusion protein immobilized on glutathione–Sepharose beads. After centrifugation, the beads were washed extensively, and bound proteins were subjected to immunoblotting with anti-Fyn antibodies.

To determine whether p250GAP interacts with Fyn in vivo, we carried out co-immunoprecipitation analysis in HEK293T cells transfected with the expression plasmids for Flag-tagged p250GAP and/or Fyn (FynY531F). p250GAP was immunoprecipitated from the cell lysates by anti-Flag mAb. Co-immunoprecipitation of Fyn with anti-Flag immunoprecipitates depended on expression of both Flag-tagged p250GAP and Fyn (Figs. 2A and B). The amounts of p250GAP and Fyn in the lysates were determined by immunoblotting (Figs. 2A and B). Therefore, we conclude that p250GAP interacts with Fyn in vivo.

Phosphorylation of p250GAP by Fyn

We next examined whether p250GAP is phosphorylated by Fyn in HEK293T cells. HEK293T cells were transfected with combinations of expression plasmids for Flag-tagged p250GAP, FynY531F, and a kinase inactive form of Fyn (FynK299M). Co-expression of FynY531F resulted in tyrosine phosphorylation of p250GAP (Fig. 3A, a), indicating that p250GAP is a substrate for Fyn in vivo. Co-expression of FynK299M did not induce tyrosine phosphorylation of p250GAP. Analyses using deletion mutants of p250GAP revealed

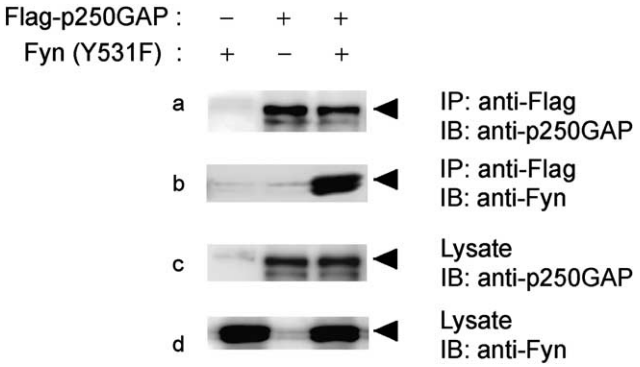

Fig. 2. Interaction between p250GAP and Fyn in HEK293T cells. HEK293T cells were transfected with expression plasmids for Flag-tagged p250GAP and/or FynY531F. Two days later, cells were lysed, and anti-Flag immunoprecipitates (IP, a and b) from cell lysates and total proteins in the cell lysates (c and d) were subjected to immunoblotting (IB) with anti-p250GAP antibodies (a and c) or anti-Fyn antibodies (b and d).

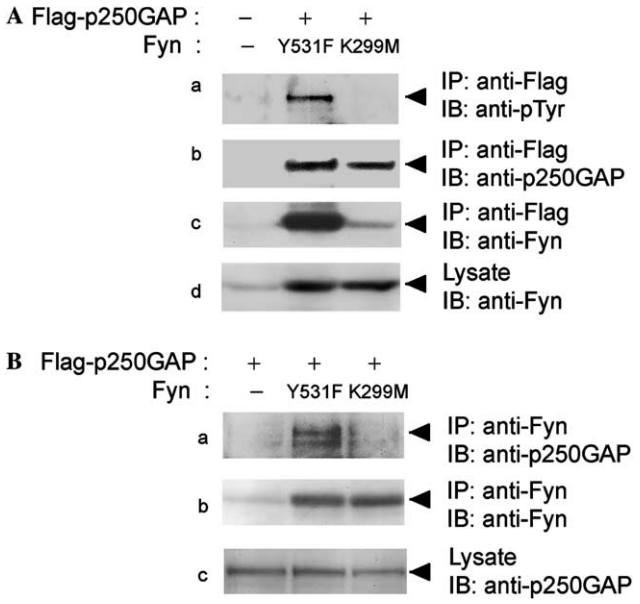

Fig. 3. Tyrosine phosphorylation of p250GAP by Fyn. HEK293T cells were transfected with combinations of expression plasmids for Flag-tagged p250GAP, FynY531F, and FynK299M. (A) Lysates of transfectants were immunoprecipitated (IP) with anti-Flag mAb. The immunoprecipitates (a–c) and total proteins in the cell lysates (d) were immunoblotted (IB) with anti-pTyr mAb (a), anti-p250GAP antibodies (b), or anti-Fyn antibodies (c and d). (B) Anti-Fyn immunoprecipitates and total proteins in the cell lysates were subjected to immunoblotting with anti-p250GAP antibodies (a and c) or anti-Fyn antibodies (b).

that multiple tyrosine residues of p250GAP were phosphorylated by Fyn (data not shown).

In order to understand the role of tyrosine phosphorylation of p250GAP, we examined whether the kinase activity of Fyn influences the interaction between p250GAP and Fyn. The association of Flag-tagged p250GAP with FynK299M was much weaker than that with FynY531F (Fig. 3A, lanes 2 and 3). Similar results were obtained when anti-Fyn immunoprecipitates were probed with anti-p250GAP antibodies (Fig. 3B). Therefore, the interaction between p250GAP and Fyn appeared to be largely dependent on the status of p250GAP tyrosine phosphorylation. The residual interaction that was seen between p250GAP and FynK299M may be due to the possible SH3 domain-dependent interaction. The results of GST-pull down assay shown in Fig. 1B support this notion. The SH3 domain–dependent interaction may be necessary for the initial step in which Fyn recognizes p250GAP, followed by subsequent phosphorylation of p250GAP.

Oligodendrocyte differentiation and p250GAP tyrosine phosphorylation

In the course of searching for neural cell lines that express p250GAP, we found that p250GAP is expressed in the oligodendrocyte cell line CG4 that arose spontaneously from rat O2A oligodendrocyte progenitor cells. Because tyrosine kinase activity of Fyn is important in oligodendrocyte differentiation [12], we examined whether p250GAP is a functional target of Fyn tyrosine kinase in oligodendrocytes. As shown in Fig. 4, levels of tyrosine phosphorylation of p250GAP increased significantly upon differentiation of CG4 cells. Activation of Fyn precedes the morphological changes that accompany oligodendrocyte differentiation, suggesting that the activation of Fyn is the earliest and the most important biochemical event in the oligodendrocyte maturation. Taken together, Fyn is likely to phosphorylate p250GAP in oligodendrocytes, and tyrosine phosphorylated p250GAP is relevant to regulation of the activity of Rho family GTPases and eventually to oligodendrocyte differentiation.

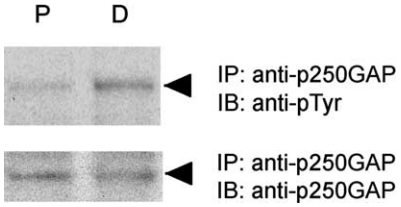

Fig. 4. Tyrosine phosphorylation of endogenous p250GAP upon differentiation of CG4 cells. p250GAP was immunoprecipitated from lysates of undifferentiated progenitor (P) and differentiated (D) CG4 cells. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-pTyr mAb (top panel) and anti-p250GAP antibodies (bottom panel). Shown are the representatives of three independent experiments.

Implication

Accumulating data indicate that activities of many GAPs are modulated by protein kinases [18]. Tyrosine phosphorylation of p190RhoGAP by Fyn causes a conformational change of the protein, leading to increases in the GAP activity and association with other molecules. This might regulate oligodendrocyte development [19]. Phosphorylation by Aurora B converts MgcRacGAP to a RhoGAP [20]. The stability and GAP activity of RGS16 are enhanced by Src-mediated phosphorylation [21]. Moreover, the GAP activity of p250GAP is inhibited by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated phosphorylation [14]. Therefore, Fyn-mediated tyrosine phosphorylation of p250GAP may regulate its RhoGAP activity, subcellular localization, or association of p250GAP with other proteins.

p250GAP has several tyrosine residues that are possibly phosphorylated by Fyn. Phosphorylation of some of these residues may regulate its RhoGAP activity. Phosphorylation of other residues may provide SH2 domain-binding sites and regulate intracellular signaling. Of special interest is the link between the activation of Fyn and oligodendrocyte maturation, including morphological changes and transcriptional regulations of various genes such as Id4, Mash1, thyroid hormone receptor subunit. p250GAP may regulate cell morphology directly by re-organizing actin cytoskeletal structure or may regulate signaling pathways to the nucleus such as MAP kinase cascades. The physiological importance of p250GAP itself and its tyrosine phosphorylation would be clarified by the analysis of p250GAP knockout mice and p250GAP knock-in mice where tyrosine phosphorylation sites are mutated.

p250GAP and Fyn are expressed not only in oligodendrocyte but also in neurons. Tyrosine phosphorylation of p190GAP is implicated in axon outgrowth and fasciculation in neurons as well as in oligodendrocyte development [22]. Therefore, tyrosine phosphorylation of p250GAP may also be important for neuronal function such as network formation and synaptic plasticity.

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