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LOCALIZATION OF Rabphilin-3A, A PUTATIVE TARGET PROTEIN FOR *Rab*3A, AT THE SITES OF Ca^{2+}-DEPENDENT EXOCYTOSIS IN PC12 CELLS[1]

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Summary: *Rab*3A/*Smg* 25A, a small GTP-binding protein, is highly concentrated in presynapse of neurons and implicated in neurotransmitter release. We have recently identified a putative target protein for *Rab*3A, isolated its cDNA, and designated it as Rabphilin-3A. To examine whether Rabphilin-3A as well as *Rab*3A is localized at the sites of Ca^{2+}-dependent exocytosis, we investigated here localization of Rabphilin-3A and *Rab*3A in comparison with the sites of exocytosis in the differentiated PC12 cells. Rabphilin-3A as well as *Rab*3A was highly concentrated at the tips of the neurites where Ca^{2+}-dependent exocytosis took place. Inversely, neither Rabphilin-3A nor *Rab*3A was concentrated at the tips of the neurites where Ca^{2+}-dependent exocytosis did not take place. These results suggest that Rabphilin-3A as well as *Rab*3A constitutes a part of the machinery necessary for neurotransmitter release.

The *Rab* family of the small G protein superfamily has been implicated ubiquitously in intracellular vesicle transport including endocytosis, exocytosis, and transcytosis (for reviews, see

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The abbreviations used are: G protein, GTP-binding protein; DβH, dopamine β-hydroxylase; NGF, nerve growth factor; Bt_2-cAMP, dibutyryl cAMP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, 50 mM Tris/HCl at pH 7.5 containing 0.2 M NaCl; BSA, bovine serum albumin.

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Refs. 1-3). *Rab*3A/*Smg* 25A is present in the cells performing Ca^{2+}-dependent exocytosis, regulated secretion, including neurons, endocrine cells, and exocrine cells (4-8). Particularly in neurons, *Rab*3A is highly concentrated in the presynapse. In the presynapse, *Rab*3A is localized on the synaptic vesicles and the presynaptic plasma membrane with the focal accumulation corresponding to active zones where voltage dependent Ca^{2+} channels cluster (9). Although the definitive role of *Rab*3A has not been established, accumulating evidence suggests that *Rab*3A is involved in regulation of neurotransmitter release through translocating and/or docking of the synaptic vesicles to the active zones.

*Rab*3A has interconvertible GDP-bound inactive form and GTP-bound active form which is assumed to interact with the possible target protein (3). We have recently identified a putative target protein for *Rab*3A which preferentially interacts with the GTP-bound form of *Rab*3A in the crude membrane fraction of bovine brain (10). We have cloned the cDNA of this protein, determined its primary structure, and named it Rabphilin-3A (11). Rabphilin-3A is a protein with an Mr of 77,976 and 704 amino acids and has two repeated C_2 domains at its C-terminal region (11). This C_2 region is found in protein kinase C, synaptotagmin, and phospholipase A_2 which are known to bind Ca^{2+} and phospholipid through this region (12-14, for a review, see Ref. 15). Consistent with this structure of Rabphilin-3A, it binds Ca^{2+} and phospholipid at its C-terminal region, whereas it binds *Rab*3A at its N-terminal region (16). Rabphilin-3A is highly expressed in rat brain but not in liver, spleen, kidney, or lung (11).

These earlier results strongly suggest that Rabphilin-3A is involved in regulation of neurotransmitter release through mediating the *Rab*3A and Ca^{2+} signals. In the present study, we have examined whether Rabphilin-3A and *Rab*3A are localized at the sites of Ca^{2+}-dependent exocytosis. The present study has shown that Rabphilin-3A as well as *Rab*3A is highly concentrated at the sites of Ca^{2+}-dependent exocytosis which are measured by DβH newly exposed to the cell surface in response to high K^+ stimulation in PC12 cells.

Materials and Methods

Materials and chemicals——A mouse anti-DβH monoclonal antibody was purchased from Chemicon International, Inc. (Temecula, CA). Fluorescein-labeled sheep anti-mouse immunoglobulin and Texas

red-labeled donkey anti-rabbit immunoglobulin were purchased from Amersham Corp. A mouse anti-*Rab*3A monoclonal antibody, mAb SG-11-7, was prepared as described previously (8). A polyclonal antibody against the N-terminal fragment of Rabphilin-3A (1-280 amino acids) was generated and purified as described (16). PC12 cells were cultured and differentiated as described (4), except that 35-mm glass-bottom dishes precoated with laminin were used.

Immunofluorescence——For double immunofluorescence with mAb SG-11-7 and with the anti-Rabphilin-3A polyclonal antibody, the cells were fixed with 2% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.5 for 20 min at room temperature. After washed twice for 10 min with TBS, the cells were incubated for 30 min with TBS containing 1% BSA and 0.05% Triton X-100. The cells were incubated for 1 h with mAb SG-11-7 at a concentration of 10 μg/ml, washed three times for 10 min with TBS, and incubated for 1 h with fluorescein-labeled sheep anti-mouse immunoglobulin at a final dilution of 1:40. After washed three times for 10 min with TBS, the cells were incubated for 1 h with the anti-Rabphilin-3A polyclonal antibody at a concentration of 10 μg/ml. The cells were washed three times with TBS and incubated for 1 h with Texas red-labeled donkey anti-rabbit immunoglobulin at a final dilution of 1:40.

The sites of Ca^{2+}-dependent exocytosis and localization of Rabphilin-3A in the differentiated PC12 cells were examined as follows. After preincubation, PC12 cells were depolarized with high K^+ (60 mM) in the presence of extracellular Ca^{2+} for 2 min at 37°C in the assay medium as previously described (17). Under these conditions, it was confirmed in the same manner as previously described that these cells released dopamine (17). For double immunofluorescence with the anti-DβH monoclonal antibody and with the anti-Rabphilin-3A polyclonal antibody, the cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.5 for 20 min and washed twice for 10 min with TBS. The cells were incubated for 30 min with TBS containing 1% BSA without 0.05% Triton X-100 in order to stain DβH on the cells surface, but not inside the cells. The cells were incubated for 1 h with the anti-DβH monoclonal antibody at a final dilution of 1:300, washed three times for 10 min with TBS, and incubated for 1 h with fluorescein-labeled sheep anti-mouse immunoglobulin at a final dilution of 1:40. The cells were washed three times for 10 min with TBS and treated with 0.05% Triton X-100. The cells were incubated sequentially with the anti-Rabphilin-3A polyclonal antibody at a concentration of 10 μg/ml and with Texas red-labeled donkey anti-rabbit immunoglobulin at a final dilution of 1:40 as described above. After incubation, the cells were washed three times for 10 min with TBS and examined by the laser scanning confocal imaging system (model MRC-600; Bio-Rad, Tokyo, Japan).

Other procedures——SDS-PAGE was performed as described (18). Protein concentrations were determined with BSA as a reference protein as described (19). Immunoblot analysis was performed as described (20)

Results

Expression of Rabphilin-3A and *Rab*3A in PC12 cells——Expression of Rabphilin-3A and *Rab*3A in the undifferentiated,

and Bt_2cAMP- and NGF-differentiated PC12 cells was studied by immunoblot analysis **(Fig. 1, A and B)**. The immunoreactive band for Rabphilin-3A was detected in the undifferentiated PC12 cells as well as the Bt_2cAMP- and NGF-differentiated PC12 cells with similar densities. The immunoreactive band for *Rab*3A was also detected in the three groups of PC12 cells and the expression level of *Rab*3A was slightly increased after both differentiations. This observation is consistent with our previous observation that the mRNA level of *Rab*3A is increased in PC12 cells by treatment either with Bt_2cAMP or with NGF (4).

Localization of Rabphilin-3A and *Rab*3A——In the Bt_2-cAMP-differentiated PC12 cells, intense Rabphilin-3A immunoreactivity was found in the cell body and the tips of the neurites which were usually more than 50 μm long and straightly growing **(Fig. 2A)**. Weak Rabphilin-3A immunoreactivity was also found in the

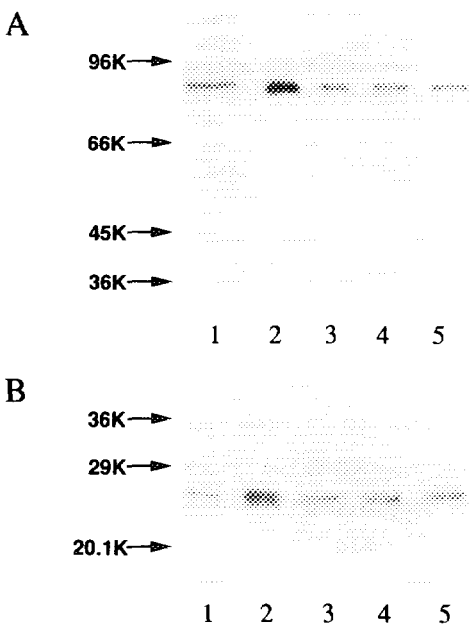

Fig. 1. Immunoblot analysis of Rabphilin-3A and *Rab*3A in PC12 cells. The homogenates of PC12 cells (50 μg each of protein) before or after differentiation were subjected to SDS-PAGE followed by immunoblot analysis by use of the anti-Rabphilin-3A polyclonal antibody or mAb SG-11-7. Immunoblot analysis of Rabphilin-3A (A) and *Rab*3A (B). Lane 1, Rabphilin-3A highly purified from bovine brain (10 ng of protein) (A) and *Rab*3A purified from bovine brain (10 ng of protein) (B); lane 2, rat brain homogenate (50 μg of protein); lane 3, undifferentiated PC12 cells; lane 4, Bt_2cAMP-differentiated PC12 cells; lane 5, NGF-differentiated PC12 cells. The results shown are representative of three independent experiments.

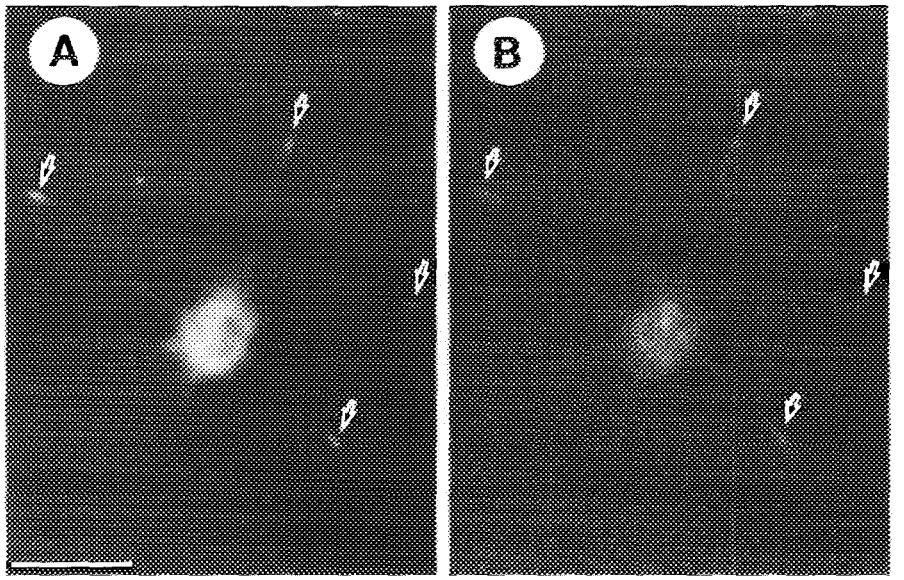

Fig. 2. Double immunofluorescence staining for Rabphilin-3A and *Rab*3A in the Bt_2cAMP-differentiated PC12 cells. The Bt_2cAMP-differentiated PC12 cells were fixed and immunostained for Rabphilin-3A and *Rab*3A. (A) Immunofluorescence staining for Rabphilin-3A. The tips with intense Rabphilin-3A immunoreactivity are indicated by arrows. (B) Immunofluorescence staining for *Rab*3A. The tips with intense *Rab*3A immunoreactivity are indicated by arrows. Bar: 25 μm.

shafts of the neurites. On the other hand, intense *Rab*3A immunoreactivity was found in the cell body and the tips of the neurites, and moderate *Rab*3A immunoreactivity was found in the shafts of the neurites **(Fig. 2B)**. Virtually all the tips of the neurites with intense *Rab*3A immunoreactivity also exhibited intense Rabphilin-3A immunoreactivity. Inversely, most of the tips of the neurites with faint or no *Rab*3A immunoreactivity exhibited no Rabphilin-3A immunoreactivity. In the NGF-differentiated PC12 cells, essentially the same findings with regard to localization of Rabphilin-3A immunoreactivity and *Rab*3A immunoreactivity were obtained (data not shown).

Comparison of the sites of Ca^{2+}-dependent exocytosis and localization of Rabphilin-3A in the Bt_2cAMP- and NGF-differentiated PC12 cells——The sites of Ca^{2+}-dependent exocytosis in PC12 cells were measured by DβH newly exposed to the cell surface in response to high K^+ stimulation. In the Bt_2cAMP-differentiated PC12 cells before high K^+ stimulation, no DβH immunoreactivity was found in any part of the PC12 cells **(Fig. 3A)**. Intense Rabphilin-3A immunoreactivity was found in the cell body and

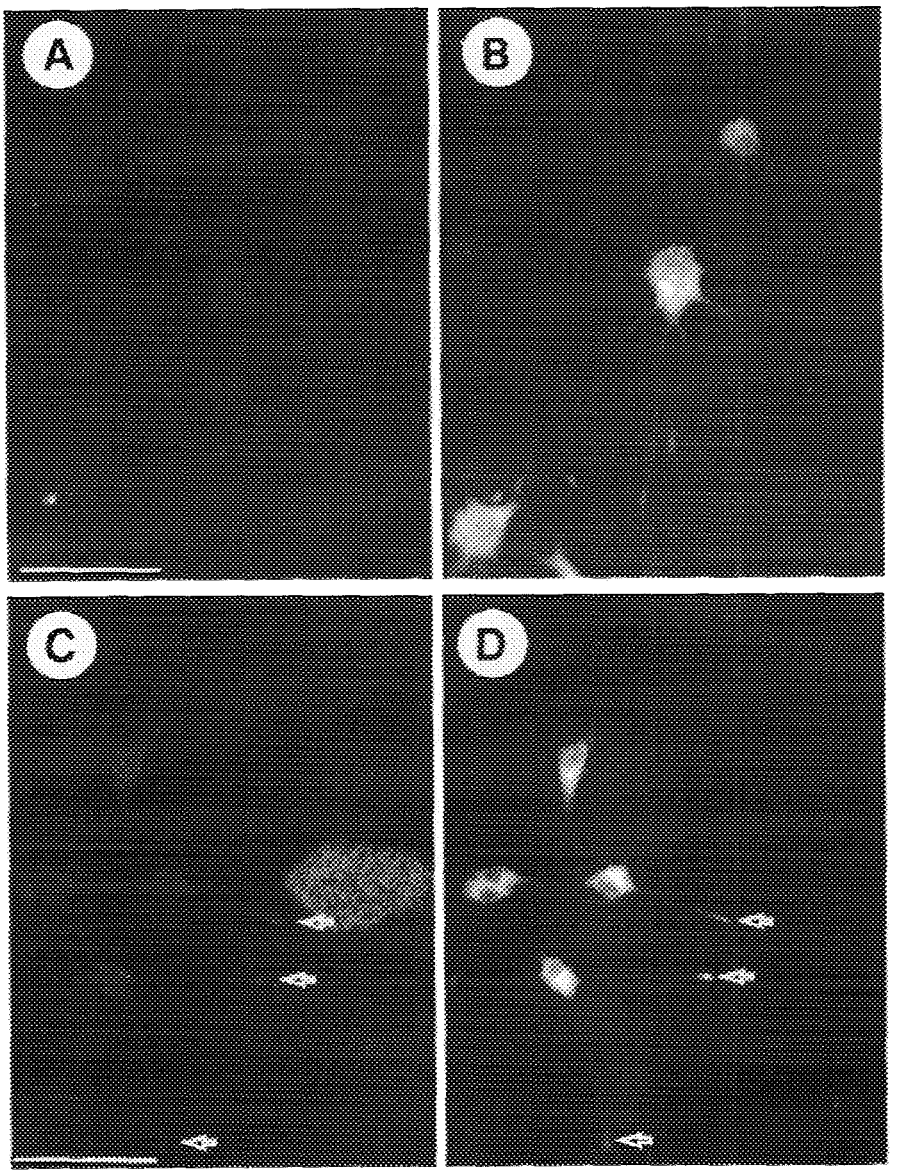

Fig. 3. Double immunofluorescence staining for DβH and Rabphilin-3A in the Bt_2cAMP-differentiated PC12 cells. The cells were depolarized with K^+ (60 mM) for 2 min at 37°C, fixed, and immunostained for DβH and Rabphilin-3A. (A) Immunofluorescence staining for cell surface DβH before high K^+ stimulation. (B) Immunofluorescence staining for Rabphilin-3A before high K^+ stimulation. (C) Immunofluorescence staining for cell surface DβH after high K^+ stimulation. The tips with intense DβH immunoreactivity are indicated by arrows. (D) Immunofluorescence staining for Rabphilin-3A after high K^+ stimulation. The tips with intense Rabphilin-3A immunoreactivity are indicated by arrows. Bars: 50 μm.

the tips of the neurites which were usually more than 50 μm long and straightly growing **(Fig. 3B)**. Weak Rabphilin-3A immunoreactivity was also found in the shafts of the neurites. After high K^+

stimulation, intense DβH immunoreactivity on the cell surface was found most commonly at the tips of the neurites and occasionally at the cell body, which was consistent with earlier observation (21) **(Fig. 3C)**. Distribution of Rabphilin-3A immunoreactivity after high K^+ stimulation was similar to that before high K^+ stimulation, and a change of the distribution was not detected **(Fig. 3, B and D)**. Virtually all the tips of the neurites with intense Rabphilin-3A immunoreactivity showed intense DβH immunoreactivity on the cell surface **(Fig. 3, C and D)**. Inversely, the tips of the neurites with weak or little Rabphilin-3A immunoreactivity, which were usually short and curved, seldom showed DβH immunoreactivity on the cell surface. In the NGF-differentiated PC12 cells, essentially the same findings with regard to localization of DβH immunoreactivity and Rabphilin-3A immunoreactivity were obtained (data not shown).

Discussion

In the present paper, we have shown that Rabphilin-3A and *Rab*3A exist in both the undifferentiated, and Bt_2cAMP- and NGF-differentiated PC12 cells. Histochemically, Rabphilin-3A and *Rab*3A are identified in the cell body, the shafts of the neurites, and the tips of the neurites of the differentiated PC12 cells. Virtually all the tips of the neurites expressing high levels of *Rab*3A express high levels of Rabphilin-3A. Inversely, most of the tips of the neurites expressing low to undetectable levels of *Rab*3A express little Rabphilin-3A. Virtually all the tips of the neurites with intense Rabphilin-3A immunoreactivity show intense DβH immunoreactivity on the cell surface after high K^+ stimulation. These results indicate that Rabphilin-3A as well as *Rab*3A is highly concentrated at the sites of Ca^{2+}-dependent exocytosis at the tips of the neurites of the differentiated PC12 cells. Therefore, it is likely that Rabphilin-3A as well as *Rab*3A may constitute a part of the machinery necessary for neurotransmitter release.

The finding that Rabphilin-3A is highly concentrated at the distal tips of the neurites is also noteworthy. The distal tips of the neurites, growth cones, are known to perform various functions including elongation, pathfinding, exocytosis, endocytosis, and synaptogenesis (for a review, see Ref. 22). Elongation of neurites is accomplished by the plasmalemmal expansion, in which the cytoplasmic membrane compartment is inserted into the plasma membrane of the growth cones in a Ca^{2+}-dependent manner (23). We have found

that the growth cones with intense immunoreactivity for Rabphilin-3A and *Rab*3A are localized at the tips of neurites which are more than 50 μm long and straightly growing. This finding suggests that Rabphilin-3A and *Rab*3A are concentrated in the actively-growing growth cones where the plasmalemmal expansion frequently takes place. Rabphilin-3A and *Rab*3A may be key molecules common to these two Ca^{2+}-dependent membrane fusion events, regulated exocytosis and regulated plasmalemmal expansion.

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