A role for the Rab6B Bicaudal–D1 interaction in retrograde transport in neuronal cells

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Abstract

The Rab6 subfamily of small GTPases consists of three different isoforms: Rab6A, Rab6A′ and Rab6B. Both Rab6A and Rab6A′ are ubiquitously expressed whereas Rab6B is predominantly expressed in brain. Recent studies have shown that Rab6A′ is the isoform regulating the retrograde transport from late endosomes via the Golgi to the ER and in the transition from anaphase to metaphase during mitosis. Since the role of Rab6B is still ill-defined, we set out to characterize its intracellular environment and dynamic behavior. In a Y–2H search for novel Rab6 interacting proteins, we identified Bicaudal–D1, a large coiled-coil protein known to bind to the dynein/dynactin complex and previously shown to be a binding partner for Rab6A/Rab6A′. Co-immunoprecipitation studies and pull down assays confirmed that Bicaudal–D1 also interacts with Rab6B in its active form. Using confocal laser scanning microscopy it was established that Rab6B and Bicaudal–D1 co-localize at the Golgi and vesicles that align along microtubules. Furthermore, both proteins co-localized with dynein in neurites of SK-N-SH cells. Live cell imaging revealed bi-directional movement of EGFP–Rab6B structures in SK-N-SH neurites. We conclude from our data that the brain-specific Rab6B via Bicaudal–D1 is linked to the dynein/dynactin complex, suggesting a regulatory role for Rab6B in the retrograde transport of cargo in neuronal cells.

Keywords:
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Introduction

Rab proteins form the largest branch of the monomeric Ras superfamily of small GTPases and are key regulators of intracellular membrane traffic. To date, more than 60 Rab family members, including isoforms, are recognized [1]. Rab proteins constantly cycle between a GTP-bound active and GDP-bound inactive state and exert their function by interacting with a wide range of Rab effector proteins. These protein complexes can regulate different membrane trafficking events such as formation of vesicles at the donor membrane, movement and subsequent tethering and fusion with the acceptor membrane [2]. Several Rab proteins are known to regulate vesicle movement along microtubules or actin filaments [3]. One example of Rab-regulated microtubule-based transport is the movement of lysosomes along...
microtubules by the Rab7–RILP complex [4]. Another Rab protein implicated in playing a role in microtubule-based transport is Rab6 [5–7].

In the past, three different isoforms of this protein were identified: Rab6A [8], Rab6A’ generated by alternative splicing of a homologues but distinct exon within the Rab6 gene [9], and a brain-specific isoform, Rab6B [10]. Initially, it was thought that Rab6A was the isoform regulating a COP-1-independent Golgi to endoplasmic reticulum (ER) retrograde transport [11–13] with an additional role for Rab6A’ in a specific endosome to Golgi transport process involving the routing of internalized Shiga toxin [14]. These latter findings have recently been challenged, proposing Rab6A’ as the isoform regulating the entire retrograde pathway from late endosomes to ER. Rab6A seems dispensable for this pathway [15] but most likely exerts its functions mainly during mitosis. Rab6A’ has also been implicated to play role in mitosis by inactivating the Mad2-spindle checkpoint during the transition from metaphase to anaphase [16].

For the other Rab6 member, Rab6B, relatively little is known and therefore is the main focus of this study. Studies from the past have shown that Rab6B binds to all, at that time known, Rab6A binding proteins such as Rabkinesin-6 (Rab6-KIFL), a Golgi localized kinesin-like protein [17], and GAPCenA, a centrosome-associated GTPase-activating protein specific for Rab6 [18]. Detailed Northern blot analysis showed that Rab6B has its highest expression in brain and is also prominently expressed in brain-derived cell lines like SK-N-SH [10]. In these cells, Rab6B is localized to the Golgi, at ER membranes and also at the ER Golgi intermediate compartment (ERGIC) [10]. While, in HeLa cells, overexpression of the GTPase-deficient Rab6A mutant induces a redistribution of Golgi-localized proteins into the ER preliminary results show that overexpression of the GTPase-deficient Rab6B does not. Its biochemical properties, specific cell- and tissue-type expression pattern and its intracellular localization at the ERGIC, suggest a specific role for Rab6B in neuronal cells. To provide us with more clues about the function of Rab6B, we performed a yeast-two-hybrid screen, using the GTPase-deficient mutant as bait, in order to identify with co-immunoprecipitation and pull down experiments. Co-localization is observed between Rab6B and Bicaudal–D1 at the Golgi and additionally at vesicular structures that align along microtubules and are found in neurites of SK-N-SH cells. These Rab6B Bicaudal–D1 vesicles co-localize also with dynein. Live cell imaging in SK-N-SH cells expressing EGFP–Rab6B revealed retrograde movement of these structures.

Although the functional relationship between Rab6B and Bicaudal–D1 has not been fully addressed yet, we conclude that the Rab6B Bicaudal–D1 interaction regulates retrograde membrane transport in neurites of neuronal cells. The additional localization of Rab6B at the ERGIC suggests a specific regulatory role for this small GTPase at this compartment in neuronal cells.

### Material and methods

#### Yeast–two-hybrid interaction trap

For Y-2H screening we used the interaction trap system as described [20]. GTPase-deficient Rab6B Q72L (Rab6B–GTP) bait was introduced in the BamHI/XhoI cloning site of the pMW101 vector by standard subcloning procedures. As prey, a human fetal brain cDNA library was used (kindly provided by Dr. Roger Brent and colleagues, Massachusetts General Hospital, Boston, MA). For two-hybrid interactions, assay plasmids were introduced in yeast strain EGY48 (Mata trp1 ura1 his3 LEU2: pLex-Aop6-LEU2) containing the plasmid pSH18–34, which includes the reporter lacZ gene, and tested for an interaction as detected by growth and blue coloring on minimal agar plates lacking histidine, tryptophan, uracil and leucine, containing 2% galactose, 1% raffinose and 80 μg/ml X-gal, buffered at pH 7.0. To confirm positive clones, the insert containing pJC4–5 plasmid DNA from positive clones was isolated, reintroduced into the yeast strain, and screened in a second round for growth and blue coloring.

#### Immunoprecipitation and immunoblotting

COS-1 cells (ATCC CRL-1650) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS) and 1% pyruvate/glutamate on 10-cm dishes. The cells were washed with OptiMEM (Gibco) and submerged with 5 ml transfection medium (4.7 ml OptiMEM, 150 μl 2 mg/ml DEAE-dextran chloride (Sigma), 5 μl 100 mM chloroquine) containing 2.5 μg of the pSG8-His plasmid vector DNA encoding full-length Myc-tagged WT’ Rab6A, Rab6A’, Rab6B or an empty pSG8-His as negative control. In addition, transfection was performed with GFP-tagged Rab4, Rab5, Rab6A’, Rab6B, Rab22B [21] or GFP alone and 2.5 μg Bicaudal–D1 in pEGFP-N2 in which the GFP was replaced by a triple HA-tagged or GFP-tagged N-terminal or C-terminal part of Bicaudal–D1 [5]. After an incubation of 3 h, the transfection medium was removed, cells were incubated for 3 min in 5 ml 10% dimethylsulfoxide (DMSO) in phosphate-buffered saline (PBS), washed, and further cultivated in DMEM. About 24 h later, cells were washed twice with PBS and lysed on the plate with 1 ml lysis buffer (100 mM Na2HPO4 pH 8.0, 1% [v/v] Triton X-100, 0.2% bovine serum albumin (BSA)) and protease inhibitor cocktail (Roche Diagnostics, GmbH, Mannheim, Germany) and put on ice for 1 h. The lysate was centrifuged for 15 min at 10,000 × g at 4 °C after which 500 μl of the supernatant was subjected to immunoprecipitation with a polyclonal antibody against Rab6B [10], GFP [22] or Bicaudal–D1 #2296 [5], non-covalently coupled to 20 μl protein-A Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden). Protein-A beads with captured protein were washed six times with high salt buffer (100 mM Tris–HCl, pH 7.4, 1.2 M KCl and 1% [v/v] Triton X-100) and twice with PBS. Then beads were taken up in 20 μl 2× sample buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and released protein was subjected to electrophoresis on a 10% polyacrylamide gel. Resolved proteins were transferred onto nitrocellulose membrane (Amersham Biosciences) by Western
blotting. After blocking with 5% non-fat dry milk in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% [v/v] Tween-20 (TBST), the blots were incubated with either anti-HA antibody 12CA5 (diluted 1:500) or anti-Myc antibody 9E10 (diluted 1:100) anti-GFP antibody B2 (diluted 1:5000; Santa Cruz) or anti-Rab6 antibody (diluted 1:5000) followed by secondary horseradish peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG antibody (diluted 1:20,000; Pierce). Incubations and subsequent washes were carried out in TBST at room temperature followed by a final rinse with TBS. Immunoreactive bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris–HCl, pH 8.5, 1.25 mM β-comucaric acid, 0.2 mM luminol and 0.009% H2O2) and signals were revealed by exposure to Kodak X-omat autoradiography films.

**Isolation of GST fusion proteins**

For the isolation of GST or GST–Rab6A, GST–Rab6A′ and GST–Rab6B fusion proteins, 200 ml of Escherichia coli DH5α cultures expressing pGEX–Rab6A, ∼6A or ∼6B were grown until an OD600 of about 0.7. Cultures where then maintained for 3 h at a non-hydrolysable variant of GTP, or GDP. Retained proteins were visualized by Western blotting using anti-HA antibody 12CA5 or anti-GFP antibody B2 (diluted 1:5000; Santa Cruz) or anti-Rab6 antibody (diluted 1:5000) followed by secondary horseradish peroxidase-conjugated AffiniPure goat anti-mouse or anti-rabbit IgG antibody (diluted 1:20,000; Pierce). Incubations and subsequent washes were carried out in TBST at room temperature followed by a final rinse with TBS. Immunoreactive bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris–HCl, pH 8.5, 1.25 mM β-comucaric acid, 0.2 mM luminol and 0.009% H2O2) and signals were revealed by exposure to Kodak X-omat autoradiography films.

**Pull down experiments**

Pull down experiments with GTPγS, a non-hydrolysable variant of GTP, or GDP loaded GST–Rab6 isoforms were done as described previously [26]. A total of 10 μg of GST–Rab6 fusion proteins were incubated in the presence of 20 μl glutathione Sepharose 4B beads in loading buffer (25 mM Tris pH 7.5, 10 mM EDTA, 5 mM MgCl2) for 1 h at 4 °C under rotation. Next the GST–Rab6 bound to the glutathione sepharose beads were loaded with GTPγS or GDP in loading buffer containing 1 mM of GTPγS or 1 mM of GDP for 1 h at 37 °C. After centrifugation, the buffer was removed and replaced by interaction buffer (25 mM Tris, pH 7.5, 10 mM MgCl2, 500 mM NaCl, 0.1% [v/v] Triton X-100) again supplemented with 1 mM GDP or 1 mM GTPγS to which 30 μl cell lysate of BHK-21 cells transfected with HA-tagged Bicaudal–D1 was added and incubated for 90 min at room temperature under rotation. After incubation, the beads were washed 4 times with interaction buffer without nucleotides and processed for Western blotting. For GST pull down experiments, BHK-21 cells were transfected with HA-Bicaudal–D1 encoding construct using Lipofectamine (Invitrogen Life Technologies) according to the manufacturer’s specifications. Subsequent cell extracts were made by lysing the PBS-washed cells in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.2% [v/v] Triton X-100, protease inhibitor cocktail).

Rab6 binding assay with purified proteins

For binding assays, ∼5 μg GST, GST–Rab6A or GST–Rab6B was immobilized on 25 μl glutathione Sepharose 4B beads and washed three times with buffer NE100 (20 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.1% [v/v] Triton X-100), two times with buffer NL100 (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1% [v/v] Triton X-100) and finally two times with 500 μl buffer NL100 GTPγS (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1% [v/v] Triton X-100, 50 μM GTPγS) as described by

Fig. 1 – Rab6 isoforms interact with Bicaudal–D1. Cell lysates of COS-1 cells transfected with Myc-tagged Rab6A, Rab6A′, Rab6B and HA-tagged Bicaudal–D1 were subjected to immunoprecipitations using protein sepharose-A beads loaded with anti-Rab6 antibody or anti-Bicaudal–D1 antibody #2296. Co-immunoprecipitated proteins were visualized by Western blotting using anti-HA antibody 12CA5 (A) or anti-Myc antibody 9E10 (B) to detect co-immunoprecipitated Bicaudal–D1 or Rab6, respectively. In the middle panels, the efficiency of the immunoprecipitation with Rab6 (A) and Bicaudal–D1 (B) is shown. In the lower panels, the input (5% of total lysate) of Bicaudal–D1 (A) and Rab6 (B) is depicted. Molecular mass markers (kDa) are shown on the left. The difference in molecular weight between Myc–Rab6A/A′ and Myc–Rab6B can be attributed to a different spacer region between the Myc tag and the Rab6 start codon. Rab6 Bicaudal–D1 interaction is specific for Rab6 (C). A number of different GFP-tagged Rab proteins were immunoprecipitated with anti-GFP antibody from COS-1 cell lysates. Co-immunoprecipitation of Bicaudal–D1 was only found with GFP–Rab6A′ and GFP–Rab6B (C). The efficiency of the immunoprecipitation and the inputs (5% of total lysate) are shown in the second and two lower panels, respectively. Molecular mass markers are shown on the left. Cell lysates of COS-1 cells expressing Myc-tagged Rab6B and full-length GFP–Bicaudal–D1, GFP–Bicaudal–C, GFP–Bicaudal–N or GFP alone were subjected to immunoprecipitation with anti-Myc antibody 9E10. Co-immunoprecipitated proteins were visualized with anti-GFP antibody (D, upper panel). Efficiency of the immunoprecipitation and input (5% of total lysate) of the different GFP-tagged proteins is shown in the middle and lower panels, respectively. Asterisk indicates an additional band often observed after GFP–Bicaudal–D1 expression. Direct interaction between Bicaudal–D1–C and Rab6. Purified GST–Rab6A, GST–Rab6B or GST alone was used in a pull down assay with purified His-tagged Bicaudal–D1–C (E). The amount of GST fusion protein is shown in the upper panel by the Coomassie staining of the SDS–PAGE gel. The amount of Bicaudal–D1–C retained by GST–Rab6 is shown in the middle panel by immunoblot and input of His–Bicaudal–D1 (5% of total) in the lower panel. Molecular mass markers (kDa) are indicated on the left. Rab6B preferentially binds to Bicaudal–D1 in the GTP-bound state (F). Cell lysates of BHK-21 cells transfected with HA-tagged Bicaudal–D1 were incubated in the presence of glutathione sepharose 4B with equal amounts of bound GST–Rab6A, GST–Rab6A′, GST–Rab6B or GST alone and loaded with either GTPγS, a non-hydrolysable variant of GTP, or GDP. Retained proteins were visualized by Western blotting using anti-HA antibody 12CA5.
Fuchs et al. [27]. For the binding reaction, loaded beads were resuspended in 200 μl NL100 GTP-γS with approximately 4 μg His-Bicaudal–D1–C and incubated on a roller for 2 h at 4 °C. After incubation, beads were pelleted by centrifugation and unbound protein was removed by washing four times with 1 ml NL100 GTP-γS. Bound protein was eluted directly in 40 μl 1× SDS sample buffer, analyzed on 12% SDS–PAGE gel and visualized on Western blot using anti-His antibody (QIAGEN).

**Anti-Rab6B antibody purification**

To purify the anti-Rab6B antibody described by Opdam and coworkers [10], a total of 50 μl of serum containing antibodies raised against GST–Rab6B were incubated O/N at 4 °C in the presence of excess amounts of GST–Rab6A and GST–Rab6A′ fusion proteins bound to glutathione Sepharose 4B beads in a total volume of 500 μl PBS with 1% BSA to bind Rab6A and Rab6A′ cross-reactive antibodies. After incubation, the beads were spun down and the remaining supernatant, containing Rab6B-specific antibodies, was tested for specificity on Western blot before use in immunofluorescence assays.

**Immunofluorescence assay**

HeLa cells (WT or stably expressing EGFP–Rab6B) or SK-N-SH cells were grown until 75% confluency in DMEM supplemented with 10% FCS, 1% pyruvate/glutamate in 24-well plates on glass coverslips with 0.1% saponin/20 mM glycine in PBS (PBSG). Some of the cells were treated with 10 μg/ml Nocodazole (Sigma) for 3 h prior to washing and fixation. Cells were incubated with anti-HA antibody 12CA5 (1:125 dilution; DBS, University of Iowa, IA) or primary antibody against Bicaudal D1 (Fig. 1C) indicating that the interaction of Bicaudal D1 with Rab6B is direct (Fig. 1E, middle panel). Specific labeling was detected by incubation with Alexa-conjugated secondary goat anti-mouse or goat anti-rabbit IgG antibodies (dilution 1:500; Jackson ImmunoResearch Laboratories Inc. West Grove, PA) for 1 h at room temperature. Finally, the glass coverslips were rinsed in PBSG/20% serum, water, dehydrated for 5 min with 100% methanol and mounted on glass slides by immersion in Mowiol (Sigma) containing 2.5% sodium azide. Cells were examined using a confocal laser scanning microscope (Biorad MRC1024).

**Live cell imaging**

SK-N-SH cells expressing EGFP–Rab6B were plated on 35-mm diameter glass-bottom Wilco-dishes (Wilco wells B.V. Amsterdam, The Netherlands). Live cell confocal microscopy was performed on Zeiss LSM510Meta equipped with a type S CO2 incubator and a temperature-controlled stage.

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**Results**

**Rab6B interacts with Bicaudal–D1**

To search for specific binding partners of Rab6B the constitutive GTP-bound form of all three Rab6 isoforms (Rab6–GTP) were used to screen a human fetal brain cDNA library in a yeast-two-hybrid interaction trap experiment. In relation to Rab6A and Rab6A′, no specific Rab6B interactors were found (see Supplemental Table 1 for details). However, we identified Bicaudal–D1 as a novel interactor for Rab6B. The binding of Bicaudal–D1 to Rab6A has previously been described by Matanis and co-workers [5]. To further characterize the nature of the Rab6B Bicaudal–D1 interaction, co-immunoprecipitation studies were performed with lysates from transfected COS-1 cells expressing all three Myc-tagged wildtype (WT) Rab6 isoforms and HA-tagged Bicaudal–D1. As a negative control, empty vectors were used showing no a-specific binding to the beads (data not shown). These experiments revealed that all Rab6 isoforms including Rab6B are able to co-immunoprecipitate Bicaudal–D1 (Fig. 1A). Reciprocally, immunoprecipitation with the anti-Bicaudal–D1 antibody #2296 resulted in the co-immunoprecipitation of all three Rab6 isoforms (Fig. 1B). The efficiency of the immunoprecipitation with anti-Rab6B and anti-Bicaudal–D1 antibodies is shown in the middle panels of Figs. 1A and B, respectively. The lower panels show the input of Bicaudal–D1 and Rab6.

To check whether the interaction between Bicaudal–D1 is specific for the Rab6B isoforms we immunoprecipitated GFP-tagged Rab4, Rab5, Rab6A, Rab6B and Rab22B or GFP alone with anti-GFP antibody and analyzed whether HA–Bicaudal–D1 was co-immunoprecipitated. In this experiment, only GFP–Rab6A′ and GFP–Rab6B were able to co-immunoprecipitate HA–Bicaudal–D1 (Fig. 1C) indicating that the interaction of Bicaudal–D1 is specific for Rab6 isoforms.

**Rab6B directly interacts with the C-terminal part of Bicaudal–D1**

To investigate which part of the Bicaudal–D1 protein is responsible for the interaction with Rab6B, co-immunoprecipitation experiments with Myc–Rab6B and the GFP-tagged N-terminal or C-terminal part of Bicaudal–D1 were performed. The results show that the C-terminal part, and not the N-terminal part of GFP–Bicaudal–D1 co-immunoprecipitates with Myc–Rab6B (Fig. 1D, upper panel). Subsequent pull down assays with purified GST–Rab6A, GST–Rab6B and His–Bicaudal–D1–C proteins showed that interaction between Bicaudal–D1 and Rab6B is direct (Fig. 1E, middle panel).

**Bicaudal–D1 preferentially binds to Rab6B in the GDP bound form**

To further characterize the Rab6B Bicaudal–D1 interaction, we investigated whether binding is determined by GTP-loading of Rab6B. Therefore, we assessed binding relative to Rab6A and Rab6A′ in pull down experiments with the use of equal amounts of Rab6A, Rab6A′ or Rab6B GST–fusion proteins coupled to glutathione Sepharose 4B beads and loaded with either GTPγS or GDP. As expected the GTPγS-loaded GST–Rab6B was far more efficient in pulling down Bicaudal–D1 than GDP-loaded Rab6B. Interestingly for Rab6A, a comparable binding was observed whereas binding to Rab6A′ appeared independent
of GTP loading (Fig. 1F). Based on this finding, we conclude that Bicaudal–D1 binding prefers the Rab6B protein to be in the GTP-bound state.

**Ectopic Rab6B co-localizes with Bicaudal–D1 at the Golgi and vesicular structures that align along microtubules**

To study whether Rab6B Bicaudal–D1 can co-localize in HeLa cells, we co-expressed EGFP–Rab6B and HA-tagged Bicaudal–D1 in HeLa cells. Cells that stably expressed EGFP–Rab6B (mild over-expression) were therefore transiently transfected with HA-tagged Bicaudal–D1 encoding plasmid and protein location was revealed with anti-HA antibody 12CA5 and direct recording of GFP fluorescence with confocal laser scanning microscopy. Ectopic Rab6B co-localizes with Bicaudal–D1 at the Golgi and at vesicular structures (arrows Figs. 2A–C). Subsequent staining with the anti-β-tubulin antibody E7 to stain the microtubule network revealed that these vesicular

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**Fig. 2 – Ectopic Rab6B and Bicaudal–D1 co-localize at the Golgi and at vesicular structures along microtubules.** HeLa cells stably expressing Rab6B–GFP were transfected with HA-tagged Bicaudal–D1, and stained with anti-HA antibody 12CA5 to visualize Bicaudal–D1 (A–C) or in addition with anti-β-tubulin antibody E7 to stain the microtubule network (D–H) and analyzed with confocal laser scanning microscopy. Co-localization of Bicaudal–D1 (A) and Rab6B (B) is observed at the Golgi and at the vesicular structures. Merged images are shown in C. The Rab6B Bicaudal–D1-positive structures align along microtubules (G, H). Merged image is shown in G. Alignment along microtubules is shown in more detail in H.
structures align along microtubules (Figs. 2D–H, indicated by arrows).

Overexpressed Rab6B–GTP and Bicaudal–D1 accumulates at the cell periphery

Since the Y–2H was performed with the constitutive GTP-bound forms, we also studied the effect of overexpression the EYFP–Rab6–GTP mutants and Bicaudal–D1 in HeLa cells. In a large number of cells, we observed co-localization between EYFP–Rab6A–GTP or EYFP–Rab6B–GTP and Bicaudal–D1 at the cell periphery (Figs. 3A–C and D–F for Rab6A and Rab6B, respectively). Interestingly this peripheral accumulation was not observed in cell overexpressing EYFP–Rab6A′–GTP (data not shown). As this accumulation was also observed in HeLa cells overexpressing only EYFP–Rab6A–GTP or EYFP–Rab6B–GTP, we conclude that this accumulation is independent of the Bicaudal–D1 interaction.

Localization of endogenous Rab6B and Bicaudal–D1 in SK-N-SH cells

To study the localization of endogenous Rab6B compared to Bicaudal–D1 we switched to SK-N-SH cells, a neuroblastoma cell line of human origin that expresses high levels of endogenous Rab6B. We started with purifying Rab6B antibodies [10] by incubating them in the presence of excess amounts of GST–Rab6A and GST–Rab6A′ to lose the cross-reactive antibodies. To confirm that no cross reacting antibodies were left, we tested the purified fraction with Western blotting using cell lysates of COS-1 cells expressing Myc-tagged Rab6A, Rab6A′ or Rab6B. Compared to the non-purified anti-Rab6B (Fig. 4A, left panel) the purified antibody only recognizes Rab6B protein (Fig. 4A, right panel).

SK-N-SH stained with anti-Rab6B antibody showed a prominent staining of the Golgi (Fig. 4B). Although visible using the non-purified anti-Rab6B antibody, we were unable to detect the punctuate pattern in the neurites with the purified Rab6B antibody as reported by Opdam and co-workers [10]. The difference may be that we now affinity-purified the antibody. This purified Rab6B antibody may have a too low affinity to detect endogenous Rab6B by immunofluorescence. Endogenous Bicaudal–D1 also localizes to the Golgi and is detected as a punctuate pattern in neurites as indicated by arrowheads in Fig. 4E (magnified in the inset). SK-N-SH cells overexpressing HA-tagged Bicaudal–D1 show the same distribution as the endogenous protein (Fig. 4C) and did not change the distribution of endogenous Rab6B compared to non-transfected cells. In SK-N-SH cells, GFP–Rab6B is mainly localized to the Golgi but also present in the...
neurites co-localizing there with endogenous Bicaudal–D1 (Fig. 4F and magnified in the inset). Localization studies addressing the co-localization of endogenous Rab6B and Bicaudal–D1 failed due to loss of affinity of the anti-Rab6B antibody after biotinylation. However, based on the individual distribution patterns of endogenous Rab6B (Fig. 4B) and Bicaudal–D1 (Fig. 4E) it is clear that they both are localized in the Golgi and in vesicles.

**Characterization of Rab6B-positive structures in SK-N-SH cells**

One function of Bicaudal–D1 is to recruit the dynein complex to different organelles [5]. We therefore checked whether dynein is also present on the Rab6B Bicaudal–D1-positive structures in the SK-N-SH neurites, where they are most easily visible and reflect structures engaged in active transport. When we double-stained SK-N-SH cells expressing EGFP–Rab6B and Bicaudal–D1 in neurites is indicated by arrowheads. Merged images are shown in panel G.

**Fig. 4 – Co-localization of endogenous Rab6B and Bicaudal–D1 in SK-N-SH cells.** Anti-Rab6B antibody purification. For studying endogenous Rab6B in SK-N-SH cells, affinity-purified anti-Rab6B antibody was used. To verify its specificity, cell extracts of COS-1 cells transfected with Myc-tagged Rab6A, Rab6A', Rab6B or mock transfected were blotted and incubated with the non-purified anti-Rab6B antibody (A, left) or with affinity-purified anti-Rab6B antibody (A, right). Molecular mass markers (kDa) are shown on the left. SK-N-SH cells transfected with HA-tagged Bicaudal–D1, stained with anti-HA 12CA5 antibody to visualize Bicaudal–D1 and purified anti-Rab6B antibody to stain endogenous Rab6B are shown in panels B and C. Merged images are shown in panel D. SK-N-SH transiently transfected with WT EGFP–Rab6B, stained with anti-Bicaudal–D1 antibody #2296 and analyzed with confocal laser scanning microscopy are shown in panels E and F. Co-localization of EGFP–Rab6B and Bicaudal–D1 in neurites is indicated by arrowheads. Merged images are shown in panel G.
Rab6B with anti-Bicaudal-D1 and anti-dynein antibody, we observed that most EGFP-Rab6B-positive structures in SK-N-SH cell extensions were also positive for Bicaudal-D1 and dynein (Figs. 5A–D, indicated by arrows). This finding suggests that the Rab6B Bicaudal-D1-positive structures in neurites are capable of being retrograde transported along microtubules.
Subsequent live cell imaging of EGFP–Rab6B in neurites of SK-N-SH cells revealed that EGFP–Rab6B-positive structures indeed move in both anterograde and retrograde directions in neurites (see Supplemental Movie 1).

From previous studies [10] it is known that, in contrast to Rab6A and Rab6A′, Rab6B co-localizes in SK-N-SH cells also with ERGIC-53, a protein that resides in the ER Golgi intermediate compartment. To see whether the pool of Rab6B present at the ERGIC also co-localizes with Bicaudal-D1, we double-stained SK-N-SH cells expressing EGFP–Rab6B with anti-ERGIC-53 and anti-Bicaudal-D1 antibody. Again co-localization between EGFP–Rab6B and Bicaudal-D1 was

![Image of Fig. 5](image_url)

**Fig. 5 – Identification of Rab6B and Bicaudal-D1-positive structures.** SK-N-SH cells were transiently transfected with WT EGFP–Rab6B (B, F) and stained with anti-dynein antibody (A) and anti-Bicaudal-D1 antibody #2296 (C, G) or anti-ERGIC53 antibody (E). Merged images are shown in panels D and H, respectively. Co-localization for a subset of Rab6B, dynein and Bicaudal-D1 vesicles are indicated by arrows. Rab6B- and ERGIC53-positive but Bicaudal-D1-negative structures are found near the cell body indicated by the arrow in panels E, F and H (magnified in the left inset in panel H), whereas Rab6B- and Bicaudal-D1-positive but ERGIC-53-negative structures in the neurites are indicated by the arrowhead in panels F, G and H (magnified in the right inset in panel H).

![Image of Fig. 6](image_url)

**Fig. 6 – Expression of Rab6B-GDP affects the localization of endogenous Bicaudal-D1 in neurites.** SK-N-SH cells were transfected with EGFP–Rab6B-GTP (B) and EGFP–Rab6B-GDP (D) encoding plasmids and stained for endogenous Bicaudal-D1 with anti-Bicaudal-D1 antibody #2296 (A and C). Localization of Bicaudal–D1 in SK-N-SH cells overexpressing GTP–Rab6B resembles the pattern found in cells overexpressing WT Rab6B i.e. co-localizing at the Golgi and along neurites. SK-N-SH cells overexpressing Rab6B–GDP (D indicated by the asterisks) show a less prominent staining of Bicaudal-D1 at the Golgi. In neurites, Bicaudal-D1 localization is also less prominent (inset in panels C and D indicated with the arrowhead) when compared to untransfected cells where Bicaudal-D1 is still present in the neurites (inset in panel C, indicated with the arrow).
observed in neurites (Figs. 5E–H, arrowhead). However, ERGIC-S3 was only found co-localizing with Rab6B in the cell body at relative close proximity of the Golgi (Figs. 5E–H, arrow). These ERGIC-S3- and Rab6B-positive structures never contained any Bicaudal-D1. It seems therefore that two pools of Rab6B in SK-N-SH cells exist, one not associated with Bicaudal-D1, present at the ERGIC, and one associated with Bicaudal-D1 present at the Golgi and vesicles in the neurites together with Rab6A.

Localization of Bicaudal-D1 to the Golgi and in SK-N-SH cell extensions is dependent on Rab6B

Finally we investigated the effect of overexpression of Rab6B-GTP or Rab6B-GDP on endogenous Bicaudal-D1 localization in SK-N-SH cells. Localization of EGFP-Rab6B-GTP resembles the wild type form as it is present at the Golgi and punctuate structures in the neurites indicated by arrows, where it co-localizes with endogenous Bicaudal-D1 (Figs. 6A, B).

In cells expressing EGFP-Rab6B-GDP, indicated by an asterisk in Fig. 6 panels C and D, the localization of Bicaudal-D1 at the Golgi is less prominent compared to cells not expressing EGFP-Rab6B-GDP. Also in neurites the localization of Bicaudal-D1 is affected by overexpression of EGFP-Rab6B-GDP. There, the localization of Bicaudal-D1 in neurites of cells expressing EGFP-Rab6B-GDP is less prominent as indicated by the arrowhead in Fig. 6 inset panels C and D compared to a neighboring cell not expressing EGFP-Rab6B-GDP indicated with an arrow in Fig. 6 inset panel C. Instead the Bicaudal-D1 protein is more evenly distributed throughout the cytosol. We therefore conclude that Rab6B is involved in the localization of Bicaudal-D1 at the Golgi and vesicles in neurites.

Discussion

In the past, three Rab6 isoforms were identified: Rab6A, Rab6A’ and Rab6B [8–10]. Both Rab6A and Rab6A’ are ubiquitously expressed and are generated via alternative splicing of a duplicated exon within the Rab6A gene [9]. Rab6B is encoded by a different gene, has 93% similarity with Rab6A, and shows a tissue and cell-type-specific expression pattern in brain. Strikingly, despite these differences between Rab6A and Rab6B, all known Rab6A effector proteins are also able to bind Rab6B. To learn more about the functional difference between Rab6A and Rab6B we searched for novel Rab6B interacting proteins. In the work described here, we identified in a Y2H screen the human homolog of the Drosophila coiled-coil protein Bicaudal-D1 [29]. This protein turned out not to be a specific partner for Rab6B as its interaction with Rab6A and to a lesser extend also with Rab6A’ had been described previously [5]. In mammalian cells, two homologs of Bicaudal-D are present, Bicaudal-D1 (at least four forms by alternative splicing) [30] and Bicaudal-D2 [30]. Previous studies have shown that in mammalian cells, Bicaudal-D2 but also Bicaudal-D1 associates with the dynein–dynactin complex and is able to induce minus end-directed transport of organelles [19,31]. The dynein–dynactin protein complex is the major minus end-directed microtubule associated motor protein complex (for review [32]). In addition, it was also established that Rab6 binds directly to p150^{glued}, a component of the dynactin protein complex [33]. Taken together, these data implicate an important role for Rab6 in the regulation of dynein-mediated processes. The fact that Rab6B is cell type specific expressed and probably regulates a different pathway from that of Rab6A/A’ prompted us to look in more detail at the interaction between Rab6B and Bicaudal-D1 in neuronal cells.

To confirm the yeast-two-hybrid data, co-immunoprecipitation experiments were performed. On the basis of the co-immunoprecipitations with the anti-Rab antibody one could assume that Rab6B binds stronger to Bicaudal-D1 compared to the other isoforms. This is however not supported by the data of the reciprocal immunoprecipitation and the pull down experiments with GTP or GDP loaded Rab6. Interestingly there was no difference in the amount of Bicaudal-D1 pulled down by GTP or GDP loaded Rab6A’. This is not uncommon as also differences in binding of the different Rab6 isoforms to Rab6IP1 were observed [26].

In our localization studies in SK-N-SH cells, we made the following observations. First, when these cells were transfected with EGFP-tagged Rab6B fluorescence was observed, as expected, at the Golgi but also in vesicles in neurites that are characteristic for SK-N-SH cells. At these EGFP-Rab6B-positive patches, also endogenous Bicaudal-D1 was detected with the Bicaudal-D1 specific antibody. The staining pattern observed for Bicaudal-D1 is not influenced by the presence of over-expressed EGFP-Rab6B. SK-N-SH overexpressing HA-tagged Bicaudal-D1 again shows the same staining pattern as observed for endogenous Bicaudal-D1. SK-N-SH cells transfected with EGFP-tagged Rab6B and stained for endogenous Bicaudal-D1 and in addition stained for dynein with an antibody against its intermediate chain shows co-localization in these cell extensions for some of these structures. Subsequent live cell imaging revealed movement of EGFP-Rab6B structures in both anterograde and retrograde directions. The bi-directional nature of these structures can be explained by the presence of both plus, perhaps Rabkinesin-6 [10] and minus end-directed dynein/dynactin motors at the same time on these vesicles [34]. The cargo of these Rab6B vesicles remains unknown, however in a recent study Rab6B was found in a purified synaptic vesicle preparation [35].

Rab6B also partially localizes to the Golgi ER intermediate compartment (ERGIC) in SK-N-SH cells, but no co-localization was observed for Rab6B and Bicaudal-D1 at the ERGIC. This is in agreement with Matanis who also did not observe Bicaudal-D1 at the ERGIC in HeLa cells [5]. Live cell imaging experiments using SK-N-SH cells expressing EYFP-Rab6B and ECFP–Rab6A revealed the presence of both Rab6 isoforms on vesicles in the neurites (data not shown). This may suggest that Rab6A and Rab6B function side by side at these vesicles. However Del Nery and co-workers [15] have shown that, although Rab6A was shown to function in retrograde transport in other cell types, this function is largely dispensable and that Rab6A most likely exerts its function during mitosis [16]. The same can hold for neuronal cells. Only at the ERGIC specific localization of Rab6B was found, although not with Bicaudal–D1. It seems therefore that Rab6B fulfills a dual role, one in ERGIC-associated COP-I- or COP-II-related traffic and another COP-independent dynein associated pathway involving Bicaudal-D1 and vesicles moving in and out neurites.
We observed that a large number of cells that co-express the GTP-bound forms of Rab6A and Rab6B together with HA–Bicaudal show an accumulation of both proteins at the cell periphery in accordance to the observations by Matanis [5] when they overexpressed only the C-terminal part of Bicaudal–D1. However, accumulation of Rab6A or Rab6B is also observed in a large number of cells only expressing Rab6A–GTP or Rab6B–GTP and seems therefore independent of the overexpression of Bicaudal–D1. This indicates that accumulation of GTP-bound Rab6A or Rab6B is induced by another process then the accumulation observed in cells overexpressing C-terminal Bicaudal–D1. In these structures also no Rabkinesin-6 was found (data not shown), ruling out any involvement of this supposed motor protein. Interestingly these peripheral accumulations are not observed in the case of GTP–Rab6A.

Our data suggest that Rab6B is involved in the recruitment of Bicaudal–D1- to Rab6B-positive structures. Cells overexpressing Rab6B–GDP showed less staining for Bicaudal–D1 at the Golgi. In addition, cells overexpressing the GDP-bound form of Rab6B lack the characteristic Bicaudal–D1 structures in the cell extensions. Overexpression of Rab6B–GTP had no observable effect on the staining pattern of Bicaudal–D1, as it resembles the pattern compared to cells overexpressing WT Rab6B.

It still remains unclear what the exact nature of the Rab6B Bicaudal–D1 interaction is. However in neurites it functions in regulating dynein-mediated retrograde movement, maybe in combination with Rab6A and/or Rab6A′, a process very important for the viability of these cells [36,37]. In addition, it might be that Rab6B regulates a neuronal cell-specific, Bicaudal–D1-independent role at the ERGIC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2007.05.032.

REFERENCES


