Rabphilin Localizes with the Cell Actin Cytoskeleton and Stimulates Association of Granules with F-actin Cross-linked by α-Actinin*

Giovanna Baldini1, Alberto M. Martelli1*, Giovanna Tabellini1, Chad Horn**, Khaled Machaca1†, Paola Narducci§, and Giulia Baldini**1

From the 1Dipartimento di Morfologia Umana Normale, via Manzoni 16, Trieste, Università di Trieste, Trieste I-34138, Italy, the 2Dipartimento di Scienze Anatomiche Umane e Fisiopatologia dell’Apparato Locomotore, Sezione di Anatomia, Cell Signalling Laboratory, Università di Bologna, via Irnerio 48, Bologna I-40126, Italy, the 3Istituto per i Trapianti d’Organò e l’Immunootologia del Consiglio Nazionale delle Ricerche, Sezione di Bologna c/o IOR, via di Barbiano 1/10, Bologna I-40136, Italy, the 4Dipartimento di Scienze Biomediche e Biotecnologie, Sezione di Citologia e Etiologia, Università di Brescia, Viale Europa 11, Brescia I-25123, Italy, and the Departments of **Physiology and Biophysics and of **Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

In endocrine cell, granules accumulate within an F-actin-rich region below the plasma membrane. The mechanisms involved in this process are largely unknown. Rabphilin is a cytosolic protein that is expressed in neurons and endocrine cells and binds with high affinity to members of the Rab3 family of GTPases localized to synaptic vesicles and dense core granules. Rabphilin also interacts with α-actinin, a protein that cross-links F-actin into bundles and networks and associates with the granule membrane. Here we asked whether rabphilin, in addition to its granule localization, also interacts with the cell actin cytoskeleton. Immunofluorescence and immuno-electron microscopy show that rabphilin localizes to the sub-plasmalemal actin cytoskeleton both in neuroendocrine and unspcialized cells. By using purified components, it is found that association of rabphilin with F-actin is dependent on added α-actinin. In an in vitro assay, granules, unlike endosomes or mitochondria, associate with F-actin cross-linked by α-actinin. Rabphilin is shown to stimulate this process. Rabphilin enhances by ~2-fold the granularity ability to localize within regions of elevated concentration of cross-linked F-actin. These results suggest that rabphilin, by interacting with α-actinin, organizes the cell cytoskeleton to facilitate granule localization within F-actin-rich regions.

Rabphilin is a cytosolic protein expressed in neurons and endocrine cells that binds with high affinity to members of the Rab3 family of proteins, namely Rab3A, Rab3B, Rab3C, and Rab3D (for review see Ref. 1). Rab3 proteins and rabphilin are localized to synaptic vesicles and dense core granules (2–5). Rabphilin has a Rab3A binding domain at the amino terminus of the protein and two C2 domains that bind to phospholipids in a Ca2+ dependent manner at the carboxyl terminus (6–9). Rabphilin localization on synaptic vesicles and granules suggests a role in regulated exocytosis of these vesicles. In PC12 cells, rabphilin overexpression increased by 30% regulated hormone secretion, whereas antisense expression of the protein decreases hormone release by 30% (10). Expression of rabphilin in insulin secreting cells enhanced by ~2-fold regulated release of insulin (11). These data indicate that rabphilin functions as a positive regulator of regulated exocytosis. Caenorhabditis elegans mutants with disrupted rabphilin function are severely lethargic in the absence of mechanical stimulation (12). Differently, mutant mice lacking rabphilin did not have obvious physiological impairments (13), indicating that other proteins can compensate for rabphilin function. Given the tight binding of rabphilin to members of the Rab3 family and the finding that both Rab3A and rabphilin dissociate from vesicles upon fusion (14), efforts have been spent to determine whether rabphilin and Rab3 effects on regulated granule release or synaptic transmission were related. However, the stimulatory effects of rabphilin on hormone release were independent of Rab3 function (15) and of association-disassociation cycles with membranes mediated by Rab3 (11). Inhibition of regulated exocytosis induced by Rab3 overexpression was also independent of a direct interaction with rabphilin (8). Both mice and C. elegans rabphilin mutants did not have the synaptic transmission defects generated by Rab3 mutants (13, 16). Therefore, Rabphilin may have functions that are not directly related to those of Rab3 and vice versa. Interestingly, neurons of mice lacking Rab3 proteins have less rabphilin than the controls. Moreover, in the mutant mice, targeting of Rabphilin to the synapse is impaired (13, 17). Thus, Rab3 function is important for rabphilin stability and targeting to the site of vesicle release. In addition to Rab3, rabphilin binds to another member of the Rab family of proteins, Rab27, which localizes to dense core granules and regulates hormone secretion (18–20). Rabphilin N terminal domain interacts with α-actinin (21, 22), a component of the actin cytoskeleton that cross-links actin filaments (23) and localizes to granules (24, 25). In endocrine cells granules are specifically accumulated in an F-actin-rich region below the plasma membrane (26, 27). Starting from these data, we hypothesized that rabphilin binds to the actin cytoskeleton in an α-actinin-dependent manner and thereby facilitates granule interactions with F-actin networks.

**EXPERIMENTAL PROCEDURES**

Materials—Enzymes for DNA modification and restriction enzymes were purchased from Promega (Madison, WI). The pEGFP-C1 vector was purchased from Clontech (Palo Alto, CA). pcDNA3.1(+), pIND, and pVGRXR vectors along with Ponasterone A, hygromycin B, and Lipofectamine were purchased from Invitrogen. Mouse monoclonal...
IgG anti-rabphilin antibody (clone 47) was purchased from BD Transduction Laboratories; anti-Myc rabbit polyclonal and mouse monoclonal antibodies, anti-actin rabbit polyclonal antibodies (H-300), and anti-Rab27a rabbit polyclonal antibodies (sc-22756) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal IgG anti-actin (clone MAB 1501) and anti-ACTH2 antibodies from Chemicon International, Inc. (Temecula, CA); mouse monoclonal IgM anti-actin from British BioCell International; enhanced chemiluminescence detection reagents (30). Brain cytosol was prepared by homogenizing one rat brain in 10 ml of Kglu buffer (20 mM Hepes, pH 7.4, 120 mM potassium glutamate, 20 mM potassium acetate, 5 mM EGTA, 1 mg/ml bovine serum albumin). The homogenate was centrifuged at 95,000 rpm for 1 h in the TL-100.2 rotor of the Beckman TLX centrifuge. The cytosol in the supernatant fraction was stored at −80°C.

Actin Polymerization—G-actin was stored at a concentration of 16 μM in G buffer containing 0.2 mM ATP, 0.2 mM CaCl₂, 0.5 mM 2-mercaptoethanol, 0.5 mM NaN₃, 20 mM Tris-HCl, pH 8.0 (29). G-actin was polymerized for 1 h at 30°C by adding one-tenth of the volume of 1 mM KCl, 20 mM MgCl₂. Cross-linking was induced by addition of 1 μM α-actinin, and samples were further incubated for 1 h at 30°C. To obtain Alexa-fluor 568-conjugated F-actin, Alexa-fluor 568-conjugated G-actin was polymerized as above and cross-linked by addition of 1 μM α-actinin.

Co-sedimentation of Rabphilin with F-actin—Recombinant glutathione S-transferase (GST) and rabphilin-GST were prepared by expression of pGEX-2T and pGEX-2T-rabphilin in Escherichia coli as previously described (30). Protein concentration was determined by using the BCA kit (Pierce). F-actin was incubated with and without 1 μM α-actinin in the presence or in the absence of different concentrations of rabphilin-GST for 1 h at 30°C. Rabphilin binding to F-actin was determined by high speed co-sedimentation assay. Samples were centrifuged at 100,000 × g for 30 min to pellet polymerized actin (31). The pellets and supernatants were collected, and one-tenth of the samples were loaded onto a 10% SDS-PAGE and analyzed by Western blot with antibodies against actin (MAB 1501) and against rabphilin. For Rab3A, Rab27, and α-actinin pull-down experiments, rabphilin-GST and GST were kept bound to the glutathione-Sepharose 4B beads (Amersham Biosciences) used for protein purification.

Cell Culture and Transfections—Neuro2A (N2A) and COS-7 cells were cultured in DMEM with 10% fetal bovine serum and penicillin/streptomycin. To transiently express rabphilin subcloned into the inducible vector pIND, COS-7 cells and N2A cells were co-transfected with rabphilin-pIND and pVGRXR or GFP-rabphilin-pIND and pVGRXR using Lipofectamine 2000 according to the manufacturer (Invitrogen) instructions. Approximately 24 h after transfection, cells were trypsinized and plated on coverslips. After 5 h cells were induced with 5 μM Ponasterone A to induce expression of rabphilin. For some experiments, N2A cells were treated with retinoic acid (10 μM from a 1 mM stock in Me₂SO) for 10 h to induce neurite formation. N2A cells were transiently transfected with POMC-pEGFP or Myc-Rab3A-pcB7 (4) 48 h before experiments. PC12 cells were cultured in DMEM with 10% horse serum and 5% fetal bovine serum. PC12 cells were transfected with Myc-Rab3B-pcDNA3 with Lipofectamine. Stably transfected colonies expressing Myc-Rab3B were selected by growth in the presence of hygromycin B.

Preparation of Dense Core Granules Derived from N2A Cells—N2A cells (two 10-cm diameter tissue culture dishes) were washed twice with Kglu buffer and scraped from plates in the same buffer. Cells were broken in 0.4 ml of Kglu buffer by passing them five times through a 27.5-gauge syringe needle, and the post-nuclear supernatants were centri-
Rabphilin and α-Actinin

**FIGURE 1. Rabphilin binding to F-actin is dependent on α-actinin and is saturable.** A, G actin was polymerized to F-actin as described under "Experimental Procedures" and incubated with and without α-actinin (1 μm) or rabphilin at the indicated concentrations. Samples were centrifuged at 100,000 × g for 30 min. Samples (1/10 of total) were loaded onto a 10% SDS-PAGE gel. Western blot analysis was done with anti-rabphilin (Rb) and anti-actin (monoclonal antibody 1501, 1:5,000) monoclonal antibodies. B, the amount of rabphilin that co-sediments with actin in the pellet at different concentrations of rabphilin added to the samples (band at 70 kDa) is shown in the graph. The data are the averages and standard deviations of three independent experiments, including the one shown in panel A. C, GST-rabphilin (GST-Rb) and GST immobilized onto Sepharose beads were incubated for 1 h at 4 °C with recombinant Rab3A at the indicated concentrations in A buffer (100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM GTP-γ-S, 5 μM ZnCl2, 1 mM dithiothreitol, and protease inhibitors) with 1 μM GTP-γ-S. The beads were washed three times with A buffer with 0.5% Triton X-100. The beads were re-suspended in sample buffer and loaded onto a 10% SDS-PAGE gel. Western blot analysis was analyzed with anti-Rab3 antibodies. D, beads were incubated without and with 10 μM Rab3A, washed three times (as described in panel C), and then mixed with 1 μM α-actinin in A buffer. After incubation for 1 h at 4 °C, the beads were washed three times, loaded onto SDS-PAGE gel, and analyzed with antibodies (H-300) against α-actinin. E, beads were preincubated with 10 μM α-actinin and washed as in panel D. Beads were then incubated for 1 h at 4 °C with rat brain cytosol (4 mg/ml) in A buffer with 1 μM GTP-γ-S, washed three times, and loaded onto the SDS-PAGE gel. The Western blot was probed with antibodies against Rab3 and Rab27. The experiments in panels C–E were done three times with similar results.

fuged at 7,200 × g for 10 min to obtain a pellet PII and a supernatant SII that contains cytosol and granule-containing fractions (32). SII was further centrifuged at 70,000 rpm for 30 min in the TLA 100.2 rotor of the Beckman TLX centrifuge to obtain the granule-containing pellet PIII and the supernatant SIII. Granules were re-suspended in GR buffer (0.1 ml) containing 100 mM KCl, 2 mM MgCl2, 10 mM Hepes pH 7.4, 1 mM GTP, and 1 mM ATP. Re-suspended granules were further centrifuged at 7,200 × g for 10 min to eliminate aggregates. The granules (40 μl) were mixed with F-actin (0.1 ml) preincubated in the presence or in the absence of α-actinin (1 μM) and rabphilin (2 μM) at 30 °C for 1 h. The mixture was incubated at 30 °C for 30 min and centrifuged at 7,200 × g for 10 min. Co-sedimentation of granules with cross-linked actin was measured by Western blot analysis of the samples.

For experiments where endosomes were loaded with human biotin-conjugated transferrin, N2A cells were washed with DMEM three times and then incubated with biotin transferrin in DMEM (5 μg/ml). After 1 h incubation at 37 °C, cells were incubated for 2 min in a mild acidic buffer (120 mM NaCl, 20 mM NaCl, pH 5.0) and then washed twice with phosphate-buffered saline to release surface bound transferrin as described by others (33, 34). Biotin-conjugated transferrin in cell fractions was detected by Western blot analysis using peroxidase-labeled streptavidin.

**Interaction of GFP-labeled Granules with Cross-linked F-actin**—For experiments where the interaction of GFP-labeled granules with cross-linked F-actin was visualized by fluorescence microscopy, granules from N2A cells transiently expressing POMC-GFP (two 10-cm diameter tissue culture dishes) were obtained as described in the section above. Resuspended granules in GR buffer (40 μl) were mixed with Alexafluor-F-actin (0.1 ml) that was preincubated in the presence or in the absence of α-actinin (1 μM) and rabphilin (2 μM). After incubation at 30 °C for 1 h, 1 ml of Antifade reagent in phosphate-buffered saline was added to 20 μl of the samples to prevent sample photobleaching. The mixture was then transferred onto a glass slide, covered with a coverslip, and observed by fluorescence microscopy.

**In Vivo Labeling of N2A Cell Mitochondria**—N2A cells plated on coverslips were washed twice with DMEM and incubated with 200 nM MitoTracker (diluted from a 0.2 mM stock solution in Me2SO). After 20-min incubation at 37 °C, cells were washed with DMEM twice and fixed for immunofluorescence with 3.7% formaldehyde solution in phosphate-buffered saline.

For the *in vitro* experiments where mitochondria were mixed with cross-linked F-actin, a crude cell fraction containing mitochondria labeled with MitoTracker (see below) was obtained from N2A post-nuclear supernatants as described (35). Briefly, the post-nuclear supernatant (0.4 ml) from a 10-cm plate incubated with 200 nM MitoTracker red as described above was centrifuged at 6,500 × g for 20 min in an Eppendorf microcentrifuge. Mitochondria in the pellet were re-suspended in 0.4 ml of GR buffer, and 40 μl of the mixture was added to cross-linked F-actin (100 μl). Samples were then incubated for 30 min at 30 °C. FITC-labeled phalloidin (1 μl) and Antifade reagent in phosphate-buffered saline (5 μl) were added to the mixture. Samples were...
Rabphilin and α-Actinin

RESULTS

Rabphilin Binds to F-actin in an α-Actinin-dependent Manner—It is unknown whether the interaction of rabphilin with α-actinin (21) leads to an association of rabphilin with F-actin. To determine whether this is the case, we investigated whether rabphilin binds to F-actin by using an in vitro assay with purified actin cytoskeleton components. Polymerization of G-actin generates actin filaments that sediment after high speed centrifugation (100,000 $\times$ g for 30 min), whereas addition of α-actinin induces formation of bundles and networks of F-actin (36) that sediment at lower speed (10,000 $\times$ g for 10 min) (37). We reasoned that binding of rabphilin to actin filaments, whether or not cross-linked with α-actinin, would lead to co-sedimentation of rabphilin and actin in the unknown whether the interaction of rabphilin with F-actin structures generated by mitochondria interaction with F-actin structures generated by and immunoelectron microscopy as described (28). Granule and mitochondrial interaction with F-actin structures generated by α-actinin were visualized by using an Olympus 1X71 inverted microscope equipped with argon laser (488 nm) and helium/neon laser (543 nm). Images were collected by using Photometrics CoolSnap HQ camera controlled by Metamorph. To measure clustering of granules within the actin structures, images of actin and granules were collected with the same exposure time (250 ms). Images were then analyzed using the “Image” software program from the National Institutes of Health. Merged images were separated into the red (actin) and blue (actinin) within areas of actin were measured and expressed as ratio granule area/actin area.

A

FIGURE 2. Rabphilin-GFP co-localizes with F-actin in N2A cells. A, post-nuclear supernatants derived from transfected N2A cells expressing GFP-rabphilin were centrifuged at 7,500 $\times$ g for 10 min to obtain a pellet PII and a supernatant SII. The supernatant SII was centrifuged at 70,000 rpm for 30 min in the Beckman 100.2 rotor to obtain a granule containing fraction (PIII) and a supernatant (SIII). Fraction PII (1/10 of total), PII (1/2 of total), and SIII (1/10 of total) were loaded onto an SDS-PAGE gel, and Western blots were done with the indicated antibodies. B–F, transfected N2A cells expressing Myc-Rab3A and GFP-rabphilin were treated with retinoic acid to stimulate formation of neurite-like processes. F-actin (red) was stained with Texas-red-phalloidin. Myc-tagged Rab3A on granules (blue) was visualized by staining with anti-Myc rabbit polyclonal antibodies and Cy5-conjugated anti-rabbit antibodies. Cells were visualized by confocal microscopy.

B

C

D

E

F

G

Immunofluorescence, Confocal Microscopy, and Immunoelectron Microscopy—Immunofluorescence staining was performed as described previously (28). Fluorescence microscopy was done using a 63× objective, on a Zeiss Axiophot fluorescence microscope. Confocal microscopy was performed with a Zeiss LSM 510 scanning laser confocal attachment mounted on a Zeiss Axiovert 100 TV inverted fluorescence microscope. Optical sections were 1-mm thick. Broken PC12 cells for immunoelectron microscopy were prepared for agarose embedding and immunoelectron microscopy as described (28). Granule and mitochondria interaction with F-actin structures generated by α-actinin were visualized by using an Olympus 1X71 inverted microscope equipped with argon laser (488 nm) and helium/neon laser (543 nm). Images were collected by using Photometrics CoolSnap HQ camera controlled by Metamorph. To measure clustering of granules within the actin structures, images of actin and granules were collected with the same exposure time (250 ms). Images were then analyzed using the “Image” software program from the National Institutes of Health. Merged images were separated into the red (actin) and green (granule) components. Images of actin and granules were processed using 200 pixels as an arbitrary threshold. The thresholded images were combined in a new image, and the areas of granule clusters (in black) within areas of actin were measured and expressed as ratio granule area/actin area.

A

B

C

D

Exogenous Rab3B and endogenous rabphilin localize to dense core granules morphologically docked to the plasma membrane. A, PC12 post-nuclear supernatants of untransfected PC12 cells and PC12 cells stably expressing Myc-Rab3B (clone 3BW1, clone 3BW2, and clone 3BW1) were loaded onto an SDS-PAGE gel. The Western blot was probed with mouse monoclonal antibodies against Myc to visualize Myc-tagged Rab3B. B, PC12 post-nuclear supernatants (0.4 ml) from untransfected PC12 cells and PC12 cells expressing Myc-Rab3B (clone 3BW1) were centrifuged at 90,000 rpm for 1 h in the MLA-130 rotor of the Beckman TL Optima MAX-E centrifuge. The pellet was resuspended in 0.4 ml of homogenization buffer, and equal volumes of supernatant and pellet were loaded onto the SDS-PAGE gel. The Western blot was probed with monoclonal antibodies against rabphilin. C, Immunogold localization of broken PC12 cells expressing Myc-Rab3B. Samples were labeled with mouse monoclonal IgG against Myc, the secondary staining was done with 5 nm colloidal gold-conjugated anti-mouse IgG. Bar, 100 nm. The inset shows the region highlighted by the asterisk at a 2× magnification. D, samples were labeled with mouse monoclonal IgG against rabphilin, the secondary staining was done with 5 nm colloidal gold-conjugated anti-mouse IgG. Arrows in B indicate rabphilin staining on dense core granules; the arrowhead in the inset (2× magnification) indicates a filament tethering a granule to the plasma membrane.

FIGURE 3. Exogenous Rab3B and endogenous rabphilin localize to dense core granules morphologically docked to the plasma membrane. A, PC12 post-nuclear supernatants of untransfected PC12 cells and PC12 cells stably expressing Myc-Rab3B (clone 3BW1, clone 3BW2, and clone 3BW1) were loaded onto an SDS-PAGE gel. The Western blot was probed with mouse monoclonal antibodies against Myc to visualize Myc-tagged Rab3B. B, PC12 post-nuclear supernatants (0.4 ml) from untransfected PC12 cells and PC12 cells expressing Myc-Rab3B (clone 3BW1) were centrifuged at 90,000 rpm for 1 h in the MLA-130 rotor of the Beckman TL Optima MAX-E centrifuge. The pellet was resuspended in 0.4 ml of homogenization buffer, and equal volumes of supernatant and pellet were loaded onto the SDS-PAGE gel. The Western blot was probed with monoclonal antibodies against rabphilin. C, Immunogold localization of broken PC12 cells expressing Myc-Rab3B. Samples were labeled with mouse monoclonal IgG against Myc, the secondary staining was done with 5 nm colloidal gold-conjugated anti-mouse IgG. Bar, 100 nm. The inset shows the region highlighted by the asterisk at a 2× magnification. D, samples were labeled with mouse monoclonal IgG against rabphilin, the secondary staining was done with 5 nm colloidal gold-conjugated anti-mouse IgG. Arrows in B indicate rabphilin staining on dense core granules; the arrowhead in the inset (2× magnification) indicates a filament tethering a granule to the plasma membrane.
Rabphilin and α-Actinin

FIGURE 4. Rabphilin associates with actin filaments in PC12 cells. A–C, broken PC12 cells expressing Rab3B were labeled with mouse monoclonal IgG against rabphilin, the secondary staining was done with 5 nm colloidal gold-conjugated anti-mouse IgG. In B the plasma membrane is indicated by arrowheads. In C a cell junction is indicated by the arrow, and a granule by an asterisk. Bar, 100 nm. D, the graph shows the average number of rabphilin gold particle/μm and standard deviations. For the quantification, five images were obtained from four independent experiments. The criterion for choosing the image was the presence of detectable actin filaments and plasma membranes in the field. Data were collected by measuring the number of gold particles within 20 nm from either the plasma membrane (outer side) or the actin filaments (filament width < 5–10 nm). The total lengths of plasma membrane and filament examined were 9.39 μm and 11.4 μm, respectively.

Rabphilin Localizes Both to the Actin Cell Cytoskeleton and to the Granules—The experiments described above suggest that rabphilin may bind to the cell actin cytoskeleton in addition to its established localizations in the cytosol and on the surface of dense core granules or synaptic vesicle (10, 14, 15, 30, 40). However, rabphilin localization to cell actin filaments has not been described. Neuroendocrine N2A cells (41) do not express endogenous rabphilin (not shown). In transfected N2A cells, rabphilin-GFP appeared as a doublet of ~100 kDa (Fig. 2A). Most of the cell rabphilin (~60%) was recovered in the supernatant (SIII) after high speed centrifugation (70,000 rpm), indicating its cytosolic localization. A fraction of cell rabphilin (20%) was found in the pellet PIII that also contains dense core granules (32). Another fraction of cell rabphilin was found in a low speed pellet (PII) where most of the plasma membrane marker Na+/K+-ATPase and 50% of the cell actin were also recovered. To determine whether rabphilin localized to the actin cytoskeleton, N2A cells transiently expressing Myc-tagged Rab3A were incubated with 10 μM retinoic acid (42). In these conditions, 30% of N2A cells formed neurite-like processes that were at least 3-fold longer than the cell body. We have shown that endogenous and Myc-tagged Rab3A localizes to dense core granules (4, 28). In the N2A cells, Rab3A fluorescence appeared as discrete puncta concentrated in the cell neurite processes, consistent with Rab3A granule localization. At the neurite margins there were spots of F-actin accumulation where Rab3A-labeled granules were excluded (Fig. 2, C–F, arrowheads). Differentially, rabphilin-GFP co-localized with F-actin at these spots. Rabphilin staining was also concentrated at zones with less F-actin that had accumulated granules (Fig. 2, C–F, arrows). In conclusion, these data indicate that exogenous rabphilin associates with actin filaments and granules in the N2A cells.
To determine the cell distribution of endogenous rabphilin, we used undifferentiated PC12 cells where granules are accumulated at the cell cortex (4, 27, 43). Because of the close spatial relationship of plasma membrane, granules, and actin network in PC12 cells, we analyzed rabphilin distribution by immunoelectron microscopy. It has been reported that expression of exogenous Rab3B in PC12 cells reorganizes the actin cytoskeleton with induction of filopodia (44) and increases the amount of rabphilin associated with the particulate cell fraction (45). In agreement with these data, the amount of endogenous rabphilin found in a high speed centrifugation pellet was higher when cell homogenates were derived from PC12 cells stably expressing exogenous Rab3B than from the control cells (Fig. 3, A and B). Thus, PC12 cells overexpressing Rab3B may have more actin cytoskeleton and associated rabphilin than parental PC12 cells and were therefore chosen for ultrastructural analysis. Immunoelectron microscopy showed that Rab3B is associated to granules docked to the plasma membrane (Fig. 3C) in agreement with another report (45) and as previously shown also for Rab3A, Rab3D, and Rab3C (3, 4, 28, 45). Rabphilin immunogold labeling was found on ~30% of the granule docked to the plasma membrane (200 docked granules examined, 2 independent experiments). Granules did not have associated gold particles when the staining was done only with the secondary antibody. Thus, granules positioned at the cell cortex have both Rab3 and rabphilin associated to their surface. A fraction of docked granules (at least 5%, 300 granules observed) were linked to the plasma membrane by filaments (Fig. 3D, arrowhead).

Rabphilin, in addition to its granule localization, was associated to thin filaments of 5- to 10-nm diameter in the proximity of the plasma membrane (Fig. 4). Rabphilin staining on the filaments, as well as that on the granules, is specific, because almost no staining was detected on the outer surface of the plasma membrane (Fig. 4, B–D) or on mitochondria (not shown). Thus, immunoelectron microscopy shows that endogenous rabphilin associates to the actin cytoskeleton in PC12 cells overexpressing Rab3B.

In transfected COS-7 cells, exogenous rabphilin and rabphilin-GFP had a diffuse fluorescence distribution that was concentrated at the cell body (Fig. 5), consistent with the finding that >50% of the protein is in the cytosol (data not shown). Rabphilin co-localization with F-actin and with α-actinin was found at the margins of flat processes (arrows). The co-localization of rabphilin with F-actin or α-actinin was detectable in ~25% of the rabphilin-transfected cells (100 cells examined). This indicates that rabphilin binds to the actin cytoskeleton in unspecialized cells.

Rabphilin Stimulates in Vitro Granule Association to F-actin Cross-linked by α-Actinin—Endocrine cells maintain a population of granules embedded within the F-actin network the cell cortex (26, 27, 46). Because rabphilin binds to α-actinin/F-actin, these interactions may
Rabphilin and α-Actinin

After centrifugation of the granule-containing supernatant SII at high speed (70,000 rpm, see Fig. 2A), both Syt1, a component of the granule membrane, and a fraction of endocytosed biotin-transferrin were recovered in the pellet PIII (not shown). Thus, PIII contains both granules and transferrin-labeled endosomes. When the re-suspended pellet PIII was incubated with cross-linked actin and re-centrifuged at low speed, a fraction (~30% of total) of Syt1 was found in the pellet P (Fig. 6, D and E), indicating that granules associate to the actin structures generated by α-actinin. When granules were added to a sample containing α-actinin, but no F-actin, the amount of granules in the pellet P was the same as in control samples with granules, F-actin, but no α-actinin (Fig. 6F). This shows that sedimentation of granule after low speed centrifugation is not the result of α-actinin-induced aggregation of the vesicles. Unlike Syt1, biotin-transferrin remained in the supernatant S whether or not actin filaments were cross-linked by α-actinin (Fig. 6D). This indicates that the population of endosomes in PIII, unlike the granules, did not associate with the cross-linked F-actin structures. Therefore, sedimentation of granules with the actin aggregates generated by α-actinin is specific for these vesicles. Electron microscopy of pellets containing granules and cross-linked actin (Fig. 6G) showed that >90% of granules (100 granules observed from 3 independent experiments) were found in the proximity (within 50 nm) of actin filaments, rather than distributed randomly. This indicates that granules co-sediment at low speed with the F-actin aggregates generated by α-actinin, because they bind to the filaments. Rabphilin (2 μM) induced an 80% increase of granule association with cross-linked actin as compared with similar samples treated without rabphilin (Fig. 6, D and E). Conversely, addition of rabphilin did not increase the amount of actin recovered in the pellet after centrifugation. Thus, the increased association of granules with
F-actin in the presence of rabphilin is not dependent upon an increased number or size of the actin aggregates in the pellet, but rather reflects an increased efficiency of granule interaction with the cross-linked F-actin. The co-sedimentation experiments described above together with the electron microscopy analysis of the granules and actin filaments in the low speed pellets indicate that granules bind to F-actin cross-linked by α-actinin. To visualize by fluorescence microscopy binding of granules to F-actin cross-linked by αH19251-actinin, we created a GFP-tagged hormone that localizes to dense core granules. When N2A cells express the exogenous ACTH precursor pro-opiomelanocortin (POMC), the precursor is converted to active hormones that are stored in granules and released in a regulated manner (41). To target GFP to the granules, N2A cells were transiently transfected with a plasmid, POMC-pEGFP, containing the cDNA of POMC linked to GFP. Transfected cells expressed a 61-kDa protein (Fig. 7A) that was detected with both anti-ACTH and anti-GFP antibodies and therefore corresponds to a fusion protein containing POMC (31 kDa) and GFP (30 kDa). In the transfected cells, another 48-kDa band was detected with both anti-ACTH and anti-GFP antibodies, indicating that POMC-GFP is cleaved to a smaller peptide. Unlike the 61-kDa POMC-GFP, most of the 48-kDa peptide fractionated with the granule-containing fraction PIII (32). In N2A cells transfected with POMC-GFP, the GFP fluorescence accumulated at the tips of the processes where it co-localized with overexpressed Rab3A (Fig. 7B) that is associated to the granule membrane (4). In conclusion, the data show that POMC-GFP precursor is processed to a cleaved product that is targeted to granules.

FIGURE 8. Rabphilin induces in vitro clustering of granules within regions of elevated actin concentration. A–F, granules derived from cells expressing POMC-GFP were incubated with Alexa-fluor 568-conjugated F-actin cross-linked by α-actinin in the absence (A–C) and in the presence (D–F) of 2 μM rabphilin (Rb). Samples were directly visualized by fluorescence microscopy. Actin (red fluorescence, A and D), granules (green fluorescence, B and E), and merged images (C and F) are shown. Bars in A and D, 10 μm. G and H, the regions indicated by the arrows in the merged images (C and F) are shown at a 3× magnification (granules, green fluorescence) in G and H, respectively. I–J, merged images shown in C and F were split into the red (actin) and green (granule) channels, thresholded, and merged again together as described under “Experimental Procedures” by using the Image J program from the National Institutes of Health. The areas of F-actin at elevated concentration are represented in blue. The areas of granule accumulation that do or do not overlap with F-actin at elevated concentration are represented in black and in red (arrow), respectively. K, the ratio, area of granule accumulation (in black, panels I and J):area of F-actin at elevated concentration (in blue, panels I and J), was derived from six thresholded images from three independent experiments. Data in the graph are expressed as averages and standard deviations. The total areas of cross-linked F-actin at elevated concentration analyzed for these experiments were 488 and 424 μm² for samples treated with and without rabphilin, respectively.
When granules labeled with POMC-GFP were mixed with cross-linked actin, they appeared as fluorescent puncta co-localized with the cross-linked structures created by α-actinin (Fig. 8, A–C). These experiments show that POMC-GFP-labeled granules bind to the cross-linked actin structures created by α-actinin. In the presence of rabphilin, granules formed larger accumulations within zones of elevated actin concentration (Fig. 8, D–F, and, at higher magnification, G and H). Rabphilin increased the area of granules clustered ~8-fold within zones of concentrated actin as compared with control samples without the protein (Fig. 8, I–K).

In PC12 cells, granules are embedded in an actin-rich region below the plasma membrane (27, 43). Similarly, in round N2A cells, granules are accumulated in a cortical actin-rich region at the base of numerous filopodia (Fig. 9, D–F). Both in N2A and PC12 cells (Fig. 9G) mitochondria are more centrally located than the granules. We reasoned that, if the granule association to cross-linked actin observed in vitro is specific for these organelles, then mitochondria may be excluded from these structures. To determine whether this is the case, mitochondria derived from cells labeled with MitoTracker were mixed with cross-linked actin in vitro and observed by fluorescence microscopy. The actin aggregates did not have co-localized mitochondria (Fig. 9H). When cross-linked actin and mitochondria were found in close vicinity, there was virtually no overlap of the mitochondria and F-actin fluorescence (Fig. 9H, inset). Thus, mitochondria, unlike granules, are excluded from F-actin networks generated by α-actinin.

DISCUSSION

Rabphilin binds α-actinin (21), a cell cytoskeleton component that cross-links F-actin into bundles and networks. Here, we have extended this observation by showing that in vitro binding of rabphilin to α-actinin leads to its association with F-actin. This observation prompted us to further study rabphilin distribution in endocrine cells where the protein localizes to dense core granules and to the cytosol (10, 15, 30). Immunofluorescence localization of rabphilin to the actin cytoskeleton in round endocrine cells is difficult to determine, because granules with rabphilin on their surface are embedded in the cortical actin network (27, 46). Therefore, we induced N2A cells to extend long neurites where by confocal microscopy we could discriminate between zones of F-actin and granule accumulation at the margins of the processes. By using this approach we found that exogenous rabphilin co-localizes with zones of F-actin that do not have granules. Immunoelectron microscopy of broken PC12 shows that endogenous rabphilin localizes to F-actin and to the granule membrane. In conclusion it is found that rabphilin associates to the actin cytoskeleton in addition to its established localization to the granule surface and in the cytosol.

It has been reported that α-actinin, in addition to cross-linked F-actin, binds to the membrane of dense core granule membrane (24, 25). It is possible that rabphilin/α-actinin association facilitates granule binding to networks of F-actin created by α-actinin. To explore this possibility, we used an in vitro approach with purified cytoskeleton components. Cosedimentation, electron microscopy and immunofluorescence assays show that granules, but not endosomes or mitochondria, associate to cross-linked actin structures generated by α-actinin in vitro. When cross-linked F-actin networks were formed in the presence of rabphilin, more granules associated with these structures and granules co-localized more efficiently with zones of elevated F-actin concentration. On the basis of these data, it is concluded that granules bind specifically to F-actin cross-linked by α-actinin and that rabphilin stimulates these interactions. It is possible that α-actinin, which exists as a divalent homodimer (23), may bind on one end to the granule membrane and
with the other end to the actin filament. This function of α-actinin as a connection between the granule membrane and F-actin may be similar to other activities of α-actinin, such as anchoring actin filaments to the plasma membrane (47, 48). In agreement with data from other groups, we find that rabphilin binds both to Rab3A and Rab27 (reviewed in Refs. 1 and 39). Differently from another report (21), we found that Rab3A did not inhibit the interaction of rabphilin with α-actinin \textit{in vitro}, nor did α-actinin inhibit Rab3A or Rab27 binding to rabphilin. Thus, our data support a model whereby activated Rab3A or Rab27 on the granule membrane bind to both rabphilin and α-actinin. Such complexes could function to connect the granule to the cortical actin cytoskeleton. In agreement with this, we have found that increased Rab3A on the granule surface enhances their positioning at the cell cortex in PC12 cells (4).

As mentioned earlier, it has been proposed that α-actinin connects granule membrane to the actin cytoskeleton (for review see Ref. 53), also bind to Rab3A (54, 55), raising the possibility thatrabphilin binding to F-actin requires a -actinin-dependent tethering of granules to actin filaments that are directly anchored to the plasma membrane. Such a tether between the granule and the plasma membrane may facilitate subsequent docking/fusion reactions even without dissociation of the vesicle from F-actin. In this respect, we have found that at least some of the granules are clearly connected to the plasma membrane by filaments (Fig. 3). More work is necessary to understand whether such filaments include rabphilin, α-actinin, and actin.

We have shown that α-actinin binds to rabphilin and that interaction of rabphilin with F-actin is α-actinin-dependent. There are four different members of the α-actinin family expressed in mammalian cells (51), skeletal muscle α-actinin-3 and α-actinin-2, which is also expressed in brain (52), and non-muscle α-actinin-1 and -4. In the future it will be interesting to determine whether rabphilin binding to F-actin requires a specific member of the α-actinin family and whether a specific isoform of the α-actinin family localizes to the granule membrane.

Interestingly, it has been recently proposed that synapsins, a family of phosphoproteins that bind both to synaptic vesicles and to the actin cytoskeleton (for review see Ref. 53), also bind to Rab3A (54, 55), raising the possibility that rabphilin/α-actinin and synapsins have similar functions. Other Rab3-binding proteins that are expressed in endocrine cells may also interact with actin cytoskeleton components to regulate granule interactions at the cell cortex. For example, Noc2 (reviewed in Ref. 56), a Rab3-binding protein involved in granule release (57, 58), has been reported to interact with Zyxin, a cytoskeleton component that binds to α-actinin (59).

In conclusion, in this report we have shown that rabphilin binds to the actin cytoskeleton and stimulates \textit{in vitro} binding of granules to cross-linked F-actin structures generated by α-actinin. More work is necessary to understand the role of rabphilin/α-actinin and other Rab3-binding proteins in the positioning of vesicles within F-actin networks.

Acknowledgments—We thank Dr. Brian Storrie for critically reading the manuscript and for helpful discussions. We thank Dr. D. Koticha for preparing the POMC-pEGFP plasmid, Dr. P. De Camilli for rabphilin-pDNAs, Dr. G. Thomas for POMC-pS65, Dr. R. Regazzi for Mγc-tagged Rab3B cDNA, and Dr. F. Bartolini and Dr. J. Schnorrer for help with fluorescence microscopy. We thank Sudhindra Swamy and Theresa Swyney of the Confocal Microscopy Facility of the Herbert Irving Comprehensive Cancer Center at the Columbia Presbyterian Medical Center for helping us with the confocal microscope.

REFERENCES

1. Gonzalez, L. Jr., and Scheller, R. H. (1999) \textit{J. Cell. Biol.} 146, 755–758
2. Li, C., Takei, K., Geppert, M., Danliel, L., Stennius, K., Chapman, E. R., Jahn, R., De Camilli, P., and Sudhof, T. C. (1994) \textit{Neuron} 13, 885–889
3. Iezzi, M., Escher, G., Meda, P., Wollheim, C. B., and Takai, Y. (1995) \textit{Cell} 80, 372–381
4. Stahl, B., Chou, J. H., Li, C., Sudhof, T. C., and Jahn, R. (1996) \textit{J. Biol. Chem.} 271, 20993–21002
5. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
6. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
7. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
8. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
9. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
10. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
11. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
12. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
13. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
14. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
15. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
16. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
17. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
18. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
19. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
20. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
21. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
22. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
23. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
24. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
25. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
26. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
27. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
28. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
29. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
30. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
31. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
32. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
33. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
34. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
35. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
36. Nakata, T., and Hirokawa, N. (1992) \textit{J. Cell Biol.} 115, 270, 283–290
36. Pelletier, O., Pokidysheva, E., Hirst, L. S., Bouxxsein, N., Li, Y., and Safinya, C. R. (2003) Phys. Rev. Lett. 91, 148102
37. Coghill, I. D., Brown, S., Cottle, D. L., McGrath, M. J., Robinson, P. A., Nandurkar, H. H., Dyson, J. M., and Mitchell, C. A. (2003) J. Biol. Chem. 278, 24139–24152
38. Shirataki, H., Kibauchi, K., Sakoda, T., Ishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993) Mol. Cell Biol. 13, 2061–2068
39. Fukuda, M. (2005) J. Biol. Chem. 270, 26868–26875
40. Lang, T., Wacker, I., Wunderlich, I., Rohrbach, A., Giese, G., Soldati, T., and Almers, W. (2000) Biophys. J. 78, 2863–2877
41. Sunshine, C., Francis, S., and Kirk, K. L. (2000) Exp. Cell Res. 257, 1–10
42. Weber, E., Illing, T., and Kirk, K. L. (1996) J. Biol. Chem. 271, 6963–6971
43. Johns, L. M., Levitan, E. S., Shelden, E. A., Holz, R. W., and Axelrod, D. (2001) J. Cell Biol. 153, 177–190
44. Otey, C. A., Paralko, F. M., and Burridge, K. (1990) J. Cell Biol. 111, 721–729
45. Wachsstock, D. H., Wilkins, J. A., and Lin, S. (1987) Biochem. Biophys. Res. Commun. 146, 554–560
46. Bader, M. F., Doussau, F., Chasserot-Golaz, S., Vitale, N., and Gasman, S. (2004) Biochim. Biophys. Acta 1742, 37–49
47. Gasman, S., Chasserot-Golaz, S., Malacome, M., Way, M., and Bader, M. F. (2004) Mol. Biol. Cell 15, 520–531
48. Otey, C. A., and Carpen, O. (2004) Cell Motil Cytoskeleton 58, 104–111
49. Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M., and Sheng, M. (1997) Nature 385, 439–442
50. Green, P., Valtorta, F., Cesareni, G., and Benfenati, F. (1993) Science 259, 780–785
51. Giovedi, S., Vaccaro, P., Valtorta, F., Darchen, F., Greengard, P., Cesareni, G., and Benfenati, F. (2004) J. Biol. Chem. 279, 43760–43768
52. Giovani, S., Darchen, F., Valtorta, F., Greengard, P., and Benfenati, F. (2004) J. Biol. Chem. 279, 43769–43779
53. Cheviet, S., Walsle, L., and Regazzi, R. (2004) Trends Cell Biol. 14, 525–528
54. Haynes, L. P., Evans, G. J., Morgan, A., and Burgoyne, R. D. (2001) J. Biol. Chem. 276, 9726–9732
55. Manabe, S., Nishimura, N., Yamamoto, Y., Kitamura, H., Morimoto, S., Inai, M., Nagahiro, S., Seino, S., and Sasaki, T. (2004) Biochem. Biophys. Res. Commun. 316, 218–225
56. Crawford, A. W., Michelsen, J. W., and Beckerle, M. C. (1992) J. Cell Biol. 116, 1381–1393

Rabphilin and α-Actinin