The Rab6 GTPase Regulates Recruitment of the Dynactin Complex to Golgi Membranes

Benjamin Short, Christian Preisinger,
Julia Schaletzky, Robert Kopajtich,
and Francis A. Barr[1]
Department of Cell Biology
Max-Planck-Institute of Biochemistry
Am Klopferspitz 18a
Martinsried 82152
Germany

Summary

Dynactin is a multisubunit protein complex required for the activity of dynein in diverse intracellular motility processes, including membrane transport [1–3]. Dynactin can bind to vesicles and liposomes containing acidic phospholipids [4], but general properties such as this are unlikely to explain the regulated recruitment of dynactin to specific sites on organelle membranes [5]. Additional factors must therefore exist to control this process. Candidates for these factors are the Rab GTPases, which function in the tethering of vesicles to their target organelle prior to membrane fusion [6]. In particular, Rab27a tethers melanosomes to the actin cytoskeleton [7–9]. Other Rabs have been implicated in microtubule-dependent organelle motility; Rab7 controls lysosomal transport, and Rab6 is involved in microtubule-dependent transport pathways through the Golgi and from endosomes to the Golgi [10–16]. We demonstrate that dynactin binds to Rab6 and shows a Rab6-dependent recruitment to Golgi membranes. Other Golgi Rabs do not bind to dynactin and are unable to support its recruitment to membranes. Rab6 therefore functions as a specificity or tethering factor controlling the recruitment of dynactin to membranes.

Results and Discussion

A number of different effectors have been described for mammalian Rab6 and its yeast counterpart, Ypt6 [12, 15, 17–19]. However, because none of these effectors explains the function of Rab6 in microtubule-dependent motility of Golgi-associated vesicles in interphase cells, we decided to screen for novel Rab6-interacting partners by using a biochemical assay [20]. Rab6 binding assays identified the p150[glued] subunit of the dynactin complex and the *trans*-Golgi network-localized dynactin-interacting protein bicaudal D2 (BICD2) [21], as preferential interactors of activated Rab6 (Figure 1A; Table S1 in the Supplementary Material available with this article online). The Rab escort protein REP-1 and the Rab6 binding protein R6IP2 were also found, although neither showed any significantly enhanced interaction with the active form of Rab6 [15]. The presence of the Rab-guanine nucleotide dissociation inhibitor (GDI) bands hindered the identification of dynactin subunits in the 50–60 kDa region of the gel by mass spectrometry,

[1]Correspondence: barr@biochem.mpg.de

and we therefore switched to Western blot analysis. Western blotting of Rab6 binding assays revealed that p150[glued] and another subunit of the dynactin complex p50-dynamitin were present, whereas tubulin and the dynactin-interacting motor protein dynein were absent (Figures 1B and 1C). Of a variety of Golgi Rabs tested, only Rab6 was able to bind the dynactin complex identified by p150[glued] and p50-dynamitin (Figure 1C). Under the same conditions, the Rab1 effector p115 [22] bound specifically to activated Rab1 (Figure 1C) and thereby validated the approach. Rab6 is therefore a specific dynactin complex binding partner that may be involved in its recruitment to Golgi membranes.

Our next step was to investigate which subunits of the dynactin complex were interacting with Rab6. A directed two-hybrid analysis with various components of the dynactin complex revealed that p150[glued] and BICD2 interacted with activated Rab6Q72L and to a lesser extent with the wild-type protein (Figure 2A). BICD1, a BICD2-related protein, showed the same interactions with activated Rab6Q72L and wild-type Rab6 as BICD2 (Figure 2A). The specificity of these interactions was confirmed by the observation that GDP-locked, and thus inactive, Rab6T27N showed no interaction with p150[glued], BICD1, or BICD2 (Figure 2A). We could also show that activated Rab6 binds directly to BICD2; this interaction can be reconstituted with only the purified proteins (Figure 2B). However, we were unable to test p150[glued] and BICD1 by using this assay because it was not possible to express soluble full-length recombinant proteins. Rab1 was used as a control in these experiments and showed an interaction with p115 as expected, but not with the other proteins tested (Figures 2A and 2B). BICD2 could therefore function as an adaptor between activated Rab6 on Golgi membranes and the dynactin complex. In addition, activated Rab6 may make interactions directly with the dynactin complex via the p150[glued] subunit.

We then addressed whether the interaction of Rab6 with BICD2 and p150[glued] can mediate the recruitment of dynactin to Golgi membranes. Prenylated Rab1 and Rab6 in the GTP form were first bound to a fixed amount of Golgi membranes (Figure 3A). The ability of these membranes to recruit either the Rab1 effector p115 or dynactin from cytosol was then tested (Figure 3B). These binding assays were performed in the presence of high salt to reduce the interaction of p115 with its receptor GM130 and thus enable visualization of the Rab1-dependent recruitment of p115. Golgi membranes loaded with Rab1 were able to recruit p115 but not the dynactin complex, whereas Rab6-loaded membranes were able to recruit the dynactin p150[glued] subunit but not p115 (Figure 3B). This binding required the presence of the respective Rab proteins because only low levels of p115 and dynactin were recruited to Golgi membranes pretreated with buffer (Figure 3B, RLG+cytosol). Dynactin is therefore specifically recruited to Golgi membranes in a Rab6-dependent fashion in vitro.

Having demonstrated a biochemical interaction between Rab6 and dynactin in vitro, we asked if Rab6 has any effect on dynactin localization in living cells. We
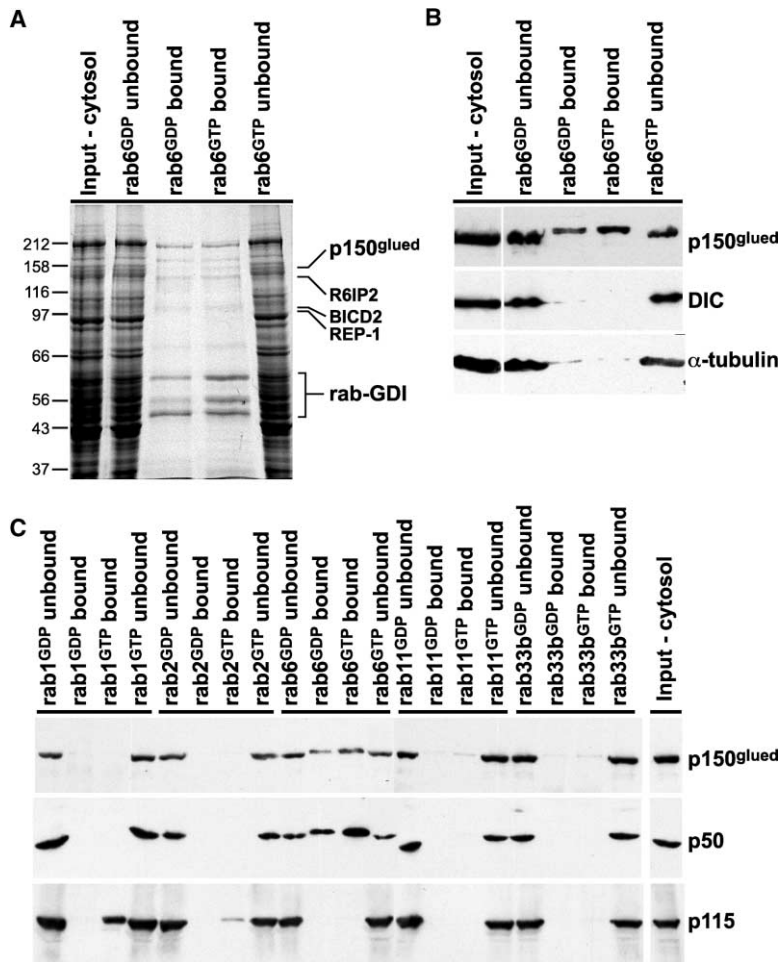
A

B

C

Figure 1. The Dynactin Complex Is a Specific Binding Partner for Rab6

(A) Rab6 beads loaded with either GDP or a nonhydrolyzable analog of GTP were incubated with a cytosolic extract of rat liver, and the specifically eluted proteins were analyzed by SDS-PAGE. A representative Coomassie blue-stained gel is shown. Rab6-interacting protein 2 (R6IP2), p150^{glued}, bicaudal D2 (BICD2), Rab escort protein (REP), and Rab-GDI are marked.

(B) Western blot analysis of proteins specifically eluted from Rab6-GDP and Rab6-GTP beads with antibodies to p150^{glued}, dynein light intermediate chain (DIC), and α-tubulin.

(C) Rab 1, 2, 6, 11, and 33b beads loaded with either GDP or a nonhydrolyzable analog of GTP were incubated as above, and the specifically eluted proteins were analyzed by Western blotting for dynactin subunits p150^{glued}, p50-dynamitin, and the Rab1 effector p115.

found that, consistent with published data, dynactin is predominantly localized to microtubule plus ends, and it is thus not possible to directly detect the pool associated with Golgi membranes. To circumvent this problem, we decided to depolymerise the microtubules with cold treatment prior to fixation, a procedure having little effect on the Golgi apparatus (Supplementary Material, Figure S1). Using these conditions, we were then able to ask if increasing cellular levels of Rab6 by transfection resulted in the recruitment of dynactin to Rab6-positive membrane structures. A clear recruitment of the dynactin complex to vesicular and tubular structures can be observed in Rab6-transfected cells, in contrast to adjacent nontransfected cells (Figure 3C). There is no obvious difference in the distribution of the Rab1 effector p115 between non-transfected and Rab6-transfected cells, and the merged image clearly demonstrates that the two proteins localize to different regions of the Golgi (Figure 3D). Rab6 can therefore enhance the specific recruitment of the dynactin complex to membranes in living cells as well as in vitro.

Our results provide a molecular basis for understanding the previously reported function of Rab6 in microtubule-dependent motility of some Golgi-associated vesicles. We have shown that Rab6 directly interacts with BICD2, which in turn is known to associate with the p50 subunit of the dynactin complex [21]. Rab6 also displays an activation state-dependent interaction with the

p150^{glued} subunit of dynactin and the BICD2-related protein BICD1 in the yeast two-hybrid system. We therefore propose a model whereby Rab6 regulates the recruitment of the dynactin complex, and thus the dynein motor, to vesicle structures or tubules (Figure 4). Our data suggest that specific dynactin recruitment involves multiple interactions between core components, such as p150^{glued}, of the dynactin complex and organelle-specific accessory subunits such as BICD2 and, potentially, BICD1, which is also a Golgi-localized protein (Supplementary Material, Figure S2). This may explain observations that blocking dynactin function with p50-dynamitin overexpression disrupts the distribution and motility of Golgi structures [23]. Interestingly, it is known that the motility of rhodopsin-containing Golgi-derived vesicles in photoreceptors involves the dynein motor complex and that Rab6 is associated with and regulates the transport of these vesicles [24, 25]. It is thought that dynein binds directly to these vesicles by a novel light chain [5], but this does not exclude a role for the Rab6 and dynactin interactions reported here. Together, these findings indicate that some Rab proteins control selective vesicle or organelle motility on microtubules by regulating the recruitment of the dynactin complex to the membrane surface.

Supplementary Material
Details about the methods used plus additional data on the mass-spectrometric identification of Rab6 binding proteins, the effects of
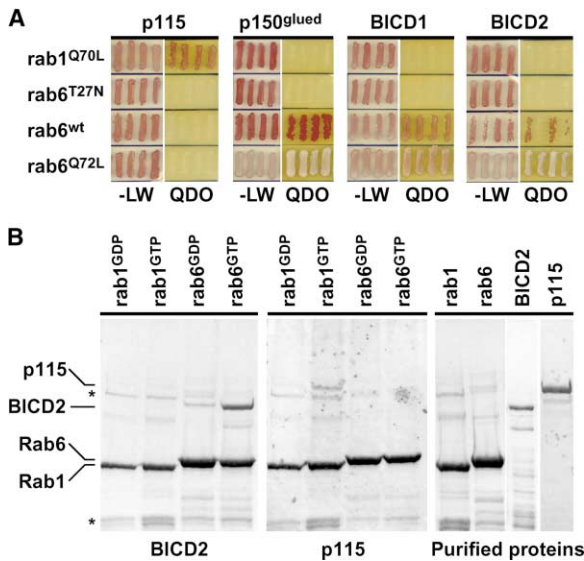
A

B

Figure 2. Rab6 Interacts with BICD2 and p150glued

(A) Rab bait constructs were tested against the indicated prey constructs in the yeast two-hybrid system for the ability to grow on selective medium (QDO), compared to nonselective medium (-LW). Lighter colony color on QDO is an indicator of a strong interaction. (B) The ability of BICD2 and p115 to bind Rab1 and Rab6 was tested with recombinant proteins. A Coomassie blue-stained gel is shown. The asterisks indicate contaminations in the recombinant Rab proteins that migrate slightly higher than purified BICD2.
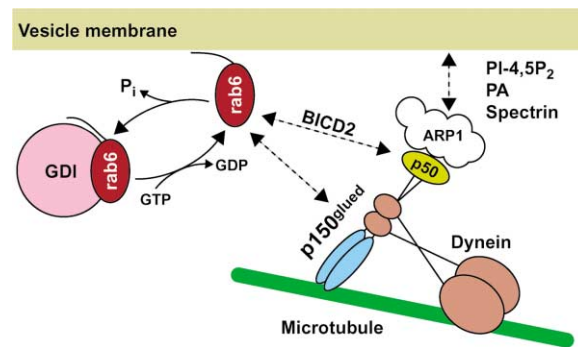

Figure 4. Recruiting the Dynein-Dynactin Complex to Specific Sites on Vesicle Membranes

Acidic phospholipids such as phosphatidyl-4,5 bisphosphate (PI-4,5P$_2$) and phosphatidic acid (PA) act in conjunction with some isoforms of spectrin to mediate attachment of dynactin to axonal vesicles. Rab6 recruitment to Golgi membranes is governed by a cycle of GTP binding and hydrolysis. When in the GTP form and associated with the membrane surface, Rab6 recruits dynactin via interactions with BICD2 and p150glued (dotted lines). This model is modified from Figure 7 of reference [4].

cold-treatment on the Golgi apparatus, and the localization of BICD proteins are available with this article online at http://images.cellpress.com/supmat/supmatin.htm.
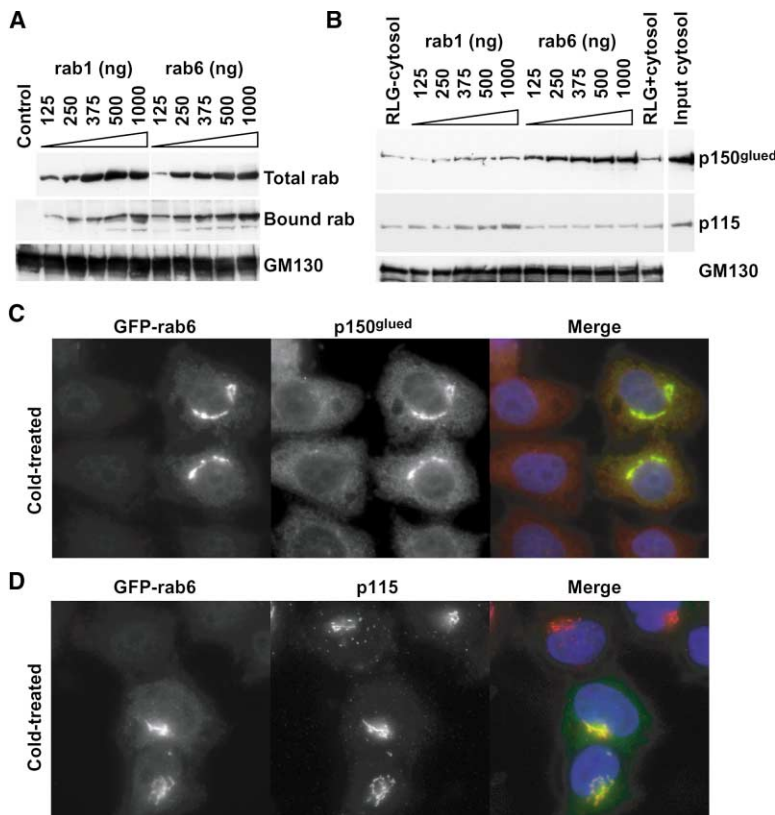
A   **B**

C

D

Figure 3. Rab6-Dependent Recruitment of Dynactin to Golgi Membranes In Vitro and In Vivo

(A) Golgi membranes were loaded with increasing amounts of prenylated Rab1 or Rab6. Western blots for the His tag detected total and bound Rab1 and Rab6, and those for GM130 demonstrated the equal recovery of the Golgi membranes.

(B) Golgi membranes preincubated with buffer (RLG+cytosol) or loaded with increasing amounts of prenylated Rab1 or Rab6 were tested for their ability to recruit Rab effectors from cytosol. A control, RLG-cytosol, in which Golgi membranes were incubated only in buffer during both reactions is also shown. Western blots were performed for p115, p150glued, and GM130.

(C and D) Cells transfected with constructs for GFP-tagged Rab6 for 18 hr were washed in ice-cold PBS and kept on ice for 1 hr prior to fixation with −20°C methanol, then stained with antibodies to GM130, p150glued, α-tubulin, p115, or GFP-Rab6. In the merged images, green is the leftmost panel, red is the center panel, and blue is DNA stained with DAPI.

References

1. Karki, S., and Holzbaur, E.L. (1999). Cytoplasmic dynein and dynactin in cell division and intracellular transport. Curr. Opin. Cell Biol. *11*, 45–53.

2. King, S.J., and Schroer, T.A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. Nat. Cell Biol. *2*, 20–24.

3. Burkhardt, J.K. (1998). The role of microtubule-based motor proteins in maintaining the structure and function of the Golgi complex. Biochim. Biophys. Acta *1404*, 113–126.

4. Muresan, V., Stankewich, M.C., Steffen, W., Morrow, J.S., Holzbaur, E.L.F., and Schnap, B.J. (2001). Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids. Mol. Cell *7*, 173–183.

5. Karcher, R.L., Deacon, S.W., and Gelfand, V.I. (2002). Motor-cargo interactions: the key to transport specificity. Trends Cell Biol. *12*, 21–27.

6. Waters, M.G., and Pfeffer, S.R. (1999). Membrane tethering in intracellular transport. Curr. Opin. Cell Biol. *11*, 453–459.

7. Hume, A.N., Collinson, L.M., Rapak, A., Gomes, A.Q., Hopkins, C.R., and Seabra, M.C. (2001). Rab27a regulates the peripheral distribution of melanosomes in melanocytes. J. Cell Biol. *152*, 795–808.

8. Wu, X., Rao, K., Bowers, M.B., Copeland, N.G., Jenkins, N.A., and Hammer, J.A., 3rd. (2001). Rab27a enables myosin Va-dependent melanosome capture by recruiting the myosin to the organelle. J. Cell Sci. *114*, 1091–1100.

9. Wu, X.S., Rao, K., Zhang, H., Wang, F., Sellers, J.R., Matesic, L.E., Copeland, N.G., Jenkins, N.A., and Hammer, J.A., 3rd. (2002). Identification of an organelle receptor for myosin-Va. Nat. Cell Biol. *4*, 271–278.

10. Jordens, I., Fernandez-Borja, M., Marsman, M., Dusseljee, S., Janssen, L., Calafat, J., Janssen, H., Wubbolts, R., and Neefjes, J. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. Curr. Biol. *11*, 1680–1685.

11. Martinez, O., Antony, C., Pehau-Arnaudet, G., Berger, E.G., Salamero, J., and Goud, B. (1997). GTP-bound forms of rab6 induce the redistribution of Golgi proteins into the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *94*, 1828–1833.

12. Echard, A., Jollivet, F., Martinez, O., Lacapere, J.J., Rousselet, A., Janoueix-Lerosey, I., and Goud, B. (1998). Interaction of a Golgi-associated kinesin-like protein with Rab6. Science *279*, 580–585.

13. White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B., et al. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. J. Cell Biol. *147*, 743–760.

14. Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T., and Pepperkok, R. (1999). Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. Nat. Cell Biol. *1*, 423–430.

15. Monier, S., Jollivet, F., Janoueix-Lerosey, I., Johannes, L., and Goud, B. (2002). Characterization of novel Rab6-interacting proteins involved in endosome-to-TGN transport. Traffic *3*, 289–297.

16. Mallard, F., Tang, B.L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B., and Johannes, L. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J. Cell Biol. *156*, 653–664.

17. Hill, E., Clarke, M., and Barr, F.A. (2000). The Rab6-binding kinesin, Rab6-KIFL, is required for cytokinesis. EMBO J. *19*, 5711–5719.

18. Fontijn, R.D., Goud, B., Echard, A., Jollivet, F., van Marle, J., Pannekoek, H., and Horrevoets, A.J. (2001). The human kinesin-like protein RB6K is under tight cell cycle control and is essential for cytokinesis. Mol. Cell. Biol. *21*, 2944–2955.

19. Siniossoglou, S., and Pelham, H.R. (2001). An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. EMBO J. *20*, 5991–5998.

20. Short, B., Preisinger, C., Korner, R., Kopajtich, R., Byron, O., and Barr, F.A. (2001). A GRASP55-rab2 effector complex linking Golgi structure to membrane traffic. J. Cell Biol. *155*, 877–883.

21. Hoogenraad, C.C., Akhmanova, A., Howell, S.A., Dortland, B.R., De Zeeuw, C.I., Willemsen, R., Visser, P., Grosveld, F., and Galjart, N. (2001). Mammalian Golgi-associated Bicaudal-D2 functions in the dynein-dynactin pathway by interacting with these complexes. EMBO J. *20*, 4041–4054.

22. Allan, B.B., Moyer, B.D., and Balch, W.E. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. Science *289*, 444–448.

23. Burkhardt, J.K., Echeverri, C.J., Nilsson, T., and Vallee, R.B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J. Cell Biol. *139*, 469–484.

24. Deretic, D., and Papermaster, D.S. (1993). Rab6 is associated with a compartment that transports rhodopsin from the trans-Golgi to the site of rod outer segment disk formation in frog retinal photoreceptors. J. Cell Sci. *106*, 803–813.

25. Shetty, K.M., Kurada, P., and O'Tousa, J.E. (1998). Rab6 regulation of rhodopsin transport in Drosophila. J. Biol. Chem. *273*, 20425–20430.