Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome

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The Rab6 GTPase regulates intracellular transport at the level of the Golgi apparatus, probably in a retrograde direction. Here, we report the identification and characterization of a novel human Rab6-interacting protein named human GAPCenA (for 'GAP and centrosome-associated'). Primary sequence analysis indicates that GAPCenA displays similarities, within a central 200 amino acids domain, to both the yeast Rab GTPase activating proteins (GAPs) and to the spindle checkpoint proteins *Saccharomyces cerevisiae* Bub2p and *Schizosaccharomyces pombe* Cdc16p. We demonstrate that GAPCenA is indeed a GAP, specifically active *in vitro* on Rab6 and, to a lesser extent, on Rab4 and Rab2 proteins. Immunofluorescence and cell fractionation experiments showed that GAPCenA is mainly cytosolic but that a minor pool is associated with the centrosome. Moreover, GAPCenA was found to form complexes with cytosolic γ -tubulin and to play a role in microtubule nucleation. Therefore, GAPCenA may be involved in the coordination of microtubule and Golgi dynamics during the cell cycle.

Keywords: centrosome/Golgi apparatus/GTPase activating protein/mitosis/Rab6

Introduction

Rab/Ypt proteins form the largest branch of the Ras superfamily of GTPases. They are localized to various compartments of both the biosynthetic/secretory and endocytic pathways of eukaryotic cells. A number of genetic and biochemical studies have shown that Rab proteins play an important role in vesicular transport and membrane traffic (for reviews see Novick and Zerial, 1997; Martinez and Goud, 1998). They participate in molecular events that underlie the targeting and fusion of transport vesicles with their appropriate acceptor membranes. For instance,

Rab proteins may modulate the assembly of the so called v/t SNARE proteins present on the cytoplasmic face of vesicles and target membranes, respectively (Rothman, 1994). However, the recent characterization of several Rab effectors points towards a more complex role of Rab proteins than previously thought. These effectors, which include a serine/threonine protein kinase, Rabphilin-3A, Rim, Rabaptin-5, EE1A, p40 and Rabkinesin-6, are not related to each other and fulfill diverse functions (Shirataki *et al.*, 1993; Stenmark *et al.*, 1995; Ren *et al.*, 1996; Diaz *et al.*, 1997; Wang *et al.*, 1997; Echard *et al.*, 1998; Simonsen *et al.*, 1998).

Like other GTPases, Rab proteins cycle between a GDP- and GTP-bound conformation. The replacement of GDP by GTP is catalyzed by guanine nucleotide exchange factors (GEFs). Several GEFs have now been identified, including Sec2p, active on Sec4p, Rabex-5, active on Rab5, and a 178 kDa cytosolic protein specific for Rab3A (Horiuchi *et al.*, 1997; Wada *et al.*, 1997; Walch-Solimena *et al.*, 1997). Since the intrinsic GTPase activity of Rab proteins is very low, GTPase activating proteins (GAPs) probably stimulate GTP hydrolysis. Although Rab GAP activities have been detected in various extracts, few GAPs have been characterized so far. In the yeast *Saccharomyces cerevisiae*, Gyp1p, Gyp6p and Gyp7p are active on Sec4p, Ypt6p and Ypt7p, respectively (Strom *et al.*, 1993; Vollmer and Gallwitz, 1995; Du *et al.*, 1998). In mammalian cells, a 130 kDa protein that acts as a GAP for the Rab3 subfamily has been isolated (Fukui *et al.*, 1997).

Work from our laboratory has focused on Rab6, a ubiquitous Rab associated with Golgi and *trans*-Golgi network (TGN) membranes (Goud *et al.*, 1990; Antony *et al.*, 1992). Rab6 is involved in transport events at the level of the Golgi complex, as shown by the fact that the overexpression of the GTPase-deficient mutant Rab6 Q72L inhibits transport of secretory markers between *cis*/medial and late Golgi compartments, without affecting their transport between the TGN and the plasma membrane (Martinez *et al.*, 1994). In addition, the overexpression of GTP-bound forms of Rab6 results in a loss of Golgi structures and a redistribution of Golgi resident enzymes into the endoplasmic reticulum (ER), pointing to a role of Rab6 in Golgi-to-ER transport (Martinez *et al.*, 1997). Several Rab6 effectors have been characterized recently. One of them is a Golgi-associated kinesin-like protein termed Rabkinesin-6, suggesting that one of the functions of Rab6 could be to regulate the binding of Golgi membranes and/or Golgi-derived vesicles to microtubules via an interaction with Rabkinesin-6 (Echard *et al.*, 1998). Another putative effector is a 150 kDa cytosolic protein with a large coiled-coil domain, but the function of this protein is currently unknown (I. Janoueix-Lerosey, F. Jollivet and B. Goud, unpublished results).

Here, we report the isolation and characterization of a

Fig. 1. Northern blot analysis of the GAPCenA mRNA expression in various human tissues. C74 mouse cDNA fragment was used as a probe. A 4.5 kb messenger RNA was detected in all tissues.

novel human protein termed GAPCenA. This protein displays a GAP activity for Rab6. Most of the protein was found to be cytosolic, but a minor pool was associated with centrosome. In addition, GAPCenA (for 'GAP and centrosome-associated') was found in complexes with cytosolic γ -tubulin. Therefore, GAPCenA could play a pivotal role in events that coordinate Golgi dynamics and the organization of microtubule cytoskeleton in interphasic and mitotic cells.

Results

Cloning of GAPCenA

To identify effector proteins of Rab6, the GTPase-defective mutant Rab6 Q72L was used as a bait in a yeast two-hybrid screen of a mouse embryo expression library. This screen yielded five independent clones that interacted specifically with Rab6 Q72L. One of them contained a cDNA insert encoding part of Rabkinesin-6 (Echard *et al.*, 1998). In this report we focus on 'clone 74' (C74), a 306 base-pair (bp) cDNA fragment encoding a 102 amino acid polypeptide. In the two-hybrid system, no interaction of the C74 polypeptide was detected with lamin or with Rab5 Q79L, the GTPase-defective mutant of a functionally distinct Rab protein. Moreover, interaction of the C74 polypeptide was detected with wild-type Rab6, but not with the GDP-bound mutant Rab6 T27N. Finally, the C74 polypeptide did not interact with the double mutant Rab6 Q72L I46E, which carries a point mutation in the effector domain of Rab6 (data not shown). Together, these results suggested a preferential interaction of the C74 polypeptide with the GTP-bound form of Rab6, probably through its effector domain.

Northern blot analysis of various human tissues using the C74 cDNA fragment as a probe indicated a predominant ubiquitously expressed 4.5 kb messenger RNA (Figure 1). We then screened a human placenta cDNA library with the C74 probe and isolated a clone encompassing a complete open reading frame (ORF). As shown in Figure 2A, this ORF encodes a protein of 1030 amino acids, with a predicted molecular weight of 118 kDa, and which we propose to name human GAPCenA (for 'GAP and centrosome-associated' protein). Primary sequence alignments indicated that GAPCenA shares 37% identity (53%

similarity) to the *Caenorhabditis elegans* F35H12_3 gene product (AC U41540). A high similarity was also found with the *Drosophila melanogaster* Pollux protein (AC U50542) (Zhang *et al.*, 1996) and the *Mus musculus* Evi5 protein (ACP97366) (Liao *et al.*, 1997) and Tbc1 protein (AC U33005) (Richardson *et al.*, 1995). Importantly, similarity of GAPCenA with these four proteins is distributed throughout the sequence, suggesting that they belong to the same family.

Three different regions can be schematically distinguished in the GAPCenA sequence (Figure 2B). The N-terminal half (domain I), from amino acids 1 to 500, contains a 120 amino acid phosphotyrosine interaction domain (PID) (amino acids 112–228), two PEST sequences (Rogers *et al.*, 1986) and several potential tyrosine phosphorylation sites. The C-terminal domain (domain III, between amino acids 750 and 1013) contains the initial C74 polypeptide, which interacts with Rab6 in the yeast two-hybrid system [Figure 2A (bold text) and B]. This region is predicted to form an α -helical coiled-coil stalk (Figure 2C). Finally, the central domain II, from amino acids 500 to 710 [Figure 2A (underlined text) and B] displays sequence similarities with the *S.cerevisiae* GTPase activating proteins Gyp1p, Gyp6p and Gyp7p (Strom *et al.*, 1993; Vollmer and Gallwitz, 1995; Du *et al.*, 1998). Black stars indicate residues especially well conserved among Gyp proteins, which could be involved in their catalytic activity (Du *et al.*, 1998). Domain II also shares homology with domains found in the spindle assembly checkpoint proteins *S.cerevisiae* Bub2p (Hoyt *et al.*, 1991) and *S.pombe* Cdc16p (Fankhauser *et al.*, 1993), as well as in a number of uncharacterized ORFs in various organisms. We indicate in Figure 2D the six motifs, named A to F, previously found to be common between Ypt GTPase activating proteins and proteins involved in spindle checkpoint assembly (Neuwald, 1997).

GAP activity of GAPCenA

The sequence similarities between GAPCenA and Gyp proteins prompted us to test whether GAPCenA could have a GAP activity on Rab6. To this end, GAPCenA was expressed in bacteria as a His-tagged protein (histidine residues were placed at the N-terminus of the protein). Figure 3 shows the protein profile of the bacterial lysates purified on nickel beads before (NI) and after (I) induction with isopropyl- β -D-thiogalactopyranoside (IPTG). GAPCenA was detected in the lysate after induction with IPTG, as indicated by Western blotting with both anti-His and anti-GAPCenA antibody (Figure 3, right panels). The fact that the antibodies detected several bands indicates that GAPCenA is sensitive to C-terminal proteolytic degradation (such a degradation was also observed for endogenous GAPCenA, see below; Figures 5 and 7A). Two prominent bands, not recognized by anti-His and GAPCenA antibodies, were present in induced and non-induced lysates. They were also present in lysates from bacteria expressing domain II and domains I + II of GAPCenA (see Figure 2), indicating that they correspond to bacterial contaminants (data not shown). A GAP assay was then performed *in vitro* on purified prenylated Rab6 expressed in baculovirus-infected Sf9 insect cells (Yang *et al.*, 1993). As shown in Figure 4A and C, Rab6 displays a very low intrinsic GTPase activity (<1 fmol/min). The

Fig. 2. Predicted amino acid sequence and structure of the GAPCenA protein. **(A)** Predicted amino acid sequence of the full-length GAPCenA protein. The region of similarity with the yeast Rab GAPs and the Bub2p and SpCdc16p proteins, corresponding to domain II, is underlined. The interaction region with Rab6 Q72L isolated from the two-hybrid screen is shown in bold type. **(B)** Predicted subdomains of the GAPCenA protein. The domain I, encompassing residues 1 to 502, is predicted to contain two PEST sequences and one PID. Sequence of the domain II (residues 502–709) is shown in D. The domain III is predicted to be an α -helical coiled coil. **(C)** Diagram of the coiled-coil conformation probability calculated for the entire GAPCenA protein. A dimer coiled-coil tertiary structure is predicted between amino acids 750 and 1013. **(D)** Sequence alignment of the GAPCenA domain II with related proteins. Identical amino acids are boxed in black, conservative substitutions are boxed in gray. *Caenorhabditis elegans* F35H12_3, *D.melanogaster* Pollux and *M.musculus* Tbc1 display the best overall homology with *H.sapiens* GAPCenA. Homology with *S.cerevisiae* Bub2p and Gyps and with *S.pombe* Cdc16p is restricted to this domain II. The letters A to F, underlined with solid bars, indicate the six homology motifs defined by Neuwald *et al.* (1997). The black stars indicate residues conserved among the Gyp family. Alignments were performed using the Pileup program of GCG (Infobiogen,Villejuif, France).

Fig. 3. Purification of bacterially expressed His-tagged GAPCenA. Bacteria expressing His-tagged GAPCenA were grown with (I, induced) or without (NI, non-induced) IPTG, and the lysates were adsorbed and eluted from nickel beads. Left panel: molecular weight markers, and NI and I eluates were resolved by SDS-PAGE and stained with Coomassie Blue. Right panel: NI and I eluates were immunoblotted with affinity-purified anti GAPCenA antibody (anti-C74) or monoclonal anti-His antibody (anti-His-Tag).

addition of GAPCenA (fraction I, Figure 3) dramatically stimulated GTP hydrolysis (Figure 4A) up to 140 fmol/min (Figure 4C). Maximal stimulation of the GTPase activity of Rab6 was obtained in the presence of ~30 ng/ μ l of the preparation of GAPCenA (corresponding to a total amount of 15 μ g of protein in the GAP assay; Figure 4B). On the other hand, GAPCenA was able to stimulate only 2- to 3-fold GTP hydrolysis of unprenylated bacterially expressed Rab6, suggesting that GAPCenA requires lipid modification on Rab6 for GAP activity (data not shown).

To test the substrate specificity of GAPCenA, we measured its activity towards a variety of prenylated Rab proteins (Figure 4C). No significant increase in GTP hydrolysis was observed when the protein was incubated with prenylated Rab5 or Rab11. However, the GTPase activities of Rab4 and Rab2 were activated by GAPCenA, although to a lesser extent than that of Rab6 (20- and 7-fold, respectively).

GAP assays were also performed using bacterially expressed domain II alone or domains I + II of GAPCenA, which do not contain Rab6 binding domain identified in the yeast two hybrid screen (Figure 2). A 30 times lower stimulation of Rab6 GTPase activity was obtained with these constructs compared with the full-length protein (data not shown).

Intracellular localization of GAPCenA

To study the intracellular localization of GAPCenA, an antibody was raised in rabbits against the bacterially expressed C74 polypeptide. Figure 5A shows that the affinity-purified antibody predominantly recognized a protein migrating with an apparent molecular mass of 150 kDa in a total lysate of HeLa cells (Figure 5A, lane 2). This is higher than the mass predicted from the nucleotide sequence (118 kDa). However, the myc-tagged GAPCenA protein expressed in HeLa cells and detected by an anti-myc antibody migrated at the same apparent molecular weight (Figure 5A, lane 1), indicating that the antibody recognizes the endogenous GAPCenA. A lower molecular

Fig. 4. GAPCenA is a GTPase activating protein. (A) Rab6 (1 μ M) was loaded with 2 μ M [γ - 32 P]GTP, diluted in buffer A (1:10, see Materials and methods) and incubated with (open squares) or without (filled squares) 50 ng/ μ l GAPCenA. Samples were taken at different times and analyzed for released [32 P]Pi. Inset, initial rate of GTP hydrolysis. (B) GTP hydrolysis of Rab6 as a function of the amount of GAPCenA. Rab6, pre-incubated with [γ - 32 P]GTP, was incubated for 30 min with different amounts of GAPCenA (from 2.2–33 ng/ μ l, corresponding to a total amount of 1–15 μ g of protein in the reaction mixture). Released [32 P]Pi was then quantitated. (C) Substrate specificity of GAPCenA. Prenylated Rab proteins were preloaded with [γ - 32 P]GTP. GAPCenA (50 ng/ μ l) or control buffer was added at time zero. Samples were taken at various time points. Initial rates of GTP hydrolysis were determined from the linear part of the curves.

weight band was detected in lysates with both anti-myc and anti-GAPCenA antibodies, which probably corresponds to a degradation product of the protein (Figure 5A, lanes 1 and 2). Fractionation of the HeLa cell lysate showed that the major pool of GAPCenA was cytosolic, with no detectable membrane association (Figure 5A, lanes 3–5).

At the immunofluorescence level on HeLa cells, the affinity-purified antibody gave a weak cytoplasmic staining, consistent with a cytosolic distribution of the protein (Figure 6, panel 1). However, a punctate staining was

Fig. 5. Endogenous GAPCenA is mainly a cytosolic protein, but a minor pool is associated with the centrosome. **(A)** Immunoblot analysis of HeLa cell extracts. Lane 1, total extract of HeLa cells transfected with myc-tagged GAPCenA; lane 2, total extract of untransfected HeLa cells; lane 3, post nuclear supernatant (PNS); lane 4, high-speed supernatant; lane 5, high-speed pellet. Proportional amounts of material were loaded in lanes 4 and 5. The samples were resolved by SDS-PAGE and immunoblotted with the 9E10 anti-myc antibody (lane 1) or with the affinity-purified anti-C74 antibody (dilution 1:1000) (lanes 2–5). **(B)** Immunoblot analysis of KE37 cell fractions. The Triton X-100 soluble fraction of 1.3×10^5 cells (Sol.), and a highly enriched centrosome preparation of 5×10^7 cells (Centr.), were resolved by SDS-PAGE and probed with the affinity-purified anti-C74 antibody (dilution 1:1000). * indicates longer exposure.

noticed in the perinuclear region of the cells, reminiscent of a centrosomal localization. To confirm such a localization, a double immunostaining was performed using a monoclonal antibody (CTR 453) directed against the pericentriolar material (PCM) (Bailey *et al.*, 1989). As shown in Figure 6 (panels 2, 3 and 4), anti-C74 staining co-localized with that of CTR 453 in HeLa cells permeabilized prior to fixation to remove the GAPCenA cytosolic pool. The co-localization of GAPCenA with the centrosomal marker was conserved throughout the cell cycle (data not shown).

To establish further that part of GAPCenA is associated with centrosomes, a fraction highly enriched in centrosomes (purification factor of 2000; see Table I in Bornens and Moudjou, 1998) was purified from human lymphoblastic KE37 cells. As shown in Figure 5B, GAPCenA can be detected in this fraction. Based on the respective amounts of soluble and centrosomal proteins loaded on the gel, we estimated that centrosomal GAPCenA represents <1% of the total cellular pool of the protein.

Association of GAPCenA with γ -tubulin-containing complexes

We wished to determine whether GAPCenA could interact with conspicuous centrosomal proteins, and in particular with those responsible for the microtubule nucleating activity of the centrosome, such as γ -tubulin (Oakley *et al.*, 1990; Joshi *et al.*, 1992). γ -tubulin-containing complexes are abundant in the pericentriolar material. However, a large fraction of γ -tubulin is not associated with centrosomes (Moudjou *et al.*, 1996), but belongs to cytoplasmic complexes (Zheng *et al.*, 1995; Martin *et al.*, 1998; Murphy *et al.*, 1998; Tassin *et al.*, 1998). Therefore, we turned to two alternative sources of γ -tubulin-containing

complexes: a bovine brain tubulin preparation obtained by two cycles of assembly/disassembly (Detraves *et al.*, 1997) and cytosol from KE37 cells.

As shown in Figure 7A, the anti-GAPCenA antibody detected a major band at 150 kDa in tubulin purified from bovine brain, suggesting that the bovine form of GAPCenA is detected with the antibody and that it is co-purified with brain tubulin. We then analyzed cytosolic fractions of KE37 cells obtained by a sucrose gradient sedimentation, previously shown to allow the separation of γ -tubulin complexes from the soluble pool (Moudjou *et al.*, 1996; Tassin *et al.*, 1998). Figure 7B shows that GAPCenA concentrated in fractions 12 to 15, corresponding to 16–22% sucrose concentrations. However, a significant part of GAPCenA entered denser fractions of sucrose gradient, in which γ -tubulin was also present. This suggested that, although GAPCenA and γ -tubulin did not seem to co-sediment precisely, a fraction of both proteins may be present in the same complex. To test this hypothesis, fractions 9–11 of the gradients were subjected to immunoprecipitation with either anti- γ -tubulin or anti-GAPCenA antibodies. As shown on Figure 7C, a small but detectable fraction of GAPCenA could be immunoprecipitated with γ -tubulin by anti- γ -tubulin antibodies (black stars). It should be pointed out, however, that anti-GAPCenA antibodies did not co-immunoprecipitate detectable amounts of γ -tubulin. The reason could be that GAPCenA is not efficiently precipitated by our antibody, as judged by the amount of GAPCenA left in the supernatant fraction of the immunoprecipitation. Alternatively, only a minor part of GAPCenA may be engaged in γ -tubulin-containing complexes.

We then attempted to obtain functional evidence for a possible role of GAPCenA in the microtubule nucleating reaction. Centrosomes isolated from KE37 cells were pre-incubated with affinity-purified anti-C74 antibody, then incubated with phosphocellulose-tubulin in a GTP-containing buffer. Microtubules were allowed to polymerize for 4 min at 37°C, as previously described (Tassin *et al.*, 1998). As shown in Figure 8 (panel 1), most centrosomes nucleated a microtubule aster in the presence of buffer alone or control antibody. In contrast, microtubules were less numerous and significantly shorter when centrosomes were pre-incubated with anti-C74 antibody (Figure 8, panel 2). Table I shows a quantification of the nucleation experiments. Both the number and the length of the microtubules were affected by the addition of anti-GAPCenA antibody, suggesting that GAPCenA play a positive role in microtubule nucleation by centrosome.

Discussion

We report here the characterization of a new human protein, GAPCenA, which acts as a GTPase activating protein for Rab6. The addition of GAPCenA led to a 140-fold increase in the GTP hydrolysis rate of Rab6 *in vitro*. Although to a lesser extent than for Rab6, GAPCenA was also found to stimulate GTP hydrolysis of two other Rab proteins acting at transport steps different from Rab6, Rab4 and Rab2 (Tisdale *et al.*, 1992; Van der Sluijs *et al.*, 1992a,b). The finding that GAPCenA can activate more than one Rab was not unexpected, since two previously characterized yeast Rab (Ypt) GAP proteins have several

Fig. 6. Immunolocalization of endogenous GAPCenA. (1) HeLa cells were fixed in methanol and immunostained with the affinity-purified anti-C74 antibody. (2, 3 and 4) Cells were permeabilized prior to fixation in methanol and double-immunostained with the anti-C74 antibody (red, image 2) and the monoclonal antibody CTR453 (green, image 3). (4) Superimposition of the two labelings.

Table I. Effect of anti-C74 antibodies on microtubule nucleation^a

	Microtubule length (μm)	Number of microtubules per centrosome		
		<5	5–15	>15
Control	9 ± 1	13	38	50
Anti-Rab6 0.25 mg/ml	8.6 ± 0.97	5	46	47
Anti-C74 0.25 mg/ml	5.2 ± 1.25	29	60	6
Anti-C74 0.4 mg/ml	5.5 ± 0.8	68	31	1

^aNucleation experiments were performed as described in Materials and methods, and in the legend of Figure 8. Photographs were taken, and number and length of microtubules were evaluated on ~100 centrosomes for each reaction condition. For the microtubule length, mean ± SD are shown.

targets: Gyp1p recently was demonstrated to be a GAP for Sec4p, Ypt1p and Ypt7p (Du *et al.*, 1998), and Gyp6p is active on both Ypt6p and Ypt7p (Strom *et al.*, 1993). In contrast to Gyp1p and Gyp6p, GAPCenA requires lipid modification (geranyl-geranylation) of Rab6 for its GAP activity, as demonstrated by the fact it does not activate unprenylated Rab6 expressed in *Escherichia coli*. Such a feature is shared by Rab3A GAP, a GAP specific for the Rab3 subfamily (Fukui *et al.*, 1997).

A sequence alignment of Gyp1p, Gyp6p and Gyp7p proteins has led to the identification of several amino acid motifs, which are potentially implicated in their catalytic activity (Du *et al.*, 1998). The fact that GAPCenA also contains these motifs strengthens the hypothesis that they define a Rab GAP protein family. However, the only other mammalian Rab GAP characterized to date, Rab3A GAP, displays no significant homology with GAPCenA and the yeast Rab GAPs. Clearly, more mammalian GAPs need

Fig. 7. Association of GAPCenA with cytosolic γ -tubulin complexes. (A) Immunoblot analysis of bovine brain tubulin purified by two cycles of polymerization/depolymerization. Lane 1, purified tubulin was resolved by SDS-PAGE and stained with Coomassie Blue; lanes 2 and 3, purified tubulin was resolved by SDS-PAGE, transferred onto nitrocellulose, and incubated with affinity-purified anti- γ -tubulin antibody (2) or affinity-purified anti-C74 antibody (dilution 1:1000) (3). (B) Immunoblot analysis of sucrose gradients fractions of KE37 cells cytosolic extracts. The sucrose concentration was measured for each fraction by refractometry. An aliquot of each fraction was resolved by SDS-PAGE, transferred onto nitrocellulose and incubated with either anti-C74 antibody (dilution 1:1000), or anti- γ -tubulin serum (dilution 1:500). (C) GAPCenA is co-immunoprecipitated by anti- γ -tubulin antibody. Fractions 9–11 of the sucrose gradients were pooled, divided into three parts, and immunoprecipitated with unrelated rabbit IgG (control), affinity-purified anti- γ -tubulin antibody (γ -tub) or affinity-purified anti-C74 antibody (GAPCenA). Proteins bound to Sepharose beads (I) or left in the supernatants (S) were resolved by SDS-PAGE and transferred onto nitrocellulose. The upper part of the blot was probed with anti-C74 antibody, and the lower part with anti- γ -tubulin serum. Black stars signify the bands corresponding to GAPCenA and γ -tubulin precipitated by anti- γ -tubulin antibodies. The bands migrating above γ -tubulin correspond to immunoglobulin heavy chains.

to be identified to determine whether these proteins share common catalytic domains, as found in Rho and Ras GAPs (Scheffzek *et al.*, 1998). It should also be pointed out that the Rab6 binding domain of GAPCenA identified in the yeast two-hybrid screen is not located in the putative catalytic domain of the protein (Figure 2B).

GAPCenA was found mostly in the cytosol of HeLa and KE37 cells. A surprising result is that a minor pool of GAPCenA was found associated with the centrosome and, furthermore, that GAPCenA was partly co-immunoprecipitated with an anti- γ -tubulin antibody. So far, the best-characterized function of γ -tubulin is to control microtubule nucleation. Although the precise mechanism of the nucleation reaction is not fully understood, a minimal complex of γ -tubulin associated with Spc97p and Spc98p is apparently sufficient to nucleate microtubules in yeast (Knop and Schiebel, 1997; Knop *et al.*, 1997). Larger cytosolic γ -tubulin complexes have been characterized in animal cells (Detraves *et al.*, 1997; Murphy *et al.*, 1998) which, in addition to γ -tubulin and the homologs of Spc97p and Spc98p, contain five or six still uncharacterized associated proteins. One hypothesis is that GAPCenA is one of them, participating directly in the nucleation reaction. A more likely possibility is that by interacting

transiently with the nucleating complex, GAPCenA brings an additional level to the regulatory process that controls microtubule nucleation in relation to other cellular events such as membrane traffic. In support of this view, one should remember that an abnormal microtubule network was a salient feature of the Ypt1p-null mutant phenotype (Schmitt *et al.*, 1986).

Insights into the role of GAPCenA at centrosomes may be gained by its homology with *S.cerevisiae* Bub2p and *S.pombe* Cdc16p. Bub2p is involved in a spindle assembly checkpoint during mitosis. Unlike wild-type cells, *bub2*-null mutants undergo nuclear DNA replication and bud emergence after benzimidazole microtubule disruption, and under normal growth conditions are unable to respond to spindle malfunctions, leading to aberrant mitosis or meiosis (Hoyt *et al.*, 1991). *cdc16* belongs to a group of genes involved in the regulation of septum formation at the onset of mitosis (Minet *et al.*, 1979). The *cdc16*-null mutants complete mitosis, but undergo multiple rounds of septation (Fankhauser *et al.*, 1993). Interestingly, Cdc16p, in association with Byr4p, has recently been shown to be a GAP for Spg1p, a small GTPase located at the spindle pole body which controls septum formation through activation of the Cdc7p kinase (Schmidt *et al.*, 1997; Furge

Fig. 8. Anti-C74 antibody inhibits microtubule nucleation *in vitro*. Centrosomes were pre-incubated with PBS (1) or with affinity-purified anti-C74 antibody (2). Microtubules were then allowed to grow for 4 min. Microtubules were visualized with monoclonal anti-tubulin antibody (red) and the centrosomes with either a polyclonal anti γ -tubulin antibody (green, image 1), or the anti-C74 antibody (green, image 2).

et al., 1998; Sohrmann *et al.*, 1998). Therefore, a tentative hypothesis is that GAPCenA could stimulate GTP hydrolysis of an as yet unidentified GTPase, homologous to Spg1p/Tem1p and located at the centrosome of mammalian cells. In this respect, it is interesting to note that among the Ras superfamily, Spg1p/Tem1p proteins show the highest similarity to members of the Rab subfamily (Schmidt *et al.*, 1997).

During mitosis, Golgi membranes are fragmented into numerous tubulo-vesicular structures. Until recently, it was believed that this process facilitated a stochastic partitioning of the Golgi apparatus into daughter cells. However, recent evidence indicates that Golgi inheritance is not a random process. In particular, the Golgi apparatus appears to use the mitotic spindle to ensure a more

accurate partitioning (Shima *et al.*, 1998). It is then likely that regulatory proteins exist which coordinate molecular events at the level of both the mitotic spindle and Golgi membranes. GAPCenA would represent an excellent candidate for such a protein, regulating the activity of a GTPase involved in Golgi dynamics (Rab6) and that of a GTPase associated with centrosome. Alternatively, one can also envision a function for GAPCenA not directly related to its GAP activity. It is noteworthy that GAPCenA displays in its C-terminal region up to 43% similarity with both GM130 and p115, two proteins involved in Golgi fragmentation at the onset of mitosis (Nakamura *et al.*, 1997). In addition, the N-terminal half of GAPCenA could be involved in regulatory protein-protein interactions since it bears a PID domain, potentially involved in the binding of tyrosine-phosphorylated proteins (Kavanaugh and Williams, 1994).

In conclusion, we have identified a novel protein that acts as a GAP for Rab6 and part of which is associated with the centrosomes. Interestingly, a new partner of the small GTPase Ran, RanBPM, has been recently localized to the centrosomes and shown to play a role in microtubule nucleation (Nakamura *et al.*, 1998). Taken together, these results suggest that still largely unknown families of proteins exist which ensure a coordination between microtubule dynamics and other cellular events such as membrane traffic or nucleocytoplasmic transport.

Materials and methods

Two-hybrid screening, cloning of the GAPCenA cDNA and constructs

All DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989), and sequence analysis was performed with the GCG Wisconsin (Genetics Computer Group, Madison, WI) software package. The two-hybrid assay using Rab6 Q72L as a bait has been described previously (Echard *et al.*, 1998). One of the positive yeast clones encoded a 306 base pair fragment that interacted specifically with Rab6 ('C74'). A 32 P-labeled C74 fragment was used as a probe to screen an oligo deoxyribosylthymine λ gt11 mouse skeletal muscle library and two overlapping cDNA inserts were isolated and sequenced on both strands by the Sanger dideoxy-termination method. The longest one encompassed ~1.3 kbp of coding sequence, a stop codon and 1.6 kbp of 3' noncoding sequence. A 32 P-labeled 400 bp fragment encompassing the 5' extremity of this clone was used in a second round of screening. However, this attempt to isolate new inserts remained unsuccessful due to yet unexplained high DNA instability leading to systematic recombination events. Nevertheless, BLAST analysis of the two clones isolated in the first round of screening identified several human ESTs, showing nucleotide conservation of >90% as well as amino acid conservation of 100% in the coding sequence. Therefore, an oligo deoxyribosylthymine and randomly primed λ pE10X cDNA library from human placenta was screened with both the initial two-hybrid clone C74 and the 5' upstream mouse cDNA fragment. Three phages were isolated out of 500 000 p.f.u., amongst which, one hybridized with both probes. Two partially overlapping cDNAs were subcloned and sequenced on both strands. One, named Hu8, was found to encompass the complete ORF. Two independent clones (IMAGE 68E07 isolated from a human lymphoblastoid cell line cDNA library, and IMAGE 25877 isolated from a human brain cDNA library) were obtained from the UK Human Genome Mapping Project Resource Centre (Hinxton, UK) and completely sequenced to confirm the coding sequence.

Human GAPCenA sequence data has been submitted to the DDBJ/EMBL/GenBank database under accession No. AJ011679.

pGEM-myc-GAPCenA was constructed by ligating the double-strand DNA linker 5'-AGCTTACCATGGAACAAAACTCATCTCAGAAG-AGGATCTGAATGACTGCAGG-3' (encoding MEQKLISEEDLN) into *Hind*III-*Pst*I-digested pGEM-4Z (Promega). GAPCenA ORF was synthesized by PCR on Hu8 phage template, using the forward primer 5'-CATGCCATGGGCTGCAGGGAAGTGATGATGAGAAAACAGGAC-

TCAAGGATTGTAGG-3', designed to insert a *Pst*I site in-frame and to eliminate the natural ATG, and a T7 λ pE10X reverse primer. The PCR product was cut by *Pst*I and *Eco*RI, and inserted into the *Pst*I-*Eco*RI sites of the pGEM-4Z-myc plasmid. The junctions of the resulting construct were verified by sequencing.

pET-15b-GAPCenA was realized by PCR synthesis on Hu8 phage template, using the 5'-ATGGTACCAGTGATGATGAGAAAACAGGA-CTCAAGGATTGTAGG-3' forward primer, designed to insert a *Kpn*I site in-frame and to eliminate the natural ATG, and a 5'-AGGGTACCT-CATCAAGTCTCTTCCCTGAAC-3' reverse primer. The PCR product was cut by *Kpn*I, and inserted at *Kpn*I site into a pET-15b vector containing a modified polylinker. The junctions of the resulting construct were verified by sequencing.

Northern blot analysis

A human multiple tissue Northern Blot purchased from Clontech Laboratories (Palo Alto, CA) was hybridized with the C74 cDNA fragment as a probe. The probe was labeled using Redivue [α - 32 P]dCTP (3000 Ci/mmol) by random priming using a Rediprime kit (Amersham).

Recombinant proteins

Lipid-modified Rab4, Rab6 and Rab11 were purified from the membrane fractions of baculovirus-infected Sf9 cells according to Yang *et al.* (1993). Prenylated Rab5 was purified according to Horiuchi *et al.* (1995). Prenylated Rab3A and Rab2 were kindly provided by Dr Y.Takai (Osaka University Medical School, Japan). To obtain recombinant GAPCenA, the BL21(DE3) *E. coli* strain was transformed with the pET-15b expression vector containing His-tagged GAPCenA. Induction was performed for 3 h at 37°C with 0.5 mM IPTG. Purification was achieved by adsorption on nickel beads (Qiagen) following the manufacturer's protocol. The concentration of the recombinant protein was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as standard.

Antibody production

The original C74 cDNA fragment encoding for a 102 amino acid polypeptide was subcloned into the pET-15b expression vector, and used to transform the BL21(DE3) *E. coli* strain. Induction and purification on nickel beads of the polypeptide were performed following the manufacturer's instructions (QIAexpressionist, Qiagen). The purified polypeptide was then injected into rabbits. Antibody was affinity-purified on nitrocellulose sheet as described previously (Goud *et al.*, 1990).

GAP assay

In all experiments, equimolar concentrations of active Rab protein were used. The concentrations of active Rab proteins were calculated on the basis of their [α - 32 P]GTP binding capacity. Rab protein (60 pmol) was incubated for 1 h at 37°C in a reaction mixture (60 μ l) consisting of buffer A (20 mM Tris-HCl pH 8, 0.5 mg/ml BSA, 0.05% CHAPS, 1 mM DTT, 2 mM EDTA) supplemented with 2 μ M [γ - 32 P]GTP. The reaction was stopped by passing the reaction mixture through a 1 ml Sephadex G-50 coarse centrifugation column followed by the addition of 30 mM MgCl₂. At t_0 , recombinant GAPCenA was added to 50 μ l of the reaction mixture and the volume was adjusted to 450 μ l with buffer A. At various time periods, 50 μ l samples were taken and added to 50 μ l of ice-cold 4% trichloroacetic acid. [32 P]Pi was then extracted from denatured samples as phosphomolybdate complexes according to Avron (1960).

Immunofluorescence microscopy

HeLa cells grown on coverslips were fixed in methanol at -20°C for 4 min. In some experiments, cells were permeabilized prior to fixation with 0.05% saponin in 80 mM PIPES pH 6.8, 5 mM EGTA, 1 mM MgCl₂, for 10 min at room temperature. Cells were then processed for immunofluorescence analysis and confocal laser scanning microscopy as described previously (Martinez *et al.*, 1994, 1997).

Cellular fractionation, tubulin purification and immunoblotting experiments

HeLa cells were mechanically broken with a barrel-type homogenizer. Total extract, postnuclear supernatant (PNS), high-speed pellet and supernatant were prepared in 50 mM HEPES (pH 7.1) and 90 mM KCl with protease inhibitors, resolved by SDS-PAGE and immunoblotted with affinity-purified anti C74 antibody as described (Roa *et al.*, 1993). The preparation of soluble and insoluble protein fractions of KE37 cells and the isolation of centrosomes from these cells have been described elsewhere (Komesli *et al.*, 1989; Bornens and Moudjou, 1998; Tassin

et al., 1998). Tubulin was purified from bovine brain with two cycles of polymerization followed by chromatography on phosphocellulose essentially as described previously (Williams and Lee, 1982).

Sucrose gradient centrifugation and immunoprecipitation experiments

Cytosolic KE37 cell extracts in 1D buffer (50 mM Tris-HCl pH 8, 0.15 M NaCl, 1% NP-40) were applied to 15–40% sucrose linear gradients essentially as described (Tassin *et al.*, 1998). Fractions from the bottom of the sucrose gradient enriched both in γ -tubulin and GAPCenA protein were pooled, and concentrated/dialyzed against 50 mM Tris pH 8, NaCl 150 mM, 1% NP-40. Primary antibody and protein G-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) were added to the sample, and the mixture was incubated for 2 h at 4°C. Protein G-Sepharose beads were sedimented, washed five times with 500 μ l of 1D buffer and three times with distilled water. The immunoprecipitates were solubilized from the Sepharose beads by incubation with the SDS-PAGE sample buffer (95°C, 5 min) and centrifuged before loading the supernatant.

Microtubule nucleation test

The microtubule nucleating activity of isolated centrosomes was tested according to Mitchison and Kirchner (1984), using bovine brain tubulin purified on phosphocellulose. Tubulin (10 mM) and 1 mM GTP were added to the centrosome suspension, and the temperature was raised to 37°C for 4 min. After glutaraldehyde fixation and sedimentation on glass coverslips, microtubules were visualized using anti- α -tubulin antibody and the centrosomes were decorated with anti- γ -tubulin antibody. To test the effect of anti-C74 antibodies on microtubules nucleation, centrosomes were pre-incubated with affinity-purified anti-C74 antibodies (0.25 and 0.4 mg/ml) for 30 min at 4°C. Microtubules were then allowed to regrow for 4 min. Microtubules were visualized with the anti- α -tubulin. Centrosomes could be detected with bound anti-C74 antibody.

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