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In its active form, the GTP-binding protein rab8 interacts with a stress-activated protein kinase

(GC kinase/rab effector/vesicular transport/Golgi apparatus/basolateral plasma membrane)

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ABSTRACT **Rab8 is a small GTP-binding protein that plays a role in vesicular transport from the trans-Golgi network to the basolateral plasma membrane in polarized epithelial cells (MDCK), and to the dendritic surface in hippocampal neurons. As is the case for most other rab proteins, the precise molecular interactions by which rab8 carries out its function remain to be elucidated. Here we report the identification and the complete cDNA-derived amino acid sequence of a murine rab8-interacting protein (rab8ip) that specifically interacts with rab8 in a GTP-dependent manner. Rab8ip displays 93% identity with the GC kinase, a serine/threonine protein kinase recently identified in human lymphoid tissue that is activated in the stress response. Like the GC kinase, rab8ip has protein kinase activity manifested by autophosphorylation and phosphorylation of the classical serine/threonine protein kinase substrates, myelin basic protein and casein. When coexpressed in transfected 293T cells, rab8 and the rab8ip/GC kinase formed a complex that could be recovered by immunoprecipitation with antibodies to rab8. Cell fractionation and immunofluorescence analyses indicate that in MDCK cells endogenous rab8ip is present both in the cytosol and as a peripheral membrane protein concentrated in the Golgi region and basolateral plasma membrane domains, sites where rab8 itself is also located. In light of recent evidence that rab proteins may act by promoting the stabilization of SNARE complexes, the specific GTP-dependent association of rab8 with the rab8ip/GC kinase raises the possibility that rab-regulated protein phosphorylation is important for vesicle targeting or fusion. Moreover, the rab8ip/GC kinase may serve to modulate secretion in response to stress stimuli.**

Rab proteins constitute a family of ras-related GTPases that have been implicated as key regulators of membrane traffic in eukaryotic cells (1, 2). Ypt1p and Sec4p, two rab proteins from budding yeast, were first shown to be essential for vesicular transport between the endoplasmic reticulum and the Golgi apparatus (3) and between the Golgi apparatus and the cell surface (4), respectively. Many rab proteins have been identified in mammalian cells that have characteristic subcellular distributions (5) and it has been shown that interference with the function of specific rabs by various means—including peptides and antibodies, or overexpression of dominant negative mutants—blocks specific transport steps (1, 2). Rab8 (5) is closely related in sequence to Sec4p, and even more so to the fission yeast Sec4p homolog Ypt2p, which also acts at the last stage in the exocytic pathway (6). Because rab8 can complement the functional loss of Ypt2p in fission yeast (6), it too is likely to function in vesicle delivery to the plasma membrane. Indeed, rab8 has been shown to play a role in vesicular transport from the trans-Golgi network to the basolateral plasma membrane in polarized epithelial cells (MDCK) (7), and to the dendritic surface in hippocampal neurons (8). However, as is the case for all rabs, the precise molecular interactions by which this protein carries out its regulatory role are yet unknown.

A variety of studies (see refs. 1 and 2) have shown that rab proteins are essential for late steps in vesicular transport, i.e., the docking on, or the fusion of the transport vesicles with, acceptor membranes. These events are dependent on interactions between complementary v- and t-SNARES, docking molecules found on the cytoplasmic surfaces of the vesicles and the corresponding acceptor membranes, respectively (9). Even though rab proteins are not part of the SNARE complexes, they appear to facilitate interactions between SNARES (10–12) by either promoting the assembly of oligomeric v-SNARES (11) or stabilizing the v-SNARE–t–SNARE interaction (12).

It is generally presumed that rab proteins exert their function in their GTP-containing "on" state by interacting with molecules that generate certain downstream events necessary for proper membrane trafficking. Therefore, there has been considerable interest in identifying and characterizing these molecules, as a way of illuminating the role of the corresponding rab proteins in vesicular transport (13–16). One such rab-interacting protein, rabphilin-3A (13), is a peripheral membrane protein of synaptic vesicles that binds to rab3a and rab3c in a GTP-dependent manner, and appears to be recruited to the vesicles by those rabs (17). Rabphilin-3A, which is also present in chromaffin granules, enhances regulated secretion, a process that appears to be controlled negatively by rab3a itself (18). Another recently identified rab-interacting protein is rabaptin-5, a presumptive effector for rab5, which is recruited by the GTP-containing form of this protein to early endosomes and is required for their fusion (14).

In this paper we describe a rab-interacting protein that interacts with the GTP-bound form of rab8 and may serve as its effector. This protein (rab8ip) is the murine equivalent of a serine/threonine protein kinase, known as the GC kinase, recently identified in human lymphoid tissue, which is a component of the stress-activated protein kinase pathway (19). It may, therefore, provide a link between the stress response and the secretory activity of the cell.

MATERIALS AND METHODS

Identification and Cloning of Rab8ip. A yeast two-hybrid screen for rab8 interacting proteins was carried out according to the procedure of Gyuris *et al.* (20). To construct "bait" plasmids, PCR-generated cDNA fragments encoding the wild-type and various mutant rab8 molecules (m1, T22N; m2, T40A; m3, Q67L; m4, N122I; m5, C205S) were each cloned in-frame downstream of the *lexA* DNA binding domain in the yeast

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Data Deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U50595).

vector pEG202 between its *Eco*RI and *Xho*I sites. The resulting constructs were used to transform *Saccharomyces cerevisiae* EGY48, a selection strain in which the chromosomal *LEU2* gene and a plasmid-borne β-galactosidase gene are each under control of *lexA* operators. An oligo(dT)-primed mouse MPC-11 myeloma cDNA expression library was constructed using a ZAP-cDNA synthesis kit (Stratagene) and inserting the cDNA downstream of the B42 transcription-activating domain in the "prey" plasmid pJG4-5 in which protein synthesis is under the control of a galactose-inducible promoter. This library was used to transfect *S. cerevisiae* EGY48 expressing the bait LexA(1-202)-rab8wt fusion protein. Positive interactions between two hybrid proteins were detected by the presence of galactose-dependent growth in leucine-free medium and galactose-dependent β-galactosidase activity. β-Galactosidase activities were quantified (21) in lysates of yeast grown in liquid culture. The 5′ portion of rab8ip cDNA, not contained in the yeast clones identified in the two-hybrid screens was obtained using a 5′ RACE kit (Clontech) with size-selected poly(A) RNA from the MPC-11 cells as template for reverse transcription. The complete cDNA for rab8ip was reconstructed by ligating overlapping 5′ and 3′ fragments at a common restriction site. The complete cDNA sequence was determined on both strands by the dideoxynucleotide-chain termination method in multiple cDNA clones and subcloned fragments.

Immunoblotting Analyses. The immunoblotting analyses were performed using affinity-purified polyclonal rabbit antibodies that were raised against the rab8 C-terminal peptide (KAKMDKKLEGNSPQGSNQGVK) conjugated to keyhole limpet hemocyanin as immunogen, or against a fusion protein produced in *Escherichia coli* consisting of the rab8-interacting domain of rab8ip (residues 430–821) linked to the C terminus of the maltose-binding protein (New England Biolabs). After probing with the first antibody, blots were incubated with the secondary peroxidase-conjugated goat anti-rabbit IgG and then for chemoluminescence using the ECL system (Amersham).

Protein Kinase Assay of Recombinant Rab8ip. A reconstructed full-length rab8ip cDNA was cloned in-frame at the 3′ end of the maltose-binding protein coding region in the plasmid pMAL-C2 (New England Biolabs) and the hybrid protein produced in transformed *E. coli* was purified on an amylose column according to the manufacturer's instructions. The *in vitro* protein kinase assay was performed by adding 2 μl of [γ-^{32}P]ATP (specific activity >5000 Ci/mmol) to a solution containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 5 μg of MBP-rab8ip, with or without 25 μg of either casein or myelin basic protein (Sigma), in a total volume of 50 μl. The reaction mixtures were incubated at room temperature for 30 min and directly analyzed by SDS/PAGE and autoradiography.

Expression of Recombinant Proteins in Transfected Mammalian Cells and Immunoprecipitation of Protein Complexes. 293T cells were transfected using the Lipofectamine reagent (Life Technologies, Grand Island, NY) with appropriate combinations of pcDNA3-based plasmids (Invitrogen) encoding rab8ip, wild-type rab8, the GTPase-deficient mutant (Q67L), or the effector domain mutant (T40A). After 48 hr, the cells were washed with ice-cold PBS, and lysed in 0.5% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, 1 mM DTT by incubation at 4°C for 10 min with occasional vortexing. Supernatants obtained after centrifugation of the cell lysates in a microfuge (2 min, 4°C) were incubated (60 min, 4°C) with affinity-purified anti-rab8 antibody together with protein-A Sepharose beads. The immunoprecipitates were washed in the lysis buffer (5 times, 1 ml each), fractionated by SDS/PAGE, and transferred onto nitrocellulose filters for western blotting.

Cell Fractionation. MDCK cells grown to confluence in DMEM containing 10% fetal bovine serum were washed twice with PBS, scraped off the dishes into ice-cold fractionation buffer (20 mM Tris, pH 7.5/100 mM NaCl/2 mM $MgCl_2$, 10% glycerol/2 mM DTT), and lysed by sonication on ice (5 times, 5 sec each). Aliquots of the lysate were adjusted to either 0.1 M or 0.5 M NaCl and centrifuged (100,000 × *g* for 1 hr at 4°C) to yield cytosolic and particulate fractions, which were analyzed by SDS/PAGE and Western blotting.

Immunofluorescence. MDCK cells were grown on coverslips to confluence and then maintained for 3 days in DMEM containing 10% fetal bovine serum. The cells were washed twice with PBS and fixed with methanol (−20°C, 3–4 min). After two washes with PBS followed by incubation with blocking buffer (1% nonfat dry milk in PBS) for 15 min, the coverslips were incubated overnight at 4°C with affinity-purified rabbit anti-rab8ip antibodies, washed three times for 10 min with blocking buffer, and then incubated (2 h, 37°C) in the same buffer with fluorescein-conjugated affinity-purified $F(ab')_2$ goat antibody fragments to rabbit IgG (Cappel). Finally, the coverslips were washed three times with blocking buffer and two times with PBS before mounting with plastic spacers for fluorescence microscopy and examination with a Zeiss Axiophot photomicroscope equipped with epifluorescence optics.

RESULTS

Using rab8 as bait in a yeast two-hybrid screen of a mouse myeloma expression library, we isolated 24 clones, all of which contained overlapping cDNAs segments derived from the same mRNA, as determined by restriction endonuclease mapping. The protein encoded by these cDNAs, designated rab8 interacting protein (rab8ip), appeared to be specific for rab8 since neither rab11 (5) nor ran/TC4, another ras superfamily member (22), when used as baits interacted with it (data not shown). Various rab8 mutants (m1, T22N; m2, T40A; m3, Q67L; m4, N122I; m5, C205S) that are analogous to well-characterized ras mutants with distinct biochemical features (23) were tested for interaction with rab8ip (Fig. 1*A* and *B*). Point mutations that were expected to confer to Rab8 the GDP-bound (m1) or nucleotide-free (m4) states abolished the interaction, whereas mutants that were expected to be either GTPase deficient (m3), or nonprenylated (m5), or had a point mutation in the effector domain (m2), interacted with rab8ip more effectively than did the wild-type rab8 (Fig. 1*B*). Since in all cases rab8ip and the individual rab8 mutants were expressed at comparable levels in the transfected yeast cells (Fig. 1*C*), as determined by Western blotting, it can be concluded that rab8ip interacts preferentially with the GTP-bound "active" form of rab8 and that the interaction might involve the effector domain of rab8.

The complete coding sequence of the cDNA for rab8ip was obtained and found to correspond to a protein of 821 amino acids (Fig. 2) that has 93% identity to a recently described human serine/threonine protein kinase, designated GC kinase, found in lymphoid germinal centers (24). The rab8ip cDNA fragment originally obtained in the yeast two-hybrid screen encoded the region of the polypeptide that extends from residues 430 to 821 which, therefore, contains the rab8 binding domain. The product of the full-length rab8ip cDNA, when assayed in the yeast two-hybrid system, interacted with rab8 as well as the original C-terminal segment. On the other hand, an amino terminal segment of 498 amino acid residues, which contains the catalytic kinase domain, did not interact with rab8 (data not shown). Like the homologous human GC kinase (24), the recombinant rab8ip was able to phosphorylate the exogenous substrates casein and myelin basic protein, as well as to undergo autophosphorylation (Fig. 3).

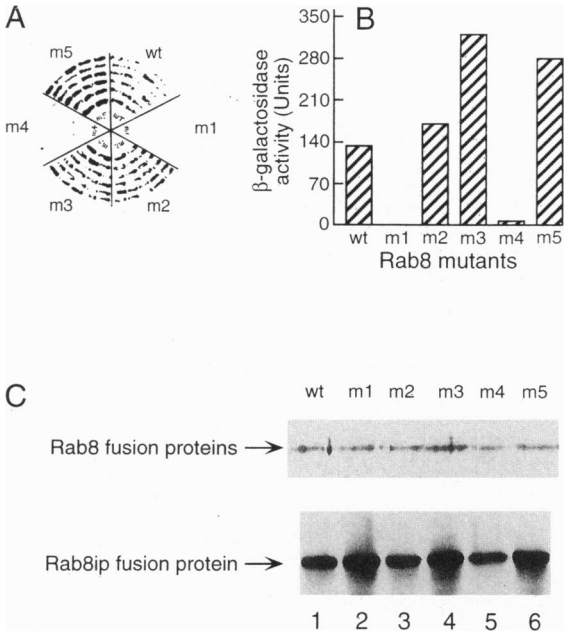

FIG. 1. Identification of a rab8-interacting protein that in the yeast two-hybrid assay interacts with rab8 only when this can achieve the "active" GTP-containing conformation. (*A*) Yeast cells were cotransfected with pairwise combinations of the plasmid that encodes the fusion protein that interacts with rab8 (rab8ip) and a series of plasmids encoding either wild-type rab8 (wt) or various rab8 mutants. Transformants expressing rab8 mutants (m2: effector domain; m3: GTPase deficient; m5: isoprenylation defective) that are expected to be present in the GTP-containing or "active" state give a positive signal (blue color on a X-gal plate), reflecting an interaction between the two coexpressed proteins. Rab8 mutant proteins that have no nucleotide binding capacity (m4) or only exist in the GDP state (m1) do not interact with rab8ip. (*B*) The extent of interaction between the various rab8 mutants and rab8ip was quantified from the β-galactosidase activities in lysates from yeast cells that were grown in liquid culture and induced with galactose. (*C*) Western blots show that, in the different yeast strains (wt, 1; m1, 2; m2, 3; m3, 4; m4, 5; m5, 6), the wild-type and mutant rab8 fusion proteins are expressed at comparable levels. The rab8ip fusion protein is also expressed at comparable levels in the various yeast strains.

When coexpressed in transfected mammalian cells rab8 and rab8ip formed a complex that could be recovered by immunoprecipitation with antibodies to rab8. In accord with the results obtained with the yeast two-hybrid assay (Fig. 1), in the transfected cells rab8ip formed complexes more effectively with rab8 mutant proteins that were either GTPase deficient (m3) or had a mutated effector domain (m2) than with the wild-type rab8 (Fig. 4).

Cell fractionation showed that although endogenous rab8ip is primarily found in the cytosol (Fig. 5*A*, lane 2), substantial amounts of the kinase are associated with sedimentable membranes in a salt-sensitive linkage (Fig. 5*A*). To determine the subcellular distribution of the membrane-associated molecules, the specific antibodies to recombinant rab8ip were used for immunofluorescence microscopy. This showed that the protein is concentrated in the basolateral plasma membrane and the Golgi region (Fig. 5*B*), which corresponds to the subcellular distribution of rab8 itself (7). These observations suggest that rab8, through protein–protein interactions, is responsible for the association of rab8ip with specific membranes.

DISCUSSION

The rab-interacting protein that we have identified has several of the properties expected of an effector for rab8. Thus, it

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Rab8ip 1 MALLRDVSLQDPRDRFELLQRVGAGTYGDVYKARDTVTSELAAVKIVKLD
         ||||||||||||||||||||||||||||||||||||||||||||||||||
GCK     1 .MELRDVSLQDPRDRFELLQRVGAGTYGDVYKARDTVTSELAAVKIVKLD

       51 PGDDISSLQQEITILRECRHPNVVAYIGSYLRNDRLWICMEFCGGGSLQE
          ||||||||||||||||||||||||||||||||||||||||||||||||||
       50 PGDDISSLQQEITILRECRHPNVVAYIGSYLRNDRLWICMEFCGGGSLQE

      101 IYHATGPLEERQIAYVCREALKGLHHLHSQGKIHRDIKGANLLLTLQGDV
          ||||||||||||||||||| ||||||||||||||||||||||||||||||
      100 IYHATGPLEERQIAYVCRERLKGLHHLHSQGKIHRDIKGANLLLTLQGDV

      151 KLADFGVSGELTASVAKRRSFIGTPYWMAPEVAAVERKGGYNELCDVWAL
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      150 KLADFGVSGELTASVAKRRSFIGTPYWMAPEVAAVERKGGYNELCDVWAL

      201 GITAIELGELQPPLFHLHPMRALMLMSKSSFQPPKLRDKTRWTQNFHHFL
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      200 GITAIELGELQPPLFHLHPMRALMLMSKSSFQPPKLRDKTRWTQNFHHFL

      251 KLALTKNPKKRPTAERLLQHPFTTQHLPPALLTQLLDKASDPHLGTLSPE
          ||||||||||||||:|||||||||:||.|||||||||||||||| |||
      250 KLALTKNPKKRPTAEKLLQHPFTTQQLPRALLTQLLDKASDPHLGTPSPE

      301 DSELETHDMFPDTIHSRSHHGPAERTPSEIQFHQVKFGAPRRKETDPLNE
          |:||||.|||||||||::|||||||||||||||||||||||||||||||
      300 DCELETYDMFPDTIHSRGQHGPAERTPSEIQFHQVKFGAPRRKETDPLNE

      351 PWEEEWTLLGKEELSGSLLQSVQEALEERSLTIRPALELQELDSPDDAIG
          ||||||||||||||||||||||||||||||||||.|  |:||||||||:|
      350 PWEEEWTLLGKEELSGSLLQSVQEALEERSLTIRSASEFQELDSPDDTMG

      401 TIKRAPFLGLPHTESTSGDNAQSCSPGTLSAPPAGPGSPALLPTAWATLK
          |||||||||  .|:. .:::: |:.||||.:||.||.|.:|||||||:|
      400 TIKRAPFLGPLPTDP.PAEEPLSSPPGTLPPPPSGPNSSPLLPTAWATMK

      451 QQEDRERSSCHGLPPTPKVHMGACFSKVFNGCPLQIHAAVTWVHPVTRDQ
          |.||.|||||||||||||||||||||||||||||.|||||||:|||||||
      449 QREDPERSSCHGLPPTPKVHMGACFSKVFNGCPLRIHAAVTWIHPVTRDQ

      501 FLVVGAEEGIYTLNLHELHEDTMEKLISQRCSWLYCVNNVLLSLSGKSTH
          ||||||||||||||||||||||:|||||:||||||||||||||||||||
      499 FLVVGAEEGIYTLNLHELHEDTLEKLISHRCSWLYCVNNVLLSLSGKSTH

      551 IWAHDLPGLFEQRRLQHQAPLSIPTNRITQRIIPRRFALSTKIPDTKGCL
          ||||||||||||||:|.|||||||:|||||||||||||||||||||||||
      549 IWAHDLPGLFEQRRLQQQVPLSIPTNRLTQRIIPRRFALSTKIPDTKGCL

      601 QCRVVRNPYTGSTFLLAALPASLLLLQWYEPLQKFLLLKNFSSPLPSPAG
          ||||||||||.||||||.|||||||||||||||||||||||||||||||
      599 QCRVVRNPYTGATFLLAALPTSLLLLQWYEPLQKFLLLKNFSSPLPSPAG

      651 MLEPLVLDGKELPQVCVGAEGPEGPGCRVLFHVLPLEAGLTPDILIPPEG
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      649 MLEPLVLDGKELPQVCVGAEGPEGPGCRVLFHVLPLEAGLTPDILIPPEG

      701 IPGSAQQVIQVDRDTVLVSFERCVRIVNLQGEPTAALAPELTFDFTIETV
          ||||||||||||||:|||||||||||:|||||||.|||||||.||||||
      699 IPGSAQQVIQVDRDTILVSFERCVRIVNMQGEPTATLAPELTFDFPIETV

      751 VCLQDSVLAFWSHGMQGRSLDTNEVTQEITDETRIFRVLGAHRDIILESI
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      749 VCLQDSVLAFWSHGMQGRSLDTNEVTQEITDETRIFRVLGAHRDIILESI

      801 PTDNPGAHSNLYILTGHQSSY 821
          |||||:|||||||||||||.|
      799 PTDNPEAHSNLYILTGHQSTY 819
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FIG. 2. Comparison of the sequences of mouse rab8ip and the homologous human GC kinase. The rab8ip sequence (821 amino acid residues) shows 93% identity with that of a human serine/threonine protein kinase, GC kinase (GenBank accession no. U07349), which was cloned from the germinal centers of lymphoid tissues. The nucleotide sequence that encodes the mouse rab8ip has been submitted to the GenBank with accession no. U50595.

interacts preferentially with the GTP-containing form of this protein and the interaction was enhanced by a mutation in the effector domain of rab8. Moreover, in polarized MDCK cells a substantial fraction of the endogenous interacting protein exists as a peripheral membrane protein with the same characteristic subcellular distribution as rab8, i.e., it is associated with the basolateral plasma membrane domains and vesicles in the region of the Golgi apparatus. This would be expected if rab8ip undergoes a functional cycle in which it is recruited by the active form of rab8 to vesicles that emerge from Golgi donor membranes and is delivered with them to the basolateral cell surface.

Rab8ip shows 93% amino acid sequence identity with—and therefore is likely to be the murine equivalent of—the GC kinase, a serine/threonine protein kinase recently identified in human lymphoid tissue (24). Indeed, as is the case with the GC kinase, recombinant rab8ip catalyzed its own phosphorylation

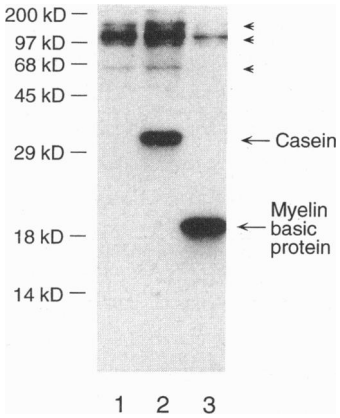

FIG. 3. Protein kinase activity of recombinant rab8ip. MBP-rab8ip, a fusion protein consisting of the full-length rab8-interacting protein linked to the C terminus of the maltose-binding protein, was purified by affinity chromatography from bacterial transformants, and incubated in a kinase reaction mixture containing [γ-^{32}P]ATP, without (lane 1) or with (lane 2) the addition of the serine/threonine kinase substrates casein or myelin basic protein (lane 3). After incubation, the reaction mixtures were analyzed by SDS/PAGE and autoradiography. The bands that are observed both in the absence and presence of exogenous substrate (arrowheads) represent autophosphorylated kinase fusion protein and its degradation products, which are visible in the Coomassie blue stained gel (not shown). The bands corresponding to phosphorylated casein and myelin basic protein are indicated.

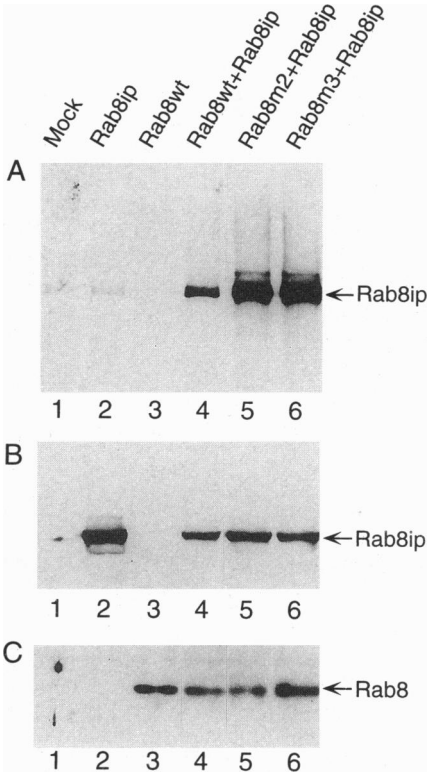

FIG. 4. Rab8 and rab8ip interact to form a complex in transfected cells that overexpress both proteins. (*A*) Rab8, together with any associated proteins, was precipitated with affinity-purified anti-rab8 antibody from lysates of untransfected 293T cells or cells transfected (as indicated above each lane) with a pcDNA3-based plasmid encoding rab8ip alone, or rab8 alone, or rab8ip together with plasmids encoding either the wild-type rab8 (wt), the GTPase-deficient mutant (m3), or the effector domain mutant (m2). The immunoprecipitates were analyzed by Western blotting using the antibody raised against the recombinant rab8ip. Western blotting analyses with anti-rab8ip (*B*) and anti-rab8 (*C*) of lysates obtained from the same singly- and doubly-transfected cultures, and untransfected control (mock) used for *A*. This shows that comparable levels of the rab8ip (*B*) and of the various rab8 mutants (*C*) proteins were present in all cases in which the cells were transfected with the corresponding expression plasmids. Cells transfected with: lane 1, no DNA (mock); lane 2, rab8ip alone; lane 3, rab8 wt alone; lane 4, rab8 wt + rab8ip; lane 5, rab8ip + rab8 m2 (effector domain mutant); lane 6, rab8ip + rab8 m3 (GTPase-deficient mutant).

and that of the exogenous substrates myelin basic protein and casein. The mRNA for the GC kinase was found to be expressed in a wide variety of tissues and cell types, but within lymphoid tissue to be preferentially expressed in B cells of germinal centers, hence its name (24). The catalytic domain of the rab8ip/GC kinase shows homology (30–35% for the yeast proteins and 60–65% for the mammalian proteins) to the corresponding domains of members of the STE20 family of protein kinases, which includes the *S. cerevisiae* STE20 (25, 26) and Sps1 (27), as well as the mammalian p65^{PAK} (28) and MST1 (29). These kinases are apparently involved in important signaling pathways and several of them have been shown to be activated by the GTP-bound forms of small GTP-binding proteins of the rho family, such as Cdc42, rac1, and rhoA (e.g., refs. 28, 30, and 31). However, the rab8ip/GC kinase lacks the Cdc42/rac1-binding sequences (31) present in those kinases.

The subcellular localization and kinase activity of the rab8-interacting protein raise the possibility that it controls a step in Golgi to plasma membrane transport through the phosphorylation of specific membrane or vesicular coat components. Protein phosphorylation–dephosphorylation events are known to be required for many stages in intracellular protein traffic. Thus, protein kinase inhibitors prevent transport between the medial Golgi and the trans-Golgi network (32), whereas the dephosphorylated state of some proteins appears to be required for endoplasmic reticulum to Golgi transport (33). Protein kinases have also been implicated in the generation of secretory vesicles in both the regulated and constitutive pathways (34), as well as in polarized secretion (35, 36) and transcytosis in epithelial cells (37, 38). The activity of a protein kinase C in particular, is required for COP-coated vesicle formation in Golgi cisternae (39) and regulates the production of post-Golgi vesicles (39–41). A possible target for the rab8ip/GC kinase in the secretory machinery remains to be identified.

A recent study (19) has shown that the GC kinase is involved in the mammalian stress response, as evidenced by the findings that it specifically activates, albeit indirectly, the stress-activated protein kinase SAPK-p46β, when coexpressed with it in transfected cells, and that the endogenous GC kinase itself is activated in cells treated with the inflammatory cytokine TNFα. The dual role of the rab8ip/GC kinase as a central element in the stress response pathway, and as a putative regulator of a step in vesicular transport to the cell surface dependent on rab8, could, therefore, provide a means to affect secretion in response to stress stimuli. Indeed, the recent observation (42) that treatment with TNFα accelerates the constitutive release of secretory proteins in several cell types, could represent a manifestation of that linkage, in which the activated rab8ip/CG kinase phosphorylates a component of the secretory machinery that stimulates secretion.

Two alternative mechanisms can be envisioned through which the rab8–rab8ip interaction may control the activity of the proteins involved. One is that the kinase serves to regulate rab8, e.g., by selectively phosphorylating active rab8 molecules and, consequently, modulating their function in vesicular transport. Although it has been reported that rab8 found on the α granules of platelets becomes phosphorylated during thrombin-induced degranulation (43), our observations with the recombinant proteins synthesized in *E. coli* (not shown) indicate that, *in vitro*, rab8 is not a substrate for the kinase rab8ip. Of course, this does not exclude the possibility that rab8ip may regulate the activity of rab8 without phosphory-

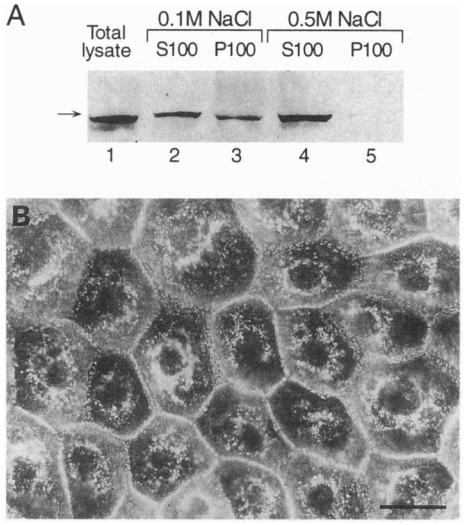

FIG. 5. Rab8ip is a peripheral membrane protein associated with the Golgi apparatus and the basolateral plasma membrane. (*A*) MDCK cell lysates prepared by sonication were adjusted to a final NaCl concentration of either 0.1 M or 0.5 M, and fractionated by differential centrifugation (100,000 × *g* for 1 hr at 4°C) into cytosolic (S100) and particulate (P100) fractions. Equivalent amounts of the total cell lysate (lane 1), the cytosol (lanes 2 and 4), and the particulate fractions (lanes 3 and 5) were analyzed by Western blotting using affinity-purified anti-rab8ip antibodies. The band corresponding to rab8ip is indicated by an arrow. (*B*) Immunofluorescence analysis of MDCK cells with antibodies to rab8ip. (Bar = 10 μm.)

lating it. The other alternative is that rab8 regulates the kinase activity of rab8ip/GC, which serves as its effector. This is consistent with the fact that rab8 interacts with the kinase only when it is in the active GTP-bound state. Rab8 could activate the kinase by two different mechanisms. It could do so directly, as is the case for the rac-1 activation of the serine/threonine kinase $p65^{PAK}$ (28), or it could serve to recruit the kinase to a specific membrane (presumably in the trans-Golgi network) where the kinase would carry out its function. The latter mechanism would be analogous to the activation of the serine/threonine kinase Raf-1 as a consequence of its ras-dependent recruitment to the plasma membrane (44, 45).

The notion that rab8 may serve to activate the rab8ip/GC kinase can be combined with current models for the regulation of vesicular transport by rab GTPases in which the latter serve to activate v-SNAREs on transport vesicles (11), or to promote the assembly of the SNARE complexes that takes place upon vesicle docking (12). This raises the possibility that rab proteins, directly or indirectly, activate specific protein kinases which, through phosphorylation, may render other protein complexes (e.g., v- and/or t-SNAREs) competent for vesicle targeting and/or fusion.

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