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Mapping of Ras-related GTP-binding proteins by GTP overlay following two-dimensional gel electrophoresis

(synaptic vesicles/vesicular transport/Rab/Rho/GDP dissociation inhibitor)

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ABSTRACT **For identification of Rab, Rac, Rho, Ral, Rap, and Arf proteins on two-dimensional polyacrylamide gels, we have expressed full-length cDNAs of members of these protein families with the T7 RNA polymerase-recombinant vaccinia virus expression system. Membrane preparations from cells expressing the cDNAs were subjected to high-resolution two-dimensional polyacrylamide gel electrophoresis followed by [α-^{32}P]GTP ligand blotting. We have mapped 28 small GTP-binding proteins relative to their isoelectric points and according to their molecular weights and by immunoblotting with specific antibodies. Rab and Rho proteins could be specifically identified by extraction of streptolysin O-permeabilized Madin-Darby canine kidney (MDCK) cells with Rab- and Rho-GDP dissociation inhibitor. We applied the reference mapping to analyze the GTP-binding patterns of synaptosome fractions from rat brain. The purified synaptosomes exhibited specific enrichment of Rab3a, Rab5a, Ral, and several other GTPases. This approach and the map we have produced should provide a useful aid for the analysis of the expression and localization of members of all families of small GTP-binding proteins in various cell types and subcellular fractions.**

Small GTPases of the Ras superfamily behave as molecular switches and regulate a diverse spectrum of intracellular processes. These roles include cellular proliferation and differentiation (Ras and Rap), vesicular trafficking (Rab and Arf), and cytoskeletal control (Rho and Rac) (1–3). The Rab family constitutes the largest (>30 mammalian members) and fastest growing branch (4–10). The role of Rab GTPases in membrane traffic was first demonstrated by studies on Sec4p and Ypt1p, which function in the exocytic pathway of *Saccharomyces cerevisiae* (11). Rab proteins are localized to distinct intracellular compartments, and a combination of *in vitro* and *in vivo* studies has demonstrated their role in the regulation of various exocytic and endocytic transport processes (3, 12).

The large number of Rab GTPases identified in mammalian cells led to the hypothesis that each step of vesicular traffic is regulated by at least one Rab protein (13). However, the picture is complicated by the existence of subgroups of Rab proteins sharing high sequence identity (e.g., Rab3a, Rab3b, Rab3c, and Rab3d; Rab5a, Rab5b, and Rab5c). Furthermore, whereas certain membrane compartments such as lysosomes still lack known Rab proteins, recent morphological data indicate that several Rab proteins localize to the same organelle. A striking example is provided by early endosomes, which contain at least three additional Rab proteins beside those belonging to the Rab4 and Rab5 subgroups (10, 14). Additional complexity is introduced by cell differentiation, as demonstrated by the recent identification of several cell- or tissue-specific Rab proteins (8, 15, 16). Since their intracellular distribution has not been defined, it is not possible to predict their functional role in membrane traffic. The localization and function of Rab proteins during cellular differentiation are far from being characterized.

To study the distribution of Rab proteins in different cells and their organelles, we have developed a mapping technique based on [α-^{32}P]GTP ligand blotting of high-resolution two-dimensional polyacrylamide gels (17). We have now extended this analysis to include 28 members of the Rab, Rac, Rho, Ral, Rap, and Arf families of proteins. We applied this technique to map small GTP-binding proteins on purified synaptosomes. Synaptosomes are easily isolated from brain tissue and are known to contain synaptic vesicles, early endosomes, and other, less defined vesicular intermediates involved in both endocytosis and exocytosis, surrounded by presynaptic plasma membrane (18).

MATERIALS AND METHODS

Cell Culture and Vaccinia Expression. MDCK-II cells and BHK-21 cells were grown and passaged as described (17, 19). Primary cultures of rat hippocampal neurons were prepared as described (20). Cells were cultured for 9 days (stage 5 cells) before use (21).

Infection with T7 polymerase-recombinant vaccinia virus (22) was carried out in serum-free medium (17). After 30 min cells were supplemented with 5% serum and transfected by using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mannheim) according to the manufacturer's instructions and incubated in the presence of 10 mM hydroxyurea for 5 hr at 37°C in a 5% CO_2 atmosphere.

Preparation of Total Cell Membrane Fractions and Synaptosomes. A postnuclear supernatant (17) was transferred to a Beckman TLA100.2 microcentrifuge tube and centrifuged at 60,000 rpm for 30 min at 4°C. The supernatant (cytosol fraction) and the pellet were separated and processed for analysis. A fraction enriched in synaptosomes (18) was isolated from 15 rat brains.

Two-Dimensional PAGE and Transfer to Nitrocellulose for [α-^{32}P]GTP Overlay and Immunoblotting. A combination of isoelectric focusing (IEF) and SDS/PAGE was used to resolve proteins in two dimensions essentially as described previously (17). For transfer to nitrocellulose for GTP overlay and immunoblotting we have modified a protocol (17)

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Abbreviations: IEF, isoelectric focusing; GDI, GDP dissociation inhibitor; GST, glutathione *S*-transferase.

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based on the method of Lapetina and Reep (23). Immunoblotting was performed as described (24).

Cell Permeabilization with Streptolysin O and Incubations with Rab- and Rho-GDP Dissociation Inhibitor (GDI). Rab-GDI cDNA was excised with *Bam*HI sites from the pGEX-2T plasmid provided by Y. Takai (25). Subsequently it was cloned into *Bam*HI-linearized plasmid pRSET A (Invitrogen) and His_6-tagged Rab-GDI was purified from transformed *Escherichia coli* by Ni^{2+}-NTA-agarose column (Qiagen Chatsworth, CA) chromatography according to the manufacturer's instructions. The recombinant glutathione *S*-transferase (GST)–Rho-GDI fusion protein was expressed in *E. coli* and purified by means of a glutathione-Sepharose 4B column (26).

Reduced streptolysin O was obtained from Wellcome. MDCK II cells were grown on polycarbonate filters (Costar, 24 mm) and permeabilized as described (27). In the permeabilization step the cells were incubated for 30 min at 37°C with ICT buffer/1 mM GDP only (ICT, 78 mM KCl/50 mM Hepes·KOH, pH 7.2/4 mM $MgCl_2$/10 mM EGTA/8.3 mM $CaCl_2$/1 mM dithiothreitol) or containing 2 μM Rab-GDI or 2 μM Rho-GDI. The media were then removed and the cells were scraped into ICT buffer and washed on a stirring wheel for 30 min at 4°C. The media containing the released cytosolic proteins and GDI were incubated for 30 min at 4°C with glutathione-Sepharose 4B (GST–Rho-GDI samples) or with Ni^{2+}-NTA-agarose (His_6-tagged Rab-GDI samples). The beads were washed three times with ICT buffer and beads and cell pellets were processed for two-dimensional PAGE analysis.

RESULTS

Two-Dimensional PAGE Mapping of Small GTPases. To identify new and to study known GTP-binding proteins we recently combined the GTP-overlay method (23) with analysis by two-dimensional PAGE and established a mapping protocol for small GTPases (17). GTP-binding proteins were transiently expressed in BHK-21 cells with the T7 RNA polymerase-recombinant vaccinia virus system (17, 22, 28). Cells were scraped and fractionated 5 hr after infection/transfection and membrane fractions were subjected to high-resolution two-dimensional PAGE. After renaturing transfer to nitrocellulose, the blots were overlaid with [α-^{32}P]GTP and the GTP-binding profiles were visualized by autoradiography.

Two examples shown here are Rab18 (Fig. 1*b*) and Rab17 (Fig. 1*c*). Overproduced Rab proteins appeared as unique spots at the expected molecular mass (arrowheads in Fig. 1 *b* and *c*; compare with Table 1). In addition, two unidentified GTP-binding protein spots of high intensity (Fig. 1 *a*, spots A and B) with apparent molecular masses of 23.5–24 kDa were found at identical electrophoretic positions in all cell types and most subcellular fractions studied, and served as internal positional markers (17). Immunoblot analysis with a specific anti-Rab17 peptide antibody confirmed the position of the overexpressed Rab17 on the same nitrocellulose sheet (Fig. 1*d*). Since in some cases specific antibodies were available (Table 1), the electrophoretic positions of small GTPases could be confirmed either after overexpression or by immunoblotting (Fig. 2).

The method of GTP overlay after renaturing transfer is suitable for small GTP-binding proteins only, and the α subunits of heterotrimeric G proteins cannot be detected (24). All GTP-binding proteins mapped here are listed in Table 1, with molecular mass and isoelectric point (pI). Isoelectric points predicted with the University of Wisconsin Genetics Computer Group program package (38) did not correspond to the detected position of a protein after IEF in some cases (e.g., Rab2, Rab8, and Rab10), presumably due to posttranslational modifications (compare Table 1 with Fig. 2). However, the same proteins (Rab2, Rab8, and Rab10) expressed and mapped in two different cell lines (MDCK-II and BHK-21) migrated at identical electrophoretic positions (17). A compilation of our mapping studies at the same scale as the blots is shown schematically in Fig. 2.

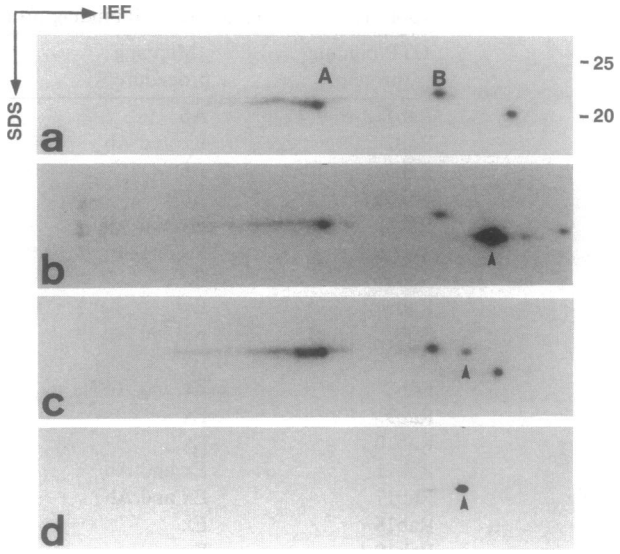

FIG. 1. [α-^{32}P]GTP overlay of overproduced Rab proteins after high-resolution two-dimensional PAGE. Rab proteins were expressed in BHK-21 cells using the T7 RNA polymerase-recombinant vaccinia virus system. Microsome fractions were prepared and equal amounts of protein (60 μg) were separated by two-dimensional PAGE, transferred to nitrocellulose paper, and then incubated with [α-^{32}P]GTP and visualized by autoradiography for 12 hr at −80°C. Arrowheads indicate overproduced Rab proteins. (*a*) VT7-infected control. (*b*) VT7-Rab18. (*c*) VT7-Rab17. (*d*) The same nitrocellulose sheet as shown in *c*, but the overproduced Rab17 protein was detected by an affinity-purified rabbit polyclonal anti-Rab17 antibody. A horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody and the blot was developed by enhanced chemiluminescence (ECL; Amersham). Proteins labeled A and B in *a* were used as internal markers with respect to their relative electrophoresis position on the blot. The area of the blot shown here represents the 20- to 25-kDa range after SDS/15% PAGE. The directions of IEF and SDS electrophoresis are indicated.

Mapping of Endogenous GTP-Binding Proteins Removed from Membranes by Rab- and Rho-GDI. To distinguish between the members of the Rab and Rho subfamilies on two-dimensional gels, we took advantage of GDI specific for either Rab or Rho. These factors induce the dissociation of Rab and Rho from cellular membranes. We studied the effects of purified and tagged Rab- and Rho-GDI on the membrane association of endogenous GTPases. We used MDCK-II cells permeabilized with the bacterial toxin streptolysin O (27). Rab-GDI (Fig. 3*b*) and Rho-GDI (Fig. 3*c*) removed a subset of small GTP-binding proteins from streptolysin O-permeabilized, filter-grown MDCK-II cells (Fig. 3*a*). The proteins removed by Rab-GDI were identified by comigration with overproduced GTP-binding proteins such as Rab5a (Fig. 4*e*) and Rab7 (Fig. 4*f*) or by immunoblotting with specific antibodies (e.g., Rab6, Fig. 4*c*). Protein spots A and B reacted with Rab-GDI but have not been identified by their sequences as members of the Rab family. One of the proteins extracted from the membranes by Rho-GDI was identified by immunoblotting as RhoA (Fig. 4*b*), whereas the second Rho protein removed (Fig. 3*c*, open arrowhead) did not react with this particular serum (Fig. 4*b*). These results demonstrate the specificity of the reference mapping and provide a tool for differentiation between Rab and Rho/Rac proteins.

Table 1. Summary of the GTP-binding proteins mapped by two-dimensional electrophoresis

GTP-binding protein	Mapping procedure*	Molecular mass, kDa	Predicted pI	Ref. for clone/Ab
Rab1a, -b	Ab	22.6	6.0	†
Rab2	Ex and Ab	23.5	6.5	28
Rab3a	Ab	25.0	4.7	29
Rab3b	Ab	24.8	4.8	29
Rab4a	Ex and Ab	23.9	5.2	30
Rab5a	Ex and Ab	23.6	8.2	28
Rab5b	Ex	23.7	8.2	‡
Rab5c	Ex	23.4	8.5	‡
Rab6	Ex and Ab	23.6	5.3	31
Rab7	Ex	23.5	6.6	28
Rab8	Ex and Ab	23.6	9.5	17
Rab9	Ex	22.8	5.2	32
Rab10	Ex	22.6	8.5	9
Rab11	Ex and Ab	24.4	6.5	9
Rab17	Ex and Ab	23.6	5.2	15
Rab18	Ex	22.8	4.8	‡
Rab19	Ex	24.4	6.6	‡
Rab22	Ex	21.7	7.3	10
RhoA, -B, -C	Ab	21.7	6.5	33
RhoD	Ex	23.6	8.1	§
Rac1, -2	Ab	21.4	7.6	33
RaIA, -B	Ab	23.6	7.1	34
Rap1a, -b	Ab	21.0	6.5	35
Rap2a, -b	Ab	20.6	4.6	35
Arf I	Ab	20.7	6.8	36
Arf III	Ab	20.6	7.55	37

All proteins mapped (compare with Fig. 2) are listed with their calculated molecular mass and predicted isoelectric points (pI). References acknowledge colleagues who provided antibodies and clones.

*Ex, expression, Ab, antibody.

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Mapping of Small GTP-Binding Proteins in a Synaptosome Preparation. Synaptosomes represent a subcellular fraction containing all components involved in synaptic vesicle docking, fusion, and recycling. Synaptic vesicles represent the most dominant organelle in this fraction. To test the practical use of the mapping technique, we investigated the presence of GTP-binding proteins in synaptosomes purified from rat brain terminals (Fig. 5*b*). Fully polarized hippocampal neurons (Fig. 5*a*) and glial cells (Fig. 5*d*) served as control preparations. The two unidentified proteins A and B were present in all membrane fractions (Fig. 5*a*, *b*, and *d*). Proteins 1–3 were identified by the reference mapping as Rab5 isoforms (protein 2 = Rab5a, compare also with Fig. 4*e*; protein 1 = Rab5b and protein 3 = Rab5c). Although the Rab5 proteins were present in all fractions, their abundance varied. Rab5a was enriched in synaptosomes when compared to neurons, and Rab5c was less abundant in glial cells. How-

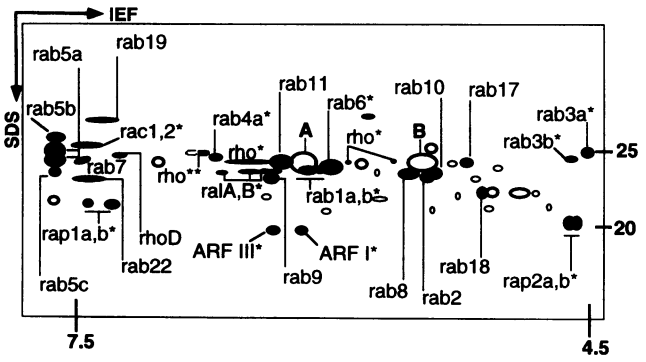

FIG. 2. Schematic image of [α-^{32}P]GTP-overlay of mapped small GTP-binding proteins after high-resolution two-dimensional PAGE. The area shown represents the 20- to 25-kDa range after SDS/15% PAGE. The pI range is shown at the bottom. *, mapped by antibodies or/and expression; **, mapped by Rho-GDI.

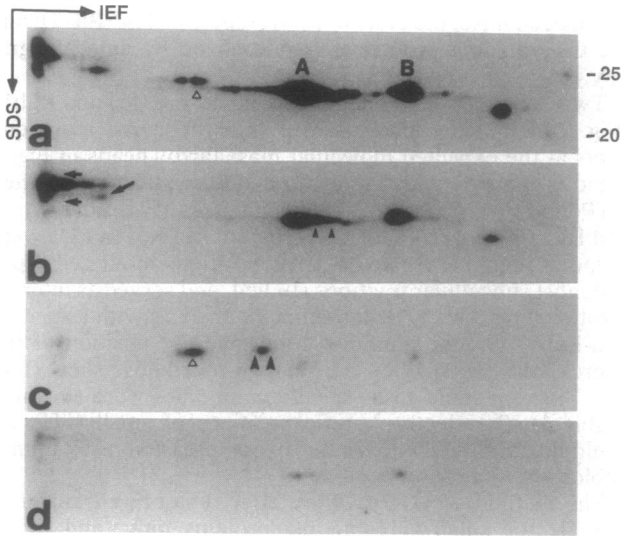

FIG. 3. [α-^{32}P]GTP overlay of small GTP-binding proteins of filter-grown MDCK II cells after permeabilization with streptolysin O. (*a*) Total membrane fraction from MDCK II cells (one-fourth of a 24-mm polycarbonate filter). (*b*) Rab-GDI beads fraction. (*c*) Rho-GDI beads fraction. (*d*) Beads control. Arrows and arrowheads indicate proteins specifically extracted by Rab- or Rho-GDI. Note that the same symbols are used for these proteins in Fig. 4.

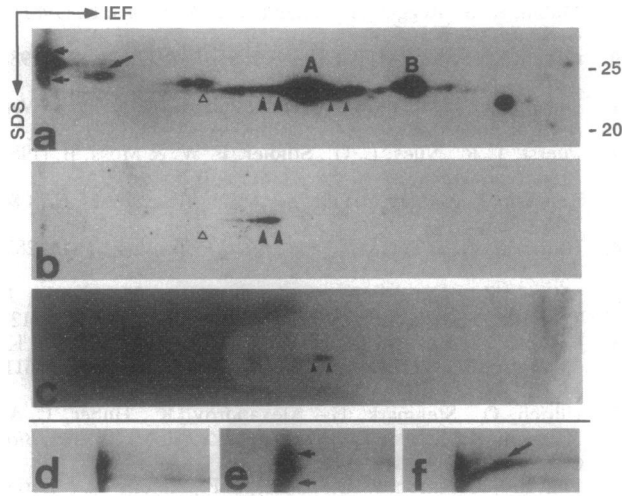

FIG. 4. Identification of GDI-extracted GTP-binding proteins after [α-^{32}P]GTP overlay of filter-grown MDCK II cells the same nitrocellulose sheet as shown in Fig. 3*a* (*a*) was probed with a polyclonal anti-RhoA antiserum (*b*) or, after stripping, with a polyclonal anti-Rab6 antiserum (*c*). Rab proteins overproduced in VT7-infected control (*d*) VT7-Rab5a (*e*), and VT7-Rab7 (*f*) cells were also detected.

ever, the most dramatic changes were observed with protein 8 (Fig. 5 *a–c*). This protein was identified as Rab3a. An immunoblot with a specific anti-Rab3a antibody of the same nitrocellulose membrane is shown in Fig. 5*c*. As expected, Rab3a was highly enriched in the synaptosomes relative to hippocampal neurons (39) but was not detected in glial cells (Fig. 5*d*). Protein 5 mapped as Rab6 in neurons and glial cells (Fig. 5 *a* and *d*), but the same isoelectric form of the protein could not be detected in synaptic vesicles (Fig. 5*b*). Protein 12 migrated at an identical electrophoretic position as Rab10 in the reference mapping and was enriched in synaptosomes. Ral was described recently as being associated with cholinergic vesicles (34) and was highly enriched in the synaptosome fraction (proteins 10 and 11; the antibody used for the

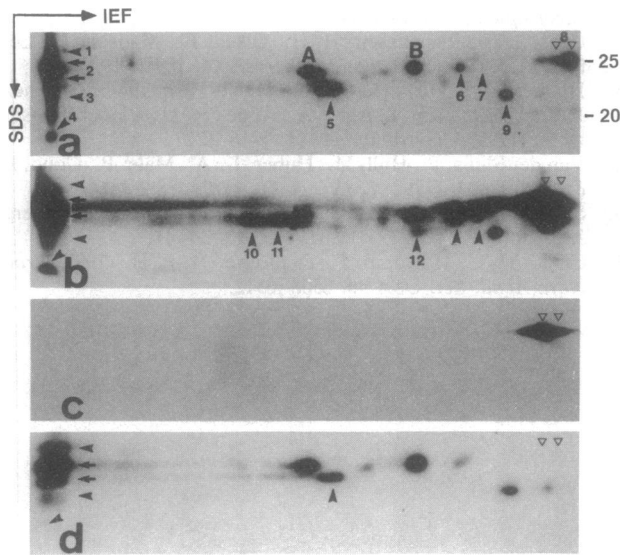

FIG. 5. Detection of small GTP-binding proteins in hippocampal neurons (*a*), synaptosomes (*b* and *c*) and glia cells (*d*) by two-dimensional PAGE followed by [α-^{32}P]GTP overlay (*a*, *b*, and *d*) or by immunoblotting (*c*). In *c*, the same nitrocellulose membrane as shown in *b* was probed with a mouse monoclonal anti-Rab3a antibody. Symbols, letters, and numbers refer to proteins described in the text.

mapping of Ral most likely recognizes both isoforms, RalA and RalB; compare also with Table 1). Rab11 was also suggested to be associated with synaptic vesicles (40) but could not be distinguished in the GTP overlay because it comigrated with protein spot A. Interestingly, protein 9, equally expressed in all fractions analyzed here, was previously found to be enriched in apical transport vesicles derived from the trans-Golgi network in polarized MDCK cells but was absent from unpolarized cells such as BHK-21 cells or other fibroblasts (17, 41). Protein 6–7 (enriched in synaptic vesicles; Fig. 5*b*) and protein 4 (missing in glial cells; Fig. 5*d*) remain unidentified.

These results demonstrate that several small GTP-binding proteins are enriched in synaptosomes, suggesting their functional involvement in membrane trafficking in nerve terminals.

DISCUSSION

High-resolution two-dimensional PAGE provides a convenient and sensitive method to analyze the protein composition of a given cell, tissue, or subcellular fraction. For instance, extensive reference maps are being compiled for the proteins found in human keratinocytes and liver (42, 43). The reference map for human keratinocytes currently lists 3038 entries of which 763 proteins have been identified (42). Two-dimensional PAGE is the ideal method for coping with the complexity of the large superfamily of small GTPases, presently comprising >50 members (7). The sensitivity of the analysis is greatly augmented by the use of [α-^{32}P]GTP ligand binding after renaturing transfer. The use of either Rab- or Rho-GDI provides an additional aid for classification. When similar binding proteins become available for other families of small GTP-binding proteins, these can be applied to characterize the proteins in question. We have in collaboration with other groups mapped GTP-binding proteins in several different systems (17, 24, 40, 41, 44, 45). Our two-dimensional gel method is the same as that used by Celis *et al.* (42) for compiling the two-dimensional-gel keratinocyte data base. Three of the proteins mapped here (Rap2, Rab2, and Rab6) were identified recently in the keratinocyte map, and they migrated at the same electrophoretic coordinates as in our reference map (42). We are using the two unidentified protein spots A and B (Fig. 2) as our internal standards. These proteins are found at practically identical electrophoretic positions for all cells and for most organelles analyzed. Alternatively, a mix of recombinant GTP-binding proteins could be developed and used as internal standards in future mapping studies. Until now the same Rab protein expressed in different cells and species has migrated with similar mobility in the two-dimensional gel. However, it is unlikely that this will be a rule without exceptions. Therefore, conclusive identification in doubtful cases will depend on antibody blotting or expression after transfection, as was done in this study.

The mapping of the GTP-binding proteins in synaptosomes illustrates the usefulness of our method. Rab3a was the major species as expected. However, Rab5 associated with early endosomes and the plasma membrane in nonpolarized cells was also found in this subcellular fraction. Rab5a has been localized to endosomes underlying both the basolateral and the apical membranes of epithelial cells as well as in dendrites and in axons (C. Bucci and M.Z., and M.J.d.H., unpublished work). Among the exocytic Rab proteins, Rab8 was excluded as shown before, and Rab10, which has been localized to the Golgi complex (46), was present, as was an unidentified GTP-binding protein (spot 9). This protein is present in apical transport vesicles immunoisolated from MDCK cells (17) and it is downregulated after transformation of mammary epithelial cells by oncogenes (41).

Much work remains to be done to place all the known and unknown small GTPases into their cellular contexts and find out what they are doing. The Rab and Arf proteins provide molecular and functional specificity to membrane traffic between different organelles. The Rho and Rac proteins are involved in regulating cytoskeletal functions and respiratory burst. It is obvious from previous studies that the expression of many of these proteins will vary with cell type. Future studies on cellular organization during embryonic development and also in different disease states will have to incorporate the analysis of small GTPases. We therefore need standardized procedures to facilitate comparison and communication. Reference two-dimensional gel mapping provides such a tool for the analysis of this superfamily of proteins.

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