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Localization of Low Molecular Weight GTP Binding Proteins to Exocytic and Endocytic Compartments

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Summary

A set of 11 clones encoding putative GTP binding proteins highly homologous to the yeast *YPT1/SEC4* gene products have been isolated from an MDCK cell cDNA library. We localized three of the corresponding proteins in mammalian cells by using affinity-purified antibodies in immunofluorescence and immunoelectron microscopy studies. One, the MDCK homolog of rab2, is associated with a structure having the characteristics of an intermediate compartment between the endoplasmic reticulum and the Golgi apparatus. The second, rab5, is located at the cytoplasmic surface of the plasma membrane and on early endosomes, while the third, rab7, is found on late endosomes. These findings provide evidence that members of the YPT1/SEC4 subfamily of GTP binding proteins are localized to specific exocytic and endocytic subcompartments in mammalian cells.

Introduction

In the eukaryotic cell, the intracellular transport of proteins and lipids between distinct subcellular compartments is mediated by carrier vesicles that bud from a donor organelle and fuse with an acceptor organelle. The molecular basis of this complex process is poorly understood. In Saccharomyces cerevisiae, two low molecular weight GTP binding proteins, SEC4p and YPT1p, have been recently demonstrated to belong to the machinery regulating membrane traffic. The *SEC4* gene encodes a 23.5 kd GTP binding protein associated with the cytoplasmic face of the plasma membrane and post-Golgi secretory vesicles (Salminen and Novick, 1987; Goud et al., 1988). Biochemical evidence shows that SEC4p is involved in regulating protein transport between the Golgi apparatus and the plasma membrane (Salminen and Novick, 1987). The *YPT1* gene product (23 kd; Gallwitz et al., 1983) is thought to act at an earlier step of the secretory pathway and is presumably associated with Golgi-like structures (Segev et al., 1988). Recently, Baker et al. (1990) have reported that an antibody directed against YPT1p could inhibit protein transport from the endoplasmic reticulum (ER) to the Golgi complex in yeast extracts.

Another line of evidence supporting a role for guanine nucleotide binding proteins in intracellular transport has been revealed by the striking inhibitory effects of a nonhydrolyzable GTP analog, GTP-γ-S, in several different cell-free transport systems. Melançon et al. (1987) first reported that GTP-γ-S inhibited the in vitro transport of a viral glycoprotein between Golgi cisternae and causes accumulation of coated carrier vesicles (Orci et al., 1989). GTP-γ-S has also been shown to affect ER to Golgi transport in both yeast and mammalian in vitro transport assays (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989). Recycling of the mannose 6-phosphate/IGF-II receptor to the *trans* Golgi network was inhibited by GTP-γ-S (Goda and Pfeffer, 1988), as was endocytic vesicle fusion in vitro (Mayorga et al., 1989; Tuomikoski et al., 1989).

How the GTP binding proteins function in membrane trafficking is not yet clear. Low molecular weight GTP binding proteins could transduce an intracellular signal needed to regulate transport between two compartments in a manner similar to G proteins (Melançon et al., 1987; Segev et al., 1988; Schmitt et al., 1988; Goud et al., 1988). Alternatively, they could act in the same way as the elongation factor Tu in protein synthesis and direct energy-dependent unidirectional delivery of vesicles to the target organelle (Bourne, 1988; Walworth et al., 1989). GTP hydrolysis would be required to trigger a conformational change of the GTP binding protein as shown for p21*ras* (deVos et al., 1988; Pai et al., 1989), and the GDP form of the protein would then return to the cytosol to target another vesicle in a cyclic process. According to Bourne's model (1988), each step in membrane traffic would require a distinct GTP binding protein. This could account for the growing number of *ras*-related GTP binding proteins identified in mammalian cells (Chardin and Tavitian, 1986; Touchot et al., 1987; Haubruck et al., 1987; Bucci et al., 1988; Matsui et al., 1988; Pizon et al., 1988; Sewell and Kahn, 1988; Zahraoui et al., 1989; Didsbury et al., 1989; Polakis et al., 1989).

With the longterm goal of understanding membrane traffic in epithelial cells (Simons and Fuller, 1985), we have screened a Madin-Darby canine kidney (MDCK) cell cDNA library with a degenerate oligonucleotide corresponding to a domain conserved between GTP binding proteins of the YPT1/SEC4 subfamily. We have isolated a set of 11 cDNAs encoding putative GTP binding proteins highly homologous to *YPT1* and *SEC4* gene products. We have raised specific antibodies against three of these proteins and studied their cellular localization. This study presents evidence that low molecular weight GTP binding proteins are associated with distinct compartments both in the exocytic and endocytic pathways in mammalian cells.

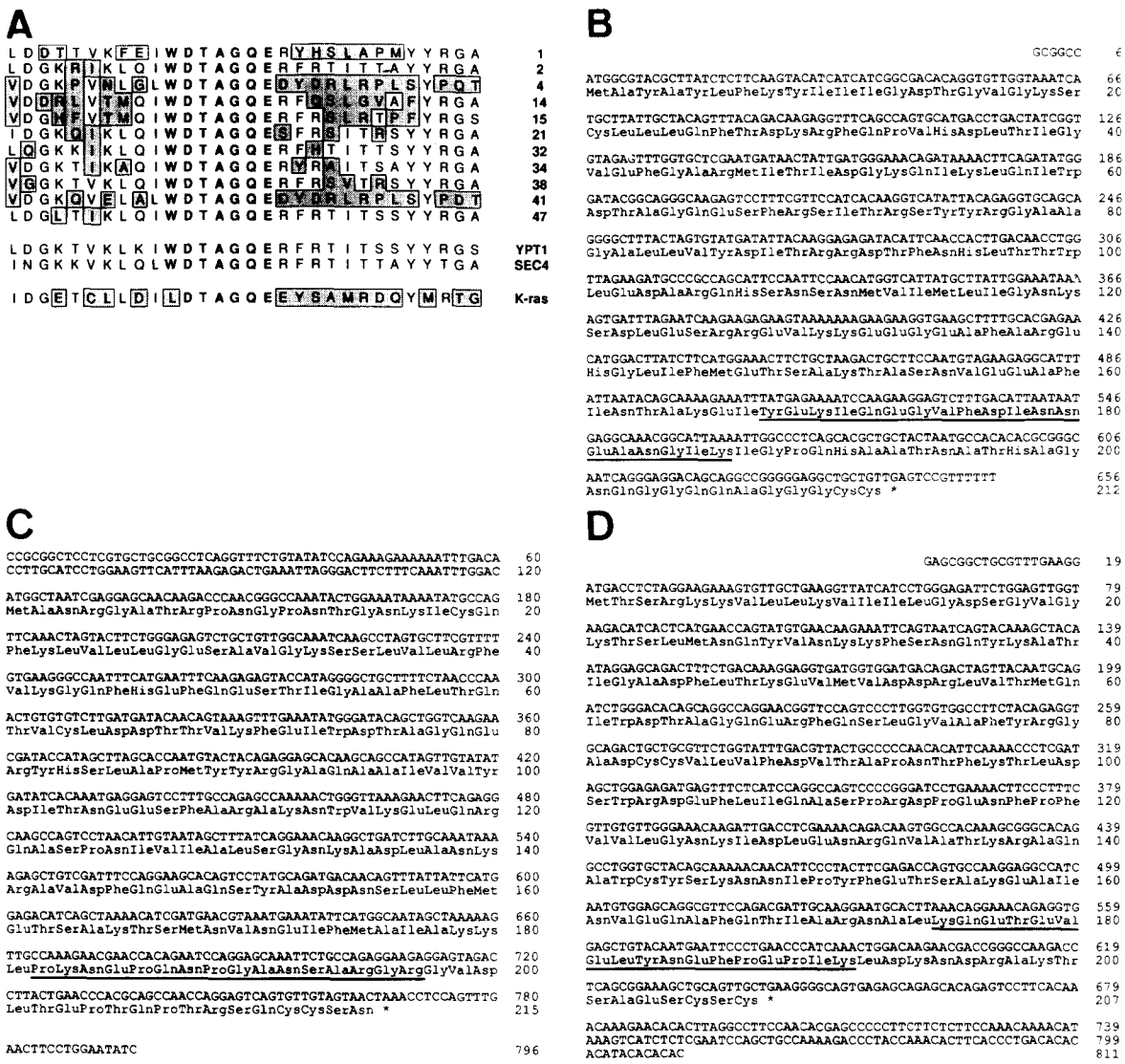

Figure 1. Comparison of Nucleotide Sequences of Low Molecular Weight GTP Binding Proteins

(A) Comparison of the WDTAGQE domain and flanking sequences of low molecular weight GTP binding proteins with YPT1 (Gallwitz et al., 1983), SEC4 (Salminen and Novick, 1987), and K-*ras* (McGrath et al., 1983). The WDTAGQE motif used to design the oligonucleotide GTP-1 is shown in bold (positions 56–62 in the K-*ras* map). Stippled areas correspond to residues that are not conserved with YPT1 and/or SEC4. Nucleotide sequences and deduced amino acid sequences of rab2 (B), rab5 (C), and rab7 (D) are shown. The sequences of the synthetic peptides used to raise antibodies are underlined.

Results

Isolation of MDCK cDNA Clones Encoding Low Molecular Weight GTP Binding Proteins Belonging to the YPT1/SEC4 Subfamily

ras and *ras*-related proteins share conserved domains that have been shown to contribute to the formation of the GTP binding site (deVos et al., 1988; Pai et al., 1989). In particular, a stretch of six residues, DTAGQE (positions 57–62 of K-*ras*), is strictly conserved and is preceded by a tryptophan residue in the case of the YPT1/SEC4 subfamily (Zahraoui et al., 1989; Didsbury et al., 1989). To study YPT1/SEC4-related proteins in an epithelial cell line, we used an oligonucleotide based on the sequence WDTAGQE (GTP-1 oligo, see Experimental Procedures) to screen 50,000 recombinant plaques of an MDCK cDNA library under low stringency conditions. From the 50 initial positive clones isolated, 19 were still positive after increasing the stringency conditions of hybridization with GTP-1 oligo and hybridized with a second oligonucleotide mixture corresponding to another conserved domain between the *ras*-related proteins (FLET-1 oligo; positions 141–146 of K-*ras*; see Experimental Procedures). Cross-hybridization analysis restricted this group to 11 nonoverlapping cDNAs whose nucleotide sequences were determined. The predicted amino acid sequences revealed that the 11 MDCK cDNAs encoded putative GTP binding proteins on the basis of the conservation of the four do-

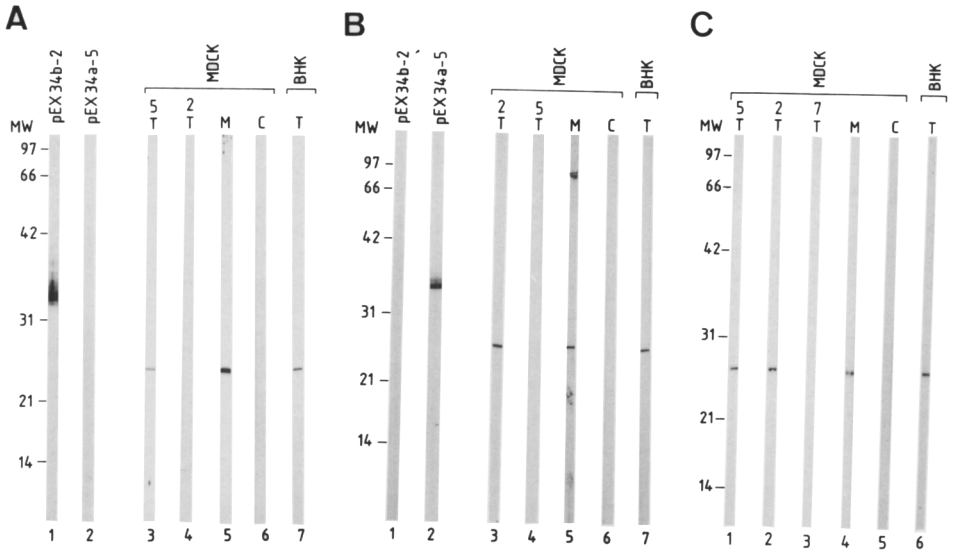

Figure 2. Immunoblot Analysis of Total Protein from E. coli Expressing MS2 Polymerase–rab Fusion Proteins and of MDCK and BHK Cell Lysates

Proteins were separated by SDS–polyacrylamide gel electrophoresis, electrophoretically transferred onto nitrocellulose filters, and incubated with affinity-purified anti-rab2 (A), -rab5 (B), and -rab7 (C) peptide antibodies. Bands were visualized using an alkaline phosphatase conjugated goat anti-rabbit antibody. The specificity of the antibodies was demonstrated by incubation with total lysates of E. coli K537 cells transformed with plasmid pEX34b-2 or pEX34a-5 induced to express MS2 polymerase–rab2 or –rab5 fusion proteins ([A] and [B], lanes 1 and 2, respectively). About 100 μg of total MDCK or BHK lysate (T), or MDCK crude membrane (M) and cytosol (C) protein fractions were incubated with the affinity-purified antibodies ([A] and [B], lanes 3–7; [C], lanes 1–6). For peptide competition experiments, antibodies were previously incubated with either of the peptides (5, 2, and 7) used for the immunization ([A] and [B], lanes 3–4; [C], lanes 1–3). Note that peptides 2 ([A], lane 4), 5 ([B], lane 4), and 7 ([C], lane 3) specifically prevent reactivity by the corresponding antisera.

mains forming the GTP binding site (data not shown; Pai et al., 1989). Figure 1A shows an amino acid sequence alignment of a 30-amino-acid long region spanning the WDTAGQE conserved domain derived from the nucleotide sequence of the 11 MDCK cDNA clones and of *YPT1*, *SEC4*, and K-*ras*. Besides a strict conservation of the WDTAGQE domain, the MDCK low molecular weight GTP binding proteins shared several common residues with YPT1p and SEC4p in the region flanking this conserved motif that were not conserved in K-*ras* (Figure 1A). When used as hybridization probes on Northern blots, none of these cDNAs appeared to be specific for MDCK cells, but recognized RNA species of the same molecular weight expressed in different cell lines (BHK, HeLa, and mouse fibroblast NIH 3T3, data not shown).

Some of the MDCK low molecular weight GTP binding proteins were identical or highly homologous to human GTP binding proteins recently isolated. The deduced amino acid sequence of protein 47 was identical to the human rab1 protein (mammalian homolog of the yeast S. cerevisiae *YPT1*; see Haubruck et al., 1987, 1989; Zahraoui et al., 1989). Protein 21 (Figure 1B) showed only one conservative change with the human rab2 protein (Ile^{144} instead of a Met in human rab2; Touchot et al., 1987), whereas protein 1 showed 98% amino acid conservation with the human rab5 protein (Zahraoui et al., 1989). Protein 14 (referred to here as rab7) showed 99% amino acid conservation with BRL-*ras* (Bucci et al., 1988) and a 6-amino-acid extension of its N-terminus (see Figure 1D for the sequence of rab7). This strong conservation prompted us to localize rab2, rab5, and rab 7 in mammalian cells using specific antibodies. All three proteins transiently expressed at high levels in BHK cells (see below) were shown to bind radiolabeled GTP when immobilized on nitrocellulose filters (data not shown).

For morphological analysis we raised antisera in rabbits against peptides located at the variable C-termini of rab2, rab5, and rab7 and that were specific for each of these proteins (Figures 1B–1D; see Experimental Procedures).

Cellular Localization of rab2 by Indirect Immunofluorescence Analysis

The affinity-purified anti-peptide antibodies raised against the C-terminus of rab2 (Figure 1B) were first characterized by immunoblot analysis. They recognized only a 22 kd species in total lysates of both MDCK and BHK cells (Figure 2A, lanes 3 and 7, respectively). This is in good agreement with the predicted molecular size of rab2 (23.545 kd). rab2 was detected in the membrane fraction of MDCK cellular extracts (Figure 2A, lane 5), whereas it was almost absent from the cytosol (Figure 2A, lane 6). This result indicates that most if not all rab2 is associated with membranes in MDCK cells.

We used this affinity-purified anti-peptide antibody for immunofluorescence microscopy of saponin-permeabilized BHK cells. The staining was restricted to vesicular structures in a small area of the cells in the vicinity and to one side of the nucleus (Figure 3A). In a few cells, the vesicular staining formed a distinct pattern around the nucleus. A similar pattern was seen in subconfluent MDCK

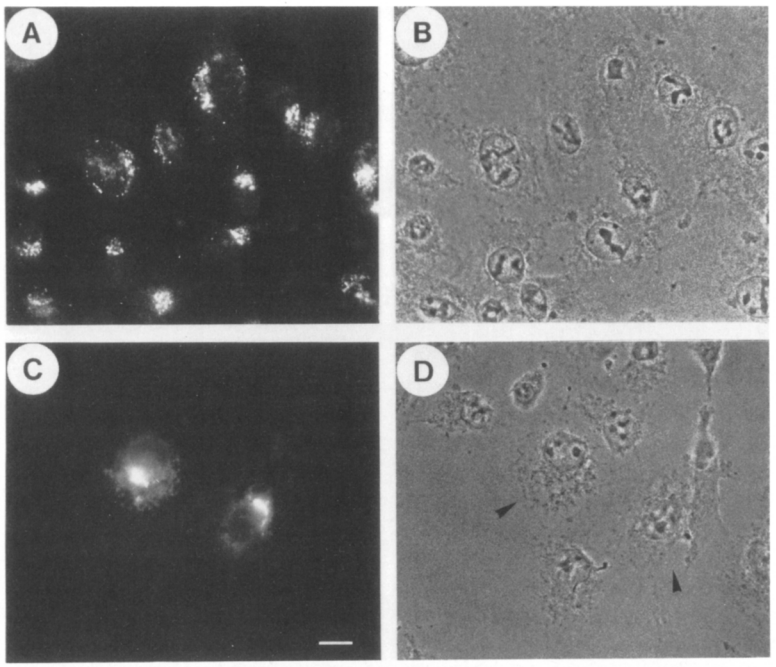

Figure 3. Immunofluorescence Localization of rab2 in Untransfected or Transfected BHK Cells

BHK cells infected with the T7 RNA polymerase–recombinant vaccinia virus and transfected with rab2 construct were permeabilized with saponin, fixed with paraformaldehyde 4 hr after transfection, and labeled with affinity-purified antibody against peptide 2. Immunofluorescence labeling of transfected cells was performed using an antibody concentration that limited detection to the cells that overexpressed the exogenous protein. After washing to remove unbound IgG, cells were incubated with rhodamine-labeled goat anti-rabbit IgG. Cells were photographed in rhodamine optics. (A) BHK cells labeled with anti-peptide 2 antibodies (dilution 1:10). (B) Corresponding phase-contrast photograph. (C) Infected/transfected BHK cells labeled with anti-peptide 2 antibodies (dilution 1:25). (D) Corresponding phase-contrast photograph. Arrowheads show positive transfected cells. Bar = 10 μm.

cells grown on cover slips (data not shown). We also used a T7 RNA polymerase–recombinant vaccinia virus (Fuerst et al., 1986) to overexpress rab2 in BHK cells to confirm its intracellular localization. For this purpose, rab2-encoding DNA sequence was inserted into plasmid pGEM1 in front of the bacteriophage T7 promoter. The exogenous protein labeled intensely structures close to the nucleus—a staining pattern similar to that of the endogenous protein. Some overexpressing cells showed an additional staining extending toward the periphery of the cell (Figure 3C). In contrast to the wild-type protein, the mutant protein rab2ΔC, lacking the C-terminal cysteines, was detected in the cytoplasm not bound to membrane structures by immunofluorescence microscopy and partitioned to the aqueous phase upon Triton X-114 extraction of the transfected cells (data not shown).

The staining pattern exhibited by the anti-peptide antibody against rab2 suggested an association of this protein with organelles in the Golgi region. This association was further studied by double immunofluorescence microscopy using a monoclonal antibody recognizing the Golgi marker 135 kd protein, which is known to be confined to

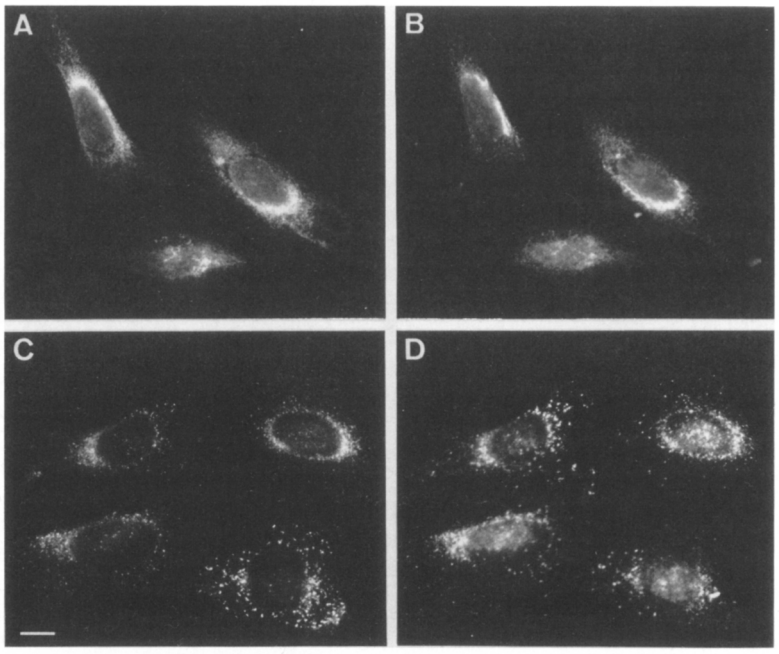

Figure 4. Effect of Nocodazole and BFA on the Distribution of 53 kd Intermediate Compartment Marker and rab2 in HeLa Cells

HeLa cells were left untreated (A and B) or incubated with nocodazole for 1 hr (C and D). Cells were permeabilized with saponin, fixed, and double-labeled for indirect immunofluorescence using monoclonal antibody G1/93 against the 53 kd protein and affinity-purified anti-peptide 2 antibodies. After washing to remove unbound IgG, cells were incubated with rhodamine-labeled goat anti-rabbit IgG to label the specifically bound anti-peptide 2 IgG and with fluorescein-labeled goat anti-mouse IgG to label specifically bound 53 kd–directed antibodies. (A and C) Cells photographed with fluorescein optics showing the distribution of the 53 kd protein. (B and D) Cells photographed with rhodamine optics showing rab2 distribution. Bar = 10 μm.

Golgi cisternae of normal rat kidney (NRK) and BHK cells (Burke et al., 1982). A comparison of the immunofluorescence patterns obtained with monoclonal antibody 53FC3 (Burke et al., 1982) and the anti-peptide antibodies against rab2 on saponin-permeabilized BHK cells showed that the staining of rab2 and 135 kd proteins overlapped in a region close to the nucleus. However, segregation of both antigens was observed in BHK cells treated with nocodazole to depolymerize the microtubules (De Brabander et al., 1976; data not shown). This result strongly argued against a localization of rab2 in the Golgi fragments stained with the monoclonal antibody against 135 kd protein, but did not rule out a possible association of rab2 with other Golgi subcompartments.

To address this possibility we performed double immunofluorescence microscopy experiments using a monoclonal antibody directed against a 53 kd protein described by Schweizer et al. (1988). This protein resides in a putative intermediate compartment composed of vesicles and tubular structures between the ER and Golgi. HeLa cells were used in this experiment since G1/93 monoclonal antibody against 53 kd protein stains only cells of human and simian origin. In HeLa cells G1/93 gave a punctate and reticular staining on one side of the nucleus (Figure 4A), in agreement with the published results of Schweizer et al. (1988). It was also possible to detect labeling of numerous small vesicles up to the periphery of the cell. A very similar pattern was observed with the anti-peptide antibody directed against rab2 (Figure 4B). In HeLa cells treated with nocodazole for 1 hr before fixation, the 53 kd protein appeared mainly in dispersed structures throughout the cytoplasm and most, although not all, of these structures also contained rab2 (compare Figures 4C and 4D). To confirm that rab2 distribution overlapped with the location of the 53 kd protein in a putative "intermediate" compartment between ER and Golgi, HeLa cells were treated with brefeldin A (BFA). BFA induces the rapid redistribution of *cis/medial/trans* Golgi proteins into the ER and causes disassembly of the Golgi apparatus (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989, 1990; Doms et al., 1989). Recent studies by Lippincott-Schwartz et al. (1990) have provided evidence that the intermediate compartment marked by the 53 kd protein is not disassembled during BFA treatment. We therefore tested whether the distribution of rab2 remained colocalized with the 53 kd protein during BFA treatment. After 1 hr of BFA treatment, the 53 kd distribution was similar to control cells, and most of these structures also showed rab2 staining (data not shown). These results suggest that at least part of rab2 is associated with a putative "intermediate" compartment on the route between ER and Golgi apparatus.

Immunoelectron Microscopic Localization of rab2 to a Tubulovesicular Compartment Close to the Golgi Apparatus

To extend the immunofluorescence microscopic localization of rab2, we carried out double-labeling immunoelectron microscopy on ultrathin frozen sections of HeLa cells with the affinity-purified antipeptide antibody against rab2 and the monoclonal antibody against the 53 kd protein (Figure 5). Labeling with the anti-rab2 colocalized precisely with the 53 kd protein in tubulovesicular structures in close proximity to the Golgi stack. Labeling was also seen on one Golgi cisterna, previously shown to be the *cis* cisterna for the 53 kd protein (Schweizer et al., 1988). Very low or negligible labeling was observed on other cisternae of the Golgi and on the *trans* Golgi network, as identified in virus-infected cells incubated at 20°C (data not shown). The specificity of the anti-rab2 labeling was shown by competition with the specific peptide to which the antibody was raised.

Cellular Localization of rab5 and rab7

The cellular localization of rab5 and rab7 was studied by indirect immunofluorescence microscopy using affinity-purified antibodies raised against specific peptides derived from the C-terminal ends of the two proteins (see Figures 1C and 1D). The specificity of both affinity-purified antibodies was demonstrated by immunoblotting analysis. Figure 2B shows that the affinity-purified antibody against peptide 5 recognized one species with an apparent molecular size of ~27 kd (predicted molecular size = 23.658 kd) in both BHK and MDCK total extracts (Figure 2B, lanes 3 and 7). rab5 could only be detected in the membrane fraction from MDCK cells (lanes 5 and 6). We also verified that the affinity-purified anti-peptide antibody raised against the C-terminal end of rab7 specifically recognized a single band of about 25 kd in total MDCK extracts (predicted molecular size = 23.519 kd; see Figure 2C, lanes 1 and 2). Although the majority of rab7 was associated with the membrane fraction, a faint signal could be detected in the soluble fraction from MDCK cells, suggesting that a minor proportion of this protein is cytosolic (Figure 2C, lanes 4 and 5).

Figure 6 shows the immunofluorescence pattern of rab5 on BHK (Figure 6A) and MDCK cells (Figure 6C). The protein was mainly localized at the plasma membrane. The peripheral staining was not uniform but rather formed a kind of fine reticular network. The plasma membrane was clearly labeled and the staining was especially intense in cellular processes. In addition, rab5 was also detected on numerous small vesicular structures of different sizes spread throughout the cytoplasm and giving rise to a punctate pattern. Those dots were particularly abundant in the region above the nucleus. In MDCK cells (Figure 6C) the lateral cell–cell contacts were clearly labeled. When rab5 was overexpressed in BHK cells using the transient T7 RNA polymerase–recombinant vaccinia virus expression system, the plasma membrane staining was not dramatically enhanced, rather the exogenous protein accumulated in slightly larger vesicular structures dispersed in the cytoplasm and concentrated above the nuclear region (Figure 6B). Similar to rab2ΔC, rab5ΔC, lacking the C-terminal cysteines (see Experimental Procedures), was found to be cytosolic (data not shown). These results indicate that the membrane association of rab2 and rab5 requires the presence of the C-terminal conserved cysteines as previously shown for the yeast YPT1p and SEC4p (Molenaar et al., 1988; Walworth et al., 1989).

The immunofluorescence pattern of rab7 was different

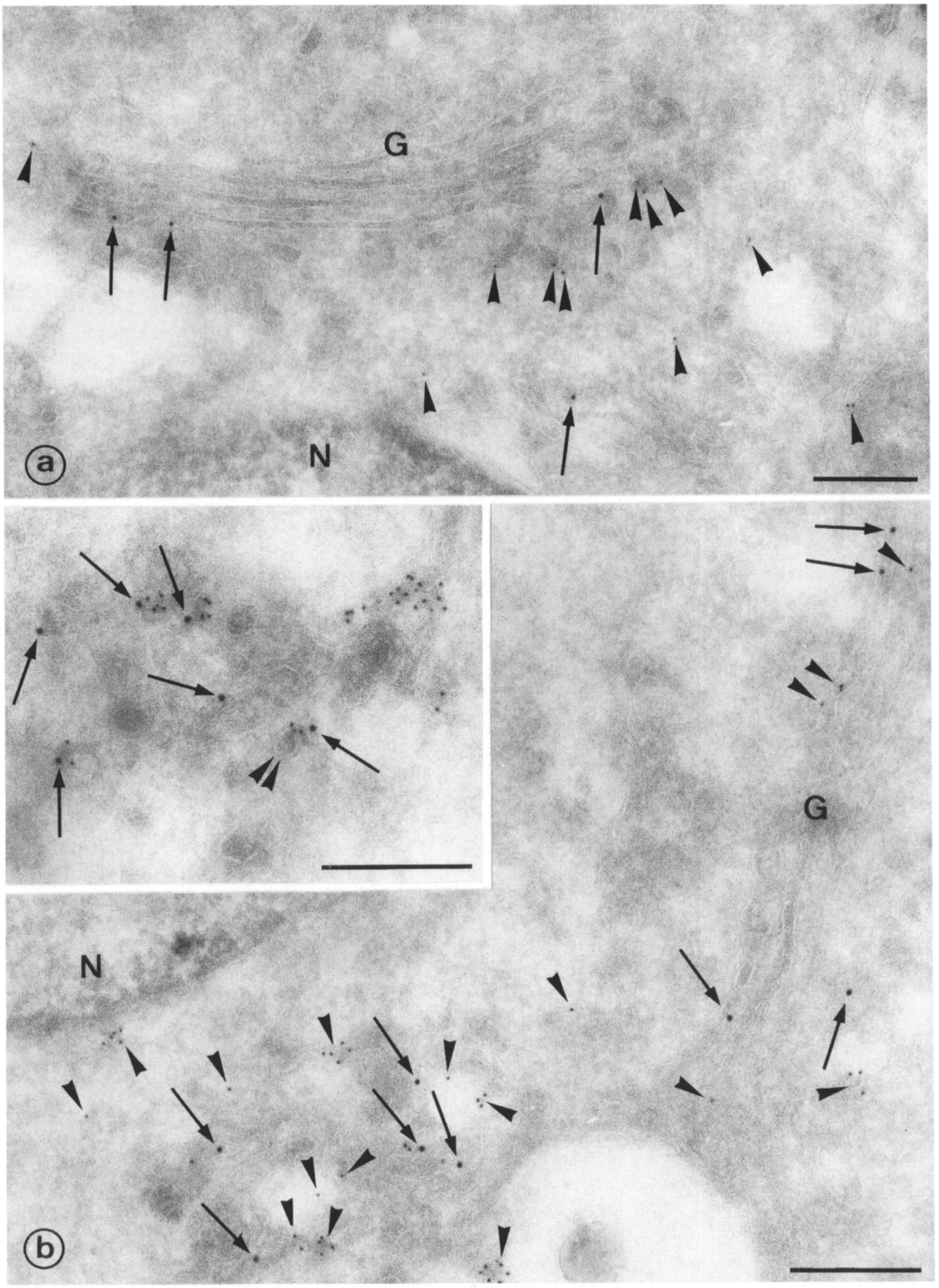

Figure 5. Immunoelectron Microscopic Localization of rab2 in HeLa Cells

Frozen sections of HeLa cells were double-labeled with affinity-purified anti-rab2 peptide antibodies followed by 9 nm protein A–gold and with G1/93 followed by a rabbit anti-mouse antibody and then 6 nm protein A–gold.

(a and b) Double-labeling of structures principally on one side of the Golgi stacks (G) and close to the nucleus (N) with the anti-rab2 (large gold, indicated by arrows) and anti-53 kd antibody (small gold; individual gold particles or groups indicated by arrowheads). Labeling with the anti-rab2 was restricted to one (*cis*) cisterna, which shows low but specific labeling with the anti-53 kd antibody, and to tubulovesicular structures (lower part of [b]) in the Golgi area, which showed heavier labeling with the anti-53 kd antibody. Such an area containing tubules and vesicular profiles (double arrowhead) is shown at higher magnification in the inset (anti-rab2 labeling indicated by arrows). Bars = 200 nm.

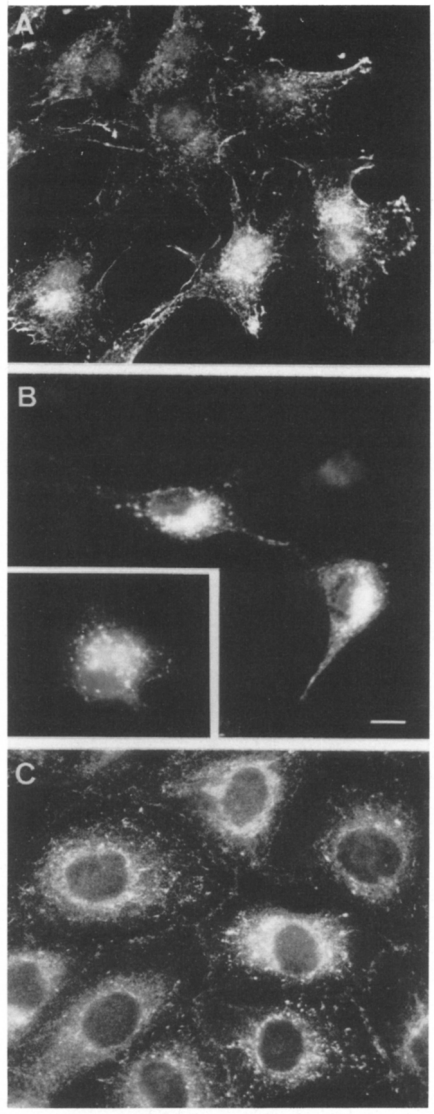

Figure 6. Immunofluorescence Microscopy Localization of rab5 in BHK and MDCK Cells

BHK cells, untransfected (A) or transfected with a construct expressing rab5 using the T7 RNA polymerase–recombinant vaccinia virus–mediated system (B) were permeabilized with saponin and then fixed with paraformaldehyde. MDCK cells (C) were fixed as described by Bacallao et al. (1989) and permeabilized with Triton X-100. Cells were incubated with affinity-purified anti-peptide 5 antibodies. Specifically, bound IgG were labeled with rhodamine-conjugated goat anti-rabbit IgG, and the cells were photographed with rhodamine optics. (B) shows two different fields. Bar = 10 μm.

from that of rab5 (Figure 7). rab7 was associated with large vesicular-tubular structures that were predominantly localized close to one side of the nucleus, in both BHK and MDCK cells (Figures 7A and 7B, respectively).

Immunoelectron Microscopy Reveals an Association of rab5 and rab7 with Two Distinct Subcompartments of the Endocytic Pathway

When we used the affinity-purified antibody specific for rab5 on frozen ultrathin sections of BHK cells, we observed a low but significant labeling on the cytoplasmic side of the plasma membrane and with peripherally located tubulocisternal structures with the appearance of early endosomes (Gruenberg et al., 1989; Griffiths et al., 1989). To examine whether the labeled compartments were indeed endosomes, two sizes of bovine serum albumin (BSA)–gold particles were internalized for different times to label early and late endosomes differentially (Griffiths et al., 1988, 1989). rab5 was specifically associated with early, but not late, endosomes and with the plasma membrane (Figure 8a). Occasionally, labeling was also seen on coated pits (data not shown). Moreover, immunogold labeling on both the plasma membrane and early endosome structures was specifically enhanced in BHK cells overproducing rab5 with the T7 RNA polymerase–recombinant vaccinia virus system as described above (Figures 8b–8e). We observed no difference in the distribution of label between untransfected and transfected cells.

In contrast, rab7 appeared predominantly in large vesicular structures and colocalized with BSA–gold particles internalized under conditions in which late endocytic compartments were labeled (Figure 9a; Griffiths et al., 1989). To investigate whether rab7 was associated with late endosomes or lysosomes, double-labeling was performed on sections of NRK cells in which the late endosomes could be identified by labeling with an antibody to the cation-independent mannose 6-phosphate receptor (CI-MPR) (Griffiths et al., 1988). rab 7 labeling colocalized precisely with the distribution of the CI-MPR (Figures 9a and 9b). rab7 was only found associated with the CI-MPR–positive late endosomes, while the CI-MPR–negative lysosomes, which contained internalized BSA–gold, were invariably devoid of labeling (an example is shown in Figure 9b).

Discussion

A number of cDNAs coding for GTP binding proteins have

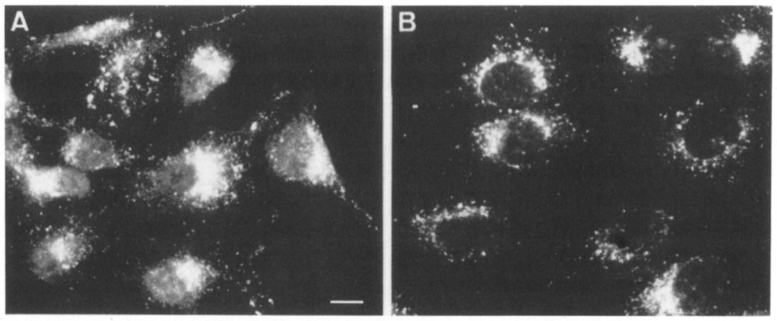

Figure 7. Immunofluorescence Microscopy Localization of rab7 in BHK and MDCK Cells

BHK cells (A) were permeabilized with saponin and then fixed with paraformaldehyde. MDCK cells (B) were fixed as described by Bacallao et al. (1989) and permeabilized with Triton X-100. Cells were incubated with affinity-purified anti-peptide 7 antibodies. Specifically, bound IgG were labeled with rhodamine-conjugated goat anti-rabbit IgG, and the cells were photographed with rhodamine optics. Bar = 10 μm.

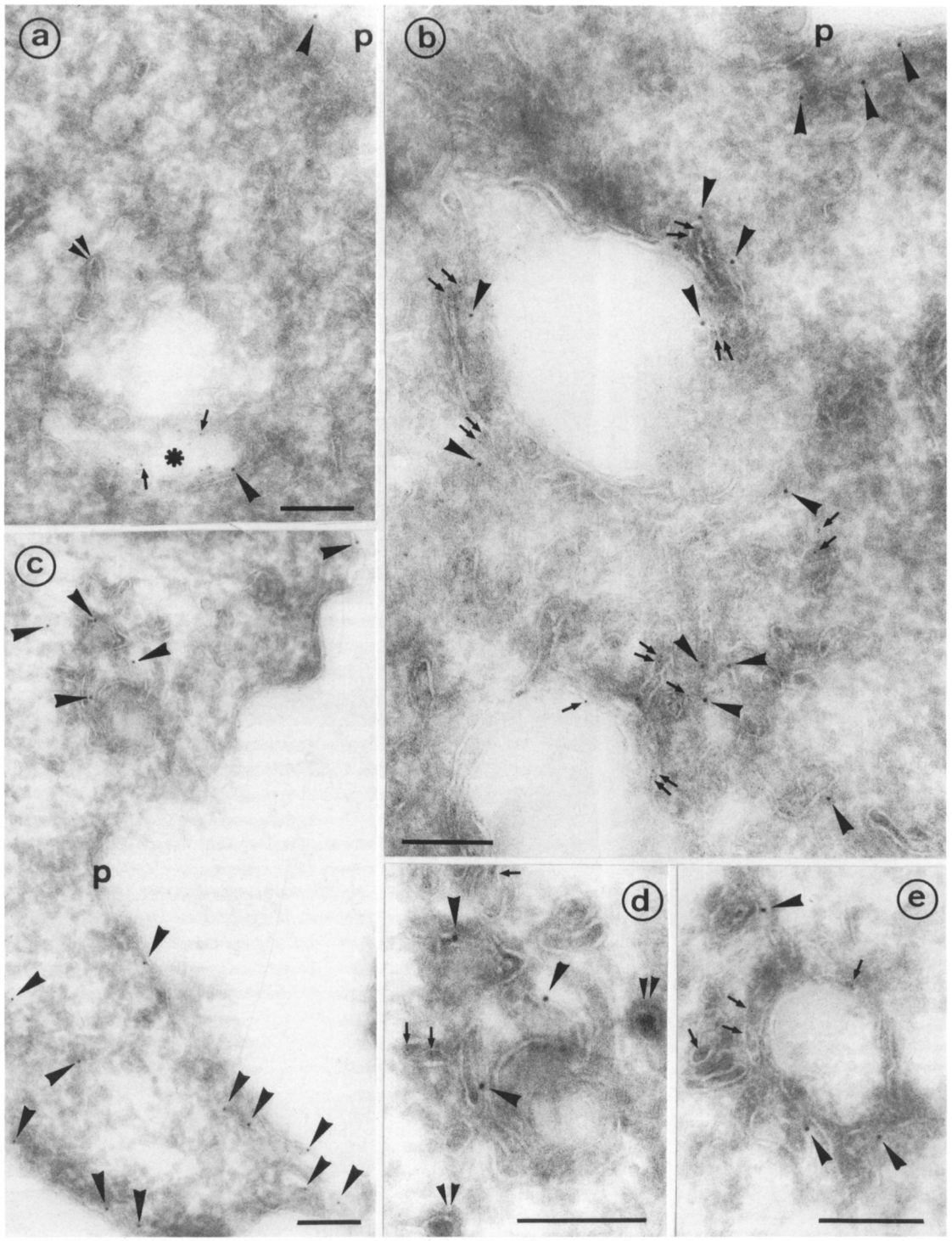

Figure 8. Immunoelectron Microscopy Localization of rab5 in Untransfected and Transfected BHK Cells

BHK cells were transfected with rab5 using a vaccinia transient expression system as described in the Experimental Procedures. The cells were incubated with 4 nm BSA–gold for 8 min to label early endosomes. Sections were then labeled with anti-rab5 (anti-peptide) and 9 nm protein A–gold.
(a) Low, but specific labeling (arrowheads) is apparent on the plasma membrane (p) and on an early endosome (asterisk) of a nontransfected cell.
(b) Increased labeling with the anti-rab5 in a transfected cell. Anti-rab5 labeling (arrowheads) is evident close to the plasma membrane (p) and associated with membranes of the early endosomal structures, which contain internalized BSA–gold (arrows).
(c) Labeling close to the plasma membrane (p) and associated with an early endosome.

A higher magnification view of the early endosome in (c) is shown in (d) and a further example is shown in (e). Note the association of gold particles with the cytoplasmic surface of the early endosomal structures, which contain the internalized BSA–gold. Double arrowheads in (a) and (d) indicate small coated buds. Bars = 200 nm.

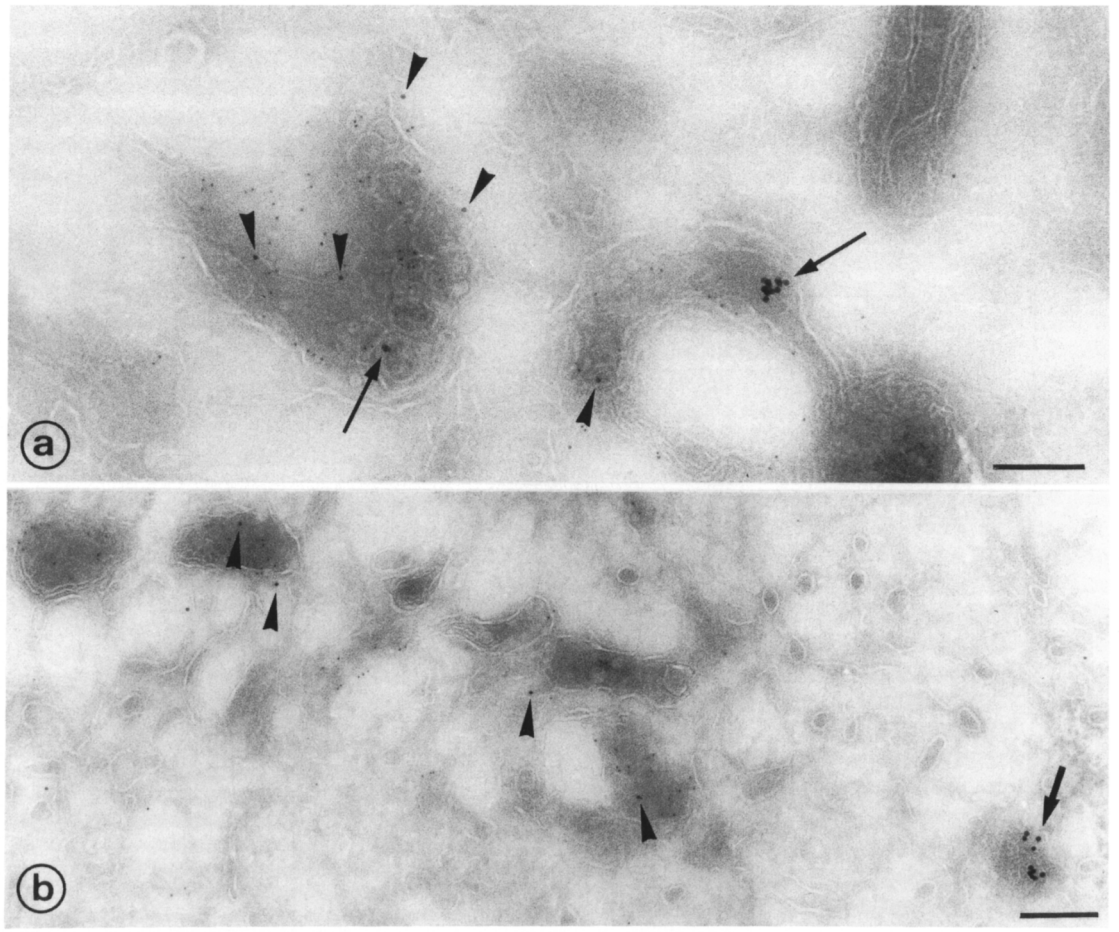

Figure 9. Double-Labeling with Anti-rab7 and Anti–CI-MPR of NRK Cells Containing Internalized BSA–Gold

NRK cells were incubated with 16 nm BSA–gold to label late endocytic structures as described in Experimental Procedures. Cryosections were prepared and double-labeled with antibodies to the CI-MPR followed by 6 nm protein A–gold and with anti-rab7 and 9 nm protein A–gold. The anti-rab7 (arrowheads) labels structures enriched for CI-MPR (small gold), some of which contain the internalized BSA–gold (arrows). As evident in (a), labeling with anti-rab7 was predominantly on the cytoplasmic surface of the late endosomes. However, specific labeling was also observed in the internal membranes of these structures (see Griffiths et al., 1988). A CI-MPR–negative lysosome containing internalized gold is indicated in (b) (arrow). Bars ≈ 200 nm.

been isolated from various mammalian systems. Some deduced amino acid sequences were found to be homologous to those of the *YPT1* and *SEC4* gene products known to regulate protein secretion in yeast (Touchot et al., 1987; Zahraoui et al., 1989; Didsbury et al., 1989). Little is known about the localization or the function of these mammalian GTP binding proteins. In this report we provide evidence that three GTP binding proteins belonging to the YPT1/SEC4 subfamily are associated with different subcompartments of the exocytic or endocytic pathways in mammalian cells. Both immunofluorescence light and immunoelectron microscopy studies revealed that rab2 was predominantly located in a putative intermediate compartment between the ER and Golgi apparatus and on the *cis* cisternae of the Golgi. In contrast, rab5 and rab7 were found associated with compartments along the endocytic pathway: the former was detected at the cytoplasmic surface of both the plasma membrane and early endosomes, whereas the latter was associated with late endosomes.

The finding of significant labeling for rab2 on the *cis*-most cisterna of the Golgi as well as on Golgi-associated tubulovesicular elements suggests that rab2 is exerting its function in the early part of the exocytic pathway. Another GTP binding protein was also found in the Golgi area in mammalian cells by fluorescence microscopy using an antibody directed against YPT1p (Segev et al., 1988). This protein probably corresponds to the mammalian counterpart of YPT1 (Touchot et al., 1987; Haubruck et al., 1987; protein 47, this report), which was recently found to be required for protein transport from ER to Golgi complex in yeast extracts (Baker et al., 1990). *SAR1*, a yeast S. cerevisiae gene identified as a multicopy suppressor of a *sec12* mutation (Nakano and Muramatsu, 1989), encodes another GTP binding protein recently implicated in early steps of protein secretion. The association of several distinct GTP binding proteins with the ER to Golgi pathway supports several recent observations that imply that traffic from the ER is more complex than previously thought.

These data indicate that there is an intermediate compartment between the ER and the Golgi complex (see Pelham, 1989). This compartment, also referred to as the salvage compartment (Warren, 1987), is thought to function in the recycling of soluble resident ER proteins to the ER. The 58 kd glycoprotein described by Saraste et al. (1987) and the 53 kd protein (Schweizer et al., 1988) have been localized to this compartment. Recent results suggest that retrograde movement of Golgi markers might occur through the intermediate compartment to the ER in the presence of BFA (Lippincott-Schwartz et al., 1990). The colocalization we observed between rab2 and the 53 kd marker suggests that rab2 plays a role in transport to or from the intermediate compartment.

It is also clear that the endocytic pathway can be subdivided into functionally distinct subcompartments (Kornfeld and Mellman, 1989; Gruenberg and Howell, 1989). These include: the plasma membrane from which coated pits bud to form coated vesicles that presumably fuse with early endosomes located at the cell periphery; early endosomes, where internalized molecules either recycle back to the plasma membrane or are delivered to the next step in the endocytic pathway, late endosomes; and the final compartment, lysosomes, where the bulk of degradation occurs. rab5, which was observed both on the plasma membrane and in early endosomes and occasionally on coated pits, might be engaged in endocytosis, in recycling from early endosomes, or both. rab7 seems to be involved in a later step of the endocytic route. It could link early to late endosomes, late endosomes to lysosomes, or connect late endosomes with the *trans* Golgi network, a transport pathway for which the involvement of GTP hydrolysis has been previously reported (Goda and Pfeffer, 1988).

Our results show that specific GTP binding proteins are associated with different steps of membrane traffic. They support Bourne's hypothesis (1988), which suggests that different transport GTPases may ensure the unidirectional transfer of carrier vesicles in protein and lipid transport. Besides the three low molecular weight GTP binding proteins characterized here, sequences encoding eight additional GTP binding proteins have been isolated from the MDCK cDNA library. If these proteins are involved in transport processes, we would end up with an unexpected complexity compared with previous results obtained using a similar oligonucleotide screening approach (Touchot et al., 1987). Moreover, these 11 cDNAs were isolated from 50,000 recombinant clones representing only 30% of a complete MDCK cDNA library, and 9 clones were found only once during our screening. Thus, the possibility exists that this family of GTP binding proteins may comprise more than 30 different members in MDCK cells. Such complexity could be explained by the special organization of epithelial cells, which are polarized into apical and basolateral surface domains (Simons and Fuller, 1985). A larger number of such GTP binding proteins would be required to generate and maintain polarity in their exocytic and endocytic pathways. An open question is whether, in MDCK cells, rab5 is expressed in the apical or the basolateral early endosomes or in both. Our previous work has demonstrated that the apical and the basolateral endocytic circuits are kept distinct at the level of the early endosomes but that the pathways meet in the late endosomes (Parton et al., 1989; Bomsel et al., 1989). We might therefore expect rab5, but not rab7, to show a polarized distribution in epithelial cells. These questions have to be addressed by future work in filter-grown MDCK cells by both immunocytochemistry and functional studies.

The availability of cDNAs and antisera should make it possible to address the function of these proteins in their specific compartments. Constructing dominant mutations of this class of GTP binding proteins will be an important tool in further studies (Schmitt et al., 1986; Walworth et al., 1989).

Experimental Procedures

cDNA Library Screening and Plasmid Constructions

We prepared an oriented lambda MDCK cDNA library cloned in UNI-ZAP™XR (Stratagene) with an average insert size of 2 kb. The library was screened with a degenerate oligonucleotide (GTP-1 oligo) based on the conserved sequence Trp-Asp-Thr-Ala-Gly-Gln-Glu between SEC4 (Salminen and Novick, 1987), YPT1 (Gallwitz et al., 1983), and the different rab proteins (Touchot et al., 1987; Zahraoui et al., 1989). The sequence of the GTP-1 oligo was as follows: 5′-TGGGA(C_{50}/T_{50})AC(A_{70}/C_{10}/T_{10}/G_{10})GC(T_{30}/A_{70})GG(A_{25}/G_{25}/C_{25}/T_{25})CA(G_{20}/A_{80})GAA-3′ (numbers in subscript refer to the relative frequency of each base at a given position). A total of 5×10^4 phage plaques were analyzed. Duplicate filters were prehybridized for 1 hr at 42°C in a 6× SSC, 5× Denhardt's, 0.05% sodium pyrophosphate, 0.5% sodium dodecyl sulfate solution, containing 100 μg/ml boiled herring sperm DNA. Hybridization was performed for 18 hr at 42°C in a 6× SSC, 1× Denhardt's, 0.05% sodium pyrophosphate solution containing 100 μg/ml yeast tRNA with 25 pmol/ml GTP-1 oligo labeled with T4 polynucleotide kinase (Biolabs). Filters were then washed for 3 hr at 44°C in a 6× SSC, 0.05% sodium pyrophosphate solution. Phage DNAs from the positive plaques were extracted, digested with EcoRI and XhoI restriction enzymes, and transferred to a Genescreen plus membrane (DuPont Co., Wilmington, Delaware). General nucleic acids methods were essentially as described in Maniatis et al. (1982). Duplicate filters were hybridized in the previous conditions with GTP-1 oligo at 42°C or at 37°C with FLET-1 oligo: 5′-TT(T_{50}/C_{50})(T_{50}/A_{50})T(G_{50}/T_{50})GA(A_{25}/G_{75}) (A_{75}/G_{25})C(A_{50}/C_{30}/G_{10}/T_{10})(A_{75}/T_{25})(G_{75} /C_{25})(T_{75}/C_{25})GC-3′. The GTP-1 oligo–hybridized filter was washed for 1 hr at 60°C in the conditions described above; in the case of the FLET-1 oligo, washing was performed at 25°C. Eleven clones hybridizing with both oligonucleotides were further characterized. Cross-hybridization analysis performed in high stringency conditions with the human rab cDNAs as probes (Touchot et al., 1987; Zahraoui et al., 1989) revealed that clones 1, 21, and 47 were highly homologous to rab5, rab2, and rab1, respectively. Rescue of the cDNA inserts from the UNI-ZAP™XR vector was performed by in vivo excision according to the manufacturer's procedure (Stratagene). Phagemid DNAs were prepared and used directly for double-stranded DNA sequencing with the T7 Sequencing™ Kit (Pharmacia) using a set of different primers including the GTP-1 and FLET-1 degenerate oligonucleotides. The complete sequences of the 11 MDCK clones will be published elsewhere and are available upon request (Chavrier et al., submitted).

Construction of the C-terminal deletion mutants was as follows. rab5 cDNA cloned in Bluescript (Stratagene) was restricted with AccI (position 716 of the cDNA) and the ends rejoined using the following oligonucleotides: 5′-AGACCTTACTGAACCCACGCAGTAA-3′/5′-CGTTACTGCCTGGGTTCAGTAAG GT-3′. This introduced a stop codon after Gln^{206} and created rab5ΔC. rab2-encoding cDNA cloned in pGEM1 (Promega) was restricted with SpeI (position 255 of the cDNA) and HindIII (in the polylinker of pGEM1) and ligated with a SpeI–HpaII fragment (positions 255–626 of the cDNA) together with the following oligonucleotides: 5′-AGGGGGAGGCTA-3′/5′-AGCTTAGCCTCCCC-3′ with HpaII–HindIII compatible ends. This introduced a stop codon after Gly^{210} and created rab2ΔC. The sequence of the carboxyl end of the mutant constructs was verified by double-stranded DNA sequencing.

Preparation of Antisera against rab2, rab5, and rab7

Polyclonal antibodies were raised against synthetic peptides covalently coupled to keyhole limpet hemocyanin (Calbiochem) (Kreis, 1986); the peptide sequence deduced from rab5 cDNA was PKNEPQNPGANSARGR (positions 182–197), from rab7 cDNA, KQETEVELYNEFPEPIK (positions 175–191), and from rab2 cDNA, YEKIQEGVFDINNEEANGIK (positions 158–176).

In the case of rab5 and rab2, affinity purification was performed by absorption onto bacterially expressed MS2 polymerase–rab5 and –rab2 fusion proteins immobilized on nitrocellulose filters. For this purpose, a 1300 bp TaqI–SacI fragment containing the coding region of rab5 starting from the Arg residue at position 3 was inserted in the EcoRI site of plasmid pEX34a (Klinkert et al., 1988) after filling in the ends of both the insert and the vector using T4 DNA polymerase and the Klenow fragment of Escherichia coli DNA polymerase I, respectively. The plasmid obtained allowed the temperature-inducible expression in E. coli K537 cells of 99 N-terminal amino acids of the phage MS2 polymerase fused at the C-terminus to the coding region of rab5. In the case of rab2, a 700 bp EcoRI fragment was inserted in the EcoRI site of the pEX34b polylinker; the fusion protein encoded by this plasmid contained 9 extra amino acids between the MS2 polymerase fragment and rab2, owing to the 5′ untranslated region of cDNA 21, downstream of its EcoRI adaptor. Both fusion proteins were expressed and extracted as described (Klinkert et al., 1988), purified on a 10% polyacrylamide gel, and electrotransferred onto nitrocellulose filters. In the case of the anti-rab7, affinity purification could be carried out over a matrix of peptide 7 coupled to Sepharose 4B. Immobilization of the peptide to cyanogen bromide–activated Sepharose 4B (Pharmacia) was performed in coupling buffer (0.2 M $NaHCO_3$ [pH 8.5], 0.5 M NaCl) at 4°C.

For affinity purification, antisera were first cleared by absorption at 4°C on a Sepharose 4B matrix coupled to a bacterial lysate of K537 cells transformed with plasmid pEX34b and temperature induced to express the MS2 polymerase polypeptide. This step was necessary to remove antibodies recognizing this and other immunologically unrelated proteins. Specific antibodies were subsequently bound at 4°C on their corresponding MS2 polymerase–rab fusion proteins or peptide column and eluted in 100 mM glycine–HCl (pH 2.8), neutralized by addition of 3 M Tris–HCl (pH 8.8). Affinity-purified antibodies were supplemented with 0.5% IgG-free BSA (Sigma) and 0.02% NaN_3 and stored at 4°C.

For immunoblots, MDCK or BHK cells were lysed in standard SDS sample buffer and extracts separated by electrophoresis on a 12% polyacrylamide gel. For crude membrane and cytosol fraction preparation, MDCK cells were resuspended in 10 mM HEPES (pH 7.0), 250 mM sucrose in the presence of a cocktail of protease inhibitors, sheared through a needle, and centrifuged at 3000 rpm for 10 min in an Eppendorf centrifuge to remove the nuclei. Supernatant was then centrifuged at 55,000 rpm in a TLA100.2 rotor at 4°C in a tabletop Beckman TL-100 ultracentrifuge. Protein content of both pellet and high-speed supernatant was determined using the Bio–Rad protein assay.

Separated proteins were transferred onto nitrocellulose filters and stained using the affinity-purified antibodies followed by goat anti-rabbit alkaline phosphatase conjugated antiserum (Bio–Rad) diluted in 5% milk powder, 0.3% Tween 20 in phosphate-buffered saline (PBS).

Cell Culture and Transfection

BHK 21 and NRK cells were grown in Glasgow's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, and 10% tryptose phosphate broth at 37°C in 5% CO_2 incubator. HeLa cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin at 37°C in 10% CO_2 incubator. MDCK strain II cells were grown as described (Bacallao et al., 1989). When required, the cells were treated with 20 μg/ml nocodazole or 10 μg/ml BFA.

The cells were split 18–24 hr before transfection so that on the day of transfection they were about 80% confluent. The cells were washed twice with serum-free medium and infected with T7 RNA polymerase–recombinant vaccinia virus (Fuerst et al., 1986). Infections were carried out with 3–5 pfu/cell at room temperature for 30 min with intermittent agitation. The cells were then washed twice with serum-free medium and transfected using Lipofectin™ reagent according to the manufacturer's instructions (Bethesda Research Laboratories). Cells were incubated for 4 hr at 37°C in 5% CO_2 incubator and processed for immunofluorescence microscopy or immunoelectron microscopy. At this stage the overexpressed proteins partitioned to the detergent phase upon Triton-X114 extraction of the transfected cells, while they were detected in the aqueous phase only after 6 hr of transfection (data not shown).

Immunofluorescence Microscopy

Cells were grown on 10 mm round cover slips for 24 hr prior to treatment. HeLa and BHK cells were washed once with PBS and permeabilized with 0.5% saponin in 80 mM K-PIPES (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$ for 5 min. The cells were fixed with 3% formaldehyde in PBS (pH 7.4) for 15 min. After fixation the cells were washed with 0.5% saponin in PBS for 5 min, and free aldehyde groups were quenched with 50 mM NH_4Cl in PBS for 10 min. The cells were washed with 0.5% saponin in PBS for 5 min and then incubated with the first antibody in PBS, 0.5% saponin for 20 min. After rinsing the cells three times (15 min total), primary antibody binding was visualized with goat anti-rabbit RITC or goat anti-mouse FITC diluted in 0.5% saponin–PBS for 20 min. After one wash in PBS–0.5% saponin and three washes in PBS (20 min total), the cover slips were mounted on glass slides in moviol and viewed with a Zeiss Axiophot Photomicroscope. MDCK cells were fixed as described by Bacallao et al. (1989) and then processed for immunofluorescence staining as described above. For double immunofluorescence, primary antibodies or secondary antibodies were added together.

Incubation with Endocytic Markers

To label early endosomes, BHK cells were incubated with 4 nm BSA–gold ($OD_{520} \approx 10$) for 8 min at 37°C (Griffiths et al., 1989). Late endocytic structures were labeled with 16 nm BSA–gold by incubation with the gold ($OD_{520} \approx 0.5$) for 3 hr followed by an overnight chase in marker-free medium. At the end of the incubations, the cells were transferred to ice and removed from the dish as described below.

Immunoelectron Microscopy

Cells were removed from the culture dishes according to Green et al. (1981). They were then fixed with 8% paraformaldehyde in 250 mM HEPES (pH 7.35), prepared for cryosections, and labeled with antibodies and protein A–gold as described previously (Griffiths et al., 1984, 1985).

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GenBank Accession Number

The accession numbers for the sequences of rab2, rab5, and rab7 are M35521, M35520, and M35522, respectively.