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The small GTP-binding protein rab4 is associated with early endosomes

(endocytosis/ras-like protein/cell cycle)

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ABSTRACT **Small GTP-binding proteins of the rab family have been implicated as playing important roles in controlling membrane traffic on the biosynthetic and endocytic pathways. We demonstrate that a distinct rab protein, rab4p, is associated with the population of early endosomes involved in transferrin-receptor recycling. An antibody to human rab4p was found to detect a doublet of ≈24-kDa proteins on immunoblots from various cell types. Seventy-five percent of these proteins were tightly membrane bound and could be released only by detergent treatment. Upon isolation of early endosomes, late endosomes, and lysosomes, by free-flow electrophoresis and Percoll density-gradient centrifugation, most (70%) of the rab4p was found to cofractionate with early endosomes and endocytic vesicles containing ^{125}I-labeled transferrin. The rab proteins previously localized to the endoplasmic reticulum and/or Golgi apparatus were not found in these fractions. We also localized rab4p to transferrin-receptor-containing early endosomes by immunofluorescence after expression of rab4 cDNA. The association of rab4p with early endosomes and other vesicles involved in the intracellular transport of transferrin receptor suggests that rab4p may play a role in regulating the pathway of receptor recycling.**

Small GTP-binding proteins of the rab family (1, 2) are the mammalian homologs of *SEC4* and *YPT1*, two genes known to play critical roles on the secretory pathway in yeast (3–8). It is likely that the rab proteins are similarly involved in biosynthetic membrane transport in animal cells, given the localization of rab1p, rab2p, and rab6p in the endoplasmic reticulum (ER) and/or the Golgi apparatus (9, 10) as well as the inhibition of ER to Golgi transport *in vitro* by rab-derived peptides (11). rab3p has also been found associated with synaptic vesicles (12, 13). Far less is known about involvement of rab proteins on the endocytic pathway. A possible role might be inferred, however, from the inhibitory action of guanosine 5′-[γ-thio]triphosphate on certain *in vitro* assays of endosome fusion (14–16) and from recent immunocytochemical data suggesting the association of rab5p and rab7p with different populations of endosomes (10).

Although their actual activities remain unknown, small GTP-binding proteins are thought to function in a variety of events during membrane transport, including the docking of a vesicle with its intended target, vesicle formation, and possibly the removal of cytoplasmic coat material (3, 17–19). Each transport step may be associated with a different small GTP-binding protein, perhaps accounting for the fact that at least 15 members of the mammalian rab family have been identified thus far (10, 20). To define the functions of these proteins, it will be necessary to first establish their intracellular distribution.

Receptor-mediated endocytosis is characterized by formation and fusion of a variety of vesicles and organelles (21, 22), suggesting that multiple rab proteins may be needed to mediate this pathway alone. In early endosomes, bound ligands are discharged and the receptors returned to the plasma membrane via recycling vesicles. In contrast, dissociated ligands are transferred to a distinct population of endosomes—late endosomes—and then to lysosomes where they are degraded. Although incompletely understood, it is clear that each step on the pathway must involve one or more fusion/fission events (21, 22). We were thus interested whether additional rab proteins were associated with specific subpopulations of organelles on the endocytic pathway.

We have found that rab4p is associated with the population of early endosomes involved in transferrin (Tfn)-receptor recycling. This association is of particular interest in that rab4p is the only known endosome-associated rab protein that contains a phosphorylation site for $p34^{cdc2}$ kinase and is, in fact, hyperphosphorylated in mitotic cells (23). Because Tfn recycling is arrested during mitosis (24, 25) and because $p34^{cdc2}$ kinase inhibits early endosome fusion *in vitro* (16), localization of rab4p to early endosomes suggests that it may play a role in regulation of endocytic activity during the cell cycle.

MATERIALS AND METHODS

Plasmid Construction, Cell Culture, and Transfection. A human rab4 cDNA in pUC8 (2) was excised with *Nde* I and *Bam*HI, ligated to *Eco*RI linkers, and inserted into pFRCM-neo, a derivative of the pFRSV expression vector (26) (constructed by Susan Stuart, University of California, San Francisco). CHO (27), HeLa (28), MDCKII (29), and NRK (30) cells were maintained as described in indicated references. HeLa cells were grown on coverslips and transfected, as described (31).

Antibodies. Antibodies against human rab proteins Hrab1p, Hrab2p, Hrab4p, Hrab5p, and Hrab6p were raised, as described (2, 9). Rabbit antisera to canine rab5p and rab7p (10) were obtained from Marino Zerial (European Molecular Biology Laboratory, Heidelberg). A monoclonal antibody against the human lysosomal membrane protein lgp-B (21) was provided by Thomas August (Johns Hopkins University School of Medicine, Baltimore, MD) (32). A mouse monoclonal antibody against the human Tfn receptor was provided by Lee Hall (Yale).

Membrane Extractions. CHO cell microsomes (200 μg), prepared as described (33), were incubated with TEAS (10

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Abbreviations: HRP, horseradish peroxidase; FFE, free-flow electrophoresis; Tfn, transferrin; ER, endoplasmic reticulum.

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mM triethanolamine/10 mM HOAc/1 mM EDTA/250 mM sucrose, pH 7.4) (control), 1 M KI, 1 M Na_2CO_3 (pH 12), 1 M hydroxylamine, 6 M urea, or 0.1% Triton X-100 for 30 min at 4°C (except incubations in hydroxylamine, which was for 60 min at 37°C). The extracted membranes were then collected by centrifugation at 150,000 × g, and rab4p was analyzed in the resulting membrane pellets and supernatants by immunoblot (see below).

Endosome Labeling. Endosome subpopulations were selectively labeled with the endocytic tracers horseradish peroxidase (HRP) or ^{125}I-labeled Tfn as described (27, 34). Briefly, early endosomes were labeled by incubating 2×10^7 monolayer CHO cells with HRP (10 mg/ml in αMEM) for 4 min at 37°C; to label late endosomes, the HRP was removed after the 4-min pulse, the cells were washed six times with cold αMEM, and then cells were incubated in HRP-free αMEM medium for an additional 11 min at 37°C before homogenization.

Cell Fractionation. Labeled cells were combined with 2×10^9 carrier CHO cells, homogenized, and fractionated by free-flow electrophoresis (FFE) (27, 33, 35). The resulting fractions were assayed for ^{125}I-labeled Tfn, HRP, protein, and marker enzymes (33). In the earlier experiments, fractions were combined into two pools, representing the anodally deflected HRP-containing membranes and the nonshifted membranes containing the major protein peak (27). The shifted pool was then centrifuged in a 22% Percoll gradient (33). High-density fractions enriched in β-hexosaminidase, low-density fractions enriched in HRP (4 min at 37°C), and fractions of intermediate density were pooled and concentrated by centrifugation. Samples were analyzed by immunoblotting.

SDS/PAGE and Immunoblotting. Samples were resolved on reducing 12.5% gels, transferred to nitrocellulose, and analyzed by immunoblotting (9). Bound antibody was detected by using ^{125}I-labeled protein A and measured by densitometry. GTP-binding proteins were detected with a [^{32}P]GTP blot overlay assay (36).

Immunofluorescence. Hela cells were fixed in paraformaldehyde/lysine/periodate (37) for 15 min at room temperature and then permeabilized for 5 min in 0.5% saponin/phosphate-buffered saline. After a 1-hr incubation in 10% goat serum/0.5% saponin/phosphate-buffered saline, cells were incubated for 1 hr with affinity-purified rabbit anti-Hrab4p IgG and double-labeled by using mouse monoclonal antibodies to human Tfn receptor or to human lysosomal membrane glycoprotein lgp-B. The coverslips were then washed four times for 5 min each time and incubated with a mixture of fluorescently labeled antibodies.

RESULTS

Membrane Association of rab4p. Rabbit antisera were prepared against the rab proteins Hrab1 (rab1p) through Hrab6 (rab6p) (9). As shown in Fig. 1*a*, the antiserum to rab4p recognized a doublet of ≈24-kDa on immunoblots of whole-cell lysates of several fibroblast and epithelial cell lines from various species (human, rodent, canine). The purified antiserum was specific for rab4p (Fig. 1*a*) and did not recognize purified, recombinant proteins corresponding to rab1, -2, -3, -5, or -6 (38).

The majority of rab4p was found to be membrane-associated, as indicated by the fact that 75% of rab4p in CHO cells could be pelleted at 150,000 × *g* (Fig. 1*a*, membr vs. cytosol; 4-fold more cytosol than membranes was used for SDS/PAGE). Membrane association was judged tight in that rab4p could not be removed by chaotropic agents, high salt, hydroxylamine, or alkaline pH (Fig. 1*b*). The protein was solubilized only by Triton X-100 (Fig. 1*b*). The stability of membrane attachment may reflect the possible fatty acylation of a cysteine at the rab4 COOH terminus, which ends with a Cys-Gly-Cys prenylation signal (39–41).

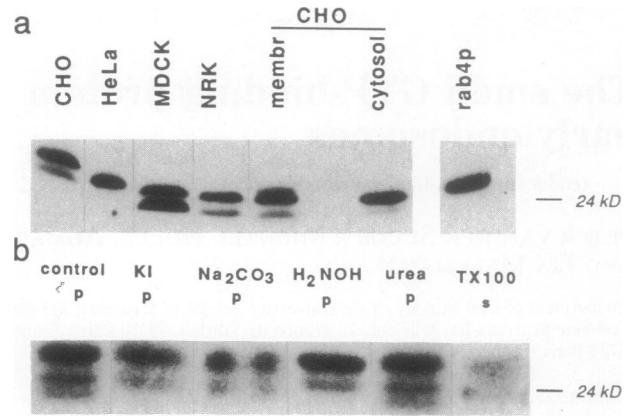

FIG. 1. Membrane association of rab4p. (*a*) Detection of rab4p by immunoblot in total, membrane (membr), and soluble fractions from different cell types. Crude lysates and CHO microsomes or cytosol were analyzed by immunoblotting with a rabbit polyclonal antiserum raised against rab4p. For a positive control, 15 ng of recombinant Hrab4p was included in the far-right lane. (*b*) Rab4p is tightly associated with membranes. CHO cell microsomes were extracted with various reagents. As shown, none of the treatments decreased the amount of rab4p recovered in the pellet (p), except Triton X-100, which quantitatively solubilized the protein into the supernatant (s).

Rab4p Is Associated with Endosomes in CHO Cells. Our strategy to determine whether rab4p or any other rab proteins were present in endocytic organelles was based on fractionation of CHO cells by FFE (33). The resulting shifted and nonshifted fractions were pooled (Fig. 2*a*) and analyzed by immunoblotting with the anti-Hrab antibodies. Only the antiserum against rab4p recognized a 20- to 25-kDa protein in the shifted pool (Fig. 2*b Left*); in contrast, antibody to Hrab2p, which has been localized by EM to ER–Golgi membranes (10) (B.G., unpublished work) was detected only in the nonshifted pool (Fig. 2*b Right*). Similar results were obtained with antibodies to Hrab1 and -6. Thus, rab4p was associated with membranes that cofractionated with endocytic organelles in a manner distinct from rab proteins known to be associated with biosynthetic compartments.

Because the anodally shifted pool contained both endosomes and lysosomes, we next separated these two organelles by centrifuging the shifted pool in a Percoll density gradient before SDS/PAGE (33). As shown in Fig. 2*c*, rab4p was detected exclusively in the low-density endosome fractions. Under the conditions used, both early and late endosomes are contained within this region of the gradient.

In the same experiment, we also investigated the distribution of total GTP-binding proteins. Both the FFE-shifted fraction and the endosome-enriched, low-density region of the gradient contained an ≈24-kDa doublet of GTP-binding proteins that comigrated with rab4p. However, [^{32}P]GTP binding was not observed in the high-density lysosomal or intermediate-density fractions (Fig. 2*b*). Thus, both [^{32}P]GTP binding and rab4p copurified with endosomes after FFE and Percoll gradient centrifugation.

Although the shifted FFE fraction is depleted relative to the homogenate with respect to Golgi markers (while endosomal markers are enriched ≈50-fold) (33), we also assayed the distribution of the Golgi marker enzyme galactosyl transferase in the gradient fractions. The remaining galactosyl transferase activity was evenly distributed between the low- and intermediate-density fractions of the gradient (data not shown). This result further indicates that the observed GTP-binding activity and the presence of immunoreactive rab4p in only the low-density region of the gradient were not from Golgi contamination.

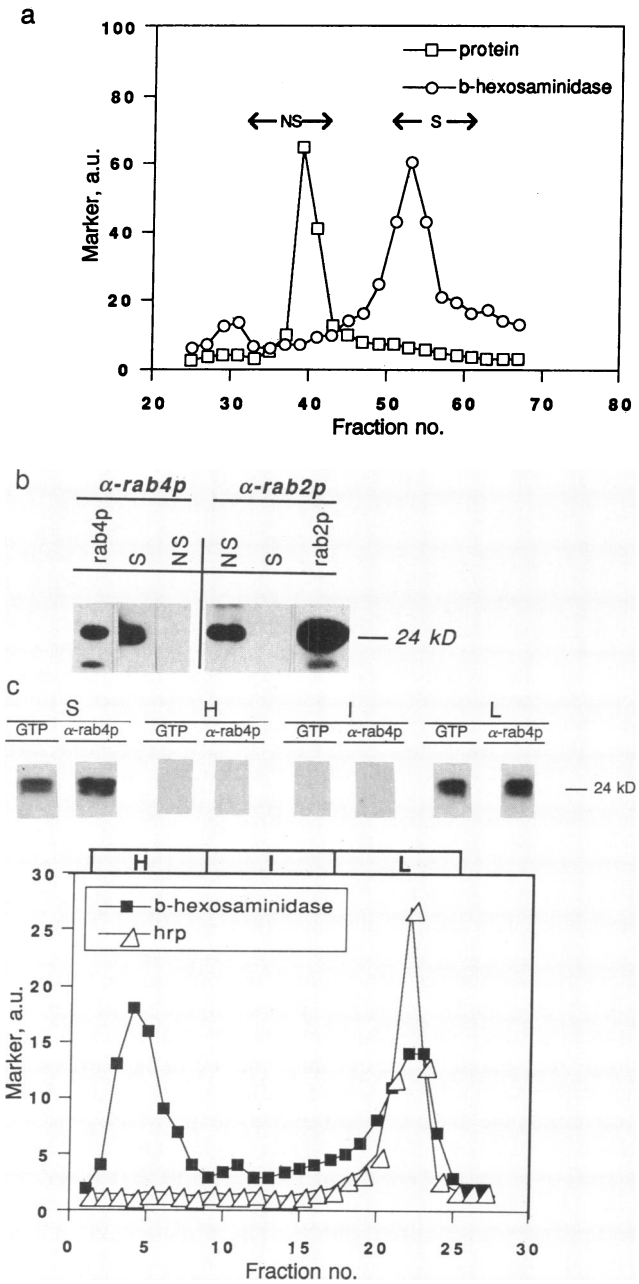

FIG. 2. Rab4p is associated with endosomes in CHO cells. (*a*) Fractionation of CHO membranes by FFE. Endosomes in CHO cells were isolated by FFE. Anodally shifted fractions containing HRP-positive endosomes and lysosomes (β-hexosaminidase marker) were pooled as indicated (S); nonshifted (NS) fractions, containing the major peak of total protein as well as most marker enzymes for ER, plasma membrane, and Golgi apparatus, were also pooled. (*b*) Rab4p is found in endosome/lysosome-containing fractions after FFE. CHO cell membranes were separated by FFE, and the resulting fractions were combined into an anodally shifted pool enriched in endosomes and lysosomes and a nonshifted pool. The shifted (S) and nonshifted (NS) FFE fractions were analyzed by immunoblotting with antibodies to rab4p (*Left*) or rab2p (*Right*). The 24-kDa doublet (more obvious on shorter exposures) corresponding to rab4p was detected in the pooled anodally shifted fractions; relatively little rab4p was detected in the nonshifted fractions. (*c*) Rab4p and GTP-binding activity copurify with low-density endosomes on density gradients. Pooled, shifted fractions (S) were centrifuged in a 22% Percoll gradient. The resulting gradient fractions were combined into three pools: a low-density, endosome-containing pool (L) corresponding with the peak of HRP; an intermediate-density pool (I); and a high-density pool (H), corresponding to the major peak of lysosomal marker β-hexosaminidase. Each pool was subjected to SDS/PAGE, and nitrocellulose replicas were probed by using antibody to rab4p or using [^{32}P]GTP. A 24-kDa doublet corresponding to rab4p was found in the shifted fraction before centrifugation and then only in the low-density fraction. The rab4p doublet also comigrated with a doublet that labeled with [^{32}P]GTP. GTP-binding activity was similarly detected only in the low-density fraction.

Rab4p Cofractionates with Early Endosomes. Endosomes can be divided into at least two functionally and biochemically distinct subpopulations (21, 27, 34, 35). To determine the subpopulation with which rab4p was associated, we next used FFE to resolve the early- and late-endosomal fractions.

As shown in Fig. 3*a*, both subpopulations were well separated from the major peak of cell protein. Although the early-endosome peak partially overlapped with the trans-Golgi marker galactosyl transferase, the two could be readily distinguished. Immunoblot of individual fractions showed that rab4p had the same distribution as ^{125}I-labeled Tfn (Fig. 3*b*). The comigration of rab4p and early endosomes was further supported by quantitative densitometry (Fig. 3*c*), strongly suggesting association with early, as opposed to late, endosomes. In addition, ≈30% of the rab4p was present, bound to membranes in the nonshifted fractions, which contained plasma membrane, ER, Golgi apparatus, and mitochondrial membranes. This relatively minor amount of rab4p was not detected in intial experiments because pooled rather than individual fractions were used (e.g., Fig. 2*b*).

Rab4p Colocalizes with Tfn Receptor-Containing Endosomes in Transfected HeLa Cells. To provide independent evidence that the rab4p-associated membranes were early endosomes, we transiently expressed rab4p in HeLa cells (31). An affinity-purified anti-rab4p antibody was used to generate the punctate immunofluorescence patterns of Fig. 4 *A* and *C*. A very similar staining pattern was observed in COS7 cells with the plasmid expression vector SRα (42).

To determine whether the rab4p-positive structures reflected early endosomes and/or recycling vesicles, the cells were then double-stained for Tfn receptor, an endogenous marker of early endosomes. Most rab4p-positive structures were also labeled with antibody to Tfn receptor (Fig. 4 *A* and *B*). As expected from the cell-fractionation experiments, the rab4p pattern was distinct from that obtained using an antibody against the lysosomal membrane glycoprotein lgp-B, which labels late endosomes and lysosomes (21) (Fig. 4 *C* and *D*). The rab4p-positive vesicles were also distinct from Golgi apparatus, as indicated by double staining with fluorescent wheat germ agglutinin (data not shown).

DISCUSSION

Our results provide two independent lines of evidence suggesting that rab4p is associated with the membranes of early endosomes. (*i*) After both FFE and Percoll density-gradient centrifugation, most endogenous membrane-bound rab4p in CHO cells was found to cofractionate with endocytic tracers that selectively label early endosomes. Little or no rab4p was detected in fractions enriched in late endosomes or lysosomes. In fact, GTP blots of Percoll gradient fractions suggested that the lysosomal membrane may be devoid not only of rab4p but of any other small GTP-binding proteins as well. Although FFE does not completely resolve Golgi membranes from early endosomes, it was clear that rab4p did not cofractionate with the Golgi marker enzyme galactosyl transferase after either electrophoresis or density-gradient centrifugation. Similarly, the fractionation of rab4p was entirely distinct from that of other rab proteins previously shown to present in Golgi apparatus and/or ER membranes. Approximately 30% of the rab4p, however, was clearly not endosome-associated and appeared in the nonshifted fractions after FFE. Although these fractions contain mitochondria, ER, plasma membrane, as well as some Golgi membranes, it

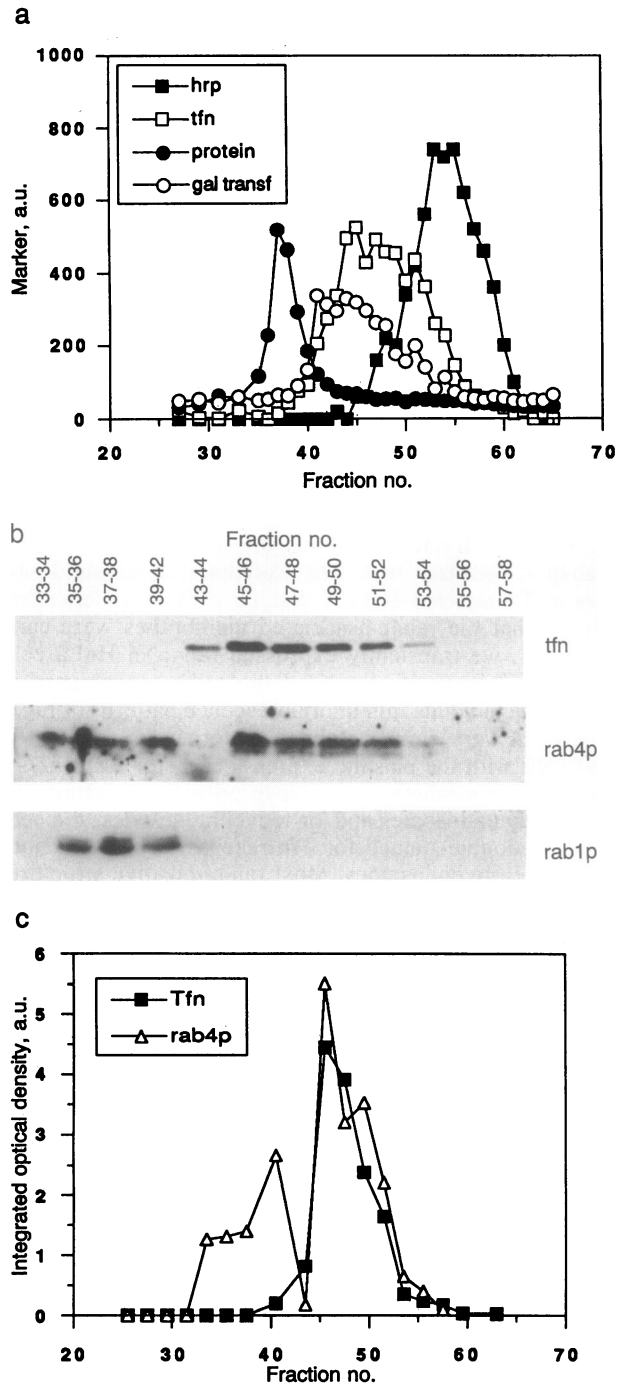

FIG. 3. Association of rab4p with enriched early endosome fractions in CHO cells. (*a*) Resolution of early and late endosomes by FFE. CHO cells were fractionated by FFE, and the resulting fractions were assayed for ^{125}I-labeled Tfn (early endosomes), HRP (late endosomes), galactosyl transferase (gal transf; trans-Golgi), and total protein, as described (27). Membranes were prepared for FFE, as described in Fig. 2, except that they were treated for 5 min at 37°C with 10 μg of trypsin per mg of protein instead of 2.5 μg of trypsin per mg of protein (followed by addition of 10-fold molar excess of soybean trypsin inhibitor). (*b*) Distribution of rab4p, rab1p, and Tfn in FFE fractions. Fractions from *a* were pooled as indicated, subjected to SDS/PAGE, and nitrocellulose replicas were probed by using antibodies to Hrab4p or Hrab1p. The distribution of internalized ^{125}I-labeled Tfn was visualized by autoradiography. (*c*) Densitometric quantitation of rab4p and Tfn from *b*. The major peak of rab4p immunoreactivity precisely comigrated with ^{125}I-labeled Tfn and was thus dissimilar from the distribution of the Golgi apparatus marker galactosyl transferase. Approximately 35% of the rab4p remained in the nonshifted fraction, however, comigrating with the major protein peak that also contains markers for the plasma membrane and ER (33). a.u., arbitrary units.

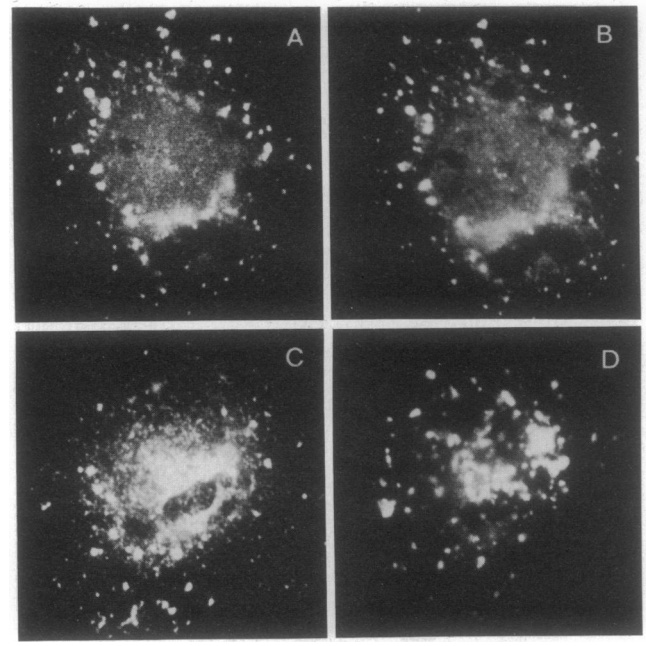

FIG. 4. Rab4p colocalizes with Tfn receptor-containing endosomes in transfected HeLa cells. Rab4p was overproduced in HeLa cells by using a vaccinia virus expression system (31). Six hours after infection and transfection, cells were fixed, permeabilized, and stained by using affinity-purified rabbit antibody to Hrab4p (*A* and *C*), a monoclonal mouse antibody to human Tfn receptor (*b*), or a monoclonal mouse antibody to a major lysosomal membrane glycoprotein (lgp-B) (*D*).

seems most likely that rab4p in the nonshifted fractions was associated with the plasma membrane, given its functional relationship with early endosomes. Because 70–75% was membrane-bound, it is apparent that ≈50% of the total-cell rab4p was actually endosome-associated; the remainder was associated with other membranes or was soluble.

(*ii*) Our second line of evidence that early endosomes are associated with rab4p was immunofluorescence. Although it was impossible to detect endogenous rab4p by this approach, transient transfection of HeLa cells with rab4 cDNA yielded a pattern of fluorescence nearly identical to that visualized with an antibody to human Tfn receptor. Thus, the transfected rab4p colocalized with a well-characterized endogenous marker of endocytic membranes involved in the pathway of rapid receptor recycling, particularly early endosomes and recycling vesicles (43). We cannot completely eliminate the possibility that overexpression of the transfected cDNA resulted in its artifactual localization to Tfn-receptor-positive structures. However, this is unlikely for several reasons. (*i*) The extent of overexpression was not enormous: HeLa cell cultures transfected by using the vaccinia vector exhibited only a 4-fold increase in amount of Hrab4p relative to controls. Because the transfections were ≈80% efficient, an individual cell, on average, would be expected to exhibit only a 5-fold increase in rab4p. (*ii*) Similar results were obtained by using two different expression systems, demonstrating that the immunofluorescence pattern was not an artifact of vaccinia virus infection. (*iii*) Localization of endogenous rab4p by cell fractionation also yielded close colocalization with Tfn-containing vesicles. (*iv*) When localization of both endogenous and overexpressed rab proteins was possible, differences in localization were not detected (10).

Rab4p is now the third small GTP-binding protein associated with endocytic organelles. Based on its colocalization with mannose 6-phosphate receptor, Chavrier *et al.* (10) have recently demonstrated that rab7p is associated with late endosomes; its distribution is thus clearly distinct from that of rab4p. EM-immunocytochemistry was also used to suggest that canine rab5p was at least partly associated with early endocytic vesicles in MDCK cells (10). Because rab5p was not colocalized with an endogenous marker of early endosomes, but with a fluid phase endocytic tracer (bovine serum albumin-gold) internalized for 8 min, whether rab5p is associated with the same structures as rab4p is difficult to know. Although our antibody to rab5p failed to detect antigen in endosome-enriched FFE fractions from CHO cells, the level of rab5p expression could be a limiting factor. Unfortunately, antibody to canine rab5p (10) did not detect antigen in CHO cells by immunofluorescence or immunoblot of crude or FFE-enriched CHO endosomes. Our antibody to Hrab5 also reacted poorly with CHO cells, suggesting that the level of expression in these cells may be low relative to the other rab proteins studied.

As for all other small GTP-binding proteins, the function of rab4p remains unknown. The fact that it is associated with early endosomes, however, suggests that it plays a role in some event on the pathway of receptor endocytosis and recycling. This role may include, however, formation of clathrin-coated vesicles, vesicle fusion with endosomes, maintenance of endosome structure, and recycling vesicle formation or fusion with the plasma membrane. Accordingly, given the possibility that individual GTP-binding proteins are required for individual steps of membrane transport (44), it is not surprising to find multiple species of rab proteins associated with endocytic organelles. These proteins are thought to be part of a complex that may cyclically associate with membranes and then dissociate upon hydrolysis of bound GTP and completion of its function (18, 45).

One feature that makes rab4p of exceptional potential interest, however, is the fact that it is one of only two known rab proteins that contain potential phosphorylation sites (Ser-Pro-Arg-Arg) for $p34^{cdc2}$ kinase (46). Recent evidence suggests that rab4p (and rab1p) are kinase substrate and are hyperphosphorylated in mitotic cells (23). Initial results also suggest that a smaller fraction of rab4p is membrane-associated in mitotic as compared with interphase cells. Given the fact that membrane traffic virtually ceases during mitosis, it is tempting to suggest that phosphorylation of rab4p by $p34^{cdc2}$ kinase underlies the mechanism by which endocytosis is arrested in mitotic cells (47).

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