A Small rab GTPase Is Distributed in Cytoplasmic Vesicles in Non Polarized Cells but Colocalizes with the Tight Junction Marker ZO-1 in Polarized Epithelial Cells

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Abstract. Small rab/Yptl/Sec4 GTPase family have been involved in the regulation of membrane traffic along the biosynthetic and endocytic pathways in eucaryotic cells. Polarized epithelial cells have morphologically and functionally distinct apical and basolateral surfaces separated by tight junctions. The establishment and maintenance of these structures require delivery of membrane proteins and lipids to these domains. In this work, we have isolated a cDNA clone from a human intestinal cDNA library encoding a small GTPase, rab13, closely related to the yeast Sec4 protein. Confocal microscopy analysis on polarized Caco-2 cells shows that rab13 protein colocalized with the tight junction marker ZO-1. Cryostat sections of tissues confirm that rab13 localized to the junctional complex region of a variety of epithelia, including intestine, kidney, liver, and of endothelial cells. This localization requires assembly and integrity of the tight junctions. Disruption of tight junctions by incubation in low Ca^{2+} media induces the redistribution of rab13. In cells devoid of tight junctions, rab13 was found associated with vesicles dispersed throughout the cytoplasm. Cell–cell contacts initiated by E-cadherin in transfected L cells do not recruit rab13 to the resulting adherens-like junction complexes. The participation of rab13 in polarized transport, in the assembly and/or the activity of tight junctions is discussed.

EUCARYOTIC cells contain a variety of small 21–27 kd guanine nucleotide-binding proteins, in addition to H-, K-, and N-p21ras. Although these proteins are structurally distinct from each other and from p21ras, they share significant homologies with p21ras, particularly in the domains involved in GTP/GDP binding and in GTP hydrolysis. Numerous members of the ras-related GTPase superfamily have been identified and classified according to their sequence similarities (for review see Valencia et al., 1991). Among them, the proteins of the rab/Ypt/Sec4 family (>30 members) have been proposed as key regulators in vesicular traffic (Touchot et al., 1987; Haubruck et al., 1987; Bucci et al., 1988; Matsui et al., 1988; Zahraoui et al., 1989; Vielh et al., 1989; Chavrier et al., 1990a, 1991a; Nimmo et al., 1991; Goldenring et al., 1993; for reviews see Bourne, 1988; Chardin, 1991; Goud and McCaffrey, 1991; Zerial and Stenmark, 1993).

In yeast, Yptl and Sec4 proteins, two small GTPases highly related to mammalian rab proteins, are required at distinct steps in transport along the secretory pathway. Conditional mutations in the *YPT1* gene cause an early block in secretion accompanied by accumulation of abnormal Golgi structures (Segev et al., 1988). In in vitro assays, the anti Yptl antibodies block transport from the endoplasmic reticulum to the Golgi (Bacon et al., 1989; Baker et al., 1990). Thermosensitive mutations in the *SEC4* gene impair the fusion of post-Golgi vesicles with the plasma membrane leading to their accumulation in the cytoplasm. Sec4 is associated with the cytoplasmic face of secretory vesicles and with the inner face of the plasma membrane, suggesting that it is required for the targeting and/or fusion of vesicles with the plasma membrane (Salminen and Novick, 1987; Goud et al., 1988).

In mammalian cells, there is increasing evidence for the involvement of rab proteins in membrane traffic. Many rab proteins have been localized to a variety of specific intracellular compartments along the endocytic and the exocytic pathways. Rab4 and rab5 have been localized to early endosomes, and rab7 to late endosomes (Van der Sluijs et al., 1991; Chavrier et al., 1990b). In the secretory pathway, rab2 is associated with the intermediate compartment between the

endoplasmic reticulum and the Golgi, rab6 with medial and *trans*-Golgi (Chavrier et al., 1990*b*; Goud et al., 1990), rab3A with synaptic vesicles and chromaffin granules (Fisher von Mollard et al., 1990; Darchen et al., 1990). Recent in vitro and in vivo studies suggest that mammalian rab proteins are involved in the regulation of several steps in the secretory and endocytic pathways. Rab1 and rab2 are required for vesicular transport in vitro between the endoplasmic reticulum and the Golgi (Plutner et al., 1991; Tisdale et al., 1992). Rab8 is involved in vesicle traffic from the TGN to the basolateral membrane in MDCK cells and to the somatodendritic plasma membrane in hippocampal neurons (Huber et al., 1993*a,b*), while rab5 appears to regulate both transport from plasma membrane to early endosomes and lateral fusion between early endosomes (Gorvel et al., 1991; Bucci et al., 1992). Overexpression of rab4 alters transferrin receptor recycling (Van der Sluijs et al., 1992). In adipocytes, insulin and okadaic acid induce a cycling of rab4 from vesicular fraction containing the Glut 4 transporter to the cytosol (Cormont et al., 1993). Rab9 stimulates the recycling of mannose 6-phosphate receptors from late endosomes to the TGN in vitro (Lombardi et al., 1993).

The exact functions of the rab protein family in vesicular traffic are not clear. However, several models have been proposed in which rab proteins interact with regulatory proteins such as GTPase-activating protein (GAP),[1] GDP dissociation stimulator (GDS), and GDP dissociation inhibitor (GDI) to regulate vesicular transport between different membrane compartments (Bourne, 1988; Walworth et al., 1989; Matsui et al., 1990; Burstein et al., 1990; Sasaki et al., 1991). Rab proteins may function as "molecular switches" to regulate the formation of protein complexes necessary for the targeting, docking and/or fusion of transport vesicles with the appropriate organelle, thereby contributing to the specificity and accuracy of vesicle targeting events.

Certain types of mammalian cells perform both constitutive secretion and either regulated secretion (e.g., endocrine, exocrine, and nerve cells) or polarized secretion (most epithelial cells). At present three rab proteins are thought to be involved in regulated secretion: rab3A has been proposed to regulate neurotransmitter release in the nerve terminals (Fisher von Mollard et al., 1991). Rab3B appears to control the Ca^{2+} induced exocytosis in anterior pituitary cells (Lledo et al., 1993) and rab3D may be involved in the insulin-induced exocytosis of GLUT-4 containing vesicles in adipocytes (Baldini et al., 1992). In platelets, rab3B, rab6, and rab8 proteins are phosphorylated upon activation by thrombin (Karniguian et al., 1993).

Delivery of membrane and secretory proteins to the apical or basolateral faces of polarized epithelial cells is well documented (Louvard et al., 1992). The apical and basolateral domains of epithelial cells are delineated by tight junctions that circumnavigate the horizontal axis of the cell. It is well accepted that different sets of plasma membrane proteins and lipids must be specifically inserted into one or other of the two distinct surface domains. Recently, a small GTPase, rab17, has been shown to be present only in epithe-

lial cells (Lütcke et al., 1993). However, whether the rab proteins are involved in the sorting of plasma membrane proteins remains to be established. As a first attempt to provide evidence supporting this possibility, we assumed, as a working hypothesis, that a human Sec4 protein homolog may perform such function. For this purpose we took advantage of an intestinal epithelial cell line (Caco-2), a well established model system for studies on polarized epithelial cells. Specific apical and basolateral exocytic and endocytic pathways have been documented in these cells as has the transcytotic route between the two surface domains that is exhibited by normal intestinal cells in vivo (Matter et al., 1990; for review see Simons and Wandinger-Ness, 1990; Louvard et al., 1992). We screened a Caco-2 cDNA library with a degenerate oligonucleotide encoding the "TIGIDFK" sequence in the effector region of the Sec4 protein. This led us to isolate cDNA clones encoding a small GTP-binding protein whose predicted protein sequence is closely related to that of Sec4. Specific antibodies raised against this protein allowed us to show that it is associated with vesicles scattered throughout the cytoplasm of non polarized cells lacking tight junctions but apparently located at tight junctions in polarized epithelial cells. Moreover, this distribution depends upon the integrity of the tight junctions which are found in simple epithelia as well as in endothelia. We discuss the possibility that this member of the rab family may participate either in the targeting of a subset of membrane proteins destined to the apical and/or basolateral cell surface and/or in regulating the tightness of the junction complex.

Materials and Methods

Cell Culture

The human colon carcinoma cell line, Caco-2, was grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, and 1% non essential amino acids. The proximal tubule cells of pig kidney, LLC-PK1, were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. CHO cells were grown in MEM alpha-medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Mouse L cells and E-cadherin transfected L cells were grown in a mixture (1:1) of DMEM and HAM F12 media supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. To remove extracellular calcium, Caco-2 cells were rinsed four times with low calcium medium (S-MEM) and incubated with S-MEM. All cultures were incubated at 37°C under a 10% CO$_2$ atmosphere.

Antibodies

The mouse monoclonal (G1/136) anti-p120 was kindly provided by Dr. Hans-Peter Hauri (University of Basel, Switzerland). The rat monoclonal antibody (R40.76) raised against ZO-1 was provided by Dr. J. Anderson (Anderson et al., 1989). The rhodamine/fluorescein-labeled goat anti-rabbit IgG and the rat monoclonal anti-E-cadherin antibody were purchased from Sigma Chem. Co. (St. Louis, MO). The fluorescein-coupled goat anti-mouse IgG were purchased from Amersham Corp. (Arlington Heights, IL). The rhodamine-linked goat anti-rat IgG were obtained from Cappel (Organ Teknika Corp., Westchester, PA).

cDNA Library Construction and Screening

A λgt10 cDNA library was constructed with polyA mRNA extracted from a human adenocarcinoma cell line (Caco-2) according to the instructions of the manufacturer (Amersham Corp.). 12 × 10^4 phage plaques were screened with the degenerate oligonucleotide 5'AC(A,C,G,T)AT(A,T)GG-(A,C,G,T,)AT(A,T)GATTTTAAG3' coding for the "TIGIDFK" sequence present in the effector domain of the yeast Sec4 protein. The oligonucleo-

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1. *Abbreviations used in this paper*: GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GDS, GDP dissociation stimulator; S-MEM, low calcium medium; TNE-NP40, TNE buffer containing 0.5% Nonidet-P40.

tides were labeled with $(\gamma\text{-}^{32}P)$ dATP by T4 polynucleotide kinase (Amersham Corp.). Filters were prehybridized for 5 h, and hybridized for 15 h at 42°C in 5× SSPE (1XSSPE: 0.15 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.4), 5× Denhardt's solution (1× Denhardt's: 0.02% polyvinyl-pyrrolidine, 0.02% Ficoll, 0.02% BSA) and 100 μg/ml of denatured salmon sperm DNA. Filters were washed in a solution containing 2× SSC, 0.1% SDS for 30 min at room temperature, and 30 min at 42°C. Two cDNA clones were isolated. Crosshybridization experiments performed in high stringency conditions (hybridization at 60°C, and washing at 65°C in 0.1× SSC, 0.1% SDS) indicated that the two cDNA clones isolated were identical, and did not crosshybridize with the human rab8 and SEC4 cDNAs. These two latter cDNAs encode proteins that possess the TIGIDFK sequence. Phage DNA was prepared and cloned in bluescript plasmid which was used for double-stranded DNA sequencing using the T7 sequencing™ Kit (Pharmacia, France).

RNA Isolation and Northern Blot Analysis

RNA was extracted from CHO, NIH3T3, Caco-2, and MDCK cells using the procedure of Chomczynski and Sacchi (1987). Total RNA (15 μg) was separated on 1% agarose gel and transferred onto Gene Screen™ membrane (New England Nuclear, Boston, MA). The rab13 cDNA probe was synthesized using the random priming procedure (Amersham) in the presence of $(\alpha^{32}P)$dCTP. Prehybridization and hybridization were performed at 42°C as described in Gene Screen™ instruction manual. The filter was washed in 0.1× SSC, 0.1% SDS at 60°C and autoradiographed.

Preparation of Antisera Against rab13

Polyclonal antibodies were raised against a synthetic peptide covalently coupled to Ovalbumin (Neosystem, Strasbourg-F). The peptide sequence was derived from rab13 cDNA COOH-terminal region: N-K-P-P-S-T-D-L-K-T-C(Acm)-D-K-K-N-T (position 182-197). The coupled peptide was emulsified in Freund's complete adjuvant and injected into the popliteal lymph nodes of a rabbit as described by Louvard et al. (1982). Affinity purification was carried out on a support of peptide covalently coupled to ACA 22 ultrogel (IBF).

Preparation of Cell Extract, Cellular Membrane, and Cytosol Fractions

Cells were scraped from plastic tissue culture dishes with a rubber policeman, washed in PBS, and lysed at 4°C in the TNE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.5% Nonidet-P40 (wt/vol), 0.5% sodium deoxycholate, 0.1% SDS and a mixture of protease inhibitors (10 μg/ml of leupeptin and of aprotainin, 1 μg/ml of pepstatin, and 1 mM PMSF). Solubilized material (cellular extract) was recovered by pelleting at 10,000 g for 10 min.

For membrane and cytosol fractions, Caco-2 cells were homogenized on ice in buffer A containing 10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 1 mM $MgCl_2$, 5 mM $CaCl_2$, and protease inhibitors (see above). After centrifugation at 600 g for 10 min to remove nuclei, the supernatant was centrifuged at 430,000 g in Beckman TL100 ultracentrifuge for 15 min to generate a cytosol (supernatant), and pellet (membrane) fractions. Protein concentrations were determined using the protein assay system (BioRad Labs., Hercules, CA).

Immunoprecipitation

Aliquots from cellular extracts, cytosol, and membrane fractions were incubated separately with specific anti rab13 antibodies in TNE buffer containing 0.5% Nonidet-P40 (TNE-NP40) for 4 h at 4°C. After addition of protein A-Sepharose, and further incubation 2 h at 4°C, the immunoprecipitate was pelleted, washed twice with TNE-NP40 containing 0.5 M NaCl, and twice with TNE-NP40. The pellet was then resuspended in Laemmli buffer and subjected to immunoblotting.

Immunoblotting

For immunoblotting, protein samples were separated on 15% polyacrylamide gels containing SDS, and then electrophoretically transferred onto nitrocellulose filters. Filters were prewashed in TBS-T (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20) including 5% (wt/vol) milk powder, for 30 min at room temperature. After three washes of 3 × 5 min in TBS-T buffer, filters were incubated for 1 h with the affinity purified anti rab13 diluted in TBS-T. After three washings of 15 min, filters were stained with

^{125}I-labeled protein A (Amersham Corp.) at 0.1 μCi/ml to detect the primary antibody bound to nitrocellulose, washed, and autoradiographed.

Immunofluorescence

Cells grown on coverslips were washed with PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$. They were then fixed and permeabilized with methanol at −20°C for 1 min. Cells were washed with 0.2% BSA in PBS. All subsequent incubations with antibodies and washes were performed in this buffer. Cells were incubated with the affinity purified anti rab13 antibodies for 30 min. After rinsing three times for 10 min, cells were incubated with rhodamine-conjugated goat anti-rabbit antibodies, washed twice in PBS-BSA and three times in PBS for 20 min. In double-labeling experiments, cells were first incubated with a mixture of anti-rab13 antibodies and mouse or rat monoclonal antibodies specific for E-cadherin and ZO-1, respectively. The primary antibodies were visualized with a mixture of goat anti-rabbit FITC and goat anti-mouse RITC or a mixture of goat anti-rabbit RITC and goat anti-rat FITC. Immunofluorescence analysis was performed with a Zeiss axioplan microscope.

The immunofluorescence microscopy was also carried out using another protocol. Cells were first permeabilized with 0.01% saponin in PBS for 5 min then fixed with 4% paraformaldehyde in PBS, washed and free aldehyde groups were quenched with 50 mM NH_4Cl in PBS for 15 min. The cells were washed with 0.01% saponin, 0.2% BSA in PBS, and then incubated with the first and second antibodies as described above.

Frozen sections (5 μm) from various mouse tissues were fixed with methanol and subsequently processed for immunofluorescence with rabbit anti-rab13 antibody according to the procedure described above. Staining was performed either with rat anti-rabbit antibody conjugated with Texas red or with biotin (Amersham Corp.). In this latter case, the staining was revealed with avidin-FITC complex (Amersham Corp.).

Results

Molecular Cloning of rab13

Ras and ras-related proteins share highly conserved domains involved in GTP/GDP binding (Pai et al., 1989). Besides these domains, additional regions are conserved in all members of rab/Ypt/Sec4 family. While a mammalian counterpart of the yeast Ypt1 has been identified (rab1A), a mammalian homolog of the Sec4 protein, which may be involved in post-Golgi transport, has not yet been identified. Rab1A displays 75% amino acid identity with its yeast homolog Ypt1 (Gallwitz et al., 1983; Zahraoui et al., 1989; Haubruck et al., 1989). This score increases up to 95% in the NH_2-terminal part, particularly in the putative effector domain. To search for Sec4 homologs in an intestinal epithelial cell line, we have screened a Caco-2 cDNA library by using a mixture of oligonucleotides coding for the "TIGIDFK" sequence present in the effector domain of Sec4 protein. Two positive cDNA clones were isolated. Crosshybridization analysis revealed that the two cDNA clones of 1.3 kb were identical. Fig. 1 *A* shows the nucleotide and the deduced amino-acid sequence of rab13. This cDNA encodes a protein that shares 92% amino acid identity with the incomplete sequence of a rat rab13 (Elferink et al., 1992). This divergence might indicate the existence of different forms of rab13 (e.g., rab3A and rab3B are 82% identical) or it might be due to species heterogeneity. Accordingly, we named the human cDNA encoded protein rab13. Rab13 has, as expected from the cloning strategy, the amino acid motif "TIGIDFK" (underlined in Fig. 1 *A*), corresponding to the effector domain of Sec4 protein. Moreover, the encoded protein displays typical structural features of ras-related proteins. We have produced rab13 protein in *E. coli* and verified that the recombinant protein binds GTP (data not shown). Besides a strict conservation of the domains that constitute the GTP-binding

A

GAATTCGAGGATCCGGGTACCATGGGAGGAAAACTTCTTCCTGGCCTGGGCTCCGTGCCGCTCTGTTTGCCA 72

ACCGTCCAGTCCCGCCTACCAGTGCCGGGCGCTCCCCACCCCTCCCCCGGCTCCCCCGGTGTCCGCC ATG 142
M 1

GCC AAA GCC TAC GAC CAC CTC TTC AAG TTG CTG CTG ATC GGG GAC TCG GGG GTG 196
A K A Y D H L F K L L L I G D S G V 19

GGC AAG ACT TGT CTG ATC ATT CGC TTT GCA GAG GAC AAC TTC AAC AAC ACT TAC 250
G K T C L I I R F A E D N F N N T Y 37

ATC TCC ACC ATC GGA ATT GAT TTC AAG ATC CGC ACT GTG GAT ATA GAG GGG AAG 304
I S T̲ I̲ G̲ I̲ D̲ F̲ K̲ I R T V D I E G K 55

AAG ATC AAA CTA CAA GTC TGG GAC ACG GCT GGC CAA GAG CGG TTC AAG ACA ATA 358
K I K L Q V W D T A G Q E R F K T I 73

ACT ACT GCC TAC TAC CGT GGA GCC ATG GGC ATT ATC CTA GTA TAC GAC ATC ACG 412
T T A Y Y R G A M G I I L V Y D I T 91

GAT GAG AAA TCT TTC GAG AAT ATT CAG AAC TGG ATG AAA AGC ATC AAG GAG AAT 466
D E K S F E N I Q N W M K S I K E N 109

GCC TCG GCT GGG GTG GAG CGC CTC TTG CTG GGG AAC AAA TGT GAC ATG GAG GCC 520
A S A G V E R L L L G N K C D M E A 127

AAG AGG AAG GTG CAG AAG GAG CAG GCC GAT AAG TTG GCT CGA GAG CAT GGA ATC 574
K R K V Q K E Q A D K L A R E H G I 145

CGA TTT TTC GAA ACT AGT GCT AAA TCC AGT ATG AAT GTG GAT GAG GCT TTT AGT 628
R F F E T S A K S S M N V D E A F S 163

TCC CTG GCC CGG GAC ATC TTG CTC AAG TCA GGA GGC CGG AGA TCA GGA AAC GGC 682
S L A R D I L L K S G G R R S G N G 181

AAC AAG CCT CCC AGT ACT GAC CTG AAA ACT TGT GAC AAG AAG AAC ACC AAC AAG 736
N̲ K̲ P̲ P̲ S̲ T̲ D̲ L̲ K̲ T̲ C̲ D̲ K̲ K̲ N̲ T̲ N K 199

TGC TCC CTG GGC TGA GGACCCTTTCTTGCCTCCCACCCCGGAAGCTGAACCTGAGGGAGACAACGG 802
C S L G * 204

CAGAGGGAGTGAGCAGGGGAGAAATAGCAGAGGGGCTTGGAGGGTCACATAGGTAGATGGTAAAGAGAATGA 874

B

Figure 1. Nucleotide sequence of rab13 cDNA and comparison of its deduced amino acid sequence with those of other rab proteins. (*A*) Nucleotide sequence of rab13 cDNA and deduced amino acid sequence of rab13 protein. The amino acid motif "TIGIDFK", encoded by the oligonucleotides used in the cloning strategy to isolate rab13 cDNA, is underlined. The sequence of the synthetic peptide located at the COOH-terminal of rab13 and used to raise antibody is also underlined. (*B*) Alignment of the rab13 protein sequence with those of human rab8, canine rab10 (Chavrier et al., 1990*a*), and the yeast Sec4 protein (Salminen and Novick, 1987). Identical residues are highlighted in reverse type. We have cloned and sequenced the human rab8 cDNA and noticed some differences with that published by Nimmo et al. (1991). Our Hrab8 cDNA encodes a protein that is 100% identical to the canine rab8 (Chavrier et al., 1990*a*). The Hrab8 Hrab13 cDNA sequences have been deposited at the EMBL Data Bank under the accession numbers X56741 and X75593, respectively.
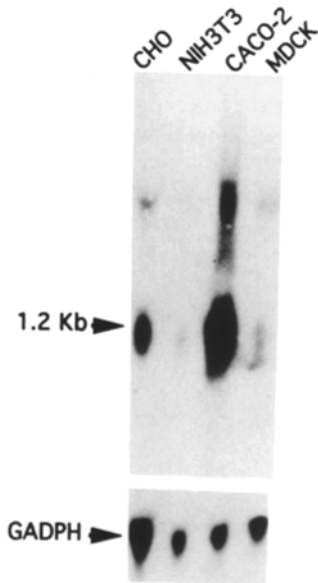
Figure 2. Rab13 mRNA expression in CHO, NIH3T3, Caco-2, and MDCK cell lines. 15 μg per lane of total RNA were probed with (α³²P)-labeled rab13 cDNA. Filter was washed at high stringency (see Materials and Methods) and exposed for 7 d at −70°C. Arrowheads indicate rab13 mRNA. The upper band represents crosshybridization of rab13 probe with the 28 S RNA. (*bottom section*) Hybridization of the same filter with a GADPH probe shows the quantity of RNA loading per lane.
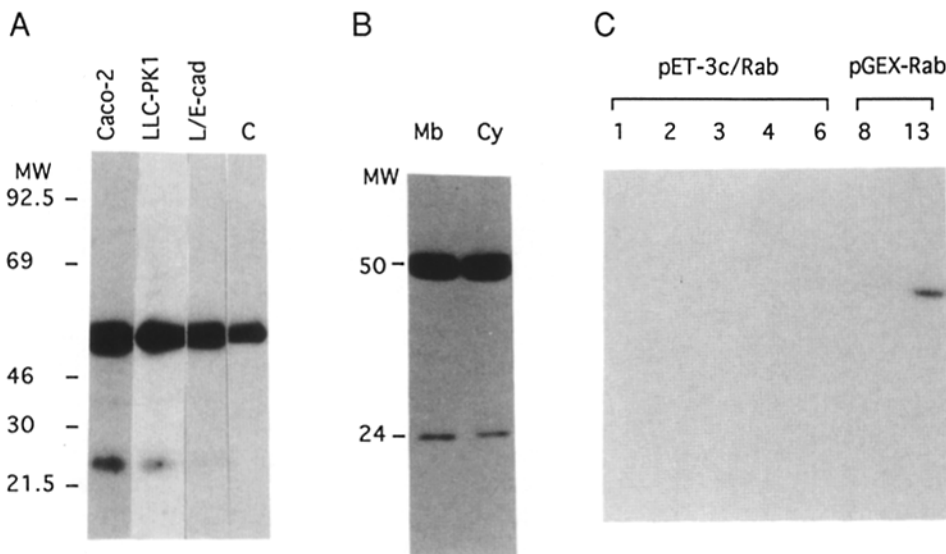
1991; Joberty et al., 1993). This modification is necessary for the attachment of rab proteins to membranes.

When used as a hybridization probe in Northern blot, rab13 cDNA detects one transcript of 1.2 kb that is highly expressed in Caco-2, moderately expressed in CHO, and weakly expressed in MDCK and NIH3T3 cells (Fig. 2). These observations prompted us to study the subcellular distribution of rab13 in cultured cell lines and particularly in the human intestinal epithelial cells (Caco-2) from which the cDNA was cloned.

## Cellular Localization of rab13 Protein by Indirect Immunofluorescence Analysis

To determine the location of rab13 protein, we raised antisera in rabbits against a peptide located near its COOH-terminus (underlined in Fig. 1 *A*). This region is highly variable between the members of the rab protein family including rab13, rab8, rab10, and Sec4 proteins and contains structural elements necessary for the association of rab proteins with their specific target membrane (Chavrier et al., 1991*b*).

The affinity purified anti-peptide antibodies were first characterized by immunoblot analysis. They are specific for rab13 since they do not recognize other rab proteins including rab1A, rab2, rab3A, rab4, rab6, and rab8 (Fig. 3 *C*). Experiments carried out with different cell lines revealed that the total amount of rab13 protein was very low and near the detection limit when a total protein extract, obtained from several cell lines, or membrane or cytosolic fractions were tested. To circumvent this difficulty, this protein was first immunoprecipitated from different fractions to enrich for rab13. The immunoprecipitated proteins were then separated on a polyacrylamide gel in presence of SDS, transferred onto nitrocellulose blots, incubated with rab13 antibodies, and visualized by autoradiography after further incubation with ¹²⁵I-labeled protein A. Using this procedure, the anti-

site, human rab13 shares several common domains with the rab protein family that were not conserved with p21ras. In Fig. 1 *B*, the deduced amino acid sequence of rab13 is aligned with those of other members of the rab family. Rab13 shares a long conserved NH₂-terminal region with rab8, rab10, and Sec4 proteins whereas their COOH-terminal parts are divergent. Overall rab13 displays 61% amino acid identity with the human rab8 and canine rab10 proteins and 56% amino acid identity with Sec4, but <47% identity with other rab proteins. Among rab proteins, only rab13, rab11, and rab8 possess at their COOH-terminal a CaaX motif which undergoes posttranslational COOH-terminal cysteine prenylation (Kinsella and Maltese, 1991; Farnsworth et al.,

Figure 3. Immunoblot analysis of proteins from different cell lines and from *E. coli* expressing rab proteins. (*A* and *B*) Proteins were first immunoprecipitated with rab13 antibodies from (*A*) Caco-2, LLC-PK1 and E-cadherin transfected L cell extracts, (*B*) Caco-2 membrane (*Mb*) and cytosol (*Cy*) fractions, and then subjected to immunoblotting. (*C*) Protein extracts from *E. coli* expressing rab1 (lane *1*), rab2 (lane *2*), rab3A (lane *3*), rab4 (lane *4*), rab6 (lane *6*), rab8 (lane *8*), and rab13 (lane *13*). The rab1A, 2, 3A, 4, and 6 were expressed in pET-3c vector under the control of the T7 promoter (Zahraoui et al., 1989). The rab8 and rab13 were cloned in pGEX expression vector and the proteins were produced as fusion with glutathione S-transferase,GST (Smith and Johnson, 1988). Proteins were separated on 10 (*A*) or 15% (*B*) and (*C*) PAGE-SDS and electrophoretically transferred onto nitrocellulose filters. The filters were incubated with the affinity purified anti-rab13 antibodies at 10 μg/ml. Bands were visualized using ¹²⁵I-labeled Protein A (Amersham Corp.). In Fig. 2 *A*, the proteins were also immunoprecipitated with rabbit nonimmune serum as control (lane *C*). (Fig. 3 *C*) arrowhead indicates rab13-GST fusion protein.
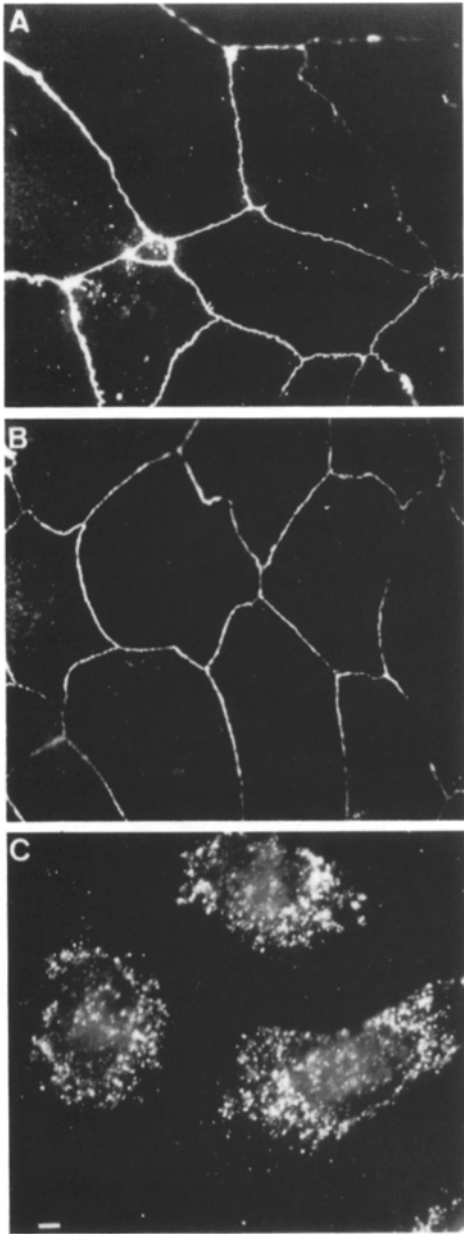
Figure 4. Immunofluorescence localization of rab13 in Caco-2 (*A*), LLC-PK1 (*B*), and CHO (*C*) cells. Caco-2 and LLC-PK1 epithelial cells were fixed and permeabilized with methanol at −20°C. CHO cells were permeabilized with saponin and fixed with paraformaldehyde. Cells were labeled with affinity purified antibodies against rab13 followed by rhodamine-labeled goat anti–rabbit IgG. Cells were viewed with a Zeiss axioplan microscope and photographed. Rab13 staining is concentrated as a sharp belt-like structure at the apex of the Caco-2 and LLC-PK1 cells in contrast to the CHO cells where rab13 labeling is restricted to vesicular structures. A similar vesicular staining was obtained when CHO cells were fixed and permeabilized with methanol. Bar, 10 μm.

peptide antibody recognized a band migrating at an apparent molecular mass of 24 kd in total lysate prepared from Caco-2, LLC-PK1, E-cadherin-transfected L (Fig. 3 *A*), MDCK, L, and CHO cell lines (data not shown). These observations were in agreement with the predicted molecular mass of 22.7 kd for rab 13. In addition, the immunoprecipi-

tated 24-kd protein was able to bind GTP (data not shown) as probed with an overlay method (Wagner et al., 1992). The additional band of 50 kd, corresponding to the heavy chain of the IgG recognized by ^{125}I-labeled protein A, was also seen in experiments carried out with nonimmune rabbit IgG. However, rab13 antibody immunoprecipitated a single band of 24 kd from [35S]methionine/cysteine (Translabel, New England Nuclear) cell extract (data not shown). Rab13 proteins present in the membrane (60%) and cytosol (40%) fractions of Caco-2 cells (Fig. 3 *B*). Its overall distribution is therefore similar to that of most other rab proteins, which are mainly associated with membranes but are also present in the cytosolic fraction.

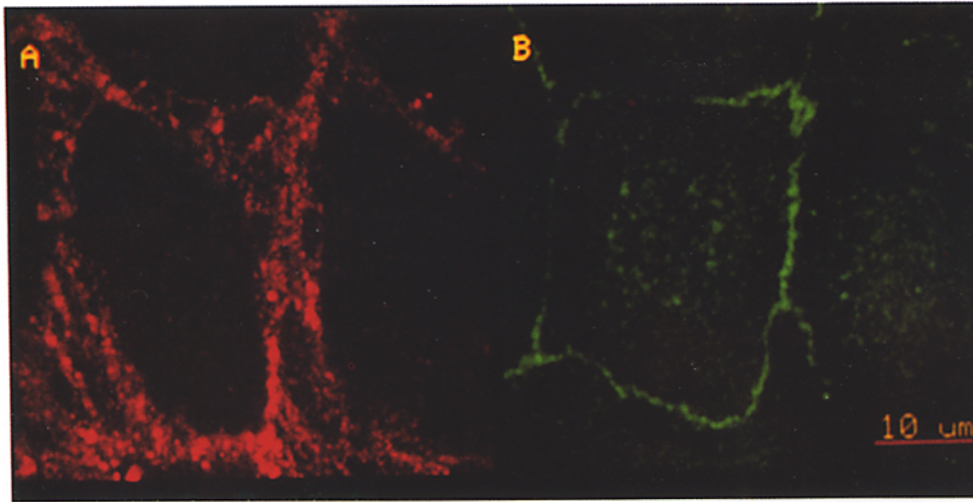### Rab13 Protein Is Localized in the Apico-Lateral Zone of Epithelial Cells

For immunofluorescence microscopy, confluent Caco-2, and LLC-PK1 cells were fixed with methanol at −20°C. Under our culture conditions, these epithelial cells form tight monolayers of differentiated polarized cells. The affinity purified anti-rab13 antibodies reacted strongly with a prominent sharp ring-like structure at the apex of the cells (Fig. 4, *A* and *B*). However, faint cytoplasmic staining was also conspicuously observed. No labeling either due to binding to apical membranes or to an overall staining of the basolateral faces could be observed. The same pattern was observed when Caco-2 cells were first permeabilized with saponin, and then fixed with 3% paraformaldehyde. Under these conditions, however, the intensity of labeling appeared weaker (data not shown). When we labeled non confluent or confluent CHO cells with rab13 antibodies, we observed specific staining of numerous vesicles spread throughout the cytoplasm (Fig. 4 *C*). A similar distribution was observed in mouse L and NIH3T3 cells, both of which are devoid of typical epithelial junction complexes (data not shown). We showed that an excess of synthetic peptide or intact recombinant rab13 protein preincubated with the antibody and subsequently applied to cells stained for immunofluorescence analysis completely abolished the patterns described above (data not shown).

To further investigate the subcellular localization of rab13, fully polarized Caco-2 cells were double labeled with anti-rab13 antibodies and a monoclonal anti p120 antibody. This latter antibody binds to a basolateral membrane marker (Eilers et al., 1989). The distributions of p120 and rab13 were visualized using a double-labeling procedure and analyzed by confocal laser microscopy. The sequence of the X-Y planes (horizontal sections) clearly showed the segregation of the two labels and the distribution of the p120 as a broad polygonal staining characteristic of a basolateral plasma membrane antigen (Louvard, 1980; Eilers et al., 1989). In contrast, rab13 staining was visible as a continuous and sharp profile outlining cell–cell contact areas at the apical borders of Caco-2 cells (Fig. 5). These data indicated that the two proteins did not colocalize. It also emphasized that rab13 was detected only in the regions of contact between neighboring cells, probably within the junction complexes.

Colocalization of rab13 with an Epithelial Tight Junction Marker: ZO-1

The apicolateral membranes of polarized cells are held to-

gether by junction complexes made of *zonula occludens* (tight junction) in the apical zone, *zonula adherens* in the intermediate zone, and belt desmosomes in the basal zone. These junctions are ultrastructurally, biochemically, and functionally distinct (Staehelin, 1974). To determine which junctional element rab13 associates, confluent monolayers of Caco-2 cells were first double stained for immunofluorescence with rab13 antibodies and a marker of *zonula adherens*, E-cadherin. The spatial distributions of rab13 and E-cadherin were analyzed by confocal laser microscopy. Affinity purified rab13 antipeptide antibodies and a rat monoclonal antibody specific for E-cadherin were applied to cells grown on glass coverslips for double immunofluorescence staining. Colocalization or superimposition of the two proteins is demonstrated by the yellow color resulting from the overlapping emissions of the two fluorophores. The X-Y views of the fluorescence showed the accumulation of the E-cadherin at the cell boundaries (Fig. 6 *A*). Rab13 appeared as a sharp profile outlining the apical borders of the cells (Fig. 6 *B*). The X-Z view (vertical section) revealed that the E-cadherin was distributed along the lateral surfaces whereas rab13 staining was found immediately above that of the E-cadherin. The staining partially overlapping near the apex (Fig. 6 *C*). These results argued against the localization of rab13 in adherens junctions, but did not exclude the possible association of rab 13 with an adherens junction subcompartment.

To obtain further insight on the location of rab13, we performed double immunofluorescence microscopy experiments using a monoclonal antibody directed against ZO-1, a 225-Kd protein located on the cytoplasmic faces of the tight junction membranes in epithelial cells (Stevenson et al., 1986). For this purpose, Caco-2 cells were fixed with methanol at −20° 10 d after confluency and processed for dual immunofluorescence analysis of rab13 and ZO-1. Cells were then analyzed by confocal laser microscopy. The X-Y views showed a very similar staining pattern for the ZO-1 and rab13 antibodies (Fig. 7, *A* and *B*). The X-Z view disclosed the colocalization of rab13 and ZO-1. We observed a perfect superimposition of the two fluorophores revealed by the yellow color (Fig. 7 *C*), indicating that rab13 and ZO-1 are in close proximity in the tight junction area.

Immunofluorescent Staining on Frozen Tissues

To confirm the rab13 localization in tight junctions of different epithelial cells, various mouse tissues were surveyed for the presence of this antigen. Fig. 8 *A* shows an immunolocalization of rab13 on a section of mouse small intestine performed along the crypt-villus axis. A strong signal is detected at the apex of mature epithelial cells. Depending on the incidence of the section, an hexagonal staining characteristic of junctional complexes was observed (Fig. 8 *B*). This apical staining was also found in crypt cells (Fig. 8 *C*). In addition, they contained a strong vesicular staining concentrated in the upper part of the cells (Fig. 8 *A*). This vesicular labeling decreased along the crypt villus axis.

This labeling of the junctional complexes was found in other epithelia. Fig. 9 *A* shows a section of kidney tubules with a characteristic staining at the apex of epithelial cells. In the liver, a labeling of the biliary canaliculi was also observed, corresponding to junctions between adjacent hepatocytes (Fig. 9 *B*). The colocalization of rab13 with tight junctions was further supported by its presence in mesothelial cells of intestine (data not shown) and in endothelial cells from various tissues as best illustrated on the section of a kidney artery (Fig. 9 *D*). This section shows very strongly labeled spots corresponding to tight junctions of endothelial cells. Underneath, a nonspecific staining of the elastic lamina can be detected due to autofluorescence. As expected, the intercalated discs of heart fibers which lack tight junctions, did not show any junctional labeling. Only the endothelial cells of capillaries showed a positive staining (Fig. 9 *C*). The absence of rab13 label from the intercalated discs indicates that rab13 is not localized at cell to cell adherens junctions in cardiac muscle cells.
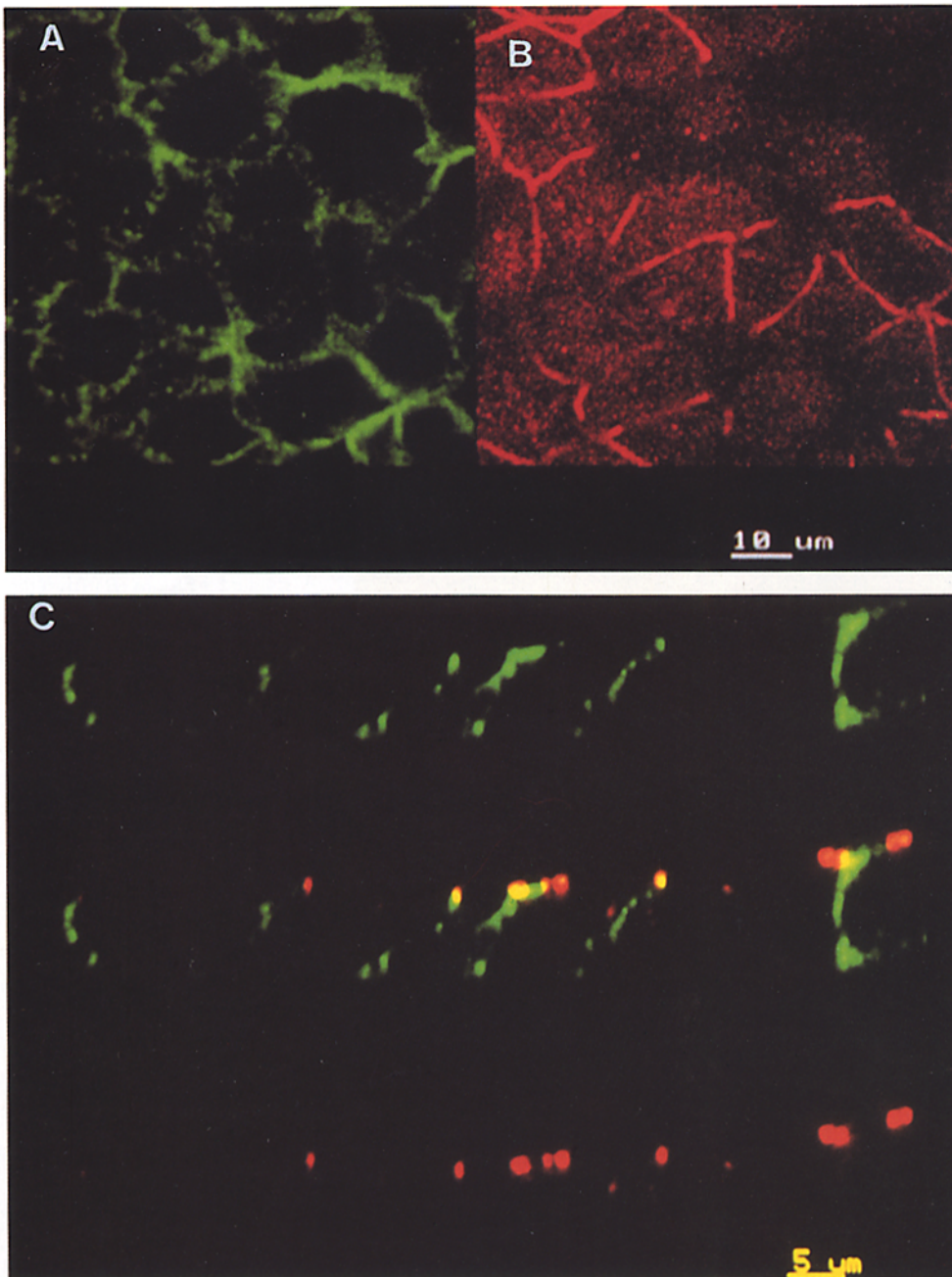
Figure 6. Confocal double immunofluorescence localization of rab13 and of an adherens junction marker, E-cadherin. Fully polarized Caco-2 cells were fixed and permeabilized as described in Fig. 4. Rab13 immunoreactivity was detected using the affinity purified anti-peptide antibody and rhodamine-coupled anti–rabbit IgG antibodies. E-cadherin was labeled with rat monoclonal antibody and fluorescein-coupled anti–rat IgG. Colocalization or superimposition of the two fluorophores was revealed by the yellow color resulting from their overlapping emissions. Specimens were scanned with the confocal microscope and photographed directly from the monitor. (*A*) Image of one optical section (*horizontal section*) taken at 1.2 μm from the base of the cells showing the distribution of E-cadherin. (*B*) Image of one focal plane (*horizontal section*) taken at 4.8 μm from the base of the cells showing the immunostaining of rab13. (*C*) X-Z views (*vertical section*) displaying the fluorescein staining of E-cadherin (*top part*), the rhodamine labeling of rab13 (*bottom part*) and the combination of the two images (*middle part*).

## Distribution of rab13 in Fibroblasts Expressing E-Cadherin

Given the vesicular appearance of the rab13 staining in non-polarized cells (Fig. 4 *C*), we next analyzed its distribution in the E-cadherin-transfected L-cell fibroblasts (L cells normally do not express E-cadherin). E-cadherin expression modifies the morphology of L cells which acquire Ca^{2+} aggregating activity and the capacity to form monolayers in which cells are tightly connected to each other (Nagafushi et al., 1987; Itoh et al., 1993). But, unlike cells in true simple epithelia, these cells completely lack tight junctions (Itoh et al., 1993). Transfected L cells were stained for E-cadherin and rab13. The E-cadherin was found concentrated in the boundary between cells and appeared to form a typical belt-like adherens junctions as in cells endogenously expressing

E-cadherin. Some labeling was also seen in Golgi structures (Fig. 10 *A*). In contrast, the antibody directed against rab13 gave rise to a staining corresponding to vesicular structures that were concentrated around the nucleus and more diffuse towards the cell periphery. Anti-rab13 immunoreactivity was not seen in the cell–cell contact sites (Fig. 10 *B*). These data demonstrate that the development of intercellular junctions mediated by E-cadherin is not sufficient to stimulate the recruitment of rab13 towards the cell surface when tight junctions are not present.

Redistribution of rab13 Protein after Removal of Extracellular Ca^{2+}

Junction complexes are dynamic structures which are repeat-
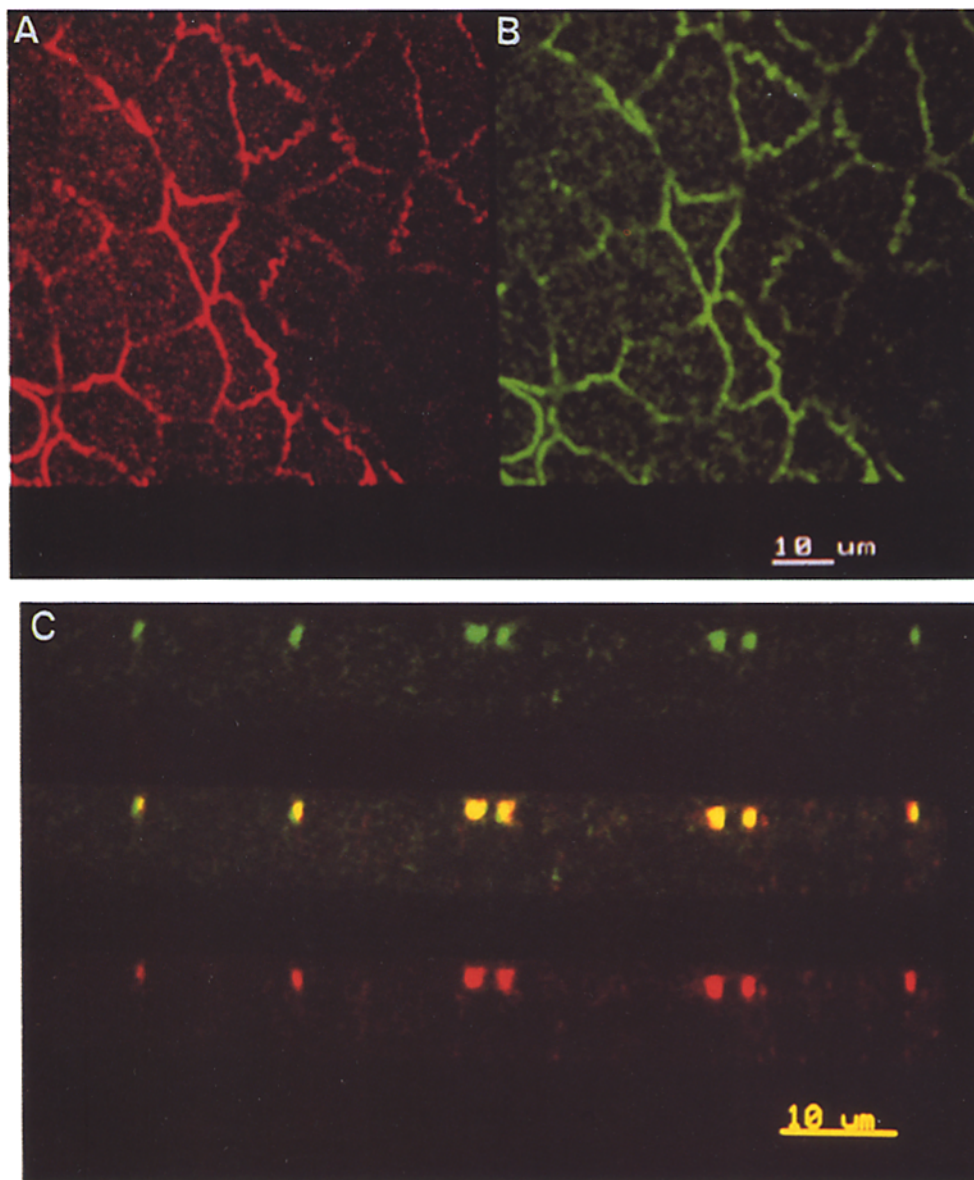
Figure 7. Confocal double immunofluorescence localization of rabl3 and of tight junction marker, ZO-1. After fixation and permeabilization of the Caco-2 cells, rabl3 was immunostained as described in Fig. 5. ZO-1 was labeled with the rat mAb R40. 76 and fluorescein-labeled anti–rat IgG. Images of one focal plane (*horizontal section*) taken at 4.2 μm from the base of Caco-2 cells and showing the immunolabeling of (*A*) rabl3 and (*B*) ZO-1. The X-Z views disclose the distribution of ZO-1 (*green color*), rabl3 (*red color*), and the combination of the two images (*yellow color*). Note the superimposition of the two fluorophores, indicating that rabl3 and ZO-1 colocalize in tight junction areas.

edly assembled and disassembled in vivo. A number of studies have shown that extracellular calcium can modulate the assembly of cell–cell contacts and affect the distribution of junction proteins (Siliciano and Goodenough, 1988; Anderson et al., 1989; Kartenbeck et al., 1991; Citi, 1992). This prompted us to study the localization of rabl3 upon removal of Ca^{2+} from the medium. Confluent Caco-2 cells were incubated for 1.5 h in low Ca^{2+} medium and double stained with rabl3 and monoclonal anti-ZO-1 antibodies. Dissociation of cell–cell contacts, changes in cell shape, and redistribution of ZO-1 and rab 13 proteins were all observed within 1 h of Ca^{2+} depletion. Furthermore, rab 13 and ZO-1 labeling of the cell periphery was reduced or eliminated after Ca^{2+} depletion, coincident with increased staining of material in the cytoplasmic compartment. In some cells, the rabl3 and ZO-1 antisera both reacted strongly with large cytoplasmic patches. As disruption of junctions became more complete, Caco-2 cells appeared retracted and began to round up at which time rabl3 and ZO-1 were diffuse throughout the cytoplasm (Fig. 11, *A* and *B*). However, the distribution of rabl3 in cytosol and membrane fractions is not affected after 1 h 30 min of removal of Ca^{2+} (results not shown). This indicates that rabl3 protein and ZO-1, are dynamically associated to tight junction structures, and that the opening of these junctions, induced by low calcium, leads to their concomittant redistribution. When subconfluent culture of Caco-2 cells were processed for immunofluorescence, careful examination of the distribution of rabl3 in small islets of differentiating Caco-2 cells revealed that the anti-rabl3 staining was restricted to the regions of cell–cell contact where neighboring cells have developed junction complexes but was absent from the edges of the islets (Fig. 11 *C*). A similar finding has also been reported for ZO-1 (Citi, 1992).
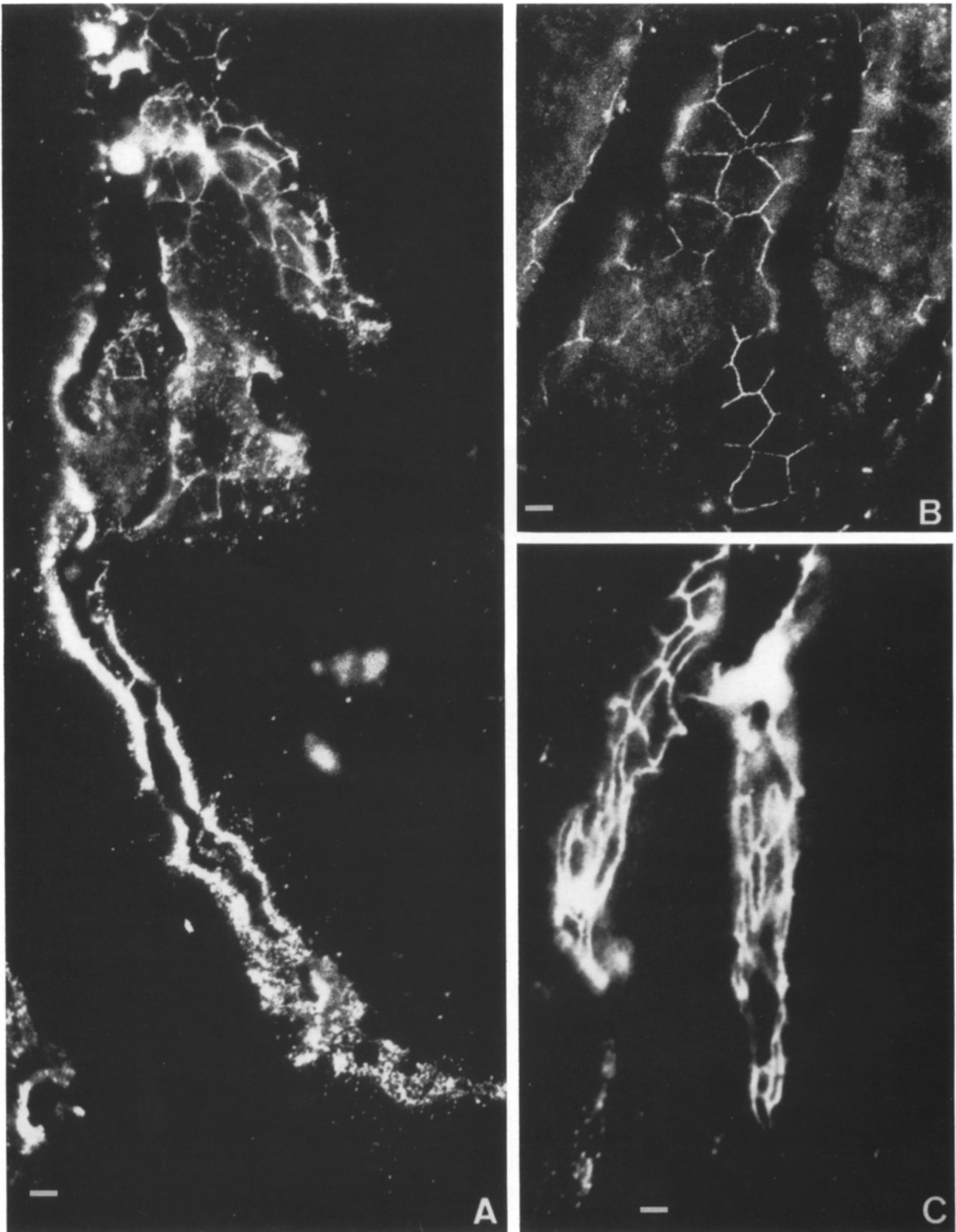
Figure 8. Immunolocalization of rab13 in frozen sections of mouse small intestine. Immunofluorescence was performed with anti-rab13 antibody and anti–rabbit antibody conjugated to Texas red (*A* and *C*) or conjugated to biotin and revealed with avidin-FITC complex (*B*). (*A*) Section of the crypt-villus axis with hexagonal apical labeling in mature cells and a strong vesicular staining in immature cells of the crypt (Bar, 10 μm). (*B*) Inset shows in greater detail the hexagonal labeling at the apex of the villus coinciding with the distribution of tight junctions. Depending on the angle of the section, discreet label is seen precisely at the site of the tight junctions (Bar, 10 μm). (*C*) Inset showing a fortuitous plane transecting the apical membranes of cells lining two adjacent crypts. The hexagonal outline of junctions is also revealed by rab13 antibody in crypt cells (Bar, 5 μm).
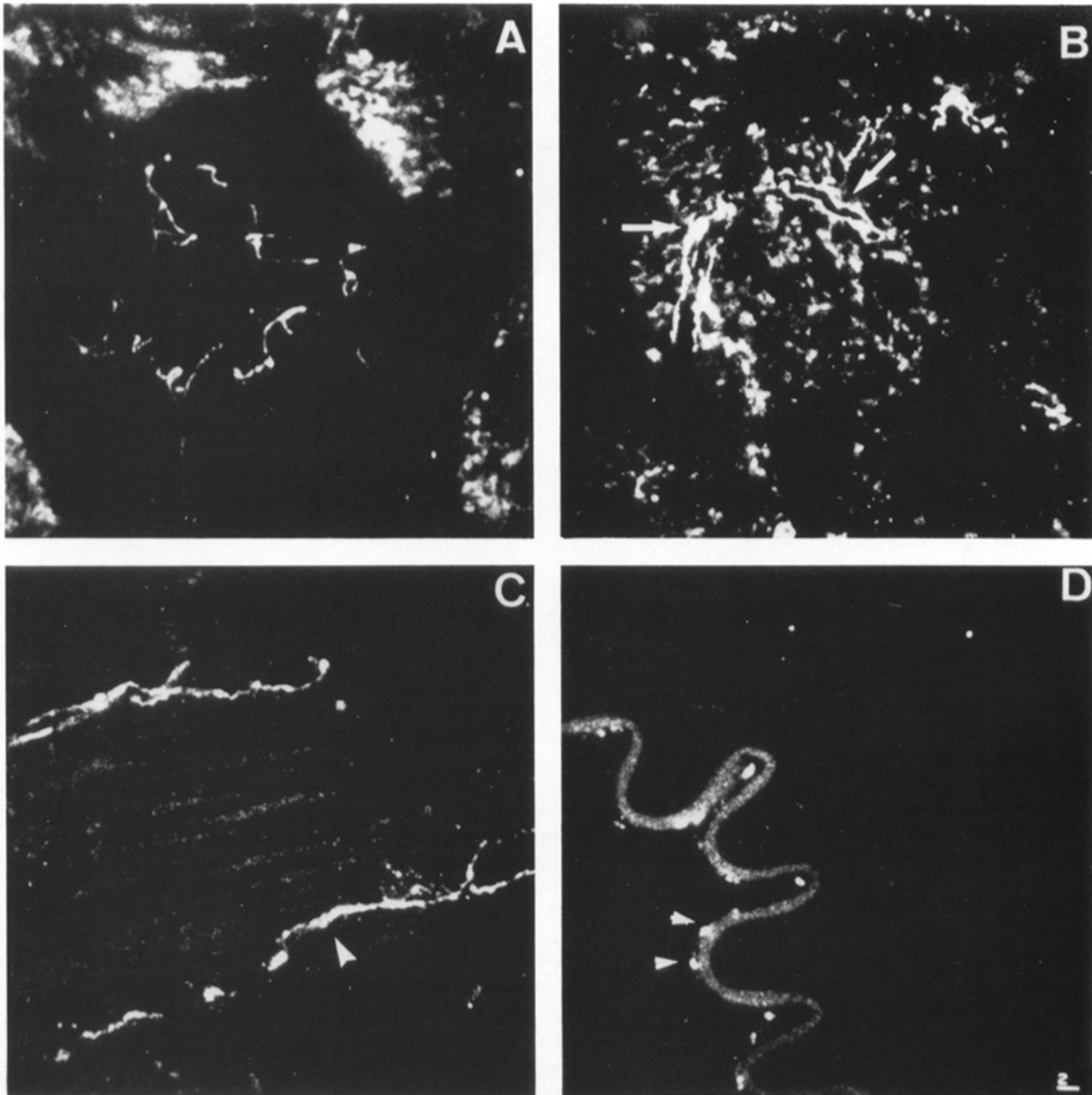
Figure 9. Confocal microscopy localization of rab13 in frozen sections of mouse tissues. Immunofluorescence was performed with anti-rab13 antibody and anti–rabbit antibody conjugated to biotin and revealed with avidin-FITC complex. Images represent one focal plane (0.7 μm). (*A*) Kidney: staining is observed in the junctional complex of tubules. (*B*) Liver: cross section shows labeling of the junctional complexes of the hepatocytes. Arrows identify bile canaliculi. (*C*) Section of heart fiber: rab13 staining is absent from myocardium cells but the internal positive control of endothelial junctions is visible (*arrowhead*). (*D*) Artery: a strong rab13 antibody immunoreactivity in endothelial junctions is shown (*arrowheads*). Unspecific staining of elastic lamina is also seen (Bar, 2 μm).

Discussion

This paper reports the identification of a human small rab13 GTPase protein highly homologous to Sec4. The rab13 protein is localized in the tight junction area of polarized epithelial cells. Our results suggest that rab13 may be involved in polarized membrane traffic and/or in tight junction structure and may control its function.

Rab13 Protein Is Closely Related to the Yeast Sec4 Gene Product

A large number of cDNAs (more than 30) coding for small GTPases of the rab family have been isolated from different

mammalian cells. We have isolated a cDNA clone from an intestinal epithelial cell line (Caco-2) library encoding a human small GTP-binding protein called rab13. Rab13 is more closely related to rab8, rab10, and Sec4 proteins than to the other members of the rab family, suggesting that these four proteins might constitute a rab subfamily. The similarity between rab8, rab10, rab13, and Sec4 is strikingly high in the effector domain, supporting the idea that these small GTPases may interact with the same or with related regulatory proteins. While a mammalian homolog of Ypt1 (rab1A) has been isolated and shown to replace functionally Ypt1 in *S. cerevisiae* (Haubruck et al., 1989), a mammalian counterpart of Sec4 has not yet been identified. Despite their high
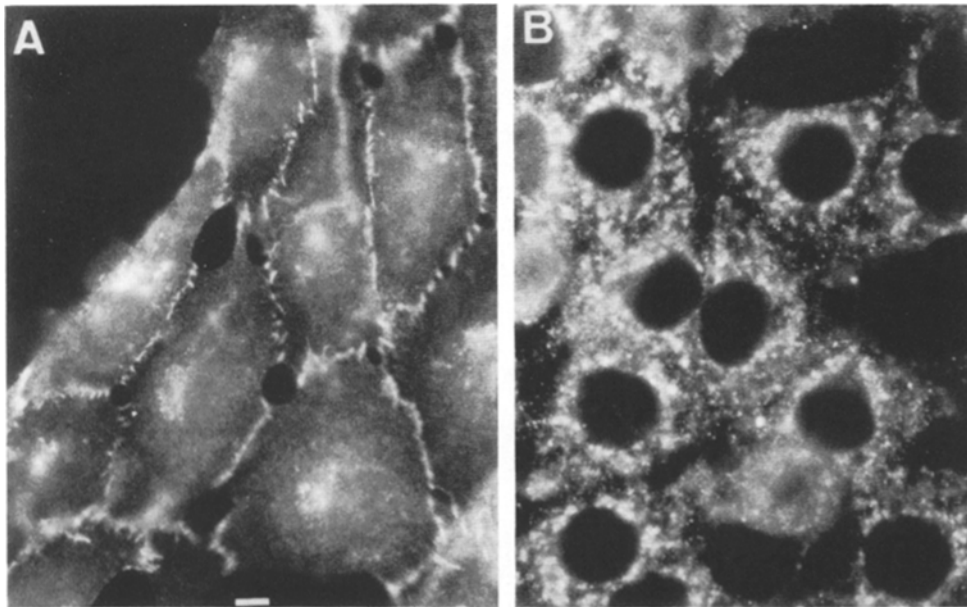
Figure 10. Distribution of rab13 in E-cadherin-transfected mouse L cells. E-cadherin-transfected L fibroblasts, fixed, and permeabilized with methanol at −20°C, were processed for staining with the affinity purified anti-rab13 and the rat mAb anti-E-cadherin antibodies. Two fluorescence micrographs of (*A*) anti-E-cadherin (fluorescein) and (*B*) rab13 (rhodamine) staining of E-cadherin transfected L cells are shown. E-cadherin is distributed at the boundaries of cells whereas anti-rab13 gave punctate staining corresponding to vesicular structures around the nucleus and throughout the cytoplasm similar to those observed in CHO cells (see Fig. 4 *C*). Bar, 16 μm.

protein sequence similarity, rab8 and rab10 proteins do not complement a *SEC4* mutation (Chen et al., 1993). It should be noted that the sequence identities between rab8, rab10, and rab13 on one hand and Sec4 on the other is not as high as that between Ypt1 and rabIA (75%). It is likely that Sec4 has undergone a high degree of specialization during evolution, and, that while *S. cerevisiae* might need only Sec4 to ensure post-Golgi constitutive transport, different mammalian cells would be expected to require several additional proteins (e.g., rab13, rab10, and rab8) to facilitate transport from the Golgi to specialized cell surface domains.

Rab13 Protein May be Essential for Protein Transport in Polarized Cells

Several lines of evidence indicate that rab proteins are involved in the control of various steps of exocytic and endocytic pathways (Plutner et al., 1991; Gorvel et al., 1991; Fisher von Mollard et al., 1991; Bucci et al., 1992; Van der Sluijs et al., 1992; Tisdale et al., 1992; Pfeffer, 1992; Lombardi et al., 1993). Candidates that contribute to specific vesicle targeting are the ras-related GTPase of the rab/Ypt/Sec4 family, together with additional proteins of the transport-fusion machinery. Recent observations suggest that the primary function of the rab proteins is to regulate interactions between components required for vesicle transport, rather than be used as labels for specifying acceptor compartment for a given vesicle (Brennwald and Novick, 1993). The specificity in membrane traffic would be ensured by a nonoverlapping distribution of receptors molecules, such as the soluble NSF attachment protein receptors, among the different subcellular compartments (Söllner et al., 1993). Moreover, in order to generate and maintain surface polarity, epithelial cells have to target proteins to both apical and basolateral membranes. Indeed, it has been proposed that the junctional complex of MDCK cells contain sites where proteins destined for the apical domain are recruited before their final localization (Louvard, 1980). Thus, the junctional complexes may provide the machinery required for docking

and fusion of some apical vesicles. This model favors a non-random transport of vesicles to cell surface domains and implies the existence of one or more small GTPases, such as rab13, performing their functions near the junctions or other specialized plasma membrane microdomains. The distribution of rab13 in polarized and unpolarized cells is consistent with a post Golgi/plasma membrane function.

Along this line, we propose that rab13 might be involved in the regulation of exocytic transport in non epithelial cells as well as in epithelial cells (apical and/or basolateral exocytic pathways). In contrast, the transcytotic pathway, for instance, would be controlled by epithelial-specific rab proteins such as rab17 (Lütcke et al., 1993).

Subcellular Distribution of rab13 Is Dependent on Tight Junctions

Confocal microscopy analysis on fully polarized Caco-2 cells showed that rab13 protein is not uniformly distributed along the lateral plasma membrane, but is codistributed with the epithelial tight junctions marker ZO-1. However, whether rab13 is attached to the inner face of the tight junction membrane, or is on the surface of vesicles that have accumulated in the vicinity of tight junctions has not been determined, since our attempt to localize rab13 by immunoelectron microscopy failed. The presence of rab13 in the junctional complexes of endothelial and epithelial cells and its absence from intercalated discs in cardiac muscle cells confirmed its association with tight junctions. It also strongly suggests that rab13 is an ubiquitous element of the tight junctions in epithelia. In intestinal crypt cells, rab13 was also detected on vesicles concentrated at the apex. This observation might correlate with the ability of these cells (contrary to the mature cells) to divide and to form new tight junctions, a process which presumably requires delivery of new junctional components. This is consistent with the role discussed below that rab13 is involved in tight junction assembly. The biogenesis of the structures that are required for the establishment and maintenance of the polarized state of epithelial cells
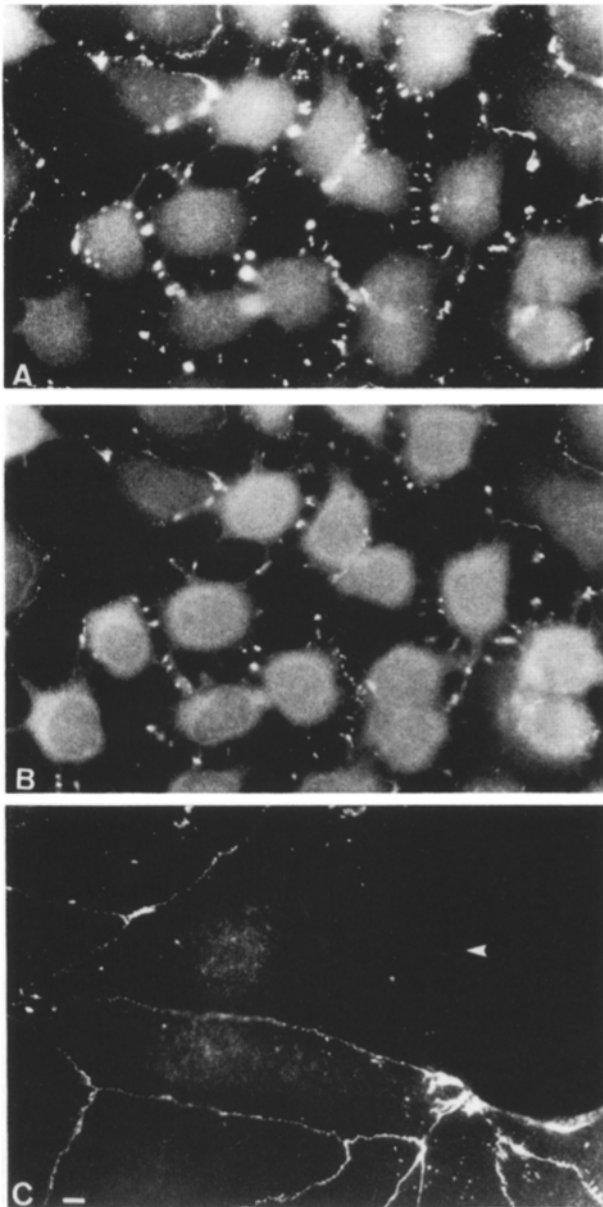
Figure 11. Dissociation of intercellular junctions induced by low Ca²⁺ leads to the redistribution of rab13. (*A* and *B*) Caco-2 cells were grown to confluency to form monolayers in which cells are tightly connected to each other, then were incubated for 1.5 h in low Ca²⁺ medium. Cells were double stained for fluorescence microscopy with (*A*) affinity purified anti-rab13 antibodies and (*B*) rat monoclonal anti-ZO-1 antibody. (*C*) Caco-2 cells were grown to subconfluency and then processed for immunostaining with rab13 antibodies. Arrowhead indicates periphery of a cell devoided of junctional complexes. Note that rab13 immunoreactivity is restricted to the regions of cells that are forming junctions with neighboring cells. Bar, 10 µm.

(Gumbiner and Louvard, 1985; Rodriguez-Boulan and Nelson, 1989; Nelson, 1992) may involve the recruitment of previously synthesized proteins.

Rab13 is associated with numerous vesicles scattered in the cytoplasm, of non polarized cell-lines (CHO, L, NIH3T3). Similarly, ZO-1 has been found in the cytoplasmic compartment in a variety of non epithelial cells unable to form tight junctions (Howart et al., 1992). These findings suggest that both proteins may have additional functions independent of the tight junction. We show here, for the first time, that the localization of a rab protein can be modulated when a specialized cell structure such as tight junction is assembled.

The molecular organization of the *Zonula occludens* is not well understood. Five tight junction proteins of unknown functions, ZO-1, ZO-2, Cingulin, 7H6, and a 130-kD protein have been identified (Stevenson et al., 1986; Gumbiner et al., 1991; Citi et al., 1988; Zhong et al., 1993; Anderson et al., 1993). In addition, actin microfilaments have been found associated with tight junctions (Madara, 1987a; Madara et al., 1987b). However, except for the association of ZO-1 with ZO-2, no other direct interaction has been demonstrated between the already known tight junction components. Tight junctions have two well studied functions. First they seal the intercellular space between adjacent cells, acting as a selective barrier restricting diffusion of molecules and ions across the "paracellular" pathway. Second, tight junctions form a "fence" within the plasma membrane preventing intermixing of apical and basolateral membrane proteins and lipids (for reviews see Schneeberger and Lynch, 1992; Citi, 1993). Two activities, which are not mutually exclusive, may be proposed for rab13. Rab13 may regulate the interactions of tight junctions components and contribute to the assembly of tight junction structure. It may be involved in the targeting/docking of a subset of vesicles transporting putative integral membrane protein(s) of tight junctions whose identification has so far remained elusive. In addition, rab13 may participate to the modulation of the permeability across the paracellular pathway and/or participate to the fence function of the tight junctions. Both hypotheses are mainly based on the finding that the localization of rab13 protein in the *Zonula occludens* depends on the occurrence of fully organized tight junctions as displayed by differentiated Caco-2 cells, rather than the mere presence of cell–cell contact mediated by an adhesion molecule such as E-cadherin. In favor of this view, it is worth recalling that transfection of cells with cadherins, although able to trigger both assembly of adherens like junctions and gap junctions, is not sufficient to induce assembly of tight junction itself (Mege et al., 1988; Itoh et al., 1993). Thus, the assembly of the junction adherens-like structure mediated by E-cadherin, in transfected L-cells, does not induce the recruitment of rab13 to the area of cell–cell contact. This indicates that specific components and signals controlling the assembly of tight junctions are probably required for rab13 localization in the *zonula occludens*. Interestingly, the disruption of tight junctions induced by low calcium concentration triggers the redistribution of rab13, as well as that of ZO-1 to the cytoplasm. Recently, the cDNAs coding for mouse and human ZO-1 proteins have been cloned and characterized (Itoh et al., 1993; Willott et al., 1993). The ZO-1 protein shows 47% homology with the drosophila discs-large tumor suppressor protein (dlg) localized in septate junctions which probably play in invertebrates, roles similar to tight junctions (Woods and Bryant, 1991; Willott et al., 1993). ZO-1, like dlg, displays SH3 domains which may interact with cytoskeletal molecules and small GTPase regulatory proteins. These findings indicate the need to investigate direct or indirect interactions between identified components of tight junctions.

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