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Molecular Cloning of YPT1/SEC4-Related cDNAs from an Epithelial Cell Line

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Molecular analysis of *Saccharomyces cerevisiae* secretion mutants has led to the identification of two Ras-like GTP-binding proteins, Ypt1p and Sec4p, which are essential for transport along the exocytic route. To study the regulation of membrane traffic in epithelial cells, a set of 11 clones encoding proteins similar to the YPT1/SEC4 products were isolated from an MDCK (Madin-Darby canine kidney) cell cDNA library. Four of these proteins, Rab8, -9, -10, and -11, are novel members of this subfamily of Ras-like proteins, and two of them are closely related to Ypt1p and Sec4p. The ratio of the number of clones isolated over the total number screened reveals a high level of complexity for this subfamily of GTP-binding proteins. This diversity supports their proposed function in controlling different steps in membrane traffic.

Ras and Ras-like proteins constitute a superfamily of GTP/GDP-binding proteins sharing regions of structural homology highly conserved through evolution (3). Different members of this superfamily can be grouped in individual subfamilies on the basis of their structural features. These characteristics suggest that the members of each subfamily, besides the common biochemical property of binding guanine nucleotides, carry out similar biological functions.

Recent in vivo and in vitro studies have indicated that the superfamily of Ras-related proteins includes proteins involved in controlling membrane traffic. Molecular analysis of *Saccharomyces cerevisiae* secretion mutants has led to the identification of two low-molecular-weight GTP-binding proteins, Ypt1p (13, 18, 19, 29–32) and Sec4p (15, 28, 39), which are structurally similar to the *ras* gene products and are essential for transport along the exocytic route.

Ypt1p is a 23-kDa GTP-binding protein which displays 38% homology with the human c-*ras* gene products (13, 30). Conditional lethal mutants of the *ypt* gene expressed at the nonpermissive temperature cause growth arrest and impaired sporulation, changes in microtubule organization, abnormal proliferation of endoplasmic reticulum and Golgi membranes, incomplete protein glycosylation, and a partial defect in secretion (29–32). Recent studies have shown that Ypt1p is involved in membrane transport from the endoplasmic reticulum to the Golgi or within the Golgi apparatus (2). Two other GTP-binding proteins, SAR1 (25) and ARF (33, 34), have also been implicated in early steps of protein secretion in *S. cerevisiae*.

Sec4p is also a Ras-like 23.5-kDa GTP-binding protein, and it controls a late step in secretion. Temperature-sensitive and dominant SEC4 mutants lead to a block in transport from the Golgi apparatus and the cells accumulate post-Golgi secretory vesicles (28, 39).

In higher eucaryotes, there is growing evidence for the involvement of GTP-binding proteins in controlling membrane traffic. The nonhydrolyzable GTP analog, GTP-γ-s, inhibits transport in several different cell-free systems assaying intracellular protein transport (14, 22, 23, 36).

Altogether, these results suggest that GTP-binding proteins are involved in distinct steps in membrane traffic (4). Indeed, a number of YPT1/SEC4-related proteins have been identified in mammalian cells (5, 7, 10, 21, 27, 35, 41, 42). Among them, a protein structurally and functionally equivalent to Ypt1p has been found in mouse (19), rat (35) and human (42) cells. However, attempts to isolate a mammalian homolog of the yeast SEC4 gene have so far failed. In this report, we describe the isolation of 11 clones encoding YPT1/SEC4-related products from a kidney epithelial (MDCK) cell cDNA library. Of the cDNAs isolated, four are new members of this subfamily of Ras-related proteins and two of these are distinguished by the high sequence similarity they share with YPT1 and SEC4. The ratio of the number of cDNAs isolated over the total number of cDNAs screened reveals a higher level of complexity for this subfamily of GTP-binding proteins than previously determined.

MATERIALS AND METHODS

Cell culture, RNA extraction, and analysis. All cells were grown in medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, and 10 μg of streptomycin (GIBCO) per ml at 37°C in 5% CO_2. MDCK strain II cells were grown in minimum essential medium plus 5% fetal calf serum, NIH 3T3 cells were grown in Dulbecco modified minimum essential medium plus 10% fetal calf serum, and BHK21 cells were grown in Glasgow minimum essential medium plus 10% fetal calf serum supplemented with 10% tryptose phosphate broth.

Total RNA was prepared from confluent cells by using the guanidine hydrochloride procedure (9). Polyadenylated RNA was purified by binding to oligo(dT) cellulose (Pharmacia). For Northern (RNA) blot analyses, polyadenylated RNAs (1 μg per slot) were separated by 1% agarose gel electrophoresis and transferred to nylon GeneScreen membranes. Filters were prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 5× Denhardt solution, and 1% sodium dodecyl sulfate at 42°C for 1 h. Hybridization was carried out in the same solution supplemented with ^{32}P-labeled probes (2×10^6 cpm/ml) for 18 h at 42°C. Washings were performed in 0.1× SSC–0.5% sodium dodecyl sulfate at 60°C. X-ray films were exposed for 1 to 2 days at −70°C with intensifying screens.

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	rab3a	rab3b	rab4	rab6	47=rab1	21=rab2	1=rab5	2=rab8	14=rab7	15=rab9	32=rab10	34=rab11	38=rab4b	YPT	YPT3	SEC4	H-ras
rab3a	100																
rab3b	85	100															
rab4	39	40	100														
rab6	42	40	39	100													
47=rab1	52	50	47	41	100												
21=rab2	42	42	59	40	49	100											
1=rab5	37	38	44	44	47	40	100										
2=rab8	56	53	44	45	59	49	41	100									
14=rab7	31	32	34	34	34	35	34	35	100								
15=rab9	29	29	34	28	32	30	27	32	54	100							
32=rab10	54	54	44	44	59	49	39	77	34	32	100						
34=rab11	41	40	52	36	52	55	46	48	34	32	47	100					
38=rab4b	38	40	87	39	47	58	43	43	33	32	44	53	100				
YPT	49	50	50	42	78	45	45	56	34	35	59	52	50	100			
YPT3	42	40	50	40	53	55	47	50	32	30	47	80	50	52	100		
SEC4	53	52	44	40	57	48	40	59	35	31	60	46	43	53	46	100	
H-ras	30	31	32	30	35	33	30	35	28	27	35	35	32	35	34	33	100

FIG. 1. Percentages of amino acid identities between MDCK proteins Rab1, -2, -5, -7, -8, -9, -10, -11, -4b; yeast YPT1, SEC4, and YPT3; and human H-*ras*, Rab3a, -3b, -4, and -6 in the sequence spanning region 1 (amino acid position 23 in the alignment of Fig. 3) to region 4 (position 187).

cDNA library screening. An oriented lambda MDCK cDNA library was constructed in the lambda cloning vector UNI-ZAP XR (Stratagene). Inserts had an average size of 2 kb. Fifty thousand bacteriophage plaques were screened by hybridization with a degenerate oligonucleotide (GTP-1 oligonucleotide) based on the conserved sequence Trp-Asp-Thr-Ala-Gly-Gln-Glu present in Sec4p (28), Ypt1p (13), and the different Rab proteins (35, 42). The sequence of the GTP-1 oligonucleotide 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). Duplicate filters were prehybridized for 1 h at 42°C in a 6× SSC–5× Denhardt solution–0.05% sodium pyrophosphate–0.5% sodium dodecyl sulfate solution containing 100 μg of boiled herring sperm DNA per ml. Hybridization was carried out for 18 h at 42°C in a 6× SSC–1× Denhardt solution–0.05% sodium pyrophosphate solution containing 100 μg of yeast tRNA per ml with 25 pmol of GTP-1 oligonucleotide labeled with T4 polynucleotide kinase (Biolabs) per ml. Filters were then washed in 6× SSC–0.05% sodium pyrophosphate for 3 h at 44°C. Phage DNAs from the positive plaques were extracted, digested with *Eco*RI and *Xho*I restriction endonucleases, and transferred to a GeneScreen Plus membrane (Du Pont Co., Wilmington, Del.). Duplicate filters were hybridized in the previous conditions with GTP-1 oligonucleotide at 42 or 37°C with FLET-1 oligonucleotide; the latter corresponded to the fourth conserved domain in Ras-like proteins, and its sequence was as follows: 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 oligonucleotide-hybridized filter was washed for 1 h at 60°C under the conditions described above, while in the case of FLET-1, oligonucleotide washing was performed at 25°C. Eleven clones hybridizing with both oligonucleotides were further characterized. Cross-hybridization analysis performed at high-stringency conditions with the human Rab cDNAs as probes (42) revealed that clones 1, 21, and 47 were highly homologous to Rab5, Rab2, and Rab1, respectively. In vivo excision of the cDNA inserts from the UNI-ZAP XR vector was performed 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 region of clone 2 upstream nucleotide 163 was obtained by using the anchored polymerase chain reaction procedure (20).

Computer methods. The multiple sequence alignment was done by using the MALI and PRALI programs (37) and was improved manually. On the basis of the alignment, the number of mismatches between the conserved parts of the sequences was calculated (Fig. 1). The phylogenetic tree was computed by using the program KITSCH from the PHYLIP package by J. Felstenstein (12). This program not only calculates the branching pattern of the tree but also estimates the evolutionary distance between adjacent branching points or end nodes. To represent the information in Fig. 1, we have depicted the tree together with a scale of sequence similarities in percent identical residues. In this way, one can read an approximate similarity between species from the height of the branching point linking them. Other methods (Fitch-Margoliash and parsimony) to calculate trees were also used and largely agree with these results.

Nucleotide sequence accession number. The sequences of Rab1, -8, -9, -10, -11, and -4b; Rac2; and Rho1 have been submitted to GenBank as accession numbers X56384 to X56391, respectively.

RESULTS

Isolation of YPT1/SEC4-related cDNAs. In order to isolate cDNA clones encoding proteins belonging to the Ypt1p/Sec4p subfamily, 50,000 recombinant plaques of an MDCK cell cDNA library were screened by using an oligonucleotide corresponding to the amino acid sequence WDTAGQE. These seven residues are strictly conserved in Ypt1p, Sec4p, Rho, and all the proteins of this subfamily identified so far (5, 7, 10, 21, 27, 35, 41, 42). Fifty positive clones were isolated under low-stringency-hybridization conditions. A second screening at higher stringency reduced the number of positive clones to 19. These also hybridized under low-stringency hybridization conditions, with a second oligonucleotide corresponding to another conserved region between the Ras-related proteins (FLET-1 oligonucleotide). Cross-hybridization analysis using the phage inserts as probes lowered the number of distinct cDNAS to 11.

Structural features of YPT1/SEC4-related proteins. We next determined the nucleotide sequences of these cDNAs and the deduced amino acid sequences corresponding to

2 = rab8

```
GAGTGTAATATGGCGAAGACCTACGATTACCTGTTCAAGCTGCTGCTGATCGGGGACTCG      60
        MetAlaLysThrTyrAspTyrLeuPheLysLeuLeuLeuIleGlyAspSer       17

GGGGTGGGGAAGACCTGTGTCCTGTTCCGCTTCTCCGAGGACGCCTTCAACTCAACTTTC     120
GlyValGlyLysThrCysValLeuPheArgPheSerGluAspAlaPheAsnSerThrPhe      37

ATCTCCACTATAGGAATTGACTTTAAAATTAGGACCATAGAGCTCGATGGCAAGAGAATT     180
IleSerThrIleGlyIleAspPheLysIleArgThrIleGluLeuAspGlyLysArgIle      57

AAGCTACAGATATGGGACACAGCTGGTCAAGAACGGTTTCGGACGATCACAACAGCCTAT     240
LysLeuGlnIleTrpAspThrAlaGlyGlnGluArgPheArgThrIleThrThrAlaTyr      77

TACAGGGGCGCAATGGGCATCATGCTGGTCTATGACATCACCAACGAGAAATCCTTTGAC     300
TyrArgGlyAlaMetGlyIleMetLeuValTyrAspIleThrAsnGluLysSerPheAsp      97

AATATCCGGAACTGGATTCGGAACATTGAGGAGCATGCTTCTGCAGATGTCGAAAAGATG     360
AsnIleArgAsnTrpIleArgAsnIleGluGluHisAlaSerAlaAspValGluLysMet     117

ATACTCGGAAACAAGTGTGATGTGAACGACAAAAGACAAGTTTCCAAGGAACGGGGAGAA     420
IleLeuGlyAsnLysCysAspValAsnAspLysArgGlnValSerLysGluArgGlyGlu     137

AAGCTGGCCCTGGACTATGGAATCAAGTTCATGGAGACCAGTGCGAAGGCCAACATCAAT     480
LysLeuAlaLeuAspTyrGlyIleLysPheMetGluThrSerAlaLysAlaAsnIleAsn     157

GTGGAGAACGCATTTTTCACTCTCGCCAGAGACATCAAAGCAAAGATGGACAAAAAATTG     540
ValGluAsnAlaPhePheThrLeuAlaArgAspIleLysAlaLysMetAspLysLysLeu     177

GAAGGCAACAGTCCCCAAGGGAGCAACCAGGGAGTCAAAATCACACCAGACCAGCAGAAG     600
GluGlyAsnSerProGlnGlySerAsnGlnGlyValLysIleThrProAspGlnGlnLys     197

AGGAGCAGCTTTTTCCGATGTGTTCTTCTGTGAGGAACACTGCCTTACTCTGAGCCTCGC     660
ArgSerSerPhePheArgCysValLeuLeu *                                 207

TCAGCTGAGCTGACTGTGCCTGTCCTGAGTGAGCCCTCCTCACTCAGCCGGGGCCCTCCC     720
CCTCCAATACTCCCTGCCGTGCCAGCCACTGGGCCCATGG                         760
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4 = rac2

```
ATGCAGGCCATCAAGTGTGTGGTGGTGGGAGACGGAGCTGTAGGTAAAACTTGTCTACTG      60
MetGlnAlaIleLysCysValValValGlyAspGlyAlaValGlyLysThrCysLeuLeu      20

ATCAGTTACACAACCAATGCATTTCCTGGAGAATACATCCCCACTGTCTTTGACAATTAC     120
IleSerTyrThrThrAsnAlaPheProGlyGluTyrIleProThrValPheAspAsnTyr      40

TCTGCCAATGTTATGGTAGATGGAAAACCAGTGAATCTGGGCTTATGGGATACAGCTGGA     180
SerAlaAsnValMetValAspGlyLysProValAsnLeuGlyLeuTrpAspThrAlaGly      60

CAAGAAGATTATGACAGATTACGTCCCTTATCCTATCCGCAAACAGATGTATTCTTAATT     240
GlnGluAspTyrAspArgLeuArgProLeuSerTyrProGlnThrAspValPheLeuIle      80

TGCTTTTCTCTTGTGAGTCCTGCATCATTTGAAAATGTTCGAGCAAAGTGGTACCCTGAA     300
CysPheSerLeuValSerProAlaSerPheGluAsnValArgAlaLysTrpTyrProGlu     100

GTGCGACACCACTGTCCCAACACCCCTATCATCTTGGTGGGGACTAAACTTGATCTCAGG     360
ValArgHisHisCysProAsnThrProIleIleLeuValGlyThrLysLeuAspLeuArg     120

GACGACAAAGACACGATTGAGAAACTGAAGGAGAAGAAGCTGACTCCCATCACCTACCCA     420
AspAspLysAspThrIleGluLysLeuLysGluLysLysLeuThrProIleThrTyrPro     140

CAGGGTCTGGCCATGGCTAAGGAGATCGGTGCTGTAAAATACCTGGAGTGCTCTGCTCTC     480
GlnGlyLeuAlaMetAlaLysGluIleGlyAlaValLysTyrLeuGluCysSerAlaLeu     160

ACGCAGCGAGGCCTCAAGACAGTGTTTGATGAAGCTATTCGAGCGGTTCTCTGCCCCCCT     540
ThrGlnArgGlyLeuLysThrValPheAspGluAlaIleArgAlaValLeuCysProPro     180

CCCGTCAAGAAGAGGAAGAGAAAATGCCTGCTGTTGTAAATGTTGGGTTCCCCTGCCCCA     600
ProValLysLysArgLysArgLysCysLeuLeuLeu *                           192

TCCCCCTCAGAACCTTTGTACGCTTTGCTCAAAAATGGTGGAGCCTTCGCACTCAATGCC     660
AAGTTTTTGTTACAGATTAGTTTTTCCATAAACCATTTGAACCAATCAGTAATTTT         716
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15 = rab9

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AATAAGTTTGATACCCAGCTCTTCCATACAATAGGTGTAGAATTTTTAAATAAAGATTTG      60
AsnLysPheAspThrGlnLeuPheHisThrIleGlyValGluPheLeuAsnLysAspLeu

GAGGTGGATGGACATTTTGTTACCATGCAGATTTGGGACACAGCCGGTCAAGAGCGATTC     120
GluValAspGlyHisPheValThrMetGlnIleTrpAspThrAlaGlyGlnGluArgPhe

AGAAGCCTGAGGACGCCGTTTTACAGAGGTTCTGACTGTTGCCTGCTCACTTTTAGTGTT     180
ArgSerLeuArgThrProPheTyrArgGlySerAspCysCysLeuLeuThrPheSerVal

GATGATTCTCAGAGCTTCCAGAACTTGAGTAACTGGAAGAAAGAATTCATATATTATGCA     240
AspAspSerGlnSerPheGlnAsnLeuSerAsnTrpLysLysGluPheIleTyrTyrAla

GATGTGAAAGAGCCCGAAAGCTTTCCTTTTGTGATTTTGGGCAACAAGATCGACATAAGT     300
AspValLysGluProGluSerPheProPheValIleLeuGlyAsnLysIleAspIleSer

GAACGACAAGTGTCTACAGAAGAAGCCCAAGCTTGGTGCAGGGACAACGGCGACTATCCT     360
GluArgGlnValSerThrGluGluAlaGlnAlaTrpCysArgAspAsnGlyAspTyrPro

TACTTTGAAACAAGTGCAAAAGATGCCACAAATGTCGCAGCAGCCTTTGAGGAAGCTGTT     420
TyrPheGluThrSerAlaLysAspAlaThrAsnValAlaAlaAlaPheGluGluAlaVal

CGAAGAGTGCTTGCTACTGAGGATAGGTCAGATCACCTGATTCAGACAGACACAGTCAGC     480
ArgArgValLeuAlaThrGluAspArgSerAspHisLeuIleGlnThrAspThrValSer

CTGCACCGAAAGCCCAAGCCTAGCTCATCTTGCTGTTGATCATTAGAGAGGTTCCCTGTG     540
LeuHisArgLysProLysProSerSerSerCysCys *

CGATCTAACCAACTTACACACACATACACGAAAATCACAGGGTGGAGAAGAGAATTAGCA     600
TTTGCAGCAATGGATCACCTACTCATAAAATTAAACTAACCATATTGCTGCTTTGTTAGT     660
GGGGTGGGAGAAGGACACATCCACTCAATGGAAGAATCAATTTACTCAGTAGTGGCACCT     720
TACATTTATAAATTGTAATGGTTGTCTAATAACGTTTAATTTAAATATGTAAGTTACAGA     780
GCTATAAACGAGATGATCAAGACTTTAATTACAATTAAAACACTTGAATATTCTGGAAGT     840
TAATGCTTTTTTTTCCCCTGGGAAAATGGAGAACTACTTTTTATATGTGTATATTTTTGT     900
GTAATTAGCATTTAATTCCTGGTTGAGGGGGGAAAAGATTCCCTAAAGCAATAATGTTAA     960
ATAATAAAGATTAAAATCTAAAAAAAAAAAAAAAAAAAAA                        1001
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32 = rab10

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GACTCGAGCCCTCGTTTTTCCCACGCTACCCCGGTCCTCCGGCCTGAGAACGCCCAAGTG      60
AGGAGTTGGCCGTCGTGAGAGGGACCGATCCCTTGGGGCCGCCGGCGGCGAGAGCCTGAG     120
CCGCTCCTCCCAATGGCGAAGAAGACGTACGACCTGCTTTTCAAGCTGCTCCTGATCGGA     180
          MetAlaLysLysThrTyrAspLeuLeuPheLysLeuLeuLeuIleGly        16

GACTCGGGAGTAGGGAAGACCTGCGTCCTTTTTCGTTTTTCGGATGATGCCTTCAATACC     240
AspSerGlyValGlyLysThrCysValLeuPheArgPheSerAspAspAlaPheAsnThr      36

ACCTTTATTTCCACCATAGGAATAGATTTTAAGATCAAAACAGTTGAATTACAAGGAAAG     300
ThrPheIleSerThrIleGlyIleAspPheLysIleLysThrValGluLeuGlnGlyLys      56

AAGATCAAGCTACAGATATGGGATACAGCAGGCCAGGAGCGATTTCACACCATCACAACC     360
LysIleLysLeuGlnIleTrpAspThrAlaGlyGlnGluArgPheHisThrIleThrThr      76

TCCTACTACAGAGGAGCAATGGGTATCATGCTAGTATATGACATCACCAATGGTAAAAGT     420
SerTyrTyrArgGlyAlaMetGlyIleMetLeuValTyrAspIleThrAsnGlyLysSer      96

TTTGAAAACATCAGCAAATGGCTTAGAAACATAGATGAGCATGCCAATGAAGATGTGGAA     480
PheGluAsnIleSerLysTrpLeuArgAsnIleAspGluHisAlaAsnGluAspValGlu     116

AGAATGTTACTAGGAAACAAATGTGATATGGACGATAAAAGAGTTGTACCTAAAGGAAAA     540
ArgMetLeuLeuGlyAsnLysCysAspMetAspAspLysArgValValProLysGlyLys     136

GGAGAGCAGATTGCAAGGGAGCATGGTATTAGATTTTTTGAGACTAGTGCAAAAGTAAAT     600
GlyGluGlnIleAlaArgGluHisGlyIleArgPhePheGluThrSerAlaLysValAsn     156

ATAAACATCGAAAAGGCTTTCCTCACATTAGCTGAAGATATCCTTCGAAAGACCCCTGTA     660
IleAsnIleGluLysAlaPheLeuThrLeuAlaGluAspIleLeuArgLysThrProVal     176

AAAGAGCCCAACAGTGAAAATGTAGATATCAGCAGTGGAGGCGGCGTGACAGGCTGGAAG     720
LysGluProAsnSerGluAsnValAspIleSerSerGlyGlyGlyValThrGlyTrpLys     196

AGCAAATGTTGCTGAGCGTTCTCCTGTTCCGTCAGTTGCCATCCACCACCCTGTTTTTTC     780
SerLysCysCys *                                                   200

CTCTTGCTGCAAAATAAACCACTCTGTCCATTTTAACTCTAAACAGATATTTTTGTTCCT     840
CATCTTAACTCTCCAATCCACCTGTTTTATTTGTTCTTTCATCCGTGA                 888
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their largest open reading frames were analyzed. The polypeptides encoded by clones 1, 21, and 47 showed 98, 99, and 100% amino acid conservation with the human Rab5, Rab2, and Rab1 proteins, respectively (8). Protein 14 showed 99% amino acid conservation with BRL-ras=Rab7 but additionally the sequence was extended in its N terminus by MTSRKKV (5, 8). Figure 2 shows the nucleotide and predicted amino acid sequences of the rest of the clones (the N terminus of protein 15 is missing).

The encoded proteins range from 192 to 216 amino acids in length, and they all contain the WDTAGQE sequence corresponding to the oligonucleotide used in the cDNA library screening. Clones 4 and 41 encode the canine homologs of Rac2 (100% sequence identity; 10) and Rho1 (99%; 41). The amino acid sequences of the other proteins were aligned with those of YPT1, SEC4, human Rab3A, -3b, -4, -6, and human H-*ras* as shown in Fig. 3. Since most of the mammalian proteins belonging to this subfamily have been called Rab, to avoid further terminology problems we use the same nomenclature for the MDCK proteins. Therefore, proteins 2, 15, 32, 34, and 38 will be referred to as Rab8, Rab9, Rab10, Rab11, and Rab4b, respectively (see below). The recently identified *Schizosaccharomyces pombe* protein Ypt3 (24) equivalent to SP8 (11) was also included in this comparison.

All proteins display the typical structural features of Ras-like GTP-binding proteins. The four regions which have been shown to participate in the formation of the GTP-binding site in the case of H-ras (26) are highly conserved

34 = rab11

```
CTCCCCGGCCGCGCAATGGGCACCCGCGACGACGAGTACGACTATCTCTTCAAAGTTGTC   60
                MetGlyThrArgAspAspGluTyrAspTyrLeuPheLysValVal   15

CTTATTGGAGATTCTGGTGTTGGAAAGAGTAATCTCTTGTCTCGATTTACTCGAAATGAG  120
LeuIleGlyAspSerGlyValGlyLysSerAsnLeuLeuSerArgPheThrArgAsnGlu   35

TTTAATCTCGAAAGTAAGAGCACCATTGGAGTAGAGTTTGCAACAAGAAGCATCCAGGTT  180
PheAsnLeuGluSerLysSerThrIleGlyValGluPheAlaThrArgSerIleGlnVal   55

GATGGGAAAACAATAAAGGCACAGATATGGGACACAGCAGGGCAGGAGCGATACCGAGCT  240
AspGlyLysThrIleLysAlaGlnIleTrpAspThrAlaGlyGlnGluArgTyrArgAla   75

ATAACATCAGCATATTATCGTGGAGCTGTAGGTGCCTTACTGGTTTATGACATTGCTAAG  300
IleThrSerAlaTyrTyrArgGlyAlaValGlyAlaLeuLeuValTyrAspIleAlaLys   95

CATCTCACATATGAAAATGTAGAACGATGGCTGAAAGAACTGAGAGATCATGCTGATAGT  360
HisLeuThrTyrGluAsnValGluArgTrpLeuLysGluLeuArgAspHisAlaAspSer  115

AACATTGTTATCATGCTTGTGGGCAATAAGAGTGATTTGCGTCATCTCAGGGCAGTTCCT  420
AsnIleValIleMetLeuValGlyAsnLysSerAspLeuArgHisLeuArgAlaValPro  135

ACAGATGAAGCAAGAGCTTTTGCAGAAAAGAATGGTTTGTCATTCATTGAGACTTCCGCT  480
ThrAspGluAlaArgAlaPheAlaGluLysAsnGlyLeuSerPheIleGluThrSerAla  155

CTAGACTCTACAAATGTAGAAGCTGCTTTTCAGACAATTCTGACAGAGATATACCGCATT  540
LeuAspSerThrAsnValGluAlaAlaPheGlnThrIleLeuThrGluIleTyrArgIle  175

GTTTCCCAGAAACAAATGTCAGACAGACGTGAAAATGACATGTCTCCAAGCAACAATGTG  600
ValSerGlnLysGlnMetSerAspArgArgGluAsnAspMetSerProSerAsnAsnVal  195

GTTCCTATTCATGTTCCACCAACCACTGAAAACAAGCCAAAGGTGCAGTGCTGTCAGAAC  660
ValProIleHisValProProThrThrGluAsnLysProLysValGlnCysCysGlnAsn  215

ATATAAGGCATTTCTCTTCTCCCCTAGAAGGCTGGGGATAGTCCATTCCCAGGTCTGAGA  720
Ile *                                                          216

TTAAATATATTTGTAATTCTTGTGGTCACTTTGTGTTTTATTACTTCCTCATACTTATGA  780
ATTTTCCCATGTCTTAAGTCTTTTGATTTTAGCTTTATAAAATCATCCACTTGTCCCGAA  840
TGACTGCAGCTTTTTTTCATGCTATGGCTTCACTAGCCTTAGTTTAATAAACTGAATGTT  900
TGGATTCCTCAAAAAAAAAAAAAAA                                     926
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38 = rab4b

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GAGTAGGAAGGAGCCGGGGCTGCAGCCGGAGTGGAGCGGCTGCCAGCCGAGGAGCAGGCG   60
CGGCCGCGGCGCCATATTGCGGCCCCGAGCGGCCGCGACCGAGTCATGGCCGAGACCTAC  120
                                        MetAlaGluThrTyr         5

GACTTCCTCTTCAAATTCCTGGTGATTGGCAGTGCAGGAACTGGCAAATCATGTCTCCTT  180
AspPheLeuPheLysPheLeuValIleGlySerAlaGlyThrGlyLysSerCysLeuLeu   25

CATCAGTTCATTGAGAATAAGTTCAAACAGGACTCCAACCACACAATCGGCGTGGAGTTT  240
HisGlnPheIleGluAsnLysPheLysGlnAspSerAsnHisThrIleGlyValGluPhe   45

GGATCTCGGGTAGTCAACGTGGGTGGGAAGACTGTGAAGCTCCAGATTTGGGACACAGCC  300
GlySerArgValValAsnValGlyGlyLysThrValLysLeuGlnIleTrpAspThrAla   65

GGCCAAGAGCGGTTTCGGTCGGTGACACGGAGTTACTACCGAGGGGCGGCTGGAGCCCTG  360
GlyGlnGluArgPheArgSerValThrArgSerTyrTyrArgGlyAlaAlaGlyAlaLeu   85

CTGGTGTACGACATCACCAGCCGGGAGACATACAACTCGTTGGCTGCCTGGCTGACGGAC  420
LeuValTyrAspIleThrSerArgGluThrTyrAsnSerLeuAlaAlaTrpLeuThrAsp  105

GCCCGCACGCTGGCTAGCCCCAACATCGTGGTCATCCTCTGTGGCAACAAGAAAGACCTG  480
AlaArgThrLeuAlaSerProAsnIleValValIleLeuCysGlyAsnLysLysAspLeu  125

GACCCTGAGCGCGAGGTCACTTTCCTGGAGGCCTCCCGCTTTGCCCAGGAGAATGAGCTA  540
AspProGluArgGluValThrPheLeuGluAlaSerArgPheAlaGlnGluAsnGluLeu  145

ATGTTCCTGGAGACTAGTGCCCTCACGGGTGAGAACGTGGAAGAGGCTTTCCTGAAGTGT  600
MetPheLeuGluThrSerAlaLeuThrGlyGluAsnValGluGluAlaPheLeuLysCys  165

GCCCGCACCATCCTGAACAAGATCGACTCAGGTGAGCTGGACCCCGAGAGGATGGGCTCA  660
AlaArgThrIleLeuAsnLysIleAspSerGlyGluLeuAspProGluArgMetGlySer  185

GGCATTCAGTACGGGGATGCTTCCCTCCGCCAGCTGCGGCAGCCTCGGAGTGCCCAGGCC  720
GlyIleGlnTyrGlyAspAlaSerLeuArgGlnLeuArgGlnProArgSerAlaGlnAla  205

GTGGCCCCCCAGCCCTGTGGCTGCTGAGACATGTGGAGCCAGCTCACCTGTTCTCCAGGA  780
ValAlaProGlnProCysGlyCys *                                    213

CCAGCCCTGCCCTTCTGGCCGGGGCCCAGACCCAGGCCC                       819
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FIG. 2. Nucleotide sequences of the MDCK YPT1/SEC4-like cDNAs and deduced primary sequences of the encoded proteins. Nucleotide and predicted amino acid (in single-letter amino acid code) sequences of cDNAs 2 (Rab8), 4 (Rac1), 15 (Rab9), 32 (Rab10), 34 (Rab11), 38 (Rab4b), and 41 (Rho2) are shown.

41 = rho1

```
ATGGCTGCCATCCGGAAGAAACTGGTGATTGTTGGTGATGGAGCCTGTGGTAAGACTTGT   60
MetAlaAlaIleArgLysLysLeuValIleValGlyAspGlyAlaCysGlyLysThrCys   20

TTGCTCATCGTCTTTAGCAAGGACCAGTTCCCAGAGGTGTATGTACCCACAGTGTTTGAG  120
LeuLeuIleValPheSerLysAspGlnPheProGluValTyrValProThrValPheGlu   40

AACTATGTGGCAGATATTGAAGTTGATGGAAAGCAGGTAGAGTTGGCTTTGTGGGATACA  180
AsnTyrValAlaAspIleGluValAspGlyLysGlnValGluLeuAlaLeuTrpAspThr   60

GCTGGGCAGGAAGATTATGATCGCTTGAGGCCTCTCTCCTATCCAGACACTGATGTTATA  240
AlaGlyGlnGluAspTyrAspArgLeuArgProLeuSerTyrProAspThrAspValIle   80

CTGATGTGTTTCTCTATTGACAGCCCTGATAGCTTAGAAAACATCCCAGAAAAATGGACC  300
LeuMetCysPheSerIleAspSerProAspSerLeuGluAsnIleProGluLysTrpThr  100

CCAGAAGTCAAGCACTTCTGTCCCAACGTGCCCATCATCCTGGTTGGGAACAAGAAGGAT  360
ProGluValLysHisPheCysProAsnValProIleIleLeuValGlyAsnLysLysAsp  120

CTTCGGAATGATGAGCACACAAGGCGGGAGCTAGCCAAGATGAAGCAGGAGCCGGTGAAA  420
LeuArgAsnAspGluHisThrArgArgGluLeuAlaLysMetLysGlnGluProValLys  140

CCGACAGAAGGCAGAGATATGGCAAACAGGATTGGTGCTTTTGGGTACATGGAGTGTTCA  480
ProThrGluGlyArgAspMetAlaAsnArgIleGlyAlaPheGlyTyrMetGluCysSer  160

GCAAAGACCAAAGATGGAGTGAGGGAGGTTTTTGAAATGGCCACGAGAGCTGCTCTGCAA  540
AlaLysThrLysAspGlyValArgGluValPheGluMetAlaThrArgAlaAlaLeuGln  180

GCCAGACGTGGGAAGAAAAAATCTGGGTGCCTTGTCTTGTGAAACCCTGCTGCAAGCACA  600
AlaArgArgGlyLysLysLysSerGlyCysLeuValLeu *                      193

GCCCTCATGCGGTTAATTTTGAAGTGCTGTTTATTAATCTTAGTGTATGATTACTGGCCT  660
TTTTCATTTATCTATAATTTACCTAAGATTACAAATC                         697
```

and are as follows: (i) the GXXXXGKS/T sequence (positions 29 to 36 of the sequence alignment) constituting the phosphate-binding loop L1 (nomenclature according to Pai et al. [26]); (ii) the DTAG sequence on L4 (positions 77 to 80) in the highly conserved WDTAGQE motif shared by Rab and Rho proteins, which interacts with the γ phosphate; (iii) the NKXD sequence (positions 139 to 142) at the junction between beta-strand 5 and loop L8, called the guanine specificity region; (iv) the SAK/L sequence in L10 (positions 169 to 171) interacting with the D residue (position 142). The region spanning these four conserved domains contains other identical residues (shown in boldface type) as well as several conservative changes.

A less conserved region is the effector loop L2 (positions 51 to 59) corresponding to amino acids 32 to 40 in the H-ras sequence which are known to mediate the interaction of $p21^{ras}$ with the GTPase-activating protein (1, 6). All these proteins share a Thr in position 54, which in $p21^{ras}$ coordinates to the Mg^{2+} ion and to the γ phosphate. The rest of this region is clearly more divergent from $p21^{ras}$ and is more similar to the Ypt1p and Sec4p effector loops. In the case of proteins Rab8 and Rab10, this similarity is striking: the FISTIGIDFKIK sequence of Rab10 almost exactly matches that of SEC4 (FITTIGIDFKIK) besides a conservative change (Ser-Thr) in the third position. Rab8 contains the sequence FISTIGIDFKIR, also very similar to that of Ypt1p and Sec4p.

The sequence identity in the region spanning the four conserved domains ranging from alignment positions 23 and 187 (the last conserved hydrophobic amino acid) is shown in Fig. 1. On the basis of these data a phylogenetic-type tree was calculated (Fig. 4) which groups the sequences depending on their sequence similarity. Interestingly, this analysis revealed that the homology between Rab8 and Rab10 proteins and Ypt1 and Sec4 proteins is not limited to the effector loop. The four proteins constitute a group together with Rab1 and, possibly, Rab3a and Rab3b. Besides Rab1, of all mammalian proteins of this subfamily identified so far, Rab8 and Rab10 are the most closely related to the yeast proteins. Within the conserved region, these two proteins share 56 and 59% sequence identity with Ypt1p and 59 and 60% with Sec4p, respectively. The similarity between the two MDCK proteins is 77%, clearly lower than for the highly related Rab3a and Rab3b.

When a mammalian sequence and a yeast sequence have more than 70% identity, one may assume that they perform analogous functions in the two species. This is the case for YPT1 and Rab1 (78%; 19) and, possibly, also for the recently

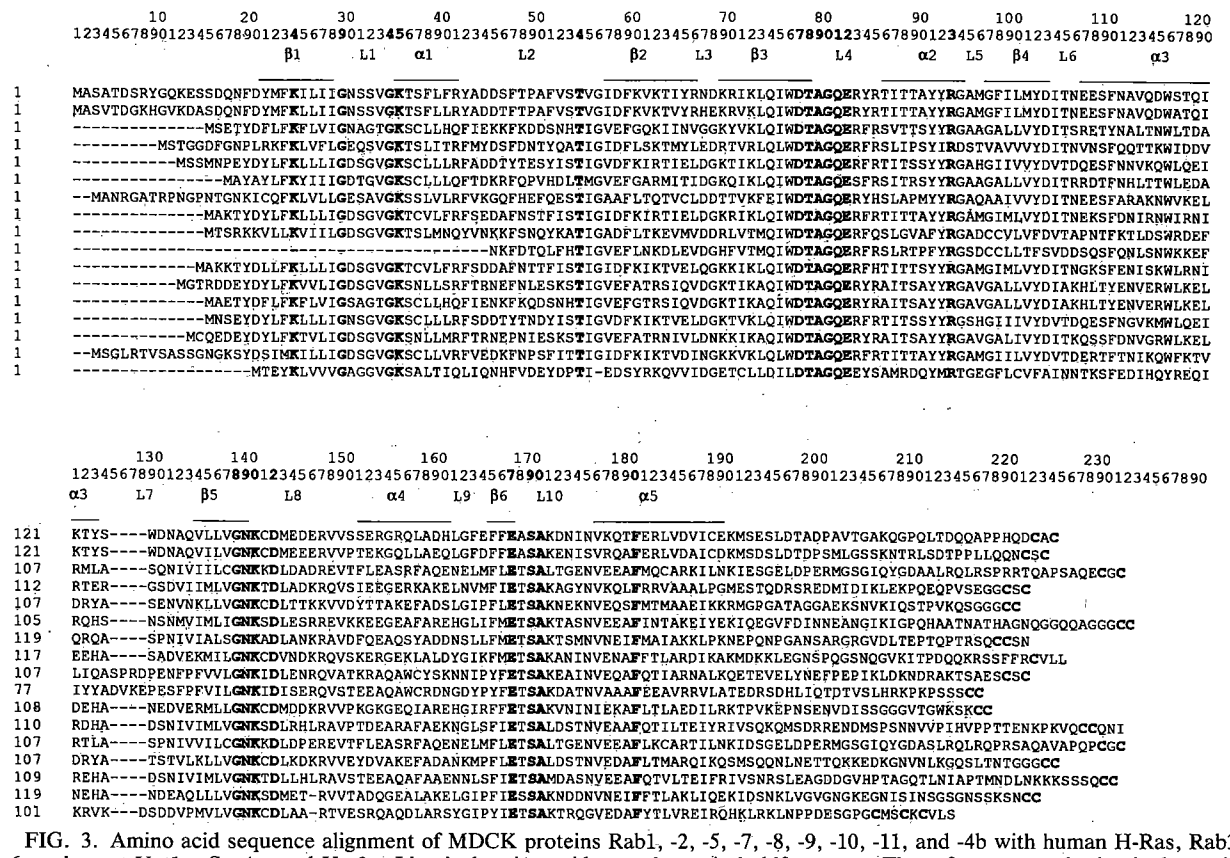

FIG. 3. Amino acid sequence alignment of MDCK proteins Rab1, -2, -5, -7, -8, -9, -10, -11, and -4b with human H-Ras, Rab3a, -3b, -4, -6, and yeast Ypt1p, Sec4p, and Ypt3p. Identical amino acids are shown in boldface type. The reference numbering is determined by the alignment. Alpha-helices, beta-strands, and loops for H-Ras taken from Pai et al. (26) are indicated.

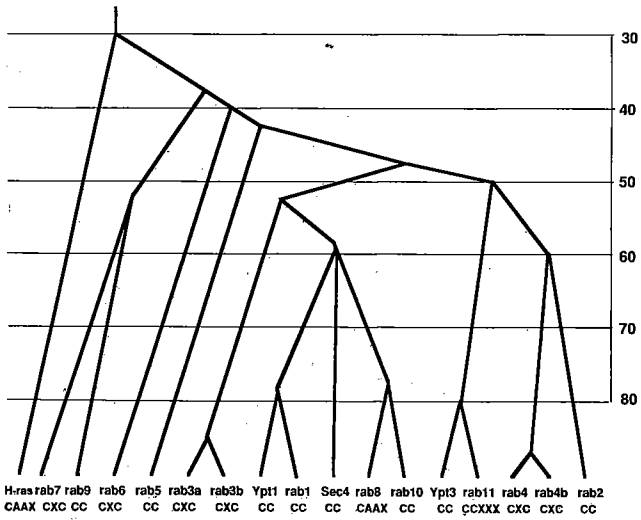

FIG. 4. Evolutionary tree of MDCK proteins Rab1, -2, -5, -7, -8, -9, -10, -11, and -4b; human Rab3a, -3b, -4, and -6; and yeast Ypt1p, Ypt3p, and Sec4p. The tree is drawn such that the height of the branching points roughly indicates the sequence similarity between the most distant sequences in the cluster defined by the branching point. A scale with percent identical residues is given next to the tree. The C-terminal cysteine motifs are also shown for each protein.

identified YPT3=SP8 and Rab11 (80%). MDCK protein 38 shares 87% identical amino acids with human Rab4 in this conserved region. It is not clear whether this divergence is due to species heterogeneity or whether it accounts for distinct, although highly related, proteins. An example of the latter case is proteins Rab3a and Rab3b (85% identity). Until this issue is clarified, we refer to protein 38 as Rab4b. Since Rab2 also shares 59% identity with Rab4 and 58% with protein 38, the three proteins are joined into a subgroup.

Also, Rab7 and Rab9 are subgrouped together due to their 54% sequence identity; in addition, they both have a 4-amino-acid insertion in loop L7 (Fig. 3). There are also proteins in the tree which are clearly separated from the others, like Rab5 and Rab6.

All proteins share one or two cysteine residues at their C termini. In the case of Ras proteins, the cysteine is followed by two aliphatic amino acids and a terminal variable residue. This C-terminal motif, the so-called CAAX box (cysteine-aliphatic-aliphatic-any amino acid), is modified posttranslationally and determines the membrane association of the Ras proteins (16, 17, 40). Analysis of the C termini of the different proteins reveals a sequence heterogeneity at the level of the cysteine motifs. Only Rab8, 4=rac2 and 41=rho1 (Fig. 2) contain the CAAX box. Rab1, Rab2, Rab9, and Rab10 have two consecutive C-terminal cysteines, similar to Ypt1p, Ypt3p, and Sec4p. In Rab5, the two cysteines are followed by serine and asparagine, while Rab11 has an unusual CCQNI C-terminal motif. In Rab3a and Rab3b, Rab4 and Rab4b, and Rab6, and Rab7, the cysteines are separated by glycine, alanine, or serine, respectively.

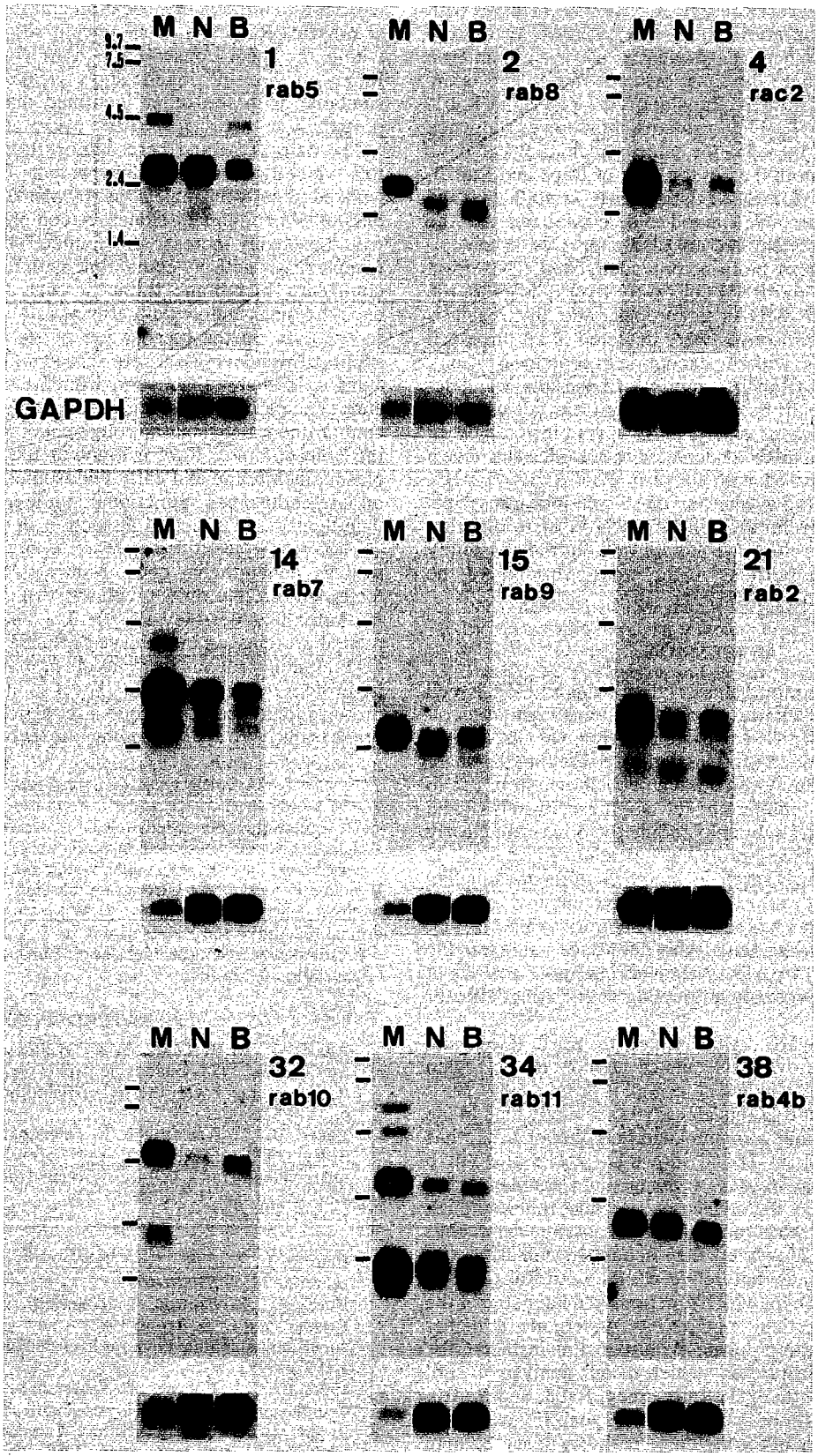

FIG. 5. Northern blot analysis of YPT1/SEC4-like genes expressed in MDCK (M), NIH 3T3 (N), and BHK (B) cells. Polyadenylated RNAs (1 μg per lane) electrophoresed on 1% agarose gels and transferred to nylon filters were hybridized with ^{32}P-labeled 1, 2, 4, 14, 15, 21, 32, 34, and 38 cDNAs as probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control probe. Sizes (in kilobases) were determined by using RNA molecular size standards.

To determine whether the cysteine motifs correlate with the sequence conservation, they were included in the tree scheme of Fig. 4. Whenever sequences are more than 80% similar, they also share the same cysteine motif. This is the case for Rab3a and Rab3b, Rab4 and -4b, YPT1 and Rab1, and YPT3 and Rab11. When proteins show lower sequence similarity, even if grouped in the same branch, they can have different cysteine motifs, as in the case of Rab8 and Rab10.

Expression of YPT1/SEC4-related genes in epithelial and nonepithelial cell lines. We wanted to determine whether the sequences we have identified are also expressed in nonepithelial cells. Northern blots of polyadenylated RNA from MDCK, NIH 3T3, and BHK cells were hybridized with the various ^{32}P-labeled cDNAs as probes, and the result is shown in Fig. 5. Transcripts were detected in all three cell types. In most cases, with the exception of proteins 4 (Rac2), 15 (Rab9), and 38 (Rab4b), multiple transcripts were detected by the same probe. This observation is not unprecedented for Rab mRNAs (35, 42). However, in the case of cDNA 2 (Rab8), the mRNAs detected in MDCK cells have a different size compared with those in NIH 3T3 and BHK cells. We do not know whether these multiple transcripts correspond to differentially spliced mRNAs or to different gene products. Therefore, we cannot rule out the possibility that highly related mRNAs might cross-hybridize to the same probe.

DISCUSSION

In this paper, we report the isolation from an MDCK cell cDNA library of 11 clones encoding proteins highly homologous to the yeast Ypt1/Sec4 proteins. Amino acid sequence analysis of the encoded proteins indicate that they all share the conserved residues which have been shown in p21^{ras} to constitute the GTP-binding site. Two of these, proteins 4 and 41, belong to the Rho subfamily, while the others are clearly Rab proteins: of these, proteins 2 (Rab8), 15 (Rab9), 32 (Rab10), and 34 (Rab11) are new members of the mammalian Rab subfamily. All these proteins have distinct effector regions and show a higher sequence similarity to Ypt1, Ypt3, and Sec4 than to Ras proteins. This difference reinforces the idea that they might perform a function similar to that of Ypt1p and Sec4p, probably interacting with distinct effector proteins.

Rab8 and Rab10 are highly related to Ypt1, Rab1, and Sec4 proteins. The overall sequence identity in the region excluding the variable N and C termini is around 60%. Most important is the fact that this identity is strikingly high in the effector domain. While a mammalian homolog of Ypt1p has been identified (Rab1=47) and shown to replace the Ypt1p functionally in *S. cerevisiae*, a mammalian counterpart of Sec4p has not been identified yet. Because of their high sequence similarity, Rab8 and Rab10 are possible candidates for such a protein. However, their sequence similarity with SEC4 is not as high as that between YPT1 and Rab1 (78%). It is possible that Sec4p, which functions at a later step of the secretory pathway than Ypt1p, has undergone a higher degree of specialization during evolution. For instance, the brain-specific Rab3a protein, which in our analysis is subgrouped together with Ypt1, Rab1, Sec4, Rab8, and Rab10, has recently been found associated to synaptic vesicles, a specialized organelle of the regulated pathway of secretion in neurons (38).

Rab11 is highly homologous to Ypt3p (24) and equivalent to SP8p (11) recently found in *S. pombe*. Deletion of the YPT3 gene causes a temperature-sensitive lethal phenotype (24), but its intracellular location and function are unknown at present. Rab9 shares significant sequence identity (54% within the most conserved region) with Rab7. Rab5 and Rab6, on the other hand, show weaker (~40%) sequence similarity with the other identified mammalian and yeast proteins.

The data presented here demonstrate the complexity of this subfamily of GTP-binding proteins. The 11 cDNAs were isolated (mostly in single copy) by a limited library screening of 50,000 recombinant clones. A complete library screening would probably increase this number about threefold. Such complexity is entirely compatible with their proposed role in controlling membrane traffic at each specific step of the exocytic and endocytic pathways (4). Furthermore, our recent data on the localization of Rab2, Rab5, and Rab7 support this view (8). These three proteins are specifically located in distinct subcompartments on the exocytic or endocytic pathway. Rab2 is associated with an intermediate compartment between the rough endoplasmic reticulum and the Golgi complex, Rab5 is found on the cytosolic side of the plasma membrane and early endosomes, and Rab7 is found on late endosomes. Functional studies will hopefully elucidate their mode of action at each of these sites.

The cDNAs identified here are expressed in epithelial as well as nonepithelial cells. Multiple transcripts were detected on Northern blots with most of these probes. We do not know whether they encode the same proteins or highly related proteins, as in the case of Rab3a and Rab3b. If these GTP-binding proteins were involved in regulating intracellular protein traffic, we would expect to find epithelial-specific proteins that specify the routes required for polarized membrane traffic. We are continuing our search for such proteins.

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