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Molecular Cloning and Characterization of Rab27a and Rab27b, Novel Human Rab Proteins Shared by Melanocytes and Platelets

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Rabs are prenylated, membrane-bound proteins involved in vesicular fusion and trafficking. We isolated the complete cDNAs of two rab isoforms, rab27a and rab27b, from human melanoma cells and melanocytes. Rab27a is the human homolog of a rat megakaryocyte rab called ram p25. Rab27b corresponds to a small GTP-binding protein, c25KG, which was previously purified from platelets but whose cDNA had not been cloned. Sequence comparisons with known rabs indicate that rab27a and rab27b comprise a melanocyte/platelet subfamily within the rab family. In addition, rab27a was expressed in a large variety of cell and tissue types, excluding brain, and rab27b manifested itself primarily in testis. Bacterially expressed and purified rab27a and rab27b exhibited GTP-binding activity and can now be used for antibody production and studies of the substrate specificities of geranylgeranyl transferase. In addition, the expression of rab27a and rab27b in both melanocytes and platelets makes them candidates for involvement in mouse and human disorders characterized by the combination of pigment dilution and a platelet storage pool defect.

Rab proteins represent a family of nontransforming monomeric GTP-binding proteins in the ras superfamily. These proteins have four highly conserved regions (I to IV) involved in GTP binding and hydrolysis. A consensus carboxy-terminal motif containing two cysteines also appears in all rabs. Geranylgeranylation of one of these cysteine residues anchors the rab to the plasma membrane or to an intracellular vesicular membrane. Subsequently, in a fashion not fully elucidated, rabs regulate membrane trafficking and vesicular fusion and targeting. Hence, a number of different rab proteins should be expressed in each eukaryotic cell, and any particular rab should be localized on the cytoplasmic surface of a distinct membrane-bound organelle (1–4).

Because melanocytes have melanosomes, i.e., intracellular vesicles specialized for pigment formation, these cells may contain unique rabs. They might also express rabs that are otherwise specific for platelets, since certain platelet organelles share membrane properties with the melanosome (5).

We identified in human melanocytes and describe in this paper the complete sequence of two rab cDNAs corresponding to sequences expressed in platelets. One cDNA, rab27a, represents the human homolog of rat ram p25, originally cloned from a megakaryocyte library (6). The other, rab27b, codes for a protein whose amino acid sequence matches the limited available peptide sequence of c25KG, a small GTP-binding protein purified from human platelets (7). Rab27a and rab27b, which comprise a rab subfamily, were characterized with respect to their homology with other rabs and their expression in various human tissues and tumor cell lines. Recombinant proteins of these rab genes were also made in bacteria, purified to near homogeneity, and demonstrated to have GTP-binding activity.

MATERIALS AND METHODS

Taq polymerase was from Boehringer-Mannheim (Indianoplis, IN). Filters for cDNA library screening

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were obtained from NEN Research Products (Boston, MA). The [α-^{32}P]dCTP and [α-^{32}P]GTP were purchased from Amersham (Arlington Heights, IL). Restriction enzymes were from New England Bio-Lab (Beverly, MA). Competent bacterial cells were from GIBCO BRL (Gaithersburg, MD). A human melanoma cDNA library was obtained from Clontech (Palo Alto, CA). Precast gels for SDS–PAGE were from Novex (San Diego, CA). Prestained protein molecular weight markers were from Bio-Rad (Hercules, CA).

Rapid Amplification of cDNA Ends (RACE)

Total RNA from pigmented human melanoma cells (American Type Cell Culture, Rockville, MD) was isolated using RNAzol (Tel-Test, Friendswood, TX) and reverse transcribed into first-strand cDNA using Superscript II reverse transcriptase (GIBCO BRL). The cDNA served as template for rapid amplification of the 3′ cDNA ends (RACE) of rab27a and rab27b. The primers for 3′-RACE were 5′-CCTCAATGTCAGAAACTGGATAAGCCAGC-3′ (external) and 5′-GCGTCTAGATAAGCCAGCTACAGATGCATGC-3′ (nested) for rab27a and 5′-GTTTGACCTCACCAGTCAACAGAGC-3′ (external) and 5′-GCGTCTAGAAACTGGATGAGCCAACTGC-3′ (nested) for rab27b. The common downstream primers were 5′-CCTCTGAAGGTTCCAGAATCGATAGGAATTC(T)$_{18}$ (A/G/C)N and 5′-TTCGGATCCTCTGAAGGTTCCAGAATCGATAG-3′. Thirty cycles of PCR were performed at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The PCR products were digested with *Xba*I and *Bam*HI, whose recognition sites were predesigned into the nested PCR primers, and subsequently inserted into pGEM11Z vector (Promega, Madison, WI). For 5′-RACE of rab27b, a gene-specific oligonucleotide, 5′-CAGCTTTCTCCACATTC-3′, was used as reverse transcription primer to synthesize cDNA. Two additional gene-specific primers, 5′-GCTTGCAGTTGGCTCATCCAG-3′ and 5′-AAGAAGCCCATGGCGTCTC-3′, served as external and nested primers, respectively, in a PCR using an anchor primer and a universal amplification primer supplied in the rapid amplification of cDNA ends kit (GIBCO BRL). The PCR was performed at 94°C, 30 s; 55°C, 30 s; and 72°C, 45 s for 35 cycles. The 5′-RACE product was cloned into the vector pCRIII from a TA cloning kit (Clontech) and sequenced.

Screening of a Human Melanocyte cDNA Library

A human melanocyte cDNA library was constructed by inserting cDNA transcribed from melanocyte poly(A)$^+$ RNA (1 μg) into a λCE27 vector as described by Miki and Aaronson (8). The 3′-RACE products of both genes were used as probes to screen the library (2×10^5 plaques). After two rounds of plaque purification, five clones of rab27a were isolated. The plasmid insertions were rescued with *Sal*I digestion as described (8), and the ligated plasmid DNA was transformed into *Escherichia coli* strain DH5α to produce enough DNA for sequencing.

Sequence comparison and alignment was achieved using the GCG program (Madison, WI).

Northern Blot Analysis of rab27a and rab27b Expression

Multiple human tissue and tumor cell line blots (Clontech) were probed with the 3′-RACE fragments of rabs27a and 27b at 65°C for 2 h in ExpressHyb solution (Clontech). The membranes were then washed at 37°C with 2× SSC, 0.1% SDS for 30 min, followed by washing at 60°C with 0.1× SSC, 0.1% SDS for 30 min. A partial cDNA fragment of human translation elongation factor-1 (900 bp) was used as control for the quantity and integrity of the RNAs loaded on the gel. The expression of rab27a and rab27b in melanocytes was examined by RT–PCR. Human melanocyte RNA, prepared from cultured normal human melanocytes (9), was reverse transcribed and used as template in PCR with the gene-specific primers 5′-CAGAAACTGGATAAGCCAGC-3′ and 5′-CGGATGCTTTATTCGTAGG-3′ for rab27a and 5′-GGCTTCTTATTAATGTTTGACC-3′ and 5′-TCACATTGGGAAATCTTTGATC-3′ for rab27b.

Bacterial Expression of rab27a and rab27b

The coding regions of rab27a and rab27b were amplified from the melanoma cDNA described above using primers 5′-CTGAGTTCTTCCATATGTCTGATGG-3′ and 5′-CCAGGCATGGCTCAGCTGAACTAC-3′ for rab27a and 5′-CAAGACCATCCATATGACCGATGGAG-3′ and 5′-CTTGATGCTCAGCTTCTATGTAGAGTC-3′ for rab27b. The PCR products were cut with *Nde*I and *Bpu*1200I and inserted into the bacterial expression vector pET15b (Novagen, Madison, WI). The constructs were sequenced and their reading frames confirmed. The plasmids were transformed into *E. coli* host strain BL21 (DE3) and grown in LB broth containing 100 mM ampicillin until the OD_{600} reached 1.0. The cells were harvested 3 h after induction with 1 mM isopropylthio-β-D-galactoside (IPTG).

Purification of the Expressed Rab Proteins and GTP-Binding Assays

The bacterial cell pellets from 50 ml of culture were resuspended in 3 ml binding buffer (Novagen) containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9, and sonicated in the presence of 1% Triton X-100 and 0.4 mg/ml lysozyme. Purification was carried out according to the instruction manual of the Novagen pET System kit. Briefly, the column (1–2 ml of resin) was charged with 50 mM $NiSO_4$ and equilibrated with the binding buffer. The bacterial sonicates were centrifuged at 12,000*g* for 20 min and the supernatants loaded on the column. The column was then washed with 10 vol of the binding buffer and 6 vol of washing buffer (60 mM imidazole). Proteins were eluted in a stepwise fashion with buffers containing imidazole concentrations increasing up to 1 M. The proteins were analyzed on 14% SDS–polyacrylamide gels and stained with Coomassie blue.

The GTP-binding assay was performed as described (10). Briefly, the crude extracts and the purified proteins were separated by 14% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were incubated with a binding buffer containing 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5, 1 mM EGTA, and 0.3% Tween 20 with 0.1 M GTP and ATP for 15 min. The membranes were then washed with the binding buffer without GTP and ATP and blotted with the same buffer containing [α-^{32}P]GTP (2 μCi/ml) at room temperature for 90 min. The membranes were washed twice with the binding buffer and exposed to X-ray films.

RESULTS

Nomenclature

Using a PCR-based strategy similar to that of Martin-Parras and Zerial (11), we previously identified in human melanoma cells 17 different partial cDNA clones of approximately 200 bp which encode small GTP-binding proteins of the rab family (D. Chen, J. Guo, and W. Gahl, unpublished work). The PCR primers used were degenerate oligonucleotides based on the sequences of GTP-binding domains II and III, conserved in all known human rab proteins. Most of the partial sequences we obtained matched perfectly with known human rab cDNAs. However, one clone was only similar, not completely identical, to the sequence of a rat rab, ram p25, isolated from megakaryocytes (6). A second clone, also homologous to ram p25, matched the limited available peptide sequence of c25KG, a small GTP-binding protein purified from human platelets (7). Our two rab sequences showed significant homology to each other, suggesting that they were likely isoforms within a rab subfamily. In accordance with the currently accepted nomenclature system (12), they were named rab27a and rab27b, respectively.

3′-RACE of Rab27a and Rab27b cDNAs

To isolate the complete cDNA sequences of rab27a and rab27b, we first determined a more gene-specific sequence outside the conserved 200-bp region by 3′-RACE. Using two gene-specific primers for each cDNA (nested PCR) and a common poly(dT) primer, fragments of 550 and 1000 bp were obtained for rab27a and rab27b, respectively. Nucleotide sequencing yielded a deduced amino acid sequence for rab27a with 97% identity to the corresponding region of rat ram p25 (Fig. 1), indicating that rab27a is the human homologue of rat ram p25.

The deduced amino acid sequence of rab27b (Fig. 2) also showed characteristic features of rab proteins, including the highly conserved GTP-binding domains III (GNKXD) and IV (ETSA) and a putative isoprenylation site, CXC, at its carboxy terminus.

Isolation of the Full-Length Rab27a and Rab27b cDNAs

To obtain the full-length cDNAs of rab27a and rab27b, we used the 3′-RACE products described above to screen a human melanocyte cDNA library. From approximately 2×10^5 phage plaques, five clones of rab27a were isolated after two rounds of screening. Two clones containing 1.3 and 1.9 kb insertions were sequenced and found to have identical coding regions, but different lengths of 3′ untranslated sequences. The open reading frame of rab27a encodes a protein of 221 amino acids with a predicted molecular weight of 25 kDa (Fig. 1). A GenBank search revealed that the nucleotide (ORF) and amino acid sequences had 87 and 96% identity to those of rat ram p25, demonstrating that rab27a is indeed its human homologue.[1] Screening the melanocyte cDNA library, as well as a human melanoma cDNA library, for rab27b yielded no positive plaques. Moreover, when phage DNA from both libraries (equivalent to 2×10^6 plaques) was used as template, a PCR prod-

[1] After the cDNA was isolated, a human cDNA rab27 was deposited with GenBank (Accession No. U38654) by Seabra *et al.* (18). The two sequences have identical ORFs, but rab27a has 89 extra bp in its 5′ UTR (nt 122–210) (Fig. 1).

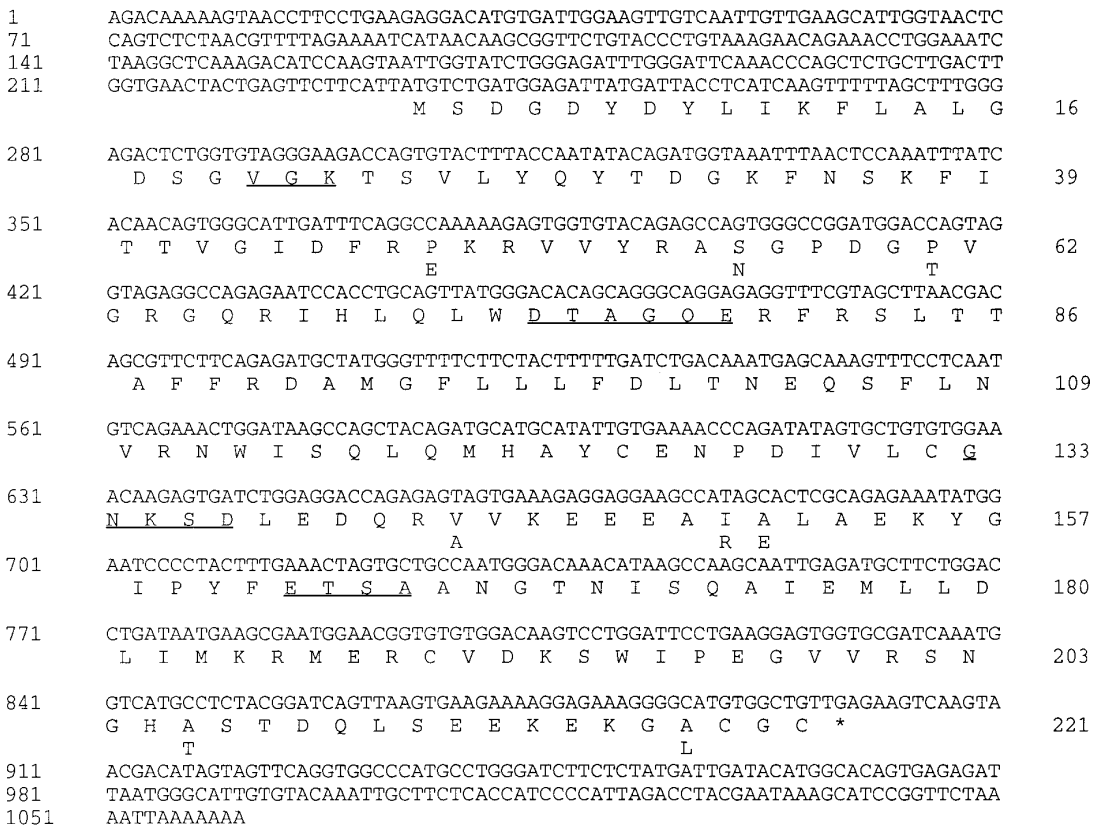

FIG. 1. Nucleotide and deduced amino acid sequences of rab27a. The highly conserved GTP-binding domains are underlined. The 8 amino acids of rat ram p25 that differ from rab27a are indicated by letter symbols under the human sequence.

uct of expected size was detected with rab27a-specific primers, but not with rab27b-specific primers (data not shown). This suggested that the copy number of rab27b was very low in both libraries. However, when cDNA synthesized from melanoma cell and human melanocyte total RNA was used as template for PCR amplification, fragments of predicted sizes were obtained with both the rab27a and rab27b primer sets (data not shown). Based on these observations, we considered that rab27b cDNA was underrepresented in both libraries. Thus, 5′-RACE was performed using two rab27b-specific oligonucleotides as internal and nested downstream primers and a poly(C) sequence as upstream primer. A product of approximately 600 bp was obtained, sequenced, and proven to be the 5′ portion of rab27b cDNA.

The complete nucleotide sequence of rab27b (Fig. 2) predicts a protein of 218 amino acids with a molecular weight of 24.7 kDa. The rab27b sequence contained all 76 known amino acid residues of c25KG, with the predicted order and spacing. Many of the amino acid residues of c25KG were also present in rab27a, but there were 12 residues that were present only in rab27b, not in rab27a, indicating that c25KG is encoded by rab27b cDNA.

Rab27a and rab27b share 66% identity at the nucleotide level in their ORFs, but low similarity in either the 5′ or 3′ untranslated regions. Amino acid sequence alignment showed 71% identity and 81% similarity between the two rabs (Fig. 3). They also share similar effector domains and carboxy-terminal isoprenylation motifs. There is very little sequence identity after GTP-binding domain IV, and rab27a has three more amino acids than rab27b (Fig. 3).

Both rab27a and rab27b showed characteristic features of rab proteins, including four conserved GTP-binding domains. Their carboxy-terminal isoprenylation motif, CXC, resembles that of human rabs 3a, 3b, 4, 6, 7, and S10 (Fig. 4). Rab27a and rab27b had only moderate homology to other human rab sequences, with the highest homology (41–44%) to members of the rab3 subfamily (Table 1). Rab27a and rab27b also bear several unique features in their primary sequences. An extra segment of 10 amino acids, in positions 57–66, is present between GTP-

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1     GGCTTGGGAAGGGGAAGGAAACTTCTCTGAAATCTGAACACCTGCTCTCCCGGCAAGGAAACTTCGAAGG
71    CTGACCGACCAAGACCATCACTATGACCGATGGAGACTATGATTATCTGATCAAACTCCTGGCCCTCGGG
                              M  T  D  G  D  Y  D  Y  L  I  K [L  L  A  L  G    16

141   GATTCAGGGGTGGGGAAGACAACATTTCTTTATAGATACACAGATAATAAATTCAATCCCAAATTCATCA
       D  S  G  V  G  K  T] T [F  L  Y  R] Y  T  D  N  K  F  N  P  K [F  I    39

211   CTACAGTAGGAATAGACTTTCGGGAAAAACGTGTGGTTTATAATGCACAAGGACCGAATGGATCTTCAGG
      T  T  V  G  I  D  F  R] E  K  R  V  V  Y  N  A  Q  G  P  N  G  S  S  G   63

281   GAAAGCATTTAAAGTGCATCTTCAGCTTTGGGACACTGCGGGACAAGAGCGGTTCCGGAGTCTCACCACT
        K  A  F  K [V  H  L  Q  L  W  D  T  A  G  Q  E  R] F  R [S  L  T  T    86

351   GCATTTTTCAGAGACGCCATGGGCTTCTTATTAATGTTTGACCTCACCAGTCAACAGAGCTTCTTAAATG
       A  F  F  R] D  A  M  G  F  L  L  M  F  D  L  T  S  Q  Q  S  F  L  N    109

421   TCAGAAACTGGATGAGCCAACTGCAAGCAAATGCTTATTGTGAAAATCCAGATATAGTATTAATTGGCAA
      V  R  N  W  M  S  Q  L  Q  A  N  A  Y  C  E  N  P  D  I  V  L  I  G  N   133

491   CAAGGCAGACCTACCAGATCAGAGGGAAGTCAATGAACGGCAAGCTCGGGAACTGGCTGACAAATATGGC
        K [A  D  L  P  D  Q  R] E  V  N  E  R  Q  A  R  E [L  A  D  K  Y  G    156

561   ATACCATATTTTGAAACAAGTGCAGCAACTGGACAGAATGTGGAGAAAGCTGTAGAAACCCTTTTGGACT
       I  P  Y  Y  F  E  T  S  A  A  T  G  Q  N  V  E  K] A  V  E  T  L  L  D    179

631   TAATCATGAAGCGAATGGAACAGTGTGTGGAGAAGACACAAATCCCTGATACTGTCAATGGTGGAAATTC
      L  I  M  K  R  M  E  Q  C  V  E  K  T  Q  I  P  D  T  V  N  G  G  N  S   203

701   TGGAAACTGGGATGGGGAAAAGCCACCAGAGAAGAAATGTATCTGCTAGACTCTACATAGAAACTGAACA
        G  N  W  D  G  E  K  P  P  E  K  K  C  I  C  *                        218

771   TCAAGAACCCCACCAAAATATTACTTTTAAAACAATGACAAACCACACAATTGTTGTTGAGTAAACCACG
841   CACAATGGCATGTCTTTCTTTTTCTGCCAGAAAATCTATTTTAAGAAACCAGAATAGTCAACAGTGTTCA
911   AAAGAATTGACTAGTTATCCCTGAGGCCCTTTCAAACATGATCAAAGATTTCCCAATGTGATCTCATCAT
981   CATGGATACTCAATTTGTTTTTTCTTATAGAGAAAATGAGTATATAGACATATACAGAGAAT
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FIG. 2. Nucleotide and deduced amino acid sequences of rab27b. The highly conserved GTP-binding domains are underlined. The peptide sequences determined from purified c25KG (7) are in brackets.

binding domains I and II; this region is not seen in other mammalian rab proteins (Fig. 4). Rab27a and rab27b also differ from other human rabs at several highly conserved positions. For example, instead of having YYRG between positions 88 and 91, both rab27a and rab27b have the amino acid sequence FFRD (Fig. 4). A Phe at position 192 is conserved in all known human rabs, but is replaced by Ile and Val in rab27a and rab27b, respectively (Fig. 4). These results suggest that the two rabs are members of a distinct subfamily which is distantly related to other members of the larger rab family.

Expression of Rab27a and Rab27b in Human Tissues and Cell Lines

The 3′-RACE products of both rab27a and rab27b were used to probe membranes blotted with 2 μg of poly(A)$^+$ RNA from different human tissues and tumor cell lines. Using the rab27a probe, three transcripts of 3.7, 2.7, and 1.3 kb were detected (Fig. 5, top panels). The 3.7-kb transcript was present in 15 of 16 tissues examined, with brain as the only exception. Quantitative variations were seen among different tissues, with thymus, kidney, muscle, and

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Rab27b   MTDGDYDYLIKLLALGDSGVGKTTFLYRYTDNKFNPKFITTVGIDFREKRVVYNAQGPNGSSGKAFKVHLQLWDT
         |  |||||||||  |||||||||||  ||  |||  |||  ||||||||||  |||||  |  ||  |   |      |||||||
Rab27a   MSDGDYDYLIKFLALGDSGVGKTSVLYQYTDGKFNSKFITTVGIDFRPKRVVYRASGPDGPVGRGQRIHLQLWDT

Rab27b   AGQERFRSLTTAFFRDAMGFLLMFDLTSQQSFLNVRNWMSQLQANAYCENPDIVLIGNKADLPDQREVNERQARE
         ||||||||||||||||||||  ||||  |||||||||  ||||  ||||||||||  |||  ||  |||  |  |   |
Rab27a   AGQERFRSLTTAFFRDAMGFLLLFDLTNEQSFLNVRNWISQLQMHAYCENPDIVLCGNKSDLEDQRVVKEEEAIA

Rab27b   LADKYGIPYFETSAATGQNVEKAVETLLDLIMKRMEQCVEKTQIPDTVNGGNSGNWDGEKPPEK-K--CIC
         ||  |||||||||||||  |  |    |  |  ||||||||||  ||  |   ||   |    |          |||  |   |  |
Rab27a   LAEKYGIPYFETSAANGTNISQAIEMLLDLIMKRMERCVDKSWIPEGVVRSNGHASTDQLSEEKEKGACGC
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FIG. 3. The alignment of rab27a and 27b sequences. Gaps (dashed lines) have been introduced to optimize the alignment. Identical amino acids are indicated by vertical bars. The effector domains and C-terminal isoprenylation domains of both proteins are underlined.

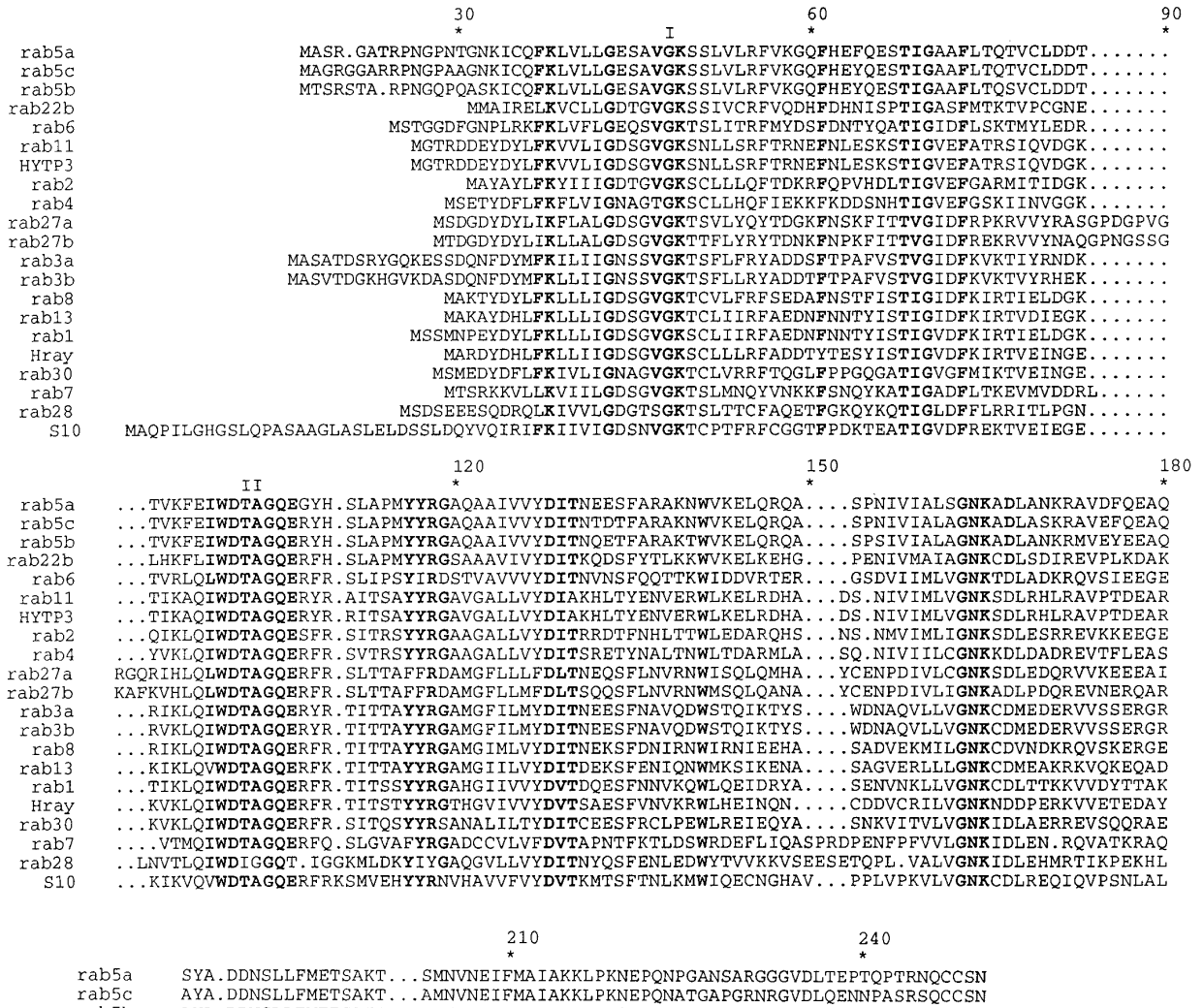

FIG. 4. The alignment of all known human rab sequences. Gaps (dotted lines) are introduced to optimize the alignment. Invariable or highly conserved amino acids are in bold. Four GTP-binding domains are indicated (I–IV). The adjacent sequences share a higher percentage of similarity to each other than distant ones. Amino acid number 1 of rab27a and rab27b corresponds to amino acid number 28 of S10.

placenta having low abundance. The smaller transcripts showed parallel but lower expression levels than the 3.7-kb transcript, except in leukocytes, where expression of the 1.3-kb transcript exceeded that of the 3.7-kb transcript (Fig. 5, top panel). Multiple transcripts have been observed with many rab

TABLE 1
Comparison of Rab27a and Rab27b and Known Human Rab Proteins

	% Sequence identity with	
	rab27a	rab27b
Rab1	40	40
Rab2	37	35
Rab3a	43	42
Rab3b	44	41
Rab4	39	40
Rab5a	30	31
Rab5b	30	30
Rab5c	31	33
Rab6	35	32
Rab7	36	36
Rab8	43	41
Rab11	37	39
HYTP3	37	38
Rab13	41	40
Hray	35	39
Rab22b	37	35
Rab30	35	39
S10	34	37
Rab28	26	26

genes, presumably due to different lengths of 3′ untranslated regions.

Rab27a was detected by Northern blots in most of the tumor cell lines examined as well. The expression levels were relatively high in two leukemia cell lines, promyelocytic leukemia HL-60 and chronic myelogenous leukemia K-562, and in Hela cells and melanoma (G361) cells, but rather low in others (Fig. 5, top panel).

In contrast to the nearly ubiquitous expression of rab27a in tissues, rab27b RNA was present in a significant amount only in testis (Fig. 5, middle panel), where the predominant transcript was 1.4 kb in size. For many tissues, an 8-kb transcript of unknown origin was also seen; this was significantly larger than the size of any reported rab mRNA (less than 4.5 kb). The 1.4-kb rab27b message was not apparent in melanoma cell line G361 RNA blotted on a membrane (Fig. 5, middle panel). However, the rab27b RNA could be detected by RT–PCR in melanoma, melanocyte, and fibroblast cells (data not shown).

The bottom panels of Fig. 5 show the signal using translation elongation factor-1 as a control for mRNA loaded on the filter. The melanoma mRNA lane contains relatively little RNA, which may account for the absent signal using rab27b as probe.

Expression and Purification of Rab27a and Rab27b

In order to carry out further biochemical studies of these small GTP-binding proteins, the coding regions of both rab27a and rab27b were inserted into a bacterial expression vector. Both rab27a and rab27b were expressed in *E. coli* at high levels after IPTG induction, with rab27b production slightly greater than that of rab27a (Fig. 6). The synthesis of each protein reached a maximal level 3 h after the addition of 1 mM IPTG (Fig. 6). A significant portion of the expressed protein was soluble, but a fraction remained insoluble (data not shown). Culture of cells at 29°C or induction with a low concentration of IPTG (0.4 mM) did not increase the solubility of these proteins, but decreased their yield (data not shown).

The expressed rab proteins were tagged with six constitutive histidine residues at their amino termini, allowing purification by nickel affinity chromatography. The purity of both proteins, examined by SDS–PAGE followed by Coomassie brilliant blue staining, exceeded 90% for both rab proteins (Fig. 6). A protein of smaller molecular weight always coeluted with rab27b. No proteins were purified from lysates of bacteria transformed with expression vector alone (Fig. 6).

An assay for GTP-binding activity was carried out using crude cell lysates and purified proteins. The proteins were first separated on SDS–PAGE, transferred to nitrocellulose membranes, and renatured. Binding of [^{32}P]GTP to both rab proteins (Fig. 7) indicated that the cDNA sequences of rab27a and rab27b encoded low-molecular-weight GTP-binding proteins and that the recombinant forms were active.

DISCUSSION

We report two complete cDNA sequences of a novel rab small GTPase subfamily from human melanocytes, with initial characterization of these cDNAs and their encoded proteins. Rab27a is the human homologue of rat megakaryocyte ram p25, and rab27b encodes human platelet c25KG (6,7). Both display all the characteristic features of small GTP-binding proteins.

Several lines of evidence support the designation of rab27a and rab27b as a distinct subfamily. First, they share a high percentage of similarity (81%) in their overall sequences. Second, both rabs have different amino acids at positions that are otherwise highly conserved among all human rabs, including Tyr at positions 88 and 89 and Phe at position 192

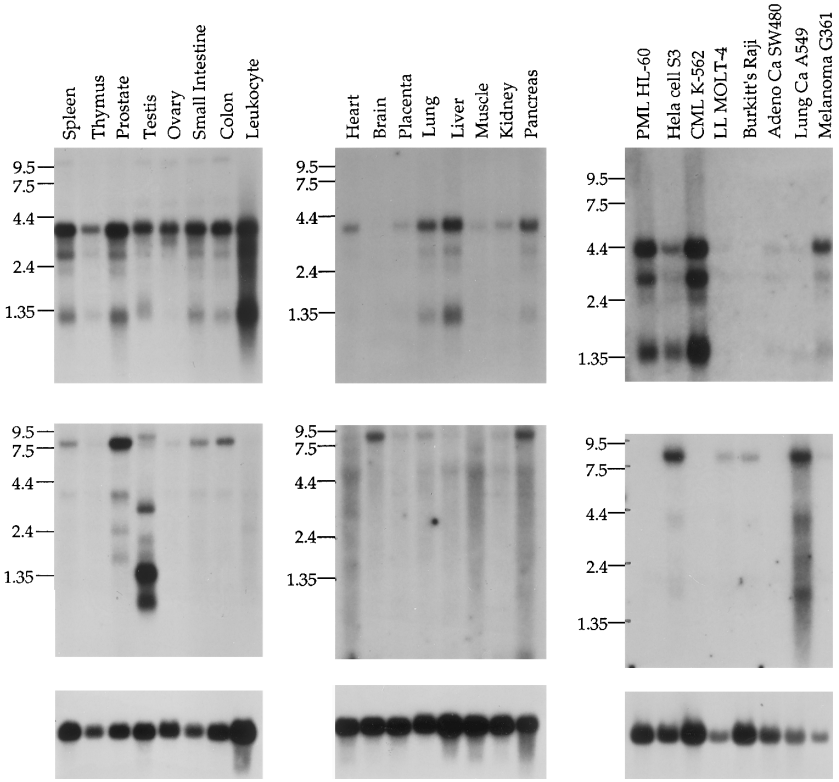

FIG. 5. Tissue distribution of rab27a and rab27b. Multiple tissue blots with 2 μg of poly(A)$^+$ RNA were probed as described under Materials and Methods. The tissue/cell source of RNA is indicated above each lane. Size markers (kb) are indicated on the left. The same set of blots was used for both rab27a (top panel) and rab27b (middle panel) probes. A partial cDNA of human translation elongation factor-1 served as a control probe (bottom panel). The labels for tumor lines are: PML, promyelocytic leukemia; CML, chronic myelogenous leukemia; LL, lymphoblastic leukemia; Adeno Ca, colorectal adenocarcinoma.

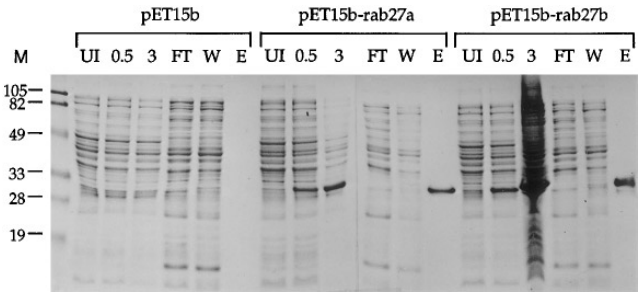

FIG. 6. Expression and purification of human rab27a and rab27b. The bacterial host strain BL21(DE3) was transformed with expression vector containing no cDNA insertion (pET15b), the rab27a coding region (pET15b-27a), or the rab27b coding region (pET15b-27b). The bacteria were harvested and protein extracts were analyzed by 14% SDS–PAGE; the gels were stained with Coomassie blue. UI, total lysate from uninduced culture; 0.5, total lysate from culture 0.5 h after 1 mM IPTG induction; 3, total lysate from culture 3 h after 1 mM IPTG induction; FT, flowthrough from nickel column after loading the 12,000*g* supernanants of total lysates; W, fraction obtained by washing the column; E, fraction eluted from the column by 0.5 M imidazole. Molecular weight markers are shown on the left in kDa.

(Fig. 4). Third, rab27a and rab27b have similar carboxy-terminal motifs, a CXC prenylation sequence, and similar effector domains. Finally, both rab27a and rab27b display a unique 10-amino-acid insertion between GTP-binding domains I and II (Fig. 4), supporting the designation of a unique subfamily for rab27.

On the other hand, compared with members of other rab subfamilies, rab27a and rab27b exhibit relatively low sequence identity (71%) and differ in size by three amino acids. Canine rab8 and rab10 have only slightly less identity with each other (65%) and are designated as separate members of the rab family, perhaps because of their different carboxy-terminal motifs (13). Rabbit rab25 and human rab11 are 63% identical, share the same prenylation motif (CCAAX), and differ slightly in size near their carboxy termini (14); they have also garnered individual rab status. If species heterogeneity is taken into consideration, these two sequences are likely to have a degree of identity similar to that between rab27a and rab27b. Hence, rab27a and rab27b may repre-

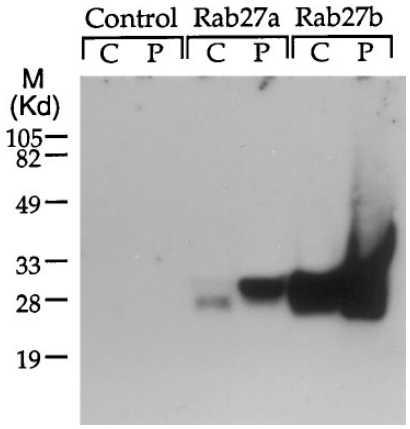

FIG. 7. GTP binding of human rab27a and rab27b. The proteins were analyzed in the same fashion as in Fig. 6, transferred to a nitrocellulose membrane, and blotted with [α^{32}P]GTP as described under Materials and Methods. C, 12,000*g* supernatants of crude lysates prepared from cells 3 h postinduction (see lane 3 in Fig. 6); P, proteins purified by elution with 0.5 M imidazole from the nickel columns (see lane E in Fig. 6). The molecular weight markers are the same as those in Fig. 6. No binding was detected with lysates prepared from uninduced cells (not shown).

sent an intermediate stage in the evolution of different members of a large family of genes.

The tissue distribution of rab proteins varies significantly. Many show nearly ubiquitous expression, including rab27a, which was expressed in several cell lines and in almost all the tissues examined (Fig. 5, top panel). Other rabs are expressed in only a few cell types or tissues. For instance, rab27b exhibits significant expression only in testis, resembling the recently isolated rat rab28, a remote member of the rab family (Fig. 4). Other examples of specialized expression include rat rab15, expressed in neuronal cells (15), rabbit rab25, expressed in chief cells (14), and mouse rab3d, expressed in adipocyte cells (16). The biological function of such tissue specific expression requires further investigation.

The tissue distribution of different isoforms within a subfamily also differs. For example, rab3a is expressed preferentially in neuronal cells (17), whereas rab3d is expressed primarily in adipocytes (16). Similarly, rab27b is expressed in at least three highly differentiated cell types, including melanocytes, testis, and platelets (as c25KG), while rab27a has an even more ubiquitous expression pattern.

In addition, rab homologues can have a different tissue distribution in different species. Rab27a, for example, is expressed in human liver (Fig. 5, top panel), while rat ram p25/rab27 was not detected in mouse liver on Northern blots (6). The absence of rab27a in mouse liver was recently confirmed by Western blot analysis (18). The recently cloned rat and human rab28 provide another example of this phenomenon, with very high expression in rat testis but very low expression in human testis (19).

The biochemical properties of rab27b (formerly c25KG) have been studied by Nagata *et al.* (7,20,21). This protein was observed as one of the major low-molecular-weight GTP-binding proteins in human platelet cytosol (7) and was also purified from the membrane fraction of human platelets (20). Nagata and Nozawa subsequently showed that rab27b was primarily associated with the plasma membrane of platelets and with the cytoplasmic side of α-granules (21). The fact that it was first purified from human platelet cytosol suggests that the protein is also present in soluble form. Whether the two forms are different in their GTP/GDP binding and prenylation status remains unknown.

Platelets contain a number of small GTP-binding proteins (MW 21–28 kDa), most of which are members of the ras superfamily, including rab1, rab3B, rab4, rab6, rab8 of the rab family (22,23). Rab6, rab8, and a few other small GTP-binding proteins have been localized on platelet secretory vesicles, in particular, α-granules (21,23,24). Whether rab27b or even rab27a corresponds to an α-granule-associated low-molecular-weight GTPase requires further study, as does the function of these proteins in vesicle exocytosis and membrane recycling. In addition, comparisons of the type of rab proteins expressed in melanocytes and platelets should be performed. Using a PCR-based strategy, we recently found that pigmented melanoma cells express at least 15 different rab proteins (D. Chen, J. Guo, and W. Gahl, unpublished work), including all those listed above.

It is not surprising that certain membrane-associated proteins are expressed in both platelets and melanocytes, since these cells possess related vesicles. Melanocytes contain melanosomes, which are now recognized to be specialized, pigment-producing organelles of lysosomal origin (25). As such, the melanosomal membrane contains lysosome-associated membrane proteins (LAMPs) (26), but it also contains ME491, which is identical to granulophysin, an integral membrane protein of the platelet dense body (5). This intracellular vesicle stores calcium, ADP, ATP, and serotonin, mediates the secondary aggregation response of platelets, and comprises the "storage pool" compartment of the platelet. Genetic evidence that melanosomes and dense bodies share membrane characteristics is provided by the existence of 12 different mutant mouse strains manifesting the combination of pigment dilution and a plate-

let storage pool defect (27). This suggests that genes controlling the biogenesis or processing of platelet dense bodies are similarly operative in melanosome formation. In many, but not all, mouse pigment dilution mutants, there are abnormalities in the lysosome as well (27).

The dual cell expression of rab27 isoforms in melanocytes and platelets makes these genes candidates for certain of the mouse genetic defects. One such mutant, the *gunmetal* mouse, provides precedent for the involvement of rab-related proteins in defects of pigment dilution and platelet storage pools. *gunmetal,* which shares phenotypic features with several human genetic diseases including the "gray platelet syndrome," displays altered expression of two unidentified small GTP-binding proteins of MW 25 and 28.5 kDa in its platelets (28). However, the identity of the overexpressed proteins remains unknown, and the observed expression pattern may reflect redistribution of these proteins due to their altered prenylation. To date, no investigations have been carried out in *gunmetal* melanocytes.

Some mouse mutants with pigment dilution and storage pool defects have human counterparts. The mouse *pale ear* corresponds both phenotypically and genetically to the human disorder, Hermansky-Pudlak syndrome (HPS). HPS consists of the triad of tyrosinase-positive albinism, storage pool deficiency due to absent platelet dense bodies, and accumulation of ceroid lipofuscin in cellular lysosomes (29). The mouse mutant *beige* serves as a model for the human disorder Chediak-Higashi disease (CHD), in which partial albinism results from giant melanosomes, platelet dense bodies are reduced in number, and frequent pyogenic infections occur due to giant, fused, dysfunctional lysosomes (29). Microscopic and histochemical observations of various CHD cell types have suggested that the CHD and *beige* mutations represent an abnormality in fusion or fission of late endocytic structures (30). The recent cloning of the CHD gene reveals that it does not code for a rab protein itself (31,32). However, since rabs affect membrane fusion and targeting, their expression, location in the membrane or cytoplasm, and function may be altered by mutations affecting related pathways. This applies to both CHD and HPS, and rabs 27a and 27b are of particular interest because they are expressed in both platelets and melanocytes.

Recently, Seabra *et al.* (18) purified a rab protein that failed to be prenylated by lymphoblasts of patients with choroideremia, an X-linked disorder of retinal degeneration due to a defective gene for the rab escort protein-1 (REP-1). REP-1 is required for the activity of geranylgeranyl transferase, an enzyme that prenylates rab proteins. The specific rab that remained unprenylated in choroideremia lymphoblasts resembled rat ram p25 and was named rab27 (i.e., rab27a). This raises two points. First, since rab27a appeared to be preferentially modified by REP-1, it may be appropriate to study rab27b for prenylation in the REP-1-deficient choroideremia cells. Second, although rab27a was expressed in retinal pigment epithelium and a variety of other tissues, its lack of prenylation was associated only with retinal degeneration. The explanation is that, while many rabs function redundantly in most tissue types, the retinal pigment epithelium depends almost exclusively on the function of rab27a (18). The same dichotomy between redundancy in most tissues and strict dependence upon specific rabs in other cells (e.g., platelets and melanocytes) may result in diseases caused by defects in rab proteins themselves. With the cloning and complete sequencing of human rab27a and rab27b, their expression and prenylation state can now be investigated in human disorders such as HPS and CHD.

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