Cloning of a Novel Family of Mammalian GTP-binding Proteins (RagA, RagB\textsuperscript{a}, RagB\textsuperscript{b}) with Remote Similarity to the Ras-related GTPases\textsuperscript{*}

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cDNA clones of two novel Ras-related GTP-binding proteins (RagA and RagB) were isolated from rat and human cDNA libraries. Their deduced amino acid sequences comprise four of the six known conserved GTP-binding motifs (PM1, -2, -3, G1), the remaining two (G2, G3) being strikingly different from those of the Ras family, and an unusually large C-terminal domain (100 amino acids) presumably unrelated to GTP binding. RagA and RagB differ by seven conservative amino acid substitutions (98% identity), and by 33 additional residues at the N terminus of RagB. In addition, two isoforms of RagB (RagBs and RagBl) were found that differed only by an insertion of 28 codons between the GTP-binding motifs PM2 and PM3, apparently generated by alternative mRNA splicing. Polymerase chain reaction amplification with specific primers indicated that both long and short form of RagB transcripts were present in adrenal gland, thymus, spleen, and kidney, whereas in brain, only the long form RagB was detected. A long splicing variant of RagA was not detected. Recombinant glutathione S-transferase (GST) fusion proteins of RagA and RagB bound large amounts of radiolabeled GTP\textsuperscript{\gamma}S in a specific and saturable manner. In contrast, GTP\textsuperscript{\gamma}S binding of GST-RagB\textsuperscript{b} hardly exceeded that of recombinant GST. GTP\textsuperscript{\gamma}S bound to recombinant RagA, and RagB was rapidly exchangeable for GTP, whereas no intrinsic GTPase activity was detected. A multiple sequence alignment indicated that RagA and RagB cannot be assigned to any of the known subfamilies of Ras-related GTPases but exhibit a 52% identity with a yeast protein (Gtr1) presumably involved in phosphate/magnesium binding sites (PM1-PM3), and the other three as guanine nucleotide binding sites (G1-G3) (Valencia et al., 1991). Therefore, the sequences of the least related GTPases comprise approximately 20–30% identical amino acids, whereas the sequence similarity is considerably higher within subfamilies (e.g. >40% identity in the Rab family). In the ARF family, >50% in the ARF family (Kahn et al., 1992).

The application of the PCR method has greatly facilitated the identification and cloning of novel cDNAs belonging to larger families of homologous genes, e.g. Ras-homologous GTPases (Chavrier et al., 1992; Clark et al., 1993). Constructing degenerate oligonucleotide primers from the conserved PM1 and PM3 domains, we have previously employed this approach to cloning of novel members of the ARF family (Schürmann et al., 1994; Cavenagh et al., 1994). In addition to the known ARF family, we isolated a cDNA fragment with overall similarity to ARF that does not resemble any of the known subfamilies. Here we report the isolation and characterization of full-length cDNAs encoding novel small GTP-binding proteins (RagA and RagB), unique among Ras-homologous GTPases. RagB is a member of the ARF-related GTPases with Remote Similarity to the Ras-related GTPases. RagA and RagB are the mammalian homologues of Gtr1 and that they represent a novel subfamily of Ras-homologous GTP binding proteins.

Ras-homologous GTPases constitute a large family of signal transducers that alternate between an activated, GTP-binding, and an inactivated, GDP-binding state. The sequences of these proteins are not only characterized by common structural features but also by a similar function, e.g. regulation of growth (Ras) (Egan and Weinberg, 1993), cytoskeleton organization (Rho) (Aktories et al., 1992), or vesicle transport (Rab and ARF) (Novick and Brennwald, 1993; Kahn et al., 1993). All GTPases of the Ras superfamily have in common the presence of six conserved motifs involved in GTP/GDP binding, three of them as phosphate/magnesium binding sites (PM1-PM3), and the other three as guanine nucleotide binding sites (G1-G3) (Valencia et al., 1991). Therefore, the sequences of the least related GTPases comprise approximately 20–30% identical amino acids, whereas the sequence similarity is considerably higher within subfamilies (e.g. >40% identity in the Rab family). In the ARF family (Kahn et al., 1992).

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MATERIALS AND METHODS

PCR Cloning—A domain of 34–40 codons of Ras-homologous GTPases was amplified with degenerate oligonucleotide primers matching the GTP-binding domains PM1 (amino acid sequence LDAAGKT) and PM3 (WD-TAGGE) of the ARF family as described previously (Schürmann et al., 1994) with cDNA from murine 3T3-L1 adipocytes (American Type Culture Collection, Rockville, MD) as the template. The reaction products were separated on 2% agarose gels and were cloned into pUC18 (Sitede-onit, Pharmacia Biotech Inc.). Plasmid DNA was isolated from 200 different clones and was characterized by sequencing or Southern blotting.

\textsuperscript{1}The abbreviations used are: ARF, ADP-ribosylation factor; PCR, polymerase chain reaction; GST, glutathione S-transferase; bp, base pairs; kb, kilobases; HPLC, high performance liquid chromatography; GTP\textsuperscript{\gamma}S, guanosine 5'-O-(3-thiotriphosphate).
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Library Screening and DNA Sequencing—PCR products subcloned into pUC18 were isolated with restriction enzymes EcoRI and BamHI and used as probes to screen a rat fat cell cDNA library (RL 1011b, Clontech, Palo Alto, CA), a λ-Zap rat brain cDNA library (937502, Stratagene, La Jolla, CA), and a human λ-Zap fetal brain cDNA library (937226, Stratagene). Positive plaques were isolated, and inserts were subcloned into the plasmid vector Bluescript (Stratagene) from plasmid DNA by exonuclease digestion in both directions, and inserts were sequenced by the method of Sanger (77 sequencing kit, Pharmacia).

PCR Amplification of Fragments Comprising the Alternatively Spliced Domain of Rag—Oligonucleotide primers were constructed that specifically matched the sequence of RagA or RagB in two domains flanking the alternatively spliced Rag domain. GTT CTG GCC GAC ACC AGG and 5′-ATT GCC AGA GAC ACA CTG-3′ were specific primers for RagA; 5′-ATT GCC AGA GAC ACA CTG and 5′-ATT GCT GTC CCA GCC ACA G; RagB-specific primers. Total RNA from rat adrenal gland, brain, thymus, kidney, and spleen was reverse transcribed and used as template. The PCR was allowed to proceed for 34 cycles of 94°C (80 s), 50°C (90 s), and 72°C (90 s). Products were separated on agarose gels, transferred on to nylon membranes, and probed with both RagA and RagB-specific probes. Parallel samples of the PCR products were isolated from the gel, subcloned, and sequenced. In a separate experiment, primers matching the sequence of RagB were used to amplify a large cDNA fragment (bp 685-1765) comprising the insertion, most of the open reading frame and 170 bp of the 3′-untranslated region (forward primer, 5′-TGG TCC ACC ACA A; reverse primer, 5′-ATT GCC AGA GAC ACA C). Total RNA from rat adrenal gland, brain, thymus, kidney, and spleen was reverse transcribed and used as template. The PCR was allowed to proceed for 34 cycles of 94°C (80 s), 50°C (90 s), and 72°C (90 s). Products were separated on agarose gels, transferred on to nylon membranes, and probed with both RagB-specific probes. Parallel samples of the PCR products were isolated from the gel, subcloned, and sequenced.

Northern Blot Analysis—Rat tissues (brain, heart, soleus muscle, adipose cells, liver, kidney, spleen, testis, adrenal gland, ovary, thymus; intestine; and lung) were homogenized with a Polytron homogenizer in ice-cold 4 M guanidine thiocyanate. The lysates were layered on a cesium chloride gradient, and the samples were again incubated at 30°C. At the desired time points, bound tracer was isolated by filtration on nitrocellulose, and the nucleotides were eluted with formic acid (2 M). Nucleotides were separated by thin layer chromatography on polyethyleneimine cellulose (1 mM lithium chloride, 1 mM formic acid) and detected by autoradiography.

RESULTS

Cloning of RagA and RagB—A cDNA fragment showing remote similarity with other members of the Ras superfamily (see Fig. 1, underlined sequence) was previously isolated in a PCR-based cloning approach designed to characterize ARF isoforms in 3T3-L1 cells (Schürmann et al., 1994). This fragment was used as a probe to screen a λ-Zap library from rat brain for full-length cDNA clones. Three clones were isolated and characterized. Two of them (sq2–13 and sq2–14, later designated RagA) were identical (1.6 kb), whereas the third (sq2–15, later designated RagB) was different in size (2.5 kb), intensity of hybridization, and pattern of restriction fragmentation.

Sequence of RagA—The nucleotide sequence of the clones sq2–13/sq2–14 contained a poly(A) tail, an open reading frame encoding a protein of 313 amino acids (Fig. 1), and a domain that was nearly identical (one mismatch) to the PCR product that had been used for screening of the library (Fig. 1, underlined). The translation start was assigned to the first AUG codon of the clone. The N-terminal region of the deduced amino acid sequence contains several structural motifs that are conserved within the Ras family (Valencia et al., 1991): the phosphoethanolamine binding motifs PM1 (14-GKSGSGKT), PM2 (61-WDCGGQ), and the guanine-nucleotide binding motif G1 (31-Y). An additional putative guanine nucleotide binding motif G2 (127-HKMD) is strikingly different from those found in all other Ras homologues (NKX). Moreover, a C-terminal domain of the protein, which is probably unrelated to GTP-binding, is considerably larger than that of all other known mammalian members of the Ras family. This domain contains a stretch of hydrophobic amino acids (259-276) that was identified as a putative membrane-associated motif. The most likely candidates being 156-ECAC and 162-TSI. Finally, it should be noted that the C-terminal domain of the protein, which is probably unrelated to GTP-binding, is considerably larger than that of all other known mammalian members of the Ras family. This domain contains a stretch of hydrophobic amino acids (259-276) that was identified as a putative membrane-associated motif.

Sequence of RagB—The nucleotide sequence of the clone sq2–15 (Fig. 2) contained a poly(A) tail and an open reading frame of 374 codons. A comparison with RagA revealed that the sequence of the clone (later designated RagB) was very similar...
but differed in an insertion of 28 codons within the reading frame between the nucleotide binding motifs PM2 and PM3 (underlined in Fig. 2, also see alignment in Fig. 3a). In order to test the possibility that two isoforms of the RagB mRNA, with and without the insertion, existed, a large portion of the cDNA including 170 bp of the 3'-untranslated region (bp 685-1765) was amplified by PCR with specific primers. With reverse-transcribed cDNA from tests as template, two cDNA fragments (1 and 1.1 kb) were isolated and subcloned. As anticipated, the sequence of the shorter fragment was identical except for the 84-bp insertion as depicted in Fig. 2. Thus, there are indeed two isoforms of RagB, RagB1 and RagB2, differing by an insertion of 28 codons between the PM2 and PM3 motifs (see Fig. 5). Because the nucleotide sequences of the two mRNA species are identical except for the insert, we assume that they are derived from the same gene, presumably by alternative mRNA splicing.

Based on the comparison with RagA, the translation start of RagB might be assigned to nucleotide 572. However, there is an alternative start codon in RagB at nucleotide 473 that extends the open reading frame by 33 codons. Therefore, a human cDNA clone was isolated and sequenced in order to compare the presumed translation starts. This clone represented the short isoform RagB2 (nucleotide sequence not shown); the sequence of the 84-bp insertion was obtained by PCR cloning. As illustrated in Fig. 3a, the open reading frame of the human sequence is homologous to that of the extended rat sequence starting at nucleotide 473, with 27 identical amino acids and five conservative substitutions in the N terminus. Furthermore, the human sequence lacks the second start codon of the rat sequence (methionine 34; Fig. 3a), which corresponds with the translation start of RagA. Thus, it appears reasonable to conclude that the N terminus of RagB is 33 amino acids longer than that of RagA.

Except for the extended N terminus and the insertion in RagB1, the deduced amino acid sequences of RagA and RagB were nearly identical (7 conservative substitution, 97.8% identity). Because of this high similarity, the two isoforms were designated A and B (instead of 1 and 2) according to previously established guidelines (Kahn et al., 1992). The identity of the nucleotide sequences was 83.8% within the coding region, but only 51% or less in the 3'-untranslated regions and before the translation start.

Homology of Rag with Other Proteins—A data base search was performed in order to determine the degree of homology of RagA/B with other G-proteins and to identify other proteins with structural similarities. The closest related protein was Gtr1 (51.6% sequence identity), a putative GTP-binding protein from yeast that is encoded by an open reading frame near the TUB3 locus on chromosome XIII (Bun-ya et al., 1992). Fig. 3b depicts the alignment of the sequences. Their highest similarity is in the N-terminal half, which comprises the domains responsible for GTP-binding. Both sequences lack a readily identifiable G3 domain; based on the assumption that this domain, if present, should be identical in Rag and Gtr1, the best candidate would be the 162-TSI motif. Except for two isolated motifs, the similarity of the two proteins within the C-terminal domain is low. Thus, it might be predicted that the GTP-binding characteristics of Rag and Gtr1 are similar, but that there are profound differences in the functions conferred by the C termini.

The alignment of the first 200 amino acids of RagA/B with prototypes of the five subfamilies of Ras-homologous GTPases revealed that the similarity was low (17.5% identity with Ras, 17.6% with Rho1, 18% with Rab1, 17.5% with Ran, 21% with ARF1, 17% with ARL1; PALIGN program). However, as is illustrated by an alignment of RagB with Ras (Fig. 3c), the GTP-binding domain of Rag is homologous to Ras in order, distance, and structure of the conserved motifs of GTP binding. Based on this similarity, Rag can be assigned to the superfamily of Ras-like GTPases.
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ily of Ras-related GTPases. The alignment also illustrates the position of the insertion in RagBl, which disrupts the PM2 motif and the $\beta_2$ strand.

The low structural similarity of Rag with other members of the Ras superfamily is illustrated by a multiple alignment and the resulting tree (Fig. 4). On the basis of this comparison, it is evident that the Rag isoforms cannot be assigned to any of the known subfamilies. Thus, the proteins appear to represent a novel subfamily of the GTP-binding proteins; we designated them Rag in order to emphasize both their unique structure and the remote similarity with the Ras, Rab, and Ran families.

Tissue Distribution of RagA and RagBIsoforms—

In order to investigate the tissue distribution of the two isoforms of RagB, and to test the possibility that an additional isoform of RagA existed, PCR primers were constructed for amplification of the domain between PM1 and PM3; these primers were specific for either RagA or RagB. cDNA from various tissues (thymus, adrenal gland, spleen, brain, and kidney) was used as template. The products were separated, blotted onto nylon membranes, and hybridized with specific probes. As is illustrated in Fig. 5, a single product hybridizing with the specific probe was obtained with RagA-specific primers; it size corresponded with the product anticipated from the sequence (Fig. 1). With primers specific for RagB, two hybridizing products (109 and 193 bp) were obtained from thymus, adrenal gland, spleen, and kidney. In contrast, brain cDNA yielded exclusively the long PCR product. The identity of the PCR products was ascertained by cloning and sequencing; they corresponded with the sequence of RagB (Fig. 2) and differed in the underlined insertion of 28 codons.

Fig. 5, upper panel illustrates a Northern blot of several rat tissues probed with a cDNA fragment of RagA. A single transcript with an approximate size of 1.8 kb was identified in most tissues with highest levels in adrenal gland; lower levels of mRNA were also detected in brain, skeletal muscle, liver, spleen, testis, ovary, thymus, and lung. When a full-length cDNA was used as the probe (data not shown), additional, presumably nonspecific bands appeared; the pattern of tissue distribution of the 1.8-kb band was identical to that shown in Fig. 5. Three different probes derived from the RagBl cDNA were employed in the Northern blot analysis. Two of these probes (full-length cDNA and 3'-untranslated region) failed to detect any specific signal. With a cDNA fragment comprising a portion of the coding region, two very weakly hybridizing transcripts (2.5 and 3.8 kb) were detected against the background in brain, testis, adrenal gland, and thymus (Fig. 5, lower panel). Thus, the mRNA of RagB appeared much less abundant than that of RagA, and exhibited a somewhat different tissue distribution.

Fig. 6, upper panel, illustrates a Northern blot of several rat tissues probed with a cDNA fragment of RagA. A single transcript with an approximate size of 1.8 kb was identified in most tissues with highest levels in adrenal gland; lower levels of mRNA were also detected in brain, skeletal muscle, liver, spleen, testis, ovary, thymus, and lung. When a full-length cDNA was used as the probe (data not shown), additional, presumably nonspecific bands appeared; the pattern of tissue distribution of the 1.8-kb band was identical to that shown in Fig. 6. Three different probes derived from the RagB cDNA were employed in the Northern blot analysis. Two of these probes (full-length cDNA and 3'-untranslated region) failed to detect any specific signal. With a cDNA fragment comprising a portion of the coding region, two very weakly hybridizing transcripts (2.5 and 3.8 kb) were detected against the background in brain, testis, adrenal gland, and thymus (Fig. 6, lower panel). Thus, the mRNA of RagB appeared much less abundant than that of RagA, and exhibited a somewhat different tissue distribution.

Fig. 2—continued

AUG codon of the done following a stop codon. The translation start matching that of RagA is boxed; the first 33 amino acids not present in RagB are highlighted by italics. The insertion of 84 nucleotides (28 amino acids), which is alternatively spliced, is underlined.

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In order to test whether the novel G-proteins do indeed bind GTP, both cDNAs (RagA and RagB) were subcloned into the expression vector pGEX. In addition, a vector comprising the RagB cDNA was prepared from the cDNA of RagB (clone sg2–15) by site-directed mutagenesis. The resulting constructs encoding fusion protein of GST and the respective Rag isoform were expressed in E. coli, and the recombinant proteins were purified by affinity adsorption on glutathione-Sepharose. HPLC separation of nucleotides bound to the native fusion proteins revealed that recombinant RagA and RagB were loaded with GTP/GDP to a ratio of 4:1, whereas no bound nucleotides were detected on RagB (data not shown). Binding of GTP was then assayed with [35S]GTPγS, and bound GTP was separated by filtration on nitrocellulose. As is illustrated in Fig. 7, specific binding of tracer GTPγS to the recombinant RagA and RagB was measurable within 5 min after addition of the ligand, and approached an equilibrium that largely exceeded that of the GST-control. In contrast, binding of GTPγS to recombinant RagB was considerably lower than that of RagA and RagB, hardly exceeding that of the GST control. This lack of specific GTP binding of RagB was also observed with a second con

Fig. 4. Dendrogram of an alignment of rag with prototypes of the other subfamilies of Ras-homologous GTPases. The dendrogram was constructed with the CLUSTAL program (gap penalty, 5; open gap cost, 10; unit gap cost, 10), Similarities were calculated from the matrix of the pairwise similarity scores. References for the known sequences: Ras, (Ruta et al., 1986); Rho, (Chavrier et al., 1990); Rab1, -2, -4 (Touche et al., 1987); Rab6, (Zahraoui et al., 1989); Ran, (Drivas et al., 1990); ARF1, (Bobak et al., 1989); ARL1, 4, (Schürmann et al., 1994); Gtr1, (Bun-ya et al., 1992)

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all Rag isoforms are denoted by asterisks; amino acids identical in the human and rat RagA are designated by periods. Note that the amino acid sequences of human and rat RagA are 100% identical and that only the long isoforms of RagB (RagBs) are given. a, sequence alignment of RagA/B with the yeast protein Gtr1. Gtr1 was identified as the closest relative of Rag by a database search (Swiss-prot, EMBL), and the deduced amino acid sequences of Rag and Gtr1 were aligned with the aid of the PALIGN program. Hyphens represent gaps introduced for optimal alignment. Amino acids identical in positions of GTP binding (PM1–3) and guanine nucleotide binding (G1–3) are depicted on the top of the alignment. Identical amino acids are denoted by vertical lines; conservative substitutions are depicted by colons.

Fig. 3. a. Sequence alignment of human and rat RagA and RagB. The alignment was performed with the CLUSTAL program. Hyphens represent gaps introduced for optimal alignment. Amino acids identical in

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or RagBl was used as template.

Fat cells; 1.1-kb (upper panel) and RagB (lower panel). Co, plasmid DNA of either RagA or RagB was used as template.

The domain including the 84-bp insertion of RagB was amplified with oligonucleotide primers specifically matching the sequences of either RagA (A) or RagB (B) as described under “Materials and Methods.” Total RNA from rat thymus (Th), adrenal gland (Ad), spleen (S), brain (B), and kidney (K) was reverse transcribed and used as template. The products were separated on agarose gels, transferred on to nylon membranes, and probed with cDNA fragments of RagA and RagB in different rat tissues. Total RNA from the indicated tissues was hybridized with probes generated from a cDNA fragment of RagA and RagB as described under “Materials and Methods.” Total RNA from rat thymus (Th), adrenal gland (Ad), spleen (S), brain (B), and kidney (K) was reverse transcribed and used as template. The products were separated on agarose gels, transferred on to nylon membranes, and probed with cDNA fragments of RagA and RagB (upper panel) and RagB (lower panel). Co, plasmid DNA of either RagA or RagB was used as template.

was no detectable decrease in the amount of bound tracer GTP, nor any formation of GDP. Thus, both RagA and RagB appeared to lack an intrinsic GTPase activity in the absence of an activating factor.

DISCUSSION

RagA and RagB are novel GTP-binding proteins with the striking feature that one isotype, RagB, was found as two mRNA species differing by an insertion of 28 codons within the reading frame. Surprisingly, the nucleotide sequence of RagB does not appear to contain the consensus sequences for splicing in the region of the insertion. However, we have repeatedly found both isoforms of RagB by PCR with cDNA from rat as well as from human tissues. The identity of the PCR products (109/153 bp and 996/1080 bp) was confirmed by sequencing; their sequences including portions of the 3′ untranslated region were identical except for the 84-bp insertion. Thus, it appears reasonable to assume that the two isoforms were derived from the same gene by alternative mRNA splicing. The splicing site near the PM2 domain of Rag (threonine 42) is not unexpected, because it corresponds exactly with well conserved exon borders of ras (McGrath et al., 1983) and ARF-2 (Serventi et al., 1993), and is only 12 codons downstream of the border between exon 2 and 3 in Rab isoforms (Wichmann et al., 1989).

Specific and saturable binding was observed with recombinant GST-fusion proteins of RagA. In contrast, preparations of GST-RagBl bound only minute amounts of GTP, which hardly exceeded those bound by GST preparations, but deletion of the 84-bp insert from the inactive RagBl generated a fully active, GTP-binding fusion protein of RagB. Thus, it is concluded that the large difference in GTP binding between RagB and RagBl is due to the insertion of 28 amino acids between PM2 and the β strand. Because the insertion might affect the orientation of PM2 (threonine 42), it appears plausible that it affects nucleotide binding, exchange, and/or hydrolysis. Furthermore, it was recently shown that the β strand in the Ras homologue Rap is involved in binding of the effector molecule Raf (Nassar et al., 1995). However, the possibility cannot be excluded that
The Ragβ1 protein is not properly folded in the bacterial expression system or that it requires the extended N terminus for proper folding and GTP binding. Thus, although it is tempting to speculate that the 28-amino acid insertion may produce an important functional alteration, the exact nature of this alteration remains to be determined.

RagA and B differ slightly in the tissue distribution of their mRNA. According to the Northern blot analysis, ragA was expressed in most tissues, but it was most abundant in adrenal gland, ovary, and testis. Rag B appeared most abundant in brain and testis, but mRNA levels were much lower than those of RagA. Like RagA, RagB isoforms could be detected by PCR amplification in all tissues investigated. However, the alternative splicing of ragB is tissue-specific in that brain expressed only its long form. This tissue specificity is an indication that the alternatively spliced insertion in RagB may confer a functional modification of the GTP-binding protein.

Several structural criteria have previously been established for entry of a novel protein into the Ras superfamily: a size of 20–29 kDa, the presence of the consensus motifs for GTP binding, the presence of other sequence motifs characteristic for one of the subfamilies, and an at least 30% identity with other members of the family (Valencia et al., 1991; Kahn et al., 1992). The novel gene product Rag comprises four of the known GTP-binding motifs (PM1-PM3 and G1). In addition, a G domain (HKMD) appears to be present but differs from that found in other Ras homologues (NKXD) by a substitution of asparagine for histidine. A potential G3 domain, tentatively assigned to the TSI motif is unrelated to those found in other isoforms. It should be noted that G3 is the domain least conserved among other subfamilies and that it appears to be only indirectly involved in GTP binding (Valencia et al., 1991). The distances between the GTP-binding domains in Rag were essentially identical to those in other members of the Ras superfamily. Because of the presence of the GTP-binding domains in conserved order and distances, it appears reasonable to assume that the tertiary structure of the main part of Rag provides the interaction with GTP/GDP (amino acids 1–200) is similar to that of Ras (Pai et al., 1990) and ARF (Amor et al., 1994) and that its basic function is that of a GTP/GDP-dependent switch. However, there are several striking differences to other members of the Ras-family. First, Rag is considerably bigger than most other known mammalian Ras homologues (calculated molecular mass 36.6 kDa (RagA), 40.2 kDa (RagB)), or 43.2 kDa (RagB1)). This extraordinary size is due to an additional domain of approximately 100 amino acids at the C terminus; similar additional domains have been found in the yeast counterparts of Ras (Powers et al., 1984; Dhar et al., 1984) and in the ARF-related GTPase ARD (Mishima et al., 1993). Second, other characteristic motifs, e.g. the lipid modification motifs commonly encountered in Ras homologues except in Ran are absent. Finally, the overall amino acid identity of Rag to other members of the Ras family is low (highest to ARF, 21%) and is restricted to the GTP binding motifs. In spite of these differences, we suggest that Rag could have identical GTP-binding characteristics, but their functions might have diverged during evolution.

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