The *ram*: a novel low molecular weight GTP-binding protein cDNA from a rat megakaryocyte library

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A novel low M_r GTP-binding protein cDNA was isolated from a rat megakaryocyte cDNA library with a synthetic oligonucleotide probe corresponding to an 8-amino acid sequence specific for c25KG, a GTP-binding protein previously isolated from human platelet cytosol fraction [(1989) J. Biol. Chem. 264, 17000–17005]. The cDNA has an open reading frame encoding a protein of 221 amino acids with a calculated M_r of 25068. The protein is designated as *ram* (*ra*s-related gene from *m*egakaryocyte) protein (*ram* p25). The amino acid sequence deduced from the *ram* cDNA contains the consensus sequences for GTP-binding and GTPase domains. *ram* p25 shares about 23%, 39% and 80% amino acid homology with the H-*ras*, *smg*25A and c25KG proteins, respectively. The 3.5-kb *ram* mRNA was detected abundantly in spleen cells.

Low M_r GTP-binding protein; cDNA cloning; Megakaryocyte

1. INTRODUCTION

There is a family of structurally homologous monomeric GTP-binding proteins with molecular weight (M_r) ranging from 20 000 to 30 000 in mammalian cells [1]. The family contains *ARF* [2], *ral* [3,4], *rho* [5,6], R-*ras* [7], *rap* [8,9], *rab* [10–12], *rac* [13], *smg* 25 [14] and *ypt*1 [15] proteins. These low M_r GTP-binding proteins show 30–50% homology to *ras* p21 [16]. Four of GTP-binding domains are highly conserved in all of the low M_r GTP-binding proteins. Among them, the phosphate-interacting domain, a stretch of six residues, Asp-Thr-Ala-Gly-Gln-Glu (in positions 57–62 of K-*ras* p21), is strictly conserved. The low M_r GTP-binding proteins also share biochemical properties, binding of GDP or GTP and GTPase activity, but the GTPase activity is lower compared to heterotrimeric G proteins such as G_s, G_i, G_o and transducin [17].

We purified two low M_r GTP-binding proteins (c21KG, c25KG) from human platelet cytosol fraction [18,19]. From the partial amino acid sequence analysis, the major component (c21KG) was found to be identical to *rap*1A protein, whereas the minor protein (c25KG) was identified as a novel low M_r GTP-binding protein. To determine its primary structure, we have attempted to clone the cDNA of c25KG. In the present studies, we have cloned from a rat megakaryocyte cDNA library a cDNA of a low M_r GTP-binding protein, which is highly homologous to but clearly distinct from c25KG. We also describe its expression in several tissues of adult rat.

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2. EXPERIMENTAL

2.1. *Design and synthesis of the oligonucleotide probe*

One 29-base antisense strand oligodeoxyribonucleotide probe (5′TG(T/C)TC(A/G)AA(A/G)TAIGGIATICC(A/G)TA(T/C)TT-(A/G)TC3′) was synthesised corresponding to the sequence Lys-Tyr-Gly-Leu-Pro-Tyr-Phe-Glu of c25KG. The oligonucleotide is a mixture of all the possible icosamers that might encode this sequence.

2.2. *Screening of the cDNA library*

Approximately 4.0×10^5 recombinant plaques were screened by plaque hybridization [20] with the synthetic probe labeled at the 5′ end with ^{32}P. Hybridization was allowed to occur overnight under a low-stringency condition at 30°C in a solution containing 30% (v/v) formamide, 5 × SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0), 1× Denhardt's solution (0.02% bovine serum albumin/0.02% polyvinylpyrrolidine/0.02% Ficoll), 20 mM sodium phosphate (pH 7.0), 100 μg/ml of heat-denatured salmon sperm DNA, 0.1% $NaDodSO_4$, and 10% dextran sulfate. Filters were washed twice at room temperature in 5× SSC/0.1% $NaDodSO_4$ for 15 min before autoradiography. The positive clones were purified by successive plaque hybridization.

2.3. *DNA sequence analysis*

DNA sequencing was performed by the dideoxynucleotide chain-termination method [21] with [α-^{32}P]dCTP. 2′-Deoxy-7-deazaguanosine 5′-triphosphate was used in place of dGTP [22]. For sequencing, cDNA fragments were subcloned into pUC118 vector and single-stranded templates were prepared with helper phage M13 KO7 [23].

2.4. *RNA blot hybridization analysis*

Total RNA was extracted by the guanidinium thiocyanate method [24]. RNA (25 μg) was denatured by heating at 60°C for 5 min in 2.2 M formaldehyde/50% (v/v) formamide and subjected to elec-

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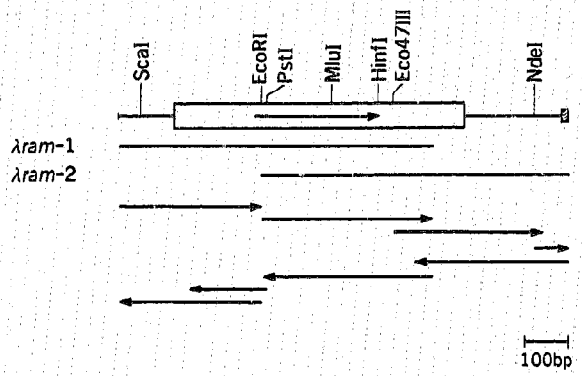

Fig. 1. Restriction maps of λ*ram*-1 and λ*ram*-2 and sequencing strategies of the cloned cDNAs. The coding region is indicated by open box. The hatched box at the right end indicates the poly(A) region. The arrow in the open box shows the direction of translation. The arrows under the map indicate the direction and the region of DNA sequencing.

trophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde [25]. The RNA was transferred to nitrocellulose membranes. Hybridization was carried out under the high-stringency condition described above. Filters were washed twice at 42°C in 0.1× SSC/0.1% NaDodSO$_4$ for 1 h before autoradiography. Mouse megakaryoblastoma cell line MK8057 was kindly supplied by Dr T. Inoue (Yokohama City University School of Medicine).

3. RESULTS AND DISCUSSION

The cDNA library was screened under the low-stringency hybridization conditions with the synthetic 29-base probe (see section 2). On screening 4 × 10^5 recombinant plaques, five positive clones were obtained. Among them, three clones had 0.8 kbp inserts, and the other two showed 0.4 kbp and 0.3 kbp inserts after *Eco*RI digestion, indicating the latter cDNA has an endogenous *Eco*RI site. Partial cDNA sequence analysis indicated that they are overlapping clones. We tentatively named them λ*ram*-1 (with 0.8 kbp insert) and λ*ram*-2 (0.7 kbp insert containing an endogenous *Eco*RI site). The restriction maps of the λ*ram*-1 and -2 inserts and the strategies for sequence determination are shown in Fig. 1. λ*ram*-1 lacked the COOH-terminal sequence corresponding to amino acid residues 192–221 of *ram* protein (*ram* p25), and λ*ram*-2 lacked the NH_2-terminal sequence corresponding to amino acid residues 1–66 of *ram* p25. The insert of λ*ram*-1 was then used for screening of the rat megakaryocyte cDNA library under the high-stringency condition, but the full-length cDNA was not obtained.

Fig. 2 shows the nucleotide and deduced amino acid sequences of *ram*. The cDNA contained an open reading frame of 221 amino acids, assuming that the in-

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                                                    -119  CGGAATTTAGAAAAGAGCAACCTCGCAGCAGGCTGGGAAT
 -79  CTAGTACTACAGGGACGCAACACCGCGGGGACAGCACCCGACAGAGCACCAAACCGGGTAAAGCAGAGAGGCCTCCATC

   1  ATG TCG GAT GGA GAT TAT GAC TAC CTC ATC AAG TTT TTG GCC TTG GGA GAC TCT GGA GTG
   1  Met Ser Asp Gly Asp Tyr Asp Tyr Leu Ile Lys Phe Leu Ala Leu Gly Asp Ser Gly Val

  61  GGG AAG ACC AGT GTA CTG TAC CAG TAC ACT GAT GGG AAG TTC AAC TCC AAA TTC ATC ACC
  21  Gly Lys Thr Ser Val Leu Tyr Gln Tyr Thr Asp Gly Lys Phe Asn Ser Lys Phe Ile Thr

 121  ACA GTG GGC ATT GAT TTC AGG GAA AAG AGA GTG GTG TAC AGA GCG AAT GGA CCA GAT GGA
  41  Thr Val Gly Ile Asp Phe Arg Glu Lys Arg Val Val Tyr Arg Ala Asn Gly Pro Asp Gly

 181  ACT GTA GGC CGA GGC CAG AGA ATT CAC CTG CAG TTA TGG GAC ACG GCG GGG CAG GAG AGG
  61  Thr Val Gly Arg Gly Gln Arg Ile His Leu Gln Leu Trp Asp Thr Ala Gly Gln Glu Arg

 241  TTT CGT AGC CTG ACC ACG GCA TTC TTC AGG GAC GCT ATG GGT TTC CTG CTT CTG TTC GAC
  81  Phe Arg Ser Leu Thr Thr Ala Phe Phe Arg Asp Ala Met Gly Phe Leu Leu Leu Phe Asp

 301  CTG ACA AAC GAG CAA AGT TTC CTC AAT GTC CGA AAC TGG ATA AGC CAG CTA CAG ATG CAC
 101  Leu Thr Asn Glu Gln Ser Phe Leu Asn Val Arg Asn Trp Ile Ser Gln Leu Gln Met His

 361  GCG TAC TGC GAA AAC CCA GAT ATA GTG CTC TGC GGA AAT AAG AGT GAC CTA GAA GAC CAG
 121  Ala Tyr Cys Glu Asn Pro Asp Ile Val Leu Cys Gly Asn Lys Ser Asp Leu Glu Asp Gln

 421  AGG GCT GTG AAA GAG GAG GAA GCC AGG GAA CTC GCA GAG AAG TAT GGA ATC CCC TAT TTT
 141  Arg Ala Val Lys Glu Glu Glu Ala Arg Glu Leu Ala Glu Lys Tyr Gly Ile Pro Tyr Phe

 481  GAA ACC AGC GCT GCC AAT GGG ACA AAC ATA AGC CAA GCA ATT GAG ATG CTC CTG GAC CTG
 161  Glu Thr Ser Ala Ala Asn Gly Thr Asn Ile Ser Gln Ala Ile Glu Met Leu Leu Asp Leu

 541  ATC ATG AAG CGG ATG GAG CGG TGT GTG GAC AAA TCC TGG ATT CCA GAG GGG GTG GTG CGG
 181  Ile Met Lys Arg Met Glu Arg Cys Val Asp Lys Ser Trp Ile Pro Glu Gly Val Val Arg

 601  TCC AAT GGC CAT ACT TCT ACA GAT CAA CTC AGT GAG GAG AAG GAG AAG GGG TTA TGC GGC
 201  Ser Asn Gly His Thr Ser Thr Asp Gln Leu Ser Glu Glu Lys Glu Lys Gly Leu Cys Gly

 661  TGT TGAGACACCGAGTGAGCATGGCGGCGGCAGTGGCGGCACACGCCTGGCCCCTGCCTGCCAAATCCTCTGTAGAGA
 221  Cys ***

 737  CCAAGCCAGCATCACGTGCCAAGTGCTGCTCCTTATCCCCAGTAGAACTGACTCAACATCCAATTGTAACTCATTGCAA
 817  CTTCATATGATTTAGTCCATGAATTGGGGGAACTATTTCACAGAGCCAAAAGTGTCTTAGAAAAAAAAAAAAAAAAA 894
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Fig. 2. The nucleotide and deduced amino acid sequence of the *ram* cDNA. Numbers indicate the positions of nucleotides or amino acid residues starting at the initiator codon.

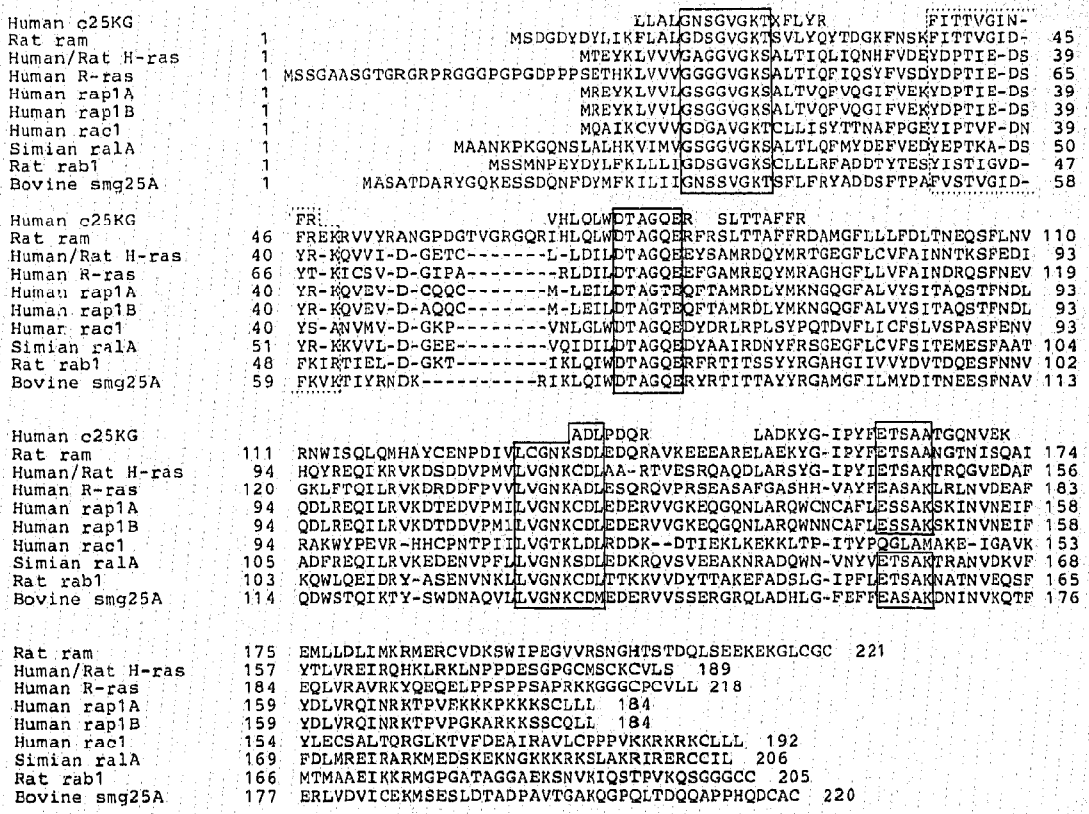

Fig. 3. Alignment of *ram* protein sequence with other low M_r GTP-binding proteins. The sequences of the GTP-binding proteins have been taken from the literature: c25KG [19], H-*ras* [16], R-*ras* [7], *rap*1A [8], *rap*1B [9], *rac*1 [13], *ral*A [3], *rab*1 [10], *smg*25A [14]. GTP-binding and GTPase regions are boxed. Effector region is boxed by dotted line. Hyphens indicate the gaps introduced for alignment. X represents unidentified residue.

itiator methionine codon is at position 1–3 and the termination codon TGA is at positions 664–666. The nucleotide sequence surrounding the first methionine codon agreed with the consensus sequence that is characteristic for the initiation codon of many eukaryotic mRNAs [26]. The calculated molecular mass of the polypeptide specified by this open reading frame (663 bp) is 25 068. The amino acid sequence corresponding to the synthetic oligonucleotide probes used for screening was partially different from the amino sequence: 2 amino acids out of 9 were different from c25KG. We screened human erythroleukemia K562 cell cDNA library by using a partial fragment of rat *ram* cDNA, and isolated two partial cDNAs of human-*ram* (data not shown). The amino acid sequence of *ram* p25 is strongly conserved between rat and human, and *ram* p25 is distinct from c25KG.

Fig. 3 compares the amino acid sequence of *ram* p25 with those of other low M_r GTP-binding proteins. *ram* p25 shares 23%, 26%, 23%, 32%, 39% and 21% amino acid identity with H-*ras*, *ral*A, *rac*1, *rab*1, *smg*25A and *rap*1A proteins, respectively. The homologies are essentially restricted to the four GTP/GDP binding domains boxed in Fig. 3. The identity between *ram* p25 and c25KG is 80%: 40 out of 70 amino acids are matched to those of *ram* p25.

On the other hand, the sequence of *ram* at the effector domain (amino acid residues 32–42 of H-*ras* p21) is different from that of H-*ras* p21 but is almost identical with c25KG. Since this region in H-*ras* p21 is essential for its transforming activity and interaction with GAP [27,28], it is likely that *ram* protein and c25KG may share the common effector and/or GAP molecules.

It has been shown that low M_r GTP-binding proteins have unique COOH-terminal amino acid sequences; they possess at least one cysteine near their COOH-terminal ends. It has been suggested that the cysteine residues are polyisoprenylated [29] and/or palmitoylated [30], and that these fatty acid moieties are essential for the proteins to attach to the inner surface of the plasma membrane and to exert their biological activity [16]. According to the COOH-terminal sequences, low M_r GTP-binding proteins can be classified into four groups: one is a Cys-A-A-X group including H-, K- and N-*ras*, and *rho*, where A is an aliphatic amino acid, and X is any one. Second is a Cys-Cys group including the *ypt*1, *rab*1 and *rab*2. Third is a Cys-Cys-X-X group including *ral*A and *ral*B, H-*rab*5. The last is a Cys-X-Cys group including *rab*3 and 4, H-*rab*3A, -3B, and -4 and *smg*25A, -B and -C. The *ram* protein belongs to the last group. The variations in the COOH-terminal structures probably reflect diverse

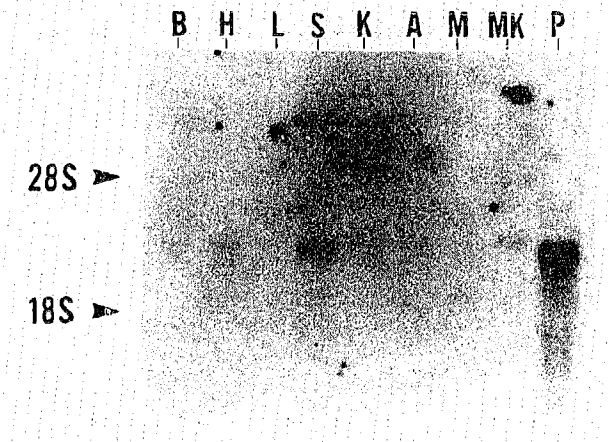

Fig. 4. Northern analysis of *ram* RNA. The following rat and mouse RNAs are in each line; brain (B), heart (H), liver (L), spleen (S), kidney (K), adrenal gland (A), skeletal muscle (M), mouse megakaryoblastoma cell line MK8057 (MK) and rat pheochromocytoma cell line PC-12 (P).

associations of the low M_r GTP-binding proteins with membranes of different organelles. As some low M_r GTP-binding proteins such as *rho*, *smg*p25A and c25KG were purified from the cytosolic fraction [19,31,32], posttranslational modifications may regulate their location.

We examined the expression of *ram* mRNA in several rat tissues and the pheochromocytoma PC-12 cell by Northern blot analysis as shown in Fig. 4. The 260-bp *Eco*RI-*Mlu*I fragment of λ*ram*-2, containing the coding region of *ram* cDNA, was used as a probe. The 3.5 kb *ram* mRNA was expressed abundantly in PC-12 cells, spleen, and weakly in kidney, adrenal gland, brain, and heart. *ram* is also expressed in mouse megakaryoblastoma MK8057 cells, though the size of the mRNA is longer than that of rat. No bands were detected in liver and skeletal muscle even after a long exposure.

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