Molecular cloning of a member of a new class of low-molecular-weight GTP-binding proteins

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We report the cloning of a low-molecular-weight GTP-binding protein that appears to be the first member of a new class of G proteins. This G protein was cloned from the HT4 neural cell line and has the closest homology to the *rab*, *sec4*, and *ypt1* members of the low-molecular-weight (LMW) G-protein family. The amino acid sequence identity is only 30% with these other LMW G proteins, but in the four conserved GTP-binding domains, amino acid identity increases to 50–100%. A unique feature that distinguishes this G protein from other LMW G proteins is its carboxy-terminal amino acid sequence -Cys-Cys-Pro. In keeping with the current nomenclature for other members of the *ras* superfamily, we will designate this new class as *rah* (*ras*-related homolog). On the basis of sequence homology, *rah* may function in vesicular trafficking and possibly in neurotransmitter secretion.

[Key Words: *ras*; *rab*; *ypt1*; *sec4*; small G protein]

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Low-molecular-weight (LMW) GTP-binding proteins are a rapidly growing family of proteins in which the *ras* oncogene is the prototype. The members of this diverse collection of proteins function in a number of cellular processes including the regulation of growth metabolism, protein sorting, and neurotransmitter secretion (see Bourne et al. 1990, 1991; Hall 1990).

These small G proteins consist of a single polypeptide chain of 21–30 kD and are able to bind and hydrolyze GTP. By analogy with the larger heterotrimeric G proteins, the binding of GTP may turn on or activate the LMW G proteins to send a signal to an acceptor molecule (Bourne 1988). The intrinsic GTPase activity would then be responsible for turning the signal off. The precise mechanism by which these small G proteins integrate into cellular metabolism remains elusive.

Of the *ras*-related small G proteins, a subset of them appear to be critical in the regulation of membrane vesicle transport. The best genetic evidence comes from two yeast mutants, *sec4* and *ypt1*. In *Saccharomyces cerevisiae*, mutations in *sec4* result in the accumulation of vesicles at the plasma membrane, suggesting that the Sec4 gene product is critical for the fusion of vesicles with the plasma membrane (Walworth et al. 1989). Disruption of *ypt1* is lethal; however, the use of temperature-sensitive mutations has shown that the Ypt1 gene product is involved in an early stage of vesicular transport, possibly between the endoplasmic reticulum (ER)

and the Golgi apparatus (Segev et al. 1988; Bacon et al. 1989).

Mammalian homologs of *ypt1* and *sec4* include the *rab* and *smg25* family of proteins. Many of these Rab/Smg25 proteins probably function analogously to *ypt1* and *sec4*, in that they are involved in protein sorting and vesicular trafficking from the ER through the Golgi and on to the plasma membrane (Segev et al. 1988; Haubruck et al. 1989). One of the Rab proteins, namely *rab3* (which is the same as Smg p25A) is found predominantly in the brain and the adrenal medulla (Burstein and Macara 1989; Darchen et al. 1990; Mizoguchi et al. 1990). A recent report has shown that Rab3A, a synaptic vesicle-associated protein (Fischer von Mollard et al. 1990), dissociates from the vesicle during the process of neurotransmitter secretion (Fischer von Mollard et al. 1991). This suggests that LMW G proteins may serve an important regulatory function in the control of neurotransmitter secretion.

The HT4 neural cell line was derived from mouse neuronal tissue and immortalized with a temperature-sensitive SV40 large T antigen (McKay et al. 1988). This neural cell line secretes excitatory amino acids in response to membrane depolarization (Morimoto and Koshland 1990a), and both short- and long-term potentiation of neurotransmitter secretion can be expressed (Morimoto and Koshland 1990b). Potentiation of neurotransmitter secretion is mediated by the elevation of cAMP (Morimoto and Koshland 1990b, 1991b).

In the process of understanding the molecular

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mechanisms responsible for potentiation of neurotransmitter secretion, we discovered a new gene whose sequence indicates that it is in the general small G-protein family but does not fit into any of the known families.

Results and discussion

In recent studies of the HT4 neural cell line, a search for a new G protein involved in neurotransmitter secretion was initiated. Two regions of conserved amino acid sequence (GNSSVGK and LVGNKCD) between *rab* (Touchot et al. 1987) and *smg25* (Matsui et al. 1988) were used to design the oligonucleotide probes, as described in Materials and methods. Approximately 500,000 recombinant phage plaques from an HT4 cDNA library (cDL39) were screened using a mixture of oligonucleotides to these two peptide sequences. After several rounds of plaque purification, a single colony gave rise to a clone with a 1-kb insert. A partial restriction map and the sequencing strategy are shown in Figure 1.

The nucleotide sequence was determined using primers to the flanking vector sequences. To facilitate the complete sequencing of this clone, a 467-bp *EcoRI* fragment was subcloned, thus generating two vectors with approximately equal size inserts. Subsequently, oligonucleotide primers internal to the cloned fragment were used to verify the nucleotide sequence of the intact clone. Both the nucleotide and predicted amino acid sequence of this clone are presented in Figure 2. The open reading frame consisted of 624 nucleotides and encodes a protein of 208 amino acids, with a putative molecular mass of 23.3 kD.

The amino acid sequence of this gene identifies it as a member of the *ras* superfamily. Three amino acids that have been shown to be critical for GTP binding in the *ras* protein are conserved in this new G protein at Lys-14, Phe-26, and Thr-33. In Figure 3, amino acid identity in the putative GTP-binding domains, at amino acid positions 3–17, 30–38, 52–63, and 111–119 (Bourne et al. 1991), is 50–100% when compared with *rab1* (Touchot et al. 1987), *sec4* (Salminen and Novick 1987), or *ypt1* (Gallwitz et al. 1983). Although the open reading frame lacks an initiator methionine, some interesting sequence comparisons to other known members of the *ras* super-

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1  AATTCCAAGGTCATCGTTGTGGGAGACCTATCTGTGGGGAAGACCTGTCTCATT 54
   N S K V I V V G D L S V G K T C L I
55  AATAGTTCCTGCAAAGACACCTTCGATAAGAATTACAAGGCTACCATCGGAGTG 108
   N R F C K D T F D K N Y K A T I G V
109 GATTTTGAGATGGAACGATTGAAGTCTTGGGTGTCCTTCAGTCTCCAACCT 162
   D F E M E R F E V L G V P F S L Q L
163 TGGGACACGGCTGGTCAGGAAAGGTTCAAGTGCATTGCTTCCACCTACTACCGT 216
   W D T A G Q E R F K C I A S T Y Y R
217 GGAGCTCAAGCCATCATCATTGTCTTCAACCTGAATGACGTGGCATCCCTGGAA 270
   G A Q A I I I V F N L N D V A S L E
271 CACACCAAGCAGTGGCTCACTGATGCCCTCAAGGAGAACGACCTTCCAACCTG 324
   H T K Q W L T D A L K E N D P S N V
325 CTCCTCTTCTTGTGGGTTCGAAGAAGGACCTGAGTACTCCTGCTCAGTATTCC 378
   L L F L V G S K K D L S T P A Q Y S
379 CTAATGGAGAAGGATGCCCTCAAGTGGCCCAAGAGATTAAGCCGAGTACTGG 432
   L M E K D A L K V A Q E I K A E T
433 GCGGTGCTCTCCCTCACTGGTGAGAATGTCGGGAATTCCTTCTTCCGTGTGGCA 486
   A V S S L T G E N V R E F F F R V A
487 GCCTAACCTTTGAGGCCAATGTCCTGGCGSACGTGGAGAAATCCGGGGCTCGG 540
   A L T F E A N V L A D V E K S G A R
541 CACATTGCAGATGTTGTTGCGCATCAACAGCGATGACAAAACCTGTACCTAACT 594
   H I A D V V R I N S D G K N L Y L T
595 GCCAGCAAGAAAAGGCCACATGTTGTCCCTGAGCAGTGAGGACTGTCAGAGA 648
   A S K K K A T C C C P
649 CTTCCCATTTCCGGGGTACCGTGCTACCTGCAACTGCTCCAGAACTCAACCCCT 702
703 GGACATTTGCACTGACTTTTCCAGACCAAGAGCTATCCATTGATGTCAGTGC 756
757 CCCCCAGAGGTGACAGGGACCATGCTAGTCAATTGCTGCCCCAGGCACCATG 810
811 CCAAAGACTGGATGACCCCTGTTCTCTGGGGCTCTCCAGGGTGCCTGGTGAG 864
865 GGGCAGTGTCTTGTGCTGCTTTGCTCAGCTGCTGGGGCTTTCATGTATAAAAA 918
919 TGGTTAATGATTGAGCCTTACACTGTGCTTATGCATTAAAGTCCATTATTAG 972
973 CAAAAAATAAAAAAAAAAAAAA 993

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Figure 2. Nucleotide and predicted amino acid sequence. Numbers indicate the positions of nucleotides starting at the first nucleotide in the open reading frame. Double underlines indicate the consensus GTP-binding and GTPase sequence domains. The carboxyl terminus is underlined.

family indicate both similarities and differences, which make this clone a novel, new entity.

This new G protein is most closely related to *rab*, *sec4*, and *ypt1*, with 32–34% amino acid identity. The next closest relatives would be *ral* (Polakis et al. 1989), *rap* (Pizon et al. 1988), *rho* (Chardin et al. 1988), *rac* (Didsbury et al. 1989), and *ras* (Capon et al. 1983), with 23–27% identity. For comparison, amino acid identity between *ras* and the other classes of LMW G proteins ranges from 21% identity for *rho* to 47% for *ral*. Within a particular class of G protein, amino acid identity can

Figure 1. Restriction map and sequencing strategy. The cDNA insert is an *EcoRI*–*XhoI* restriction fragment and is aligned 5' → 3'. Restriction sites are indicated above the map; the numbers denote the positions of digestion. The open reading frame is indicated by the solid double lines and extends from bp 1 to 624. The arrows indicate the direction and region of DNA sequencing.

Figure 3. Comparison of amino acid sequences in the conserved GTP-binding domains. Identical amino acids to *rah1* are highlighted in boxes. The sequences are ordered with respect to the weighted identical amino matches in the GTP-binding domains compared with *rah1*. The numbers to the left of each domain indicate the beginning amino acid position for that sequence. Consensus sequence is based on amino acid homology; (X) Any amino acid; (O) a hydrophobic amino acid (V, L, I, M, C); (Z) an aromatic amino acid (F, Y, W); (B) serine (S) or threonine (T).

		G-1		G-2		G-3		G-4
consensus		K O O O G X X X O G K B X L		Z X X T O X X X Z		O X O X D T A G X E X Z		X L V G X K X D X
<i>rah1</i>	3	K V I V V G D L S V G K T C L	30	Y K A T I G V D F	52	L Q L W D T A G Q E R F	111	F L V G S K K D L
<i>rab1a</i>	13	K L L L I G D S G V G K S C L	40	V I S T I G V D F	62	L Q I W D T A G Q E R F	120	L L V G N K C D L
<i>ypt1</i>	13	K L L L I G N S G V G K S C L	40	V I S T I G V D F	62	L Q I W D T A G Q E R F	120	L L V G N K C D M
<i>sec4</i>	22	K I L L I G D S G V G K S C L	49	F I T I G I D F	71	L Q L W D T A G Q E R F	129	L L V G N K S D M
<i>racA</i>	5	K C V V V G D G A V G K T C L	32	V I P T V F D N Y	53	L G L W D T A G Q E D Y	111	I L V G T K L D L
<i>rhoB</i>	7	K L V I V G D G A C G K T C L	34	V V P T V F E N Y	55	L A L W D T A G Q E D Y	113	I L V G N K K D L
<i>rap2</i>	5	K V V V L G S G G V G K S A L	32	Y D P T I E D F Y	53	L E I L D T A G T E Q F	112	I L V G N K V D L
<i>H-ras</i>	5	K L V V V G A G G V G K S A L	32	Y D P T I E D S Y	53	L D I L D T A G Q E E Y	112	V L V G N K C D L
<i>ral</i>	16	K V I V V G S G G V G K S A L	43	V E P T K A D S Y	64	I D I L D T A G Q E D Y	123	L L V G N K S D L

vary from 39% to 89%. For example, the homology of *rab1a* to *rab1b* is 89%; to *rab2*, 44%; to *rab3*, 45%; and to *rab4*, 39%. To evaluate the significance of these amino acid identities, the amino acid sequence for each of these LMW G proteins was placed in random order and subjected to the same homology criteria. This analysis maintains the amino acid composition and provides an estimate of random chance for amino acid identity. By randomizing the order of amino acids, homology between members of the LMW G proteins gave an apparent amino acid identity score of $9 \pm 1\%$ (mean $\pm$ S.E., for $n = 9$). Thus, the amino acid sequence of the HT4 G protein is sufficiently different from the other known members of the *ras* superfamily to qualify this gene as a member of a new class of LMW GTP-binding protein, designated *rah* for *ras*-related homolog.

A unique structural feature of *rah1* is the carboxy-terminal amino acids (Table 1). The *ras* superfamily of G proteins is known to be post-translationally modified by isoprenoid groups at a cysteine residue (see Maltese 1990; Gibbs 1991). The carboxyl terminus of these LMW G proteins can be classified into four subgroups according to their amino acid sequences. The first group includes Ras p21, Rho, and G25K, with a terminus of -Cys-A-A-X, where A is an aliphatic amino acid and X is any amino acid. The cysteine residue is either farnesylated (Hancock et al. 1989) or geranylgeranylated (Yamane et al. 1991), and the -A-A-X amino acids are then removed. The free carboxyl group on the carboxy-terminal cysteine is then methyl-esterified (Clarke et al. 1988; Stephenson and Clarke 1990).

The second and third groups of carboxyl termini have either the -Cys-Cys or -Cys-X-Cys motif, and the cysteines are modified with geranylgeranyl isoprenoid

groups (Farnsworth et al. 1991; Khosravi-Far et al. 1991; Kinsella and Maltese 1991). The fourth group includes *arf* (Sewell and Kahn 1988) and contains no carboxy-terminal cysteine. The carboxyl terminus of *rah1* is -Cys-Cys-Pro. This carboxyl terminus is related most closely to the Rab family of -Cys-Cys and -Cys-X-Cys, but the carboxy-terminal proline is a novel structure for a LMW G protein.

To confirm the expression of this new G-protein clone, first-strand cDNA was generated from poly(A) mRNA isolated from either undifferentiated or differentiated HT4 cells. Two sets of primers were used to perform polymerase chain reaction (PCR) amplification of this G-protein transcript. Primer set 1 consisted of oligonucleotides to an amino-terminal amino acid sequence (KVIVVGD) and to a region within the third conserved GTP-binding domain (TAGQERF). In Figure 4 (lane 2), a 183-bp PCR product was generated using the LMW G-protein plasmid as template for amplification. First-strand cDNA from undifferentiated (lane 3) and differentiated (lane 4) HT4 cells also gave rise to a single 183-bp product. By making use of the oligonucleotide to the amino-terminal sequence KVIVVGD and to one in the carboxy-terminal region (FEANVLAD), a second set of primers defined a 511-bp fragment to the plasmid template (lane 5). Through the use of this second set of primers, amplification of the cDNA from undifferentiated (lane 6) and differentiated (lane 7) HT4 cells also produced a PCR product of 511 bp. Thus, this new LMW G protein is expressed in both the undifferentiated and differentiated state, consistent with the ability of this neural cell line to secrete excitatory amino acids.

Although the cellular function of *rah1* is unknown, homology to *rab*, *sec4*, and *ypt1* classes of LMW G pro-

Table 1. Carboxyl termini of various LMW GTP-binding proteins

new -C-C-P terminus		-C-A-A-X termini		-C-C termini		-C-X-C termini		No Cys terminus	
<i>rah1</i>	-T-C-C-P	<i>Ha-ras</i>	-C-V-L-S	<i>ypt1</i>	-G-G-C-C	<i>rab3</i>	-D-C-A-C	<i>arf</i>	-R-N-Q-K
		<i>ralA</i>	-C-C-I-L	<i>sec4</i>	-S-N-C-C	<i>rab4</i>	-E-C-G-C		
		<i>rhoA</i>	-C-L-V-L	<i>rab1a</i>	-G-G-C-C				
		<i>rac1</i>	-C-L-L-L	<i>rab1b</i>	-G-G-C-C				
				<i>rab2</i>	-G-G-C-C

Figure 4. RNA analysis using PCR. Poly(A) mRNA was isolated from undifferentiated and differentiated HT4 cells. First-strand cDNA was generated and used as a template for PCR amplification. Two sets of primers were used: Primer set 1 defines a 183-bp fragment; primer set 2 defines a 511-bp fragment. (Lane 1) 500 ng of a *HaeIII* digest of Phi-X 174 DNA; the size fragments are denoted in the left margin. (Lane 2) *rah* plasmid as template and oligonucleotides to primer set 1. (Lane 3) Undifferentiated HT4 mRNA as template and oligonucleotides to primer set 1. (Lane 4) Differentiated HT4 mRNA as template and oligonucleotides to primer set 1. (Lane 5) *rah* plasmid as template and oligonucleotides to primer set 2. (Lane 6) Undifferentiated HT4 mRNA as template and oligonucleotides to primer set 2. (Lane 7) Differentiated HT4 mRNA as template and oligonucleotides to primer set 2.

teins suggests a possible role in vesicular secretion, most likely functioning in the fusion of secretory vesicles with the plasma membrane. HT4 cells have been shown to express both short- and long-term potentiation of neurotransmitter secretion (Morimoto and Koshland 1990b, 1991a), and *rah1* or possibly some other LMW G protein may function as an important signal transduction component in the expression of potentiation.

Materials and methods

Cell growth

HT4 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 33°C. Cells were differentiated by growth at the nonpermissive temperature for the SV40 large T antigen for 5–7 days. HT4 cells were obtained from Ronald McKay at MIT (Cambridge, MA).

Construction of cDNA libraries

cDNA libraries were constructed from poly(A) mRNA isolated from either differentiated or nondifferentiated HT4 cells. mRNA was isolated in a single-step extraction with guanidinium thiocyanate/phenol/chloroform (Chomczynski and Sacchi 1987). Poly(A) RNA was purified on oligo(dT) cellulose, and 10 µg was used for first-strand cDNA synthesis. Template mRNA was primed with a poly(dT) oligonucleotide containing an internal *XhoI* restriction site. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and a nucle-

otide mixture containing 5-methyl dCTP. During second-strand synthesis, RNase H and DNA polymerase I were used to replace the RNA strand with deoxynucleotides. T4 DNA polymerase was then used to blunt the ends of the cDNA, and *EcoRI* adaptors were ligated to the blunt termini. The cDNA was digested with *XhoI* to expose the *XhoI* site at the 3' end of the cDNA. The incorporation of 5-methyl dCTP during the first-strand synthesis prevented the internal restriction of the cDNA inserts. The cDNA was directionally cloned into Lambda Zap II (Stratagene). Size fractionation of the cDNA gave inserts of sizes ranging from 0.5 to 3.5 kb. Phage DNA was packaged using Giga-pack II Gold packaging extracts (Stratagene), which gave rise to ~10⁶ recombinant clones. The library was amplified once in the PLK-F' *Escherichia coli* strain. The cDNA library constructed from undifferentiated HT4 cells was designated cDL33, and the library constructed from differentiated HT4 cells was designated cDL39.

Isolation of cDNA clones

A mixture of two oligonucleotides with the sequence 5'-CT-TGCCCCACGCTGCTGTTTCC-3' and 5'-GTCACACTTGT-TCCCTACCAG-3' was end-labeled with T4 polynucleotide kinase and [γ -³²P]ATP (Amersham, 6000 Ci/mmol). These oligonucleotides were used to screen the cDL39 cDNA library. Hybridization was conducted under low stringency in 6× SSC, 5× Denhardt's solution, and 0.05% NaPP_i at room temperature for 24 hr. Filters were washed initially in 6× SSC containing 0.05% NaPP_i and 0.1% SDS, and the wash stringency was increased to 2× SSC containing 0.05% NaPP_i and 0.1% SDS. Ten independent clones were isolated, and pBluescript phagemids were excised *in vivo* by coinfection with R408 helper phage (Stratagene).

DNA sequence analysis

The nucleotide sequence was initially determined using a modification of the Sanger dideoxy method (Sequenase, U.S. Biochemical). Sequence data were verified using the linear amplification DNA sequencing method (dsCycle Sequencing, GIBCO/BRL). Briefly, 1 pmole of the appropriate sequencing primer was end labeled with T4 polynucleotide kinase and [γ -³²P]ATP. The end-labeled primer was combined with 290 ng of plasmid DNA in *Taq* sequencing buffer (30 mM Tris-HCl at pH 7.8, 5 mM MgCl₂, 30 mM KCl, and 0.5% W-1) containing 2.5 units of *Taq* polymerase. Four separate termination reactions were thermal cycled 30 times at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, with a final elongation step of 72°C for 5 min. The termination mixes contained as the final concentration 10 µM each of dATP, dCTP, dTTP, and c⁷-deaza-dGTP. The dideoxy nucleotide concentrations in the respective termination reactions were 0.4 mM ddATP, 0.2 mM ddCTP, 0.04 mM ddGTP, and 0.4 mM ddTTP. The reactions were stopped with one-third volume of 95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. This linear amplification method allowed sequencing of relatively impure double-stranded template DNA. Nucleotide chain elongation and termination at temperatures >70°C prevented the formation of secondary structures, which often resulted in premature chain termination.

Sequence comparisons were performed by using the Intelligenetics Suite on a Sun 3/50 computer.

RNA analysis

Poly(A) mRNA was isolated from both differentiated and undifferentiated HT4 cells. First-strand cDNA was synthesized by

AMV reverse transcriptase in the presence of methyl-mercury hydroxide (cDNA cycle, Invitrogen). The *rah* G-protein transcripts were amplified using the GeneAmp PCR (Perkin-Elmer Cetus). Two primer combinations were used to generate 183- and 511-bp fragments. Primer set 1 consisted of the oligonucleotides 5'-AAGGTCATCGTTGTGGGAGAC-3' and 5'-AACCTTTCCTGACCAGCCGTG-3'. Primer set 2 consisted of 5'-AAGGTCATCGTTGTGGGAGAC-3' and 5'-CCGCCAG-GACATTGGCCTCAAAG-3'. The amplification reactions were cycled 30 times with the following temperature profile: 95°C, 30 sec; 50°C, 30 sec; 72°C, 1 min. Nucleic acid fragments were separated on a 1.7% agarose gel and stained with ethidium bromide.

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