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, 1990b), 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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mechansims 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. A partial restriction map and complete sequencing of this clone, a 1-kb insert. A partial restriction map and sequencing strategy are shown in Figure 1. 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, Arg-17, and Thr-20. In Figure 3, amino acid identity in the putative GTP-binding domains, at amino acid positions 3–17, 22–38, and 52–63, and 111–119 (Bourne et al. 1991), is 50–100% when compared with tabl (Touchot et al. 1987), sec4 (Salminen and Novick 1987), or vpt1 (Gallwitz et al. 1983). Although the open reading frame lacks an initiator methionine, some interesting sequence comparisons to other known members of the ras superfamily indicate both similarities and differences, which make this clone a novel, new entity.

This new G protein is most closely related to ras, sec4, and vpt1, with 32–34% amino acid identity. The next closest relatives would be rab (Polakis et al. 1989), sec4 (Pizon et al. 1988), ras (Chardin et al. 1988), and the other classes of LMW G proteins ranges from 21% identity for rho to 47% for ras. Within a particular class of G protein, amino acid identity can

![Figure 1](genesdev.cshlp.org)  
**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 show the direction and region of DNA sequencing.

![Figure 2](genesdev.cshlp.org)  
**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.
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Figure 3. Comparison of amino acid sequences in the conserved GTP-binding domains. Identical amino acids to rahl are highlighted in boxes. The sequences are ordered with respect to the weighted identical amino matches in the GTP-binding domains compared with rahl. 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, I, L, M, C); (Z) an aromatic amino acid (F, Y, W), (B) serine (S) or threonine (T).

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

<table>
<thead>
<tr>
<th>new -C-C-P terminus</th>
<th>-C-A-A-X termini</th>
<th>-C-C termini</th>
<th>-C-X-C termini</th>
<th>No Cys terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>rahl</td>
<td>-T-C-C-P</td>
<td>Ha-ras</td>
<td>-C-V-L-S</td>
<td>ypt1</td>
</tr>
<tr>
<td>ralA</td>
<td>-C-C-I-L</td>
<td>sec4</td>
<td>-S-N-C-C</td>
<td></td>
</tr>
<tr>
<td>rhoA</td>
<td>-C-L-V-L</td>
<td>rab1a</td>
<td>-G-G-C-C</td>
<td></td>
</tr>
<tr>
<td>rac1</td>
<td>-C-L-L-L</td>
<td>rab2</td>
<td>-G-G-C-C</td>
<td></td>
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</tbody>
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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 differentiated by growth at the nonpermissive temperature.

Materials and methods

Cloning of a new G protein

A mixture of two oligonucleotides with the sequence 5'-CTTGCCCCAGGCTGTCTTCCC-3' and 5'-GTCACACCTTGT-TCCCTACAGG-3' was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP [Amersham, 6000 Ci/mmole]. 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% NaPPi at room temperature for 24 hr. Filters were washed initially in 6× SSC containing 0.05% NaPPi, and 0.1% SDS, and the wash stringency was increased to 2× SSC containing 0.05% NaPPi, 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 and a mixture of two oligonucleotides with the sequence 5'-CT-

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.

oligonucleotide 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 adapters 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 Gigapack 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.

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 and a mixture of two oligonucleotides with the sequence 5'-CT-
AMV reverse transcriptase in the presence of methyl-mercury hydroxide [cDNA cycle, Invitrogen]. The \( \text{tab} \) 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'-AAGGTCATCGTTGAGGAC-3' and 5'-AACCTTTTCCTGACCCCGTG-3'. Primer set 2 consisted of 5'-AAGGTCATCGTTGAGGACGAC-3' and 5'-CCGCCAG-GACTTGGCCCTAAAG-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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