Abundant Expression of ras Proteins in Aplysia Neurons

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Abstract. We have cloned a DNA fragment from the marine mollusc Aplysia californica, which contains sequences homologous to mammalian ras genes, by screening a genomic library with a viral Ha-ras oncogene probe under conditions of low stringency hybridization. Nucleotide sequencing revealed a putative exon that encodes amino acids sharing 68% homology with residues 5 to 54 of mammalian p21\textsuperscript{pp60\textasciitilde}s polypeptides, and which therefore is likely to encode a ras-like Aplysia protein. The cloned locus, designated Apl-ras, is distinct from the Aplysia rho (ras-homologue) gene and appears to be more closely related to mammalian ras. We used a panel of monoclonal antibodies raised against v-Ha-ras p21 to precipitate an M,

21,000 protein from extracts of Aplysia nervous tissue, ovotestis, and, to a much lesser degree, buccal muscle. Fluorescence immunocytochemistry revealed that ras-like protein is most abundant in neuronal cell bodies and axon processes, with staining most prominent at plasma membranes. Much less was present in other tissues. The prominence of ras protein in neurons, which are terminally differentiated and non-proliferating, indicates that the control of cell division is not the sole function of this proto-oncogene. The large identified neurons of Aplysia offer the opportunity to examine how ras protein might function in mature nerve cells.

Ras proto-oncogenes are expressed throughout mammalian embryonic and fetal development (47) and ras proteins can be detected in almost all human fetal and adult tissues examined (Furth, M. E., T. A. Aldrich, C. Cordon-Cardo, unpublished data). Because they induce neoplastic transformation (8, 56) and stimulate synthesis of DNA in quiescent cells (10, 50), activated ras genes have been associated with uncontrolled cell proliferation. There is also strong evidence implicating ras proto-oncogene proteins in the control of normal cell division (34). Surprisingly, ras proto-oncogenes are also expressed in non-proliferating, terminally differentiated cells; for example, in humans it has recently been found that ras proteins are abundant in nerve cells (Furth, M. E., T. A. Aldrich, C. Cordon-Cardo, unpublished data). The product of the src proto-oncogene, pp60v-src, also has been found to be abundant in developing neurons as well as in some mature nerve cells that are postmitotic (7, 15, 24, 48).

Ras proteins were first identified as products of the transforming genes of the Harvey and Kirsten murine sarcoma viruses (v-Ha-ras and v-Ki-ras) (9). Three classes of proto-oncogenes (Ha-ras, Ki-ras, and N-ras) have been recognized in normal mammalian cells (9, 17, 46). These genes each encode similar p21 proteins (6, 32, 45, 52) that are localized to the cytoplasmic face of the plasma membrane (55), bind guanine nucleotides (43), and hydrolyze GTP (16, 28, 31, 51). Ras genes and proteins also have been found in Drosophila (33, 35), Dictyostelium (37, 39), and yeast (13, 36, 38). Although ras proteins have not yet been identified in molluscs, Madaule and Axel (26) isolated an Aplysia gene that encodes an M, 21,000 polypeptide whose predicted amino acid sequence shares ~35% homology with mammalian ras proteins; they found similar ras-homologous (rho) genes in man, rat, Drosophila, and yeast.

Their widespread phylogenetic and tissue distribution suggests that ras proteins participate in some general cellular mechanism. Although their biochemical function has not yet been determined, ras proteins have been shown to share homology with the family of guanine nucleotide-binding proteins that transduce signals from receptors on the cell membrane to the cAMP and other intracellular second messenger systems (19, 25). Genetic and biochemical studies have demonstrated that ras proteins are required for GTP-stimulated adenylate cyclase activity in the yeast, Saccharomyces cerevisiae (53), but there is little evidence for this action in other eucaryotes (2, 11, 14, 40).

We are interested in the function of ras proteins in neurons. The marine mollusc, Aplysia, has large nerve cells in which the cAMP and other second messenger cascades have been characterized (see reference 41). Because invertebrate neurons have been useful for studying how molecules operate to mediate signal transduction, we have begun to examine ras proteins and the sequences encoding them in Aplysia. We find that Aplysia contains genomic DNA sequences homologous to vertebrate ras genes and that neurons contain high concentrations of immunoreactive ras p21 protein.
Materials and Methods

Animals and Cells

Tissues from *Aplysia* weighing from 150 to 400 g, purchased from California collectors (Marinus Inc., Inglewood, CA; Pacific Bio-Marine Laboratories Inc., Venice, CA; and Sea Life Supply, Sand City, CA) or grown at the Howard Hughes Medical Institute's Mariculture Resource Facility at the Woods Hole Oceanographic Institute (Woods Hole, MA) were obtained as described (42). Mammalian cell lines have been described previously (12).

Antibodies

Anti-ras monoclonal antibodies were purified from supernatant fluids of hybridoma cells grown in serum-free medium (12). Rabbit anti-rat IgG, rhodamine-conjugated goat anti-rabbit IgG, rat IgG, and rabbit serum were purchased from Cappel Laboratories, West Chester, PA. Anti-serotonin antisera (lot SER-7-7) was kindly provided by Dr. H. M. B. Steinbusch, the Free University, Amsterdam; this serum's immunoreactivity in *Aplysia* has previously been characterized (22).

ras-homologous Aplysia Clones

An *Aplysia* genomic library in bacteriophage lambda J1 was provided by R. H. Scheller, Department of Biology, Stanford University. Screening of plaques was performed according to Benton and Davis (3). Low stringency hybridization was performed as described by Shilo and Weinberg (44) using a nick-translated, 32P-labeled v-Ha-ras probe. The fragment spanning the entire v-Ha-ras coding sequence was obtained from plasmid pB185 (56) by digestion with restriction endonuclease Bam HI. The fragment was separated from vector sequences by gel electrophoresis on 1% (wt/vol) agarose, and isolated by electroelution. Plate lysate stocks were prepared (27), phage purified (57), and phage DNA extracted (27) as previously described. To subclone the 2-kb Hind III fragment, which hybridizes to the v-Ha-ras probe, Hind III-digested DNA from phage B2 was ligated with Hind III-digested pBR322 (5) and the mixture used to transform *Escherichia coli* strain HB101. A plasmid (designated pB2104) containing the 2-kb Hind III fragment of phage B2 in pBR322 was identified by screening Hind III-digested DNA from individual transformants. Nucleotide sequence was determined (29) on fragments that were end labeled after removal of phosphate with bacterial alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, MD) and phosphorylated with γ-[32P]-ATP (New England Nuclear, Boston, MA) and polynucleotide kinase (P. L. Biochemicals, Inc., Milwaukee, WI). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Beverly, MA.

Immunoprecipitation

Proteins were labeled by incubation of ganglia and other *Aplysia* tissues for 15 h at 15°C in a supplemented sea water containing [35S]methionine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL); under these conditions isolated ganglia are viable for at least 12–24 h (42). Nervous tissue incorporated approximately twice the amount of radioactivity per mg protein (104 cpm) as ovotests. Labeling of v-Ha-ras-transformed HD8 cells with [35S]methionine, preparation of cell lysates, immunoprecipitation of proteins from both vertebrate and *Aplysia* tissue extracts, and gel electrophoresis of the precipitates are described by Furth et al. (12). SDS (0.25%, wt/vol) was included in immunoprecipitation reactions to reduce nonspecific binding of labeled proteins.

Immunocytochemistry

Tissue was either rapidly frozen, sectioned, and then fixed in 1% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) containing 30% (wt/vol) sucrose, or first fixed in 4% paraformaldehyde and then frozen and sectioned. Although fixation before sectioning improves the preservation of the tissue's morphology, no differences either in intensity or specificity of staining were found in tissues fixed in 4% paraformaldehyde and then frozen and sectioned. Morphology, no differences either in intensity or specificity of staining were observed. 16-μm cryostat sections were rinsed in PBS (0 mM Na phosphate, 0.9% NaCl, 0.3% sodium azide, pH 7.4) and incubated for 1–2 h at room temperature in dilutions of antibody Y13-259 in PBS containing bovine serum albumin (1 mg/ml). Sections were rinsed, incubated for 1 h in PBS containing rabbit anti-rat IgG (0.1 mg/ml), rinsed again in PBS, and incubated for 40 min in rhodamine-conjugated goat anti-rabbit IgG (0.3 mg/ml). After they were washed and coverslipped under glycerol, sections were viewed by epifluorescence with a Leitz microscope (Filter Pack N-2) and photographed with high-speed Tri-X (Eastman Kodak Co.). Serotonin immunocytochemistry was performed as described (22). No differences in staining resulted when we used this procedure with the ras monoclonal antibody.

Results

Cloning ras-homologous Sequences in Aplysia

We screened an *Aplysia* genomic library using low stringency conditions that allowed a v-Ha-ras gene probe to hybridize to discrete DNA restriction fragments from both mouse and *Aplysia* by Southern blotting (49; data not shown). Three of the 300,000 phage clones screened (A18, A34, B2) hybridized to the probe. Restriction endonuclease and Southern blot analysis of DNA prepared from each of these three clones revealed that although each was unique, all shared a 2-kb Hind III restriction fragment that hybridizes to the v-Ha-ras probe. We have also found this restriction fragment in high molecular weight *Aplysia* sperm DNA using high-stringency Southern blotting with the cloned fragment as probe (data not shown).

The 2-kb Hind III restriction fragment from phage clone B2 was subcloned into the single Hind III restriction site of plasmid pBR322 (designated pB2104). Sequences hybridizing to the v-Ha-ras probe were localized to within a 0.8-kb Hind III–Sal I subfragment by plasmid Southern blot analysis of pB2104 after digestion with several combinations of restriction endonucleases (data not shown). We determined the nucleotide sequence of the entire 0.8-kb Hind III–Sal I restriction fragment.

Nucleotide sequence determination precisely localized the *Aplysia* DNA sequences homologous to mammalian ras genes. The first 248 bases downstream of the Hind III site are shown in Fig. 1 (top). Residues 1 to 150 (ApI-ras) share 65% homology with sequences encoding the amino-terminal portion of the c-Ha-ras gene. The inferred amino acid sequence of the longest possible reading frame displays 68% amino acid homology from residue 5 to 54 of the c-Ha-ras protein. In 7 of 9 nucleotides, the sequence ATGGTAAGA is conserved (12), and suggests an exon/intron junction. We detected no significant homology to the c-Ha-ras gene between this putative splice junction and the Sal I restriction site.

Immunoprecipitation of Aplysia ras Proteins

To identify ras proteins in *Aplysia*, we used eight monoclonal antibodies prepared against ras p21 encoded by Harvey murine sarcoma virus (12) to immunoprecipitate ras-like polypeptides from extracts of isolated *Aplysia* ganglia labeled in organ culture with [35S]methionine. Antibodies Y13-4, Y13-259 (Fig. 2 B, lanes 2 and 4) and Y13-128 (not shown) precipitated a major labeled Mr, 21,000 polypeptide (Fig. 2 B, lanes 2 and 4). In contrast, Y13-238, Y6-40, Y6-92, Y6-165, Y6-172 (12), and normal rat IgG failed to react with any of the labeled polypeptides. Each of the three monoclonal antibodies precipitated the p21 ras from HD8 canine kidney epithelial cells transformed by Harvey murine sarcoma virus (Fig. 2 A, lanes 1–4) that characteristically
migrates as a doublet. The species that migrates more slowly is the phosphorylated form of v-Ha-ras p21 (43).

In addition to the $M_r$ 21,000 polypeptide, a less abundant $M_r$ 26,000 polypeptide was also precipitated from the extracts of Aplysia ganglia by antibody Y13-259 (Fig. 2 B, lane 4). We have observed a similar protein in some mammalian cells. A second antibody, Y13-4, also precipitated $M_r$ 37,000 and 90,000 polypeptides from extracts of both mammalian cells and Aplysia neural components (Fig. 2, A and B, lanes 2). These larger polypeptides were observed only when we

![Diagram of nucleotide and amino acid sequences](image_url)

**Figure 1. (Top)** Comparison of the nucleotide and the deduced amino acid sequence of Apl-ras with those of c-Ha-ras (6). Apl-ras nucleotide sequence between the Hind III and Nsi I restriction sites was determined as described in Materials and Methods. We show the predicted amino acid sequence from Hind III to a putative splice junction (arrow); a consensus 5' splice donor sequence is underlined. Boxed regions indicate conserved amino acid residues between Apl-ras and c-Ha-ras. Numbers above the sequences refer to nucleotide residues of Apl-ras; numbers below refer to amino acid residues of c-Ha-ras.

**Figure 1. (Bottom)** Comparison of the amino-terminal residues of Apl-ras with c-Ha-ras and Aplysia rho (26). Boxed regions indicate conserved residues between Apl-ras (A-ras) and c-Ha-ras (H-ras). Underlined residues of rho (A-rho) are conserved in Apl-ras. Numbers refer to the amino acid residues of c-Ha-ras.
Figure 2. Immunoprecipitation of *Aplysia* ras proteins. [³⁵S]methionine-labeled proteins from extracts of v-Ha-ras-transformed HD8 cells (A) and of *Aplysia* central ganglia (B) were precipitated with monoclonal ras antibodies Y13-4 (lanes 2), Y13-238 (lanes 3), Y13-259 (lanes 4), or normal rat IgG (lanes 1). In a separate experiment, Aplysia proteins precipitated from the ganglia (C) are compared to proteins precipitated from ovotestis (D). Equal amounts of radioactive protein from each tissue were subjected to the precipitation. A fluorogram of the precipitated proteins separated by electrophoresis on an SDS/12% polyacrylamide gel is shown above. Arrows indicate the migrations of the major immunoprecipitated proteins; the positions of protein standards are also indicated.

**Immunocytochemistry with *Aplysia* Tissues**

We used indirect immunofluorescence with antibody Y13-259 to determine where ras proteins are localized within the *Aplysia* tissues. This antibody reacts with the products of all three known mammalian ras proto-oncogenes as well as with the products of ras genes in *S. cerevisiae*, *Dictyostelium*, and *Drosophila* (36, 37, 38). The most abundant fluorescence was observed in neurons; little or no fluorescence was detected in the connective tissue sheath that surrounds the neuronal components (Fig. 3A). Little immunoreactivity was seen in buccal muscle exposed photographically for the same short period of time (Fig. 3C). No immunofluorescence was detected in sections of ganglia and muscle when the antibody was replaced by bovine serum albumin or normal rat IgG. Ras immunofluorescence was also abundant in sections of ovotestis (Fig. 4B, shown compared to a section of cerebral ganglion, Fig. 4A). Bright fluorescence was also detected in sections of fertilized eggs; sperm and salivary gland were less reactive (data not shown). Much longer photographic exposure revealed that muscle also contains some immunoreactive material (Fig. 4C) compared to a control section in which Y13-259 was replaced with normal IgG (Fig. 4D).

The neuronal components of all central *Aplysia* ganglia were highly immunoreactive. We studied the regional distribution of ras immunoreactivity in nervous tissue of the abdominal ganglion in greater detail. All of the neurons seen by phase-contrast microscopy (Fig. 5A) stained brightly in both their cell bodies and axon processes (Fig. 5B). Immunoreactive material appeared more concentrated at cell membranes and was excluded from cell nuclei. The connective tissue sheath surrounding the ganglion was not stained. Ras immunoreactive material also appeared in the sensory cells of the pleural ganglia (Fig. 5C). This population of neurons has been shown to mediate simple forms of learning through the activation of a serotonin-sensitive adenylate cyclase (20, 21, 41). Alternate staining of consecutive sections of a pleural sensory cell cluster with antibodies to serotonin (Fig. 5D) and ras (Fig. 5C) shows that serotonin immunoreactivity (and, by inference, serotonergic input) is restricted to specific sensory neurons, as described previously (22). Since ras immunofluorescence was more evenly distributed, the location of ras protein does not appear to be congruent with serotonergic innervation to *Aplysia* sensory cells.
Discussion

Identification and Characterization of ras in Aplysia

We have isolated Aplysia genomic DNA sequences (Apl-ras) containing a 150-bp region that shares 65% nucleic acid homology with a segment of mammalian c-Ha-ras. Although the entire Apl-ras gene has not yet been characterized, a putative coding region predicts an amino acid sequence similar to that from residues 5 to 54 of c-Ha-ras p21. The overall amino acid homology is 68% with no gaps necessary in either sequence. Amino acid residues 5 to 22 of Apl-ras and c-Ha-ras are 94% homologous, differing by only a single amino acid substitution. These residues include one loop of the proposed binding site for the phosphoryl group of GTP in the mammalian ras protein (18, 30), suggesting that the Apl-ras product binds GTP, as do other ras proteins. Apl-ras was the only gene that we isolated from a genomic library by low stringency hybridization with the v-Ha-ras probe. As in other animals, additional ras genes may be present in Aplysia.

The gene that we have identified is distinct from the Aplysia rho gene (26). As shown at the bottom of Fig. 1, Apl-ras encodes a protein that contains greater amino acid homology (identity in 34 of 50 residues) with the amino-terminal portion of mammalian ras proteins than does the predicted protein encoded by Aplysia rho (identity in 22 of 50 residues). Interestingly, Apl ras and Aplysia rho proteins do share common amino acids at positions that are not also conserved in the mammalian ras protein.

Because we had found ras sequences in the genome of Aplysia, we expected that ras proteins are present in Aplysia tissues. We used a panel of monoclonal antibodies that react with vertebrate ras proteins for immunoprecipitation studies to identify an Aplysia ras p21 and to determine its tissue distribution. Ras is most abundantly expressed in the neuronal components of nervous tissue. It is also plentiful in ovotestis and fertilized egg, but only small amounts are present in muscle, salivary gland, and sperm.

Immunocytochemical analysis of Aplysia ganglia with antibody Y13-259 allowed us to localize ras proteins within nervous tissue. We found ras epitopes in high concentration in all neuronal cell bodies, axons, and neuropil. This ubiquitous distribution differs from that described for pp60^sv in the vertebrate nervous system. Abundant immunocytochemical staining for pp60^sv was observed only in specific cell types in developing cerebellum and retina; in the mature ani-

Figure 3. ras immunoreactive material in Aplysia nervous tissue. Indirect immunocytochemical analysis with ras antibody Y13-259 (22 μg/ml) on sections of (A) cerebral ganglion and (C) buccal muscle. Sections of cerebral ganglion (B) and buccal muscle (D) were incubated with bovine serum albumin (1 mg/ml) in place of the antibody. The photographic exposure time was optimized for the cerebral ganglion, and all other sections were then exposed for the same length of time. Bar, 100 μm.
Figure 4. ras-immunoreactive material in Aplysia ovotestis and muscle. Indirect immunocytochemical analysis using antibody Y13-259 on (A) cerebral ganglion, (B) ovotestis, and (C) buccal muscle. (D) A section of muscle incubated with normal rat IgG (18 μg/ml) in place of the antibody. Photographic time exposures were matched between A and B, and between C and D. The amount of antibody Y13-259 used was 45 μg/ml with the ganglion and ovotestis, and 18 μg/ml with muscle. Bar, 100 μm.

mal, expression of pp60c-src disappeared in the cerebellum, but persisted in the retina (15, 48). Staining in Aplysia with the antibody against ras appeared brightest at the nerve cell's external membrane, both somatic and axolemma, consistent with the localization to the inner surface of the plasma membrane demonstrated previously in mammalian cells (55). Because of the specificity of the antibody, which precipitated a major p21 and a minor p26 from the neuronal components (Fig. 2), the immunostaining obtained most likely reflects the content of ras p21. Antibody Y13-259 has been shown to react with an epitope contained within amino acid residues 70-81 of ras p21 which is highly conserved in ras proteins in yeast, Drosophila, Dictyostelium, and human (23). This region of the protein is not homologous to the corresponding domain in the protein encoded by the Aplysia rho gene, and therefore it is unlikely that the immunostaining observed was caused by a rho p21. The Apl-ras exon identified here does not contain the nucleotide sequence that codes for amino acid residues 63 to 73, however. Further characterization of the Apl-ras gene will be required to be confident that it encodes the p21 ras protein immunoprecipitated by the ras monoclonal antibodies.

Possible Function of ras in Neurons

We find that ras is plentiful in some Aplysia cells (eggs and neurons), and much less abundant in others (muscle, sperm, and salivary gland). Eggs are totipotent and ready to proliferate. Aplysia neurons in the mature specimens used here appear to be terminally differentiated like their mammalian counterparts: they do not increase in size or number, nor do they proliferate in culture. The distribution in Aplysia conforms to that found in a more extensive study of human tissues (Furth, M. E., T. A. Aldrich, C. Cordon-Cardo, unpublished data): in both man and Aplysia, ras p21 can be detected in almost all cells, but the extent of its expression is not simply correlated with potential to proliferate or with state of differentiation. Although ras proto-oncogenes may play distinctly different roles in mature neurons and in proliferating cells, it is attractive to think that ras might serve the same function in both kinds of cells.

Two recent reports, which show that ras oncogene products can circumvent the cAMP cascade, may provide a clue as to what that function might be. First, treatment with analogues of cAMP or NGF induce rat pheochromocytoma (PC12) to differentiate rapidly but transiently into sympathet-ic neuron-like cells (1). Microinjection of ras p21 oncogene protein induces a longer-lasting differentiation of PC12 cells without detectable changes in cAMP. Induction of PC12 cells by either nerve growth factor or ras requires synthesis of both RNA and protein whereas induction with cAMP does not. In the second example (4), Xenopus oocytes undergo meiosis when microinjected with ras proteins. Meiosis can also be induced by changes in the concentration of cAMP. No change in cAMP was observed after microinjection of ras proteins, however, again suggesting that the mechanisms involving ras and the cAMP-cascade operate separately. We suggest that both proliferating cells that are undifferentiated, and neurons, even though terminally differentiated, share the potential for making persistent responses that are only tran-
siently induced by extracellular signals through transducing (conventional) guanine nucleotide-binding proteins. Persistence would be achieved through an alternate (and as yet unidentified) second messenger pathway involving ras proteins, possibly leading to changes in gene expression.

In Aplysia sensory neurons, a rise of cAMP produced by a serotonin-sensitive adenylate cyclase brings about the increased release of neurotransmitter that underlies simple forms of short-term learning (see references 20, 21, 41). It has been proposed that long-term learning in these sensory cells is mediated by changes in gene expression (see reference 21). The large identified neurons of Aplysia allow us to test the hypothesis that ras proteins function in neurons in a parallel pathway to make durable transient changes produced by cAMP-dependent protein phosphorylation.

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**Figure 5.** Regional distribution of ras immunoreactive material in the abdominal ganglion of Aplysia. (A) Section of the ganglion in phase-contrast microscopy. (B) Immunocytochemical analysis with ras antibody Y13-259 (45 µg/ml) in the same section of the ganglion shown in A, and (C) in a sensory cell cluster dissected from the pleural ganglion. (D) Serotonin immunofluorescence in the next consecutive section of the same sensory cell cluster shown in C. No staining was observed when normal rat IgG was used in place of the ras antibody or with normal rabbit serum in place of the serotonin antiserum (not shown). Bar, 100 µm.

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