

# R2D5 Antigen: a Calcium-binding Phosphoprotein Predominantly Expressed in Olfactory Receptor Neurons

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**Abstract.** R2D5 is a mouse monoclonal antibody that labels rabbit olfactory receptor neurons. Immunoblot analysis showed that mAb R2D5 recognizes a 22-kD protein with apparent pI of 4.8, which is abundantly contained in the olfactory epithelium and the olfactory bulb. We isolated cDNA for R2D5 antigen and confirmed by Northern analysis and neuronal depletion technique that R2D5 antigen is expressed predominantly, but not exclusively, in olfactory receptor neurons. Analysis of the deduced primary structure revealed that R2D5 antigen consists of 189 amino acids with calculated  $M_r$  of 20,864 and pI of 4.74, has three calcium-binding EF hands, and has possible phosphorylation sites for  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaM kinase II) and cAMP-dependent protein kinase (A kinase). Using the bacterially expressed protein, we directly examined the biochemical proper-

ties of R2D5 antigen. R2D5 antigen binds  $Ca^{2+}$  and undergoes a conformational change in a manner similar to calmodulin. R2D5 antigen is phosphorylated *in vitro* by CaM kinase II and A kinase at different sites, and 1.81 and 0.80 mol of Pi were maximally incorporated per mol of R2D5 antigen by CaM kinase II and A kinase, respectively. Detailed immunohistochemical study showed that R2D5 antigen is also expressed in a variety of ependymal cells in the rabbit central nervous system. Aside from ubiquitous calmodulin, R2D5 antigen is the first identified calcium-binding protein in olfactory receptor neurons that may modulate olfactory signal transduction. Furthermore our results indicate that olfactory receptor neurons and ependymal cells have certain signal transduction components in common, suggesting a novel physiological process in ependymal cells.

**A**n enormous variety of odor molecules are recognized by olfactory receptor neurons in the olfactory epithelium, and the activated receptor neurons send the spike signal to the olfactory bulb via their axons. Recent studies have elucidated molecular components and physiological processes underlying the detection of odor molecules and the transduction of the odor signal into electrical responses (for reviews see Anholt, 1991; Reed, 1992; Ronnett and Snyder, 1992). Odor molecules bind to specific receptor proteins on the cilia of olfactory receptor neurons (Buck and Axel, 1991; Raming et al., 1993), thereby activating an olfactory-specific adenylyl cyclase (Bakalyar and Reed, 1990) through a GTP-binding protein,  $G_{olf}$  (Jones and Reed, 1989). This leads to an increase in intracellular cAMP (Pace et al., 1985; Shirley et al., 1986; Sklar et al., 1986; Breer et al., 1990; Boekhoff et al., 1990), which opens cyclic nucleotide-gated cation channels and eventually causes depolarizing inward membrane current (Nakamura and Gold, 1987; Dhallan et al., 1990). Another signal transduction cascade involving inositol 1,4,5-trisphosphate ( $IP_3$ )<sup>1</sup> may be

coupled to some odor receptor proteins (Huque and Bruch, 1986; Breer et al., 1990; Boekhoff et al., 1990; Restrepo et al., 1990; Fadool and Ache, 1992; Raming et al., 1993).

Besides the transduction of odor signal, olfactory receptor neurons exhibit adaptive properties such as response desensitization, termination of signal transduction, and recovery from the excited state. For example, prolonged odor stimulation causes a decrease in odor-evoked response of olfactory receptor neurons.  $Ca^{2+}$  inflow into the cell (Kurahashi, 1989, 1990; Kurahashi and Shibuya, 1990; Zufall et al., 1991; Kramer and Siegelbaum, 1992) and cAMP-dependent protein phosphorylation (Firestein et al., 1991; Boekhoff and Breer, 1992) are thought to be responsible for this desensitization.

In photoreceptor cells, the adaptive properties are known to be mediated by several processes. They include phosphorylation of rhodopsin by rhodopsin kinase (Miller et al., 1986), binding of arrestin to the phosphorylated rhodopsin (Wilden et al., 1986), and modulation of transducin GTPase activity by  $\gamma$ -subunit of cyclic GMP phosphodiesterase and cyclic GMP (Arshavsky et al., 1991; Arshavsky and Bownds, 1992). Furthermore,  $Ca^{2+}$  modulates the activity of guanylate cyclase (Dizhoor et al., 1991; Lambrecht and Koch, 1991), cyclic GMP phosphodiesterase (Kawamura and Murakami, 1991), and cyclic GMP-gated cation channel (Steele et al., 1992; Gordon et al., 1992; Hsu and Molday, 1993). In olfactory receptor neurons, however, molecular basis for the adaptive properties is poorly understood.

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1. *Abbreviations used in this paper:*  $IP_3$ , inositol 1,4,5-trisphosphate; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside.

In previous works, we described mAb R2D5 which recognizes an antigen (R2D5 antigen) highly localized to the olfactory receptor neurons (Mori et al., 1985, 1987, 1990). Immunocytochemical analysis with mAb R2D5 revealed that R2D5 antigen is present in all population of olfactory receptor neurons, and for individual receptor neurons, in their entire cytosolic parts including the cilia at the dendritic terminals, the sites for olfactory sensory transduction. Within the rabbit central nervous system, R2D5 antigen is present in olfactory receptor axons and in a limited population of non-neuronal epithelial cells, i.e., the ventricular ependymal cells and epithelial cells of the choroid plexus. However virtually no other neurons express the R2D5 antigen. In the peripheral tissues examined, the R2D5 antigen was also expressed in a limited population of the nasal respiratory epithelial cells.

In the present study we have determined the identity of the rabbit R2D5 antigen by molecular cloning. We examined its biochemical characteristics using the bacterially expressed R2D5 antigen. The rabbit R2D5 antigen is a calcium-binding protein which can be phosphorylated by CaM kinase II and A kinase. As  $\text{Ca}^{2+}$  and cAMP play crucial roles in olfactory adaptation, these results suggest possible functions of R2D5 antigen in the adaptive properties of olfactory receptor neurons.

## Materials and Methods

### Antibody

The procedure for the production of hybridoma secreting mAb R2D5 has been fully described in our previous reports (Fujita et al., 1985; Mori et al., 1985, 1987). In brief, mAb R2D5 is a mouse monoclonal antibody (IgG<sub>1</sub>,  $\kappa$ ) produced by fusion of myeloma X63-Ag8-653 with splenocytes of a BALB/c mouse immunized with the homogenate of adult rabbit olfactory bulbs. The ascitic fluid was produced by injecting  $10^7$  myeloma cells intraperitoneally into mice primed with an injection of pristane (2,6,10,14-tetramethylpentadecane) 1 wk before. The ascitic fluid was used at 1:1,000 dilution in immunoblot analysis and cDNA library screening.

### Animals and Surgical Manipulations

Adult rabbits used in this study were of the Japanese White strain (Oriental Bioservice, Kyoto, Japan) weighing 2–3 kg. Procedures for the anesthesia, tissue dissection, and surgical manipulation for the olfactory bulbectomy were as described (Mori et al., 1985).

### cDNA Cloning

Total RNA was isolated from the dissected rabbit tissues by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)<sup>+</sup>RNA was selected by chromatography on oligo(dT) cellulose (Aviv and Leder, 1972).

cDNA was synthesized by using oligo(dT) primer from poly(A)<sup>+</sup>RNA of the rabbit olfactory epithelium according to the method of Gubler and Hoffman (1983), and ligated with EcoRI-digested and phosphatase-treated  $\lambda$ gt11 vector via EcoRI adaptors. The recombinant  $\lambda$ gt11 phage DNA was packaged in vitro by using Gigapack II Gold Packaging Extract (Stratagene, La Jolla, CA) and introduced directly into *E. coli* Y1090r-. The recombinants carrying R2D5 antigenicity were identified by screening with mAb R2D5 by the standard procedures (Huynh et al., 1985) and plaque-purified. The cDNA inserts of the isolated  $\lambda$ gt11 phages were subcloned into pBluescriptII SK(+) at EcoRI site for subsequent analysis. Plasmids were propagated in *E. coli* DH5 $\alpha$  (Sambrook et al., 1989). Nucleotide sequence was determined by the modified dideoxy chain termination method (Sanger et al., 1977) by the use of Sequenase (Un. States Biochem. Corp., Cleveland, OH). The cDNA inserts were sequenced entirely on both strands using specific oligonucleotides synthesized on a model 392 DNA synthesizer (Appl. Biosystems Inc., Foster City, CA).

### Northern Blot Analysis

A 0.6-kb PvuII fragment of the cDNA insert (see Fig. 2 A) was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by the oligolabeling method (Feinberg and Vogelstein, 1983, 1984), and used as a hybridization probe to detect R2D5 antigen mRNA. 10  $\mu$ g each of total RNA extracted from various rabbit tissues was resolved on a formaldehyde/1.2% agarose gel and transferred to a nylon membrane filter. The filter was hybridized at 42°C in a solution containing 50% formamide, 5  $\times$  SSC, 5  $\times$  Denhardt's solution, 0.05% (wt/vol) sodium pyrophosphate, 0.5% (wt/vol) SDS, 100  $\mu$ g/ml heat-denatured salmon sperm DNA, and 100  $\mu$ g/ml baker's yeast tRNA with  $10^6$  cpm/ml <sup>32</sup>P-labeled DNA probe. The filter was washed at 50°C in 0.2  $\times$  SSC, 0.5% (wt/vol) SDS, and exposed to a Kodak X-Omat x-ray film at -80°C for 24 h.

### Expression of R2D5 Antigen in *E. coli*

To the 5' and 3' ends of the coding region of the R2D5 antigen cDNA, the new restriction enzyme sites (NdeI and BamHI, respectively) were added by PCR with the cDNA as a template and synthetic oligonucleotides, 5'-TGACCCCATATGGACACTGCGGACGCCAT-3' and 5'-GCCAGGGATCCTATTACAGCTGCCAGGCGCTGCT-3', as primers. The amplified product was cleaved with NdeI and BamHI and ligated with NdeI- and BamHI-digested pET-3a vector. After confirming the integrity of the coding region by DNA sequencing, the recombinant plasmid, named pET-3a/R2D5Ag, was introduced into *E. coli* BL21(DE3)pLysS. The following procedures for expression of the recombinant R2D5 antigen were essentially as described (Studier and Moffatt, 1986; Rosenberg et al., 1987).

### Phenyl-Sepharose Chromatography and <sup>45</sup>Ca<sup>2+</sup> Autoradiography

The *E. coli* lysate prepared from 1 liter of the culture carrying pET-3a/R2D5Ag was centrifuged at 10,000 g for 30 min, and the supernatant was dialyzed against three changes of 2 liters of 20 mM Tris-HCl, pH 7.5, 1 mM DTT (buffer A) containing 1 mM EDTA and 1 mM EGTA. To the dialysate CaCl<sub>2</sub> was added to a final concentration of 4 mM, and the mixture was applied to a column of phenyl-Sepharose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ) (1.5 cm i.d.  $\times$  10 cm) previously equilibrated with buffer A containing 0.1 mM CaCl<sub>2</sub>. The column was washed with the equilibration buffer until absorbance at 280 nm reached baseline. Then the column was washed first with 100 ml of buffer A containing 0.1 mM CaCl<sub>2</sub> and 0.2 M NaCl, and next with 100 ml of buffer A containing 0.1 mM CaCl<sub>2</sub> and 0.5 M NaCl, to elute any proteins electrostatically bound to the column. Finally, proteins bound hydrophobically in a Ca<sup>2+</sup>-dependent manner were eluted with buffer A containing 1 mM EGTA. All phenyl-Sepharose chromatography was performed at room temperature. Fractions of 20 ml each were collected and analyzed by Coomassie staining and immunoblotting with mAb R2D5 after SDS-PAGE. The EGTA-eluted fractions contained the R2D5 antigen which was more than 95% pure. These fractions were combined and dialyzed against buffer A containing 0.1 mM EGTA. The dialysate was stored at -80°C until use.

Detection of Ca<sup>2+</sup> binding by <sup>45</sup>Ca<sup>2+</sup> blotting and autoradiography was performed according to Maruyama et al. (1984).

### Phosphorylation of R2D5 Antigen by CaM Kinase II and A Kinase

The purified CaM kinase II and catalytic subunit of A kinase were most generous gifts from Professor H. Fujisawa of Asahikawa Medical College. The standard reaction mixture contained the kinase (0.54  $\mu$ g of CaM kinase II or 1.4  $\mu$ g of A kinase), 0.2  $\mu$ g of the purified recombinant R2D5 antigen, 50 mM Hepes-Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, [ $\gamma$ -<sup>32</sup>P]ATP (at 30  $\mu$ M or 0.5 mM, final concentration), and either 0.1 mM CaCl<sub>2</sub> or 2 mM EGTA in a total volume of 70  $\mu$ l. One half  $\mu$ g of bovine calmodulin (Sigma Chem. Co., St. Louis, MO) was included in the reaction for CaM kinase II. The reaction was initiated by adding [ $\gamma$ -<sup>32</sup>P]ATP, incubated at 30°C for the indicated time, and terminated by the addition of cold TCA. The precipitates were collected by centrifugation and subjected to gel electrophoresis and autoradiography. The amount of <sup>32</sup>Pi incorporation into the R2D5 antigen was determined by liquid scintillation counting of the corresponding radioactive band excised from the gel.

### Immunological Techniques

Tissue preparation and immunostaining by the indirect immunofluorescence

method using mAb R2D5 and fluorescein-conjugated goat anti-mouse IgG (H+L) from Cappel as second antibody were conducted as described previously (Mori et al., 1985).

mAb R2D5-conjugated Sepharose 4B was prepared by coupling of mAb R2D5 (20 mg) to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology; 4 ml bed vol) according to manufacturer's instruction.

Samples for gel electrophoresis were prepared from the dissected rabbit tissues by homogenization in 3 vol of 0.32 M sucrose, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM EGTA, and 0.5 mM PMSF using Potter-Elvehjem homogenizer. The homogenates were boiled in SDS sample buffer and electrophoresed on 10–20% (wt/vol) linear gradient SDS-polyacrylamide slab gels according to the method of Laemmli (1970). Alternatively the homogenates were subjected to isoelectric focusing using PhastSystem (Pharmacia LKB Biotechnology) according to manufacturer's instruction. The proteins were stained with Coomassie brilliant blue, or transferred to a nitrocellulose membrane after the standard procedures for immunoblot analysis (Burnette, 1981). The membrane was incubated with 10% (wt/vol) non-fat milk containing 0.05% (vol/vol) Tween 20 for 2 h, and then with mAb R2D5 for 30 min at room temperature. The bound mAb R2D5 was visualized by using an alkaline-phosphatase-conjugated goat anti-mouse IgG antibody system from Promega Corp. (Madison, WI). Protein amounts were determined by the method of Bradford (1976) with BSA as standard.

### Purification and Amino Acid Sequencing of R2D5 Antigen

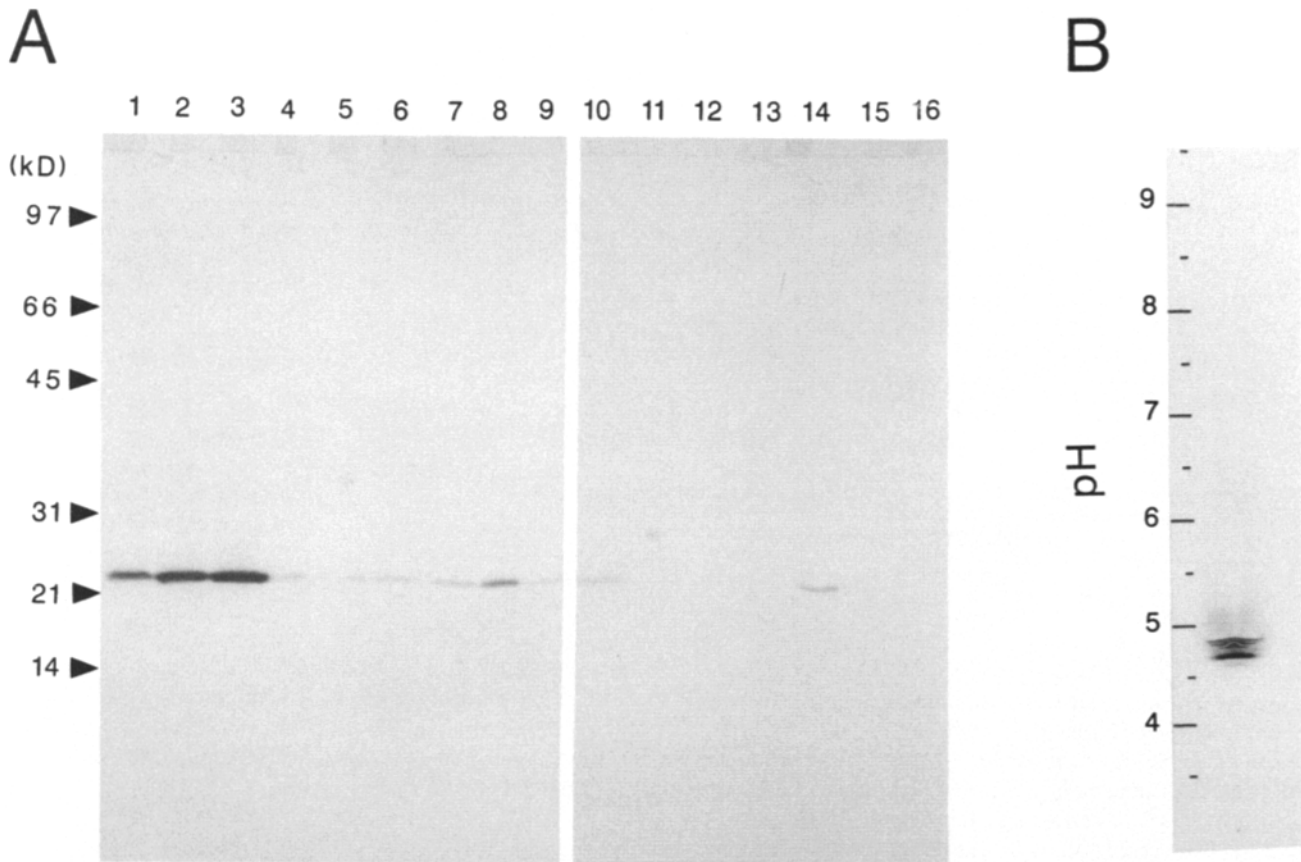
Olfactory epithelia and olfactory bulbs were isolated from adult rabbits and

homogenized in 10 vol of 0.32 M sucrose, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM EGTA, and 0.5 mM PMSF. The homogenate (2 g of protein) was centrifuged at 100,000 g for 1 h. The supernatant was applied to a DE 52 column (Whatman; 1.5 cm i.d. × 6 cm) equilibrated with 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 1 mM EGTA. Elution was performed with stepwise increment of NaCl concentration (0–2 M). R2D5 antigen was detected in the fraction eluted with 0.15 M NaCl by immunoblot analyses using mAb R2D5. The active fraction (50 ml) was then loaded on to a mAb R2D5-conjugated Sepharose 4B column (1.5 cm i.d. × 2.3 cm). The bound R2D5 antigen was eluted with 50 mM DEAE, pH 11.5, and immediately neutralized with 0.5 M NaH<sub>2</sub>PO<sub>4</sub>. The R2D5 antigen preparation thus obtained (20 μg of protein) was highly purified; the only observable band after protein staining was at 22 kD. The purified R2D5 antigen was digested with lysyl endopeptidase. The generated peptides were purified by reversed-phase HPLC and applied to an automated amino acid sequencer (Appl. Biosystems model 477A with an on-line 120A PTH analyzer) as described (Nemoto et al., 1992).

## Results

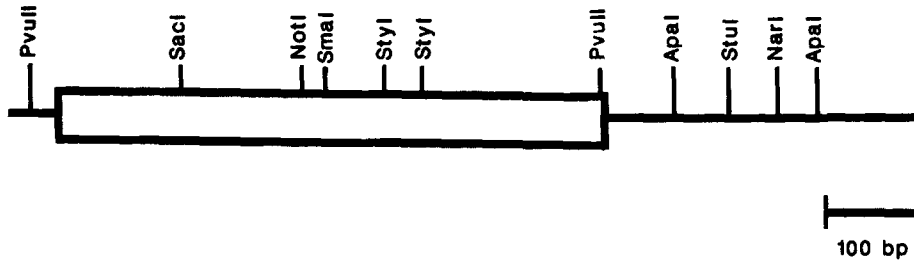
### Tissue Distribution of Rabbit R2D5 Antigen

To see the distribution of the R2D5 antigen, homogenates prepared from various rabbit tissues were subjected to immunoblot analysis with mAb R2D5 (Fig. 1 A). mAb R2D5



**Figure 1.** (A) Immunoblot analysis of the tissue distribution of the rabbit R2D5 antigen. The homogenates of various rabbit tissues (5 μg of protein for the olfactory epithelium and the olfactory bulb and 50 μg of protein for other tissues) were electrophoresed on a 10–20% (wt/vol) SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with mAb R2D5. (Lane 1) nasal respiratory epithelium; (lane 2) olfactory epithelium; (lane 3) olfactory bulb; (lane 4) cerebrum; (lane 5) cerebellum; (lane 6) brain stem; (lane 7) thyroid gland; (lane 8) lung; (lane 9) heart; (lane 10) stomach; (lane 11) liver; (lane 12) spleen; (lane 13) pancreas; (lane 14) kidney; (lane 15) intestine; (lane 16) skeletal muscle. Positions of protein molecular size markers are shown in kD at left. Note the difference in the amounts of applied protein and the abundance of the R2D5 antigen in the olfactory tissues. (B) Isoelectric focusing of the rabbit R2D5 antigen. The soluble fraction of the rabbit olfactory bulb was subjected to isoelectric focusing and transferred to a nitrocellulose membrane. The membrane was probed with mAb R2D5. Note that the R2D5 antigen is electrophoresed at apparent pI of 4.8.

**A**



**B**

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1          GGACTGCGGGACACACAGGCCAGCTGCCACAAAGCCCTGACCCGGC
50  ATG GAC ACT GCG GAC GCC ATT GTG GAG AAG CTC CGG GCA CAG TGC CTG GCC CGA GGG GCA   20
    Met Asp Thr Ala Asp Ala Ile Val Glu Lys Leu Arg Ala Gln Cys Leu Ala Arg Gly Ala
110  GCA GGC ATC CAG GGC CTG GCC AGG TTT TTC CGC CGC CTG GAC CGG GAT GGG AGC CGA TCC   40
    Ala Gly Ile Gln Gly Leu Ala Arg Phe Phe Arg Arg Leu Asp Arg Asp Gly Ser Arg Ser
170  CTG GAC GCT GGG GAG CTC CGG CAG GGC CTG GCC AAG CTG GGG CTG GCC CTG GAC GAG GCT   60
    Leu Asp Ala Gly Glu Leu Arg Gln Gly Leu Ala Lys Leu Gly Leu Ala Leu Asp Glu Ala
230  GAG GCC CAG AGT GTG TGC AGA CGC TGG GAC CGC AAC GGC AGC GGG AGC CTG GAC CTG GAG   80
    Glu Ala Gln Ser Val Cys Arg Arg Trp Asp Arg Asn Gly Ser Gly Thr Leu Asp Leu Asp
290  GAG TTC TTG CCG GCG CTG CCG CCG CCC ATG TCC CAG GCC CGG GAG GCA GTC ATT GCC GCC   100
    Glu Phe Leu Arg Ala Leu Arg Pro Pro Met Ser Gln Ala Arg Glu Ala Val Ile Ala Ala
350  GCC TTI GCC AAG CTG GAC CGC AGT GGG GAC GGC GTG GTG ACC TTG GGT GAC CTC CGC GGG   120
    Ala Phe Ala Lys Leu Asp Arg Ser Gly Asp Gly Val Val Thr Leu Gly Asp Leu Arg Gly
410  GTG TAC AGT GGC CGC AAC CAC CCC AAG GTG CGC ACG GGG GAG TGG ACA GAG GAC GAG GTG   140
    Val Tyr Ser Gly Arg Asn His Pro Lys Val Arg Thr Gly Glu Trp Thr Glu Asp Glu Val
470  CTC CGC CGC TTC CTG GAC AAC TTT GAC TCC TCG GAG AAG GAC GGG CAG GTC ACG CTG GCC   160
    Leu Arg Arg Phe Leu Asp Asn Phe Asp Ser Ser Glu Lys Asp Gly Gln Val Thr Leu Ala
530  GAG TTC CAG GAT TAC TAC AGC GGC CTG AGC GCG TCC GTG GAC ACA GAC GAG GAG TTC GTG   180
    Glu Phe Gln Asp Tyr Tyr Ser Gly Leu Ser Ala Ser Val Asp Thr Asp Glu Glu Phe Val
590  GCC ATG ATG AGC AGC GCC TGG CAG CTG TGA
    Ala Met Met Ser Ser Ala Trp Gln Leu *
620  GCAGCCGCTGGCTGGCTGTGCCAGCCAGGCCCTCCCGTCCCATGCCCTACTGGGCCAGGCAGGGCCCGACACG
699  GGGGTGGGGTGGGGTGGGGTGGCTGAGGCTGCAGAACCGCAGGACCAGGCCCTCCCTGCTGGGCCACTAGGGGAGCTGG
778  AACAGCACCCAGGGGAGGGGGCCCGCTGCGTGCCTCTGCCTGGAGCAGCCTGGGGGCCCTGGGGCACCCAGGAG
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936  GAAAAAAAAAAAAA
  
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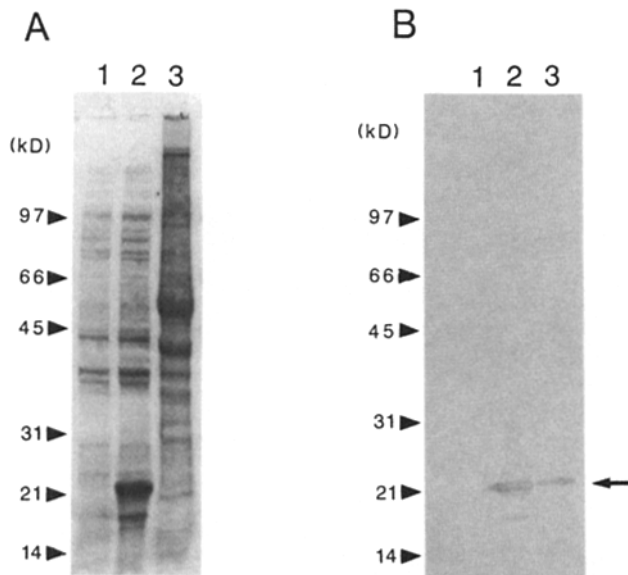
**Figure 2.** (A) Restriction map and schematic representation of the rabbit R2D5 antigen cDNA. Restriction enzyme recognition sites and the open reading frame (indicated by a horizontal open bar) in the longest of the isolated cDNA inserts are shown. (B) Nucleotide and the deduced amino acid sequence of the rabbit R2D5 antigen cDNA. Nucleotide sequence of the rabbit R2D5 antigen cDNA is numbered on the left side. An in-frame termination codon preceding the initiator codon and the polyadenylation signal are indicated by an *overline* and a *double underline*, respectively. The deduced amino acid sequence of the coding region is shown below the nucleotide sequence and numbered on the right side, beginning with the initiator methionine. Three calcium-binding loops in the R2D5 antigen are *underlined*. Partial amino acid sequences of the purified R2D5 antigen are indicated by *dashed-underlines*. These sequence data are available from EMBL/Genbank/DBJ under accession number X72874.

specifically recognized a protein with an apparent  $M_r$  of 22,000, which was heavily concentrated in the olfactory epithelium and the olfactory bulb. Weaker but significant immunoreactive bands of the same size were observed in the homogenates of the nasal respiratory epithelium, lung, and kidney. Much fainter bands were also detected in the homogenates of the cerebrum, cerebellum, brain stem, thyroid gland, heart, and stomach. No immunoreactivity was detected in the liver, spleen, pancreas, intestine, and skeletal muscle. In a separate subcellular fractionation experiment, the R2D5 antigen was recovered in the soluble fraction after centrifugation at 100,000  $g$  for 1 h, indicating that the R2D5 antigen is a cytosolic protein (data not shown). In addition, we analyzed the soluble fraction of the olfactory bulb by isoelectric focusing and immunoblotting with mAb R2D5. The result indicated that the R2D5 antigen is an acidic pro-

tein with apparent pI of 4.8 (Fig. 1 B). No change was observed in the mobility of the R2D5 antigen upon SDS-PAGE under the reducing or nonreducing conditions, suggesting that there is no inter- or intradisulfide bond in the R2D5 antigen (data not shown).

#### Isolation of cDNA Encoding Rabbit R2D5 Antigen

To determine the primary structure of the rabbit R2D5 antigen, we screened with mAb R2D5 a rabbit olfactory epithelium cDNA library constructed in the expression vector  $\lambda$ gt11. Out of  $6.0 \times 10^5$  independent clones we found four positives. Restriction mapping and DNA sequencing of the cDNA inserts revealed that all these cDNAs were derived from the same mRNA species and harbored an open reading frame which can encode a polypeptide of 189 amino acid



**Figure 3.** Expression of the rabbit R2D5 antigen in *E. coli*. The lysates prepared from IPTG-induced cultures of *E. coli* BL21(DE3)-pLysS transformed with pET-3Ta vector alone (lane 1), or with expression plasmid pET-3a/R2D5Ag (lane 2), and the homogenate of the rabbit olfactory bulb (lane 3) were electrophoresed on an SDS-polyacrylamide gel and analyzed by Coomassie staining (A), or immunoblotting with mAb R2D5 (B). The position of the R2D5 antigen is marked by an *arrow* at right. The lower immunoreactive bands were degradation products of the R2D5 antigen in the preparation.

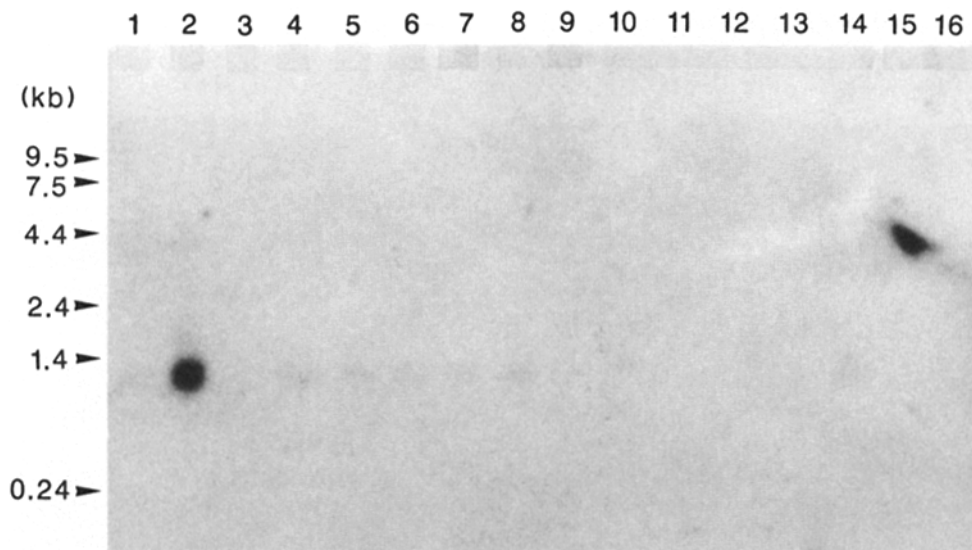
residues with calculated  $M_r$  of 20,864 and pI of 4.74 (Fig. 2, A and B). These values are in close agreement with those obtained by the prior analysis of the R2D5 antigen. Furthermore all the peptide sequences obtained from the purified R2D5 antigen were found in the deduced amino acid sequence of the isolated cDNA (indicated by *dashed-under-*

*lines* in Fig. 2 B). The nucleotide sequence surrounding the initiator codon for the open reading frame matches well with the functional initiation consensus proposed by Kozak (1987, 1989). An in-frame termination codon TGA located just 6-nucleotide upstream of the initiator ATG triplet (*overlined* in Fig. 2 B). While no canonical polyadenylation signal AATAAA was found in the 3' untranslated region, the sequence CATAAA (*doubly underlined* in Fig. 2 B) may possibly function as such (Proudfoot and Brownlee, 1976; Wickens and Stephenson, 1984).

To prove that the isolated cDNA represents the R2D5 antigen mRNA, we constructed a plasmid pET3-a/R2D5Ag that can dictate the synthesis of the open reading frame-product in *E. coli*. The expression plasmid was introduced into host strain BL21(DE3)pLysS, and the protein synthesis was induced with isopropyl- $\beta$ -D-thio-galactopyranoside (IPTG). After gel electrophoresis, the lysate of the *E. coli* was either stained with Coomassie blue or immunoblotted with mAb R2D5. As shown in Fig. 3 A, a prominent protein band appeared at 22 kD in the lysate of the *E. coli* transformed with pET3-a/R2D5Ag. This protein was immunoreactive with mAb R2D5 and comigrated with the rabbit R2D5 antigen, while no immunoreactivity was observed in the lysate of the *E. coli* transformed with the vector pET-3a alone (Fig. 3 B). This result directly demonstrates that the isolated cDNA encodes the rabbit R2D5 antigen with the primary structure deduced from the open reading frame.

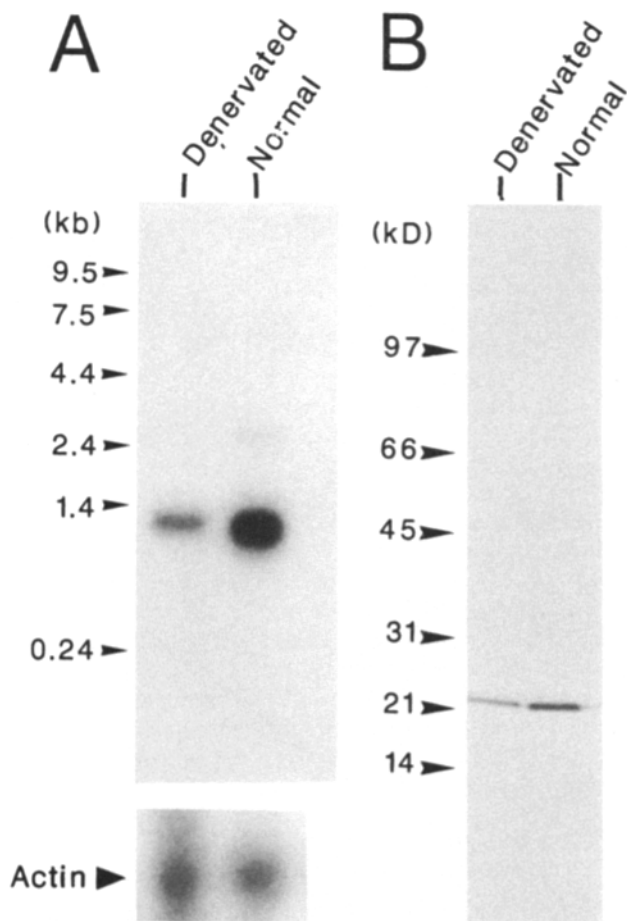
#### Northern Blot Analysis of R2D5 Antigen mRNA Expression

To analyze the tissue distribution of the R2D5 antigen mRNA, we performed Northern blot experiments. As shown in Fig. 4, the radiolabeled cDNA probe hybridized to a single mRNA species of 1.1 kb which is expressed most abundantly in the olfactory epithelium. Much weaker signals of the same size were detected in RNAs of the nasal respiratory epithelium, cerebrum, cerebellum, brain stem, lung, thyroid



**Figure 4.** Northern blot analysis of the tissue distribution of the rabbit R2D5 antigen mRNA. Total RNA (10  $\mu$ g) from various rabbit tissues was resolved on a 1.2% agarose gel and transferred to a nylon filter. The filter was hybridized with a rabbit R2D5 antigen cDNA probe. (Lane 1) nasal respiratory epithelium; (lane 2) olfactory epithelium; (lane 3) olfactory bulb; (lane 4) cerebrum; (lane 5) cerebellum; (lane 6) brain stem; (lane 7) thyroid gland; (lane 8) lung; (lane 9) heart; (lane 10) stomach; (lane 11) liver; (lane 12) spleen; (lane 13) pancreas; (lane 14) kidney; (lane 15) intestine; (lane 16) skeletal

muscle. Positions of RNA size markers are shown in kb at left. Equal amounts and quality of RNA were loaded in each lane as judged by the amounts of 28S and 18S ribosomal RNAs (data not shown). Note that the R2D5 antigen mRNA (1.1 kb) is predominantly expressed in the olfactory epithelium and virtually absent in the olfactory bulb.



**Figure 5.** Effect of bulbectomy on the expression of the rabbit R2D5 antigen in the olfactory epithelium. Unilateral olfactory bulbectomy was performed on an adult rabbit. The olfactory epithelium was taken from the normal and denervated sides after 7 d and the expression of the R2D5 antigen was examined. (A) Northern blotting. Total RNA (10 µg) from each side was hybridized with the R2D5 antigen cDNA probe (top panel). The blot was rehybridized with a  $\beta$ -actin probe to ascertain that equal amounts of RNA were placed in each lane (bottom panel). (B) Immunoblotting. The homogenate (5 µg protein) from each side was probed with mAb R2D5.

gland, heart, stomach, and kidney. This pattern of expression is largely consistent with that of the rabbit R2D5 antigen as probed by immunoblot analysis in Fig. 1 A, providing an additional support for the authenticity of the isolated cDNA.

Our previous immunohistochemical study has shown that R2D5 immunoreactivity in the rabbit olfactory bulb mostly originates from the axons of olfactory receptor neurons (Mori et al., 1985). This observation is corroborated by the Northern analysis. No R2D5 antigen mRNA expression was detected in the olfactory bulb, while much amount of R2D5 antigen was contained in the homogenate of the olfactory bulb (see Figs. 1 A and 4).

#### **Effect of Bulbectomy on R2D5 Antigen Expression**

The removal of the olfactory bulb causes the degeneration of olfactory receptor neurons after 6–8 d, leaving other types of cells in the olfactory epithelium such as sustentacular

cells and basal cells largely intact (Monti Graziadei and Graziadei, 1979). We used this neuronal depletion technique to ascertain whether the receptor neurons express the R2D5 antigen mRNA. As shown in Fig. 5, the level of the R2D5 antigen mRNA declined significantly (A), with concomitant decrease of the R2D5 antigen (B), in the bulbectomized side of the olfactory epithelium. The results indicate the neuronal origin of the R2D5 antigen in the olfactory epithelium.

#### **Ca<sup>2+</sup>-binding Properties of R2D5 Antigen**

Analysis of the primary structure of the R2D5 antigen has revealed significant similarities between the R2D5 antigen and most of the calmodulin-related calcium-binding proteins with the 'EF hand,' which is a Ca<sup>2+</sup>-binding motif composed of helix-loop-helix structure with 29 amino acids (Kretsinger, 1980). In particular, the R2D5 antigen shares extensive amino acid identity (86%) with calcyphosine, a protein of unknown function in the dog thyroid (Lecocq et al., 1979; Lamy et al., 1986; Lefort et al., 1989) (Fig. 6). We tested by two methods whether the R2D5 antigen actually binds Ca<sup>2+</sup>.

The first method employs Ca<sup>2+</sup>-dependent hydrophobic chromatography on phenyl-Sepharose. Several calcium-binding proteins including calmodulin, troponin C, and S100 proteins exhibit Ca<sup>2+</sup>-dependent binding to hydrophobic matrices and EGTA-dependent elution (Gopalakrishna and Anderson, 1982; Marshak et al., 1981; Endo et al., 1981; Walsh et al., 1984). For these proteins, particularly for calmodulin, it has been well established that the binding of Ca<sup>2+</sup> leads to a conformational change that expose a hydrophobic site, which in turn is capable of interacting with appropriate receptor sites on other proteins as well as certain amphipathic molecules (Strynadka and James, 1989; O'Neil and DeGrado, 1990). The lysate of the *E. coli* carrying the expression plasmid for the R2D5 antigen and induced with IPTG was applied to a phenyl-Sepharose column. As shown in Fig. 7, the R2D5 antigen was adsorbed to the column in the presence of Ca<sup>2+</sup>, while most proteins in the column load passed straight through the column. The bound R2D5 antigen was not released by high salt wash, but specifically eluted with EGTA. The column chromatography provided evidence for the Ca<sup>2+</sup>-binding nature of the R2D5 antigen as well as a reliable means to obtain highly homogeneous preparation of the R2D5 antigen whose purity exceeds 95% as judged by gel electrophoresis and Coomassie staining.

In the next experiment, the binding of Ca<sup>2+</sup> to the R2D5 antigen was directly probed by <sup>45</sup>Ca<sup>2+</sup> blotting. The R2D5 antigen purified by the above chromatography was electrophoresed on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with <sup>45</sup>Ca<sup>2+</sup>. The R2D5 antigen bound Ca<sup>2+</sup>, as presented in Fig. 8.

#### **Phosphorylation of R2D5 Antigen by CaM Kinase II and A Kinase**

The primary structure analysis of the R2D5 antigen revealed the presence of possible phosphorylation sites for CaM kinase II, namely Ser<sup>38</sup> and Ser<sup>74</sup>. In addition, calcyphosine, which shows the highest degree of homology with the R2D5 antigen, was reported to be phosphorylated in response to the elevation of intracellular cAMP level in the dog thyroid presumably at Ser<sup>40</sup> (Lecocq et al., 1979; Lamy et al.,

			En**nn**nO*O*OG*nO**On**nn**n	
Calmodulin		1	ADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSL	39
R2D5 Antigen	1		MDTADAIVEKLRQAQCLARGAAGIQGLARFFRRLDRDGSRSLDAGELRQGLAKL	53
Calcyphosine	1		MDAVDATVEKLRQAQCLSRGALGIQGLARFFRRLDRDRSRLDSRELQRLAEL	53
			En**nn**nO*O*OG*nO**On**nn**n	
Calmodulin		40	GQNPTEAELQDMINEVDADGNGITDFPEFLTMMARK	75
R2D5 Antigen		54	GLALDEAEAQSVCRWRDRNGSGTLDLDEFLLRALRPP	89
Calcyphosine		54	GLVLDTAAEAGVCRWRDRDGSGLDLEEFLLRALRPP	89
			En**nn**nO*O*OG*nO**On**nn**n	
Calmodulin		76	MKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNL	112
R2D5 Antigen		90	M-SQAREAVIAAAFAKLDKRSRSGDGVVTLGDLRGVYSGR	125
Calcyphosine		90	M-SQAREAVIAAAFAKLDKRSRSGDGVVTVDDLRGVYSGR	125
Calmodulin		113	GEKLTDEEV--DEMIREADIDGDGQVNY-----EEFVQMMT-AK	148
R2D5 Antigen	126		NHPKVRTGE-WTEDEVLRRLDNFDSSEKDGQVTLAEFQDYSGLSASVDTDEEFVAMMSSAWQL	189
Calcyphosine	126		THPKVQSGE-WTEEEVLRRLDNFDSSEKDGQVTLAEFQDYSGVSSASMDTDEEFVAMMSSAWQL	189

**Figure 6.** Homology of the rabbit R2D5 antigen with calcium-binding proteins. The deduced amino acid sequence of the rabbit R2D5 antigen (*middle row*) is aligned with those of canine calcyphosine (*lower row*; taken from Lefort et al., 1989) and bovine calmodulin (*upper row*; taken from Watterson et al., 1980) in one-letter code. EF-hand motif is marked above that in the R2D5 antigen: E, glutamate; n, nonpolar residue; \*, any residue; O, Ca<sup>2+</sup>-chelating residue containing oxygen in the side chain. The *lines* and *dots* represent the identical and homologous amino acids, respectively.

1986; Lefort et al., 1989). Thus the R2D5 antigen might also serve as a substrate for A kinase, although difference in a preceding amino acid (Gly<sup>37</sup> of the R2D5 antigen vs Arg<sup>37</sup> of calcyphosine) renders Ser<sup>40</sup> of the R2D5 antigen in the less favorable context for phosphorylation by A kinase. We therefore examined whether the R2D5 antigen can be phosphorylated by these kinases using the purified recombinant protein. As shown in Fig. 9 A, the R2D5 antigen was phosphorylated by CaM kinase II in the presence of Ca<sup>2+</sup>. The R2D5 antigen was also phosphorylated by A kinase, but in this case, irrespective of the presence of Ca<sup>2+</sup> (Fig. 9 B). We further analyzed the time courses for the phosphorylation reactions (Fig. 9 C). The kinetics of phosphorylation of the R2D5 antigen by A kinase was not affected by Ca<sup>2+</sup> under the experimental condition and 0.80 mol of Pi was maximally incorporated per mol of the R2D5 antigen, while 1.81 mol of Pi was maximally incorporated per mol of the R2D5 antigen in the CaM kinase II-catalyzed reaction. Fig. 9 C also shows that phosphorylation of the R2D5 antigen by these protein kinases is additive, indicating the sites in the R2D5 antigen modified by the two kinases are different.

#### Expression of R2D5 Antigen in Ependymal Cells of Rabbit Central Nervous System

Our previous work has shown that R2D5 antigen is expressed in ependymal cells in addition to olfactory receptor neurons (Mori et al., 1985). To examine the detailed distribution of cells that express the R2D5 antigen in the central nervous system, we performed an immunohistochemical study on a series of sections using mAb R2D5. As exemplified in Fig. 10, mAb R2D5 labeled virtually all types of

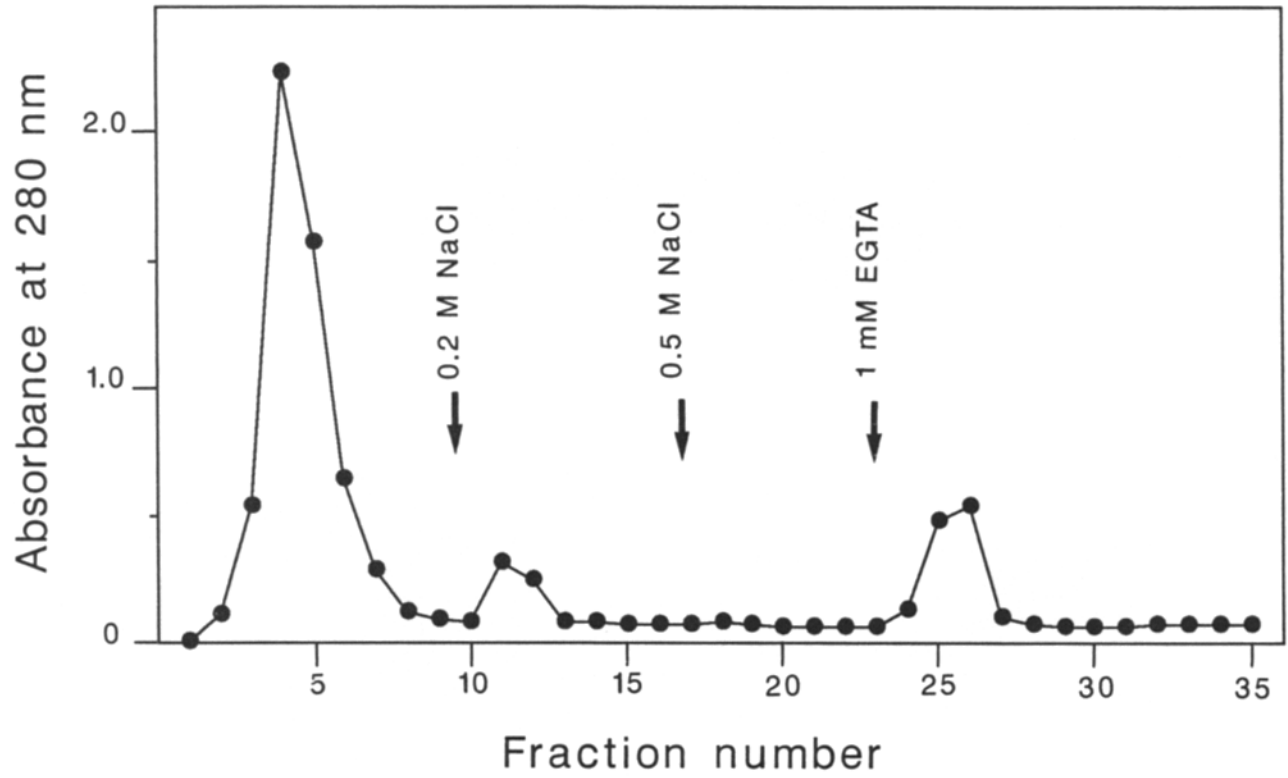
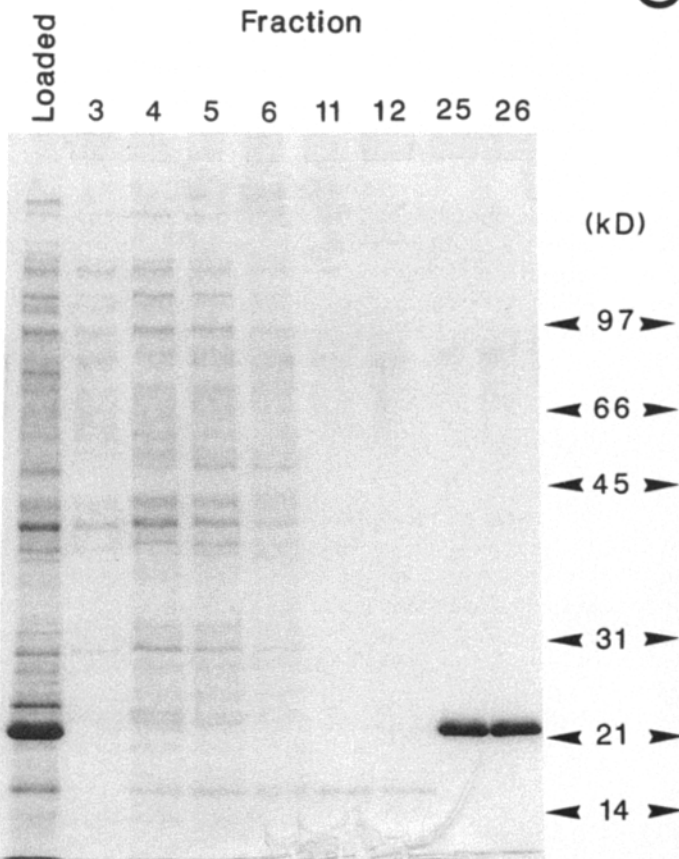
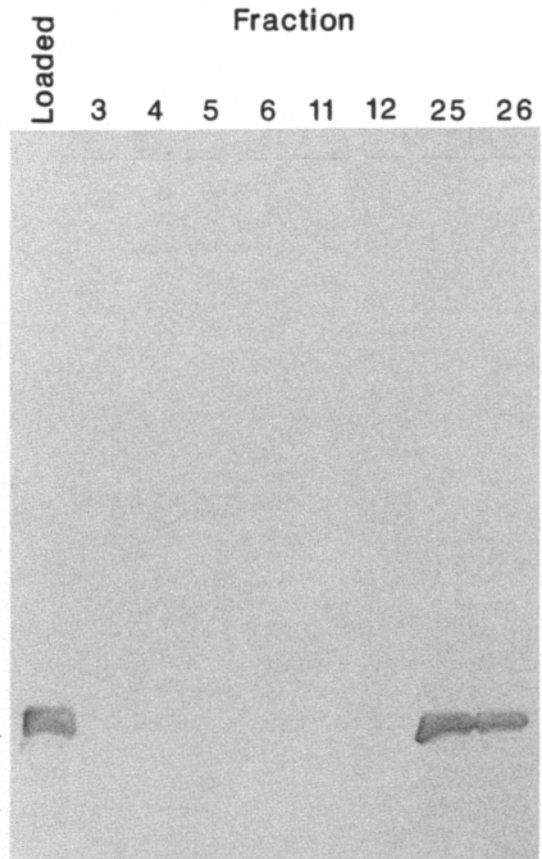
ventricular ependymal cells as well as olfactory receptor neurons (A). The R2D5-positive cells include common ependymal cells lining the ventricles, epithelial cells of the choroid plexus (B), ependymal cells in the organum vasculosum laminae terminalis located at the most rostral part of the third ventricle (C), tanycytes at the third ventricle with their processes distributing in the hypothalamus (D), ependymal cells in the subcommissural organ (E), and ependymal cells and their processes near the area postrema at the most rostral part of the central canal of the spinal cord (F).

We prepared the homogenates of ependymal cell-enriched fraction from the ventricular surface of the forebrain and the choroid plexus, and examined the immunoreactivity by immunoblotting with mAb R2D5. mAb R2D5 recognized a protein band which comigrates with the R2D5 antigen in the olfactory tissue (data not shown). This result suggests that the same R2D5 antigen is expressed both in olfactory receptor neurons and in ependymal cells.

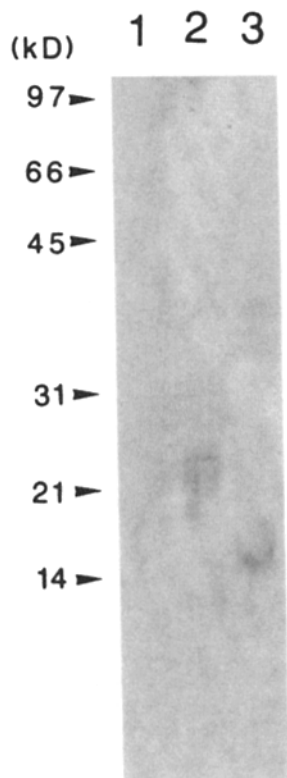
#### Discussion

##### R2D5 Antigen Is an EF-hand Calcium-binding Protein

The present study showed that the rabbit R2D5 antigen belongs to a superfamily of EF-hand calcium-binding proteins. Besides ubiquitous calmodulin (Biffo et al., 1991; Borisy et al., 1992), this is the first to show the presence of the calcium-binding protein in olfactory receptor neurons. EF-hand calcium-binding proteins are considered to be broadly classified into two functional subgroups, the 'trigger' and the 'buffer' proteins (Levine and Dalgarno, 1983). The trigger

**A****B****C**





**Figure 8.**  $^{45}\text{Ca}^{2+}$  blotting. The recombinant R2D5 antigen (2  $\mu\text{g}$ ) (lane 2), the lysate of *E. coli* without the expression plasmid (50  $\mu\text{g}$ ) (lane 1), and bovine calmodulin (2  $\mu\text{g}$ ) (lane 3) were subjected to gel electrophoresis, transferred to a nitrocellulose membrane, and probed with  $^{45}\text{Ca}^{2+}$ . The positions of the R2D5 antigen and calmodulin are marked by an open and a closed arrow, respectively, at right.

proteins, such as calmodulin, troponin C, or myosin light chains, change their conformation upon  $\text{Ca}^{2+}$  binding, and in turn modulate the activity of various enzymes and ion channels. On the other hand the buffer proteins, such as parvalbumin in muscle or calbindin in gut enterocytes, are believed to make up a more passive system, limiting a stimulated rise in intracellular  $\text{Ca}^{2+}$  concentration. The overall structural similarity of the R2D5 antigen to calmodulin and the biochemical data indicating a conformational change of the R2D5 antigen upon  $\text{Ca}^{2+}$  binding, strongly suggest that the R2D5 antigen is a trigger calcium-binding protein and bears some regulatory role(s).

#### ***R2D5 Antigen Is a Phosphoprotein Closely Related to Canine Thyroid Calcyphosine***

The R2D5 antigen has possible phosphorylation sites by CaM kinase II and A kinase, and our results demonstrated that the R2D5 antigen can be phosphorylated by these protein kinases *in vitro* (Fig. 9).

As shown in Fig. 6, the rabbit R2D5 antigen is closely related to calcyphosine, a protein found in the dog thyroid by Dumont and his colleagues (Lecocq et al., 1979; Lamy

et al., 1986; Lefort et al., 1989). Calcyphosine is shown to be phosphorylated in the dog thyroid in response to an increase in the intracellular cAMP concentration. In olfactory receptor neurons, a marked rise of the cAMP level is evoked by odor stimulation, which presumably results in the activation of A kinase and phosphorylation of the R2D5 antigen. Notably, the existence of characteristic proteins which undergo cAMP-dependent phosphorylation in frog olfactory cilia has been described and the most prominent phosphoprotein (p24) has a molecular size similar to the R2D5 antigen (Heldman and Lancet, 1986).

It is evident from their extensive amino acid identity (86%) that the rabbit R2D5 antigen and the canine thyroid calcyphosine have a common ancestor in evolution. However, the precise relationship between the two proteins is unclear. The R2D5 antigen may be the rabbit version of calcyphosine. Alternatively a single species may express multiple R2D5 antigen/calcyphosine-related proteins, each with specific tissue distribution. To address this issue, we performed Southern blot analyses of rabbit genomic DNA with the R2D5 antigen cDNA as a probe under a low stringent condition which should allow detection of the related genes. Only single bands were detected in the EcoRI, PstI, and XhoI digests (data not shown), suggesting that the R2D5 antigen/calcyphosine is encoded by the same gene. In addition, we designed a set of oligonucleotides based on the sequences conserved between rabbit R2D5 antigen and canine calcyphosine, and carried out PCR analyses using cDNA from various rabbit tissues, including the thyroid gland, as template. DNA sequencing revealed that the same product was amplified (data not shown). These results suggest that the R2D5 antigen is a rabbit homologue of the canine calcyphosine.

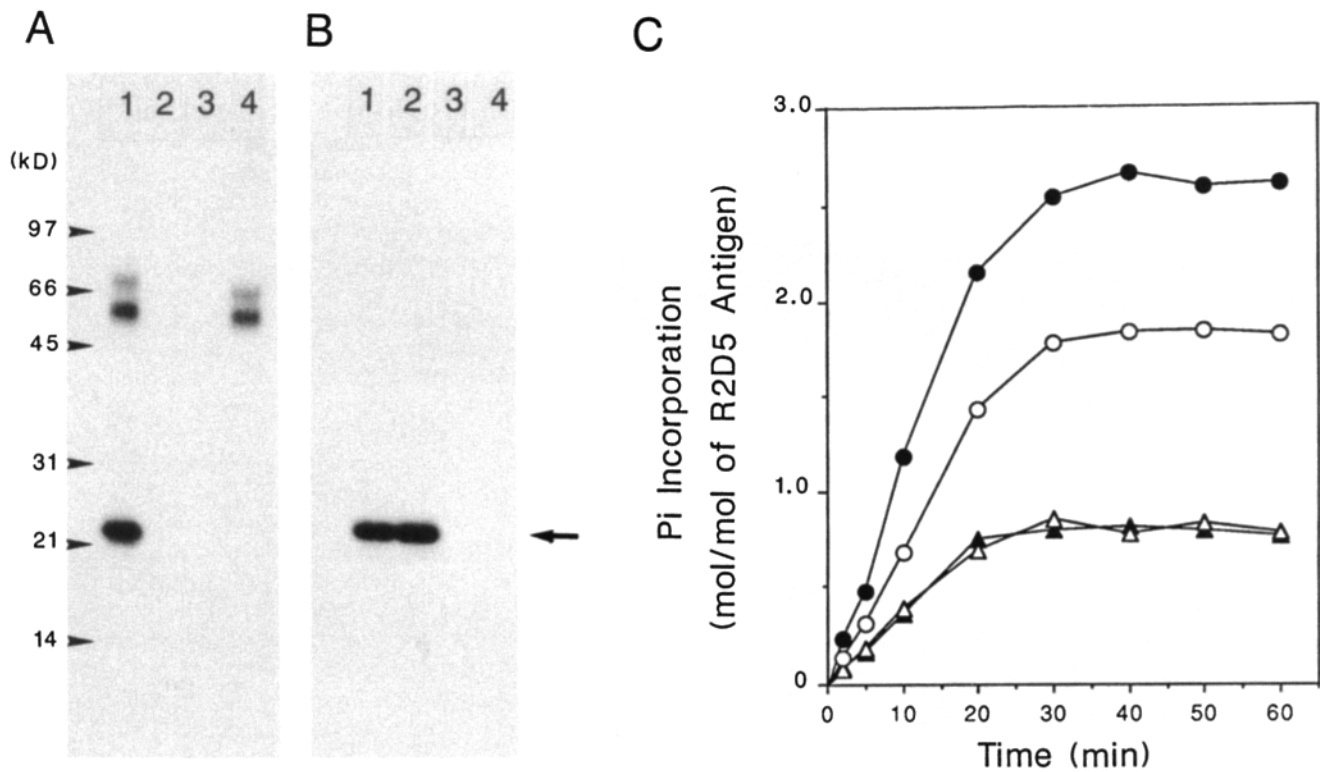
#### ***Possible Biological Functions of R2D5 Antigen in Olfactory Receptor Neurons; Its Involvement in Adaptive Processes***

Although the physiological function of the R2D5 antigen remains unknown, its biochemical properties and unique localization invite several speculations.

Several members of the EF-hand calcium-binding proteins (e.g., calbindin-D28K, calretinin, parvalbumin, visinin, and recoverin) are expressed in a cell-type specific manner within the nervous system (Celio, 1990; Braun, 1990; Jande et al., 1981; Jacobowitz and Winsky, 1991; Arai et al., 1991; Résiibois and Rogers, 1992; Yamagata et al., 1990; Dizhoor et al., 1991). These proteins are believed to be involved in  $\text{Ca}^{2+}$ -mediated processes within the cells and contribute to the neuronal type-specific characters (for reviews see Heizmann and Hunziker, 1991; Baimbridge et al., 1992).

Photoreceptor cells in the visual system and olfactory receptor neurons in the olfactory system show striking

**Figure 7.**  $\text{Ca}^{2+}$ -dependent hydrophobic chromatography on phenyl-Sepharose. (A) Protein elution profile.  $\text{CaCl}_2$  was added to the lysate of *E. coli* introduced with pET-3a/R2D5Ag, and the mixture was applied to a phenyl-Sepharose column equilibrated with a buffer containing 0.1 mM  $\text{CaCl}_2$ . After washing with the same buffer, the column was washed with a buffer containing 0.1 mM  $\text{CaCl}_2$  and 0.2 M NaCl (indicated by the first arrow), and then with a buffer containing 0.1 mM  $\text{CaCl}_2$  and 0.5 M NaCl (indicated by the second arrow). Finally the column was eluted with a buffer containing 1 mM EGTA (indicated by the third arrow). (B and C) The loaded sample and the indicated fractions were subjected to SDS-PAGE and stained with Coomassie blue (B) or probed with mAb R2D5 (C). Note that the R2D5 antigen was adsorbed to phenyl-Sepharose in the presence of  $\text{Ca}^{2+}$  and specifically eluted with EGTA.



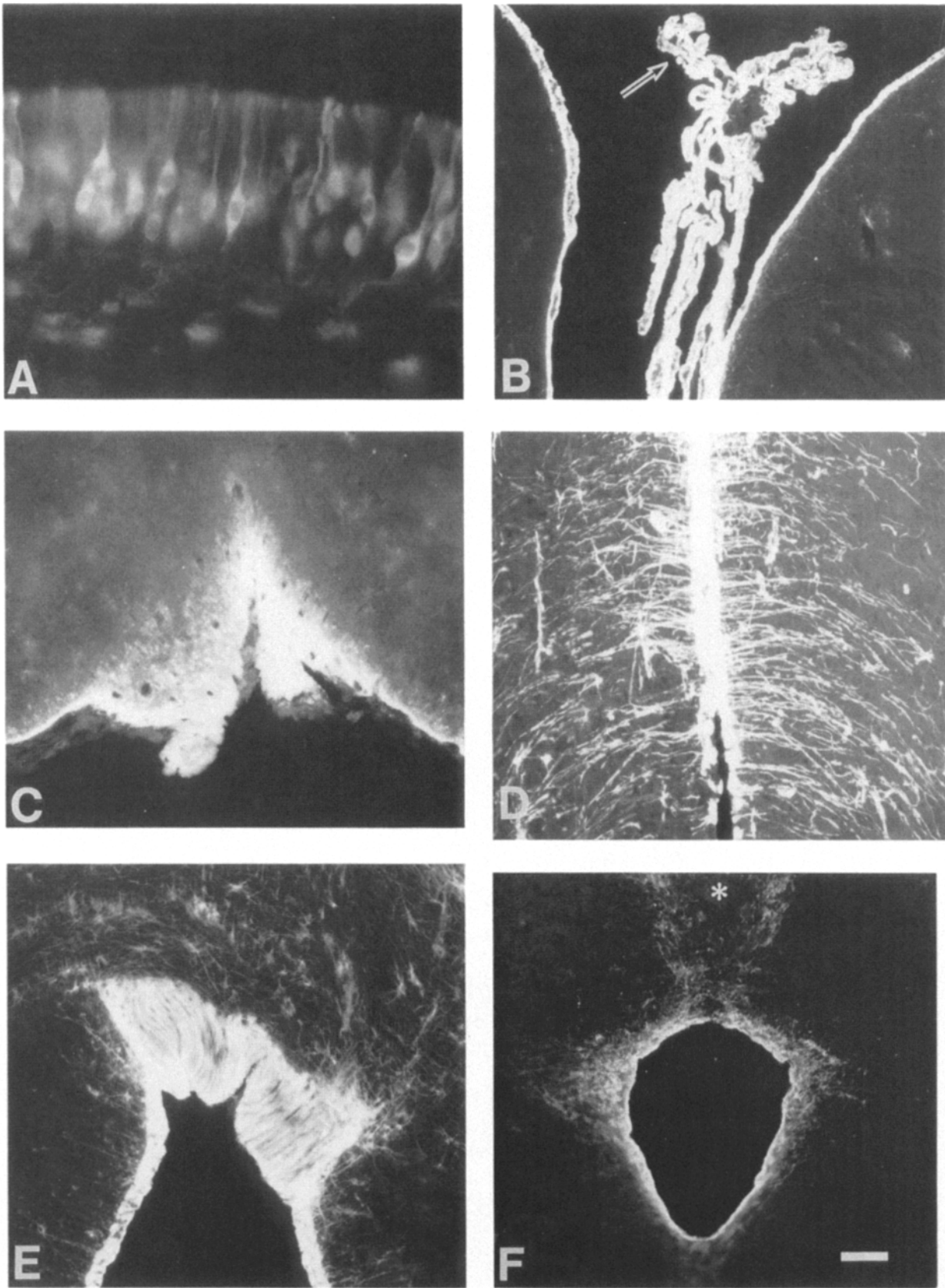
**Figure 9.** Phosphorylation of the recombinant R2D5 antigen by CaM kinase II and A kinase. (A and B) The rabbit R2D5 antigen was expressed in *E. coli* and purified by phenyl-Sepharose chromatography. The purified protein was incubated with 30  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP and CaM kinase II (A) or A kinase (B), at 30°C for 10 min and subjected to SDS-gel electrophoresis and autoradiography. In both panels, (lane 1) complete mixture with Ca<sup>2+</sup>; (lane 2) without Ca<sup>2+</sup>; (lane 3) without the protein kinase; (lane 4) without the R2D5 antigen. The position of the R2D5 antigen is marked by an arrow at right. The upper radioactive bands in A were due to CaM kinase II autophosphorylation. (C) Time courses of phosphorylation. The purified recombinant R2D5 antigen was phosphorylated with CaM kinase II (○), with A kinase in the presence (▲) or in the absence (△) of Ca<sup>2+</sup>, or with both of the protein kinases (●), in 0.5 mM [ $\gamma$ -<sup>32</sup>P]ATP at 30°C. Aliquots were taken at the indicated times, and radioactivity incorporated into the R2D5 antigen was determined by liquid scintillation counting after gel electrophoresis.

similarities in the molecular machineries for signal transduction (Stryer, 1991). In photoreceptor cells, Ca<sup>2+</sup> is a component of central importance in the adaptation processes (Hodgkin and Nunn, 1988; Koch and Stryer, 1988; Matthews et al., 1988; Nakatani and Yau, 1988), which is mediated through several calcium-binding proteins. Recoverin is a Ca<sup>2+</sup>-dependent modulator of guanylate cyclase activity in rod photoreceptor cells (Dizhoor et al., 1991; Lambrecht and Koch, 1991). Visinin seems to play a similar role in avian cone photoreceptor cells (Yamagata et al., 1990). In addition, Ca<sup>2+</sup> regulates activity of cyclic GMP phosphodiesterase via a recoverin/visinin-related protein (S-modulin) in frog retinal rod cells (Kawamura and Murakami, 1991; Kawamura et al., 1992). Ca<sup>2+</sup> also modulates the affinity of cyclic GMP-gated cation channel toward cyclic GMP via a protein phosphatase with EF hands (Steele et al., 1992; Gordon et al., 1992) and via calmodulin (Hsu and Molday, 1993). Thus Ca<sup>2+</sup> acts at several sites within the phototransduction cascade to attain the fine-tuning or the adaptation of light response.

By analogy, Ca<sup>2+</sup> is anticipated to play an important role in the olfactory adaptive processes possibly via calcium-binding proteins. Indeed it has been shown that inactivation of membrane conductance of olfactory receptor neurons upon prolonged odor exposure is dependent on Ca<sup>2+</sup> influx

(Kurahashi, 1989; Kurahashi and Shibuya, 1990; Zufall et al., 1991), and the desensitization is caused by reduction of cAMP sensitivity of cyclic nucleotide-gated cation channel, which is mediated through a putative calcium-binding protein distinct from calmodulin (Kurahashi, 1990; Kramer and Siegelbaum, 1992). In addition, the odor-evoked increase in intracellular cAMP is followed by a rapid decline of the cAMP level for which cAMP-dependent protein phosphorylation is in part responsible (Breer et al., 1990; Boekhoff et al., 1990; Firestein et al., 1991; Boekhoff and Breer, 1992). It is tempting to postulate the R2D5 antigen to be a mediator of one or both of these processes (Fig. 11), because this molecule is at the same time a calcium-binding protein and a substrate for A kinase. The R2D5 antigen might also affect the activity of other components of olfactory transduction such as odor receptor proteins, G<sub>olf</sub> adenylyl cyclase or phosphodiesterase. With the R2D5 antigen expression system available, each of these possibilities can be tested.

Recent studies have shown that several odor stimuli elicit IP<sub>3</sub>-mediated Ca<sup>2+</sup> influx through plasma membrane IP<sub>3</sub>-gated Ca<sup>2+</sup> channel in olfactory receptor neurons (Breer et al., 1990; Boekhoff et al., 1990; Restrepo et al., 1990; Fadool and Ache, 1992; Raming et al., 1993). Opening of cyclic nucleotide-gated cation channel by cAMP also causes Ca<sup>2+</sup> influx. Thus there should exist coordinated control or



**Figure 10.** Localization of R2D5 antigen in olfactory receptor neurons and ependymal cells in the rabbit central nervous system. Coronal sections through the olfactory epithelium of a newborn rabbit (A) and ventricles of adult rabbits (B–F) were stained with mAb R2D5 followed by fluorescein-conjugated secondary antibody. Note the immunoreactivity in epithelial cells of the choroid plexus (indicated by an arrow in B) and the common ependymal cells lining the lateral ventricle (B), ependymal cells associated with the organum vasculosum laminae terminalis at the most rostral part of the third ventricle (C), tanycytes in the wall of the third ventricle projecting the basal processes into the hypothalamus (D), ependymal cells associated with subcommissural organ in the wall of the cerebral aqueduct (E), and ependymal cells lining the most rostral part of the central canal of the spinal cord at the level of the area postrema (\*) (F). Bars: (A) 25  $\mu\text{m}$ ; (D and E) 100  $\mu\text{m}$ ; (B, C, and F) 250  $\mu\text{m}$ .

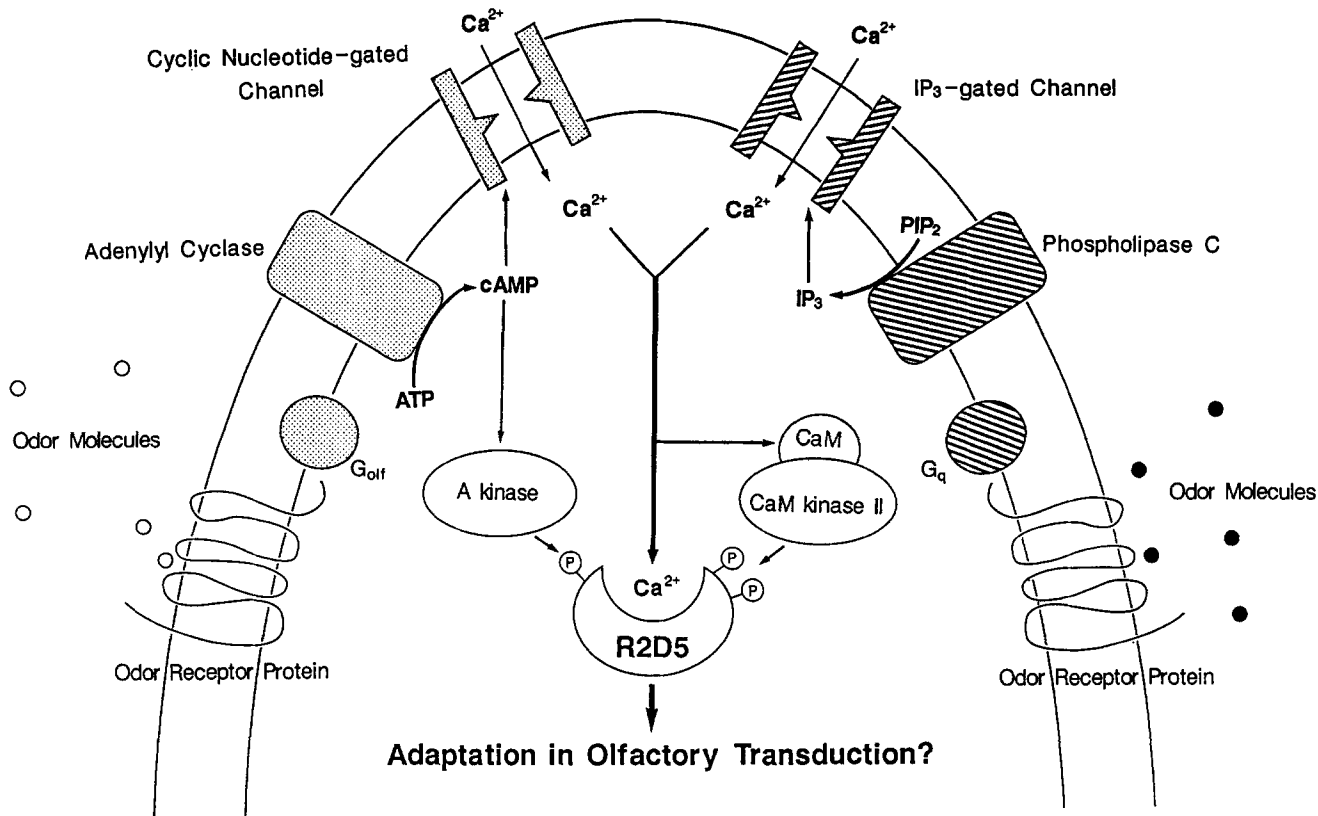


Figure 11. A schematic diagram of possible interactions between the R2D5 antigen and second messenger systems in olfactory receptor neurons. See text for further explanation.

cross-talk of cAMP-mediated and  $IP_3/Ca^{2+}$ -mediated pathways in olfactory receptor neurons. The R2D5 antigen and its phosphorylation by CaM kinase II and A kinase may be involved in this control. Further research will clarify the functional role of the R2D5 antigen in the interplay of the two signaling pathways in olfactory transduction.

Our previous and present results have shown that in the central nervous system the R2D5 antigen is highly enriched in a variety of ependymal cells including common ependymal cells, the choroid epithelial cells, specialized process-bearing ependymal cells called tanycytes, and those associated with circumventricular organs (Hofer, 1958; Weindl, 1973). It would be of interest to note the notion that ependymal cells might be more than protective and subservise a sensory function; ependymal cells might detect chemical substances in the cerebrospinal fluid and somehow transmit the signals to the neighboring neurons (for reviews see Rodríguez, 1976; Bruni et al., 1985). However, there are at present few data that ependymal cells are equipped with components required to perform such function. The presence of the R2D5 antigen in ependymal cells most likely means that the biochemical pathway mediated by this protein is shared by ependymal cells and olfactory receptor neurons. Therefore the identification of the molecule(s) interacting with the R2D5 antigen may eventually lead to elucidation of a novel physiological role of ependymal cells, as well as the functional role of the R2D5 antigen in olfactory receptor neurons.

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