Identification of a Group of Novel Membrane Proteins Unique to Chemosensory Cilia of Olfactory Receptor Cells†

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ABSTRACT: We have used a library of monoclonal antibodies (mAbs) against chemosensory cilia of the olfactory epithelium of *Rana catesbeiana* to identify proteins that are unique to the ciliary membrane. Five different antibodies (mAb 8, 26, 34, 42/45, and 43) identify novel proteins in olfactory cilia that are not detected in olfactory nerve membranes, nonchemosensory cilia from respiratory epithelium, or membranes from brain, heart, liver, kidney, and lung. Deglycosylation of olfactory cilia with endoglycosidase H shows that most of these antibodies (mAb 8, 42/45, 43, and possibly 26) react with antigenic determinants comprised partially or entirely of carbohydrate, while only one (mAb 34) recognizes an 87-kDa protein that is resistant to endoglycosidase H treatment. Furthermore, a 59-kDa glycoprotein visualized by mAb 8 exists as membrane-associated oligomers connected via intermolecular disulfide bonds. These proteins, tagged with distinct high-mannose-containing carbohydrate moieties and found only in chemosensory cilia of olfactory receptor cells, may be involved in odorant recognition and/or olfactory transduction.

Olfactory reception occurs after airborne odorants enter the nasal cavity and partition in the mucus where they interact with chemosensory cilia that protrude from the dendritic tips of olfactory receptor cells (Lancet, 1986; Anholt, 1987, 1989; Getchell & Getchell, 1987). The molecular pathways that mediate odorant recognition and signal transduction at the chemosensory membrane followed by excitation of the appropriate olfactory receptor cells remain to be elucidated. Studies on partially purified preparations of isolated olfactory cilia from frog and rat olfactory epithelium revealed a high activity of adenylate cyclase (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986), GTP-binding proteins (Pace et al., 1985; Anholt et al., 1987; Pace & Lancet, 1986; Jones & Reed, 1987, 1989), and several classes of ion channels including cation-selective channels that are gated directly by nanomolar concentrations of odorants (Labarca et al., 1988). The olfactory adenylate cyclase is stimulated by micromolar to millimolar concentrations of some, primarily hydrophobic, odorants (Pace, 1985; Sklar et al., 1986; Shirley et al., 1986; Anholt, 1987). The physiological significance of this stimulation and the precise function of cyclic AMP in olfaction remain to be established. However, patch-clamp studies on isolated olfactory receptor cells revealed cyclic AMP activated currents in the cilia, the dendritic knob, and soma, indicating that cyclic AMP profoundly affects the excitable properties of the olfactory receptor cell (Nakamura & Gold, 1987).

Despite these important advances, the molecular nature of odorant receptors, odorant-gated channels, and cyclic AMP activated channels remains unknown. Polyacrylamide gel electrophoresis of olfactory cilia revealed a prominent, diffuse 95-kDa wheat germ agglutinin (WGA)[1]-binding region, which led to the hypothesis that this region may harbor a family of odorant receptor proteins (Chen & Lancet, 1984; Lancet, 1986; Chen et al., 1986a). Evidence to support this hypothesis is, however, still lacking.

Monoclonal antibodies (mAbs) are powerful probes for the identification, purification, and characterization of proteins unique to olfactory tissue. A mAb against the 95-kDa WGA binding protein of olfactory cilia has been described (Chen et al., 1986b). In addition, several laboratories have generated mAbs that recognize different components of the olfactory neuroepithelium, including glands, sustentacular cells, and olfactory neurons (Hempstead & Morgan, 1985; Morgan, 1988). Another study described a mAb obtained after immunization of Balb/c mice with rat pheochromocytoma cells that reacts in the central nervous system only with the glomerular and olfactory nerve layers of the olfactory bulb (Allen & Akeson, 1985). This antibody recognizes a family of surface glycoproteins which in the olfactory system are present only on olfactory receptor neurons. Furthermore, an antibody generated against a homogenate of olfactory bulb differentiates subclasses of olfactory receptor cells, labeling only those olfactory axons that project to the lateral, but not those that project to the medial, portion of the olfactory bulb (Fujita et al., 1985).

Here, we report the generation of a library of mAbs against chemosensory dendritic cilia isolated from the olfactory epithelium of *Rana catesbeiana*. Antibodies were identified that react with proteins found only in olfactory cilia and not in membranes from olfactory nerve, respiratory cilia, brain, heart, lung, liver, or kidney. Further characterization of the antigenic determinants for these mAbs demonstrates the occurrence of unique carbohydrate structures on membrane proteins of olfactory cilia. Most of these mAbs recognize carbohydrate moieties, and one recognizes an 87-kDa protein. Furthermore, one of the glycoproteins identified exists as disulfide-linked oligomers. Since the proteins identified by these mAbs are detected only on the dendritic cilia of olfactory receptor cells, they are likely to be involved in odorant recognition and/or olfactory transduction.

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[1] Abbreviations: mAb, monoclonal antibody; WGA, wheat germ agglutinin; Con A, concanavalin A; Ringer's solution, 2 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid], pH 7.4, 112 mM NaCl, 3.4 mM KCl, and 2.4 mM $NaHCO_3$; SDS, sodium dodecyl sulfate.

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Materials and Methods

Preparation of Olfactory Cilia and Membranes from Frog Tissues. Bullfrogs (*Rana catesbeiana*) were obtained from Acadian Biological (Rayne, LA), Lemberger Co. (Oshkosh, WI), and Amphibians of North America (Nashville, TN). Frogs were killed by decapitation, and the ventral and dorsal olfactory epithelia were dissected. Olfactory cilia were detached from the epithelia by a calcium shock and subjected to sucrose gradient centrifugation as described previously (Anholt et al., 1986). Respiratory cilia were obtained from the palate of the frog following a similar procedure (Anholt et al., 1986). To prepare olfactory nerve membranes that contain the axons of olfactory receptor cells, stretches of olfactory nerve between the olfactory epithelium and the cribriform plate were dissected and homogenized in Ringer's solution (2 mM HEPES, pH 7.4, 112 mM NaCl, 3.4 mM KCl, and 2.4 mM $NaHCO_3$) with a glass homogenizer. The homogenate was filtered through a double layer of surgical gauze and centrifuged for 20 min at 6500*g* at 4 °C. The pellet was resuspended in Ringer's solution and centrifuged once again as before. Membranes from brain, liver, heart, kidney, and lung were prepared in the same way except that homogenization was performed in a Polytron for 2 × 30 s on ice. All membranes were suspended in Ringer's solution, and the concentration of protein was measured by the method of Lowry et al. (1951). The membrane suspensions were stored at −80 °C.

Production of Monoclonal Antibodies. Five–six-week-old female Balb/c mice, obtained from Charles River Laboratories, Inc. (Wilmington, MA), were injected subcutaneously behind the scapulae with 200 μg of ciliary protein emulsified with an equal volume of incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Some mice were immunized with ciliary proteins prepared from 200 μg of a Triton X-100 extract of olfactory cilia by adsorption to Con A or WGA conjugated to Sepharose C14B (Vector Laboratories, Burlingame, CA), followed by elution with 0.25 M methyl α-D-mannoside or *N*-acetylglucoseamine, respectively, in 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100. The mice received two similar booster injections with olfactory cilia, Con A binding proteins or WGA-binding proteins at 2-week intervals. Four days after the last injection, the spleens were removed, and the spleen cells were fused to P3×653.Ag8 myeloma cells by standard procedures using poly(ethylene glycol) (PEG 1500, Boehringer Mannheim, Indianapolis, IN). The cells were plated in microtiter wells in high glucose and L-glutamine-containing Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY), supplemented with 20% horse serum (PelFreeze Biologicals, Rogers, AK); 0.1 mM nonessential amino acids (GIBCO); 1 mM pyruvate (GIBCO); 0.1 mM hypoxanthine, 4.0×10^{-7} M aminopterin, and 2×10^{-5} M thymidine, all from Hazleton Biologics, Inc. (Lenexa, KS); and 100 units/mL pennicilin and 100 μg/mL streptomycin (GIBCO). Cells were grown at 37 °C in a humidified atmosphere containing 8% CO_2. Antibody-producing wells were selected by a solid-phase enzyme-linked immunosorbent assay in 96-well Immulon II microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) coated with 1 μg of ciliary protein per well. To coat the plates, ciliary membranes were resuspended in 0.1 M sodium bicarbonate buffer, pH 9.6, at a protein concentration of 20 μg/mL, and 50-μL aliquots were placed in the wells. The plates were incubated for 16 h at 37 °C, washed extensively with 10 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, and 0.05% Tween 20, and blocked with 10 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 10 mM NaN_3, 0.05% Tween 20, and 0.2% bovine serum albumin for 30 min at 37 °C. The wells were washed as before and incubated with hybridoma supernatants followed by incubation with a mixture of biotinylated horse anti-mouse IgG and goat anti-mouse IgM and an avidin-biotinylated horseradish peroxidase system (Vector Laboratories) using 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (Sigma Chemical Co.) as the chromogenic substrate. Hybridomas from positive wells were collected and subcloned twice in soft agarose (SeaPrep, FMC BioProducts, Rockland, ME) to ensure stability and monoclonality. To collect mAbs, either cells were grown to high density and supernatants were collected or the cells were grown as ascites in 2,6,10,14-tetramethylpentadecane (pristane; Aldrich Chemical Co., Milwaukee, WI) primed female Balb/c mice. Supernatants or ascites fluids were subjected to precipitation with 45% ammonium sulfate, followed by extensive dialysis of the resuspended precipitates against 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, and 10 mM NaN_3. Aliquots of the antibody solutions were stored at −80 °C.

Immunoblotting. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed on 10% slab gels in the discontinuous buffer system of Laemmli (1970). Electrophoretic transfer onto nitrocellulose membranes was performed as described previously (Anholt et al., 1987). All electrophoresis chemicals were purchased from Bio-Rad Laboratories (Richmond, CA). Samples were heated for 3 min at 90 °C with 2% (w/v) SDS and 10% (v/v) 2-mercaptoethanol, unless indicated otherwise, and 200 μg of protein was applied to a 6-cm-wide well. After electrophoresis and electrophoretic transfer, the nitrocellulose membrane was incubated for 30 min at 4 °C in 50 mM Tris·HCl, pH 7.7, 1 mM ethylenediaminetetraacetic acid, 0.1% (w/v) gelatin, and 0.1% Triton X-100. Subsequently, 5-mm-wide strips were cut and incubated with the desired mAbs at the indicated dilutions in 10 mM sodium phosphate buffer, 150 mM NaCl, and 0.05% (v/v) Tween 20 (Aldrich Chemical Co.) for 2 h at 4 °C. Bound antibodies were visualized with biotinylated secondary antibody complexed to avidin and biotinylated horseradish peroxidase (Vector Laboratories) using 3,3′-diaminobenzidine (Sigma Chemical Co.) as the chromogenic substrate. Soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (42.7 kDa), bovine serum albumin (66.2 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116.25 kDa), and myosin (200 kDa), all from Bio-Rad, were used as molecular weight standards and visualized with Amido Black 10B. Biotinylated molecular weight markers (Bio-Rad), including all of the above except β-galactosidase and myosin, were sometimes used instead and visualized with avidin and biotinylated horseradish peroxidase.

Deglycosylation of Olfactory Cilia. To remove N-linked oligosaccharides from ciliary membranes, 200 μg of ciliary protein was treated at 37 °C for the indicated incubation times with 30 units/L endoglycosidase H (recombinant endoglycosidase H without detectable exoglycosidase or protease activities and with a specific activity of approximately 25 units/mg of protein; Boehringer Mannheim) in 10 mM sodium phosphate/citrate buffer, pH 5.5, 75 mM NaCl, 0.05% SDS, and 5 mM NaN_3. The reactions were stopped by the addition of an equal volume of 10 mM Tris·HCl, pH 7.0, 6% SDS, 28.5% 2-mercaptoethanol, and 20% glycerol followed by heating for 3 min at 90 °C. Reactivity of antibodies with the deglycosylated cilia was analyzed by Western blotting after polyacrylamide gel electrophoresis in SDS using 8 μg of ciliary protein per lane.

Sucrose Gradient Centrifugation. To analyze different molecular forms of the 59-kDa glycoprotein identified by mAb 8, samples were subjected to sucrose gradient centrifugation. Ciliary membrane proteins (300 μg of protein/gradient) were solubilized by incubation with 1% Triton X-100 in Ringer's solution for 10 min at 4 °C followed by centrifugation in a microcentrifuge for 15 min at 4 °C at 16600*g*. Extracts were incubated in the presence or absence of 0.14 M (1% v/v) 2-mercaptoethanol for 15 min at 37 °C, and 100 μL of each sample was layered on 680 μL of a linear gradient of 6–15% (w/w) sucrose in Ringer's solution supplemented with 0.5% Triton X-100. The gradients were prepared in 0.8-mL Beckman Ultra-Clear centrifuge tubes (Beckman Instruments, Inc., Fullerton, CA) by sequentially layering sucrose solutions of decreasing density and allowing the gradients to diffuse for 2 h at 4 °C. The gradients were centrifuged for 16 h at 4 °C at 40000 rpm in appropriate adaptors in a Beckman SW50.1 rotor. Fractions of 55 μL were collected after puncturing the bottom of the tube. Samples (40 μL) of the fractions were subjected to polyacrylamide gel electrophoresis in SDS and electrophoretic transfer onto nitrocellulose membranes, as described above, followed by staining of the membranes with a 10-fold dilution of an ammonium sulfate fractionated hybridoma supernatant containing mAb 8. Sucrose concentrations in the fractions were measured with a refractometer.

Results

Frog olfactory cilia are highly immunogenic in mice and elicit high-titer antiserum. Several prominent immunoreactive regions are evident on Western blots at high dilutions of mouse antiserum, revealing areas of diffuse staining along with several sharply defined bands (Figure 1). Most prominent staining occurs in a region between 53 and 62 kDa, in the 100–150-kDa region, and in a region just below the top of the gel (>200 kDa). When bound antibody is visualized with biotinylated secondary antibodies, nonspecifically staining bands are observed at 74 and 78 kDa, as previously described (Anholt et al., 1986), along with a band at about 160 kDa. At the same dilution of antiserum, few bands are seen when respiratory cilia are applied to the gel at the same amount of ciliary protein. Compared to olfactory cilia, respiratory cilia contain little membrane glycoprotein (Chen & Lancet, 1984; Anholt et al., 1986) and few intramembranous particles as detected by freeze–fracture electron microscopy studies (Menco, 1980). More of the protein content of respiratory cilia than of olfactory cilia appears to be represented by cytoskeletal proteins (Chen & Lancet, 1984; Anholt et al., 1986). Only one band at 69 kDa reacts prominently with mouse antiserum raised against frog olfactory cilia in both olfactory and respiratory cilia (Figure 1). Thus, mice immunized with frog olfactory cilia generate high-titer antiserum primarily directed against ciliary membrane proteins.

Of 36 antibody-secreting hybridomas initially generated, 19 produce mAbs that react with proteins immobilized on nitrocellulose (Table I). About 50% of mAbs generated were reactive with immunoblots, independent of whether immunizations were performed with olfactory cilia, Con A binding proteins, or WGA-binding proteins. Failure of the remaining 17 mAbs to react on Western blots can be attributed to low binding affinity and/or denaturation of conformationally dependent determinants after treatment with SDS. We focused on the characterization of only those antibodies that react with immunoblots. Reactivity of these antibodies with olfactory cilia, olfactory nerve membranes, respiratory cilia, and brain membranes is shown in Figure 2 (left and middle panels). Three patterns of reactivity are evident: (1) antibodies

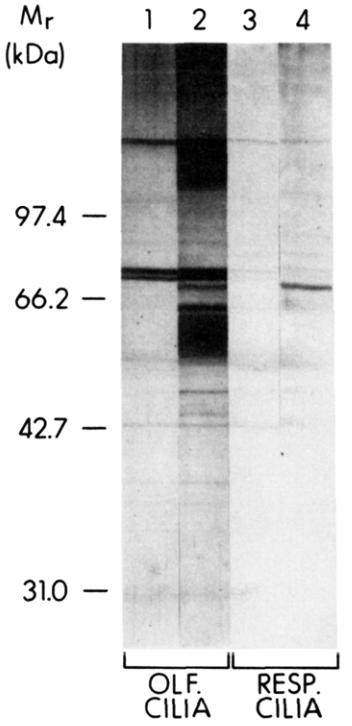

FIGURE 1: Reactivity of mouse antiserum with cilia from olfactory and respiratory epithelium. Olfactory cilia (lanes 1 and 2) and respiratory cilia (lanes 3 and 4) were subjected to polyacrylamide gel electrophoresis in SDS followed by immunoblotting, as described under Materials and Methods, and incubated with 10000-fold-diluted normal mouse serum (lanes 1 and 3) or antiserum pooled from five mice immunized with frog olfactory cilia (lanes 2 and 4). Incubation with the primary antiserum was for 16 h at 4 °C. Each lane contains 40 μg of protein.

that visualize one distinct band (e.g., mAb 34; Figure 2, left panel, A); (2) antibodies that visualize multiple distinct bands, most likely reflecting proteolysis and/or reactivity with carbohydrate (e.g., mAbs 6, 10, and 18; Figure 2); and (3) antibodies that stain one or more diffuse bands, suggestive of reactivity with carbohydrate (e.g., mAbs 2, 20, and 26; Table I, Figure 2). Six antibodies (~17% of all mAbs) displayed specificity toward olfactory cilia, specifically mAbs 8, 26, 34, 42, 43, and 45 (Figure 2). These mAbs were obtained after immunization of mice with olfactory cilia, except mAb 26 which resulted from immunization with WGA-binding proteins. mAbs 42 and 45 show identical staining patterns, and we have, therefore, characterized only mAb 42. mAb 8 visualizes mainly a 59 ± 2 (n = 7) kDa band. Patterns of immunoreactivity observed with this mAb depend on the extent of reduction of the samples prior to electrophoresis, as described in detail below. mAb 26 visualizes several broad bands in olfactory cilia with prominent staining in regions between 56–68 and 108–142 kDa. In olfactory nerve membranes, it stains a more restricted diffuse region within the 56–68-kDa region. The higher molecular weight region is not detected in olfactory nerve membranes, and no immunoreactivity is observed with this antibody in respiratory cilia and brain membranes. mAb 34 visualizes a distinct band at 87 ± 4 (n = 7) kDa in olfactory cilia. mAb 42 reveals two major molecular weight regions of immunoreactive bands, a region between 60K and 66K and a high molecular weight region with intense staining between 120K and 140K along with several bands at molecular weights above 200K. This antibody stains in olfactory nerve membranes one band at about 250 kDa, but does not react with respiratory cilia or brain membranes. Finally, mAb 43 identifies in olfactory cilia diffuse regions of staining at molecular weights from 150K to well above 200K

Table I: Monoclonal Antibodies against Olfactory Cilia

mAb	antigen	reactivity with Western blots	category	olfactory cilia	olfactory nerve	respiratory cilia	brain	peripheral tissues[c]	sensitivity to endoglycosidase H
1	O								
2	O	+	3	+	+	–	+	ND[e]	+
3	C	+	2	+	+	+	+	ND	ND
4	C	+	2	+	+	+	+	ND	ND
5	C	–							
6	C	+	2	+	+	+	+	+	+
8	O	+	1	+	–	–	–	–	+
10	C	+	2	+	+	+	+	ND	+
11	C	–							
12	C	–							
13	O	–							
14	O	+	2	+	+	+	+	ND	ND
15	O	–							
18	C	+	2	+	+	+	+	ND	+
19	C	–							
20	W	+	3	+	+	+	+	+	ND
21	W	+	2	+	+	+	+	+	ND
22	W	–							
23	W	–							
24	O								
26	W	+	3	+	+[d]	–	–	–	+
27	W	–							
28	O	+	2	+	+	+	+	ND	ND
30	O	–							
31	O	–							
32	O	–							
34	O	+	1	+	–	–	–	–	–
35	O	–							
37	O	+	2	+	+	+	+	+	+
38	O	–							
40	O	–							
42	O	+	3	+	+[d]	–	–	–	+
43	O	+	3	+	–	–	–	–	+
45	O	+	3	+	–	–	–	ND	ND
47	O	+	2	+	+	+	+	ND	ND
49	O	+	2	+	+	+	+	ND	+

[a] O, olfactory cilia; C, Con A binding proteins; W, WGA-binding proteins. [b] Categories: (1) stains one distinct band; (2) stains multiple distinct bands; (3) stains one or more diffuse bands. [c] Peripheral tissues, liver, lung, heart, and kidney. [d] Stains fewer or different proteins in olfactory nerve membranes than in olfactory cilia. [e] ND, not determined. Determination of isotypes of mAbs reactive with Western blots using isotype-specific rabbit-anti-mouse antisera (Bio-Rad) show that all mAbs tested belong to the IgM subclass, except mAb8, which is IgG_1. Isotypes for mAbs 14, 18, 20, 21, 26, and 47 were not determined. mAbs 61 and 82 raised against endoglycosidase H treated cilia that are not included in the table, but are mentioned in the text, are IgM and IgG_1, respectively. All light chains are of the κ type. Isotype frequencies in mouse antisera collected at the time of fusion were as follows: IgA, 1%; IgG_1, 29 ± 2%; IgG_{2A}, 7 ± 4%; IgG_{2B}, 19 ± 3%; IgG_3, 8 ± 2%; IgM, 36 ± 7%; κ chains, 78 ± 6%; λ chains, 22 ± 6%; $n = 5$.

near the top of the resolving gel. No immunoreactivity of mAbs 8, 34, and 43 is detected in olfactory nerve membranes, respiratory cilia, and brain membranes (Table I, Figure 2, left and middle panels).

To further assess whether mAbs 8, 26, 34, 42, and 43 are directed against proteins unique to olfactory receptor cells, we investigated the reactivity of these antibodies with membranes from several peripheral tissues of the frog, including kidney, lung, liver, and heart (Figure 2, right panel). None of these five antibodies showed reactivity with any of these tissues, indicating that under the conditions used they react only with olfactory tissue.

To assess whether the mAbs are directed against carbohydrate or protein determinants, we removed N-linked oligosaccharides by incubation of olfactory cilia with endoglycosidase H. Of 12 antibodies tested, 11 appeared to recognize a determinant that is composed entirely or largely of carbohydrate, since immunoreactivity is lost after deglycosylation (Figure 3). Thus, carbohydrate determinants dominate the antigenicity of membrane proteins of olfactory cilia. The two upper left panels in Figure 3 show that binding of Con A and WGA is abolished after deglycosylation except to a region of 59–66 kDa. It is possible that binding in this region occurs to endoglycosidase H resistant lectin-binding sites or that the binding is nonspecific. We favor the latter explanation, since binding of Con A and WGA to this region cannot be blocked by high concentrations of the sugars methyl α-D-mannoside and *N*-acetylglucosamine, respectively (data not shown). It is of interest to note that many antibodies that react with multiple well-defined bands (e.g., mAbs 6, 10, and 18) are also sensitive to deglycosylation. This reflects either the occurrence of specific carbohydrate configurations on different proteins or the proteolysis of one or few proteins carrying an antigenic carbohydrate determinant. Disappearance of antigenic sites after treatment with endoglycosidase H is not due to proteolysis during the incubation time because the epitopes remain intact when cilia are incubated under the same conditions in the absence of the enzyme or in the presence of neuraminidase or α-mannosidase (data not shown). Of the mAbs that uniquely react with proteins of olfactory cilia, only one, mAb 34, recognizes its epitope after treatment with endoglycosidase H (Figure 3). Immunoreactivity of mAbs 8, 42, and 43 is abolished by deglycosylation. The deglycosylation pattern of the protein recognized by mAb 26 is particularly intriguing. After incubation for 2 h with endoglycosidase H, staining in the 108–142-kDa region disappears concomitant with the appearance of a prominent diffuse band in the 34–43-kDa region which is resistant to further endoglycosidase H treatment. This may reflect either binding of mAb 26 to similar protein structures displaying heterogeneity, which appear at a lower molecular weight after removal of the carbohydrate, or binding to endoglycosidase

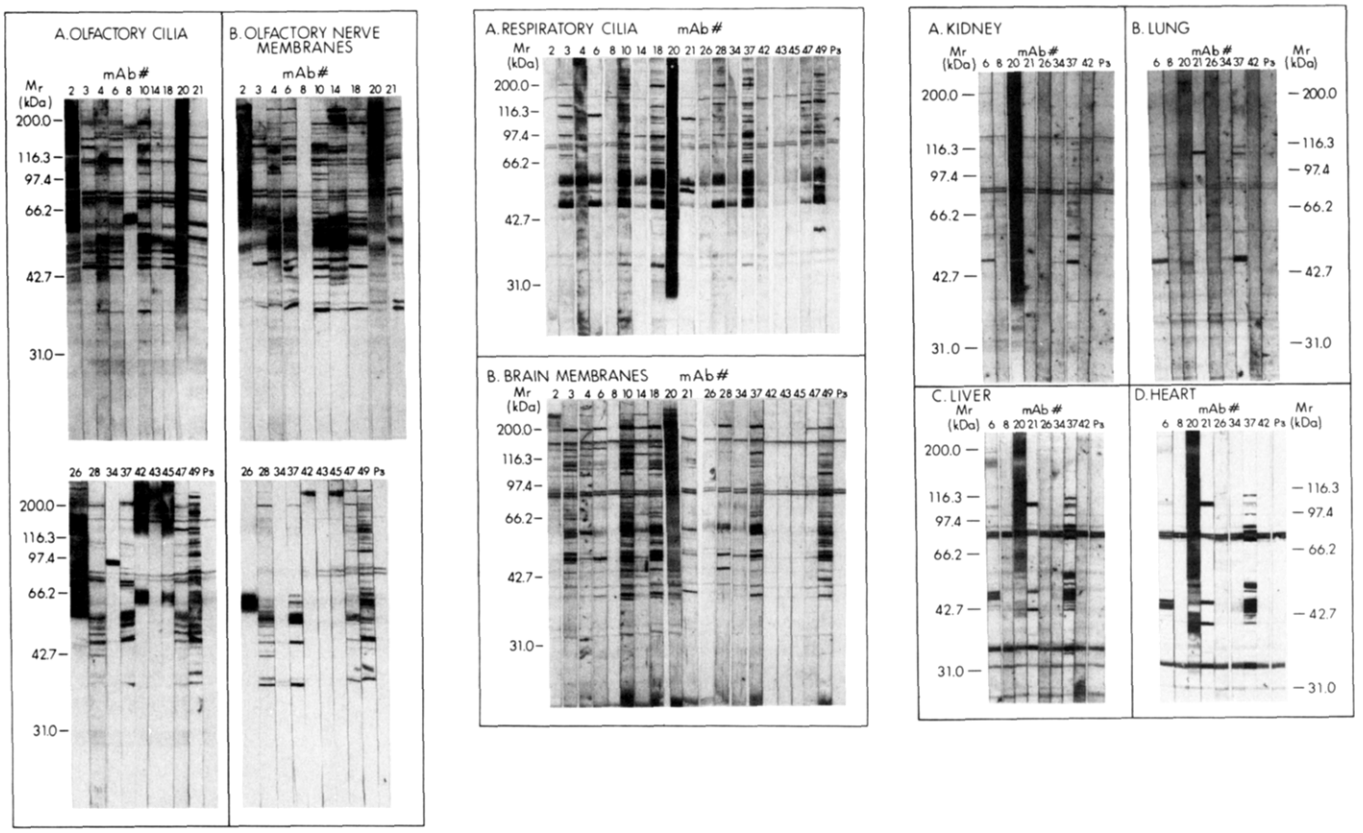

FIGURE 2: Immunoreactivity of monoclonal antibodies against chemosensory cilia of olfactory receptor cells. (Left panel) Reactivity of mAbs with olfactory cilia and olfactory nerve membranes. Olfactory cilia or olfactory nerve membranes were subjected to polyacrylamide gel electrophoresis in SDS followed by electrophoretic transfer onto nitrocellulose membranes, as described under Materials and Methods. The membranes were cut in strips which were stained with ammonium sulfate fractionated hybridoma supernatants containing the indicated mAbs. The antibody-containing stock solutions were present at 25-fold dilution for all mAbs, except mAbs 2, 20, 42, 43, 45, and 49, which were used at 250-fold dilution. mAb 26 was applied as a 1000-fold dilution of ammonium sulfate fractionated ascites fluid. The strip marked P3 was incubated with a 25-fold dilution of ammonium sulfate fractionated supernatant of P3×653.Ag8 myeloma cells to assess nonspecific binding. Note the staining of mAbs 8, 26, 34, 42, 43, and 45. (Middle panel) Reactivity of mAbs with respiratory cilia and membranes from frog brain. The conditions were the same as in the left panel. Note the absence of specific staining for mAbs 8, 26, 34, 42, 43, and 45. (Right panel) Reactivity of selected mAbs with membranes from frog kidney, lung, liver, and heart. The conditions were the same as in the left panel. Note the absence of specific staining for mAbs 8, 26, 34, and 42.

H resistant oligosaccharide moieties that remain after most of the carbohydrate determinants have been digested and that are not recognized by either Con A or WGA. The broadness of the band leads us to favor the latter possibility.

We have produced 11 mAbs by immunizing mice with cilia treated with endoglycosidase H. Seven of these reacted on Western blots, and two (mAbs 61 and 82) reacted exclusively with olfactory cilia. Both of these mAbs showed patterns of immunoreactivity before and after endoglycosidase H treatment that are identical with those observed with mAb 26 (data not shown).

The epitope recognized by mAb 8 appeared to be especially interesting. When electrophoresis followed by immunoblotting with mAb 8 was performed without prior reduction with 2-mercaptoethanol, the usually observed 59-kDa band (Figures 2 and 3) was absent, but immunoreactivity was found at higher molecular weight regions (Figure 4, lane 1). Incubation of olfactory cilia for 15 min at 37 °C in the presence of 2-mercaptoethanol results in the appearance of the 59-kDa band. Under these conditions, immunoreactive species are also observed in the 120- and 180-kDa regions (Figure 4, lanes 2–4). Staining in the 120-kDa region often appears as a doublet band. After treatment with 2-mercaptoethanol at concentrations above 1 M, staining of the 59-kDa band and especially of the higher molecular weight bands is greatly reduced (Figure 4, lane 5) and is sometimes entirely abolished (not shown). These observations suggest that the protein recognized by mAb 8 exists as oligomers linked together via disulfide bonds.

To present further evidence for the oligomeric nature of the protein recognized by mAb 8, we solubilized ciliary membranes with Triton X-100 and subjected untreated extracts or extracts reduced with 2-mercaptoethanol to sucrose gradient centrifugation. Fractions were collected and analyzed for immunoreactivity with mAb 8 after reduction with 2-mercaptoethanol by polyacrylamide gel electrophoresis in SDS. Under the conditions used, samples treated with 2-mercaptoethanol prior to sucrose gradient centrifugation prominently display the characteristic immunoreactive 59-kDa band at a sucrose density of 10–12% (w/w) (Figure 5A,C). Immunoreactive species in the 120-kDa region are recovered near the center of the tube at ~12–14% (w/w) sucrose. High molecular weight material that runs near the top of the gel is recovered in the bottom fractions of the gradient (Figure 5A,C). Samples, not treated with 2-mercaptoethanol prior to sucrose gradient centrifugation, do not display the 59-kDa band even though the fractions were subjected to reduction with 2-mercaptoethanol prior to electrophoresis. Immunoreactive species are observed here in the 120-kDa region, and a large amount of high molecular weight material, much of which is retained in the stacking gel, is evident in the bottom of the gradient (Figure 5B,C). Thus, mAb 8 recognizes a unique membrane glycoprotein, tagged with distinct carbohydrate moieties, which is found exclusively in dendritic olfactory cilia

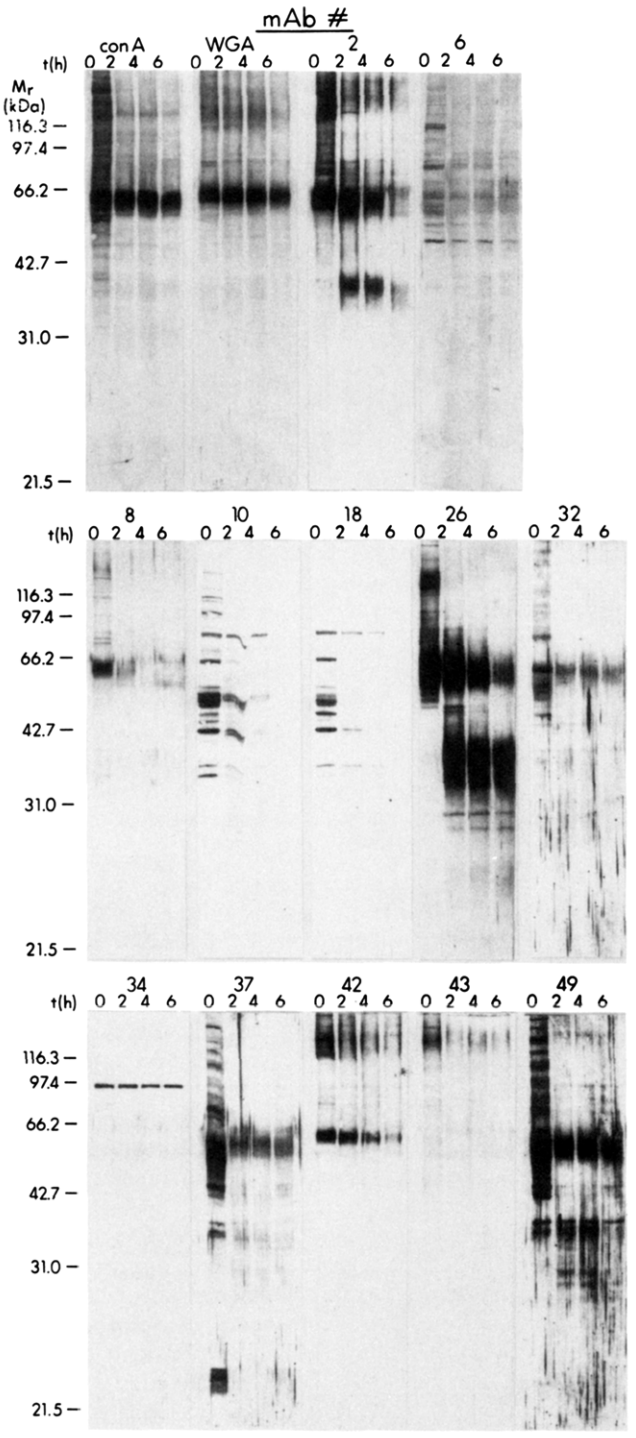

FIGURE 3: Deglycosylation of olfactory cilia with endoglycosidase H. Olfactory cilia were treated with endoglycosidase H, as described under Materials and Methods, for 0, 2, 4, or 6 h, as indicated in the figure, and reactivity with the indicated lectins or mAbs was assessed after polyacrylamide gel electrophoresis in SDS and electrophoretic transfer. Con A and biotinylated WGA, both from Vector Laboratories, were used at 10 and 20 μg/mL, respectively. mAb 6 was used as a 10-fold dilution of ammonium sulfate fractionated cell supernatant; mAbs 8, 10, 18, 32, 34, 37, and 49 were used as 25-fold dilutions of ammonium sulfate fractionated cell supernatants; mAbs 2, 42, and 43 were used as 250-fold dilutions of ammonium sulfate fractionated cell supernatants; and mAb 26 was used as a 2000-fold dilution of ammonium sulfate fractionated ascites fluid.

and which exists as disulfide-linked oligomers.

Discussion

We have generated a library of mAbs against chemosensory cilia of olfactory receptor cells. About half of our mAbs recognize proteins on Western blots, and, initially, we have

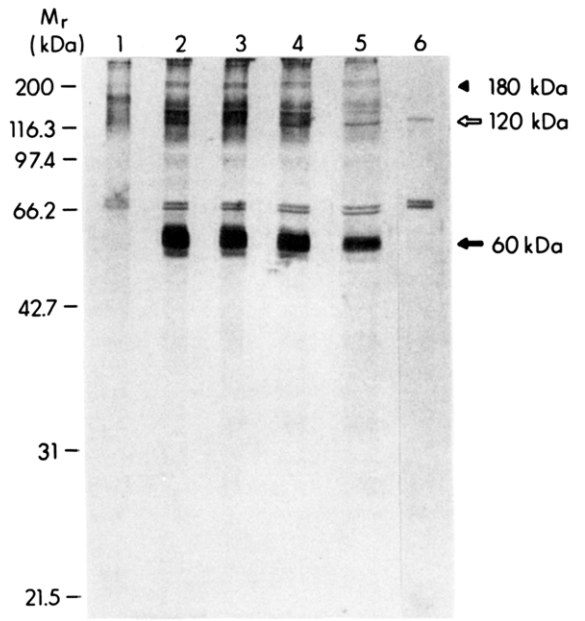

FIGURE 4: Effect of reduction of ciliary proteins by 2-mercaptoethanol on immunoreactivity of mAb 8. Olfactory cilia were incubated for 15 min at 37 °C in the absence of 2-mercaptoethanol (lane 1) or in the presence of 0.5% (v/v, lane 2), 1.0% (v/v, lanes 3 and 6), 5% (v/v, lane 4), and 10% (v/v, 1.4 M, lane 5) 2-mercaptoethanol prior to electrophoresis and immunoblotting. All lanes contained 25 μg of protein. Lanes 1–5 were stained with a 10-fold dilution of ammonium sulfate fractionated supernatant of mAb8, and lane 6 was stained with a 25-fold dilution of ammonium sulfate fractionated supernatant of P3×653.Ag8 myeloma cells to visualize nonspecific staining. Immunoreactive species in the 60- and 120-kDa regions are indicated by the closed and open arrow, respectively. The arrowhead indicates the 180-kDa region. Note that staining of the 59-kDa band visualized by mAb 8 is decreased at the highest concentration of 2-mercaptoethanol (lane 5).

focussed our attention on these antibodies. Most of these antibodies react with multiple bands on Western blots, suggesting that many proteins undergo proteolysis during the preparation of olfactory cilia. Inclusion of a cocktail of protease inhibitors in the initial buffers during the preparation of olfactory cilia, including aprotinin (20 units/L), benzamidine (5 mM), iodoacetamide (5 mM), and phenylmethanesulfonyl fluoride (1 mM), does not significantly alter the patterns of immunoreactive products, indicating that proteolysis occurs rapidly after killing the frog during the dissection of the tissue. Differential sensitivity to proteolysis in this tissue has been observed previously. For example, GTP-binding proteins are detected intact in olfactory cilia, whereas protein kinase C is highly sensitive to proteolysis (Anholt et al., 1987).

Our mAbs (mAbs 8, 26/61/82, 34, 42/45, and 43) recognize proteins that appear unique to olfactory cilia and are not detected in membranes prepared from the olfactory nerve that contain the axons of olfactory receptor cells or in cilia prepared from respiratory epithelium that do not perform a chemosensory function. Neither are they detected in membranes from brain and in membranes from four peripheral tissues examined. However, we cannot exclude that some or all of these antigens may be present in membranes from these tissues at concentrations too low to be detected by our procedures. Nonetheless, their absence on blots prepared with olfactory nerve membranes or respiratory cilia and their prominence in olfactory cilia indicate that, if not unique, they are at least highly enriched in olfactory cilia.

Studies with endoglycosidase H indicate that carbohydrate determinants dominate the immunogenicity of olfactory cilia.

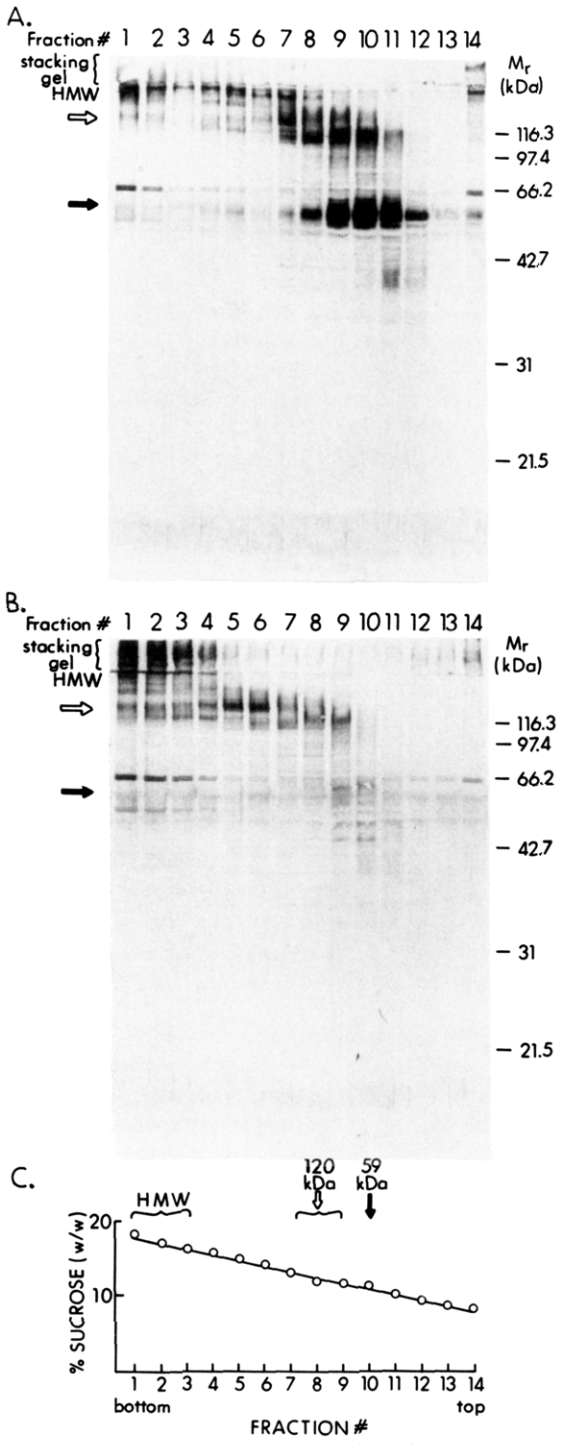

FIGURE 5: Analysis of 2-mercaptoethanol-sensitive proteins immunoreactive with mAb 8 by polyacrylamide gel electrophoresis following sucrose gradient centrifugation of reduced (panel A) and nonreduced (panel B) samples. The experiment was performed as described under Materials and Methods. Panel C shows the sucrose concentrations in the fractions. Note the virtual absence of the 59-kDa immunoreactive species in panel B as compared to panel A (fractions 8–12; closed arrow) and the accumulation of high molecular weight material (HMW), much of which does not enter the resolving gel, in fractions 1–3. Immunoreactive material in the 120-kDa region (open arrow) is evident in both gradients, but more of it appears in the heavier fractions in panel B as a result of limited reduction of the high molecular weight material after centrifugation and prior to electrophoresis. The tendency of the protein recognized by mAb8 to aggregate even in detergent solution was frequently observed.

This is in line with a previously reported study describing a mAb reactive with proteins in the 58- and 95-kDa region and a mAb reactive with the 95-kDa WGA-binding protein of olfactory cilia, both of which react with carbohydrate determinants (Chen et al., 1986b). Studies with WGA and Con A have revealed an abundance of glycoproteins on olfactory cilia (Chen & Lancet, 1984; Chen et al., 1986b; Anholt et al., 1986). Lectins also inhibit olfactory responses as measured by the electroolfactogram (Wood et al., 1983; Shirley et al., 1983a) and inhibit binding of odorous amino acids to chemosensory membranes from catfish (Kalinoski et al., 1987). The uniform sensitivity of our mAbs to endoglycosidase H is remarkable and may reflect a preponderance of high-mannose-containing carbohydrates. The different patterns of reactivity of our mAbs with endoglycosidase H sensitive determinants underscore the wide variety of carbohydrate structures expressed on proteins of olfactory cilia. MAbs 8, 42/45, 43, and possibly mAb 26 recognize distinct carbohydrate determinants that appear unique to olfactory cilia. Preliminary studies suggest that terminal galactose residues may contribute to the unique antigenic structure of the determinant recognized by mAb8 (D. A. Snyder and R. R. H. Anholt, unpublished observations). The function of these carbohydrate moieties is not clear. They may serve as signals for the insertion of certain proteins into the ciliary membrane. Alternatively, they could contribute to the structure of the mucus surrounding the dendritic cilia and aid in providing a favorable medium for interactions between odorants and the chemosensory membrane. Furthermore, they could also represent receptor sites for viruses, known to be capable of gaining entry into the central nervous system via the olfactory nerve (Barthold, 1988; Perlman et al., 1989).

The functions of the proteins detected by mAbs 8, 26/61/82, 34, 42/45, and 43 remain to be established. Of particular interest is the 59-kDa protein detected by mAb 8. This protein is found only in olfactory cilia, contains a unique carbohydrate determinant, and appears to exist as oligomers linked via disulfide bonds. The antigenic determinant detected by mAb 8 becomes accessible after reduction with 2-mercaptoethanol, but is less reactive after treatment with high concentrations of this reducing agent and sometimes disappears entirely under this condition. This may be due to reduction of an internal disulfide bond which is essential for maintaining the conformation of the antigenic determinant and is less sensitive to treatment with 2-mercaptoethanol than the intermolecular disulfide bonds that are reduced at lower concentrations of this reducing agent. Partial reduction of intramolecular disulfide bonds may also explain the often observed doublet band in the 120-kDa region, if it is assumed that only one of the constituent monomers has undergone reduction at an intramolecular disulfide bond, which is reflected in slightly altered electrophoretic migration of the dimer. At present, we have no explanation for the observation that the intermolecular disulfide bonds become resistant to reduction following sucrose gradient centrifugation other than that this may be a consequence of delipidation of the protein. It is also intriguing that conformational changes affect binding of mAb 8 to its antigenic site, even though this epitope may be entirely, or at least partially, composed of carbohydrate. We can, however, not fully exclude the possibility that the antibody may recognize a determinant carried on the protein and that deglycosylation by endoglycosidase H leads to conformational changes that are reflected in loss of immunoreactivity after subsequent electrophoresis in SDS.

It is of interest to note that treatment of olfactory epithelium with sulfhydryl reagents abolishes the electroolfactogram (Getchell & Gesteland, 1972; Shirley et al., 1983b). Furthermore, odorant-activated channels from olfactory cilia show

several conductance states that are multiples of a unitary conductance and that open in a concerted manner (Labarca et al., 1988). Although the 2-mercaptoethanol-sensitive oligomeric glycoprotein detected by mAb 8 has structural properties that could account for some of the observed electrophysiological phenomena, it would at this point be premature to speculate on the function of this protein in olfaction.

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