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Membrane proteins unique to vertebrate olfactory cilia: Candidates for sensory receptor molecules

(chemoreception/sensory neurons/olfactory epithelium/glycoproteins/membrane tubulin)

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ABSTRACT **In search for olfactory receptor molecules, we carried out comprehensive electrophoretic mapping of membrane proteins in the cilia of frog olfactory epithelium. Seven polypeptides, extracted from isolated cilia by nonionic detergent, were unique to the sensory organelles, compared to nonsensory (respiratory) counterparts. Olfactory cilia contained 3–10 times more membrane-associated protein as compared to respiratory cilia, in agreement with reported densities of freeze-fracture intramembranous particles. Four of the olfactory polypeptides were major constituents of the ciliary membrane, each amounting to >10% of its total protein. Three major and one minor specific polypeptide were glycosylated, whereas membranes of nonsensory cilia were practically devoid of glycoproteins. A clear difference in surface composition was also shown by microscopic visualization of fluoresceinated lectin bound to intact isolated cilia. Two of the olfactory glycoproteins displayed pronounced heterogeneity of apparent molecular weight, which could partly be due to protein sequence diversity, as expected for odorant receptor molecules. The recently described inhibition of odorant-evoked sensory potentials by the lectin concanavalin A is consistent with the hypothesis that one or more of the specific glycoproteins described here plays a role in olfactory reception.**

The sense of smell in higher vertebrates is capable of extremely sensitive detection and accurate identification of practically all airborne chemical compounds. While extensive research has been aimed at the anatomy and electrophysiology of this sensory pathway (1–3), relatively little is known about its molecular mechanisms. Recent studies suggest that the first step in vertebrate chemoreception involves membrane-receptor proteins that reside on the cilia of sensory neurons in the olfactory epithelium (4–6). However, the biochemical identity and properties of these putative receptor molecules remain largely unknown.

The olfactory system appears to share many structural and functional attributes with the visual system. In both, sensory reception takes place at ciliary organelles having large membrane area and containing the molecular apparatus that mediates stimulus-evoked changes of membrane potential. While the structure and function of rhodopsin, the photoreceptor protein of retinal rods, and several enzymes coupled to it are known in detail (7), virtually no equivalent information is available for the olfactory counterparts.

Previous biochemical studies of olfactory reception relied on techniques that involve odorant binding. This approach has been successful in fish, which are sensitive to a limited number of odorants (mainly amino acids), and presumably have relatively few olfactory receptor types (4). In contrast, amphibia and terrestrial vertebrates are capable of detecting a much wider range of chemical compounds and their olfactory receptors may have undergone a more extensive diversification (2–6). It has recently been suggested (8, 9) that the olfactory system of higher vertebrates is analogous to the immune system, both having evolved large receptor repertoires to recognize a practically unlimited variety of extraneous chemical determinants. Although it is possible in the immune system to enhance specific receptor subpopulations through immunization, no equivalent process has been clearly demonstrated in the olfactory system. Olfactory epithelial preparations should thus be similar to nonimmune serum, containing small and approximately equimolar amounts of all receptor types. Because any ligand may bind with appreciable affinity to only a minute fraction of the total receptor population, ligand-binding techniques could not be readily used for receptor characterization and isolation (cf. refs. 5 and 6).

We decided to use an alternative approach, aimed at "common denominator" properties (e.g., polypeptide structure and glycosylation pattern) that may be shared by many olfactory receptor types irrespective of ligand specificity, as exemplified by the immunoglobulin heavy- and light-chain structure. Because membranes of nonsensory cilia are relatively protein poor (10, 11) while those of olfactory cilia contain considerably more protein (11), it is possible to identify candidate components of the "generalized" olfactory-receptor molecule through comparison of polypeptide profiles of ciliary membranes, and later attempt to establish their function. This strategy resembles that used in studies of erythrocyte-membrane proteins (12) and of tissue-specific polypeptides such as olfactory marker protein (13).

The frog was chosen as the experimental animal because of its easily accessible epithelium and extremely long cilia and because of its use in numerous electrophysiological studies (2, 3).

MATERIALS AND METHODS

Frogs (*Rana ridibunda*) were caught in the wild and grown locally and kept at 4°C until used (<7 days). After decapitation, olfactory epithelia and palate respiratory epithelia were dissected, and deciliation was carried out by the calcium pulse and 10% ethanol method (14, 4). Isolated cilia were examined by dark-field or electron microscopy to control their purity. Ciliary membrane proteins were solubilized by 1% Triton X-100, where detergent-insoluble axonemes could be separated by centrifugation (10,000 × *g*) as described (14).

Electrophoresis in 10% polyacrylamide gel in the presence of 2-mercaptoethanol was done according to ref. 15. Protein bands were visualized by Coomassie brilliant blue or by silver staining (16). Glycoproteins were identified by direct binding of ^{125}I-labeled lectins (Sigma) to the NaDodSO$_4$ gel (17). Immunoreactivity of electrophoretic bands was examined by the nitrocellulose immunoblotting method (18), using

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Abbreviation: Con A, concanavalin A.

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mouse monoclonal antibody TUB 2.1 (ref. 19; gift of I. Gozes) and rabbit anti-chicken brain tubulin antiserum (gift of B. Geiger). Protein radioiodination was by the chloramine-T method. Gel patterns were quantified by a Beckman DU-8 spectrophotometer.

Fluorescence-microscopy visualization of lectin binding was carried out on isolated cilia adhered to glass coverslips coated with poly-L-lysine and incubated with lectins derivatized with fluorescein isothiocyanate.

RESULTS

Identification of Olfactory-Specific Polypeptides. Fig. 1*A* depicts $NaDodSO_4$ gel electrophoresis patterns of membrane-associated (Triton X-100 soluble) and axonemal (Triton X-100 insoluble) proteins of olfactory and respiratory cilia, visualized with Coomassie brilliant blue. Both types of axoneme contain >95% tubulin, identified by apparent molecular weight and by immunoreactivity (Fig. 1*B*). Quantitation of axonemal tubulin serves as a criterion for purity and allows calibration of the amounts of different cilia. Densitometric analyses of Fig. 2*A* show that the amount of protein in the membrane of olfactory cilia is 5–10 times higher than in the membrane of respiratory cilia and is comparable to the amount of axonemal tubulin. Coomassie brilliant blue staining reveals two major olfactory specific proteins, gp58 and p53, and one minor specific protein, p78 (see Table 1). The polypeptides migrating at $M_r < 42,000$ are found to vary considerably among preparations and some may represent degradation products.

Fig. 1 also shows the glycoprotein patterns of the cilia, visualized by binding of the mannose-specific lectin concanavalin A (Con A) to the electrophoretic gel. Olfactory cilia have four membrane glycoproteins (Table 1), while the proteins of respiratory cilia show no detectable glycosylation.

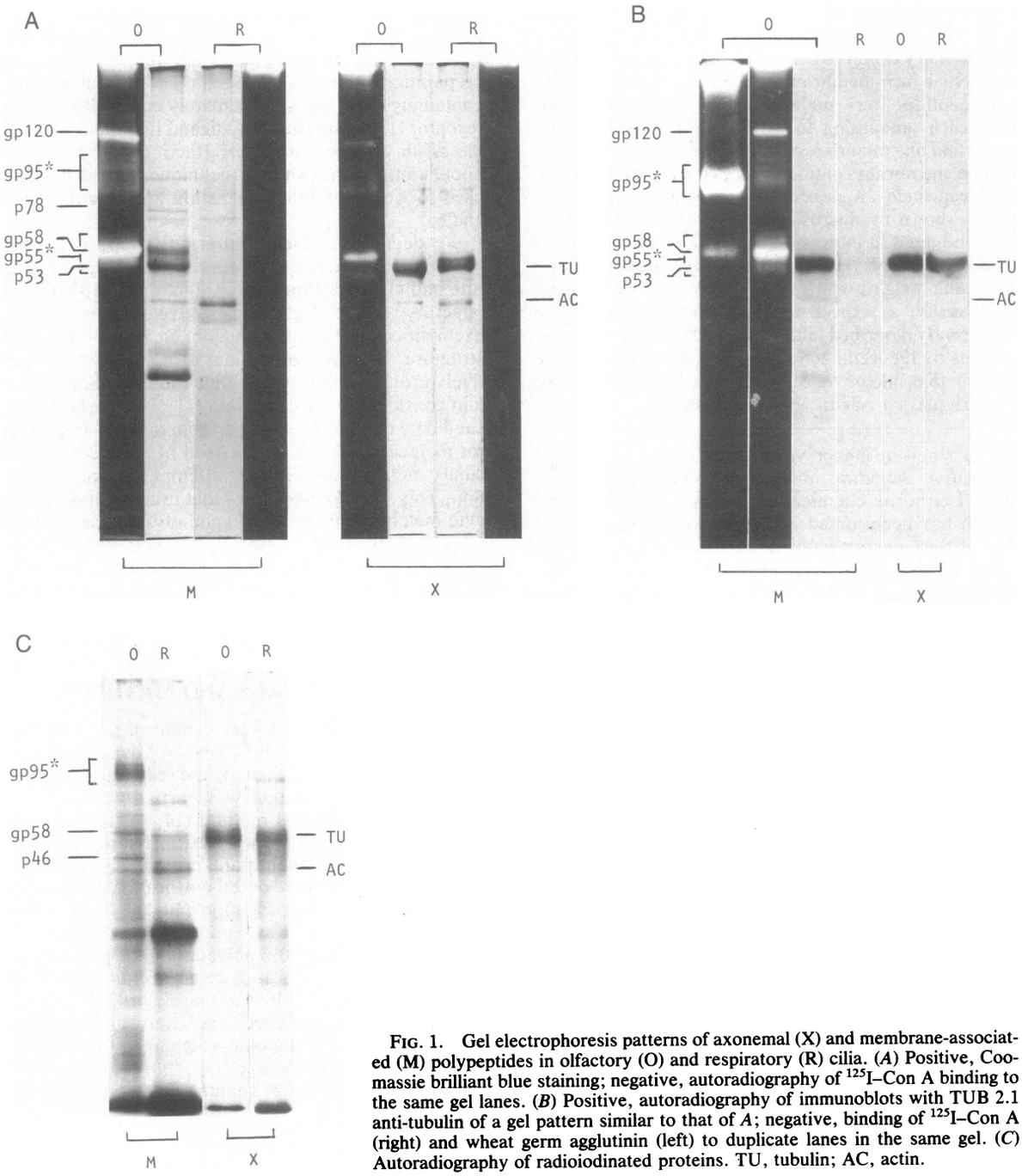

FIG. 1. Gel electrophoresis patterns of axonemal (X) and membrane-associated (M) polypeptides in olfactory (O) and respiratory (R) cilia. (*A*) Positive, Coomassie brilliant blue staining; negative, autoradiography of ^{125}I–Con A binding to the same gel lanes. (*B*) Positive, autoradiography of immunoblots with TUB 2.1 anti-tubulin of a gel pattern similar to that of *A*; negative, binding of ^{125}I–Con A (right) and wheat germ agglutinin (left) to duplicate lanes in the same gel. (*C*) Autoradiography of radioiodinated proteins. TU, tubulin; AC, actin.

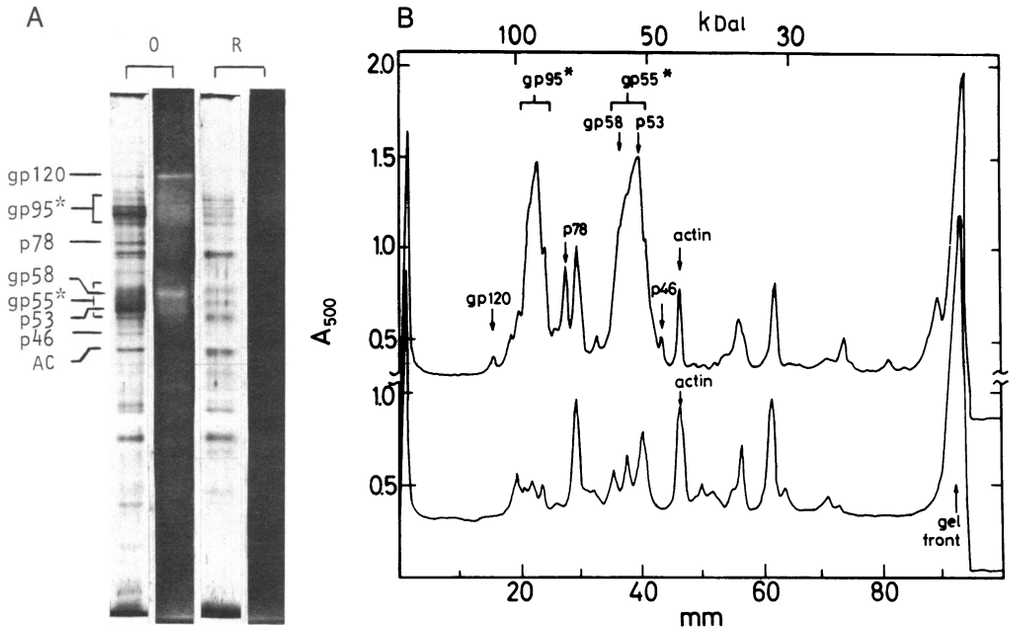

FIG. 2. (*A*) Silver-stained gel electrophoretic patterns of membrane proteins from olfactory and respiratory cilia. Negative, Con A binding to the same gel lanes. (*B*) Densitometric scans of olfactory (upper) and respiratory (lower) silver-stained lanes of *A*. Notations are as in Fig. 1.

The polypeptides gp95* and gp55* display broad apparent molecular weight ranges, while gp120 is sharply defined. The narrowly migrating gp58, which coincides with a major Coomassie brilliant blue-stained band, is separable from the overlapping gp55* by gel filtration (not shown). Olfactory (but not respiratory) axonemes contain residual amounts of membrane glycoproteins, probably due to incomplete solubilization.

Fig. 1*B* depicts immunoreactivity of ciliary proteins with mouse monoclonal antibody TUB 2.1 specific mainly to β-tubulin. It is shown that olfactory membranes contain a specific species of tubulin (p53), which is absent in respiratory membranes, and is distinct from the glycoprotein gp58. Rabbit antiserum to chicken α and β brain tubulin gave identical results (not shown).

Ciliary proteins were also visualized by radioiodination (Fig. 1*C*; Table 1). The polypeptide gp95* now appears as a major component, while the proteins around gp55* are not as prominent. A minor olfactory polypeptide (p46) is seen, and respiratory cilia appear to contain a highly iodinated species at M_r 33,000.

Table 1. Membrane proteins unique to olfactory cilia

Band	Label				
	Coomassie blue	Silver stain	^{125}I	Con A	WGA
gp120	—	0.6	—	**18.0**	—
gp95*	—	27.0	26.0	25.3	**80.0**
p78	4.8	**5.8**	—	—	—
gp58	15†	↑	11.0	**38**†	19.0
gp55*	17†	**32.3**‡	—	14†	—
p53§	15†	↓	—	—	—
p46	—	1.3	**3.7**	—	—

Notation of proteins (p) and glycoproteins (gp) is according to apparent M_r ($\times 10^{-3}$) calculated from calibration curves using protein standards. The symbol * denotes polypeptide species displaying a broad electrophoretic migration range. Numbers represent percent of total labeled protein, measured by densitometry of gels shown in Figs. 1 and 2. The best labeling method for each polypeptide is indicated by boldface type. —, Not readily measurable. WGA, wheat germ agglutinin.

†Rough estimate obtained by multiple gaussian analysis of composite densitometric peak.

‡Gaussian analysis not feasible, number representing sum of three species.

§Best defined by anti-tubulin binding (Fig. 1*B*).

Fig. 2 shows a comprehensive protein profile of ciliary membranes obtained by silver staining, which affords 10–100 better sensitivity than Coomassie brilliant blue. All seven olfactory-specific proteins identified by the other methods are revealed, and the profile is correlated with Con A binding. Olfactory-specific proteins are readily identifiable on a background of minor polypeptides common to both ciliary types. Quantitative densitometric analysis (Fig. 2*B*; Table 1) shows that the olfactory-specific proteins amount to more than two-thirds of the silver-stainable polypeptides of olfactory cilia. About 60% of this specific protein is in gp95* and the complex around M_r 55,000 containing gp58, gp55*, and p53. The remainder is in minor olfactory-specific polypeptides (gp120, p78, p46). A polypeptide at M_r 17,000 may be impurity of olfactory marker protein, a cytoplasmic protein specific to olfactory receptor neurons (13).

Further Characterization of Olfactory Glycoproteins. Fig. 1*B* and Table 1 provide a comparison of labeling patterns of olfactory-membrane proteins with two lectins: Con A and wheat germ agglutinin (specific to *N*-acetyl glucosamine and to sialic acid). The differences are attributed to variation in glycosylation pattern in the different glycoproteins and can assist in protein identification and purification.

Olfactory cilia are normally embedded in a mucus layer containing glycosaminoglycans. To eliminate the possibility that these components contribute to the lectin labeling, we subjected detergent extracts of olfactory cilia to proteolytic cleavage by trypsin and Pronase (not shown). All the olfactory glycoconjugates were completely degraded, confirming their glycoprotein nature.

To further establish the glycosylation differences between olfactory and respiratory cilia and to verify the surface disposition of the olfactory glycoproteins, we examined the binding of fluorescein-conjugated wheat germ agglutinin to intact isolated cilia by fluorescence microscopy (Fig. 3). Olfactory cilia were labeled, but respiratory cilia were not labeled. Binding of fluoresceinated Con A and wax bean ag-

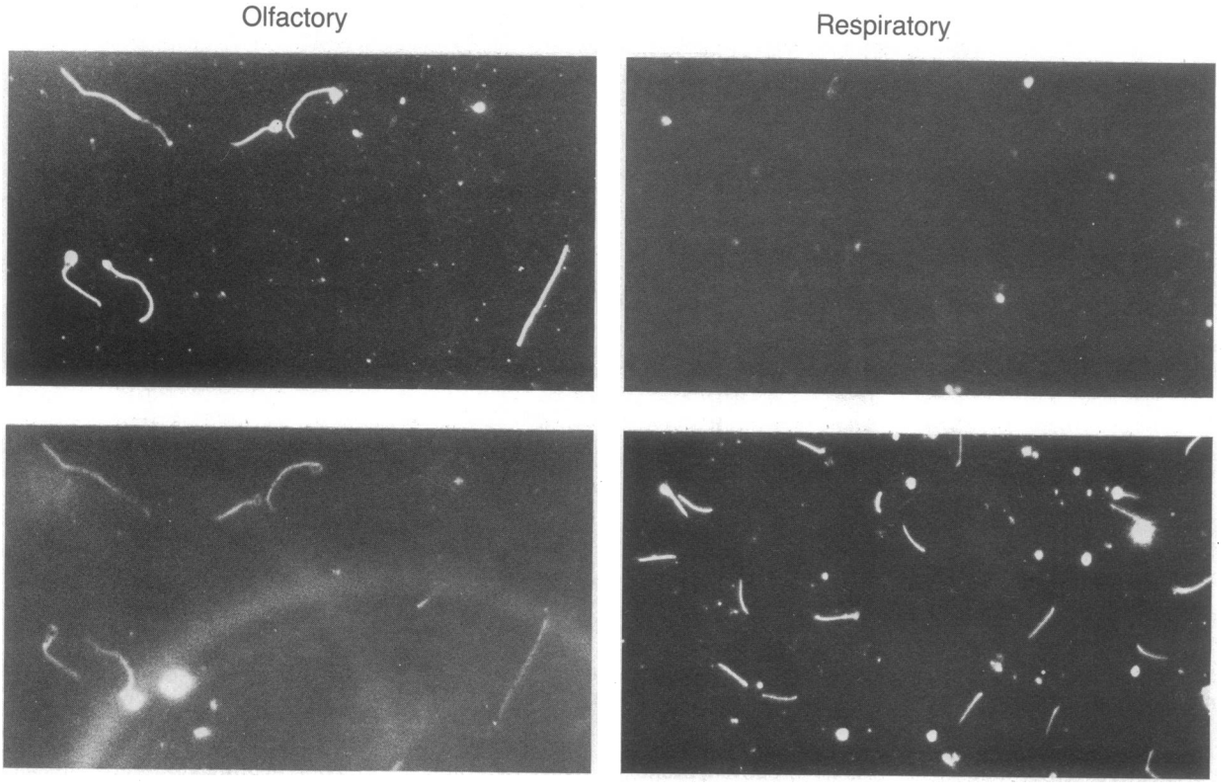

FIG. 3. (*Upper*) Fluorescence microscopy visualization of fluoresceinated wheat germ agglutinin binding to isolated intact olfactory and respiratory cilia. (*Lower*) Dark-field visualization of cilia in the same fields. Scale: lower right-hand corner olfactory cilium is 25 μm long.

glutinin (data not shown) gave similar results, although the intensity of labeling was considerably lower.

DISCUSSION

The main point of this study was to create a basis for molecular biological investigation of proteins involved in vertebrate olfaction. We have shown the relative simplicity of protein composition in frog olfactory cilia and identified olfactory-specific ciliary membrane proteins. A major future task would be to establish the physiological role and biochemical properties of each of these proteins.

The membrane-associated polypeptide gp95* is a most prominent component of olfactory cilia, constituting about one-quarter of the silver-stainable protein, and also being the most heavily radioiodinated species. Interestingly, it does not stain with Coomassie brilliant blue, and this (as well as other cases of differential staining) may be attributed to peculiarities of amino acid composition or carbohydrate content. The findings that intact olfactory cilia bind wheat germ agglutinin considerably better than other lectins, and that gp95* is most heavily labeled with wheat germ agglutinin, imply that gp95* is a dominant surface component of the sensory organelles.

There are three olfactory-specific membrane-associated polypeptides migrating near axonemal tubulin, gp58, gp55*, and p53, together constituting at least 35% of the ciliary membrane protein. The protein p53 is established as olfactory-specific membrane-associated tubulin and is clearly nonglycosylated, and in this respect it may be different from previously described membrane-associated species of tubulin that occur in relatively low amounts in cilia of other species (10). It has been suggested that membrane tubulin is involved in chemoreception (10), and this could also be true for frog olfaction, particularly in view of a model proposing a role for axonemal microtubules in chemosensory signal transfer (20). Alternatively, p53 could mediate membrane–axoneme interactions that might be important for the structural integrity of the long olfactory cilium or for confinement of specific proteins to its specialized sensory membrane.

Membranes of vertebrate olfactory cilia have been probed by freeze-fracture electron microscopy (11, 21). Densities of 1000–2500 per μm^2 of intramembranous particles have been reported, contrasting with the low densities (300–800 per μm^2) in nonsensory cilia. It was suggested that the abundant sensory-specific particles represent olfactory receptor molecules (11). The present study provides possible biochemical counterparts for these microscopically observed particles. A straightforward calculation shows that the major olfactory-specific proteins, but not the minor specific or nonspecific proteins, could account for the documented particle densities. Based on our finding that the total membrane protein in an olfactory cilium equals approximately the amount of axonemal tubulin, and because the number of tubulin molecules per μm of ciliary length is about 60,000 (22), there should be $\approx$100,000 membrane polypeptides (average M_r 60,000) in a segment of cilium (radius, 0.1 μm) having a 1-μm^2 membrane area. Each of the major proteins (constituting 10–20% of the total membrane protein) will thus be represented at 10,000–20,000 molecules per μm^2. Because the membrane particles are 8–12 nm in diameter (11), corresponding to 3–6 polypeptides of M_r 60,000–90,000, the major proteins account nicely for the observed particle density of several thousand per μm^2. In contrast, minor ciliary membrane proteins could only constitute a few hundred particles per μm^2, in agreement with the density in nonsensory cilia.

The olfactory system is capable of responding to odorants at relatively low concentrations (1–100 nM, and often considerably lower; cf. ref. 6). While for some odorants (most notably behaviorally significant ones) the receptors might have high affinity, this could not possibly be true for the entire range of odorous compounds (see ref. 11). An alternative means of augmenting sensory acuity would be provided by enhancing the bilayer concentration of the receptors. An

analogous situation prevails for rhodopsin, the major membrane protein of retinal rod outer segments, in which high concentration enhances photon-capture efficiency (7). It therefore appears that major olfactory membrane proteins, such as gp95* or the M_r 55,000 complex, are better candidates for odorant receptor molecules, while minor olfactory proteins could play a role in sensory transduction, similar to the membrane enzymes of retinal rods (7).

In addition to being much richer in protein, frog olfactory ciliary membrane also differs from the respiratory counterpart in having several specific glycoproteins. We now have evidence (unpublished observations) that a similar glycoprotein pattern occurs also in mammals. Glycosylation serves to indicate that the proteins involved are located on the ciliary surface, as also shown by our fluorescence microscopy experiments. Lectins could thus be used to probe vertebrate chemosensory function. Indeed, it has recently been reported that the lectin Con A diminishes the electro-olfactogram response (a summed epithelial potential) to many odorants (23). This, together with the complete absence of glycoproteins in nonsensory cilia, may be taken to suggest that one or more of the olfactory glycopeptides described here is involved in vertebrate olfactory reception.

Since olfactory receptor molecules are expected to form a large group of proteins with different binding specificities (8, 9), the broadly defined electrophoretic mobility of gp95* and gp55* could serve as an additional suggestion for a possible role in odorant recognition. However, further biochemical characterization would be necessary to find out whether such heterogeneity is due to amino acid sequence diversity.

Involvement of ciliary polypeptides in odorant reception may also be verified (*i*) by gene cloning methods, in which olfactory receptors could be revealed as a multigene family, similar to that of immunoglobulins (24); and (*ii*) by immunological techniques, in which antibodies directed either against variable determinants (at the odorant binding site) or against constant determinants (common to all receptor specificities) may serve to identify odorant receptors and probe their structure and function.

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