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Robert R. H. Anholt

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, NC 27710

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13. ABSTRACT (Maximum 200 words)

This project was designed to characterize olfactory tissue-specific proteins previously identified with a library of monoclonal antibodies raised against frog olfactory cilia. First, we showed that our olfactory tissue-specific monoclonal antibodies all recognize different molecular forms of the same protein. We named this protein "olfactomedin". We then proceeded to purify olfactomedin and characterize it biochemically and immunohistochemically. We showed that olfactomedin is produced only in olfactory tissue by sustentacular cells and Bowman's glands and that it is deposited in the lower mucus layer of olfactory neuroepithelium. Next, we extracted mRNA from olfactory tissue and constructed a cDNA library. We obtained partial sequence information of the N-terminus of purified olfactomedin and used these data to design a degenerate oligonucleotide probe for the identification of a full-length cDNA clone which encodes olfactomedin. This clone was sequenced and found to encode a 448 amino acid protein preceded by a leader peptide of 16 amino acids. Analysis of the sequence showed no homologies with any known protein. Examination of its amino acid sequence by Chou-Fasman analysis in combination with biochemical and immunochemical evidence obtained previously enabled us to construct a model for olfactomedin which identifies this protein as a novel olfactory tissue-specific extracellular matrix protein, which forms the main structural component of the extracellular mucous matrix of olfactory neuroepithelium and which may trigger the growth and differentiation of the dendritic knob and its olfactory cilia that house the olfactory transduction machinery.

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A. Background and Perspective

Odor recognition and olfactory transduction occur at chemosensory cilia that protrude from the dendritic tips of olfactory receptor neurons. These cilia are surrounded by a layer of mucus that lines the olfactory neuroepithelium. Odorants that enter the nasal cavity with the inspired air partition into and diffuse through this aqueous mucus layer on their way to odorant receptors on the ciliary membrane. The interaction between an odorant and its receptor sets in motion a cascade of transduction mechanisms, which results in the generation of second messengers, cyclic AMP and calcium, which trigger excitation of the cell by regulating the activity of ion channels. These events result in a generator potential which ultimately evokes action potentials.

Prior to the initiation of contract DAAL03-89-K-0178 we have contributed to the elucidation of second messenger pathways involved in olfactory transduction and, in an effort to identify olfactory tissue-specific proteins likely to play key roles in olfaction, we established a library of monoclonal antibodies against olfactory cilia from the bullfrog, *Rana catesbeiana*. We identified a set of antibodies that revealed immunoreactivity only in olfactory cilia, but not in membranes from other tissues, including olfactory nerve and cilia from respiratory epithelium. Project DAAL03-89-K-0178 was designed to characterize these olfactory tissue-specific proteins.

First, we showed that our olfactory tissue-specific monoclonal antibodies all recognize different molecular forms of the same protein. We named this protein "olfactomedin". We then proceeded to purify olfactomedin and characterize it biochemically and immunohistochemically. We showed that olfactomedin is produced only in olfactory tissue by sustentacular cells and Bowman's glands and that it is deposited in the lower mucus layer of olfactory neuroepithelium. Next, we extracted mRNA from olfactory tissue and constructed a cDNA library. We obtained partial sequence information of the N-terminus of purified olfactomedin and used these data to design a degenerate oligonucleotide probe for the identification of a full-length cDNA clone which encodes olfactomedin. This clone was sequenced and found to encode a 448 amino acid protein preceded by a leader peptide of 16 amino acids. Analysis of the sequence showed no homologies with any known protein. Examination of its amino acid sequence by Chou-Fasman analysis in combination with biochemical and immunochemical evidence obtained previously enabled us to construct a model for olfactomedin which identifies this protein as a novel olfactory tissue-specific extracellular matrix protein, which forms the main structural component of the extracellular mucous matrix of olfactory neuroepithelium and which may trigger the growth and differentiation of the dendritic knob and its olfactory cilia that house the olfactory transduction machinery.

Contract DAAL03-89-K-0178 has produced five original publications and ten communications in abstract form, and supported four invited review articles and book chapters. A more detailed account of this project follows below.

B. Summary of Research Findings

1. Identification of monomeric and polymeric forms of olfactomedin

Some of our monoclonal antibodies raised against frog olfactory cilia showed immunoreactivity with a 57 kd polypeptide. Others reacted with both this 57 kd and a 120 kd polypeptide. One antibody reacted only with the 120 kd species. Further studies revealed that the 120 kd protein was a dimeric form of the 57 kd polypeptide generated through the formation of intermolecular disulfide bonds. In addition, polymeric aggregates were observed which were excluded from 10% polyacrylamide gels. These polymers would sediment through the bottom of sucrose gradients and treatment of these polymers with 2-mercaptoethanol would release the 57 kd monomer. Competition studies between monoclonal antibodies and polyclonal rabbit antiserum against olfactomedin and reactivity of all our antibodies with the purified protein (see below) verified the notion that all our antibodies indeed recognized different molecular forms of the same protein, which we named "olfactomedin". Our studies further indicated that our monoclonal antibodies recognize carbohydrate groups that dominate olfactomedin's immunogenicity. The conclusion of these studies was the following:

Olfactomedin is a glycoprotein which is uniquely expressed in olfactory tissue where it occurs as a 57 kd polypeptide which via intermolecular disulfide bonds forms polymers that upon reduction will generate monomers as well as a relatively stable 120 kd dimeric form.

2. Purification of olfactomedin

Studies with lectins, such as wheat germ agglutinin and *Ricinus communis* agglutinin I (RCA) indicated that olfactomedin contains N-acetylglucosamine and galactose sugars, but no sialic acid. This allowed us to design a purification protocol based on affinity chromatography using RCA conjugated to agarose. This procedure required reduction of olfactomedin, since RCA was found to bind only to the monomeric form. Purification of olfactomedin allowed us to quantitate the amount of olfactomedin in olfactory tissue. We found that olfactomedin is extremely abundant representing approximately 1-5% of total tissue protein. We generated rabbit antisera against the purified protein and confirmed that olfactomedin is produced only in olfactory tissue. Using conventional microsequencing techniques the N-terminal amino acid sequence of olfactomedin was determined. A single N-terminal amino acid was identified (phenylalanine) suggesting that olfactomedin occurs as a single protein species. Positions 5 and 18 could not be determined and later were found to be asparagine, which is part of a consensus N-linked glycosylation site, and cysteine, respectively. Position 16 was initially misidentified as aspartic acid and later correctly identified as cysteine. The cysteines at positions 16 and 18 appear to be important for dimer formation, as described below. A synthetic peptide corresponding to this N-terminal region of the sequence TGILAGKDHD was synthesized and an antibody raised against this peptide was generated and found to be useful in identifying precursor forms of olfactomedin (see below). The conclusions from this part of the project were as follows:

Olfactomedin is produced in abundant quantities in olfactory tissue and can readily be

purified by lectin affinity chromatography.

3. Immunohistochemical localization of olfactomedin

Immunohistochemical studies with monoclonal and polyclonal antibodies visualized olfactomedin in the acinar cells of Bowman's glands and near the surface of the epithelium. Patches of reaction product were often seen associated with olfactory cilia. Immuno-gold labeling studies on freeze-substituted material at the electron microscopic level demonstrated that olfactomedin was present in secretory granules of sustentacular cells and in the mucus which lines the olfactory epithelium. Olfactomedin appears to be confined exclusively to the lower, viscous, granular layer of the mucus immediately adjacent to the surface of the neuroepithelium. Our conclusions from our immunohistochemical studies were as follows:

Olfactomedin is produced by sustentacular cells and Bowman's glands and deposited into the lower mucus layer on top of the chemosensory neuroepithelium in the vicinity of the proximal regions of olfactory cilia.

4. Glycosylation of olfactomedin

To study glycosylation of olfactomedin we used rabbit antibodies against the mature protein and antibodies against the synthetic peptide which corresponds to the N-terminal region adjacent to an N-linked glycosylation site (see above). The latter antibodies cannot bind when this site is glycosylated and reveal precursors of olfactomedin in the perinuclear regions of Bowman's glands. This staining contrasts with that observed with antibodies against the mature protein (mostly directed against carbohydrate moieties) which stain the ciliary surface and the rims of acinar cells. Enzymatic deglycosylation of olfactomedin showed stepwise removal of carbohydrate groups and revealed a 51 kd deglycosylated form. Later studies identified six potential N-linked glycosylation sites in the complete sequence of olfactomedin. We derive from our studies on the glycosylation of olfactomedin the following conclusion:

Prior to secretion, most, if not all, of the six potential N-linked glycosylation sites of olfactomedin are glycosylated with carbohydrate moieties of about 8-10 sugar residues devoid of sialic acid, but containing N-acetylglucosamine and terminal galactose.

5. Molecular cloning of olfactomedin

We extracted mRNA from olfactory epithelium of *Rana catesbeiana* and constructed a cDNA library in Lambda ZAPII. Using a 16-fold degenerate oligonucleotide probe corresponding to part of olfactomedin's N-terminal sequence, we isolated and sequenced two clones. Figure 1 shows the nucleotide sequence and deduced amino acid sequence of a 2.0 kb clone. It contains 93 bases of 5'-untranslated region, 1,392 bases of coding region, and 517 bases of 3'-untranslated region including a poly-A tail. The 464 amino acid open reading frame includes a 16 amino acid leader peptide with the initiating methionine. The mature protein which consists of 448 amino acids has a calculated molecular weight of 50,873, which is in close agreement with the experimentally observed size of deglycosylated olfactomedin (51,000; see above). Olfactomedin contains six potential N-linked glycosylation sites, evenly distributed

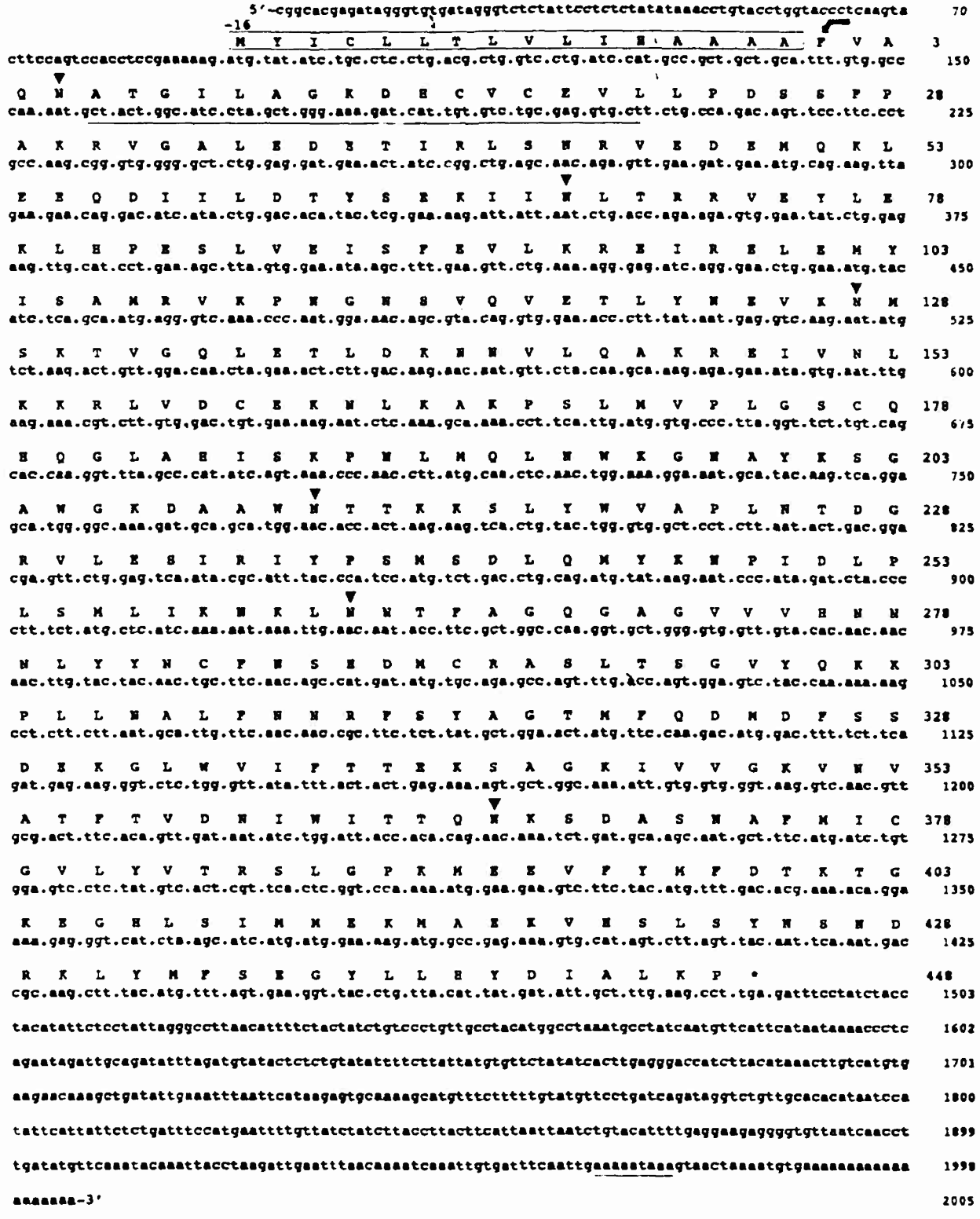

Figure 1: Amino acid sequence of olfactomedin deduced from the nucleotide sequence of a corresponding cDNA clone. The N-terminus of the mature protein was identified by comparing the translated open reading frame to 21 amino acids of the N-terminal sequence of purified olfactomedin obtained by conventional protein sequencing. The N-terminal amino acid of mature olfactomedin is the phenylalanine indicated by the arrow. It is preceded by a 16 amino acid-long leader peptide, indicated by the boxed area, which contains the initiating methionine. Olfactomedin contains six potential N-linked glycosylation sites, indicated by arrowheads. The nucleotide sequence which corresponds to the degenerate oligonucleotide probe initially used to screen the cDNA library is underlined. A characteristic polyadenylation signal near the 3'-end is also underlined. Amino acids are indicated by single letter code. The asterisk indicates the termination codon.

throughout its sequence, and seven cysteines which can form disulfide bonds. Comparison of olfactomedin's amino acid sequence to sequences stored in the GenBank, EMBL and Swissprot databases did not reveal any significant homologies. Thus, the sequence of this novel olfactory tissue-specific protein is apparently unique.

In agreement with the previously observed abundance of olfactomedin in olfactory tissue (see above), we found 0.6% positive clones when we screened the olfactory cDNA library with a random primer-labeled probe corresponding to olfactomedin's open reading frame. Hybridization of olfactomedin-encoding cDNA to restriction endonuclease-treated genomic DNA indicates that olfactomedin is likely to be the product of a single gene. Northern blot analysis confirms that mRNA for olfactomedin is produced exclusively in olfactory tissue. These experiments lead us to the following conclusion:

A single gene expressed only in olfactory tissue encodes olfactomedin, a secretory glycoprotein with a unique amino acid sequence which is produced in massive quantities and deposited at the chemosensory surface.

6. A structural model for olfactomedin

Chou-Fasman analysis of olfactomedin's amino acid sequence predicts a predominantly alpha-helical structure of the N-terminal 150 amino acids of olfactomedin, mostly beta-sheet configuration near its carboxyl terminal third and a region characterized by several turns in the center. This analysis and previous biochemical observations support a structural model in which cysteines at positions 160, 177, 283 and 290 form intramolecular disulfide bonds, as shown in figure 2. This generates a large central "head" domain consisting of a loop of 106 amino acids, a "neck" region consisting of a loop of 26 amino acids and two "legs" of equal length. As mentioned above, olfactomedin forms disulfide-linked polymers which can be reduced to monomers. However, even under reducing conditions, a relatively stable 120 kd dimer is observed, which may result from the formation of adjacent intermolecular disulfide bridges via cysteines at positions 16 and 18. This is in line with the observation that a site-directed antibody against a synthetic peptide corresponding to amino acids 7-16, described above, can bind only to the unobstructed monomer. The remaining cysteine at position 377 on the C-terminal leg is proposed to form an intermolecular disulfide bond with its counterpart on a neighboring molecule to give rise to disulfide-linked polymers as observed experimentally and as illustrated in figure 3. The identification of a monoclonal antibody that recognizes a carbohydrate determinant and reacts only with dimers and polymers, but not with monomers (see above) supports this model, since it requires the proposed symmetry of apposed carbohydrate attachment sites to form antigenic determinants through interactions between sugar groups present on adjacent polypeptide chains. The conclusion of our analysis of the structure of olfactomedin can be summarized by the following statement:

Olfactomedin is a novel olfactory tissue-specific extracellular matrix protein which forms disulfide-linked polymers that represent the primary building blocks for the architecture of the lower mucus layer of olfactory neuroepithelium.

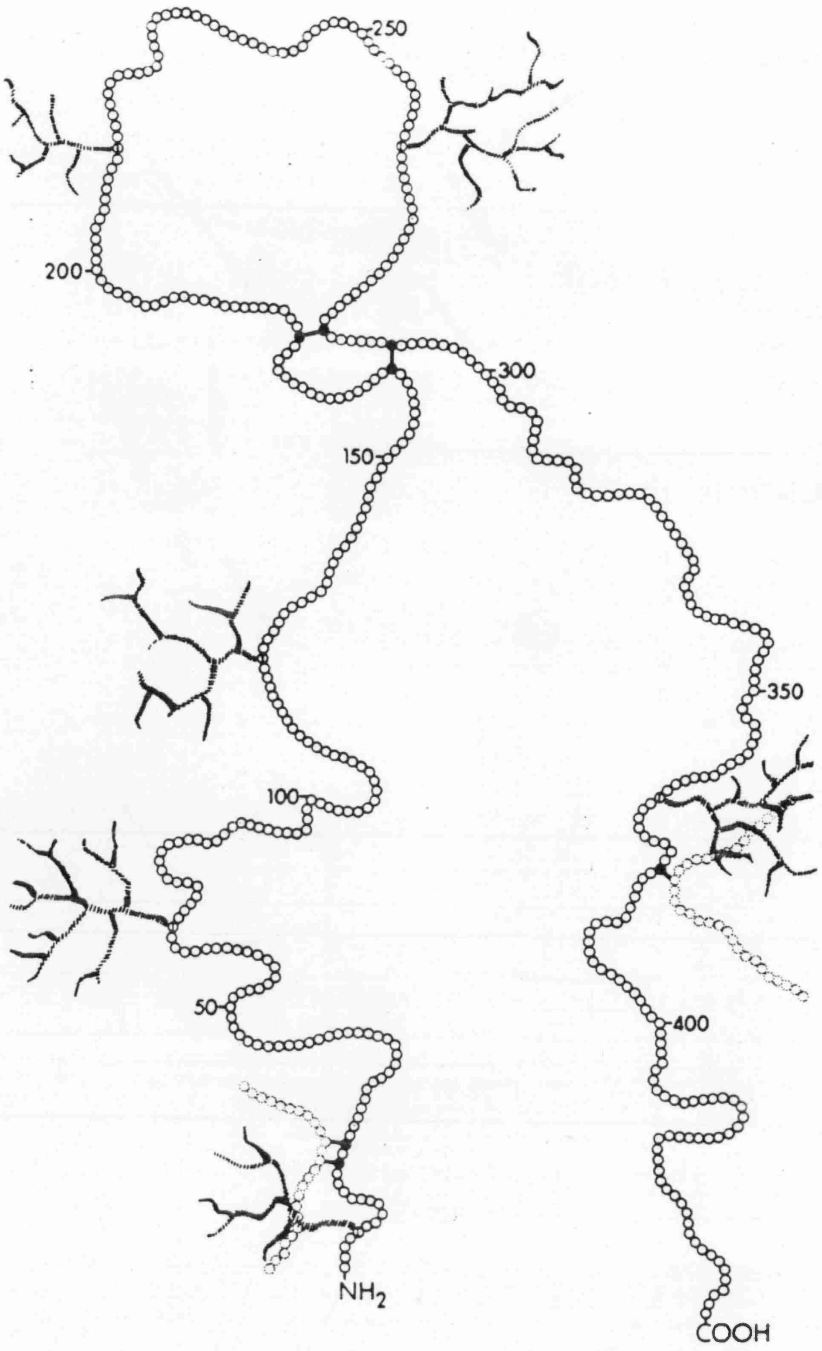

Figure 2: A model for the structure of olfactomedin. Each amino acid is represented by a circle. Cysteines are represented by solid circles and attachment sites for carbohydrates are indicated at positions 5, 69, 137, 212, 263, and 367. parts of neighboring olfactomedin molecules are depicted by dotted lines.

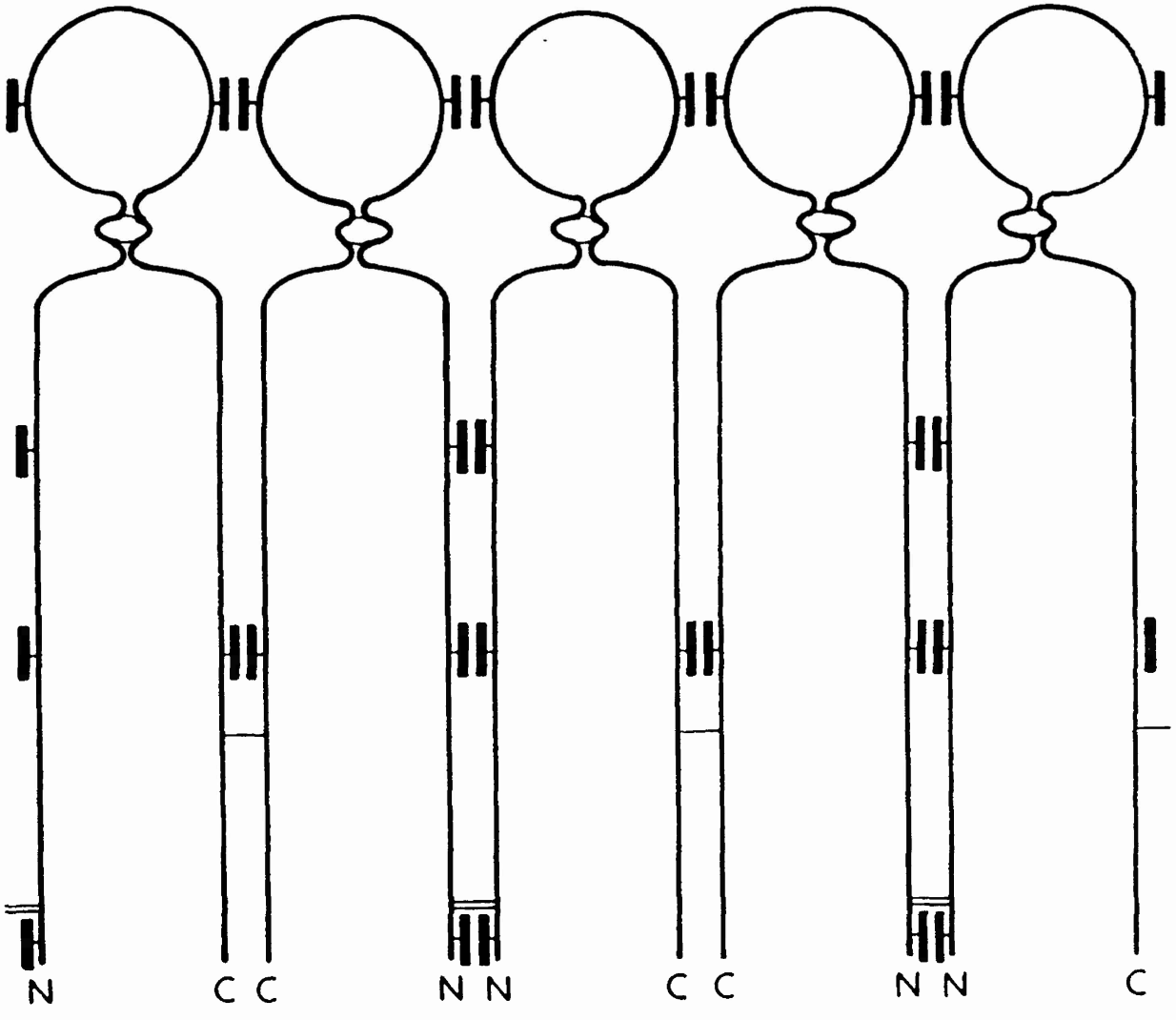

Figure 3: Diagrammatic representation of disulfide-linked polymers of olfactomedin. Olfactomedin may generate long disulfide-linked polymers which represent the building blocks for the olfactory extracellular matrix. Solid rectangles indicate carbohydrate moieties.

7. Is olfactomedin a dendritic differentiation factor?

The extracellular mucous matrix of olfactory neuroepithelium is a highly organized structure in intimate contact with chemosensory cilia that house the olfactory transduction machinery. Olfactory neurons are continuously replaced throughout the lifespan of the animal from neurogenic stem cells in the base of the epithelium. The axons of newly generated olfactory neurons establish functional connections with postsynaptic cells in the olfactory bulb of the brain while their dendrites grow toward the nasal lumen; when they reach the chemosensory surface they form a dendritic knob that carries a group of chemosensory cilia. The signals which trigger dendritic differentiation of developing neurons are unknown, but most likely reside in the mucus which lines the chemosensory surface.

Elucidation of the primary structure of olfactomedin establishes this protein as the newest member of an expanding group of extracellular matrix proteins which includes N-CAM, laminin, fibronectin and proteoglycans. The monomer of olfactomedin is smaller than any of these proteins, but olfactomedin, nevertheless, can generate an extracellular matrix through the formation of polymers. The size of its proposed intramolecular loop and the conformational importance of its disulfide bonds are reminiscent of the immunoglobulin superfamily, which includes cell adhesion molecules like N-CAM. The tissue-specific expression of olfactomedin contrasts with the widespread occurrence of other extracellular matrix proteins. N-CAM, laminin, fibronectin and proteoglycans have all been implicated in adhesion, maintenance, migration, growth or differentiation of neurons. Olfactory neuron-specific genes, including those that encode olfactory marker protein and transduction components such as adenylate cyclase type III, G_{olf}, and the cyclic nucleotide-activated channel are all under the control of a tissue-specific promoter, which is activated by a recently identified transcription factor, designated "olf-1". It is likely that olfactomedin triggers the expression of olf-1 when a newly emerging olfactory dendrite reaches the chemosensory surface and that this signal induces differentiation of the dendritic knob and concomitant expression of the olfactory transduction machinery. The availability of a cDNA clone that encodes olfactomedin now enables us in future experiments to validate this hypothesis, which is illustrated in figure 4 and which can be summarized as follows:

Olfactomedin is an olfactory tissue-specific extracellular matrix protein which makes close contact with the highly specialized chemosensory surface of the olfactory neuroepithelium and which, by analogy to other extracellular matrix proteins, may contribute to the maintenance, growth and differentiation of olfactory cilia.

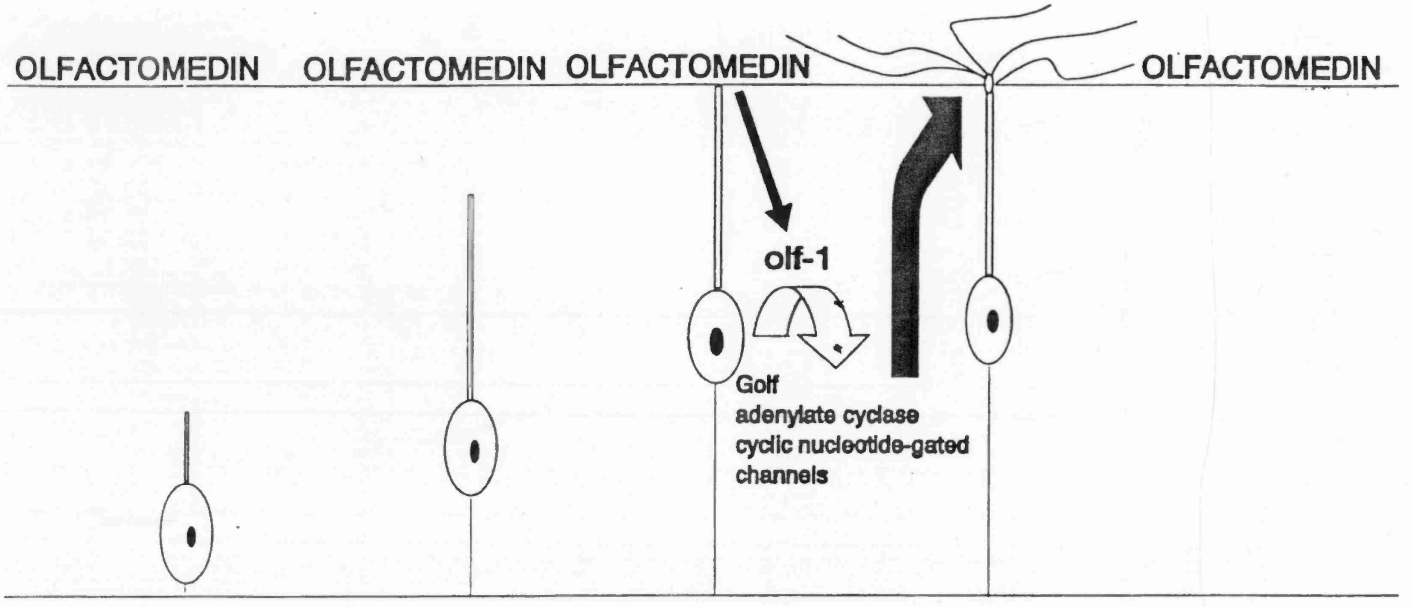

Figure 4: Schematic representation of the "olfactomedin hypothesis". It is proposed that olfactomedin may deliver to the emerging dendrite of a newly developing olfactory neuron a differentiation signal that triggers the expression of olf-1, a recently discovered transcriptional activator which controls expression of olfactory neuron specific genes, including components of the olfactory transduction machinery.

C. Publications

1. Journal articles

1. Anholt, R. R. H., Petro, A. E. and Rivers, A. M. (1990) Identification of a group of novel membrane proteins unique to chemosensory cilia of olfactory receptor cells. *Biochemistry* **29**: 3366-3373.

2. Anholt, R. R. H. and Rivers, A. M. (1990) Olfactory transduction: Crosstalk between second messenger systems. *Biochemistry* **29**: 4049-4054.

3. Snyder, D. A., Rivers, A. M., Yokoe, H., Menco, B. Ph. M. and Anholt, R. R. H. (1991) Olfactomedin: Purification, characterization and localization of a novel olfactory glycoprotein. *Biochemistry* **30**: 9143-9153.

4. Bal, R. S. and Anholt, R. R. H. (1993) Formation of the extracellular mucous matrix of olfactory neuroepithelium: Identification of partially and non-glycosylated precursors of olfactomedin. *Biochemistry*, in press.

5. Yokoe, H. and Anholt, R. R. H. (1993) Molecular cloning of olfactomedin: A novel extracellular matrix protein specific to olfactory neuroepithelium. *Proc. Natl. Acad. Sci. U.S.A.*, submitted.

2. Invited reviews

1. Anholt, R. R. H. (1991) Odor recognition and olfactory transduction: The new frontier. *Chemical Senses* **16**: 421-427.

2. Anholt, R. R. H. (1993) Molecular neurobiology of olfaction. *Critical Rev. Neurobiol.*, in press.

3. Book chapters

1. Anholt, R. R. H. (1992) Molecular aspects of olfaction. In: *The Science of Olfaction* (eds. M. Serby and K. Chobor) Chapter 3, pp.51-79, Springer-Verlag, New York, NY.

2. Menini, A.and Anholt, R. R. H. (1992) Cyclic nucleotide-activated channels. In: *Endocrinology and Metabolism. Progress in Research and Clinical Practice. Vol. 7. Ion Channels and Ion Transport. Structure, Function and Endocrine Regulation* (eds. M. F. Walsh and P. P. Foa), in press, Springer-Verlag, New York, NY.

4. Abstracts

Ten abstracts describing ARO-sponsored research were presented at various national and international meetings, including the Association for Chemoreception Sciences meetings in 1989, 1990, 1991 and 1993, the Society for Neurosciences in 1992, the American Society for

Neurochemistry in 1991 and 1993, the Third IBRO World Congress in 1991, the Columbus Conference on Physics of Matter (Genova, Italy) in 1992, and the U. S. Army CRDEC conference in 1991.

D. Personnel

The Principal Investigator, Dr. Robert R. H. Anholt, a postdoctoral fellow, Dr. David A. Snyder, and two research technicians, Ann M. Rivers and Hiroko Yokoe, were the primary participants in this project. Five undergraduate independent study students, Kevan Mann, Dragon Dimitrov, Lydia Coulter, Alison Steube and Rupinder Bal, and three undergraduate work-study students, Sosena Kebede, Karen Ulmer and Cristina Davis have also participated in this project.