Formation of the Extracellular Mucous Matrix of Olfactory Neuroepithelium: Identification of Partially Glycosylated and Nonglycosylated Precursors of Olfactomedin†

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ABSTRACT: Olfactomedin is the major glycoprotein of the extracellular mucous matrix of frog olfactory neuroepithelium. It is responsible for the primary architecture of this extracellular matrix by forming via intermolecular disulfide bonds polymers, which are covered with evenly spaced carbohydrate groups. To study glycosylation of olfactomedin, we raised antibodies against the mature protein and antibodies against a region adjacent to an N-linked glycosylation site near its amino terminus. The latter antibodies cannot bind when this site is glycosylated and reveal precursors of olfactomedin in the perinuclear regions of Bowman's glands. In contrast, antiserum against the mature protein stains acinar regions of glands and the ciliary surface. Enzymatic deglycosylation of olfactomedin shows stepwise removal of carbohydrate and reveals a 51-kDa deglycosylated form. Our results indicate that, 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.

Recognition of chemical signals, which provide, among others, information about the localization of food and the availability of reproductive partners, is essential for the survival of most animals. This important function is mediated via the olfactory system, where the nature and concentration of the chemical stimulus are encoded in a pattern of neural activity which arises from differential activation of olfactory receptor neurons. These neurons are bipolar cells, which send an axon to the olfactory bulb of the brain and a dendrite to the nasal lumen. The dendrite forms a dilatation, the dendritic knob, which carries a group of chemosensory cilia. These cilia are the sites where odor recognition and olfactory transduction occur (Getchell et al., 1984; Getchell, 1986; Lancet, 1986; Anholt, 1989, 1992).

The olfactory neuroepithelium is lined with a layer of aqueous mucus which odorants must partition into and traverse in order to reach the chemosensory membrane (Getchell et al., 1984; Carr et al., 1990). The composition of the mucus may influence access of odorants to the chemosensory membrane and affect the threshold for odorant recognition (Senf et al., 1980; Getchell et al., 1984; Lancet, 1986). Odorant binding proteins have been identified in several species as prominent components of the mucus (Pelosi et al., 1982; Bignetti et al., 1985; Pevsner et al., 1985, 1986, 1988a; Dear et al., 1991). These proteins are members of a family of hydrophobic ligand carrier proteins (Pevsner et al., 1988b) and are secreted by Bowman's glands of the neuroepithelium (Pevsner et al., 1986; Lee et al., 1987) or, in rat, by the lateral nasal gland (Pevsner et al., 1988a). In addition to facilitating access of odorants to the chemosensory membrane, the mucus contains a diverse array of immunoglobulins and bacteriocidal proteins (Getchell & Getchell, 1991; Dear et al., 1991; Mellert et al., 1992) and serves as an immune barrier, which prevents microorganisms from gaining access to the central nervous system via the olfactory nerve (Tomlinson & Esiri, 1983; Lundh et al., 1987; Perlman et al., 1989). Finally, the mucus may play an important role in promoting the growth and integrity of the apical dendritic specializations of olfactory neurons and may contain as yet unidentified growth factors.

The olfactory mucus consists of two distinct layers, a superficial aqueous layer, which has a granular appearance on electron micrographs, and a deeper, more viscous layer, which has a smooth appearance (Reese, 1965; Getchell et al., 1984; Carr et al., 1990; Snyder et al., 1991; Menco & Farbman, 1992). Localization of components of the mucus with lectins and antibodies has demonstrated that the mucus represents a highly structured extracellular matrix, of which the different constituents are organized in distinct compartments (Foster et al., 1991, 1992; Menco & Farbman, 1992).

One of the major structural components of the olfactory neuroepithelium of frog is a 57-kDa, olfactory tissue-specific glycoprotein, which has been named "olfactomedin" and which has been estimated to represent up to 5% of total tissue protein in membrane homogenates from frog olfactory tissue (Snyder et al., 1991). It is produced in massive quantities by sustentacular cells and Bowman's glands and secreted into the mucus, where it remains confined to the deeper mucus layer. Olfactomedin via intermolecular disulfide bonds forms dimers and larger polymers, which represent the primary building blocks of the extracellular mucous matrix (Snyder et al., 1991; H. Yokoe and R. Anholt, unpublished observations).

Olfactomedin, like most components of the mucus, is glycosylated (Strous & Dekker, 1992). Studies with monoclonal antibodies have demonstrated that olfactomedin's carbohydrate dominates its immunogenicity (Anholt et al., 1990; Snyder et al., 1991). Wheat germ agglutinin (WGA)[1] and *Ricinus communis* agglutinin I (RCA) bind to olfactomedin, which indicates the presence of *N*-acetylglucosamine and terminal galactoside sugars. Since olfactomedin is one of only a few proteins of olfactory tissue that binds to RCA,

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[1] Abbreviations: WGA, wheat germ agglutinin; RCA, *Ricinus communis* agglutinin I.

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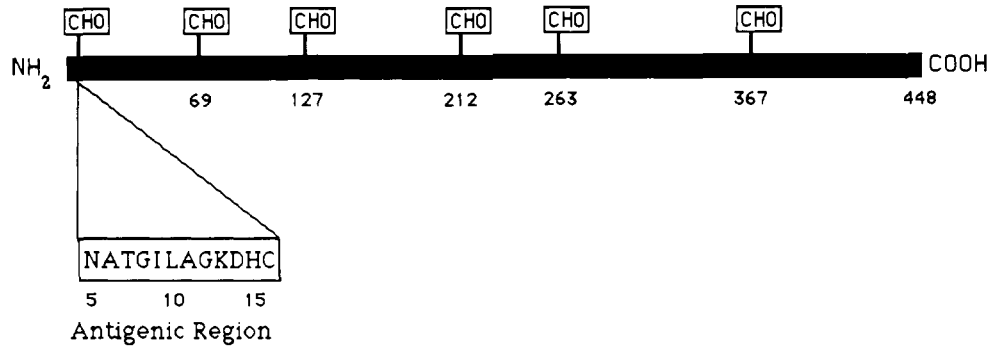


FIGURE 1: Schematic representation of the distribution of N-linked glycosylation sites in olfactomedin. Olfactomedin contains six evenly spaced consensus N-linked glycosylation sites (CHO). Carbohydrate attached to these sites dominates its immunogenicity and accounts for most of the immunoreactivity in antiserum raised against purified olfactomedin. The diagram shows the sequence TGILAGKDH between positions 7 and 15 which is recognized by a site-directed antibody, described in the text. This sequence is adjacent to a glycosylation site, which includes asparagine 5. The cysteine at position 16 is thought to participate in the formation of olfactomedin dimers.

it could be purified to homogeneity by affinity chromatography using this lectin (Snyder et al., 1991). Recently, the amino acid sequence of olfactomedin has been deduced from the nucleotide sequence of a cDNA clone and revealed a protein which contains six potential N-linked consensus glycosylation sites evenly spaced throughout its sequence (H. Yokoe and R. Anholt, unpublished observations; Figure 1).

To study the glycosylation of olfactomedin, we raised two antisera, one against the mature protein and another against a synthetic peptide, which corresponds to a consensus N-linked glycosylation sequence adjacent to aspargine 5 (Figure 1). This site is inaccessible to the antibody when carbohydrate is attached to asparagine 5. Thus, this antiserum, in contrast to the antiserum raised against the mature protein, recognizes only precursor forms of olfactomedin, which lack carbohydrate on at least one N-linked glycosylation site. Using these antibodies, we report the identification of precursors of olfactomedin in frog olfactory neuroepithelium and the detection of partially and completely deglycosylated forms of olfactomedin after enzymatic deglycosylation.

MATERIALS AND METHODS

Preparation of Olfactory Membranes. Bullfrogs, *Rana catesbeiana*, were obtained from Acadian Biological (Rayne, LA), Lemberger (Oshkosh, WI), and Amphibians of North America (Nashville, TN). Frogs were killed by decapitation, and the dorsal and ventral olfactory epithelia were dissected. Olfactory cilia were detached from the epithelia by calcium shock and subjected to sucrose gradient centrifugation, as described previously (Anholt et al., 1986). After deciliation the remaining olfactory membranes were homogenized in ice-cold Ringer's solution (2 mM HEPES, 112 mM NaCl, 3.4 mM KCl, and 2.4 mM $NaHCO_3$, pH 7.4) by intermittent 30-s bursts of homogenization in a Tekmar Tissumizer and filtered through a double layer of surgical gauze. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. The cilia preparations and the deciliated olfactory membranes were divided into aliquots and stored at −80 °C.

Preparation of Antisera. The polyclonal rabbit antiserum against purified olfactomedin used in these studies has been described previously (Snyder et al., 1991). To make the site-directed antibody used in these experiments, the decapeptide TGILAGKDHD was synthesized by Research Genetics, Inc. (Huntsville, AL), and conjugated to ovalbumin (Sigma Chemical Co., St. Louis, MO) via a standard glutaraldehyde cross-linking procedure (Reichlin, 1980). A 10–12-week-old female New Zealand White rabbit, purchased from Myrtle's Rabbitry (Thompson Station, TN) and maintained in a pathogen-free facility, was immunized in the muscles along the back with 160 μg of the conjugate suspended in 10 mM sodium phosphate buffer and 150 mM NaCl, pH 7.4, and emulsified in an equal volume of complete Freund's adjuvant. The rabbit was boosted five times at biweekly intervals with antigen emulsified in incomplete Freund's adjuvant, and antiserum was collected 4 days after the final booster injection. Titers of antisera were monitored by ELISA, as described previously (Snyder et al., 1991).

Polyacrylamide Gel Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on 10% slab gels in the discontinuous buffer system of Laemmli (1970) after treatment of the samples with 2-mercaptoethanol. Prestained standards were obtained either from Sigma Chemical Co. or from Bio-Rad Laboratories (Richmond, CA). The former include triosephosphate isomerase (38.9 kDa), lactic dehydrogenase (44.6 kDa), fumarase (64.4 kDa), pyruvate kinase (80.6 kDa), fructose-6-phosphate kinase (98 kDa), β-galactosidase (119 kDa), and α-macroglobulin (211 kDa), whereas the latter include soybean trypsin inhibitor (24 kDa), carbonic anhydrase (33 kDa), ovalbumin (47 kDa), and bovine serum albumin (84 kDa). Electrophoretic transfer onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and immunoblotting were performed exactly as described previously (Anholt et al., 1987). The membranes were blocked by incubation for at least 30 min in 50 mM Tris-HCl, 1 mM EDTA, 0.1% gelatin, and 0.1% Triton X-100 (Boehringer Mannheim, Indianapolis, IN), pH 7.5. The nitrocellulose membrane was, when desired, cut into strips, which were incubated for 1–2 h at 4 °C with anti-olfactomedin antiserum, preimmune serum, or biotinylated RCA (Vector Laboratories, Burlingame, CA) at the desired concentration in 10 mM sodium phosphate buffer, 150 mM NaCl, and 0.05% (v/v) Tween 20 (Aldrich Chemical Co., Milwaukee, WI), pH 7.5. Bound antibodies were visualized with biotinylated goat anti-rabbit antibody complexed to avidin and biotinylated horseradish peroxidase (Vector Laboratories) using 3,3′-diaminobenzidine (Sigma Chemical Co.) as chromogenic substrate in the presence of 0.015% (v/v) H_2O_2.

Immunohistochemistry. Immunohistochemical localization of olfactomedin and its precursors was performed on 6 μm thick coronal sections of formalin-fixed decalcified nasal sacs of *R. catesbeiana* embedded in paraffin and mounted on chromalum–gelatin-coated slides. The sections were deparaffinized in xylene and rehydrated through a graded series of alcohols from absolute ethanol to water. The sections were

washed with 10 mM sodium phosphate buffer and 150 mM NaCl, pH 7.4, and incubated for 90 min at ambient temperature with anti-olfactomedin antisera in the same buffer, supplemented with 0.05% Tween 20. Bound antibodies were visualized as described above with biotinylated goat anti-rabbit antibody complexed to avidin and biotinylated horseradish peroxidase in the presence of 0.015% (v/v) H_2O_2 and 3,3′-diaminobenzidine as the chromogenic substrate. The stained sections were observed under a Nikon Optiphot microscope equipped with an FX-35 camera and photographed with Kodak Ektar 100 color film. To evaluate nonspecific staining, sections were incubated with preimmune sera instead of antisera, or incubation with the secondary antibody was deleted from the staining procedure. Control sections were processed together with the sections of interest in the identical solutions for the same incubation periods.

Enzymatic Deglycosylation of Olfactomedin. To remove N-linked carbohydrate groups from olfactomedin, olfactory cilia were suspended in 10 mM potassium phosphate buffer, 50 mM EDTA, and 0.1% sodium dodecyl sulfate, pH 7.0, at a concentration of 6.0 mg of protein/mL and boiled for 2 min. The sample was cooled to room temperature, and 1-*O*-*n*-octyl β-D-glucopyranoside (Boehringer Mannheim) was added to a final concentration of 0.3%. The final volume of the reaction mixture was 100 μL, containing 300 μg of ciliary protein in 10 mM potassium phosphate buffer, 50 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.3% 1-*O*-*n*-octyl β-D-glucopyranoside, pH 7.0. Deglycosylation was initiated with the addition of 0.15 unit of endoglycosidase F/*N*-glycosidase F (*N*-glycanase) (Boehringer Mannheim; Elder & Alexander, 1982; Tarentino et al., 1985), and the deglycosylation reaction was allowed to proceed at 37 °C. To stop the reaction, aliquots were removed after 5, 10, 15, 30, 60, and 120 min and immediately placed in a boiling water bath for 2 min. The samples were analyzed by polyacrylamide gel electrophoresis followed by immunoblotting with anti-olfactomedin antisera, preimmune serum, or biotinylated RCA, as described above. Just before the addition of enzyme, a control aliquot was collected from the reaction mixture and incubated for 2 h at 37 °C alongside the tube in which the deglycosylation reaction proceeded. This nontreated control sample was placed in the boiling water bath at the same time as the final sample, which was collected after 120 min.

RESULTS

Immunoreactivity of Antisera against Olfactomedin. Previously, we described an antiserum raised against purified olfactomedin (Snyder et al., 1991). On western blots this antiserum reveals in homogenates from whole olfactory tissue a prominent diffuse band centered around 57 kDa, but ranging between 53 and 61 kDa, which represents olfactomedin (Figure 2, lane 1). Immunoreactivity is also detected around 120 kDa due to residual dimers of olfactomedin. Partial sequence analysis of the N-terminus of purified olfactomedin by conventional protein sequencing procedures yielded the sequence FVAQXATGILAGKDHDVXEVL. We raised an antiserum against the synthetic decapeptide TGILAGKDHD contained within this sequence. Subsequent identification of a cDNA clone encoding olfactomedin showed that position 5 is asparagine (N), which forms part of the consensus glycosylation sequence NAT, and that position 16, like position 18, is a cysteine (C) instead of an aspartate (Figure 1). Despite the difference between the terminal amino acid of the synthetic peptide (D) and its corresponding region of olfactomedin (C), the antiserum raised against the synthetic peptide recognized

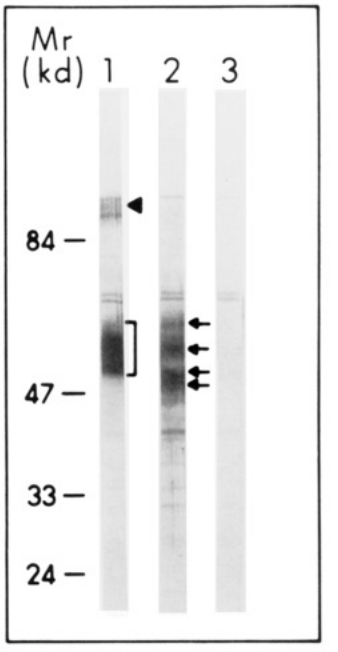
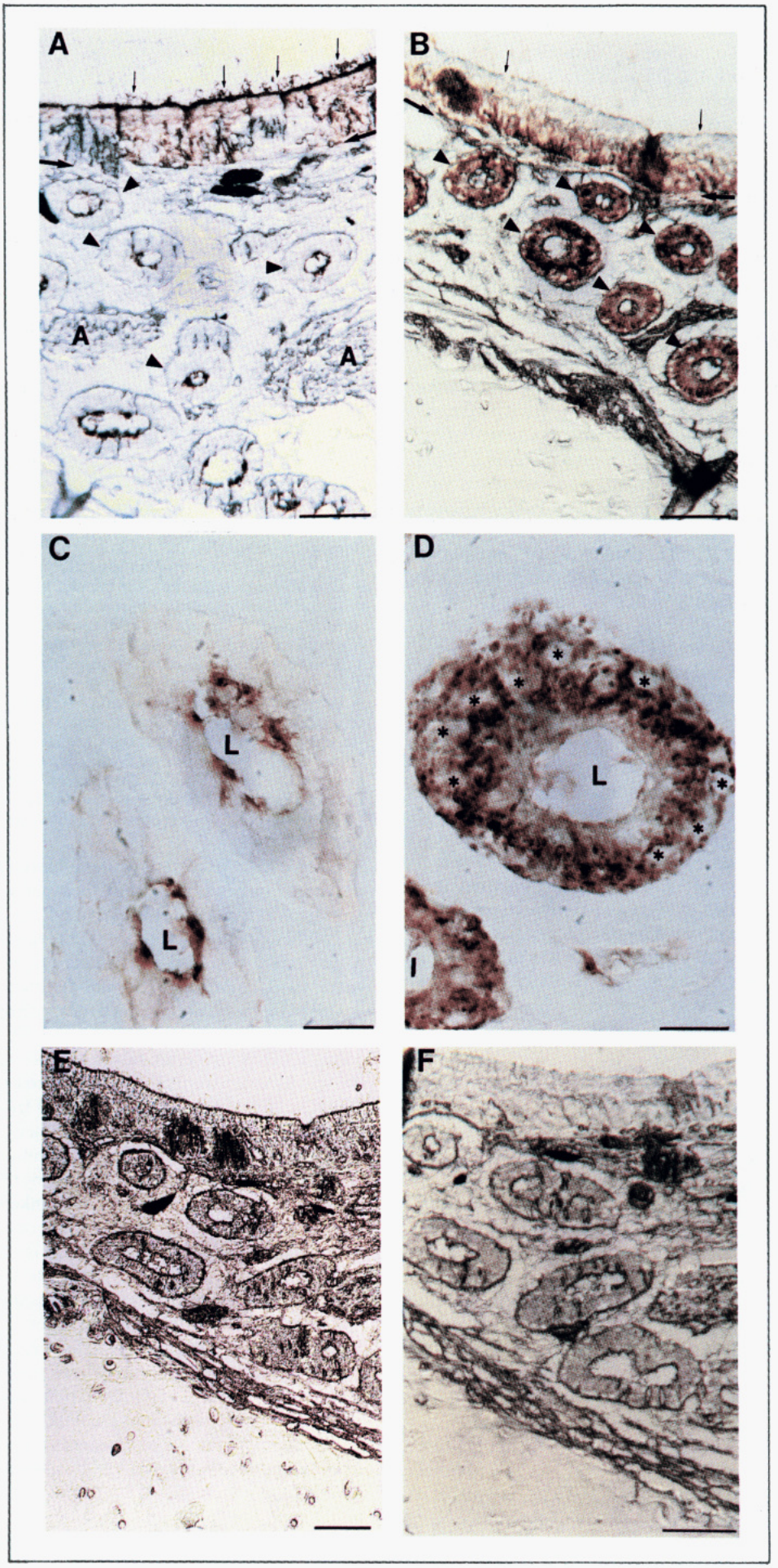


FIGURE 2: Identification of olfactomedin in olfactory membranes by immunoblotting. Deciliated olfactory membranes were subjected to polyacrylamide gel electrophoresis and electrophoretic transfer onto a nitrocellulose membrane. The membrane was cut into strips, each of which contained approximately 20 μg of protein, and the strips were stained with a 1000-fold dilution of ammonium sulfate fractionated antiserum raised against purified olfactomedin (lane 1), a 500-fold dilution of antiserum raised against the synthetic peptide TGILAGKDHD corresponding to the region of olfactomedin illustrated in Figure 1 (lane 2), and a 500-fold dilution of preimmune serum from the same rabbit used to generate the site-directed antibody (lane 3). Immunoreactive species are indicated by the bracket in lane 1 and by arrows in lane 2. Bands at 72 and 74 kDa are also detected in lane 3 and are, therefore, considered nonspecific. Immunoreactive material of higher molecular weight in lane 1 (arrowhead) most likely represents residual dimers of olfactomedin.

olfactomedin. However, western blots revealed a marked difference in banding patterns between this antiserum and antiserum raised against the purified protein (Figure 2, lane 2). Although staining appeared in the same region, the diffuse band pattern observed with antiserum against the purified protein was, in the case of the site-directed antiserum, broken down into at least four distinct bands with apparent molecular masses of approximately 50, 52, 56–59, and 62 kDa. In contrast to the antiserum raised against the purified protein, the site-directed antibody never stained residual dimers at 120 kDa. Preimmune serum obtained from the same rabbit in which the antiserum against the synthetic peptide was raised at the same dilution did not reveal immunoreactivity (Figure 2, lane 3). Since the region recognized by the antiserum raised against the synthetic peptide is flanked by a consensus glycosylation site and by a cysteine proposed to be involved in intermolecular dimer formation (Figure 1), the observed pattern of immunoreactivity suggests that this region is inaccessible to the antibody when the protein is dimerized or when a carbohydrate group is present on asparagine 5. Thus, only nonglycosylated or partially glycosylated forms of olfactomedin carrying carbohydrate moieties on any of the other potential N-linked glycosylation sites are detected. This leads to clearer resolution of bands of individual partially glycosylated precursors of olfactomedin, which are obscured when olfactomedin is visualized by antiserum raised against the purified protein. Antiserum raised against the purified protein shows equally strong immunoreactivity with preparations of partially purified olfactory cilia as with membrane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homogenates from the entire olfactory epithelium. In contrast, the site-directed antiserum shows hardly any reactivity with olfactory cilia preparations at the same concentrations at which reactivity can readily be detected with homogenates from the entire olfactory neuroepithelium, in line with immunohistochemical observations, described below.

Immunohistochemical Localization of Precursors of Olfactomedin in Frog Olfactory Tissue. To localize olfactomedin and its precursors in situ, we stained coronal sections through the olfactory cavity of *R. catesbeiana* with both of our antisera. Antiserum raised against purified olfactomedin stains the ciliary surface of the olfactory neuroepithelium and the acinar regions of submucosal glands, as described previously (Figure 3, panels A and C; Snyder et al., 1991). In contrast, antiserum raised against the synthetic peptide reveals granular staining in the perinuclear regions of the acinar cells of Bowman's glands (Figure 3, panels B and D). This antibody does not show staining in the vicinity of the lumina of the glands. Neither is immunoreactivity detected at the ciliary surface of the neuroepithelium (Figure 3, panel B). Some reactivity in the lower third of the neuroepithelium may reflect staining in or near the basal processes of sustentacular cells. The intense staining of this antiserum with immunoreactive material exclusively in the perinuclear regions of acinar cells suggests that it recognizes endoplasmic reticulum- and Golgi apparatus-associated precursor forms of olfactomedin which lack carbohydrate on asparagine 5 and which have not yet undergone dimerization. Since antiserum raised against purified olfactomedin recognizes predominantly carbohydrate determinants (Anholt et al., 1990), our observations indicate that virtually all of the olfactomedin synthesized by Bowman's glands is posttranslationally modified before it is transported to the lumina of the acinar cells and secreted to become part of the extracellular mucous matrix. No staining is observed with the preimmune counterparts of either antiserum at equivalent dilutions (Figure 3, panels E and F).

Enzymatic Deglycosylation of Olfactomedin. To further characterize the glycosylation of olfactomedin, we subjected preparations of olfactory cilia to enzymatic deglycosylation with endoglycosidase F/*N*-glycosidase F (*N*-glycanase; Elder & Alexander, 1982; Tarentino et al., 1985). Within 15 min this treatment completely abolishes staining of olfactomedin with biotinylated RCA (Figure 4, panel A). Removal of carbohydrate from olfactomedin proceeds in a stepwise fashion during which at least four intermediate forms of partially deglycosylated olfactomedin appear between 52.5 and 59 kDa when visualized with antiserum raised against the purified protein (Figure 4, panel B). During this deglycosylation process after 10 min a distinct species of approximately 51 kDa appears, which remains stable even after 2 h of enzymatic deglycosylation (Figure 4, panel B, arrow). The apparent electrophoretic migration of this band is identical to the molecular weight of the nonglycosylated protein, predicted from its amino acid sequence (50 873; H. Yokoe and R. Anholt, unpublished observations). Staining of the products of the deglycosylation reaction with antiserum raised against the synthetic peptide reveals the same deglycosylated 51-kDa band, which accumulates with an identical time course (Figure 4, panel C, arrow). This antiserum needs to be used at high concentrations to detect native olfactomedin in olfactory cilia prior to enzymatic treatment (Figure 4, panel C). At these concentrations this antiserum generates nonspecific background artifacts, which appear also in a lane to which only sample buffer without ciliary protein has been applied (Figure 4, panel C, lane B). Although these artifacts obscure some of the partially deglycosylated reaction products, observed in panel B of Figure 4, they still allow visualization of native olfactomedin and its fully deglycosylated 51-kDa precursor. These immunoreactive bands are not observed when the samples are stained with preimmune serum (Figure 4, panel D).

The difference between the apparent molecular weight of the mature protein and that of its deglycosylated counterpart indicates that approximately 15% of the molecular weight of olfactomedin is contributed by carbohydrate moieties. They also suggest that most, if not all, of its six potential N-linked glycosylation sites carry carbohydrate. Thus, we can estimate that each carbohydrate moiety would consist of 8–10 sugar residues.

DISCUSSION

The mucus layer of the olfactory neuroepithelium is a highly organized extracellular matrix (Foster et al., 1991, 1992; Menco & Farbman, 1992). Olfactomedin, at least in frog, is one of the major structural components of the deep mucus layer, which is in intimate contact with the surface of the chemosensory epithelium (Snyder et al., 1991; Menco & Farbman, 1992). Like other components of the mucus, olfactomedin is glycosylated, and this has given us the opportunity to characterize in detail the glycosylation of one well-defined major component of the extracellular mucous matrix of the olfactory neuroepithelium.

Using antibodies against the purified protein and against a sequence adjacent to an N-linked glycosylation site near its amino terminus, we have been able to localize mature forms as well as nonglycosylated and partially glycosylated precursors of olfactomedin (Figure 3). Precursors of olfactomedin are detected in the perinuclear regions of acinar cells of Bowman's glands, most likely associated with the endoplasmic reticulum and Golgi apparatus (Roth, 1991). Failure of the antiserum raised against the synthetic peptide to stain the ciliary surface and the luminal regions of the acini (Figure 3, panels B and D) suggests that the mature protein is fully glycosylated, at least on asparagine 5, prior to being exported. Previously, we performed immunohistochemical studies with a monoclonal antibody that reacts exclusively with the dimeric form of olfactomedin (Snyder et al., 1991). This antibody revealed

FIGURE 3: Immunohistochemical localization of olfactomedin and its precursors. Coronal sections through the olfactory cavity of *R. catesbeiana* were stained with a 1000-fold dilution of ammonium sulfate fractionated antiserum raised against the purified protein (panels A and C) or its corresponding preimmune serum (panel E) or with a 500-fold dilution of antiserum raised against the synthetic peptide corresponding to the antigenic region, illustrated in Figure 1 (panels B and D), or its corresponding preimmune serum (panel F). The large arrows in panels A and B indicate the position of the basement membrane, which separates the neuroepithelium from the lamina propria. Small arrows indicate the ciliary surface of the neuroepithelium. Vertical columns of heavy staining across the neuroepithelium reflect ducts of Bowman's glands. Arrowheads point to submucosal glands, A designates axon bundles, and dark pigmented heteromorphous cells underneath the basement membrane are melanocytes. Panels C and D show a closeup of individual Bowman's glands. Note that staining in panel C is confined to the apical regions of the acinar cells immediately adjacent to the lumen (L), whereas staining in panel D appears to be granular in appearance and localized to the perinuclear regions of the cells, suggesting localization to membrane vesicles of the endoplasmic reticulum or Golgi (Roth, 1991). The positions of some of the nuclei are indicated by asterisks. The bars in panels A, B, E, and F correspond to 40 μm. The bars in panels C and D correspond to 10 μm.

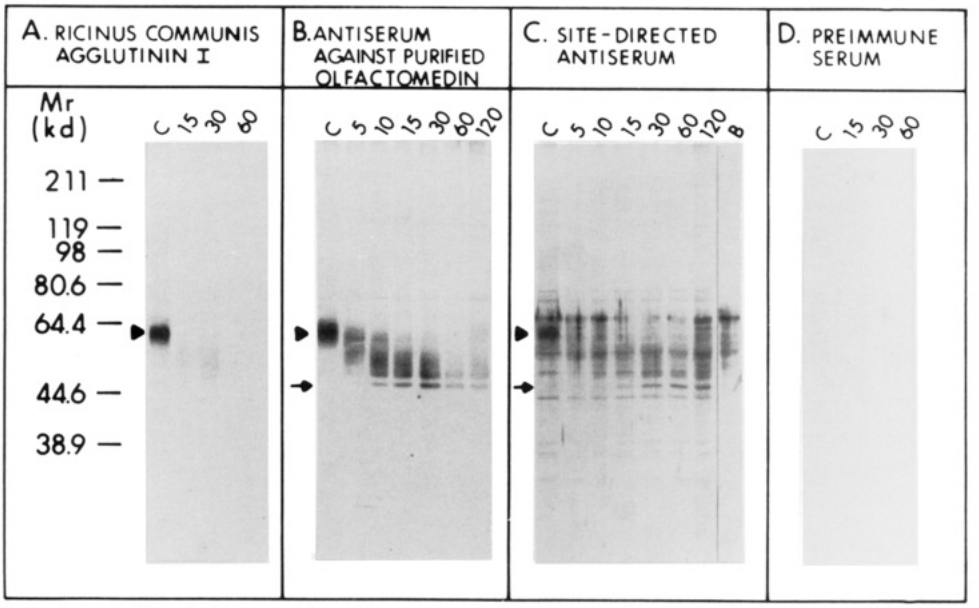

FIGURE 4: Enzymatic deglycosylation of olfactomedin. Aliquots containing 10 μg of ciliary protein were removed at the indicated time points from the deglycosylation reaction and subjected to polyacrylamide gel electrophoresis and electrophoretic transfer onto nitrocellulose membranes. The membranes were stained with 20 μg/mL biotinylated RCA (panel A), a 1000-fold dilution of ammonium sulfate fractionated antiserum raised against purified olfactomedin (panel B), a 100-fold dilution of the site-directed antiserum (panel C), and a 100-fold dilution of preimmune serum obtained from the same rabbit used to generate the site-directed antiserum (panel D). The arrowhead indicates the position of native olfactomedin, and the arrow indicates its fully deglycosylated 51-kDa precursor. The lanes labeled C show immunoreactivity of samples not treated with endoglycosidase. The lane labeled B in panel C contained only sample buffer without ciliary protein and shows staining artifacts due to reactivity of components in the antiserum with components of the sample buffer (most likely 2-mercaptoethanol). The prominent band below the 51-kDa band in panel C is also observed as a faint band in panel D and is, therefore, also considered a nonspecific staining artifact.

the same immunohistochemical staining pattern as that observed with antiserum raised against the purified protein, which suggests that dimer formation and polymerization of olfactomedin also occur prior to its secretion.

The diffuse staining in the 57-kDa region observed on immunoblots with antiserum raised against purified olfactomedin suggests that there is some heterogeneity in the number of carbohydrate groups attached to the mature protein or in their sugar composition. Resolution of individual electrophoretic bands in the 57-kDa region is increased when the site-directed antiserum is used, since this antiserum does not detect olfactomedin molecules which are glycosylated on asparagine 5. It is not known which of the potential five N-linked glycosylation sites besides asparagine 5 are glycosylated for each of the immunoreactive species detected with antiserum raised against the synthetic peptide. However, since asparagine 5 must be devoid of carbohydrate in order for the antibodies to bind and since this antiserum fails to stain the ciliary surface of the olfactory neuroepithelium, it appears that at least asparagine 5 is fully glycosylated in the mature protein. We can, however, not exclude the possibility that the observed lack of immunoreactivity at the ciliary surface is due to dimer formation and polymerization, which would also interfere with binding of this antibody.

Lack of staining of the perinuclear regions of the glands by antiserum raised against the purified protein is surprising, since this antiserum is able to detect the deglycosylated 51-kDa band on immunoblots. However, we have previously shown that olfactomedin's carbohydrate dominates its immunogenicity (Anholt et al., 1990), and competition studies between monoclonal antibodies directed against carbohydrate determinants and polyclonal antibodies raised against the purified protein showed that most of the immunoreactivity in polyclonal antiserum is directed against carbohydrate moieties (Snyder et al., 1991). Thus, at the concentrations used in our immunohistochemical studies the titer of antibodies within this antiserum directed against protein determinants of olfactomedin may have been too low to enable detectable visualization of olfactomedin precursors in the perinuclear compartment of the acinar cells of Bowman's glands.

Enzymatic deglycosylation reveals a 51-kDa immunoreactive form of olfactomedin (Figure 4). This polypeptide is detected by two different antibodies against olfactomedin, and its accumulation follows the disappearance of reactivity with RCA. Furthermore, its molecular weight resembles the molecular weight predicted for the native form of olfactomedin calculated from its amino acid composition (H. Yokoe and R. Anholt, unpublished observations). These observations support the notion that this polypeptide indeed represents the fully deglycosylated form of olfactomedin.

Immunoblots with antiserum raised against the synthetic peptide reveal at least four distinct immunoreactive bands (Figure 2). Furthermore, during enzymatic deglycosylation intermediate forms are evident en route to the appearance of the fully deglycosylated 51-kDa band (Figure 4). These data suggest that most, if not all, of the six N-linked glycosylation sites of mature olfactomedin carry carbohydrate moieties. On the basis of the observation that at least 15% of the molecular weight of olfactomedin is contributed by its carbohydrate and on the assumption that its different carbohydrate groups are similar in size, we can estimate that each carbohydrate moiety consists of about 8–10 sugar residues. Reactivity of olfactomedin with WGA and RCA indicates that these carbohydrate groups contain *N*-acetylglucosamine and galactoside sugars (Snyder et al., 1991). Since partially deglycosylated intermediates are virtually nondetectable with biotinylated RCA during deglycosylation of olfactomedin (Figure 4, panel A), it is possible that not all of its carbohydrate groups contain terminal galactose sugars. Treatment with neuraminidase does not affect reactivity of olfactomedin with monoclonal antibodies directed against carbohydrate determinants. In addition, lectins, which recognize sialic acid, such as elderberry bark lectin (Vector Laboratories, Burlingame, CA), do not react with olfactomedin

(unpublished observations). Therefore, carbohydrate moieties on olfactomedin appear not to contain terminal *N*-acetylneuraminic acid residues but rather to be truncated complex carbohydrates, at least some of which are characterized by terminal galactose.

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