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A human axillary odorant is carried by apolipoprotein D

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ABSTRACT **The characterization of the source of the odor in the human axillary region is not only of commercial interest but is also important biologically because axillary extracts can alter the length and timing of the female menstrual cycle. In males, the most abundant odor component is known to be *E*-3-methyl-2-hexenoic acid (*E*-3M2H), which is liberated from nonodorous apocrine secretions by axillary microorganisms. Recently, it was found that in the apocrine gland secretions, 3M2H is carried to the skin surface bound to two proteins, apocrine secretion odor-binding proteins 1 and 2 (ASOB1 and ASOB2) with apparent molecular masses of 45 kDa and 26 kDa, respectively. To better understand the formation of axillary odors and the structural relationship between 3M2H and its carrier protein, the amino acid sequence and glycosylation pattern of ASOB2 were determined by mass spectrometry. The ASOB2 protein was identified as apolipoprotein D (apoD), a known member of the $\alpha_{2\mu}$-microglobulin superfamily of carrier proteins also known as lipocalins. The pattern of glycosylation for axillary apoD differs from that reported for plasma apoD, suggesting different sites of expression for the two glycoproteins. *In situ* hybridization of an oligonucleotide probe against apoD mRNA with axillary tissue demonstrates that the message for synthesis of this protein is specific to the apocrine glands. These results suggest a remarkable similarity between human axillary secretions and nonhuman mammalian odor sources, where lipocalins have been shown to carry the odoriferous signals used in pheromonal communication.**

Several studies in humans have suggested that axillary odors and secretions from both males and females are a source of chemical signals containing physiologically active components capable of altering the menstrual cycle. These alterations include the well-accepted menstrual synchrony affect first documented by McClintock (1) in an all female living group and later replicated by others in coeducational facilities (2, 3). In nonhuman mammals such as rodents, estrus synchrony has been shown to be mediated by airborne chemical signals (4).

The involvement of axillary components in mediating menstrual synchrony was first suggested by Russell *et al.* (5). Studies by Cutler *et al.* (6) and Preti *et al.* (7) were the first prospectively conducted double-blind studies to attempt menstrual cycle alterations by using axillary extracts from male and female donors. The above studies suggest that certain axillary components function as chemical signals involved in the regulation of reproductive function via alteration of the hypothalamic pituitary-gonadal axis; chemical signals with this mode of action are termed primer pheromones (8).

Axillary secretions and odors are derived from an area of the body with exceptional odor-producing capabilities. Several types of skin glands, including apocrine, eccrine, sebaceous, and apoeccrine glands, contribute moisture and substrate to a large permanent population of cutaneous microflora (9). These consist of lipophilic and large colony diptheroids as well as micrococci. These microorganisms generate a variety of odoriferous compounds that characterize the axillary region. *In vivo* correlations of odor quality and axillary bacterial populations have demonstrated that the aerobic diptheroids are associated with the stronger, more distinct axillary odor (9).

A number of investigations of axillary constituents have focused upon the interesting steroidal molecules found there (10, 11). Volatile odoriferous steroids such as 5α-androst-16-en-3β-ol (androstenol) and 5α-androst-16-en-3-one (androstenone) as well as nonvolatile steroid sulfates were identified and quantitated by radioimmunoassay and gas chromatography/mass spectrometry (GC/MS) (10, 11), The urine/musky-like odors of androstenone and androstenol were thought by some investigators to be suggestive of axillary odor (9–11). However, recent studies (12, 13) have presented both organoleptic and analytical evidence that a mixture of C_6–C_{11}, straight-chain, branched, and unsaturated acids constitute the characteristic axillary odor. In combined male samples, the *E*-isomer of 3-methyl-2-hexenoic acid (3M2H) is the dominant analytical component of the mixture, while in combined female samples the straight-chain acids are present in greater relative abundance (14). The *Z*-isomer is also present in both genders, however in different relative abundance: 10:1 (*E*/*Z*) in males (12) and 16:1 (*E*/*Z*) in females (14).

More than 30 years ago, it was demonstrated that the odorless precursors of axillary odor are present in apocrine gland secretions and that the characteristic odor arises from interaction of the odorless apocrine secretion precursors with the axillary microflora (15). The water-soluble components of apocrine secretion were found to contain the odorless precursors of the characteristic odor (13). Furthermore, it was recently shown that 3M2H is carried to the skin surface bound to two proteins, which have been designated as apocrine secretion odor-binding proteins 1 and 2 (ASOB1 and ASOB2), with molecular masses of 45 and 26 kDa, respectively, as determined from SDS/PAGE (16). Spielman *et al.* (16) also partially characterized ASOB1 and -2 and produced polyclonal antibodies to each purified protein isolated from male subjects. Antisera raised against ASOB1 demonstrated reactivity to both proteins; however, antisera to ASOB2 reacted only with ASOB2, suggesting relatedness or perhaps a precursor–product relationship. In addition, both ASOB1 and -2 were found to be glycosylated (16).

Although the structures of the physiologically active molecules in the axillae that alter menstrual cycle length and timing are not known, the above results demonstrate that the chem-

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Abbreviations: 3M2H, 3-methyl-2-hexenoic acid; ASOB1 and ASOB2, apocrine secretion odor-binding proteins 1 and 2, respectively; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; RP-HPLC, reversed-phase high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; PSD, postsource decay; apoD, apolipoprotein D; ACN, acetonitrile.

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istry of axillary odor production is similar to that found in sources of chemical signals in nonhuman mammals. In the hamster (17, 18) and house mouse (19), volatile signals found in vaginal secretions and urine, respectively, are bound to proteins. To better understand the mechanism of axillary odor production and the structural relationship between the ASOB proteins and 3M2H, we used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in conjunction with specific enzymatic cleavages to determine the structure of ASOB2 and the extent of glycosylation of this protein.

MATERIALS AND METHODS

Apocrine Secretion Donors. Healthy male paid volunteers (ages 21–40) were employed as apocrine secretion donors. The criteria for donor selection has been reported (6, 7, 12, 13). Each donor had large numbers of lipophilic diphtheroids in their axillary region. These microorganisms have been associated with the production of the strongest odors (9). To complete the experiments described below, a total of eight separate collections of apocrine secretions were made from 47 different subjects over a period of 2 years. As previously described (13, 14), the volume from any given subject ranged from a trace to ≈50 μl.

Collection of Apocrine Secretions. The axillary regions of the donors were first washed with 0.1% Triton X-100, then with water, dried, and finally washed with hexane. Apocrine secretions were stimulated by intradermal injection of 0.1 ml of 1:10,000 epinephrine in sterile saline. Secretion was collected with a glass microcapillary and samples were stored at −20°C until used.

Protein Purification. The secretions collected from several individuals were combined and separated either on 5–15% gradient or 12% SDS/PAGE (8 cm × 10 cm × 0.75 cm) under reducing conditions (20). Following SDS/PAGE, the proteins were electroeluted by the method of Hunkapiller *et al.* (21). Unbound SDS and Coomassie blue were removed as described (16).

Assay for 3M2H Bound to Proteins. Electroeluted protein (ASOB2) was dissolved in 0.5 ml of 5% aqueous NaOH and heated to 100°C for 20 min under nitrogen. The reaction mixture was then cooled to room temperature and acidified with ≤0.1 ml of 6 M HCl and extracted with 3 × 0.15 ml of $CHCl_3$ (Nanograde, Mallinkrodt). The extract was concentrated to 10 μl and analyzed by GC/MS for the presence of 3M2H. Organoleptic analysis of samples of concentrated hydrolyzate extracts were carried out as described by Zeng *et al.* (13).

GC/MS. A Finnigan–MAT 4510 GC/MS system equipped with a split/splitless injector was used. The 30 m × 0.32 mm (interior diameter) fused silica column with a 1.0 μm coating of Stabilwax (cross-bonded polyethylene glycol; Restek, Port Matilda, PA) was programmed as follows: 100°C for 1 min, then 6°C/min to 220°C, and held there for 30 min. A typical experiment produced 2000, 1-sec scans from m/z 40 to m/z 400. The 3M2H was identified by matching the retention times and mass spectra with those of authentic material (12, 13).

***In Situ* Hybridization.** An antisense probe (bases 420–391: CTGGTTTATG CCATCGGCAC CGTACTGGAT) for apolipoprotein D (apoD) was synthesized based on the previously published sequence (22) and by selection using the primer selection program, OSP (23). Axillary tissue was obtained by 4-mm punch biopsy by using local anesthesia (lidocaine with 1:100,000 epinephrine). The tissue was snap frozen in liquid nitrogen and stored at −80°C.

Tissue was embedded in optimal cutting media (OCT). Eight micrometer sections were cut and placed on diethyl pyrocarbonate-treated slides. The sections were washed in 0.1 M Tris·HCl, treated with 10 μg of proteinase K per ml for 20 min at room temperature, again washed in 0.1 M Tris·HCl followed by phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde. These sections were prehybridized at room temperature for 2 h, hybridization solutions containing digoxigenin-labeled oligonucleotide probe for apoD added, and incubated at 45°C for 18 h. The sections were then subjected to a series of posthybridization washes in decreasing concentrations of saline sodium citrate buffer at various temperatures, incubated with an alkaline phosphatase antidigoxigenin antibody, and following washing were exposed to the chromogen to allow color development. An irrelevant oligonucleotide probe that consisted of a 26-base sequence of a bacterial antibiotic gene (*erm*C) was used as a negative control (CAA TGG CAG TTA CGA AAT TAC ACC TC).

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC). Enzymatic digests were separated by RP-HPLC on a Vydac C_4 column (4.6 × 250 mm) at a flow rate of 1 ml/min and using a gradient of 100% solvent A (0.05% trifluoroacetic acid in deionized, distilled water) to 60% solvent B [0.035% trifluoroacetic acid in acetonitrile (ACN)] over 60 min using a UV detector (214 nm).

Enzymatic Digests. Approximately 200–300 pmol of ASOB2 in 100 μl PBS directly eluted from SDS/PAGE was taken to dryness in a Speed-Vac. It was digested using 3% (wt/wt) endo Lys-C (Wako Pure Chemical, Dallas) in 60 μl 2.5 M urea, 0.025 M Tris·HCl (pH 9.0) at room temperature for 5 h, followed by RP-HPLC fractionation. The HPLC fractions containing glycopeptides (as judged from their mass spectra) were deglycosylated with 0.5 units PNGase F (Boehringer Mannheim) in 45 μl 13% ACN and 0.025 M NH_4HCO_3 (pH 7.8) at 37°C for 9 h, further digested with 10% (wt/wt) endo Arg-C (Boehringer Mannheim, sequencing grade) in 10 μl 30% ACN, 20 μl buffer [0.1 M Tris·HCl (pH 7.6)/0.01 M $CaCl_2$] and 10 μl activation solution (Boehringer Mannheim) at 37°C for 18 h.

Another HPLC fraction containing the glycopeptide with a complex-type oligosaccharide was treated sequentially with (*i*) 10 mU neuraminidase in 12 μl 0.02 M NH_4OAc (pH 5.0) at 37°C for 5 h, (*ii*) 1 mU β-galactosidase in 11 μl 0.02 M NH_4OAc (pH 5.0) at 37°C for 5 h, and (*iii*) 0.6 units PNGase F in 16 μl 0.02 M NH_4HCO_3 (pH 7.5) incubated at 37°C overnight. The sugar-free peptide was again further cleaved to smaller peptides by Arg-C digestion under the conditions described above.

In a separate experiment, another batch of approximately 300–500 pmol of ASOB2, collected from five male donors and dissolved in PBS, was divided into two equal portions; one was hydrolyzed with 5% NaOH, as described above, and analyzed organoleptically and by GC/MS for the presence of 3M2H (12, 16). The other portion was digested with endo Glu-C [about 20% (wt/wt)] in 50 μl 6% ACN, 0.01 M NH_4OAc (pH 4.0) at room temperature overnight in a sealed tube. Immediately after opening, the content was first evaluated organoleptically and then extracted with 3 × 150 μl $CHCl_3$. The extract was concentrated and analyzed by GC/MS for 3M2H. The aqueous phase was injected onto an HPLC C_4-column and fractions analyzed by MALDI-TOF-MS.

MALDI-TOF-MS. The molecular weights of ASOB2, deglycosylated ASOB2, and proteolytic peptides formed by enzymatic digestions were determined by MALDI-TOF-MS by using a Voyager Elite (PerSeptive Biosystems, Framingham, MA) or a VT2000 (Vestec, Houston) TOF mass spectrometer. α-Cyano-4-hydroxycinnamic acid was used as the matrix. The sample on the target was irradiated with 3 nsec pulses of a 337 nm N_2 laser at a 5-Hz repetition rate. The accelerating voltage was 30 kV [except in the postsource decay (PSD) mode]. Angiotensin I and bovine insulin were used as external calibrants for peptides and trypsinogen for intact and deglycosylated proteins, resulting in ±0.1% mass accuracy at a resolution m/Δm of about 300–500.

To obtain the sequence of some of the proteolytic peptides, their PSD mass spectra (24) were measured with the Voyager Elite TOF spectrometer, operated in the reflector mode at an

accelerating voltage of 20 kV. To cover the entire spectrum of fragment ions, the mirror voltage was decreased from 20 to 1.2 kV in 9–11 steps.

RESULTS

Attempts to determine the N-terminal sequence of ASOB2 by the Edman method had failed, indicating that the N terminus was blocked. Consequently, the structure of this protein was determined by mass spectrometry. The molecular weights (M_r) of three independently isolated ASOB2 samples were found to be ≈22,582, 23,396, and 22,920 by MALDI-TOF-MS. Upon deglycosylation with PNGase F, the M_r shifted to 19,359, 19,428, and 19,400, respectively. These results suggested that each of the three isolates consisted of the same glycoprotein differing slightly in the size of the attached *N*-linked oligosaccharides. The variations in the molecular weights exceed the 0.1% of the method and are due to the common inhomogeneity of the carbohydrate portion and an artifact of the SDS/PAGE separation (see below).

To determine the primary structure of the ASOB2 protein, ≈250 pmol (based on the intensity of Coomassie blue staining of the SDS gel from which it was eluted) was digested with endo Lys-C. The digest was fractionated on RP-HPLC and 28 fractions were collected. The more abundant $(M + H)^+$ ions in the MALDI mass spectra of these fractions were subjected to PSD in an effort to obtain sequence information. Two of the spectra are shown in Fig. 1. Each of these provided a definite partial sequence based on the *m/z* values of consecutive fragment ions differing from another by one of the amino acid residue masses (NHCHRCO). For example, the spectrum in Fig. 1*a* would fit a peptide with a partial sequence ... VQENFDVYPNP..., if one assumes that all the ions of that series are of the N-terminal type b_n (25). The N-terminal fragment (*m/z* 195.1) could correspond to PP, but this peak may also possibly be related to a form of ligand covalently bound to valine. Similarly, a definite sequence ... NVQDTVT... can be deduced from the other spectrum (Fig. 1*b*). Whether it is to be read from the N to the C terminus or the reverse depends on the type of fragments, b_n or y_n (25). Rather than attempting a more complete interpretation, which was hampered by some unassignable mass differences in the low and high mass region (i.e., the N and/or C termini of the peptides that later turned out to be due to an unexpected modification of a cysteine), we decided to first check whether the protein is a known one. When independently searching (26) the SwissProt protein data base (containing 43,470 protein sequences) using the BLITZ server of the European Molecular Biology Laboratory (EMBL) for a match with ... VQENFDVYPNP..., as well as ...NVQDTVT... (both forward and reverse), human apolipoprotein D (22) was found to be a perfect fit for the latter (in reverse) heptapeptide sequence and for the first seven amino acids of the former, respectively. The mass spectra (Fig. 1) turned out to represent peptides 8–21 and 157–167 of apoD, both of which contained a cysteine that had been unexpectedly *S*-carbamidoethylated by addition of monomeric acrylamide still present in the SDS/PAGE system (27). The major ion-series (single-letter code) shown in the spectrum (Fig. 1*a*) and used for data base matching turned out, in retrospect, to correspond to a set of "internal" fragment ions (25) representing the sequence PPVQENFDV and triggered by the preferential primary cleavage at the N—P peptide bond. This result is an impressive example of the power of protein data base searching utilizing partial sequence information derived from the mass spectra of peptides, even if they contain an unexpected and unknown modification of one of the amino acids (see Fig. 1). In addition, the sequences deduced from the PSD spectra of the other peptides were also found to fit apoD, including a putative blocked N-terminal fragment, <QAFHLGK (Fig. 2). The partial carbamidoethylation of at least two cysteines explains the variations in the measured $(M + H)^+$ ions of the PNGase F-deglycosylated ASOB2, which were higher than 19,289.1 as calculated for apoD (fully reduced, Asp in positions 45 and 78). Finally, it should be noted that a minor component of $(M + H)^+ = m/z$ 1676.85 was found that corresponded to peptide 8-21, where Cys^8 had formed a disulfide bond with 2-mercaptoethanol as deduced from the N-terminal ions in the PSD spectrum, which otherwise was very similar to that shown in Fig. 1*a*. Its presence indicated the formation of yet another artifact of the isolation procedure.

The MALDI mass spectrum of fraction 12 exhibited a multiplet of peaks spaced 191 Da apart (Table 1), indicative of

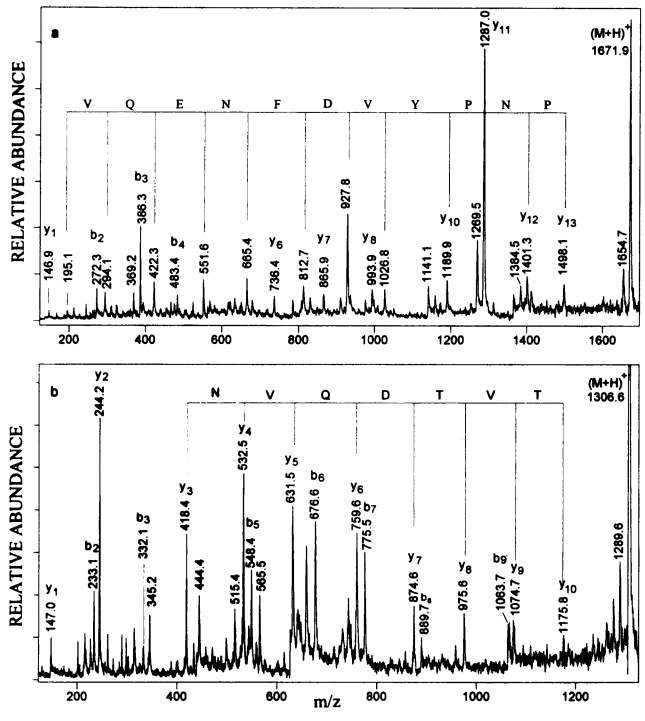


FIG. 1. PSD mass spectrum of the (*a*) $(M + H)^+$ ion (*m/z* 1671.9) of C*PNPPVQENFDVNK and (*b*) $(M + H)^+$ ion (*m/z* 1306.6) of MTVTDQVNC*PK (C* = Cys-CH_2-CH_2-$CONH_2$). The notations b_n, y_n, etc. refer to fragment ions corresponding to these sequences. The single letter notation in the figure refers to the preliminary partial interpretation discussed in the text and used for data base matching. The mass differences (Δ = 174) between y_{13} and $(M + H)^+$ in (*a*) and y_2 and y_3, as well as b_8 and b_9, in (*b*) are due to the unexpected carbamidoethylated cysteines (C*).

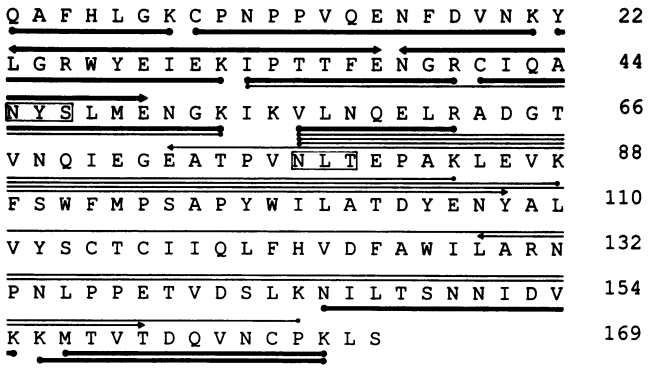


FIG. 2. Amino acid sequence of human apoD (22). ·–·, Peptides observed in endo Lys-C digest; ↔, peptides observed in endo Glu-C digest. Heavy lines indicate sequences confirmed by MALDI-PSD mass spectra. The two glycosylated sequons (N-X-S/T) are boxed.

a carbohydrate moiety terminating in sialic acids. Successive treatment with neuraminidase, β-galactosidase, and PNGase F produced single peaks at m/z 4182, 3858, and 2559, respectively. These results indicated that this glycopeptide spans the region of amino acids 32–53, $(M + H)^+$ calc. m/z 2559.9 (for the deglycosylation product), which must bear a complex oligosaccharide of the composition $(HexNAc)_4$ $(Hex)_5$ $(NeuAc)_{0-2}$ at the glycosylation site Asn-45. The identity of the deglycosylated peptide was further confirmed by digestion with Arg-C, producing $(M + H)^+$ ions at m/z 1035 (calc. 1035.1) and m/z 1543 (calc. 1542.7), respectively, corresponding to peptides 32-40 and 41-53. Their PSD spectra corroborated the expected amino acid sequences.

The MALDI mass spectrum of fraction 13 consisted of a pattern of peaks (Table 1) spaced about 162 Da apart, typical of glycopeptides differing in the number of hexose residues. Upon deglycosylation with PNGase F, the multiplet collapsed to m/z 3109, which best fit the peptide 56-84 (calc. 3109.9) and includes the other glycosylation site, Asn-78. This identification was further confirmed by digestion of the peptide with Arg-C, producing the expected smaller peptides 56-62 of $(M + H)^+ = m/z$ 872 (calc. 871.7) and 63-84 of $(M + H)^+ = m/z$ 2257 (calc. 2257.2). The PSD spectrum of the former revealed the expected sequence, VLNQELR.

Because we did not observe any Lys-C peptides of a mass indicating the covalent attachment of 3M2H, it was important to prove that it was still present in the protein sample being analyzed. Therefore, another batch of ASOB2 (estimated to represent 300–500 pmol from Coomassie blue staining of the SDS gel from which the protein had been eluted) dissolved in PBS was divided into two equal portions. One portion was subjected to base hydrolysis and the hydrolyzate analyzed by GC/MS for the presence of 3M2H, which was indeed detected. To liberate and detect noncovalently bound odor components, the remaining portion of unhydrolyzed ASOB2 was digested with endo Glu-C at pH 4.0, conditions under which ester bonds would remain intact. A $CHCl_3$ extraction of the digest was concentrated and analyzed for free 3M2H, both organoleptically and by GC/MS. The latter indicated the presence of ≈280 pmol 3M2H, with the *E*-isomer predominating over the *Z*-isomer. The $(M + H)^+$ ions of the peptides and some of their PSD spectra from this digest further confirmed that the carrier protein is apoD (Fig. 2). In addition, the strength of the signals in the HPLC, the abundance of the $(M + H)^+$ ions of the intact ASOB2 in this sample, and the abundance of those peptides present in the digest suggested that the amount of ASOB2 digested in this experiment was ≈100–150 pmol instead of 150–250 pmol as the Coomassie stain would have indicated.

To determine if expression of apoD occurred in axillary tissue, an antisense oligonucleotide probe for apoD was synthesized. It was found that hybridization occurred exclusively in the apocrine gland. The reactivity was predominately in the cytoplasm; however, in some cells, a slight degree of nuclear staining was evident (see Fig. 3). There was no reactivity in any other cell type in the epidermis or dermis including keratinocytes, fibroblasts, eccrine glands, sebaceous glands, vascular epithelium, and perivascular mononuclear cells. In addition, no reactivity was observed using the irrelevant oligonucleotide probe referred to earlier (data not shown).

DISCUSSION

To date, the role, if any, of apoD in the plasma lipoprotein system is not known. Several hydrophobic ligands for apoD have been suggested by both *in vitro* studies (progesterone type steroids) (28, 29) and theoretical considerations (bilirubin) (30). Our data identify, for the first time, a ligand (3M2H) that apoD carries in apocrine secretion. These results further demonstrate a remarkable similarity between human axillary secretions and odors and nonhuman mammalian odor sources used in chemical signaling. It is known that chemical signals, which modify reproductive endocrinology and behavior, are carried by proteins of the lipocalin family in the female golden hamster (aphrodisin) (18), house mouse (major urinary proteins) (19), and possibly the pig (pheromaxein) (31). As discussed above, human axillary extracts contain components that act as primer pheromones because the extracts can alter the length and timing of the female menstrual cycle, presumably by alteration of neuroendocrine pathways.

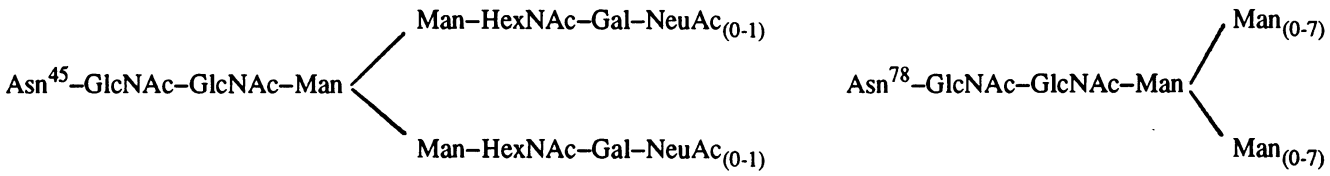
Both the three-dimensional structure (from x-ray crystallography) and amino acid sequence of other known lipocalins—e.g., retinol-binding protein and bilin-binding protein—suggest that the interactions between a member of the lipocalin protein superfamily and its ligand(s) are noncovalent (32). Our results also show that although 3M2H was associated with the ASOB2 samples analyzed, none of the proteolytic peptides was

Table 1. Summary of the characterization of the carbohydrate moieties at Asn-45 and Asn-78

HPLC fraction	m/z of $(M + H)^+$ intact glycopeptide	m/z of $(M + H)^+$ after digestion with				Glycosylation site	Amino acid position	Proposed carbohydrate composition†
		Neuraminidase	β-Galactosidase	PNGase F	Endo H*			
12	4182							$HexNAc_4Hex_5NeuAc_0$
	4473	4182	3858	2559		Asn-45	32-53	$HexNAc_4Hex_5NeuAc_1$
	4764							**$HexNAc_4Hex_5NeuAc_2$**
13	3839							$HexNAc_2Hex_2$
	4001							$HexNAc_2Hex_3$
	4163							$HexNAc_2Hex_4$
	4325	No change	No change	3109	3312	Asn-78	56-84	**$HexNAc_2Hex_5$**
	4488							$HexNAc_2Hex_6$
	4652							$HexNAc_2Hex_7$
	4817							$HexNAc_2Hex_8$
	4980							$HexNAc_2Hex_9$

*For fraction 13 only.

†Structures proposed based on these data and known biogenesis of glycosylation:



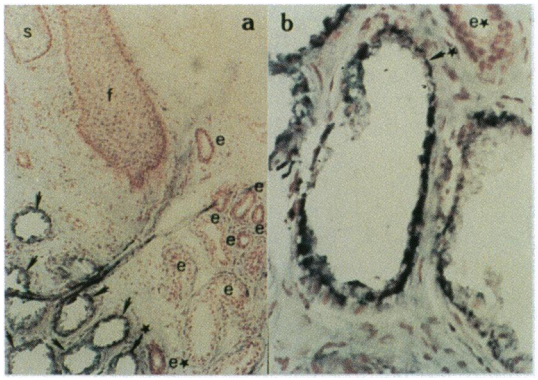


FIG. 3. ApoD mRNA is expressed in apocrine glands of the axillae. *In situ* hybridization using a digoxigenin-labeled oligonucleotide probe shows localization of apoD mRNA expression to the aprocrine gland (blue-purple color). Sections are counterstained with nuclear fast red. (*a*) ApoD mRNA expression in apocrine glands (arrowheads); there is no expression in hair follicle (f), sebaceous (s), or eccrine (e) glands. (×200.) (*b*) Enlargement (×600) of apocrine and eccrine glands indicated by asterisks in *a*.

found to be 3M2H adducts by mass spectrometry, indicating that the ligand is not covalently bound to the protein.

Although first isolated from mixtures of plasma lipoproteins, apoD is not a member of the protein family containing the other apolipoproteins that include apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, apoB-100, and apoE. The complete amino acid sequence of apoD was first inferred from its cDNA and then confirmed by partial sequencing using the Edman degradation (22). The mature protein is cleaved from the 20-amino acid leader peptide between glycine and glutamine, which converts to pyroglutamic acid, thus blocking the N terminus. The 169-amino acid sequence of apoD is homologous with members of the $\alpha_{2\mu}$-microglobulin or lipocalin superfamily of proteins (22). In contrast to the other apolipoproteins that are synthesized primarily in the liver and intestines, apoD is produced in a variety of organs including the liver, intestines, pancreas, kidney, placenta, adrenals, spleen, and fetal brain (22, 28). The amount of apoD mRNA varies from organ to organ and is cell-type specific (22, 28, 29, 33); in addition, the mRNA appears to be transcriptionally inducible because its amount increases several-fold in regenerating rat sciatic nerve (33) and breast tumors under the influence of retinoic acid (29). Because apoD is expressed in a variety of tissues and under different circumstances, it has been suggested that the ligands associated with this protein may differ with tissue and circumstances—e.g., regenerating nerves and breast tumor cells in the presence of retinoic acid (28). However, when apoD is expressed in apocrine glands the *E*- and *Z*-isomers of 3M2H are the major ligands. As outlined above, it is difficult to know the amount of protein actually used in our experiments, but the best estimates indicate that there may be two moles of 3M2H for each mole of apoD in apocrine secretions.

Previous studies to localize expression of apoD mRNA in human tissue have not specifically included skin; however, it appears that the majority of cells expressing apoD *in vivo* are fibroblasts (28). In five separate axillary tissues examined, no expression of apoD was observed in fibroblasts. In agreement with these results, an *in vitro* study of a human diploid fibroblast line indicated that apoD mRNA occurred in quiescent and senescent fibroblasts but not in actively proliferating cells (34).

It should be noted that the glycosylation pattern of the apoD from axillary glands differs significantly from that recently found for apoD from serum (35). In serum, the carbohydrate moieties are more complex and extended. Furthermore, not only the Asn-45 site but also the Asn-78 site terminates in sialic acids. Clearly, the sites of expression (or, at least, of posttranslational glycosylation) differ for plasma apoD and apocrine apoD.

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