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An alternatively spliced Muc10 glycoprotein ligand for putative L-selectin binding during mouse embryonic submandibular gland morphogenesis

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Abstract

Late-gestation (embryonic day 18; E18) mouse submandibular glands (SMG) comprise a network of large and small ducts that terminate in lumen-containing, presumptive acini (terminal buds) expressing unique, cell membrane-associated embryonic mucin. The objective here was to clone and sequence embryonic low molecular-weight SMG mucin, predict its secondary structure, and begin to investigate its possible role in SMG development. Evidence was found that: (1) embryonic low molecular-weight mucin is an alternatively spliced *Muc10* gene product, 220 amino acids in size (approximately 25 kDa), rich in potential *O*-glycosylation sites, and variably glycosylated (approximately 40 and 68 kDa); (2) consensus secondary-structure prediction for embryonic low molecular-weight mucin is consistent with a molecule that is anchored to the plasma membrane, directly or indirectly (via a glycolipid), and has a protein core that serves as a scaffold for carbohydrate presentation; (3) embryonic L-selectin is immunolocalized to the plasma membrane region of terminal-bud epithelial cells in a pattern similar to that seen for embryonic mucin; (4) embryonic, but not adult, mucin is able to bind L-selectin and does so endogenously in E18 SMG. As the primary role of L-selectin is to mediate cell adhesion and its ligands are mucin-like glycoproteins, it is suggested that this embryonic low molecular-weight mucin be termed MucCAM.

Keywords: Submandibular salivary gland; Embryo; Mouse; Mucin sequence; L-selectin binding

1. Introduction

The mouse submandibular gland begins as an outgrowth of the oral epithelium into the underlying mandibular mesenchyme on E11–11.5. The initial bud of the gland elongates to form a solid epithelial cord with a bulb at its end. Clefts subdivide the initially solid epithelial bulb to begin a process of repetitive, self-similar furcations that serve as branch points for new epithelial outgrowths and result in the gland's ultimate bush-like morphology (Spooner et al., 1989). As the embryonic development of the submandibular gland differs between gland regions, depending on the time of branch formation, it is often more informative to speak of developmental stage than of gestational age (i.e. initial bud, pseudoglandular, canalicular, and terminal bud) (Jaskoll and Melnick, 1999). Late-gestation submandibular glands (mostly late terminal bud stage)

Abbreviations: BSA, bovine serum albumin; E, embryonic day; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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comprise a network of large and small ducts that terminate in lumen-containing, presumptive acini expressing a unique, cell membrane-associated embryonic mucin (Jaskoll et al., 1998).

Our objective now was to clone and sequence embryonic low molecular-weight submandibular gland mucin, predict its secondary structure, and begin an investigation of its possible role in gland development.

2. Materials and methods

2.1. Tissue collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as described by Jaskoll et al. (1994); plug day = day 0 of gestation. Newborn, 21-day old postnatal, and adult non-pregnant and pregnant females were anaesthetized with methoxyflurane (Metafane) and killed by cervical dislocation. Embryos were dissected in cold PBS containing 0.02% diethyl pyrocarbonate and staged according to Theiler (1989). Embryonic, neonatal and adult submandibular glands were dissected, processed for histology, or stored at −70°C. For the cDNA library, five litters of E18 submandibular glands were dissected, snap-frozen in liquid nitrogen, and stored at −70°C.

2.2. cDNA cloning and sequence analysis

Poly(A)+ RNA was isolated from five litters of 90 pooled E18 submandibular glands using the MessageMaker mRNA Isolation System (Life Technologies, Inc., Gaithersburg, MD). Total RNA was isolated using Trizol Reagent, which is a modification of the Chomzynski and Sacchi (1987) method. Then, poly(A)+ was isolated from total RNA using oligo(dT) cellulose in a filter syringe with a double-purification method. cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies) and oligo(dT).

An E18 cDNA library was constructed using pCMVSPORT6 vector plasmid and the SuperScript System (Life Technologies). Approximately 1.5×10^8 colonies, with an average insert size of 1.9 kb, were isolated and screened according to the GeneTrapper cDNA Positive Selection System (Life Technologies) using PCR primer pairs GGTTTCATTCCAAGCTCTCC (forward primer) and TTAGGAGAACGGCGACTGAT (reverse primer) for library and colony screening. The sequences of the primers was based on a 160 bp region of the adult *Muc10* sequence (bp 212–372; Denny et al., 1996) shown by BLAST to be unique to *Muc10*. In brief, the oligonucleotide was biotinylated at the 3′ end with biotin-14-dCTP using terminal deoxynucleotidyl. Simultaneously, a complex population of double-stranded phagemid DNA containing cDNA inserts was converted to single-strand DNA using Gene II (phage F1 endonuclease) and *Escherichia coli* exonuclease III. Hybrids between the biotinylated oligonucleotide and single-strand DNA were formed in solution and then captured on streptavidin-coated paramagnetic beads. A magnet was used to retrieve the beads from the solution and the captured single-strand DNA target was released from the biotinylated oligonucleotide by sequentially incubating beads in 1 × elution buffer and removing them with a magnet; the captured cDNA remains in the supernatant. After release, the cDNA clone was further enriched by using a non-biotinylated target oligonucleotide to prime conversion of the recovered single-strand DNA target to double-strand DNA. The repaired DNA was transformed into *E. coli* DH5 α-competent cells and plated. The colonies were screened by PCR using primer pairs GGTTTCATTCCAAGCTCTCC (forward primer) and TTAGGAGAACGGCGACTGAT. (reverse primer). One positive clone of approximately 1.1 kb was identified and its nucleotide sequence was determined by sequencing both strands by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using an ABI 317 DNA Sequencer (Perkins Elmer) and primers M13F and M13R synthesized on an ABI 392 DNA/RNA Synthesizer. Sequence assembly and analysis was performed using the Sequencer 4.0.2 (Gene Codes).

2.3. Submandibular gland mucin antibody and Western blot

A polyclonal antibody to Muc10 protein isoforms was generated by Zymed (South San Francisco, CA) using standard methods. The antigen used was a 22-amino acid peptide [amino acids 22–42: (C)SHHKSRSSQFPVRKYLEDPRY–COOH] from the 5′, non-repeat region of the protein (which lacks glycosylation sites) (Denny et al., 1996; present study); this peptide was shown by PSI-BLAST (Altschul et al., 1997) to be unique to Muc10 protein. The antibody was affinity-purified with the antigen peptide and its peptide specificity was determined by ELISA.

To characterize age-specific differences in mucin protein, Western blot analyses of E18, 1-day neonatal, 21-day-old postnatal and adult submandibular glands were conducted as described by Jaskoll et al. (1998). Each independent sample consisted of E18 (18–20 glands from one litter), 1-day-old neonate (four glands/two animals), 21-day-old postnatal (four glands/two animals) and adult (two glands/one animal) material. Equivalent amounts of total protein (20 μg) were resolved on 8% SDS–PAGE gels, transferred to Immunobilon membranes (Pierce Chemical Co.,

Rockford, IL), and immunoblotted (Jaskoll et al., 1998). Blots were blocked overnight at 4°C with Blotto and then incubated overnight at 4°C with antimucin antibodies (1:100 in Tris-buffered saline). After several washes in Tris-buffered saline containing 0.05% Tween 20, the blots were then incubated in antirabbit IgG–alkaline phosphatase conjugate (Pierce Chemical Co.). Immune complexes were visualized with the Pierce 1-step alkaline-phosphatase substrate kit or Enhanced Chemiluminescent Plus (Amersham Pharmacia Biotech, Arlington Heights, IL) and autoradiography. Controls consisted of blots incubated in preimmune rabbit serum or in the absence of primary antibodies; controls were routinely negative. Six independent experiments were conducted. To demonstrate the specificity of the mucin antibody, 390 μg/100 μl rabbit antimucin IgG was neutralized by preabsorption with 20 μg of the 22-amino acid peptide for 30 min at room temperature before membrane incubation; no mucin bands were immunodetected using the preabsorbed antibody (data not shown).

2.4. Immunohistochemistry

E18 and adult submandibular glands were fixed in Carnoy's fixative, processed, embedded in low melting-point paraplast, and immunostained as described by Jaskoll et al. (1998). In brief, the sections were incubated overnight with the primary antibody [goat anti-L-selectin (1:10) (Santa Cruz)]. Sections were then incubated with fluoroscein isothiocyanate-labelled, antigoat IgG (1:10; ICN). In all experiments, negative controls were incubated with preimmune serum or in the absence of primary antibody; controls were routinely negative. A minimum of six submandibular glands was evaluated for each age.

2.5. Immunoprecipitation and L-selectin-binding studies

To determine if L-selectin binds to embryonic mucin, mucin protein was immunoprecipitated from E18 or adult submandibular glands lysated with NP-40 lysate buffer (1% SP-40, 1 mM phenylmethylsulphonyl fluoride, 1 μM leupeptin and 0.05 M Tris–HCl) at room temperature for 2 h. Each independent E18 sample consisted of four litters of approximately 80 submandibular glands or one adult submandibular gland; three independent samples were analysed. The lysate was incubated with 3.9 mg/100 μl antimucin antibody overnight at 4°C with gentle mixing and then dialysed against ImmunoPure IgG Binding Bugger (Pierce). The immunocomplexes were collected using carbohydrate-free, protein A - Sepharose beads (Pierce). The beads were collected by centrifugation three to five times in binding buffer. After each centrifugation, the beads were collected and washed several times in buffer, transferred to new tubes, and immune complexes eluted by boiling in SDS sample buffer.

The immunoprecipitated sample was cleared by high-speed centrifugation and both pellet and supernatant were collected. After resuspending the pellet in SDS sample buffer, equivalent amounts of both fractions were resolved on 8% SDS–PAGE gels, transferred to Immunobilon membranes (Pierce) and immunoblotted (Jaskoll et al., 1998). The presence of mucin protein was confirmed by incubation with antimucin antibody for each sample as described above and the M_r calculated.

The ability of exogenous L-selectin to bind to mouse E18 and adult submandibular gland mucin was determined according to the method of Prakobphol et al. (1998). In brief, mouse L-selectin/human IgG (4 μg/ml in PBS/3% BSA) was incubated with 1:500 biotinylated goat antihuman IgG (Caltag Laboratories, Burlington, CA) and 1:500 streptavidin–alkaline phosphatase (Caltag) for 20 min at room temperature, then diluted 1:1 in PBS/3% BSA (experimental) or 20 mM EDTA (control). The mouse L-selectin/human IgG chimera was generously provided by Dr Steven D. Rosen (University of California, San Francisco). Immunobilon membranes containing immunoprecipitated soluble (cytosolic) and membrane fractions were incubated with preformed L-selectin/human IgG chimeras–biotinylated goat antihuman IgG/streptavidin–alkaline phophatase complex overnight at 4°C; control blots were incubated in the presence of 20 mM EDTA to confirm the specificity of Ca^{2+}-dependent L-selectin binding. The L-selectin-reactive bands were visualized by 1-step alkaline phosphatase substrate kit (Pierce). The M_r of the L-selectin-reactive band was calculated and compared to an immunoblot from the identical sample stained with antimucin antibodies. This experiment was conducted three times, each experiment consisting of an independent E18 or adult submandibular gland sample.

To determine if endogenous L-selectin binds to E18 or adult submandibular gland mucin, E18 and adult gland samples were immunoprecipitated with antimucin antibodies, electrophoresed on reducing gels, and immunoblotted as described above. Immunobilon membranes containing immunoprecipitated soluble (cytosolic) and membrane fractions were preincubated overnight in Blotto and incubated overnight in goat anti-L-selectin (1:50; Santa Cruz) at 4°C. Biotinylated swine antigoat IgG (1:500; Caltag) and streptavidin–alkaline phosphatase (1:500; Caltag) were preincubated for 30 min at room temperature with shaking,

and the membranes were then incubated in biotinylated antigoat IgG/streptavidin–alkaline phosphatase complex for 1 h at room temperature. The L-selectin reactive band was visualized by 1-step alkaline phosphatase substrate kit (Pierce) and the M_r of the L-selectin-reactive band was calculated. Controls consisted of blots incubated with preimmune serum or in the absence of primary antibody. Controls were routinely negative.

3. Results

3.1. cDNA sequence analysis of embryonic Muc10

As noted above, we identified a single positive clone from our E18 submandibular gland library. The nucleotide sequence encoded by this full-length cDNA and its deduced amino acid sequence is shown in Fig. 1

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1   CCACGCGTCCGAGCTAACCAAACTGANTATTTGGCAAAAGTAAGCTTAAGCAACAACTG

60  ACTGTTCCAAAGGAGAATCTCCTACCCAGGAGCAACATTAAGAAATG AAA AAT GAA
                                                    M   K   N   E     4

116 ATG TTC ATC TTG GGC CTG TTG GCT CTT ACT TCA TAT TTC ATG CCT
     M   F   I   L   G   L   L   A   L   T   S   Y   F   M   P    19

161 GGT GAA AGT CAT CAC AAG TCA AGA AGT TCA CAA TTC CCT GTC AGA
     G   E   S   H   H   K   S   R   S   S   Q   F   P   V   R    34

206 AAA TAT CTA GAA GAC CCA AGA TAT CCA AGA TAT CCA AGA CCA CAT
     K   Y   L   E   D   P   R   Y   P   R   Y   P   R   P   H    49

251 TAT TCC TAT GGT TTC ATT CCA AGC TCT CCA AAG TTT CCA AAA GAC
     Y   S   Y   G   F   I   P   S   S   P   K   F   P   K   D    64

296 AAT CAA TGG TAT AAG ATG TGC CCT CCA GGA ACT ACC TTA ATG CTG
     N   Q   W   Y   K   M   C   P   P   G   T   T   L   M   L    79

341 ATC AGT CGC CGT TCT CCT AAA TTT CTA TGC ATT CCT AAA AGA CAG
     I   S   R   R   S   P   K   F   L   C   I   P   K   R   Q    94

386 ATT ATC TCT GAT AAA ACG AAA CCA AAT GCA ACC ACA CCA GCA CCT
     I   I   S   D   K   T   K   P  *N   A   T   T   P   A   P   109

431 ACC ACC AAA CCT ACC ACA AAT GCA ACT ACA CCA GCA CCA ACC AAC
     T   T   K   P   T   T  *N   A   T   T   P   A   P   T   N   124

476 AGA ACT ACC ACA AAT GCA ATC ACA CCA GCA CCG ACC CCC AAA CCT
     R   T   T   T  *N   A   I   T   P   A   P   T   P   K   P   139

521 ACC ACA AAT GCA ACC ACA CCA GCA ACA ACC ACA AAT GCA ACC ACA
     T   T  *N   A   T   T   P   A   T   T   T  *N   A   T   T   154

566 CCA GCA ACA ACC ACC AAA TCT ACA ACA AAA GAA CCT ACT ACT TCA
     P   A   T   T   T   K   S   T   T  ▲K   E   P   T   T   S   169

611 CCT AAG CCT AGC ACC TCT ACA GCC ATA CCT ACA ACA ACT AAG TCT
     P   K   P   S   T   S   T   A   I   P   T   T   T   K   S   184

656 GCA AAT AGT ACT TCC TCT ACT ACA ACT AGT ACT ACC ATC CAA ACT
     A   N   S   T   S   S   T   T   T   S   T   T   I   Q   T   199

701 ACA GCT CCA ACA TTT GCG GAA GTG TTT TGG AAG TTC TTT CAG CAG
     T   A   P   T   F   A   E   V   F   W   K   F   F   Q   Q   214

746 ATC TTT AGG TTA AAG AAA TGATAGGATAGTTCCAAACTTTTCTGATCTCTTAG
     I   F   R   L   K   K                                      220

799 GATAAAAAAGCCTCAATGATGATTTTGAAGAAATCAACCTGATATATAATTGGAAAAAT

858 CAAACAAATAAAAATAATTTGAGCAAGGAAAAAAAAAAAAAAAAAAA
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Fig. 1. Mouse embryonic submandibular salivary gland mucin sequence: The 5′ and 3′ non-translated regions are represented by continuous sequence. The coding region is presented in codon triplets with their deduced amino acid below in single-letter notation. The most probable initiation codon, stop codon, and polyA signal are underlined. The five repeats in the repeat domain are designated by *; the start of the non-repeat C-terminal domain is designated by △.

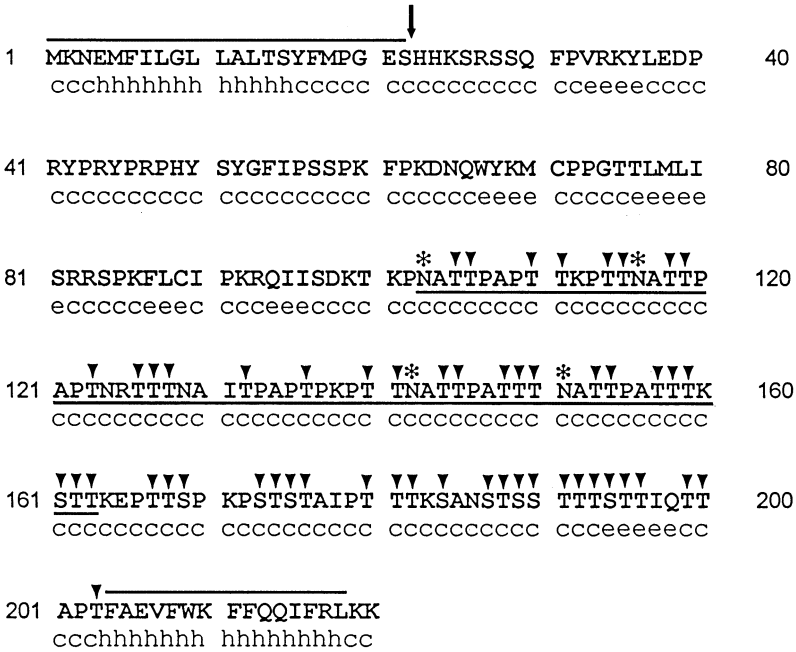

Fig. 2. Mouse embryonic submandibular salivary gland mucin amino acid sequence: The overlines designate the N-terminal signal sequence (arrow = signal peptide cleavage site) and the C-terminal α-helix. The underlines designate the tandem-repeat domain; * designate putative *N*-glycosylation sites and ▽ designate putative *O*-glycosylation sites. Other secondary-structure designations: *h*, α-helix; *c*, random coils; *e*, extended strands.

(GenBank no. AF247816). The cDNA encodes an 885-bp sequence (lacking the polyA tail) that contains a most probable translation start site at bp 104–106, followed by a single open reading frame of 219 amino acids (approximately 24600 Da). The coding sequence contains three domains: N-terminal, repeat, and C-terminal. The total amino acid composition is rich in serine (approximately 9%), threonine (approximately 22%), and proline (approximately 14%); the repeat domain is particularly rich in threonine (approximately 46%) and proline (approximately 18%).

3.2. Signal sequence, glycosylation sites, and putative secondary structure

Signal-peptide prediction was performed using the Nielsen neural network and hidden Markov model algorithms (Nielsen et al., 1997; Nielsen and Krogh, 1998). With a probability of 87%, the signal peptide was predicted to span amino acid position 1–22 (Fig. 2). It has long been known that potential *N*-glycosylation sites are specific to the consensus sequence *N*–(X)–[S/T]–(X), (X) being any amino acid but proline and *N* being the glycosylation site (Marshall, 1972; Gavel and von Heijne, 1990). ScanProsite analysis (*www.expasy.ch/cgi-bin/scanprosite*) identified potential *N*-glycosylation sites at the initial amino acid of each of four repeats in the repeat domain (Fig. 2). *O*-glycosylation sites were predicted using the NetOglyc algorithm of Hansen et al. (1995, 1997, 1998); 53 *O*-glycosylation sites were identified, all in the repeat and C-terminal domains (Fig. 2).

Consensus secondary-structure prediction for the non-glycosylated peptide was made with the NPS@ algorithm (*pbil.ibcp.fr/cgi-bin/npsa*), which presents a meta-analysis of results from analyses of the query sequence by eight independent prediction algorithms. From the signal sequence cleavage site at amino acid 23–203, the secondary structure is essentially random coils interspersed by short extended strands (Fig. 2). The C-terminus (amino acid 204–218) is a putative α-helix (Fig. 2). This amino acid sequence was examined with the helical-wheel algorithm of Turcotte (*www.bmm.icnet.uk/people/turcotte/Java/HelixWheel/*), which is based on Benner et al. (1994) and Naor et al. (1996), and confirmed by the algorithm HELICALWHEEL (*www.gcg.com*). The helical-wheel diagram (Fig. 3) consists of a projection of the side-chain orientation on to a plane perpendicular to the long axis of the helix. This sequence (amino acid 204–218) appears to form an amphipathic α-helix, with one face of the helix containing polar residues and the other containing non-polar residues (Segrest et al., 1990), a finding of probable significance to this membrane-associated molecule (discussed below).

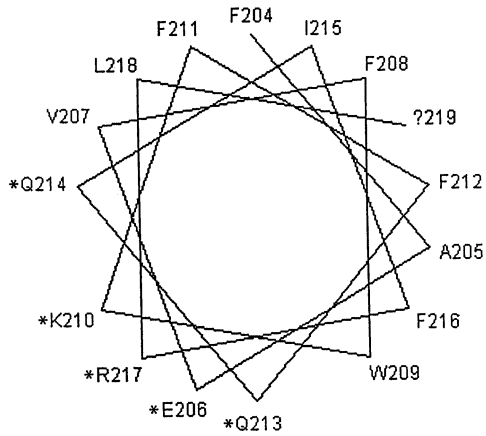

Fig. 3. The predicted C-terminal α-helix (amino acid 204–218) was analysed using the helical-wheel program of M. Turcotte (*www.bmm.icnet.uk/people/turcotte/Java/HW/*). This program displays a view down the barrel of the α-helix and designates the amino acid residues surrounding the helix. Polar amino acid residues are indicated by *; all other residues are non-polar. This is an amphipathic helix. Using the method of Eisenberg et al. (1984), we calculate the mean hydrophobicity of the non-polar face to be 1.09/amino acid residue and the polar face to be −1.29/amino acid residue.

3.3. Alignment with adult Muc10

There is 80% identity of the sequence reported here (Fig. 1) with that of adult Muc10 (Denny et al., 1996). The N- and C-terminal domains are 100% identical. Major differences exist in the repeat domain. Indeed, it is highly probable that embryonic Muc10 is an alternative splicing variant of adult Muc10 (Fig. 4). This was determined using the IRACE algorithm (*www.labonweb.com*; Compugen, Ltd., Tel Aviv, Israel), which queries a proprietary database of clustered and assembled expressed sequences. The algorithm that builds this database models alternative splicing based on the alignment characteristics of individual expressed sequences (full-length mRNAs) belonging to the same cluster. The alternatively spliced embryonic Muc10 repeat domain has approximately 50% of the potential *N*- and *O*-glycosylation sites seen in the adult Muc10 repeat domain. Additionally, other mucin and mucin-like proteins of high identity and similarity are presented in Table 1.

3.4. Relative expression of embryonic and adult Muc10

Western blot analysis was used to characterize age-specific differences in submandibular gland mucin protein using an antibody specifically directed against a unique 21-amino acid sequence (amino acid 22–42) common to both embryonic and adult Muc10. Because it lacks potential glycosylation sites, it is not likely to be masked by carbohydrate chains. As shown in Fig. 5, notable mucin protein differences were seen between embryonic, postnatal and adult submandibular glands.

In E18 submandibular glands, several small proteins were identified with our peptide-specific antibody: approximately 25, 40, and 68 kDa. By contrast, the adult submandibular gland exhibits a large band spanning from approximately 130–200 kDa, as previously reported (Jaskoll et al., 1998). The smaller proteins seen in E18 submandibular glands are absent from adult submandibular glands. In newborn and 21-day postnatal submandibular glands, one sees a transition from embryonic to adult protein.

408- AAT GCA ACC ACA CCA GCA CCT ACC ACC AAA CCT ACC ACA (repeat 1)

447- AAT GCA ACC ACA CCA GCA CCG ACC CTC AAA CCT ACC ACA (repeat 2)

486- AAT GCA ACC ATA CCA GCA CCA AAC AAC AGA ACT ACC ACA (repeat 3)

525- AAT GCA ACC ACA CCA GCA CCG ACC ACA AAA CCT ACC ACA (repeat 4)

564- AAT ACA ACC ACA CCA GCA CCG ACC ACC AAA TCT ACC ACA (repeat 5)

603- AAT GTA ACC ACA CCA GCA ACA ACC ACA (repeat 6)

630- AAT GTA ACC ACA CCA GCA ACA ACC ACA (repeat 7)

657- AAT GCA ACC ACA CCA GCA ACA ACC ACA (repeat 8)

684- AAT GCA ACC ACA CCA GCA ACA ACC ACA (repeat 9)

711- AAT GCA ACC ACA CCA GCA ACA ACC ACC AAA TCT ACA ACA (repeat 10)

Fig. 4. The repeat domain of the adult submandibular salivary gland mucin (Denny et al., 1996): shaded areas represent sequence spliced out of the embryonic mucin, as determined by the IRACE algorithm (*www.labonweb.com*).

Table 1
Most probable sequence alignments with embryonic submandibular salivary gland (SMG) mucin

Name (accession no.)	E-score[a]	% identity[b]	% similarity[c]
Muc10: adult mouse SMG mucin (U37531)	2.4×10^{-81}	79.9	79.9
Adult rat apomucin precursor (U03407)	1.8×10^{-33}	40.5	54.2
D. virilis salivary larval glue protein (A60095)	7.7×10^{-11}	40.5	60.1
C. parvum mucin-like glycoprotein (AF06065)	1.1×10^{-10}	36.4	64.7

[a] E-score, the expected frequency of the chance occurrence of a sequence with a specific Z-score in a random database of the length equal to the length of the database searched (*www.labon.web.com*: a comprehensive and non-redundant protein database created from all publicly available databases).
[b] % identity, the ratio of the number of identical residues in aligned sequences to the alignment length.
[c] % similarity, the ratio of the number of residues in the alignment that are assigned a positive score in the comparison matrix to the alignment length.

3.5. Embryonic Muc10 L-selectin-binding activity

Given that substantial qualitative differences exist between mRNA and cell localization for embryonic and adult submandibular gland mucin protein (Jaskoll et al., 1998; present study), we speculated that the embryonic mucin has a morphogenetic role during cell–cell interactions. Such speculation was encouraged by studies showing that cell membrane-associated proteins with a mucin-like domain have functional roles in cell–cell adhesion and interactions. For example, a membrane-associated, low molecular-weight, mucin-like glycoprotein (GlyCAM) has been shown to be an endothelial ligand for L-selectin (Lasky et al., 1992); the high-affinity binding of L-selectin is due to the extracellular presentation of the carbohydrates in the mucin-like domain of the GlyCAM. By analogy, we sought evidence that L-selectin was specifically present in E18 mouse submandibular glands and that it was capable of binding embryonic low molecular-weight mucin.

Antibodies to L-selectin immunodetected protein expression in ductal and terminal bud epithelia of E18 submandibular glands (mostly late terminal bud stage) (Fig. 6A–C). Of note, the immunolocalized L-selectin protein was often cell membrane-associated, i.e. as it is with embryonic submandibular gland mucin (Jaskoll et al., 1998). By contrast, no L-selectin was immunodetected in adult submandibular glands (Fig. 6D). Next we assayed the ability of a mouse L-selectin/IgG chimera to bind to immunoprecipitated E18 or adult submandibular gland mucin (Fig. 7A). L-selectin interacted exclusively with the membrane fraction of the immunoprecipitated embryonic mucin, and not with the soluble (mostly cytosolic) fraction. Specifically, binding was only to the putatively glycosylated, approximately 68 kDa embryonic mucin protein present in the membrane fraction (Fig. 7A) and not the approximately 25 and 40 kDa species found in the E18 soluble fraction (not shown). No L-selectin binding was observed when the membrane fraction was in the presence of 20 mM EDTA, demonstrating the specificity of the Ca^{2+}-dependent L-selectin binding. In addition, L-selectin did not bind to adult mucin, neither membrane nor soluble fraction (Fig. 7A).

Finally, we sought to determine if endogenous L-selectin is bound to embryonic mucin. E18 or adult submandibular gland extracts were immunoprecipitated with antimucin antibodies to isolate mucin proteins and all mucin-bound proteins. With anti-L-se

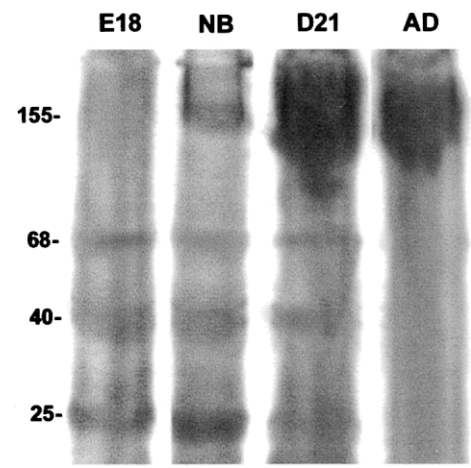

Fig. 5. Western blot analysis of mucin protein. Representative immunoblots of E18, newborn (NB), 21-day-old (D21) and adult (AD) submandibular salivary glands (SMGs) demonstrate marked age-specific differences in mucin protein. E18 SMGs exhibit several small proteins of approximately 25, 48, 68 kDa. Adult SMG mucin is seen as a large band spanning from approximately 130–200 kDa. Both adult and embryonic variants are seen in newborn and 21-day old SMGs, with a substantial increase in adult mucin being seen between newborn and 21 days.

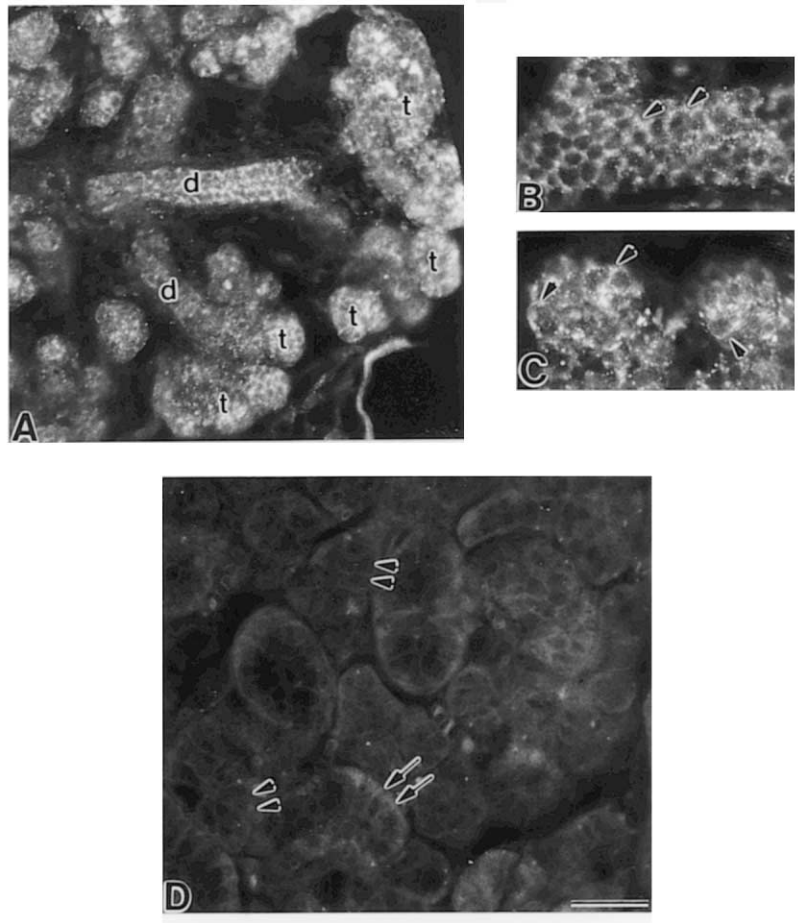

Fig. 6. Immunolocalization of L-selectin in embryonic submandibular salivary glands (SMGs). (A–C) E18 SMGs. L-selectin is immunodetected in E18 ductal (d) and terminal bud (t) epithelia in association with cell membranes, as well as in the cytoplasm. Higher-magnification views of E18 SMG ductal; (B) and terminal bud; (C) epithelia demonstrate that L-selectin protein expression is cell-membrane associated (arrowhead); (D) Adult SMG. L-selectin is not immunodetected in adult SMG ducts (double arrows) or acini (double arrowheads). Bar: (A) 50 μm; (B–D) 20 μm.

lectin antibodies, Western blot analysis of immunoprecipitated proteins showed a single, approximately 74 kDa band in the membrane fraction of E18 immunoprecipitated proteins (Fig. 7B). This was consistent with L-selectin protein (Nicholson et al., 1998). There was a similar, but weaker, band in the soluble fraction [unlike the preceding experiment (Fig. 7A)] because some of the endogenous L-selectin is released from mucin protein into the buffer. Demonstration of L-selectin protein among proteins immunoprecipitated with anti-mucin antibodies is probative of endogenous embryonic mucin/L-selectin complexes in E18 submandibular glands. By contrast, Western blot analysis of adult immunoprecipitated proteins demonstrated no L-selectin protein in either the membrane or soluble fraction, indicating that L-selectin is not bound to adult submandibular gland mucin (Fig. 7B).

4. Discussion

Mucins have typically been thought of as the major organic component of saliva or mucus, viscous substances that serve as a protective barrier between epithelial cells and the extracellular milieu of many organ systems (mouth, lungs, reproductive tract, etc.) (Gendler and Spicer, 1995). These secretory mucins are large molecules (>200 kDa) that are highly *O*-glycosylated and form oligomers via disulphide bonds. There is, also, another class of mucins, and other glycoproteins with mucin-like domains, that are membrane-associated and serve to modulate cell adhesion (Gendler and Spicer, 1995). These include Muc1 (MUC1 in humans) (Braga et al., 1992; Hilkens et al., 1992), CD43 (leukosialin) (Ardman et al., 1992; Park et al., 1991; Rosenstein et al., 1991); sialomucin complex (MUC4 in

humans) (McNeer et al., 1998; Moniaux et al., 1999), GlyCAM (Lasky et al., 1992), and *Drosophila melanogaster* mucin-D (Kramerova and Kramerov, 1999).

Some of these mucin glycoproteins are detected during mammalian embryogenesis. For example, Muc1 is found in pseudoglandular-stage (approximately E15) submandibular gland ducts and terminal bud-stage (approximately E18) ducts and presumptive acini (Braga et al., 1992). Muc1 is also seen in developing lungs, pancreas, and kidneys. Braga et al. (1992) propose that Muc1 participates in epithelial sheet differentiation (branching) and lumen formation, most likely by inhibiting cell adhesion due to its large size (Hilkens et al., 1992). We report here an embryonic mucin of smaller size, which is likely to promote cell adhesion by lectin binding. As such, we suggest that it be termed mucin cell adhesion molecule or MucCAM.

Using a ribroprobe generated from a plasmid containing a 160-bp fragment of the 5' non-repeat domain of the full-length, 1.01-kb adult submandibular gland mucin (Muc10) (Denny et al., 1996), we recently identified a smaller (0.85 kb) transcript of embryonic submandibular gland mucin (Jaskoll et al., 1998). This embryonic transcript is first detected in the pseudoglandular stage (approximately E14) and significantly increases by the late terminal bud stage (approximately E18). Sequencing of the clone identified from our E18 cDNA library indicates that this smaller transcript is an alternatively spliced variant of adult Muc10. Using an antibody specifically directed against a unique 22-amino acid sequence from the N-terminal, non-repeat region encoded by the cDNA, we identified several variants of mucin protein (approximately 25, 40 and 68 kDa) in E18 submandibular glands. The approximately 25-kDa protein was similar in size to that predicted from our cDNA-encoded sequence (24600) and most probably represents the native, unglycosylated peptide. The larger variants probably represent glycosylated forms of the protein (Denny et al., 1996; Jaskoll et al., 1998), or less probably dimerization/trimerization of the native unglycosylated peptide. It is interesting to note that both embryonic and adult mucin variants are immunodetected in newborn and 21-day submandibular glands, with a marked increase in adult mucin forms being seen between the newborn and 21-day-old gland. The predominance of the adult mucin proteins is correlated with the appearance of acinar cell histodifferentiation, which occurs 2–3 weeks after birth (Srinivasan and Chang, 1979). As our E18 mouse submandibular gland library contains only the MucCAM clone and the adult mouse submandibular gland library contains only Muc10 clones (Denny et al., 1996), it is almost certain that adult mucin protein is not a more mature, highly glycosylated form of MucCAM.

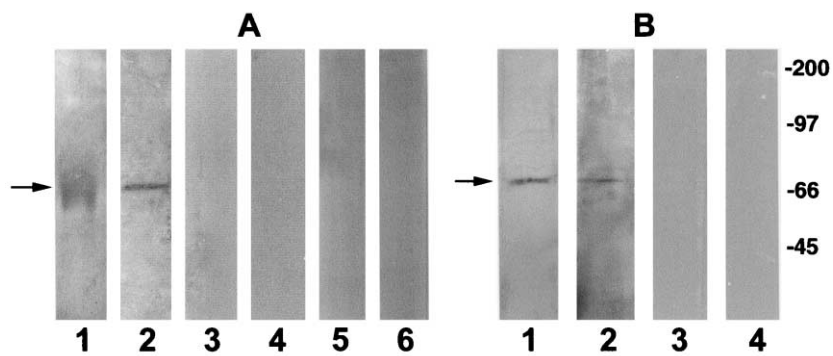

Fig. 7. L-selectin studies. (A) Exogenous L-selectin-binding studies. Proteins were immunoprecipitated from E18 and adult submandibular salivary glands (SMGs) with antimucin antibody and the membrane and soluble fractions were electrophoretically separated and transferred to Immunobilon membranes. Lanes 1–3. E18 SMG membrane fractions. Lane 4. E18 SMG soluble fraction. Lane 5. Adult SMG membrane fraction. Lane 6. Adult SMG soluble fraction. Lane 1, incubated with antimucin antibody, demonstrates the presence of the approximately 68 kDa mucin form (arrow) in the E18 membrane fraction. Lanes 2–6 were incubated with a preformed complex of mouse L-selectin/human IgG chimera, biotinylated goat antihuman IgG, and streptavidin–alkaline phosphatase, in the absence (lanes 2, 4–6) or presence (lane 3) of 20 mM EDTA. L-selectin chimera binds only to membrane-associated mucin protein in the presence of Ca^{2+} (lane 2, arrow); EDTA eliminated L-selectin binding (lane 3); L-selectin chimera does not bind to the embryonic soluble fraction (lane 4), adult membrane fraction (lane 5) or adult soluble fraction (lane 6). This L-selectin/mucin complex at approximately 68 kDa correlates with the mucin variant of similar size seen in the immunoprecipitated protein incubated with antimucin antibody (compare lanes 1 and 2). (B) Endogenous L-selectin studies. E18 and adult proteins were immunoprecipitated with antimucin antibody and immunoblotted as described in (A), then evaluated by Western blot for the presence of L-selectin protein using an anti-L-selectin antibody. A single L-selectin protein band of approximately 74 kDa (arrow) is seen in both E18 membrane (lane 1) and soluble (lane 2) immunoprecipitated fractions; L-selectin protein is not detected in adult membrane (lane 3) or soluble (lane 4) fractions.

Although we have previously reported mucin proteins of approximately 110 and 152 kDa in E17 submandibular glands (Jaskoll et al., 1998), these protein forms were not seen here. It is unclear why the embryonic mucin proteins detected by Western blot differ notably between our two studies while the immunohistology is identical. The most likely explanation is the difference between the antibodies used. In our present study, we employed a peptide-specific antibody made against a 22-amino acid peptide from the 5′, non-repeat region of the protein. This peptide was chosen because it lacks potential glycosylation sites. Thus, it would be less likely for this region of the mucin protein antigen to be masked by carbohydrate chains. It is for this reason that we were now able to detect the native mucin protein (approximately 25 kDa). By contrast, our previous experimentation utilized polyclonal antibodies directed against the adult-secreted, glycosylated submandibular gland mucin protein. This antibody most probably identified highly glycosylated proteins and/or their associated carbohydrate chains, including some glycoproteins other than mucin.

4.1. L-selectin binding

The need for cells to adhere to one another or to the extracellular matrix is fundamental to organogenesis (Edelman, 1988). It has long been recognized that lectins and their cognate carbohydrates play an important part in this process (Mann and Waterman, 1998). We present evidence that at least one possible function of the embryonic, low molecular-weight mucin is to bind the L-selectin expressed in terminal bud epithelium. The primary role of L-selectin is to mediate cell adhesion; its ligands are mucin-like glycoproteins (Kaltner and Stierstorfer, 1998; Shimizu and Shaw, 1993); hence the suggestion that this embryonic *Muc10* gene product be termed mucin cell adhesion molecule or MucCAM.

L-selectin has an N-terminal, C-type lectin domain that recognizes carbohydrate ligands on apposing cells (Bird et al., 1997). C-type lectins are characterized by their dependence on Ca^{2+} ions for carbohydrate binding and their carbohydrate ligands include the tetrasaccharide sialyl Lewis^x for selectin binding (Kaltner and Stierstorfer, 1998). At present, all the known ligands for L-selectin are mucins or mucin-like molecules, e.g. GlyCAM (Lasky et al., 1992), PSGL-1 (Aeed et al., 1998), MUC7 (Prakobphol et al., 1998, 1999), and MucCAM (present study). During morphodifferentiation there is a close relation between the expression of cell-adhesion molecules, cell adhesion and boundary formation between cell collectives (Edelman, 1988). Interestingly, although L-selectin has been shown to bind to adult human submandibular gland *MUC7* gene products, we demonstrate that L-selectin does not bind to adult mouse Muc10 protein.

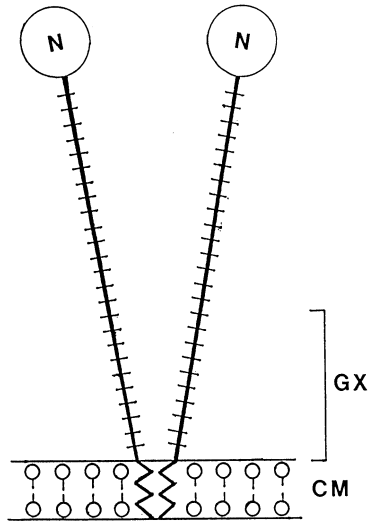

Fig. 8. Proposed structure of MucCAM: attachment is by dimerization of C-terminal amphipathic α-helices and insertion into the cell membrane (CM) such that the polar faces interact with one another and the non-polar faces interact with the lipid bilayer; cross-hatches along the protein core represent sites of *O*-glycosylation; N is the N-terminal spherical domain; GX, glycocalyx. This model is similar to one proposed for GlyCAM, another mucin-like glycoprotein ligand for L-selectin (Lasky et al., 1992). As noted in the text, the cell membrane-association of MucCAM may, alternatively, be by glycolipid anchoring (Cross, 1990).

Currently, it is the consensus that selectins initiate a transient, weak cell adhesion before the strong adhesion mediated by integrins (Shimizu and Shaw, 1993; Kaltner and Stierstorfer, 1998). Although it is difficult to be certain without three-dimensional reconstruction of developing submandibular glands, the immunohistology reported here and elsewhere (Jaskoll et al., 1998; Melnick and Jaskoll, 2000) suggests that L-selectin/MucCAM binding may be a transient mechanism of weak cell adhesion preceding either the integrin binding of boundary cells or the anoikis (apoptosis) of interior cells that makes possible lumen formation. The absence of L-selectin/Muc10 binding in the adult gland supports this hypothesis.

4.2. Proposed MucCAM structure

The primary role of MucCAM in cell adhesion would be to present the *O*-linked carbohydrates to the lectin domain of L-selectin. As such, we propose a structure for MucCAM as shown in Fig. 8. The α-helix at the C-terminus is an amphipathic helix with opposing polar and non-polar faces oriented along the long axis of the helix. The non-polar face has twice the number of residues as the polar face; the non-polar face has a high mean hydrophobicity of 1.09 per residue and the

polar face has a low mean hydrophobicity of −1.29 per residue. This C-terminal, amphipathic α-helix has a length of about four turns (Eisenberg et al., 1984). Given the above, we propose that MucCAM is plasma-membrane-bound such that the polar faces interact with each other to form a dimer and the non-polar faces interact with the lipid bilayer. This is similar to that proposed for GlyCAM, another mucin-like glycoprotein ligand for L-selectin (Lasky et al., 1992).

Alternatively, MucCAM may be membrane-associated by covalent linkage to the glycolipid anchor, glycosyl-phosphatidylinositol. Many glycoproteins, including cell-adhesion molecules, appear to attach to cell surfaces via glycosyl-phosphatidylinositol anchor structures that are preassembled and added to nascent proteins by C-terminal cleavage and attachment in the endoplasmic reticulum (Cross, 1990). Coyne et al. (1993) have defined a core consensus signal in the C-terminal domain for glycosyl-phosphatidylinositol anchoring: (1) a C-terminus of at least 11 residues with weak to moderate hydrophobicity, and without a cytoplasmic tail; (2) a cleavage/attachment tripeptide domain with small amino acids such as Ser, Ala, or Gly preferred at the first (attachment) and third positions; (3) a spacer domain between the tripeptide and the hydrophobic C-terminus of more than seven and less than 20 residues in size. MucCAM presents all characteristics of the core consensus signal for glycosyl-phosphatidylinositol anchoring: (1) there is a 15-residue, hydrophobic C-terminus without a cytoplasmic tail (amino acid 204–218); (2) probably cleavage/attachment tripeptides exist at residues 184–186 (SAN), 185–187 (ANS), and 187–189 (STS); (3) there is a spacer domain of 14–17 residues, depending on the cleavage site. Given the characteristics of greater anchoring efficiency (Coyne et al., 1993), the most likely structure would be cleavage between amino acids 187 and 189 and attachment of the glycosyl-phosphatidylinositol to the Ser residue at amino acid 187.

Mucin glycoproteins are relatively inflexible because of steric interactions between the peptide-linked GalNac residue (*O*-glycosylation) and adjacent amino acids in the peptide core (Jentoft, 1990). The repeat domain of MucCAM has 29 putative *O*-glycosylation sites and the C-terminal domain has 24. In all, more than 50% of amino acid residues 103–203 are possible *O*-glycosylation sites. At 0.25 nm per amino acid residue (Jentoft, 1990), this glycosylated region would be expected to be a rather rigid rod with a persistence length of approximately 25 nm. In effect, this extends the MucCAM molecule well above the approx imately 10 nm glycocalyx (extracellular surface), allowing this glycoprotein to interact with extracellular macromolecules (such as L-selectin) that might otherwise be unable to penetrate the relatively crowded glycocalyx [in a manner similar to other membrane glycoproteins such as decay-accelerating factor and LDL receptor (Jentoft, 1990)]. Further, it is reasonable to expect that the close clustering of carbohydrate epitopes will result in rapid on-rates and enhanced avidity of mucin/selectin binding (Lee, 1989; Williams, 1991).

The 102-amino acid, N-terminal domain of MucCAM (exclusive of the signal sequence) has a predicted secondary structure of random coils interrupted by short extended strands and is without putative glycosylation sites. Unlike tightly folded globular peptides, which have a relatively fixed and compact tertiary structure, those with a random coil conformation form a spherical domain with variant configuration (Jentoft, 1990).

Each region of the MucCAM protein, then, has a proposed function. The amphiphathic α-helix anchors the molecule to the plasma membrane. The protein core of the glycosylated region serves as a scaffold for carbohydrate presentation to L-selectin and, perhaps, other cognate extracellular molecules. The function of the spherical domain, however, is obscure.

4.3. Evolution and class considerations

Many investigators have utilized specific carbohydrate/lectin staining patterns in defining the dynamics of morphodifferentiation in various embryonic models of diverse species and tissues (see Mann and Waterman, 1998). It is thought that the sugar/lectin complex 'information management system' is evolutionarily primitive relative to more specific peptide ligand/receptor mechanisms (Mann and Waterman, 1998). In this context, it is not rare to see common genetic and epigenetic mechanisms at work in completely disparate developmental processes, a kind of co-option of developmental genetic mechanisms (Raff, 1996). This often gives rise to an evolutionarily related class of genes across widely divergent species (Hutter et al., 2000).

Of particular interest here is the relation between MucCAM and the salivary glue proteins of *Drosophila*. In the third instar larvae of *Drosophila*, the major function of the salivary glands is the production of a mucoprotein glue (Swida et al., 1990). This glue is extruded shortly after puparium formation and serves to attach the pupal case to the substrate. Multiple related salivary glue proteins are expressed by *D. melanogaster* and *D. virilis*. It is more than interesting that there is a 60% similarity ($E = 7.7 \times 10^{-11}$) between the salivary glue protein lpg-1 (Swida et al., 1990) and MucCAM. As large portions of these two sequences contain close residues, they may belong to the same protein family. It is reasonable to speculate, then, that additional MucCAMs in diverse species and disparate developing organ systems await discovery.

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References

Aeed, P.A., Geng, J.G., Asa, D., Raycroft, L., Ma, L., Elhammer, A.P., 1998. Characterization of the O-linked oligosaccharide structures on P-selectin glycoprotein ligand-1 (PSGL-1). Glycoconjugate J. 15, 975–985.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST. Nucleic Acid Res. 25, 3389–3402.

Ardman, B., Sikorski, M.A., Staunton, D.E., 1992. CD43 interferes with T-lymphocyte adhesion. Proc. Natl. Acad. Sci. USA 89, 5001–5005.

Benner, S.A., Badcoe, I., Cohen, M.A., Gerloff, D., 1994. Bone fide prediction of aspects of protein conformation. J. Mol. Biol. 235, 926–958.

Bird, M.I., Foster, M.R., Priest, R., Malhorta, R., 1997. Selectins: physiological and pathophysiological roles. Biochem. Soc. Trans. 25, 1199–1206.

Braga, V.M.M., Pemberton, L.F., Duhig, T., Gendler, S.J., 1992. Spatial and temporal expression of an epithelial mucin, Muc-1, during mouse development. Development 115, 427–437.

Chomzynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinuime thyocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Coyne, K.E., Crisci, A., Lublin, D.M., 1993. Construction of synthetic signals for glycosyl-phosphatidylinositol anchor attachment: analysis of amino acid sequence requirements for anchoring. J. Biol. Chem. 268, 6689–6693.

Cross, G.A.M., 1990. Glycolipid anchoring of plasma membrane proteins. Annu. Rev. Cell. Biol. 6, 1–39.

Denny, P.C., Mirels, L., Denny, P.A., 1996. Mouse submandibular gland salivary apomucin contains repeated *N*-glycosylation sites. Glycobiology 6, 43–50.

Edelman, G.M., 1988. Topobiology. Basic Books, New York.

Eisenberg, D., Schwarz, E., Komaromy, M., Wall, R., 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. 179, 125–142.

Gavel, Y., von Heijne, G., 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng. 3, 433–442.

Gendler, S.J., Spicer, A.P., 1995. Epithelial mucin genes. Annu. Rev. Physiol. 57, 607–634.

Hansen, J.E., Lund, O., Engelbrecht, J., Bohr, H., Nielsen, J.O., Hansen, J.-E.S., Brunak, S., 1995. Prediction of *O*-glycosylation of mammalian proteins: specificity patterns of UDP-GalNAC: polypeptide *N*-acetylgalactosaminyltransferase. Biochem. J. 308, 801–813.

Hansen, J.E., Lund, O., Rapacki, K., Brunak, S., 1997. *O*-GLYCBASE VERSION 2.0-A revised database of *O*-GLYCOSYLATED Proteins. Nucleic Acids Res. 25, 278–282.

Hansen, J.E., Lund, O., Tolstrup, N., Gooley, A.A., Williams, K.L., Brunak, S., 1998. NetOglyc: prediction of mucin type *O*-glycosylation sites based on sequence context and surface accessibility. Glycoconjugate J. 15, 115–130.

Hilkens, J., Ligtenberg, M.L.L., Vos, H.L., Litvinov, S.V., 1992. Cell membrane-associated mucins and their adhesion-modulating property. Trends. Biochem. Sci. 17, 359–363.

Hutter, H., Vogel, B.E., Plenefisch, J.D., Norris, C.R., Proenca, R.B., Spieth, J., Guo, C., Mastwal, S., Zhu, X., Scheel, J., Hedgecock, E.M., 2000. Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. Science 287, 989–994.

Jaskoll, T., Chen, H., Denny, P.C., Denny, P.A., Melnick, M., 1998. Mouse submandibular gland mucin: embryo-specific mRNA and protein species. Mech. Dev. 74, 179–183.

Jaskoll, T., Melnick, M., 1999. Submandibular gland morphogenesis: stage-specific expression of TGF-a/EGF, IGF, TGF-β. TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF-β2, TGF-β3, and EGF-R null mutations. Anat. Rec. 256, 252–268.

Jaskoll, T., Choy, H.A., Melnick, M., 1994. Glucolcorticoids, TGF-Beta, and embryonic mouse salivary gland morphogenesis. J. Craniof. Gent. Dev. Biol. 14, 217–230.

Jentoft, N., 1990. Why are proteins *O*-glycosylated? Trends. Biochem. Sci. 15, 291–294.

Kaltner, H., Stierstorfer, B., 1998. Animal lectins as cell adhesion molecules. Acta. Anat. 161, 162–179.

Kramerova, I.A., Kramerov, A.A., 1999. Mucinprotein is a universal constituent of stable intercellular bridges in *Drosophila melanogaster* germ line and somatic cells. Dev. Dyn. 216, 349–360.

Lasky, L.A., Singer, M.S., Dowbenko, D., Imai, Y., Henzel, W.J., Grimley, C., Fennie, C., Gillett, N., Watson, S.R., Rosen, S.D., 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69, 927–938.

Lee, Y.C., 1989. Binding modes of mammalian hepatic Gal/GalNAc receptors. In: Bock, G., Harnett, S. (Eds.), Symposium on Carbohydrate Recognition in Cellular Function. Wiley, New York, pp. 80–95.

Mann, P.L., Waterman, R.E., 1998. Glycocoding as an information management system in embryonic development. Acta Anat. 161, 153–161.

Marshall, R.D., 1972. Glycoproteins. Annu. Rev. Biochem. 41, 673–702.

McNeer, R.R., Huang, D., Fregien, N.L., Carraway, K.L., 1998. Sialomucin complex in the rat respiratory tract: a model for its role in epithelial protection. Biochem. J. 330, 737–744.

Melnick, M., Jaskoll, T., 2000. Mouse submandibular gland morphogenesis: a paradigm for embryonic signal processing. Crit. Rev. Oral Biol. Med. 11, 199–215.

Moniaux, N., Nollet, S., Porchet, N., Degand, P., Laine, A., Aubert, J.-P., 1999. Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. Biochem. J. 338, 325–333.

Naor, D., Fischer, D., Jernigan, R.L., Wolfson, H.J., Nussinov, R., 1996. Amino acid pair interchanges at spatially conserved locations. J. Mol. Biol. 256, 924–938.

Nicholson, M.W., Barclay, A.N., Singer, M.S., Rosen, S.D., van der Merwe, P.A., 1998. Affinity and kinetic analysis of L-selectin (CD62L) binding to glycosylation-dependent cell-adhesion molecule-1. J. Biol. Chem. 273, 763–770.

Nielsen, H., Krogh, A., 1998. Prediction of signal peptides and signal anchors by a hidden Markov model. In: Glasgow, J. (Ed.), Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology, AAAI, Menlo Park, California, pp. 122–130.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Park, J.K., Rosenstein, Y.J., Remold-O'Donnell, E., Bierer, B.E., Rosen, F.S., Burakoff, S.J., 1991. Enhancement of T-cell activation by the CD43 molecule whose expression is defective in Wiskott–Aldrich syndrome. Nature 350, 706–709.

Prakobphol, A., Thomsson, K.A., Hansson, G.C., Rosen, S.D., Singer, M.S., Phillips, N.J., Medzihradszky, K.F., Burlingame, A.L., Leffler, H., Fisher, S.J., 1998. Human low-molecular-weight salivary mucin expresses sialyl Lewisx determinant and has L-selectin ligand activity. Biochemistry 37, 4916–4927.

Prakobphol, A., Tangemann, K., Rosen, S.D., Hoover, C.I., Leffler, H., Fisher, S.J., 1999. Separate oligosaccharide determinants mediate interactions of the low-molecular-weight salivary mucin with neutrophils and bacteria. Biochemistry 38, 6817–6825.

Raff, R.A., 1996. The Shape of Life. Genes, Development, and the Evolution of Animal Form. University of Chicago Press, Chicago, p. 431.

Rosenstein, Y., Park, J.K., Hahn, W.C., Rosen, F.S., Bierer, B.E., Burakoff, S.J., 1991. CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. Nature 354, 233–235.

Sanger, F., Wicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Segrest, J.P., De Loof, H., Dohlman, J.G., Brouillette, C.G., Anatharamaiah, G.M., 1990. Amphipathic helix motif: classes and properties. Proteins 8, 103–117.

Shimizu, Y., Shaw, S., 1993. Mucins in the mainstream. Nature 336, 630–631.

Spooner, B.S., Bassett, K.E., Spooner, B.S. Jr., 1989. Embryonic salivary gland epithelial branching activity is experimentally independent of epithelial expansion activity. Dev. Biol. 133, 569–575.

Srinivasan, R., Chang, W.W.L., 1979. The postnatal development of the submandibular gland. Cell. Tissue Res. 198, 363–371.

Swida, U., Lucka, L., Kress, H., 1990. Glue protein genes in Drosophila virilis: their organization, developmental control of transcription and specific mRNA degradation. Development 108, 269–280.

Theiler, K., 1989. The House Mouse. Atlas of Embryonic Development. Springer-Verlag, New York.

Williams, A.F., 1991. Out of equilibrium. Nature 352, 473–474