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Mucin-Type Glycoproteins

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ABSTRACT: Considerable advances have been made in recent years in our understanding of the biochemistry of mucin-type glycoproteins. This class of compounds is characterized mainly by a high level of O-linked oligosaccharides. Initially, the glycoproteins were solely known as the major constituents of mucus. Recent studies have shown that mucins from the gastrointestinal tract, lungs, salivary glands, sweat glands, breast, and tumor cells are structurally related to high-molecular-weight glycoproteins, which are produced by epithelial cells as membrane proteins. During mucin synthesis, an orchestrated sequence of events results in giant molecules of M_r 4 to $6 \cdot 10^6$, which are stored in mucous granules until secretion. Once secreted, mucin forms a barrier, not only to protect the delicate epithelial cells against the extracellular environment, but also to select substances for binding and uptake by these epithelia. This review is designed to critically examine relations between structure and function of the different compounds categorized as mucin glycoproteins.

KEY WORDS: structure, biosynthesis, genes, function, cell surface mucin.

I. INTRODUCTION

All living cells interact with their environment by a selective barrier separating the cell interior from the extracellular milieu. The interactions are mediated by the plasma membrane, composed of lipids organized in a bilayer and integral membrane glycoproteins, which are imbedded in the lipid bilayer. A portion of these membrane proteins reaches several tens of nanometers into the pericellular space and contains considerable quantities of carbohydrate organized in many oligosaccharides. For most cell types in the animal body this barrier is sufficient for maintaining cellular integrity. However, many epithelia are in contact with environments, which necessitates additional protection. Therefore, they are covered by a protective secretion, which also serves as a selective physical barrier between the plasma membrane and the environment of the cell. This secretion, usually called slime or mucus, is produced by specialized epithelial cells and is a highly hydrated, slippery gel comprising a large number of constituents. The most prominent macromolecules in these gels are the mucins, which are responsible for the specific protective properties of mucus-gels. The capacity of mucins to protect epithelial surfaces depends largely on their high content of oligosaccharides and on their ability to form this protective gel.

This review will focus on the molecular structure and function of mucin-type glycoproteins. Knowledge about function of these complex molecules is limited to the generally ac-

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cepted idea that these molecules serve primarily for protection of cells and epithelia. Recent studies on mucin cDNAs using molecular cloning techniques have greatly increased insight into the primary structure of mucins.

Considering the various aspects of this type of glycoproteins enables a proper **definition** of mucus glycoproteins. Obviously, they are glycoconjugates specified as glycoproteins.[1] Mucus glycoproteins are best defined as a subclass of glycoproteins, as are the proteoglycans. Their specific character is expressed in two features: first, a high percentage of their weight consists of oligosaccharides, O-linked to serine or threonine residues in the polypeptide backbone, and second, the protein backbone contains a number of repeating sequences, including virtually all the O-linked oligosaccharide attachment sites. An accurate lower limit for the percentage of O-linked sugars to comply with the definition is difficult to give, but in general it amounts to 50% or more. This definition differs from the earlier definition of Pigman, who considered absence of N-linked oligosaccharides as a distinct mucin feature.[2]

A further subdivision of mucin-type glycoproteins is of potential importance for their function: there are secretory or soluble and membrane-bound mucin-type glycoproteins. *Secretory* mucins constitute the viscous gel that covers most mucosal surfaces of respiratory, gastrointestinal, and reproductive tracts. In addition to mucin, the gel contains water, cellular macromolecules, electrolytes, and remnants of cells. The protective and lubricative properties of mucus are determined to a large extent by the viscous and viscoelastic properties of mucins. Essential for gel formation is the capability of secretory mucins to form intermolecular disulfide bridges, resulting in oligomeric mucin. The *membrane-bound* mucins are similar to the secretory mucins in the presence of repeating amino acid sequences containing all the attachment sites for O-linked oligosaccharides, but they differ from the secretory mucins in that they contain a hydrophobic stretch of amino acid residues anchoring the long filamentous molecules in the plasma membrane. Moreover, they probably lack intermolecular disulfide bridges. The function of membrane-bound or cell-associated mucins is unclear. The expression of some of these mucins is tremendously increased in transformed cells. In case of epithelial tumors, high titers of these mucins appear in soluble form in the bloodstream, apparently originating from molecules proteolytically clipped from the tumor cells.

II. PHYSICAL AND CHEMICAL CHARACTERIZATION

Secretory mucins are by far the most abundant structural components of mucus-gels. The mucus consists of about 95% water and about 5% mucus glycoproteins, plus a large number of minor components, like electrolytes, various cellular and serum proteins, lipids, and nucleic acids.[3,4] Due to the elaborate work of Allen and co-workers it is generally accepted that all the essential physicochemical properties of the mucus-gel are associated with intact mucus glycoproteins.[5-14] These authors showed that at physiological concentrations (about 50 mg/ml) the purified mucins can be reconstituted into a gel with physicochemical properties identical to the native mucus-gel.[10,14-16] The specific gel-forming ability of mucins is due to a combination of structural features of the mucin molecules, which will be discussed in detail in the next sections.

Organs, e.g., the eyes, gastrointestinal tract, trachea, lungs, bladder, pancreatic duct, gallbladder, and the reproductive tracts, protect their epithelia by the production of secretory mucins.[4,17,18] These mucins constitute a distinct group of glycoconjugates, differing structurally from serum glycoproteins and proteoglycans. Most oligosaccharides are attached to the polypeptide by *O*-glycosidic linkages between *N*-acetylgalactosamine residues (GalNAc) and the hydroxyl groups of serine and threonine residues. In addition, a growing number of mucins are reported to contain a small number of N-linked glycans[19-23] The mucins are distinguished from proteoglycans by the absence of D-glucuronic acid and L-iduronic acid residues.[24]

In general, native secretory mucin molecules have an oligomeric structure containing several monomeric glycoproteins. These monomers vary in size between 250,000 and 10^6 D. More than 50% of their dry weight consists of carbohydrates organized in oligosaccharides, O-linked to serine

or threonine with sugar residues varying between 1 and 20 sugar residues per carbohydrate chain. The oligomers are jointed by disulfide bridges. The backbone proteins contain regions rich in serine and threonine residues, clustered in the central part of the polypeptide. Virtually all the hydroxylated amino acids in this central region are O-glycosylated. In cervix and tracheo-bronchial mucin the heavily O-glycosylated part is subdivided into sequences of about 600 to 1200 amino acids, which appear to be separated by short nonglycosylated domains.[25,26] The heavily glycosylated region is negatively charged, due to the presence of sialic acid residues and to the frequent, but not universal, presence of sulfate ester residues attached to carbohydrate. In addition, these glycosylated regions are very resistant to proteolysis. The terminal parts of the backbone proteins contain a "normal" distribution of amino acid residues. In these parts cysteine residues are located that provide for the intra- and intermolecular disulfide bonds. If present, N-linked oligosaccharides are also located in these regions. As oligomerization is essential for the rheological properties of mucins, both reducing agents and proteases can destroy the mucus-forming properties of mucins. The variation in number of linked monomers, combined with the variation in O-linked oligosaccharides, causes the extreme heterogeneity of oligomeric mucins.

The membrane-bound mucins share the oligosaccharide cluster with the secretory mucins. Oligomerization does not seem to be the rule in this class of mucins. This is inferred from the absence of cysteine residues in the luminal portion of the mucins MUC1 and rat leucosialin.[27-29]

The rheological properties of mucins are the result of disulfide bridges between monomers, as well as noncovalent intermolecular interactions between carbohydrate moieties. Both are essential for gel formation. The mucin molecules form a viscoelastic gel with specific physical properties, which allows the gel to flow and, if severed, anneal.[3] The immobile mucus adhering to the epithelium forms an unstirred layer on the plasma membrane of mucosal cells.

A. Purification

The isolation of mucins is usually based on the large size of the molecules and their characteristically high-buoyant density (around 1.40 g/ml). These characteristics allow mucins to be isolated using Sepharose CL-2B gel filtration and CsCl density gradient centrifugation, respectively.[30,31] Isolation can be performed following two different strategies: first, for compositional analysis, mucus glycoprotein can be isolated under nonreducing conditions to preserve all covalent bonds between polypeptides present in the native gel; second, disulfide bonds can be reduced before isolation to purify the monomeric mucin moieties. As N- and C-terminal unglycosylated peptides of mucins are extremely sensitive to proteolysis, purification must occur in the continuous presence of inhibitors of proteases. If no precautions are taken, most purified mucin molecules lack their "naked" terminal protruding peptides.[32] Carlstedt and Sheehan[33] and Carlstedt et al.[34] used 6 *M* guanidinium chloride with low-molecular-weight protease inhibitors during solubilization and purification of human cervical mucin. For most mucins, isopycnic density gradient centrifugation in CsCl/guanidinium chloride gradients is most appropriate to isolate and purify mucin in high yields and to a high degree of purity. A disadvantage of this procedure is the highly denaturing character of the solvents. Mucin purified in this way might not regain the ability to form gels with the same rheological properties as the originally secreted molecules.[35]

Another important point is the choice of starting material. Mucin polypeptides, present in a mucus gel, may be damaged partly by the influence of proteases or shear forces at the gel/lumen interface or by influences of secretory products from epithelial cells beneath the mucus layer. If mucin is isolated from secreted material, there is also a potential risk of contamination with mucus glycoproteins originating from other organs along the same tract. Absolutely intact and pure molecules can therefore only be isolated from intracellularly stored mucin. The best procedure is to immediately immerse the tissue after cutting off

the blood supply and removal of extracellular mucin into 6 *M* guanidinium chloride and to homogenize it. This is illustrated in a study of Fahim et al., which indicated clearly that intestinal mucin obtained from intra- as well as extracellular sources shows surprising differences in amino acid composition.[36] CsCl density gradient centrifugation, originally reported by Creeth and Denborough,[30] has been used to isolate mucin glycoprotein from many epithelia, e.g., stomach,[31,37,38] small intestine,[39] colon,[40] cervix,[41] and trachea.[26,42,43] The same procedure has been applied successfully for the purification of membrane-bound mucin glycoproteins from mammary adenocarcinoma and pancreatic cancer.[44-46] In earlier studies, column chromatography was used for the isolation of submaxillary and gastric mucin.[47,48] Gel filtration can be used for further characterization of mucin oligo- and monomers, but is less suitable for purification, as nonmucin polypeptides as well as DNA are in general not completely removed from the mucin.

B. Size and Structure

Due to the complexity, details of molecular size and structure of mucins are diverse and difficult to reconcile. An abundance of data are available about molecular sizes of a number of mucus glycoproteins in either monomeric or native form after a variety of isolation procedures. Studies in this field are hampered by the fact that the molecular weight of oligomeric mucins can neither be assessed by regular gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) nor by gel filtration. Analysis of monomeric mucin molecules, although they can enter low-percentage polyacrylamide gel, is complicated by their extreme heterogeneity. In Table 1, a selection of data on the molecular weights of mucins has been compiled.

As little information is available regarding the size of intact mucin protein cores, most estimates of the percentage carbohydrate per monomeric unit are too high. Data on mucin prepared by CsCl density gradients in the absence of protease inhibitors underestimate the amount of amino acid residues. Rat gastric mucin isolated without protease inhibitors contains 10% (w/w) protein,[37] while the same mucin contains about 50% (w/w) protein after isolation in the presence of guanidinium chloride and the protease inhibitor phenylmethyl sulfonylfluoride.[63] Extending these calculations with the assumptions that the protein backbone is 300,000, including N-linked oligosaccharides,[33] that all serine and threonine residues are *O*-glycosylated, and that 3% (w/w) of the monomer consist of sulfate, one can calculate that one gastric mucin monomer contains about 800 O-linked chains, with an average length of 5 sugar residues and one sulfate per 3 O-linked chains.

The monosaccharide composition of the isolated mucins shows large amounts of fucose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine (GlcNAc) and small amounts of sialic acid (SA) and, often, mannose residues. The presence of mannose is characteristic for the occurrence of N-linked glycans, as this monosaccharide is absent in O-linked oligosaccharides. Mucins are negatively charged molecules, due to the presence of sialic acid and sulfate residues on the oligosaccharides. Serine, threonine, proline, glycine, and alanine residues dominate the amino acid composition. Almost all serine and threonine residues bear O-linked glycans.[4,19,29,62,64-66] The O-linked glycans in mucins are very heterogeneous and vary in length between 1 and 20 residues.[4,6,9,18,67-69] The large abundance of closely packed O-linked glycans protects a large part of the mucin polypeptide against proteases. As indicated above, this protection is not perfect, as mucin oligomers can be degraded by proteolytic enzymes into fragments, which no longer possess the ability to form a viscoelastic gel.[11,13]

An essential feature of the rheological characteristics of secretory mucins is their oligomeric structure.[4,7] The presence of subunits, linked by disulfide bridges, was first reported by Snary et al.[70] Reduction of the disulfide bonds between cysteine residues causes an abrupt loss of viscosity of the mucus gel.[5,11,13,71] In 1981, Allen proposed a model for the oligomeric structure of pig gastric mucin.[6] In this so-called "windmill" model, four identical mucin monomers are joined to one central link protein by disulfide bonds. Except for gastric mucins, potential link proteins have been suggested in salivary mucin and intestinal mucin.[36,72,73] These polypeptides emerge

TABLE 1
Size (10^6 kDa) and Organization of Mucin Glycoproteins

Mucin	Backbone	Monomer	Oligomer	Ref.
Cervix	0.18	2.0—2.5	10—15	25
Rat gastric	0.27	0.9	1.8—3.6	49
Human gastric	0.9	2.0	4.0—6.0	63
Intestine (inc. MUC3)	0.162	0.25—0.5	2.0	19,33,51,52
Submaxillary	0.06—0.1	0.5—2.5	2.0—5.0	53-57
Trachea	0.1	0.5—0.6	9.0—16.0	26,58,59
Sialomucins (ASGP1)	0.2	0.6—0.7	No	60
MUC1	0.12—0.31	0.45—0.65	No	20,27,28,61
PSGP	0.05	0.2	No	62

when purified oligomeric mucin is reduced and analyzed by SDS-PAGE. Polypeptides of 70,000 for gastric mucin,[74] 90,000 for pig small intestinal,[52] 118,000 for rat intestinal,[75] human intestinal,[76] and human respiratory mucin were found.[77] Solid proof for the "windmill" model is lacking. However, the consistent finding of a 118-kDa glycoprotein in a number of mucin preparations by Robertson et al. indicates that nonmucin proteins may be closely associated with native mucin oligomers.[73] Slomiany and coworkers demonstrates close relationship between human and rat "118-kD link protein" and a fibronectin fragment.[73a] More recent experiments using rotary shadowing techniques in combination with electron microscopy, physicochemical experiments, or *in vitro* metabolic pulse-labeling studies favor another overall mucin structure.[26,43,49,50,63,78-81] This model, first put forward by Carlstedt and Sheehan, who studied human cervical mucin, denies the existence of link proteins.[33] Although a role for these proteins in oligomerization of some mucins cannot be excluded, mucin monomers are, most probably, linked together directly by disulfide bonds, without "linking" proteins present in the oligomer.

Controversy exists about the amount of lipid molecules associated with mucins. Slomiany and co-workers have found large quantities of lipids, using a mucus isolation technique in which the mucus-gel is scraped from the mucosa using a blunt spatula.[68,82] These authors have found a mucus composition comprising 70% protein, 15% carbohydrate, and 15% lipids. The lipids include phospholipids, glycolipids, mono-, di-, and triglycerides, fatty acids, cholesterol, and cholesterolesters. The lipid composition detected in the mucus preparations was similar to the overall lipid composition of cells, indicating that these lipids probably originate from epithelial cells, damaged by the isolation technique. This is also indicated by the extremely high protein content found by these authors.[83] If one assumes that all carbohydrates in their mucus preparation arises from mucin, then the mucin would only account for about 20% (w/w) of the mucus constituents. However, it is generally accepted that mucin is the main constituent of mucus.[4,7,18,84] Therefore, these findings most likely overestimate both protein and lipid content of mucus.

Membrane-bound mucins do not seem to have functional intermolecular disulfide bonds. This is inferred from molecular mass analysis of mucin under reducing and nonreducing conditions.[85] For pancreatic and mammary mucins, it has been shown that no cysteine residues are present in the polypeptide extending from the cell surface; all (three) cysteine residues are present in the region of the protein spanning the lipid bilayer.[27,28,61] These residues are potential attachment sites for fatty acids, as has been reported for other transmembrane proteins.[86]

The significance of the nonmucin components in the mucus-gel is poorly understood. Part of these components have been shown to influence the *in vitro* rheological properties of mucin-gels. Some components, like cellular proteins and nucleic acids, arise from contamination of the gel by sloughed cells and may not have specific functions. Certain proteins, like lysozyme and secre-

tory IgA, are secreted into mucus as part of the defense mechanism against viruses and bacteria.[87] It has been demonstrated that albumin and cations, like calcium, influence the *in vitro* rheological properties of isolated mucus.[35,39,87-92] However, the roles of these components in *in vivo* mucus function remains to be elucidated.

1. Protein Backbone

An estimate of the size of the protein backbone is difficult to obtain. Most data have been achieved after chemical deglycosylation using hydrogen fluoride, trifluoromethane sulfonic acid, or a mixture of hydrolases (Table 1).[54,58,93,94] The molecular weights of the protein backbone of ovine and bovine submaxillary mucins range from 58.3 to 96.5 kDa.[54,93] However, these methods may suffer from a high degree of peptide-bond breakage or enzymatic proteolysis.

The presence of a larger backbone protein was indicated by studies by Marianne et al., who, using antibodies, estimated the size of the human bronchial mucin backbone peptide to be about 400,000.[95] Antibodies raised against backbone proteins of different types of mucus glycoproteins have been of great value for identification of mucins. Antibodies against the N- or C- terminal (unglycosylated) portions of the backbone are most suitable for biochemical and morphological studies, as they recognize both precursor and mature mucin molecules. The molecular weight of the polypeptide backbone of human pancreatic tumor mucin has been determined after Western blot analysis, using an antibody raised against deglycosylated mucin.[96] The antibody reacted with a M_r >200,000 species. After cell-free translation of mRNA from bovine submaxillary glands and immunoprecipitation using an antibody raised against the deglycosylated mucin, the resulting primary translation product was a 60,000-Da protein.[57] Reliable figures can preeminently be achieved by studying the biosynthesis of mucus glycoprotein. By this method the backbone protein of human and rat gastric mucin was estimated to be 900 kDa and 270 kDa respectively (vide infra).[23,63]

The backbone sizes of several membrane-bound mucins were assessed after removal of oligosaccharides. Burchell et al. reported a 68-kDa backbone for human epithelial sialomucin (ESM).[97] M_r analyses of backbone polypeptides measured as biosynthetic intermediates indicated a size of at least 200,000 for human ESM.[29,98,99] Using Western and dot blot assays, Lan et al.[96] showed that human pancreatic apomucins share similarities with human breast apomucins and that related precursors are present in various pancreatic and breast mucin-producing carcinoma cell lines.

Reactivity of antibodies raised against sugar determinants of different mucus glycoproteins is beyond the scope of this review. These studies have been very important for diagnosis and detection of cancer. For a comprehensive study see Zotter et al.[100]

Recent investigations using molecular biological techniques have brought new insights into the structure of backbone polypeptides of mucus glycoproteins. The number of sequenced cDNAs of mucus glycoproteins is growing fast. The studies thus far have confirmed and extended the concept that backbone proteins contain high percentages of serine, threonine, alanine, glycine, and proline. These five amino acid residues usually account for more than 50% of total amino acid residues. Serine and threonine residues, especially, dominate the amino acid composition, together comprising 25 to 40% of total amino acids. The O-linked glycans are attached to the serine and threonine residues only one or two residues apart and often even on adjacent residues.[4,19,29,62,64,65,101] The interjacent amino acids in the *O*-glycosylated region of the amino acid sequence are usually small residues like proline, glycine, and alanine. As proline residues can induce β-turn conformations, it is likely that the high percentage of this amino acid allows close packing of O-linked oligosaccharides. In 1973, Pigman and co-workers hypothesized that the backbone protein of bovine submaxillary mucin is composed of a number of repeating amino acid sequences.[102] This concept has been confirmed recently for all mucus glycoproteins hitherto known. In general, these repeating sequences are located in the central part of the polypeptide chain and contain all attachment sites for O-linking oligosaccharides. DNA analysis of porcine submaxillary mucin confirmed that this mucin con-

tains tandemly repeated identical amino acid sequences of 81 amino acid residues with high percentages of serine, threonine, and glycine residues.[22] Gum et al., studying a cloned human intestinal/colonic mucin gene, have reported a repeated sequence of 23 amino acids in a backbone protein of 162 kDa.[19] A compilation of repeating amino acid sequences found in cloned mucins is shown in Table 2.

In contrast to the special amino acid composition of the central region, containing the tandem repeats, the N- and C-terminal ends of the backbone protein of secretory mucins contain amino acid residues with a "normal" composition. At present, only the complete amino acid sequence of a major tumor-associated membrane-bound mucin is known.[27,28,61] Membrane-bound mucins from human pancreas and breast cancer cells have a common backbone containing 20-amino acid repeats and differ only in glycosylation patterns; they have a 69-amino acid long cytosolic tail. This mucin, originally designated by various authors as DU-PAN-2, episialin, MAM-6, PEM, and PUM, is in this review termed MUC1, after the proposed name for the gene coding for this mucin.[106]

2. Carbohydrate Chains

Roughly 50% of the dry weight of mucins consists of carbohydrate chains, O-linked to serine or threonine residues, in the central "repeating sequence-containing" region of the protein backbone. O-linked oligosaccharides can be easily removed from the peptide by mild alkaline treatment. If this reaction proceeds in the presence of borohydrate (β-elimination reaction), O-glycosidic linked oligosaccharides are obtained intact.[108] This finding has been of importance for our present knowledge of the composition and structure of O-linked sugars. Five different monosaccharides are commonly found: GalNAc, GlcNAc, galactose, fucose, and SA. The latter sugar confers a negative charge to oligosaccharides. In addition to these five sugars, O-linked oligosaccharides may contain sulfate residues. According to charge, oligosaccharides can be subdivided in neutral and acidic glycans. This differentiation is especially important in defining onco-developmental markers.[109] A further subdivision is based on structure. In O-linked glycans three domains can be distinguished: a core, a backbone, and a peripheral region, each with its own specific set of antigens or tumor-associated determinants.

The core structure — The GalNAc residue directly attached to the hydroxyl group of serine or threonine of the protein backbone and the sugar residue(s) directly linked to it are defined as core structures. An *O*-glycosidically linked oligosaccharide might contain GalNAc as the only sugar or be terminated with a sialic acid residue, as in ovine submaxillary mucin.[47] There are at least six core classes known (Table 3). The most simple structures (GalNAc-, and Gal β(1-3)-GalNAc) constitute the T blood group antigens, Tn and T.[110] This structure is recognized by peanut ag-

TABLE 2
Tandemly Repeated Amino Acid Sequences Found in Mucins

Mucus	Repeated amino acid sequence	Ref.
MUC1	PGSTAPPAHGVTSAPDTRPA	27,28,61,103
BSM	GTTVAPGSSNT	56
Fim A.1	VPTTPETTT	104
Fim B.1	GESTPAPSETT	105
PSM	GAGPGTTASSVGVTETARPSVAGSGTTGTVSGASGSTGS- SSGSPGATGASIGQPETSR IRVAGSSGAPAVSSGASQAAGTS	22
SIB	HSTPSFTSSITTTETTS	106
SMUC	PTTTPITTTTTVTPTPTPTGTQT	19
Trachea	PTPTPITTTTTVTPTPTPT(G/S)TQT	107
PSGP	DDATSEAATGPSG	62

TABLE 3
The Core Regions of Mucin O-Linked Oligosaccharides

Core 1	Gal β(1-3)-GalNAc α(1-O)-Ser/Thr
Core 2	Gal β(1-3)-[GlcNAc β(1-6)]-GalNAc α(1-O)-Ser/Thr
Core 3	GlcNAc β(1-3)-GalNAc α(1-O)-Ser/Thr
Core 4	GlcNAc β(1-3)-[GlcNAc β(1-6)]-GalNAc α(1-O)-Ser/Thr
Core 5	GalNAc α(1-3)-GalNAc α(1-O)-Ser/Thr
Core 6	Gal β(1-3)-[Gal β(1-6)]-GalNAc α(1-O)-Ser/Thr

glutinin. The core structures 1 to 4 are most common (Table 3). In most instances these blood group activities are masked by carbohydrate from the backbone or peripheral region. In humans, T antigen is found in "nonsecretors" and in meconium extracts; it is "cryptic" in colonic mucin of adults and can be demonstrated only after treatment with neuraminidase.[111] Mucus glycoproteins can have any combination of the different core structures.

Backbone regions — This region consists of a series of Gal β(1-3) and GlcNAc β(1-4) units; they constitute the i, I, and IMa antigens. The two most common backbones are Gal β(1-3)-GlcNAc (type 1) and Gal β(1-4)-GlcNAc β(1-3)- (type 2, blood group i).[67] The type 2 structure is often repeated, while type 1 is never repeated.[112] Type 1 and type 2 structures can be branched in a β(1-6), β(1-3) branchpoint to galactose; this structure is associated with "I" antigenic activity.[67] In the majority of the human secretor population the backbone antigens are masked by the blood group antigens A, B, or H.

Peripheral regions — The backbone chains are usually terminated by α-glycosidic-linked galactose, GalNAc, fucose, sialic acid, or sulfate. These peripheral sugars determine most of the characteristics of the mucins as a whole. In adults the expression of ABH and Lewis antigens is found only in secreted mucins of "secretor" persons. For some time it was assumed that blood group H activity also determined the secretor status H (Se-gene).[113] More recently, it was suggested that this gene also encoded an activity different from H activity.[114] In recent studies novel carbohydrate structure-related antigens are described, some of them being expressed in specific stages during embryonic development.[115,116] During carcinogenesis the structure of O-linked oligosaccharides in the peripheral regions changes; in most cases blood group activities present during fetal development, but absent in adult epithelia, return during carcinogenesis.[117]

Mucin may contain variable amounts of sulfate. This is in most instances linked to galactose, GalNAc or GlcNAc. The degree of sulfation is locally determined: in gastric glands mucus-secreting cells lose their capacity to add sulfate to mucins while migrating from deeper locations in the foveola towards the surface positions.[118] The role of sulfate residues is unclear. There are indications that sulfated mucins inhibit peptic activity in stomach by binding to the substrate of pepsin and that mucin adhesion to the cell surface is enhanced by sulfation.[119,120] Another possible role for sulfate residues in mucins could be the induction of condensation in secretory granules (Section III.C).[121-123]

For a long time the presence of **N-linked** oligosaccharides on mucin glycoproteins has been doubted. Initially, mucins were defined as mannose-free glycoproteins.[2] In gastrointestinal mucus glycoproteins, trace quantities of mannose and glucose have been reported.[124] Mannose was also found in submandibular mucin by Malinowski and Herp,[125] Van Nieuw Amerongen et al.,[21] and by Tabak et al.[126] More recently, Hilkens and Buijs have clearly shown that in mammary tumor, mucin MUC1 is provided with several N-linked oligosaccharides.[20] Five potential attachment sites are recently inferred from the nucleotide sequence of the cloned gene.[28] Evidence for the presence of N-linked oligosaccharides on gastric secretory mucus glycoproteins comes from biosynthetic studies by Dekker et al.[23,49] About 10 N-linked oligosaccharides are cotranslationally attached to the backbone moiety of rat gastric mucin. N-linked oligosaccharides

are transferred from dolichol to the glycoprotein in the RER in a "high mannose" configuration.[127] Most N-linked oligosaccharides of glycoproteins are converted to a "complex" configuration in the Golgi complex. The N-linked oligosaccharides of mature MUC1 are of the complex type.[20] In the gastric mucin glycoprotein this conversion has not been shown, due to the poor resolution of the high-molecular-weight mucins on SDS-PAGE.[49] Mouse submandibular mucin contains "high mannose" N-linked oligosaccharides, as has been shown by structural analysis.[21] Thus, it is uncertain so far whether all secretory mucins have N-linked oligosaccharides and whether "high mannose" oligosaccharides present on fully *O*-glycosylated secretory mucins are processed to "complex"-type glycans.

3. Subunit Structure

The basic subunit of a secretory mucin (designated in this review as oligomeric mucins) is a single polypeptide chain that consists of three regions: a densely glycosylated "central" region and the two extending (N- and C-terminal) peptides. The central region is rich in serine and threonine residues and contains no cysteine residues. The most prominent feature of functional mucin is the imperative relation between gel formation and intactness of disulfide bridges.[128] The macromolecules participating in these bridges have been a matter of controversy. As noted above, Allen and co-workers proposed a model in which four mucin subunits are joined together by a central nonmucin protein via disulfide bridges: the so-called "windmill" model.[6] Stoichiometry for such heterooligomeric structure has never been determined. However, observations using electron microscopy indicated that mucins are organized in a linear array (Figure 1). In these initial studies, human bronchial mucin was dried on grids for electron microscopy and visualized by rotary shadowing using platinum.[79,129,130] These findings were confirmed for other secretory mucins like human cervical,[78] human tracheo-bronchial,[26] and rat gastric mucin.[50,63,131] Carlstedt and Sheehan[25] and Thornton et al.[26] have comprehensively studied tracheo-bronchial and cervical mucins in the electron microscope after several purification and modification procedures. At present, there is little doubt that most, if not all, secretory mucins are organized as homooligomers in a linear array (Figure 1). In the case of cervical and tracheo-bronchial mucin, the "central" part of a mucin subunit seems to consist of alternating protease-resistant and protease-sensitive stretches. Proteolytic digestion of one subunit results in 3 to 5 so-called (protease-resistant) T-domains. In electron microscopy the subunits have a weight-average contour length of 490 nm, while the T-domains are 160 nm.[26] The consequence of this arrangement is that the amino acid sequence in the central region of tracheo-bronchial and cervical mucins consists of serine and threonine-rich stretches alternated by stretches of naked peptide sequences composed of "normal" amino acid residues. Independent molecular weight estimates indicated a molecular weight for the T-domains of 0.3 to 0.4 $\times$ 10^6 and 2 to 2.5 $\times$ 10^6 for the subunits.[25] Another important conclusion drawn from these results is that mucin subunits are *assembled* end-to-end into large, linear, and flexible macromolecules. Dekker et al. have studied the length and subunit composition of rat and human gastric mucin.[50,63,131] In line with the results of Carlstedt and co-workers, they reported a linear subunit arrangement in oligomeric mucin (Figure 2). Recorded oligomers with lengths up to 1000 nm consisted mainly of three subpopulations, which demonstrated mean lengths similar to 1, 2, or 3 times the length of the monomeric subunit. These recordings indicate that the monomer is the smallest unit in the mucin oligomer, as the length of the oligomer is a multiple of the monomer length. In addition, rat oligomers showed small globular structures (diameter about 15 nm) at regular intervals, which were absent on the monomeric subunit. The mean distance between the globular domains on the oligomers (350 nm) was virtually identical to the mean length of the reduced monomer (280 nm). This suggests that the globular structures indicate attachment sites of two subunits in the rat oligomers. Recorded electron micrographs of intact human gastric mucin oligomers and reduced monomeric subunits showed filamentous molecules similar to the rat mucin, whereas no globular structures were present in the human oligomers.[131]

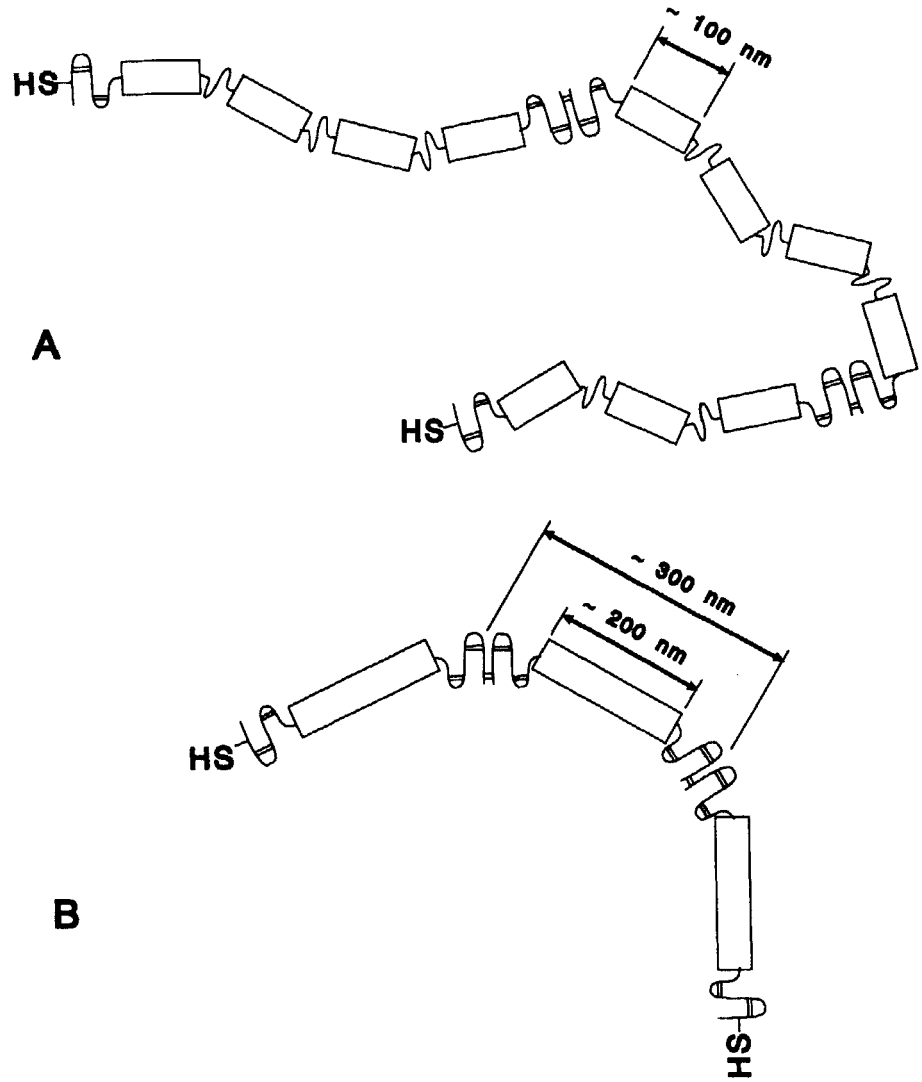

FIGURE 1. Model for mucin oligomers. (A) Human cervical mucin.[33,61,132] The structure, originally proposed in 1984 for cervical mucin by Carlstedt and Sheehan, is based on physicochemical data and electron microscopical recordings of isolated mucin oligomers and monomers, and proteolytically digested mucin. These data reveal that the monomers with a uniform length of about 500 nm are attached end-to-end to form oligomers with a heterogenous length distribution and a maximal length of about 3000 nm. The mucin monomer contains several glycopeptides, i.e., the central, *O*-glycosylated region is intersected by small, nonglycosylated, protease-sensitive sequences. Proteolytic digestion yields 3 to 4 glycopeptides of about 100 nm per monomer. (B) Rat gastric mucin.[32,63,131] Oligomeric structure of gastric mucin based on the data on rat mucin. The general structure of the oligomers, which demonstrate considerable length heterogeneity, is represented by the commonly observed mucin trimer. In contrast to the findings in the cervical mucin, the central *O*-glycosylated region of the molecule contains no protease-sensitive domains; only the terminal regions of the polypeptide are protease susceptible. The length of oligomeric mucins is very heterogenous and extends up to 3000 nm, whereas the monomers have a uniform length of about 300 nm. The glycosylated region of the monomer comprises about 72% of the length of the monomer. The attachment site of two adjacent monomers is indicated by a globular domain of about 15 nm, probably representing a structure in the terminal, nonglycosylated region of the monomer (see Figure 2). (□), glycosylated region of the polypeptide, containing all glycans; (——), nonglycosylated, protease-sensitive peptide regions; (=), disulfide-bond; -SH, thiolgroup.

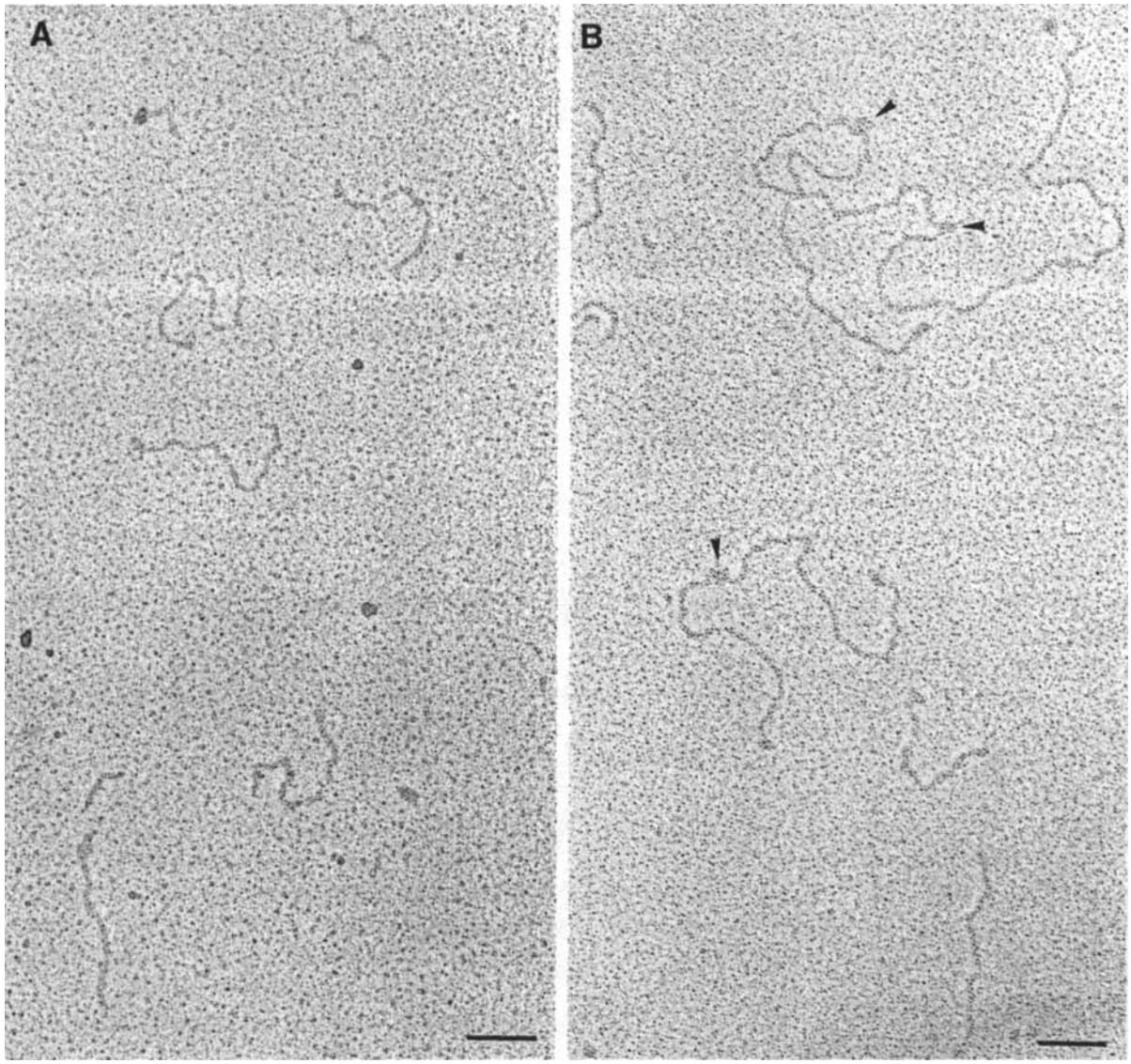

FIGURE 2. Rat gastric mucin visualized by electron microscopy. The rat gastric mucin was isolated using CsCl/guanidinium chloride density gradient centrifugation.[32,63] Panel A, mucin after reduction and carboxymethylation; panel B, mucin before reduction. Globular structures on the oligomers, which probably indicate the attachment site of two adjacent monomers, are indicated by arrowheads. (Bar = 200 nm.)

The presence of more than two mucin monomers in one oligomer indicates that subunits contain linking sequences at both the N- and C-terminal ends of the polypeptide. As no other (nonmucin) proteins were detectable in purified oligomeric mucin, the globular domain on rat gastric oligomers most likely represents a structure in the molecule which is abolished upon reduction. Clearly different from the cervical mucin was the absence of molecules with T-domain characteristics in both human and rat gastric mucins. In these mucins the central region apparently lacks proteolytically degradable segments. As will be discussed later (Section III), the electron microscopic observations are in full agreement with biosynthesis studies that show that the

oligomeric structure is most likely already shaped in a very early stage during biosynthesis of the mucin.

III. BIOSYNTHESIS OF MUCINS

Secretory mucins are produced by specialized exocrine epithelial cells in mucosae.[133-135] The cells are highly polarized and show specializations typical for the massive production of mucus glycoproteins. The rough endoplasmic reticulum (RER) is generally concentrated in the basal cytoplasm of the cells. The Golgi complex is well developed, consistent with the high glycosylation activity required for mucin synthesis. Although the morphological features of mucus-producing cells are obvious, the biosynthesis of mucins is not studied extensively. This is mainly due to the lack of proper antisera against the mucins, needed to identify the various biosynthetic intermediates. The biogenesis of mucins is complex, as it involves both *N*- and *O*-glycosylation, protein oligomerization, intracellular storage, and both nonregulated and stimulated secretion. The combination of these features, as described in Sections II.B and IV, is essential for the production of functional mucin. The individual biosynthetic processes, including their roles in the production of functional mucin, are described below.

A. *N*- and *O*-Glycosylation

The protein backbones of the mucins are synthesized on polyribosomes on the cytoplasmic face of the RER and cotranslationally translocated across the membrane into the lumen of the RER, by intervention of a, presumably cleavable, N-terminal signal sequence. Membrane-associated mucins, such as MUC1 and leukosialin, contain a hydrophobic amino acid sequence, which functions as a stop-transfer signal for protein translocation, and thus renders a transmembraneous protein.[28,29] Most mucin polypeptides are ***N*-glycosylated** during translocation by *en bloc* transfer of a "high mannose" oligosaccharide. ($Glc_3Man_9GlcNAc_2$) from a lipid donor to an asparagine residue.[136] Asparagine residues in glycoproteins are only *N*-glycosylated if they are part of the amino acid sequences Asn-X-Ser or Asn-X-Thr, in which "X" can be any amino acid except proline and aspartic acid.[134] *N*-Glycosylation has been demonstrated in MUC1, rat gastric mucin, and in murine submandibular mucin.[20,21,23] As the consensus sequence for *N*-glycosylation was found in most amino acid sequences deduced from cloned mucin-cDNAs, *N*-glycosylation is probably an event common for most mucins (Section II.B).[19,22,27,56,62,104-106]

After polypeptide synthesis and *N*-glycosylation is completed, the mucin is transported to the Golgi complex by vesicular transport. During its passage through the cisternae of the Golgi complex, the mucin acquires its final glycosylation. Generally, the N-linked glycans are processed in a well-defined way, clearly different from the mechanism of *O*-glycosylation.[136] In the Golgi complex the N-linked glycans are converted from "high mannose" to "complex" oligosaccharides by removal of all (three) glucose and four mannose residues in the medial-cisternae of the Golgi complex. First, a GlcNAc residue is added to the glycan, followed by removal of two more mannose residues. All "complex" N-linked glycans are characterized by the presence of the three remaining mannose residues. The hexasaccharide ($GlcNAc_3Man_3$) is the acceptor for a large number of glycosyltransferases in the *trans*-cisternae of the Golgi complex. The N-linked glycans can be supplied with monosaccharides, linked one after another. As discussed in Section II.B.2, it is not known whether all N-linked glycans on mucins acquire a complex configuration.

The onset of the ***O*-glycosylation** of glycoproteins is less well defined. No amino acid sequence requirements are known at *O*-glycosylation sites. The tandemly repeated amino acid sequences of the various mucin protein backbones, which are the target for *O*-glycosylation, show no homology (Table 2). Therefore, the specificity of the GalNAc-transferase toward its protein substrate is thus far unexplained. However, the presence of proline residues in the vicinity of serine and threonine results in a partially rigid peptide conformation, which may provide the best access for the GalNAc-transferase.[137,138]

The signals for *O*-glycosylation are probably conserved throughout vertebrate evolution, as was demonstrated for lipoprotein receptors.[139]

There is evidence that the addition of GalNAc to serine and threonine residues can occur cotranslationally.[140] However, the bulk of GalNac addition is most likely a posttranslational event.[141-145] Most of the initial *O*-glycosylation of rat gastric mucin occurs after protein-oligomerization, and after the oligomerized precursors have left the RER.[49] The addition of GalNAc to serine and threonine residues is most likely located in the "transitional elements" of the RER or in the *cis*-cisternae of the Golgi complex.[49,141-145] The initial *O*-glycosylation of rat gastric mucin depends neither on *N*-glycosylation nor on the protein-oligomerization.[49]

The GalNAc-transferase involved in initial *O*-glycosylation adds a vast number of tightly packed GalNAc residues to the central region of the protein. As a result, the central *O*-glycosylated region of the polypeptide in the mature mucin is transformed into an elongated, stretched conformation.[101,146-149] After the initial addition of GalNAc residues to hydroxylated amino acid residues, the *O*-glycosylation proceeds by sequential addition of monosaccharides by specific glycosyltransferases. The mucin precursors are transported to the *trans*-cisternae of the Golgi complex, where the biosynthesis of the O-linked glycans is completed. The sequence of the sugar additions in the *O*-glycosylation follows a general pattern, as reviewed by Hounsell and Feizi,[67] Feizi et al.,[150] and Schachter and Williams.[151] The resulting structures are outlined in the section on oligosaccharide structure (Section II.B.2). The O-linked oligosaccharides show heterogeneity arising from a number of causes: (1) chain elongation is dependent on the expression of the proper glycosyltransferases, which is cell-type specific; (2) addition of a monosaccharide to the oligosaccharide chain is intrinsically determined by the nature of the last-added, nonreducing residue; (3) the degree of "branching", i.e., addition of more than one sugar to the same monosaccharide in the chain; (4) some glycosyltransferases compete for the same oligosaccharide substrate, usually mutually excluding each other's actions; (5) addition of a monosaccharide to a particular, nonterminal, sugar residue in a growing oligosaccharide chain sometimes prevents further elongation. The interlocked, complex processing steps may culminate in numerous different oligosaccharides, consisting of 1 to 20 monosaccharides, on one mucin molecule.[4,18,67] During the final steps in oligosaccharide synthesis, variable amounts of sialic acid as well as sulfate residues are added, giving rise to a molecule with heterogeneity in negative charge.[118,120,152-154] The heterogeneity in carbohydrate structure and charge most likely regulates the properties of the mucins (Section IV.B).

In summary, the biogenesis of mucins depends on both *N*- and *O*-glycosylation. In gastric mucin, the *N*-glycosylation occurs early in the biogenesis of glycoproteins and is likely essential for oligomerization and thus for further transport along the secretory pathway (Sections III.B and III.C). The O-linked glycans are responsible for the specific interactions between the mucin oligomers within secretory granules and within the extracellular gel, and give the mucin its protease resistance (Sections II and V). In addition, they have a profound effect on protein conformation. Thus, both the N- and O-linked glycans are essential in establishing the final functional conformation of the oligomeric mucins.

B. Oligomer Formation

Most secretory mucins, like gastrointestinal and cervical mucins, consist of covalently linked oligomers (Section II.B). For some other mucins, like submaxillary mucins, it is not certain whether they form disulfide-linked oligomers. The transmembrane mucins MUC1 and leucosialin do not form covalently linked oligomers.[20,29] The covalent oligomerization by disulfide bond formation during biosynthesis has only been described for rat gastric mucin.[49] Therefore, this section will mainly deal with the biosynthesis of this mucin (Figure 3).

In the rat stomach, two independent messenger RNAs are transcribed from the two codominant mucin genes, which are both translated at the RER.[23] The two mRNAs are most likely simultaneously translated on polyribosomes on the RER, because the monomeric precursors, of slightly different molecular weight, are mixed

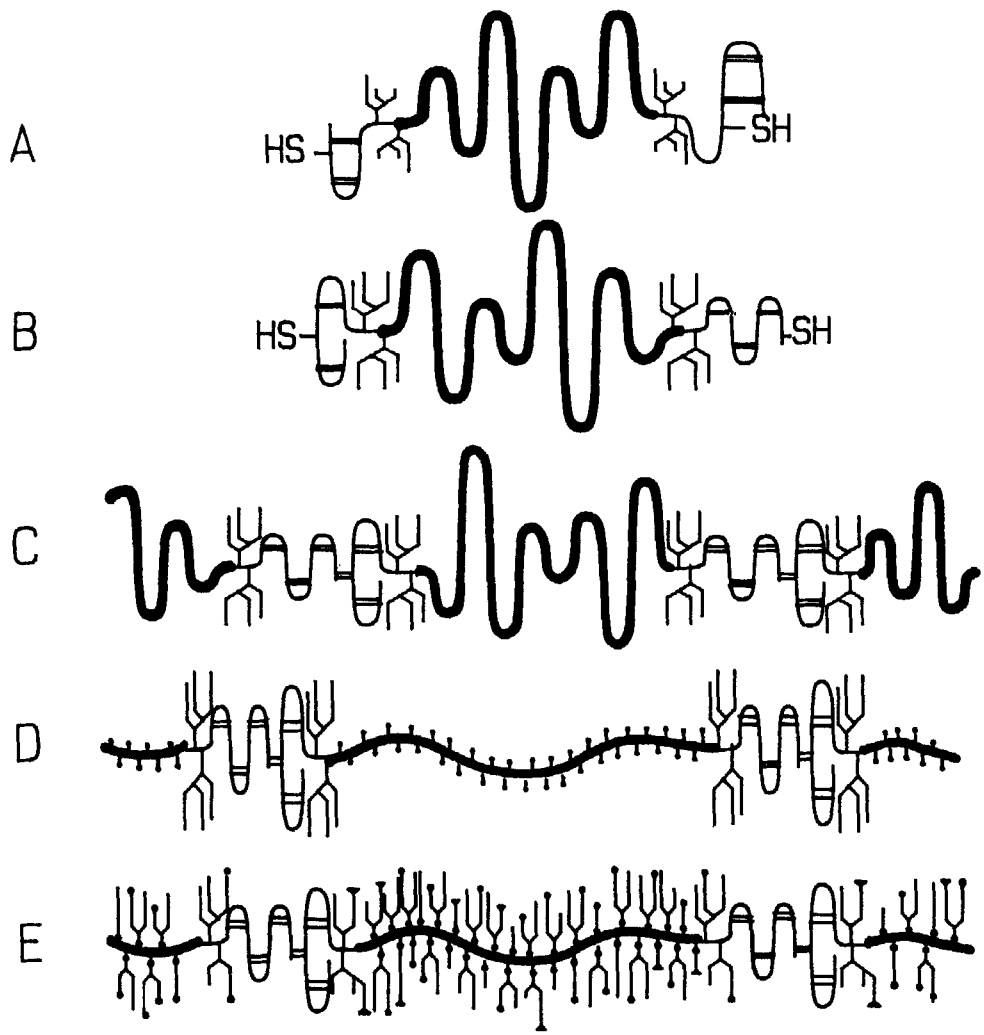

FIGURE 3. Model for the biosynthesis of mucin, based on the studies of rat gastric mucin. (A) The mucin precursor is synthesized in the RER. In this early stage the N-linked glycans are in "high mannose" configuration ($Glc_3Man_9GlcNAc_2$). The central part of the polypeptide has no rigid tertiary structure and behaves like a random-coil structure. The peripheral parts of the polypeptide do not possess their final disulfide-bond configuration. During this stage the mucin precursor may be bound to a binding protein (BiP or grp78), which retains it in the RER. (B) The precursor is still present in the RER and the N-linked glycans have a "high-mannose" configuration ($Man_8GlcNAc_2$). The random coil conformation of the central part of the polypeptide is unaltered. However, the peripheral parts of the protein have acquired their proper conformation for oligomerization, facilitated by the N-linked glycans. (C) The precursors oligomerize in the RER. The central part is still unaltered. First, the properly folded peripheral parts of the polypeptide have established a specific, noncovalent interaction. Then, the intermolecular disulfide-bonds are established, catalyzed by protein disulfide isomerase. It is unknown whether the N terminus of the polypeptide is predestined to interact with a C terminus, as is suggested by the illustration. On the other hand, the N terminus of the precursor might interact only with another N terminus. As only oligomers are transported to the Golgi complex for *O*-glycosylation, this might be the stage in biogenesis when the oligomerized precursor loses its affinity for the binding protein. (D) The oligomers arrive in a compartment where GalNAc-transferase is located. This initial *O*-glycosylation occurs in a late RER compartment ("transitional elements") or in the *cis*-cisternae of the Golgi complex. The central part of the polypeptide is provided with GalNAc residues. These residues are added to serine and threonine residues in the tandemly repeated part of the amino acid sequence. The addition of GalNAc residues alters the conformation of the central polypeptide region into an elongated random-coil structure. The conformation of the N- and C-terminal parts of the molecule is unaltered. (Notice, that the central region of the mucin in diagrams D and E is not drawn to scale, when compared with diagrams A, B, and C.) (E) The mucin intermediates reach the *trans*-cisternae of the Golgi complex shortly after addition of the O-linked GalNAc residues. Here the oligosaccharide synthesis of the mucin is completed. During the last steps in the oligosaccharide synthesis, sulfate and sialic acid residues are added to the glycans, giving the mucin oligomer its overall negative charge. In this compartment the N-linked glycans probably acquire a "complex" configuration. The conformation of the protein moiety is not affected by the terminal glycosylation steps. ▬ Tandemly repeated amino acid sequence; ─, unique, nonrepeated amino acid sequence; ❡, GalNAc linked to serine or threonine residue; -SH, thiol-group; ●, sulfate residue; ▲, sialic acid residue; =, disulfide bond; Ψ, "high mannose" N-linked glycans; ΨΨ, "complex" N-linked glycans; ΨΨ!Ψ, O-linked glycans.

during oligomerization.[49] This heterogeneity in polypeptide size is caused by the codominant expression of two mucin genes with a variable number of tandemly repeated sequences (Section IV.A). Thus, this heterogeneity in protein backbone size adds further heterogeneity to the oligomeric mucins.

Oligomerization of rat gastric mucin does not start immediately after the protein synthesis is completed.[49] Apparently, the N- and C-terminal polypeptide regions do not have the proper conformation for subunit-subunit interactions. This "improper" conformation is likely stabilized by intramolecular disulfide bonds, as was suggested for other proteins, like T-cell antigen receptor, influenza HA, and VSV G-protein.[155,156] The appropriate protein conformation is slowly acquired by refolding (of the peripheral parts) of the polypeptide. During refolding, the disulfide bonds are probably rearranged by the enzyme protein disulfide isomerase, a protein retained in the RER by the amino acid motive KDEL, which catalyzes disulfide bond formation.[157]

The presence of N-linked oligosaccharides, cotranslationally added to the polypeptide, is essential for efficient oligomerization of the rat gastric mucin precursors in the RER.[49] These glycans likely facilitate the formation of a tertiary structure of the peripheral polypeptide regions favorable for intermolecular interaction as was suggested for other proteins.[155,156] When this structure is formed, a specific recognition between the peripheral polypeptide regions of two adjacent precursor molecules is presumably established. Then, intermolecular disulfide bonds are formed. In the absence of the N-linked glycans this tertiary conformation is acquired only very slowly, as both the oligomerization of the rat gastric mucin precursors and their subsequent transport to the Golgi complex were severely retarded.[49]

With a half-time of about 30 min, a mixture of di- and trimers is formed in molar ratio 3:2, whereas small amounts of larger oligomers are formed. The dimers are not converted into larger oligomers in the RER and the various precursor oligomers are transported to the Golgi complex with identical rates.[49] Oligomerization clearly precedes transport to the Golgi complex, because transport of monomeric precursor to the Golgi complex was not observed.[49,50,63]

It is presently unknown what difference in function exists between dimeric, trimeric, or higher oligomers. But it is clear that the final size of oligomeric mucin is determined in the RER by the number of oligomers linked end to end. As the presence of intermolecular disulfide bridges is the primary factor in gel formation, it is clear that the process of refolding and processing by the protein disulfide isomerase must be highly regulated to ensure the final quality of the mucin.

C. Intracellular Transport and Sorting

Like most aspects in mucin biosynthesis, the transport through the cell during and after biosynthesis of the mucins is a largely unexploited area of research. This transport includes two main features: first, the mucins are secreted at the apical side of the cells, and must therefore be unidirectionally transported; and second, the gel-forming, secretory mucins are stored in storage granules prior to secretion. After the biosynthesis is completed the secretory mucins are sorted in the *trans*-Golgi reticulum (TGR) into secretory granules, which are transported toward the apical side of the cell and eventually fuse with the apical plasma membrane.[4] The transmembrane mucins are not stored intracellularly, and are presumably transported to the apical plasma membrane directly. Thus, the transport of the mucins through the cell is partly identical to the general route of secreted and membrane proteins, and partly mucin specific.

The signal for the RER-to-Golgi transport of rat gastric mucin resides most likely in the conformation of the properly folded polypeptide, rather than in the sugar structure.[49] As the *configuration* of the N-linked glycans is irrelevant to this transport, and the *O*-glycosylation is initiated only after precursor oligomerization is completed, the glycans do not contribute to the transport signal.[49] It is generally believed that improperly folded, newly synthesized proteins are retained in the RER by a binding protein that recognizes the improperly folded protein conformations.[155,156,158] This binding protein (BiP or

grp78) is retained in the RER by a specific amino acid sequence (KDEL).[159] The binding protein has been shown to bind various improperly folded secretory and membrane proteins in the RER, whereas this binding was abolished when the proteins attained their proper, functional conformation.[160-163] The rat gastric mucin precursor may be retained in the RER by this mechanism. Once the peripheral peptide regions have acquired their proper conformation, facilitated by the N-linked glycans, they presumably lose their affinity for the folding proteins and are transported to the Golgi complex.

Upon completion of the biosynthesis in the Golgi complex, the mucins are sorted into so-called condensing vacuoles, which are formed at the *trans*-Golgi cisternae. The contents of the condensing vacuoles undergo further concentration, which is reflected by the increasing electron density of the granules seen in electron microscopy. A large number of storage granules are very tightly packed against the apical plasma membrane. The molecular signal for storage of secretory proteins is thought to reside within the primary structure of the proteins. Generally, these signals are dominant, since the fusion of a constitutively secreted protein to a protein secreted in the regulatory pathway results in regulated secretion of the hybrid protein.[164-166] The negative charge of the mucin is thought to be essential for its storage. The calcium content of the mucin granules is very high, and it is generally assumed that calcium ions form complexes with mucin.[122,167-170] However, sulfation of mucin is probably not a prerequisite for storage and secretion, as unsulfated rat gastric mucin is stored and secreted *in vitro* to the same extent as the sulfated mucin.[23] Greve and co-workers showed in similar experiments that unsulfated proteoglycan was stored and secreted normally by human fibroblasts.[171]

Sorting of membrane-bound mucins is probably programmed in the peptide portion present in the cytosol (cytosolic tail of the mucin). Generally, cytosolic sequences of proteins contain information for their targeting to certain organelles or for their presence in certain plasma membrane domains. Lysosomal membrane proteins have the motif Gly-Tyr in their tails, which is important for their sorting to lysosomes.[172] The presence of one or more tyrosine residues in cell surface receptors is crucial for their endocytosis via coated pits.[172] If tyrosine, present in the cytoplasmatic tail of mannose phosphate receptors, is converted to phenylalanine, the receptor is no longer taken up by the cells.[173] The current view is that amino acid motifs containing tyrosine are recognized by a specialized set of proteins (adaptins) present in the coated pits at the plasma membrane and the Golgi complex. This recognition is a prerequisite for proteins to be endocytosed via the clathrin-coated pathway.[172,174] Membrane-bound mucins from human pancreas and breast cancer cells (MUC1) have a common backbone, including a 69-amino acid long cytosolic tail. The MUC1 cytosolic tail contains several tyrosine residues. Indeed, Calafat et al. have recently reported the recycling of MUC1 after uptake via the coated as well as the uncoated pathway.[175] The extent of recycling seems relatively low and the biological significance is unclear at present. Preliminary experiments indicate that after synthesis and transport to the cell surface, sialylation of MUC1 is incomplete. Recycling via coated vesicles and the Golgi complex might provide for a mechanism that enables completion of sialylation.[176] Another feature of the cytoplasmic tail of MUC1 is the presence of three cysteine residues in the lipid bilayer-spanning amino acid sequence. So far it can be concluded that the presence of cysteine residues in this part of a transmembrane protein gives rise to palmitation.[86] Although acylation of MUC1 has not been established so far, it may stabilize the binding of the mucin in the membrane.

Studies on the biology of the membrane-bound mucin MUC1 have concentrated on carcinoma cells.[177] Although this polymorphic mucin is present on these cells in high amounts, it is not exclusive for this class of (transformed) cells. In epithelial cells of most glandular epithelia it is present in the apical and absent in the baso-lateral membrane.[100] This location is probably also imposed by signals in the cytoplasmic tail peptide. After transformation, junctional complexes disappear and MUC1 can freely diffuse at the whole surface of these cells. Although the information present in the tail of membrane-

bound mucins is not yet fully understood, it is certainly of high importance for determining their function.

Exocytosis of the secretory, gel-forming mucins occurs by fusion of the membrane delimiting the mucin storage granules with the apical plasma membrane. Secretion can be stimulated rapidly, under conditions when the epithelium needs extra protection. Mucus secretion is probably under both neural and hormonal control.[4] Under experimental *in vitro* conditions the basal secretion is generally low.[178,179] Exocytosis is an energy-dependent process, occurring at specific sites of the plasma membrane. The membrane delimiting the mucus granule is brought into very close contact with the apical plasma membrane, where a so-called fusion pore is formed.[180] This pore consists most likely of specific proteins present in the granule membrane.[180] After triggering by a messenger, these proteins interact with the plasma membrane, forming a pore through both membranes into the extracellular space. One of the signals implicated in this process is an increase in cytoplasmic Ca^{2+} concentration. Interestingly, Ca^{2+} release was noted prior to the release of mucins from mucus granules of the slug *Arioli-max columbianus*.[122] As mucus granules generally contain high Ca^{2+} concentrations, release of calcium from mucin granules may be the trigger for the formation of fusion pores.

After formation of the exocytosis-fusion pore, the highly concentrated mucin is rapidly hydrated and expands to 400 to 600 times the granule volume within milliseconds.[122,123] Mucus expansion is initiated by loss of calcium ions from the granule, whereby the polyanionic mucin loses the shielding cations.[123] The expansion of the mucin upon exocytosis is likely driven by electrostatic repulsion between the negatively charged molecules and the hydrophilic nature of the abundant oligosaccharides.[123]

Extracellularly, the negative charge of the molecules, in conjunction with low concentrations of cations, partly determines the viscosity of the extracellular mucus-gel. In the presence of cations, the negatively charged mucin molecules in the mucus-gel condense and partly exclude water, thereby influencing the viscoelastic properties of the gel.[35,88,181] Increased sulfate and sialic acid contents of mucin are correlated with increased viscosity of the mucin gel.[181] Thus, the negative charge imposed by sialic acid and sulfate residues functions initially in complexation of the mucin with calcium during storage. Then, upon release of the calcium during exocytosis, the charge is responsible for the rapid expansion of the mucin to form a highly hydrated gel. Finally, the charge partly determines the rheological properties of the mucus-gel by electrostatic interaction with low concentration of cations (Section V.A).

IV. REGULATION OF BIOSYNTHESIS

The biogenesis of mucins is a very complex process, involving transcription of the mucin gene, mRNA processing, mRNA translation, peptide oligomerization, *N*-glycosylation, *O*-glycosylation, intracellular transport, storage, and secretion. The regulation of each of these processes in mucin synthesis, as for the synthesis of many other glycoproteins, is virtually unknown. The amount of mucin expressed at the cell surface is likely to be controlled at the transcriptional level. However, it is evident from the described structure/function relationship of the mucins (Sections II and V), that the structure of the secreted mucin, e.g., the extent of the glycosylation and the length of the oligomers of the secretory mucins, is crucial for its function. The regulation of the synthesis and secretion of the secretory mucins is regulated in such a way that the thickness of the protective mucus layer is maintained. On the other hand, the functions of mucins may change throughout life, as is the case in the developing gastrointestinal tract. Thus, while probably the same mucin polypeptide is expressed, different posttranslational processing may result in mucin with other gel-forming properties. In disease, the regulation of mucin expression may be affected, resulting in abnormal quantities of mucin or in mucin with an abnormal structure.

A. Organization of the Gene

The mucins were early recognized as a distinct group of glycoproteins with a characteristic high molecular weight, chemical composition, and molecular structure (Section II.B). However,

the first partial cDNA sequence of a mucin was only published in 1987.[182] Since then, a number of cDNA sequences were determined, which are accumulated in Tables 2 and 4. These data reveal that the mucins do not show sequence homology in either nucleotide or amino acid sequence. A number of features are characteristic for all mucins studied thus far. The primary amino acid sequence can be divided into three regions. The central region, carrying all the O-linked oligosaccharides, contains no *N*-glycosylation and is devoid of cysteine residues. The N- and C-terminal regions contain all the *N*-glycosylation, all cysteine residues, and most likely no *O*-glycosylation. The central region consists of short, tandemly repeated amino acid sequences, as shown in Table 2, whereas the peripheral regions consist of unique amino acid sequences. This subdivision of the mucin polypeptide into a large region, containing all O-linked glycans, flanked by two non-*O*-glycosylated regions, was also inferred from a large number of proteolytic digestion studies. All mucins yielded a protease-resistant glycopeptide, containing all *O*-glycosylation, whereas cysteine residues were only found in the proteinase-sensitive part of the molecule (Figure 1 and Section II). The only exceptions so far are the structures of the cervical and tracheo-bronchial mucins, which contain more than one proteolysis-resistant glycopeptide (Figure 1, and Section II.B). As neither of these mucins has been sequenced so far, the character of the intercepting amino acid sequences is unknown.

The location of the mucin genes (MUC) is known for three human mucins: *MUC1* (coding for MUC1 mucin) is located on chromosome 1;[183] *MUC2* (coding for a secretory intestinal mucin) is located on chromosome 11;[184] *MUC3* (coding for another secretory intestinal mucin) is located on chromosome 7.[106] Thus, the known mucin genes are probably not clustered in the human genome. Human epithelia possess two MUC1 genes, which are codominantly expressed.[182] Codominant expression of genetically polymorphic mucin genes was also demonstrated for the rat gastric mucin.[23] Swallow and co-workers elegantly demonstrated that the variation in alleles coding for the MUC1 mucin correlated directly with protein polymorphism.[182] The allelic variation in this gene likely arises from unequal crossing-over events in the homologous multiple tandem repeats in the coding sequence of the mucin gene.[182] This genetic variation in the number of nucleotide repeats has been described for the noncoding "minisatellite" DNA commonly found in eukaryotic cells.[185] The tandem repeats of the mucins are unique in the fact that they are

TABLE 4
Sequenced Mucins

Name	m-b	Repeats AA	p/c	bp	No.	Origin	*N*-glyc	Ref.
PEM (MUC1)	Yes	20	p	60	21—125	Human	Yes	27
Episialin (MUC1)	Yes	20	p	60	40—90	Human	Yes	28
HPTM (MUC1)	Yes	20	p	60	42	Human	Susp	61
BSM	nk	11	c	33	3—4	Bovine	Susp	56
FIM A.1	No	9	p	27	14	*X. leavis*	Susp	104
FIM B.1	No	11	p	33	>6	*X. leavis*	Susp	105
Leucosialin	Yes	9	c	27	3	Rat	No	29
PSM	nk	81	p	243	>8	Porcine	Susp	22
SIB	nk	17	c	51	>7	Human	nk	106
SMUC	nk	23	c	69	>7	Human	Susp	19
Trachea	nk	23	c	69	>14	Human	nk	107
PSGP	No	13	p	39	10—200	*S. gard.*	No	62

Note: The data are, except for MUC1, derived from sequenced cDNAs. m-b, Membrane-bound; *N*-glyc, *N*-glycosylated; p, perfect repeat; c, consensus repeats; susp, suspected; nk, not known; AA, amino acids.

part of the coding sequence of the mucin genes; in MUC1 all repeats are found within one exon.[28] The function of noncoding "minisatellite" sequences in the genome is not known. The only other example of a human gene that shows a similar genetic polymorphism, due to the presence of tandemly repeated sequences, is the family of salivary proline-rich proteins. In these genes, located on chromosome 12, 5 to 15 tandem repeats of 63 bp are located in one exon.[186-188] The 21-amino acid repeats in the polypeptide of these proteins are very proline rich, but they are not glycosylated. These genes show individual polymorphism, most likely due to unequal crossing-over events similar to the ones postulated for "minisatellite" DNA and for the mucin genes.

The crossing-over events in this tandemly repeated sequence of the mucin genes make these genes hypervariable, as shown for MUC1. The variations in the repeated nucleotide sequences affect exclusively the number of repeats (Figure 4). Due to the repeated homology within this part of the gene, the nucleotide sequence of the repeats itself is unaltered by the unequal crossing-over event. The number of repeats in different alleles varies greatly. The range in number of repeats found for MUC1 mucin and PSGP is 20 to 125 and 10 to 200, respectively, whereas other mucin genes may contain a much smaller number of repeats (Table 4). The crossing-over events occurring during the formation of the haploid gametes result in stable mutations, which are inherited following Mendel's laws.[65,182] Due to the variety in MUC1 genes, nearly all individuals express their own set of mucin precursors: in 69 persons, 30 different alleles were found.[65,182] The number of nucleotides in the repeated nucleotide sequence in all known mucin genes is a multiple of three. In other words, the nucleotide repeats are within the same translational reading frame; each repeat in the gene codes for the same unique repeated amino acid sequence. The length of the tandem repeats in the amino acid sequence of different mucins comprises 9 to 81 amino acid residues (Tables 2 and 4). These repeated sequences in the DNA code for the *O*-glycosylated region of the mucin.[19,22,29,62,65,104-106] The maximal difference in expression of MUC1, between the smallest and the largest observed polypeptide,

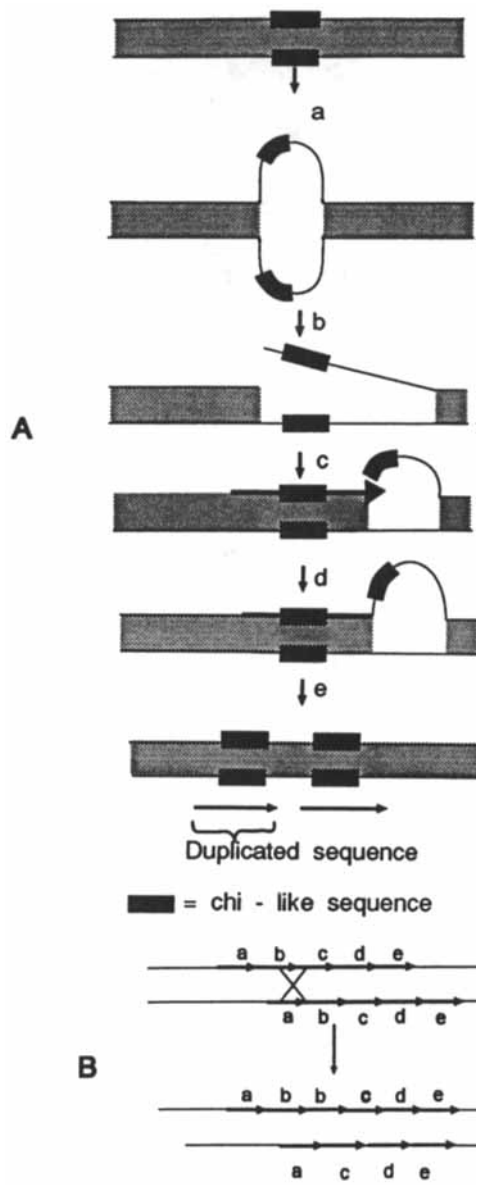

FIGURE 4. Model for the genetic polymorphism in mucin genes. The mucin genes show remarkable polymorphism in allele size, due to a variable number of tandemly repeated nucleotide sequences in the central part of the gene. These tandem repeats are either perfect, as in MUC1 and PSM, or display small variations around a consensus sequence, as in intestinal mucins and tracheal mucin (Table 4). The generation of these tandem repeats may be related to the presence of Chi-like sequences. These sequences, originally found in *Escherichia coli* and bacteriophage lambda, are thought to function as signals for homologous recombination.[189,190] Moreover, these sequences, also found in the eukaryotic genome, probably promote the generation of tandem repeats in DNA. Panel A shows a tentative model, adapted from Jeffreys et al.,[189] explaining this phenomenon. The expansion and contraction of the number of repeats in the DNA is further generated by unequal crossing over. Crossing-over events occurring between chromosomes, which are "out of register", results in changes in the number of repeats. These events lead to mutations in the length of the mucin alleles (Panel B). This type of crossing over may occur between identical as well as nonidentical alleles. These mutations result in a considerable genomic heterogeneity; 69 individuals tested showed 30 different MUC1-alleles.[27] The mutations, once established, are stably inherited between generations, following Mendel's laws.[182]

is about 200 kDa.[182] As the repeated amino acid region contains a number of O-linked glycans, the final difference in molecular weight of the mature mucins is considerable. A model for the expression of mucin genes is depicted in Figure 5. As noticed, mucins are filamentous molecules, which form filamentous, covalent oligomers. The number of tandemly repeated elements in the polypeptide directly effects the length of the oligomers and the length of the *O*-glycosylated regions. As both these features effect the gel-forming properties of the mucins, the rheological properties of the gel are directly affected by the genetic polymorphism of the mucin genes.

The region in the gene containing the repeated nucleotide sequence is flanked at both sides with nonrepetitive nucleotide sequences.[19,22,29,61,62,65,104-106] These nucleotide sequences code for the N- and C-terminal part of the polypeptide. The nucleotide sequences coding for the N- and C-terminal pat of MUC1 consist of several exons.[28,191]

The genetic polymorphism was also suggested for other mucins. The gene for human intestinal mucin (MUC2) was found to be genetically polymorphic.[19] The alleles for the PSGP gene of the rainbow trout were estimated to comprise 100 different forms.[62] As the tandemly repeated sequences are located within the coding sequence, the genetic polymorphism can readily be detected in the expressed mucin polypeptide.[65] Gastric mucous cells of the rat produce two mucin precursors, which are both expressed equally, whereas individual rats show expression of mucin precursors of variable molecular weight.[23] Preliminary results of the biosynthesis of the human gastric mucin showed the expression of mucin precursors of a molecular weight of about 900 kDa.[63,131] These individuals equally expressed two mucin precursors with a variable molecular weight, similar to the variations observed in the rat.[192] These results suggest that gastric mucous cells express two codominant mucin genes of similar structure, as outlined above.

B. Regulation of Expression

Secretory mucins are produced by highly differentiated cells within epithelia. In rat gastric mucous cells, the mucin polypeptide comprises at least 10% of the total protein synthesis, and nearly all glycosylation and sulfation in these cells is dedicated to the production of mucin.[23] The turnover of the mucous cells in the gastrointestinal tract is 4 to 7 d. The mucous cells differentiate from a pluripotent stem cell within the epithelium. The regulation of the turnover and of the number of mucous cells developing from these stem cells is unknown. However, it is evident that the number of mucous cells forms the basis for the mucosal protection by mucus. Epithelia are very frequently exposed to hostile environments. As these mucins primarily serve to protect the epithelia, the mucin synthesis will be basically constitutive. The mucous cells have a large storage capacity for mucin, in the form of mucous granules, which can be secreted very rapidly in case extra protection of the epithelium is needed. There is a continuous, baseline secretion of mucin, which results in an average release time for newly formed granules in goblet cells of the intestine of: 4 to 8 h in rat *in vivo*; 8 to 12 h in rabbit *in vitro*; and 20 to 24 h in human *in vitro*.[4] The turnover of the mucin granules is not uniform, as the granules at the periphery of the mucous cells tend to migrate at a much higher speed, bypassing the more centrally located granules.[193] The centrally located granules can be released within minutes under the influence of secretagogues, in case extra protection of the epithelium is needed.[4] The very rapid secretion of mucin from these granules is possible because they do not individually fuse with the apical plasma membrane. Instead, many granules fuse with other granules that have already fused with the plasma membrane. This so-called compound exocytosis leaves the cells with a characteristic apical cavitation. The signals triggering the secretion can be both neuronal and hormonal, although the cellular response by the mucous cells is not mediated by cyclic nucleotides.[4] The secretion may be triggered by increase of the Ca^{2+} concentration in the cytoplasm, inducing the formation of fusion pores (Section III.C).

The most prominent feature of the mucin genes is the occurrence of multiple tandem repeats in the coding sequence. This structure causes the major genetic polymorphism of these genes, presumably by unequal crossing-over events be-

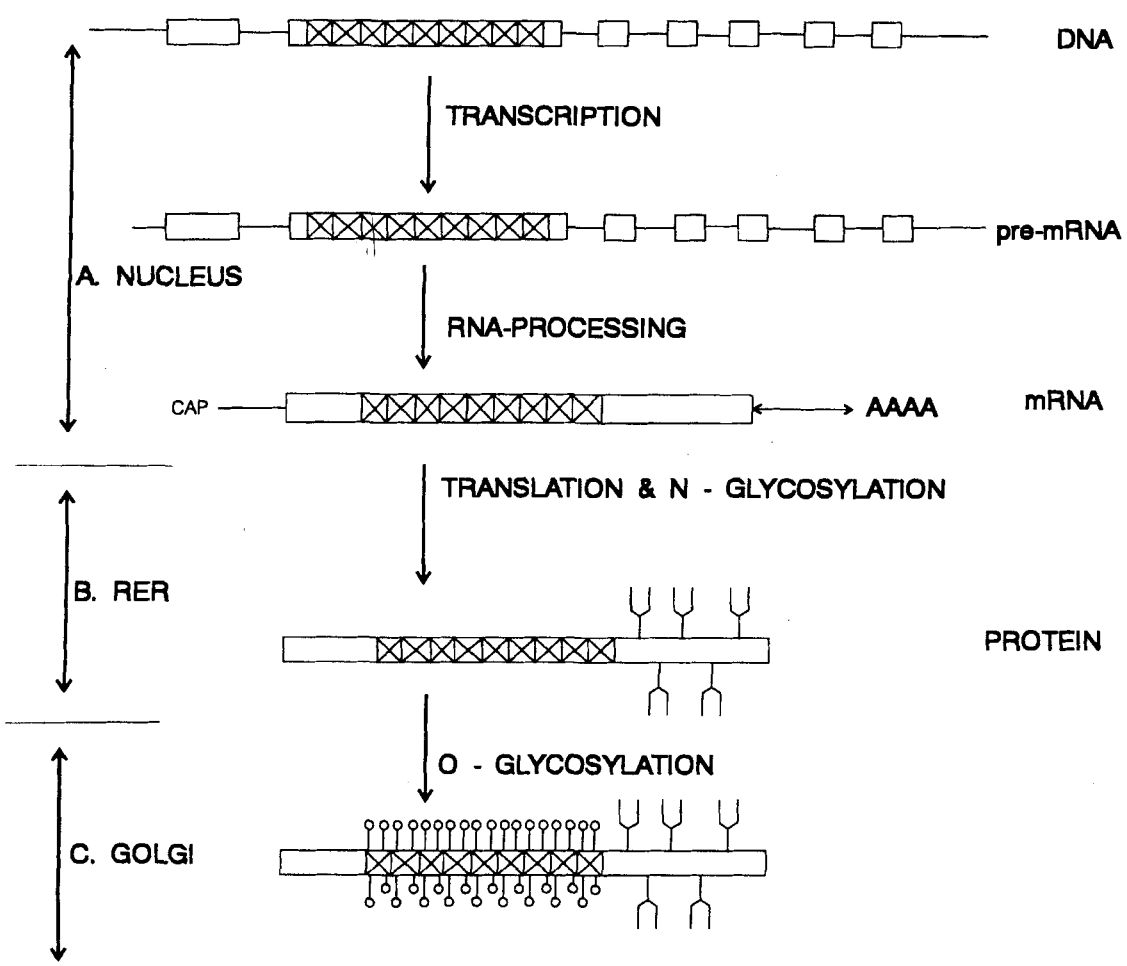

FIGURE 5. Model for the expression of mucin genes, based on the studies on MUC1. (A) The MUC1 gene is composed of 7 exons, and located on chromosome 1.[28,191] All tandemly repeated sequences, composed of 60 bp, are located within exon 2. These repeats in exon 2 are flanked at both sides by nonrepeating sequences, whereas the other exons contain only nonrepeating sequences. The genomic size comprises about 6 to 12.3 kb, depending on the number of tandem repeats; 21 to 125, respectively. The depicted gene contains the most common allele found in the human population, which contains 40 tandem repeats.[27] The primary transcript of the gene is spliced to yield two different mRNAs, which are both translated. These splice variants arise by two different splice acceptor sites in exon 2, resulting in two mRNAs one of which is 27 bp longer than the other.[28] These 27 bp are part of the coding sequence, and are located very close to the amino-terminal end of the polypeptide, resulting in two polypeptides with distinct N termini. (These two different mRNAs are not shown in this figure, as the resulting proteins differ only by 9 amino acids in the N-terminal region.) (B) The translation of the mRNA results in polypeptides containing the number of tandem repeats in the amino acid sequence corresponding to the number of nucleotide repeats present in the allele. The nonrepeating sequences at the N- and C-terminal ends of the polypeptide contain the *N*-glycosylation sites, whereas all *O*-glycosylation is present on the central, repeated amino acid sequences. (C) The variation in number of tandem repeats in the gene directly affects the length of the polypeptide and the relative amount of oligosaccharides present on the mucin. Mucins are filamentous molecules as a consequence of their *O*-glycosylation, and therefore the length of the whole molecule is affected by the genomic heterogeneity of the gene.

tween homologous genes. An interesting finding in this respect is the occurrence of a Chi- or Chi-like sequence in the tandemly repeated base sequences.[191] These short sequences, originally found in bacteriophage lambda and in *Escherichia coli*, are thought to function as signals for homologous recombination.[189] These sequences are usually found in noncoding, G+C-rich, "minisatellite" regions of the genome, and are known to be hypervariable within the human population, making them particularly useful in analysis of human disease.[189,190] A speculative model for the generation of the tandem repeats, based on the presence of Chi-like sequences, has been proposed by Jarman and Wells (Figure 4).[190] The DNA duplex near the Chi sequence is nicked; repair synthesis and ligation of the nicked strand results in the duplication of the Chi-like sequence, which subsequently promotes mispairing and unequal exchange, leading to amplification of this sequence. Thus, the Chi sequence might be responsible for the multiplication of the tandem repeats. Moreover, this sequence enhances the chance of crossing over between the tandemly repeated sequence of the mucin genes. This indicates that this mucin gene-specific structure is intended to generate genetic polymorphism within this region of these genes. The genetic polymorphism has direct consequences for the size of mucins produced. This process of homologous recombination is thought to be mediated by a specific nuclear factor.[190] Therefore, control of these events, leading to the generation of different alleles, influences the expression of mucins with different physicochemical properties.

The mucin genes expressed in the various tissues show no homology in either nucleotide or amino acid sequence (Tables 2 and 4). Therefore, the expression of the mucin genes is apparently tissue specific. However, one human mucin was found to be expressed in small intestine, colon, and trachea.[19,106,107] So far, no mucin sequences are known from homologous organs in various species. These data would reveal whether the mucins expressed in a homologous organ in specific species have evolved from a common ancestral gene. The available data make it very unlikely that the mucin genes expressed in various organs within one species have developed from a common ancestral gene. At present only the genomic structure of MUC1 has been determined.[191] As noted, a major genetic polymorphism is found in one exon, leading to the expression of a polymorphic polypeptide (Section VI.A). This polymorphism does not affect the 5′ or the 3′ regulatory sequences of the mucin gene. The preliminary analysis of the promotor of the MUC1 gene shows several potential regulatory elements in the 5′ flanking region of the gene. This analysis showed the presence of potential binding sites for steroid receptor sequences and cellular transcription factors like SP-1 and AP-2.[191] Until now, no studies have been undertaken to measure the transcriptional activation of mucin genes, in order to find potential (tissue-specific) gene activators or inhibitors.

The translational regulation of genes may occur at two different levels. First, there is regulation during the process of pre-mRNA splicing in the nucleus, possibly resulting in different mRNAs from the same genomic sequence. Second, there is regulation of the factors determining the mRNA concentration in the cytoplasm. An example of regulation at the pre-mRNA splicing of a mucin gene was found in the mRNA processing of the MUC1 gene. Two different splice variants of this mRNA were found, resulting in two proteins differing in their signal sequence and in their extreme amino-terminal parts of the mature proteins.[28,191] This event leads to a polypeptide polymorphism not dictated by the variation in the tandemly repeated nucleotide sequence. It might be that this mRNA splicing-induced heterogeneity of the polypeptide results in two proteins with different functions. The speed of mucin production in a mucous cell is subjected to large variations. When the epithelium is forced to release the stored mucin, in response to an extracellular signal, the loss of mucin has to be restored within a minimum of time. Generally, the activation of genes takes several hours to produce enhanced quantities of protein. Increase of the translational efficiency of the mucin mRNAs can provide a much faster answer to the need for additional mucin. In several studies of various mucins it proved difficult to isolate intact mucin mRNA, whereas other mRNAs of the same molecular size could be isolated intact from the same tissue[19,56,106,107] The polydispersity of the mucin mRNAs isolated from tissue is probably

characteristic, and might indicate the high turnover of these mRNAs. The tandem repeats, which are specific for these mRNAs, may influence the stability of the mRNA negatively. The biosynthesis of the rat gastric mucin protein backbone could be stimulated or inhibited by prostaglandin E_2 and indomethacin, respectively, within 30 min.[194] The speed of these effects indicates that translational regulation of this mucin is very likely, suggesting that this mode of regulation might be essential in the rapid restoration of the mucus-secreting capacity of the mucous cells after a challenge.

The length of the oligomers is very heterogeneous in all mucins studied so far. It is always difficult to reconcile whether this heterogeneity arises from partial degradation of secreted mucin, heterogeneous biosynthesis, or a combination of both. This dilemma is largely caused by the inability to differentially isolate secreted and intracellular mucin from tissue. Metabolic labeling studies with radioactive amino acids are in this respect the only reliable method to study the process of oligomerization. Such experiments have shown that the oligomerization of rat gastric mucin within the RER yields heterogeneous oligomers.[49,50,63] Thus, the intracellular mucin is heterogeneous, whereas the regulation of the length of the oligomers remains obscure. The length of the oligomers will have its effect on the properties of the gel formed. Longer oligomers will form more viscous gels, as these molecules are larger and have more possibilities to associate with other mucin molecules within the gel. The genetic polymorphism of the mucin genes affect the central, *O*-glycosylated region of the mucin molecules (Section IV.A). This region forms the largest part of the filamentous shape of the molecule. Therefore, the genetic polymorphism will affect the length of the filamentous oligomers directly. This implies that the properties of the gel formed by gel-forming mucins are, at least in part, inherited in the coding sequence of the mucin gene.

V. FUNCTION

The various characteristic features of mucin-type glycoproteins confer different functions, some of which are more clear than others. It is generally accepted that mucin-type molecules exert an effective shielding of cells against surrounding molecules. In epithelial cells this function is in effect at the apical plasma membrane and is accomplished by a continuous mucous layer. Membrane-bound mucins, present in small amounts on many cells, may function especially during development and carcinogenesis in cell migration, adhesion, and recognition. The carbohydrate structure largely determines the specificity of binding to other molecules. Thus, binding to cells, extracellular matrix, or pathogenic organisms can occur. In the following sections, functional aspects of both secretory and membrane-bound mucins will be elaborated.

A. Protection of Epithelia by Mucin-Gels

As mentioned before, the functional configuration of the secretory mucins of the gastrointestinal, cervical, and tracheo-bronchial tract is a disulfide-linked oligomer.[4,7,25,26,43,49] The gel-forming ability of isolated gastric mucins is completely lost upon reduction of the disulfide bonds. Optimal protection is regulated by: (1) the amount of mucin; (2) its specific structure; (3) the presence of other molecules like nonmucin, noncovalently bound proteins; (4) lipids; and (5) ionic conditions.

Independent of the kind of epithelium, secretory mucin is always covering the apical cell membrane. In epithelia like stomach and cervix, the environmental conditions may require a thick mucin layer, while in esophagus or gallbladder a thin layer may provide sufficient protection against the environment. Also the kind of protection varies among different epithelia. In stomach, with its extremely low pH and high concentrations of pepsins, mucins must be highly resistant to these agents and the layer must be tightly closed, while in small intestine, with its slightly alkaline pH and a variety of different hydrolytic enzymes, the mucous layer must allow easy access of nutrients to the enterocytes. To maintain an intact mucous layer, which wears away due to the action of hydrolytic enzymes and shear forces, surface mucous cells must produce sufficient amounts of mucin molecules to account for this. Moreover, like in the gastrointestinal

tract, where challenge of the mucous layers fluctuates considerably during a day, there is a strong need for regulatory mechanisms to keep the mucin synthesis and secretion at the necessary level. Cholinergic innervation was first implicated in the regulation of mucus secretion.[195] Topical application of acetylcholine or carbachol produces an increase in thickness of the mucus-gel in gastric mucosa of dogs and rats.[8,196,197] Intestinal crypt cells, including goblet cells, are clearly sensitive to acetylcholine and its analogs, while goblet cells of the villi and colonic mucus-producing cells are insensitive.[179,198,199] Secretion in tracheal mucin-producing cells can be induced by cAMP.[200] Prostaglandins are also indicated to play a role in the rapid increase in mucin secretion. In tracheal, gastric, and gallbladder epithelia the amount of mucin either increases after addition of PGE_2 or decreases after application of indomethacin or aspirin, which both inhibit cyclooxygenase, the enzyme transforming arachidonic acid into the endoperoxides.[196,201-205]

How do the structural features of the oligomeric secretory mucins relate to their protective functions? In the majority of mucins present along the epithelia, oligomeric mucin molecules form mucus with viscous and elastic properties due to physical entanglement. As stated above, the oligomeric configuration is essential: both reduction of the disulfide bonds in the oligomers and proteolytic degradation into glycosylated fragments result in complete loss of gel formation.[3,15] If total shielding of the epithelium is required, like in stomach, the mucous layer is continuous and impermeable to large molecules like pepsin. On the other hand, ions like H^+ secreted by the parietal cells, can diffuse through the mucous layer. This acid from the lumen is neutralized within the stable "unstirred" layer by bicarbonate secreted from the surface mucous cells.[206] This model is supported by a number of studies on bicarbonate secretion by surface mucous cells and pH measurements across the mucous layer.[207-211]

Mucin molecules are stiff, random-coil polymers. Shogren and co-workers studied the conformations of desialylated and deglycosylated submaxilliary sheep mucin.[101,148] It has been shown that the chain-stiffening effect of glycosylation is primarily due to steric interactions between the peptide-linked GalNAc residues and adjacent amino acids in the polypeptide. The rigidity is somewhat increased by one extra sugar added to the GalNAc, but further elongation of the O-linked side chains has little or no additional effect.[212,213] Based on such a model, the mucin chain has an average length of 2.5 Å per amino acid residue. For rat gastric mucin with a backbone of 270 kDa and a length of about 300 nm, as measured by electron microscopy, it can be calculated that, if half of the backbone peptide has all the GalNAc binding sites, 1200 amino acids shape the rigid part, while the remaining 1200 amino acid residues are in the N- and C-terminal extensions, spanning less than 30 nm. Thus, only the first one or two sugar residues of O-linked oligosaccharides contribute to the rigidity and persistence-length of mucin molecules. However, secretory mucus glycoproteins in most epithelia have far longer O-linked oligosaccharides (5 to 10 sugar residues). In the case of rat gastric mucin, mature tri- or tetramers are probably 1.8 to 2.4 $\times$ 10^6 kDa. The radius of gyration, defined as the statistical average distance of all elements of the molecule from its center, is in the order of 500 nm.[148,149] This implies that the domains of these molecules begin to overlap in solution at concentrations of less than 1 mg/ml. Mucin concentrations in normal mucus up to 50 mg/ml can only be understood if one assumes stable intertangled networks in which the rigid chains hinge at the disulfide-connected peptide extensions. In these networks the oligosaccharide chains contribute to the viscosity not only by their size, but may also participate in stronger intermolecular interactions that enhance gel formation.[214] Such a network offers perfect protection for the protease-sensitive hinge regions as they are all well inside the network, which is impermeable for proteases. Only peptide ends extending from the entanglement into the lumen can be degraded.

Another feature of mucins, closely related to gel formation, is their polyanionic structure. The overall negative charge is due to the presence of abundant sulfate and/or sialic acid residues on the oligosaccharides. The charge of the molecules contributes probably in two ways to the gel formation. First, the repulsive forces between the mucin oligomers result in a highly hydrated gel structure. Second, it is known that cations, like

calcium, can induce electrostatic interactions between negatively charged groups on separate mucin oligomers.[35,88] The highly hydrated state of the molecules giving the gel its volume is due to the hydrophilic nature of the oligosaccharides of the mucins and to the electrostatic repulsion between the oligomers. The interactions between the mucin oligomers within the gel are established by specific interactions between the oligosaccharides and by electrostatic linkages between the mucin oligomers mediated by cations.

Apart from its function as a protective layer, mucus also acts as a selective barrier. In intestine, nutrients such as small peptides, sucrose, and lactose must first pass the mucous layer before they can be hydrolyzed by intestinal membrane-bound hydrolases like dipeptidases, sucrose-isomaltase, and lactase. In general, mucus retards the free diffusion not only of small nutrients but also of antibiotics, pharmaca, and even water.[215,216]

B. Functions of Mucins at the Cell Surface

Membrane-bound mucins may have protective functions comparable to the secretory mucins. Most, if not all, epithelial cells have low concentrations of mucin-like glycoproteins like MUC1 at their apical membrane.[100,217] Although they are not covalently linked into homooligomers, like the secretory mucins, they may serve as a last protective barrier against extracellular environmental factors like low pH or hydrolytic enzymes. In line with such a task are the same considerations as given above concerning the length of these giant molecules. The polypeptide chain of MUC1 ranges between 120 and 300 kDa, of which about 25% of the amino acid residues are serine and threonine.[27] If all hydroxyl amino acids contain O-linked oligosaccharides, which is expected on the basis of the molecular weight of mature membrane-bound mucin, the same rigidity and persistence length as in secretory mucin is likely. It implies that such a molecule extends at least 150 nm into the lumen as a rigid rod. If sufficient amounts of these glycoproteins are present, they certainly would shield some of the cell surface antigens or receptor binding sites for high-molecular-weight ligands. As the apical cell surface of most epithelial cells has mainly absorptive functions (for low-molecular-weight molecules) it could well be that in normal cells the class of membrane-bound mucins has an important shielding function, as illustrated in Figure 6. Recently, it was found that the purified MUC1 mucin inhibited the cytotoxic action of eosinophils toward target cells.[218] This inhibition was due to a specific interaction between this mucin and a component on the cytotoxic eosinophil surface. This may explain the inability of cytotoxic cells to kill epithelial tumor cells, as these cells overexpress this mucin on their entire surface. On the other hand, the normal expression of MUC1 mucin on the epithelial plasma membrane may serve to protect these cells against damage caused by inflammatory cells.

Many epithelial cells, for example, of the small intestine or the mouth, function not only in defense, but also support numerous selective uptake and binding processes. Most of the molecules responsible for the latter processes are membrane-bound enzymes or receptors (like in taste buds on the tongue) with their active center close to the membrane. Sucrose-isomaltase, one of the small intestinal microvillar proteins, has a relatively short *O*-glycosylated sequence separating the membrane-bound domain from the functional domain by 35 Å.[219,220] In this way the glycocalyx of epithelial cells consists of several functional layers (Figure 6): (1) immersed in the membrane are the transporter molecules for uptake or release of ions and small nutrients; (2) at 100 to 150 Å above the cell are the enzymes and receptors; and (3) at >150 Å the protective membrane-bound mucins. Thus, the ability of epithelial cells to interact with their environment may be regulated by the density of the membrane-bound mucins: at higher density access to receptors and enzymes may be limited or impeded.

C. Pathology

Many diseases affect the production of mucus. In a number of cases the biochemical characteristics of the mucins are altered; in other instances the amounts and properties of the mucus are changed, and sometimes both effects occur

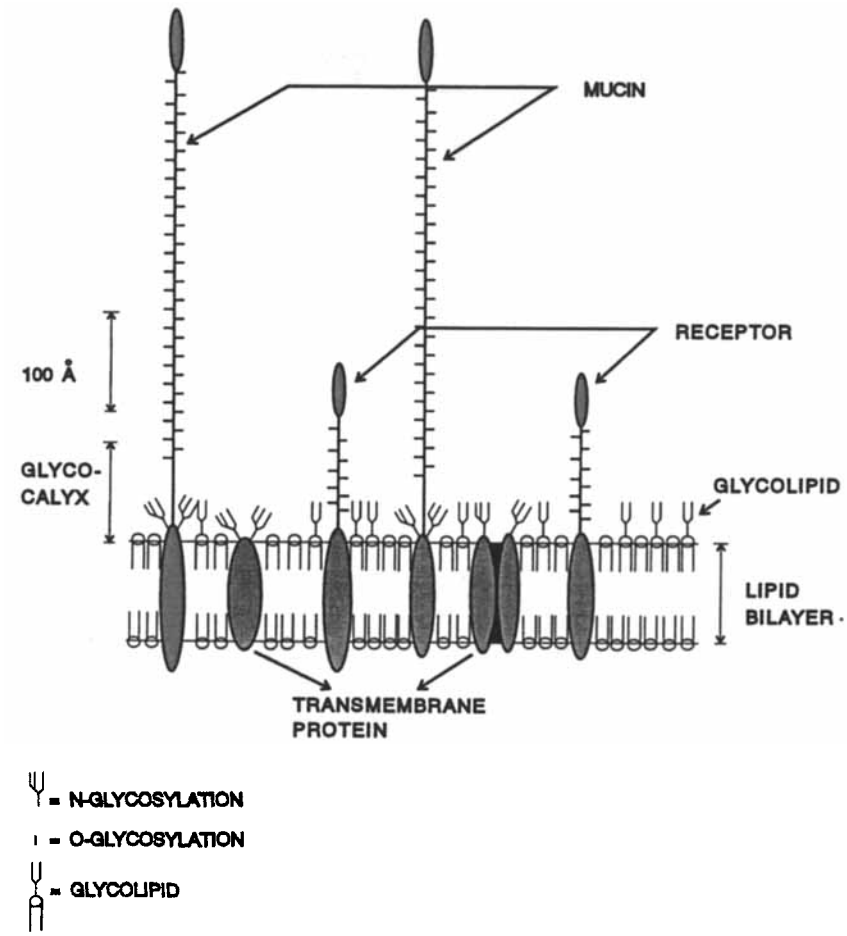

FIGURE 6. Model for the structure and function of transmembrane mucins at the cell surface. All the components of the plasma membrane are drawn to scale. Shown are the lipid bilayer with its (glyco)lipids and transmembrane (glyco)proteins; the glycocalyx, composed of the saccharide moieties of the glycolipids, and the extracellular portions of the transmembrane glycoproteins; and the membrane-bound mucins.

simultaneously. The *in vivo* quality of the mucus is determined by a complex of factors (Section V.A). Not only the structure of the secretory mucins is essential, but also the ionic environment and the presence of other nonmucin components might determine the mucus properties. Usually, when mucus is studied in relation to disease, only a few of the many characteristics of the mucus, mucins and nonmucin components, are determined. Thus, although the quality and quantity of the mucus produced is certainly of great importance in the history of many diseases, they are often a consequence of other (primary) causes.

In cystic fibrosis, characteristic changes in mucin occur: in addition to dramatic changes in rheological properties of mucus, an increase in buoyant density and a higher degree of total glycosylation is accompanied by higher amounts of Fuc, Gal, GlcNAc, and sulfate per molecule. This is consistently found in intestinal and meconial mucin.[221-224] Recently it was reported that cystic fibrosis is caused by a defective membrane protein involved in Cl^- transport activity.[225] It is tempting to speculate about the effect of Cl^- transport on the activity of glycosyltransferases in the Golgi complex. One possibility could be that cell surface Cl^- channels recycle via the clathrin-coated endocytic pathway. As these channels are then present in vesicles that are aci-

dified by the endosomal proton pump, the Cl^- channels can well be involved in compensating for the extra electrogenic potential due to proton transport by transport of Cl^- ions. A defected or closed channel might hamper proper functioning of the pump, leading to a higher than normal pH in the endosomes and the *trans*-Golgi reticulum. As the pH in the *trans*-Golgi reticulum that harbors terminal glycosyltransferases is lower than in the Golgi stack, it might be that in the Golgi complex of cystic fibrosis patients the balance for addition of terminal sugar residues changes in favor of fucose, galacatose, and GlcNAc.[226-228] Thus, the most severe direct effects of cystic fibrosis are on the rheological properties of mucus, due to the change in ionic environment at the epithelial surface, while the variations in sugar composition of mucin are probably secondary effects.

In malignancy, gastrointestinal or bronchial secretory mucins undergo dramatic changes at the nonreducing termini of O-linked oligosaccharides. These changes can easily be detected using lectins or monoclonal antibodies. The status of these very important aspects of regulation of mucin synthesis is reviewed by Zotter et al.[100]

In colon and intestine the effects of various pathological conditions on the composition and quality of mucin is not at all clear. In gastric adenocarcinomas, ulcerative and ischemic colitis, and Crohn's disease, mucins with higher percentages of sulfate and less sialic acid are induced.[229] Whether these effects are directly linked to genomic defects or are due to secondary causes is unknown. Numerous reports show changes in histochemical carbohydrate staining in Crohn's disease and in ulcerative colitis.[229] In addition, alterations in sugar composition include increase in sialomucin relative to sulfomucin, altered *O*-acylation of sialic acid, diminished fucose, and increased exposure of galactose.[296-231] As the causes of these conditions are unclear it is hard to classify these observations. It is important to realize that the status of a mucous layer is the result of a dynamic equilibrium between synthesis and degradation. Quantitative analysis of mucin isolated from diseased tissues is only one of the parameters. Assessment of, for example, the role bacteria play in the composition and quality of mucin is hard to establish in such an environment.

There is growing evidence that mucin contributes to gallstone formation. Although stone formation is dependent on a complex of different factors, there is consistency in the observation that mucin hypersecretion accompanies cholesterol stone formation.[232,233] Another important observation is that factors that inhibit this secretion suppress stone formation. When aspirin was added to a cholesterol-rich diet in the prairie dog, mucin secretion decreased and at the same time gallstone formation was suppressed.[232,234] Whether mucin only acts as a nucleating agent for cholesterol to be deposited or whether gel-forming properties are important as well in this respect is unclear. Thus, in future approaches stone formation may be slowed either by inhibition of arachidonate metabolite formation or by enhancement of mucin degradation.

Most, if not all, epithelial cells express the membrane-bound mucin-like MUC1 at their apical cell surface. In most carcinomas, expression of MUC1 is increased considerably.[100] The glycosylation pattern of this polypeptide differs among carcinomas from different tissues and specific antibodies have therefore been used as tumor markers.[96,177,235,236] The mechanism of this increased expression is unknown. However, this class of mucins has been implicated in the metastatic potential of carcinoma cells. Primary tumor cells lose their polarized character and, as a consequence, high concentrations of MUC1 might spread out over the whole cell surface. This mucin may protect the tumor cells against low pH, due to the high concentration of lactate caused by the high glycolytic activity of these cells.[237] If these cells leave the epithelial layer they may resist lysis by natural killer cells due to the high concentration of sialomucins.[215,238-240] Alternatively, these cell surface mucins may interfere with immune surveillance by T lymphocytes, by causing steric hindrance of surface antigen presentation.[241] Another way to block the immune system might be by the high concentration of mucin molecules shed from the carcinoma cells by proteolysis. These circulating mucin fragments form immune complexes, which act as blocking factors for the immune system.[215,242] Fi-

nally, dependent on its lectin-binding character, cancer cells may adhere only to specific other categories of cells in the organism, giving cancer cells of different origin their typical metastatic pattern.[243,244]

VI. CONCLUSIONS AND PERSPECTIVES

Mucin-type glycoproteins are mainly used by the organism for protective purposes. This conclusion has been drawn from the start of mucus research.[2,4,7] However, the prospective for specifying this further is promising. The major recent advances in this respect are (1) the number of available cDNA clones for glycosyltransferases is rapidly increasing; (2) new model cell culture systems are available, capable of synthesizing and storing mucin-type glycoproteins; (3) sequence data from several mucins are emerging, and their gene structures will soon be known. All these possibilities will be used to express mucin molecules into cell lines of choice; these cell lines can be manipulated by transfecting varying glycosyltransferases, resulting in synthesis of mucins with special sugar epitopes. All this together will answer questions like: What are the critical steps in mucin biosynthesis? How are mucins stored intracellularly? How are mucin genes regulated? How do cells adapt their mucins to the needs of local epithelia? What is the real function of MUC1 and why is it massively expressed in malignancy? A number of answers concerning the expression of mucins will be found when the structure of the mucin genes is determined and their regulation is fully understood. However, the full expression of the mucins requires the coordinate expression of a large number of other genes, e.g., glycosyltransferases. The methods to do this are at hand.

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