Review

Gel-forming mucins. Notions from in vitro studies

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Summary. Mucus secretions form a protective barrier in the mucosa of the auditory, gastrointestinal, respiratory, and urogenital systems, and the conjunctiva in the eyes. A family of glycoproteins known as gel forming mucins is the major component of the mucus. Gel-forming mucins are among the largest and most complex proteins known. Their polypeptide chains comprise thousands of amino acid residues organized into different domains with diverse post-translational modifications, including O- and N-glycosylation, sulfation, proteolysis, and likely C-mannosylation. Moreover, these glycoproteins form disulfide-linked oligomers/multimers with molecular weights in the millions. Molecular polydispersity in terms of length, carbohydrate content and composition, is an invariable feature of purified mucins. This structural complexity makes it technically very difficult to study mucin biochemical and physical properties. It is not surprising, therefore, that our knowledge on mucin structure, biosynthesis and function still is incomplete. During the last decade, the use of recombinant mucins has allowed researchers to study the biochemical properties of protein domains, peptide motifs and amino acid residues common to all gel-forming mucins, and to propose specific roles for them. We review here the relative impact that these in vitro studies have had for our current understanding of two of the most important features of these macromolecules: formation of disulfide linked oligomers and mucin intragranular packaging.

Key words: Mucins, Mucin, Mucin assembly, Secretory granule, Mucin granule, Goblet cell

The Gel-forming mucin family of glycoproteins

Five secreted gel-forming mucins, named MUC2, MUC5AC, MUC5B, MUC6 and MUC19 have been identified in humans (Dekker et al., 2002; Chen et al., 2004). Structurally related mucins have been described in other mammalians, amphibians and fishes and even in

invertebrates. This family of glycoproteins is the major components of the gel-like mucus that covers epithelial cells in different organs in all vertebrates, the amphibian epidermis, and the gills in fishes. Because of their large sizes, high carbohydrate content, extended solution structure and polymeric nature, gel-forming mucins endow mucus secretions with high viscosity and the necessary chemical diversity to interact, entrap and transport microorganisms, particles and noxious chemicals. Moreover, in the gastric mucosa, the hydrodynamic properties of gel-forming mucins prevent mucosal damage by hydrochloric acid (Bhaskar et al., 1992). It is not surprising that deregulation of mucins/mucus production can have serious health consequences. For instance, reduction in the synthesis of intestinal mucins has been associated with predisposition to colitis (Van der Sluis et al., 2006), and overproduction and accumulation of gel-forming mucins/mucus is a common feature in the lung of patients with obstructive lung diseases, including COPD, cystic fibrosis and asthma (Rose and Voynow, 2006). Besides lubricating and forming a protecting sheet, gelforming mucins have other pivotal roles. Some gastric mucin oligosaccharides have an antibiotic activity against Helicobacter pylori, the etiological agent responsible for gastric ulcer (Kawakubo et al., 2004). Moreover, gel-forming mucins might interact and retain important antimicrobial peptides in the airway mucus (Felgentreff et al., 2006). Furthermore, the major gelforming mucin in the colon likely protects against cancer development in an environment where potentially carcinogenic products are present (Velcich et al., 2002).

Common to all mucins is a large, centrally located and highly glycosylated protein domain, named the mucin-, O-glycosylation-, or tandem repeat domain, which comprises most of the mucin polypeptide chains (Perez-Vilar and Hill, 1999; Dekker et al., 2002) (Fig. 1A). These domains are made of several repeats of a unique sequence rich in threonine and/or serine residues, whose hydroxyl groups are in O-glycosidic linkage with oligosaccharides. O-linked oligosaccharides are very diverse and provide mucins with the capability to interact with microorganisms and many compounds (e. g., Roussel and Lamblin, 2003). The amino acid

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sequence, length and number of repeats vary among mucins. In all, but MUC6 and likely MUC19, the mucin domains are interrupted by several copies of a 90-100 amino acid residues cysteine-rich domain, denoted as the CS (Cys-rich Subdomain)-domain (Desseyn et al., 1997; Escande et al., 2001). All mucins have signal peptides at their N-termini followed by three homologous, ~ 320-400 amino acid residues each, cysteine-rich domains known as the D1, D2 and the D3 (Disulfide-rich)domain (Perez-Vilar and Hill, 1999). A fourth D-domain (D4) is located after the corresponding mucin domains in all mucins but MUC6 and MUC19 (Chen et al., 2004, Rousseau et al., 2004). A potential domain, known as TIL (Trypsin Inhibitor-Like Cysteine rich domain), has been recognized within the D-like domains (Lang et al., 2004). Other protein domains, which include the C (Cysrich) and CK (Cystine Knot) domains at the C-termini (Perez-Vilar and Hill, 1999), are usually underglycosylated and rich in cysteine residues.

Gel-forming mucins are synthesized in and secreted from specialized cells known as mucous/goblet cells (Neutra et al., 1984; Specian and Oliver, 1991; Rogers, 2003). These cells are characterized by apically-located large secretory granules (the mucin granules) where mucins are accumulated. Mucin biosynthesis and intracellular trafficking involve three major cellular compartments: the endoplasmic reticulum, the Golgi complex and the mucin granules. In the endoplasmic reticulum mucins are folded, N-glycosylated and likely C-mannosylated, and form disulfide-linked dimers (e. g., Perez-Vilar and Hill, 1999; Asker et al., 1998; Perez-Vilar et al., 2004). The bulk of O-glycosylation occurs once the mucin precursors reach the Golgi complex, where sulfation, formation of mucin disulfide-linked oligomers/multimers and likely proteolysis also take place (e. g., Roth et al., 1994; Perez-Vilar et al, 1998; Perez-Vilar and Hill, 1999; Gold et al., 2002; Brockhausen, 2003; Lidell et al., 2003a; Lidell and Hansson, 2006). Inside mucin granules, mucins are accumulated and stored until proper regulatory signals triggers granule exocytosis (e. g., Li et al., 2001).

The study of native mucins. The difficulties

Native gel-forming mucins are difficult to study for several major reasons. First, a typical human mucin, e.g., MUC5B, has around 5,000-6,000 amino acid residues, of which ~3,000 residues comprise the highlyglycosylated mucin domain, i.e., Ser/Thr-rich sequences. O-glycosylated mucins reach an average molecular weight of more than 1×10^6 d, which is increased by a factor of two and up when disulfide-linked species are



assembled. At this range of molecular weights, the use of classical biochemical techniques is mainly limited by the lack of resolution. Second, the abundance of Olinked oligosaccharide chains endows mucins with anomalous behavior in commonly used techniques like size-exclusion chromatography or SDS-PAGE (e.g., Tytgat et al., 1995). Third, molecular polydispersity due to genetic polymorphism, proteolysis and/or differential glycosylation is a common feature of gel-forming mucins (e.g., Vinall et al., 2000; Wickstrom and Carlstedt, 2001; Veerman et al., 2003). Fourth, mucus varies from a viscous fluid to a strongly viscoelastic gel, not readily dispersible in non-denaturing buffers (Bansil et al., 1995; Bansil and Turner, 2006). Indeed, reduction of disulfide bonds and extraction in solutions containing high concentrations of chaotropic agents such as guanidine are usually required to disperse and get mucins into solution. These protocols, however, result in the disappearance of important structural information and, ultimately, limit the usefulness of these preparations to perform functional studies (e.g., Bansil et al., 1995; Raynal et al., 2003). Fifth, when extraction of mucins under non-denaturing conditions is possible (e.g., from saliva), mucin preparations are usually "contaminated" with cellular and bacterial proteins that to some extent can mask or alter some of the mucin properties. Sixth, only a few effective inhibitors of O-glycan biosynthesis are available (e.g., Hang et al., 2004; Patsos et al., 2005). Altogether the above difficulties have prevented the mucin field to advance at the same pace than related fields (e.g., proteoglycans). From the technical point of view, the use of recombinant mucins overcomes some of the inconveniences related to the large sizes, extent of glycosylation, and molecular polydispersity of native mucins.

Recombinant mucins as structural model systems. The assumptions

Any recombinant mucin project first assumes that the overall properties of native mucins are the sum of the properties of their protein domains. Accordingly, the study of an isolated (recombinant) domain should provide truthful information on its function in the native mucin. Many of the protein domains present in mucins are also found in other, functionally diverse, proteins (Perez-Vilar and Hill, 1999). In a few cases it has been demonstrated that a single domain has a similar structural function in two or more proteins. For instance, the CK-domain found in mucins, TGF-B1, von Willebrand factor (VWF) and norrin, among others, is involved in the formation of disulfide-linked species, mainly dimers, of the corresponding proteins (e.g., Sun and Davies, 1995; Perez-Vilar and Hill, 1997, 1999). However, this is not always the case. For example, the D-domains in mucins and von-Willebrand factor are involved in formation of interchain disulfide bonds while the D-domains in α -tectorins are not (Kevin Legan et al., 1997; Sadler, 1998; Perez-Vilar, 1999). In other

instances, a protein domain may not have a known function, even when present in different proteins, although clues of its function might be obtained from analysis of its primary sequence. Perhaps the best case of this type is the CS domain, which has a single conserved C-mannosylation acceptor site (WXXW) (Spiro, 2002) that is likely C-mannosylated (Perez-Vilar et al., 2004), and accordingly could be expected to be involved in lectin-type interactions.

The second assumption is also originated from another technical limitation, i. e., the low number of physiologically relevant cellular systems to express and study recombinant mucins. Mucins are synthesized in and secreted from mucous/goblet cells, a highlyspecialized cell type that is usually found inter-dispersed among other cell types in epithelial tissues. In appropriate primary cultures (for instance, human bronchial epithelial cells; e.g., Perez-Vilar et al., 2003), mucous cells are scarce and resilient to transformation/transfection and, hence, are at present of limited value for expression studies. Indeed, the biochemistry of recombinant mucins is often studied in non-native permanent cell lines under the primary assumption that their posttranslational modifications will be similar to those of the native mucins. The fact that all modifications described in mucins (e.g., N- and Oglycosylation, C-mannosylation, sulfation, disulfide bond formation, proteolysis, etc) are widespread posttranslational protein modifications not restricted to certain cell types (Spiro, 2002) lends support to this assumption. Whether gel-forming mucins have other specific post-translational modifications is yet to be determined. Although there is always the possibility that common post-translational modifications are differentially regulated in mucous/goblet cells, the current evidences suggest that this is not the case. For instance, the CK domain of pMuc19/PSM, the porcine counterpart of MUC19 (Chen et al., 2004), expressed in mammalian cells, including non-mucin- and mucinproducing cell lines, insect or yeast cells, is secreted as a mixture of unglycosylated and N-glycosylated disulfidelinked dimers (Perez-Vilar and Hill, 1998a, Perez-Vilar et al., 2005a; Perez-Vilar, unpublished observations). However, when the same domain is expressed in bacteria, only high molecular weight disulfide-linked, likely unfolded, aggregates are observed (Perez-Vilar, unpublished observations). These results suggest that the mechanism operating during the folding and dimerization of the mucin CK domains is the same functioning throughout the folding and disulfide bonding of other secretory proteins in yeast, or different mammalian cell types. It is reasonable to expect that this is also the case for other post-translational modifications in mucins. Conversely, the study of certain properties of mucins, like those related with mucin sorting, intragranular packaging or regulated secretion, for instance, necessarily requires expression of recombinant mucins in cell lines with regulated secretory pathways.

Gel-forming mucin covalent assembly

The major steps

By the early 1980s, it was increasingly clear that gelforming mucin polypeptides had non-glycosylated, or "bare", protein domains/regions besides the prominent Ser/Thr-rich glycosylated ones, and that the former were very likely linked by disulfide bonds to form larger molecules (e.g., Roberts, 1976; Allen, 1978; Boat and Cheng, 1980; Silberberg and Meyer, 1982; Carlstedt et al., 1983; Shogren et al., 1984). Consistent with this notion, purified mucins observed under the electron microscope appeared as long linear structures that could be reduced to smaller subunits on reduction of disulfide bonds (Carlstedt et al., 1983; Rose et al., 1984). Pulsechase studies with mucin-producing cell lines and/or tissue explants showed that mucin oligomerization likely begins in the endoplasmic reticulum with the formation of disulfide-linked dimers and/or trimers and continues in the Golgi complex (e.g., Dekker et al., 1989; Dekker and Strous, 1990; Sheehan et al., 1996; Asker et al., 1998; Van Klinken et al., 1998; Sheehan et al., 2004). Questions like the degree of oligomerization and the identification of the specific domains and peptide motifs involved in the assembly process are out of the reach of these kinds of studies. These restrictions and the inherent confusion they create have been the norm over the years and can be seen in recent manuscripts dealing with mucin biosynthesis and/or assembly. Nevertheless, the research direction for overcoming these limitations became clear once molecular procedures were widely used by biologists. In their 1990 manuscript on gastric mucin covalent assembly (Dekker and Strous, 1990), Dekker and Strous stated that "The nature of the mechanism of oligomerization and of the signal for RER-to-Golgi transport can only be elucidated by cloning and manipulating the gene of this intriguing molecule".

Molecular aspects

The cloning of frog FIMB1, pMuc19/PSM and MUC2 cDNA sequences (Probst et al., 1990; Eckhardt et al., 1991; Gum et al., 1992) not only permitted to design experiments with recombinant mucins, but revealed that protein domains in mucins were also present in other proteins as well. Among these proteins, VWF, an important blood protein, was also assembled into disulfide-linked species (Sadler, 1998). Moreover, since the domains involved in VWF multimerization were present in mucin polypeptides, it was possible that the homologous domains mediated mucin oligomerization (Gum et al., 1992). This notion was initially corroborated by studies with recombinant domains of pMuc19/PSM (Perez-Vilar et al., 1996, 1998; Perez-Vilar and Hill, 1998a, 1998b), the porcine counterpart of human MUC19 (Perez-Vilar and Hill, 1998c), and later other animal and human mucins (Bell et al., 1998, 2001, 2003; Xu et al., 2000; Godl et al., 2002; Lidell et al., 2003b; Perez-Vilar et al., 2005; Lidell and Hansson, 2006). Altogether these studies provided the experimental basis for the widely-accepted current paradigm explaining the covalent assembly of gelforming mucins, including the likely roles of different protein domains, peptide motifs and/or specific amino acid residues (Perez-Vilar and Hill, 1999) (Fig. 1). Most important, these studies give a reasonable molecular basis for the studies with native mucins. The assembly is characterized by four major features that highlight the complexity associated with the cellular synthesis of large macromolecules like gel-forming mucins.

First, mucin assembly is a sequential process, which begins with the formation in the endoplasmic reticulum of interchain disulfide bonds that link the CK-domains, and ends in the acidic compartments of the Golgi complex, where additional disulfide bonds are established among the N-terminal D-domains. It is important to note that protein disulfide bonding in the endoplasmic reticulum is facilitated by the: a) luminal pH, which is slightly basic; b) ratio between reduced and oxidized glutathione; and c) presence of thiol/disulfide oxido/reductases (Wei and Hendershot, 1997; Frand et al., 2000). In contrast, formation of protein disulfide bonds in the trans-Golgi compartments is an uncommon modification that only have been described for mucins and VWF (Perez-Vilar et al., 1998; Perez-Vilar and Hill, 1998b; Sandler, 1998; Godl et al., 2002), which likely reflects the fact that: a) acidic pH does not chemically favor formation of disulfide bonds; and b) no thiol/disulfide oxido/reductases residing in the trans-Golgi have been reported. Second, formation of interdimeric disulfide bonds connecting the N-terminal Ddomains requires an acidic pH, as judged by its sensitivity to agents that increase the intraluminal pH (Perez-Vilar et al., 1998; Gold et al. 2002). Third, the CGLC motifs in the mucin D1 and D3 N-terminal domains are required for inter-dimeric disulfide bonding (Perez-Vilar and Hill, 1998b). CXXC motifs provide the critical residues in the catalytic center of thiol/disulfide oxido/reductases (Noiva, 1994). Fourth, formation of disulfide bonds among the mucin D-domains takes place when purified recombinant mucins comprising the Nterminal D-domains are incubated in slightly acidic buffers (Perez-Vilar and Boucher, 2004a), consistent with similar studies with purified VWF (Mayadas and Wagner, 1989). Altogether these properties support the notion that mucin oligomerization/multimerization in the trans-Golgi compartments is a pH-dependent, selfcatalyzed mechanism, in which the CGLC motifs at the D1 domain provide the necessary enzymatic activity (Perez-Vilar and Hill, 1998b). Moreover, inside the endoplasmic reticulum lumen, i.e., at neutral pH, the CGLC sequence at the D3 domain will prevent formation of interchain disulfide bonds, as judged by mutagenesis studies (Perez-Vilar and Hill, 1998b). Thus, the mucin N-terminal D-domains, and the corresponding domains in other macromolecules, would have CXXC-

dependent thiol/disulfide oxido/reductase activities like protein disulfide isomerases, thioredoxins, etc. In particular, the D-domains would reduce interchain disulfide bonds at neutral pH and catalyze their formation at acidic pH. This notion implies that formation of disulfide-linked mucin oligomers/ multimers in the endoplasmic reticulum is actively prevented by goblet/mucous cells, and other cells specialized in the secretion of CXXC-containing macromolecules.

There are two reasonable explanations for the evolutionary emergence and conservation of this complex mechanism of multimerization. First, in vitro studies with recombinant pMuc19/PSM suggest that mucin oligomers assembled in the endoplasmic reticulum would not be efficiently transported out of this organelle because of their large sizes (Perez-Vilar and Hill, 1998b). Second, disulfide-linked multimers, rather than monomers or dimers, of gel-forming mucins could be the more biologically active forms (see below), as is evident by the number of mutations in patients with von Willebrand disease that alter VWF covalent assembly mechanism (Sandler, 1998). Hence, in evolutionary terms, by acquiring the capability to self-reduce/oxidize disulfide/thiol groups, the N-terminal D-domains in mucins have made possible an efficient flow of mucin precursors and the formation of large oligomers/ multimers in a chemically unfavorable, but distal, compartment.

Biologic significance

As judged by the degree of conservancy of the key peptide motifs, the assembly mechanism of mucins is extremely well conserved among vertebrates. Moreover, the length of the mucin strands is critical for mucus viscoelastic properties, as the degree of entanglement and the viscosity of random coil polymers like gelforming mucins is directly proportional to their lengths (Doi and See, 1995). In fact, the viscosity of mucin solutions and mucus can be efficiently diminished with disulfide reducing agents, or protease digestion (Bansil et al., 1995). Hence, it is reasonable to presume that the degree of mucin covalent oligomerization is critical for the formation of a protective mucus blanket. Recent studies have shown that cysteine proteases of Entamoeba histolytica, an intestinal protozoan parasite, cleave the C-terminal region of MUC2 at two sites (Lidell et al., 2006). Cutting in one of these sites, which is located in the D4 domain, predicts the fragmentation of mucin oligomers into smaller chains, containing only O-glycosylated polypeptides linked by the N-terminal side, i.e., lacking continuity through their respective Cterminal CK-domains. Thus, this parasite appears to traverse the intestinal mucus protective barrier by fragmenting the mucin oligomeric strands, which ultimately reduces the degree of entanglements of the mucus network and its viscosity. Of course, as any parasite-host pair relationship, fixation of certain mutations during evolution can restore the lost function, as attested by the fact that mMuc2, the mouse counterpart of MUC2, does not have the cleavage site (Lidell et al., 2006), which would explains that rodents are resistant to infections by this protozoan.

Gel-forming mucin intragranular packaging. The organization of the granule lumen

Once mucin disulfide-linked oligomers are assembled in the trans-Golgi compartments, they are sorted and accumulated into mucin granules. The molecular and cellular aspects of this process are not fully understood (Perez-Vilar, 2006). This is a fascinating research area for biochemists for two main reasons. First, gel-forming mucins form rather linear oligometric strands with contour sizes of 16 µm or more, which in view of the average diameter of the mucin granule ($\sim 1 \mu m$), suggests intragranular mucins must be highly condensed. Second, mucins are highly hydrophilic polyanionic macromolecules, meaning that any condensation, or accumulation process, must explain the tendency of mucins to absorb large amounts of water. That gel-forming mucins are the major component of the mucin granule matrix (Velcich et al., 2002) suggest that sorting and matrix assembly are equivalent processes in contraposition to other regulated secretory granules in which the secretory product and the granule matrix are different (Burgoyne and Morgan, 2003).

The core matrix paradigm

Macromolecules like mucins, which behave in aqueous solutions like polyanionic random coils (Verdugo, 1990; Bensil et al., 1995), can be simplified and reasonable understood by looking into basic principles of polymer physic theory. This was first exploited by Verdugo to explain mucin intragranular packaging on the basis of studies that measured the degree of expansion of the mucin matrix on exocytosis (Verdugo, 1984, 1990, 1991; Espinosa et al., 2002; Kuver et al., 2006). This model suggests that mucin oligomers form an entangled polymeric network, i. e., a gel, that at some point during granule formation undergoes a pH/Ca²⁺-dependent volume phase transition. Similar to other regulated secretory granules, the mucin granules have high intraluminal $[Ca^{2+}]$ and [H⁺] (Kuver et al., 2000; Chin et al., 2002). This volume transition results in the formation of a condensed granule core matrix comprising entangled mucin strands stabilized by Ca²⁺. Secretory proteins (e.g., trefoil factors, defensins, etc) are entrapped within the collapsed immobile mucin matrix. Mucins in the matrix core would have just a hydration shell that would not allow diffusion of ions and proteins. Additional studies have shown that the mucin core is likely surrounded by a fluid with free Ca²⁺, which can be released from the granule by diffusion during signal transduction (Nguyen et al., 1998). Because secretory products entrapped by

the immobile matrix cannot diffuse they are osmotically inert and, accordingly, intragranular concentrations of these proteins are very high without resulting in granule swelling.

It is noteworthy to mention here that volume transitions have been thoroughly characterized in synthetic polymer gels, and are presumed to occur in all gels, irrespective of their chemical nature (Li and Tanaka, 1992). Thus, small changes in temperature, ionic concentrations, electric field, etc are able to cause a total collapse (or expansion if the gel is already collapsed) of the polymer network. In the case of the granule lumen, increased [Ca²⁺] and [H⁺] would be the triggering factors. These ions would bind to sialic and sulfate groups in mucin O-glycans, promoting strand cross-linking and further entanglement (Verdugo, 1990). Though numerous studies suggest that mucins aggregate in the presence of high [Ca²⁺] and/or [H⁺] (e. g., McCullagh et al., 1995; Cao et al., 1999), compelling evidence showing total mucin collapse is lacking. Therefore, either intragranular packaging takes place through another mechanism, or other intragranular compounds drastically increase the inter-chain affinity of the highly-hydrophilic mucin oligomers. In this respect, it has been suggested that intragranular lipids could have such role (Verdugo, 1990).

The meshwork matrix paradigm

To better understand the organization of the mucin granule and to directly test the core matrix paradigm, we generated a mucous/goblet cell system, named HT29SHGMUC5AC/CK cells, consisting of HT29-18N2 cells (Phillips et al., 1988), a human colon, mucin-producing adenocarcinoma cell line, permanently expressing a fusion protein between EGFP (Enhanced Green Fluorescent Protein) and the CK-domain of MUC5AC (Perez-Vilar et al., 2005a). When seeded and maintained in a defined serum-free culture medium, up to $\sim 30\%$ of the HT29-SHGMU5AC/CK cells in the cultures differentiated into mucous/goblet cells within two weeks, as attested by histochemical, immunocytochemical and ultrastructural analyses. The fusion protein (SHGFP-MUC5AC/CK) co-localized with the native MUC5AC in the mucin granules where they could be detected with specific antibodies, although covalent linkage to the endogenous mucin could not be demonstrated (Perez-Vilar et al., 2005a). Consistent with these observations, in living cells the fusion protein mainly resided in vesicular structures with diameters (0.5-2.0 mm) similar to the mucin granules observed in chemically-fixed cells, and that occupied most of the cytoplasm (Fig. 2)(Perez-Vilar et al., 2005a). Endoplasmic reticulum, endosome/lysosomes or Golgi complex fluorescent markers did not co-localized with the SHGFP-MUC5AC/CK-containing granules in living or chemically fixed cells (Perez-Vilar et al., 2005b). Moreover, the fluorescent protein in the granules was discharge by incubating the cells in the presence of ATP, a well-established mucin secretagogue, although only a fraction of the mucous cells and often their granules were responsive (Perez-Vilar et al., 2006). Further, inhibition of protein synthesis did not diminish the intragranular SHGFP-MUC5AC/CK fluorescence,

Fig. 2. HT29-SHGFP-MUC5AC/CK cells. A. XY confocal images of live differentiated HT29-SHGFP-MUC5AC/CK cells observed with a laser confocal microscope at 37°C on the stage using the 488 nm laser excitation. Scale bar: 5 µm. B. XY confocal image of a single live mucous cells with numerous granules containing fluorescent fusion protein. Scale bar: 3 µm. (Reproduced from Perez-Vilar et al., 2005a).



showing its long term accumulation, as expected for a regulated secretory product. These results indicated that these cells are a suitable model system to study goblet cells and their mucin granules.

The availability of this novel mucous cell model system made possible a direct experimental testing of the core matrix paradigm using FRAP (Fluorescent Recovery After Photobleaching) methods. In a typical GFP-based FRAP experiment, a subcelular area of interest is bleached at high laser power and the recovery of the fluorescence, i. e., the diffusion of the nonbleached molecules inside the bleached area followed over time (Lippincott-Schwartz and Patterson, 2003). Important biophysical information can be derived from the fluorescence recovery curves regarding the environment in which the tracer is diffusing, and indirectly on its organization. In our samples, the equatorial plane of the largest mucin granules in live mucous cells were imaged with a laser confocal microscope and a circular spot with the diameter of the laser beam was irreversibly bleached (Perez-Vilar et al., 2005a). The bleached spot and the subsequent recovery of the fluorescence in the bleached region could be clearly visualized in all granules analyzed. Most important, the fluorescent signal recovered in the bleach area, although not completely (Fig. 3). These results showed that: a) the fusion protein was able to diffuse, though very slowly (~0.001 µm²s-¹; Perez-Vilar et al., 2005a); and b) a significant proportion of intragranular SHGFP-MUC5AC/CK was immobilized during the time scale of the FRAP analysis. It can be concluded that the

granule lumen comprises, rather than a non-penetrable mucin matrix core, a matrix meshwork, where proteins are able to diffuse and to interact with the meshwork components. The slow diffusion of the protein in the granule lumen in comparison to its diffusion in the endoplasmic reticulum lumen or the extracellular medium (Perez-Vilar et al., 2005a), suggest that the intragranular matrix is partially condensed though. Hence, a pH/Ca²⁺-dependent volume phase transitionlike mechanism of mucin strands to form the meshwork was possible.

As expected for a matrix meshwork model, subsequent studies showed that alterations of intralumenal pH or modifications pertinent to mucins, including O-glycosylation, sialylation, sulfation or disulfide-bonding, did alter the protein tracer diffusion and/or mobilization in the matrix (Perez-Vilar et al., 2006). The emerging notion from these studies is that the mucin granule lumen is organized in a pH-dependent, partially condensed, mucin meshwork embedded in a fluid phase (Perez-Vilar, 2006). Because reduction of intragranular protein disulfide bonds did not disorganize the granule, the matrix meshwork is likely maintained at acidic pH by inter-strand interactions mediated by Ca²⁺ and the sialic and sulfate rests in mucin O-glycans (Perez-Vilar et al., 2006). The degree of mucin sulfation and sialylation, and also the length of the O-linked oligosaccharides in the matrix, determine the mobility of secretory proteins through the meshwork pores. Secretory proteins can also bind the meshwork, which has protein- and O-glycan-rich regions. Mucin-type O-



Fig. 3. FRAP analysis of mucin granules in live mucous cells. **A.** Representative FRAP analysis involving bleaching of a circular spot in a mucin granule of a live mucous/goblet cell. Shown are pre-bleaching (left), immediately post-bleaching (center), and 24-s post-bleaching (right) images at two different magnifications. A low magnification of the cell is also shown (right cell in the inset). Scale bars: 0.5 or 1 μm (inset). **B.** Averaged (mean ± S.E.) normalized bleaching/recovery curve for 21 granules in 21 mucous/goblet cells analyzed as described for A. Error bars indicate S.E. (Reproduced from Perez-Vilar et al., 2005a).

glycans in the latter limit the interactions of secretory proteins with the protein-rich regions (Perez-Vilar et al., 2006). The meshwork model clearly points toward a more sophisticated assembly mechanism during the early stages of mucin granule biogenesis prior inter-strands cross-links via Ca^{2+} are definitely formed. Perhaps the multi-modular nature of gel-forming mucins might hold the explanation for such a mechanism. In any case, these and other important questions, like the recruitment of membrane and luminal proteins, are currently under investigation.

Conclusions and future directions

Molecular biology in general and expression studies in particular have brought to light the similarities and differences within the gel-forming mucin family members. Above all, these studies have shown us a structural complexity well beyond the simple definition of mucins as mucoproteins rich in carbohydrates that can be stained with cationic dyes or the acid-Schiff reagent. Indeed, nowadays mucin research has wide open implications for our understanding of the structure function relationship of large multi-modular proteins, the topological problems that cells must face to synthesize and process them, and the biochemical constrains that prevents the structural diversification of certain protein domains. Most important, we are starting to provide direct proof for the roles of mucins in specific diseases or, at the very least, anticipate their roles with certain confidence (e.g., Perez-Vilar and Boucher, 2004b). Although, we have reviewed here two research areas, mucin assembly and intragranular packaging, in which recombinant approaches have been particularly fruitful, similar strategies are beginning to provide significant and novel information on mucin proteolytical processing (Xu et al., 2000; Lidell et al., 2003a; Lidell and Hansson, 2006), and mucin O-glycosylation (e.g., Silverman et al., 2002, 2003; Parry et al., 2005) and its status in obstructive lung diseases (Leir et al., 2005). Moreover, it seems reasonable to foresee that in vitro studies in combination with functional genomic and proteomic approaches will be the source of the next wave of novel information on mucins, their mechanisms of secretion and their roles in health and disease.

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