pH-dependent Intraluminal Organization of Mucin Granules in Live Human Mucous/Goblet Cells*

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To study the mechanism of gel-forming mucin packaging within mucin granules, we generated human mucous/goblet cells stably expressing a recombinant MUC5AC domain fused to green fluorescent protein (GFP). The fusion protein, named SHGFP-MUC5AC/CK, accumulated in the granules together with native MUC5AC. Inhibition of protein synthesis or disorganization of the Golgi complex did not result in diminished intragranular SHGFP-MUC5AC/CK signals, consistent with long-term storage of the fusion protein. However, SHGFP-MUC5AC/CK was rapidly discharged from the granules upon incubation of the cells with ATP, an established mucin secretagogue. Several criteria indicated that SHGFP-MUC5AC/CK was not covalently linked to endogenous MUC5AC. Analysis of fluorescence recovery after photobleaching suggested that the intragranular SHGFP-MUC5AC/CK mobile fraction and mobility were significantly lower than in the endoplasmic reticulum lumen. Incubation of the cells with bafilomycin A1, a specific inhibitor of the vacuolar H+-ATPase, did not alter the fusion protein mobility, although it significantly increased (~20%) the intragranular SHGFP-MUC5AC/CK mobile fraction. In addition, the granules in bafilomycin- incubated cells typically exhibited a heterogeneous intraluminal distribution of the fluorescent fusion protein. These results are consistent with a model of mucin granule intraluminal organization with two phases: a mobile phase in which secretory proteins diffuse as in the endoplasmic reticulum lumen but at a lower rate and an immobile phase or matrix in which proteins are immobilized by noncovalent pH-dependent interactions. An intraluminal acidic pH, maintained by the vacuolar H+-ATPase, is one of the critical factors for secretory protein binding to the immobile phase and also for its organization.

The mucous blanket that covers the gastrointestinal, tracheobronchial, urogenital, auditory, and conjunctiva epithelia and also the gills of fishes and the epidermis of amphibians lubricates these mucosas and creates a protective barrier against pathogenic and noxious agents (1). This layer is a component of the mucosal innate host response that is regulated in response to inflammation and infection (2, 3). Conversely, overproduction of mucus can be detrimental to health, as is evident in diseases characterized by a mucous hypersecretory phenotype such as cystic fibrosis (4). Proteins, glycoproteins, and phospholipids are an integral part of mucus, but current evidence suggests that gel-forming mucins, together with water and salts, largely determine the viscoelastic and adhesive properties of mucus.

Only five different but structurally related gel-forming mucins (MUC2, MUC5B, MUC5AC, MUC6, and MUC19) are known in humans (5–12). These glycoproteins are synthesized in and secreted from specialized cells known as mucous/goblet cells. In the endoplasmic reticulum, mucin precursors are N-glycosylated and likely C-mannosylated (6, 13) and form disulfide-linked dimers (14–17). In the different compartments of the Golgi complex, mucin dimers are O-glycosylated and sulfated (6). Once they reach the acidic trans-Golgi compartments, mucins are assembled into large covalent disulfide-linked oligomers/multimers (6, 18, 19). Certain mucins are proteolyzed to some extent in the latter compartments (20, 21).

Fully processed mucins are stored in large secretory vesicles known as mucin granules, which occupy the majority of the cytoplasm in mucus/goblet cells (22, 23). A combination of mucin granules released at a very low rate and/or small secretory vesicles departing from the trans-Golgi compartments might mediate constitutive mucin secretion (23, 24). In addition, Ca2+-regulated discharge of mucin granules can be rapidly triggered by a wide variety of physiological agents, including cytokines/chemokines, bacterial exoproducts, nucleotides, neurotransmitters, and proteases (25). Regulated secretion guarantees a high supply of mucins in a minimal period of time, e.g. in response to acute microbial infection or the sudden presence of noxious chemicals. An efficient mechanism of mucin packing within secretory granules is critical to attain this function.

The mechanism of protein concentration in secretory granules has attracted great attention over the years (26). It has been proposed that secretory products in granules form insoluble aggregates and/or are entrapped in a condensed matrix that fully constrains their mobility and ultimately permits high concentrations of the product to be attained (27, 28). In the case of mucin granules, the matrix is postulated to be constituted by condensed mucin multimers (29), which are also the major secretory product. In other cell types, however, the major secretory products might co-aggregate with intraluminal matrix proteins.

Morphological evidences suggest that, upon fusion of the granule with the plasma membrane, the mucin matrix undergoes massive swelling with the discharge of accompanying secretory products (30). Because mucin matrix swelling can be prevented by increasing the extracellular Ca2+ concentration and decreasing its pH, it was proposed that these two factors are critical for...
mucin intragranular condensation (29, 30). Consistent with this notion, electron microscopy microanalysis suggests the existence of a high concentration of Ca\textsuperscript{2+} in the mucin granules, whereas in vivo measurements with pH-dependent fluorescent dyes suggest an acidic intraluminal environment (31–33). Alternatively, an ion exchange-triggered phase transition from a dehydrated/condensed mucin matrix to fully hydrated mucus might explain the experimental observations (30).

The large size, polymeric nature, high charge density, and hydrodynamic radii of mucins suggest the existence of a complex mechanism of intragranular accumulation. These macromolecules have multiple specific domains and post-translational modifications (6). It is likely that they are critical for mucin condensation and play specific roles in the overall process. As a first step to study these issues, we modified an existing human colon mucous/goblet cell line to stably express a recombinant mucin domain fused to green fluorescent protein (GFP).\textsuperscript{1} We report the characterization of this system and the analysis of mucin granule lumen by photobleaching techniques in live cells. The results strongly suggest that the mucin granule lumen comprises two components: a pH-dependent immobile phase and a fluid/mobile phase. The biological significance of granule compartmentalization is discussed.

EXPERIMENTAL PROCEDURES

Cloning of MUC5AC and MUC5B CK Domains, Construction of Plasmid Expression Vectors, and Murine Leukemia Virus Vector Production—\textsuperscript{1}cDNAs encoding MUC5AC and MUC5B CK (cystine-knot) subdomains were generated by PCR using human airway cDNA as template and oligonucleotide pairs (MGW Biotech Inc.) based on their reported sequences (GenBank U26255/EBI accession numbers AJ001402 and XM695877). Airway cDNA was prepared with SuperScript II RNase H reverse transcriptase (PerkinElmer Life Sciences) from RNA purified from well differentiated human primary airway epithelial cells as described (34). The amplified mucin cDNA fragments were initially cloned in the vector pCR2.1TOPO (Invitrogen) and then subcloned into pSH-GFP, a plasmid that encodes a His-tagged secreted form of GFP (13), to create plasmids pSHGF-MUC5AC/CK and pSHGF-MUC5B/CK. These plasmids encode fusion proteins (denoted SHGF-MUC5AC/CK and SHGF-MUC5B/CK, respectively) consisting of an N-terminal signal peptide sequence, the poliovirus internal ribosome entry site sequence, and the first 340 residues of the CK domain. A schematic structure of the protein encoded by pSH-GFP-MUC5AC/CK is shown in Fig. 1.

To visualize the ultrastructure of cells under an electron microscope, cells were briefly washed with PBS containing calcium and magnesium (1 mM each) and fixed in 2% (w/v) freshly prepared glutaraldehyde in 0.1 M Na\textsubscript{2}PO\textsubscript{4} (pH 7.4). Cells were deparaffinized with xylene; rehydrated; and stained with rabbit polyclonal antibody against GFP (Abcam) following standard procedures, including antigen retrieval with citric acid, sequential incubations with appropriate goat biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.), horseradish peroxidase (HRP)-conjugated streptavidin, and the HRP substrate diaminobenzidine and H\textsubscript{2}O\textsubscript{2}. Sections were counterstained with 1% (w/v) methyl green in methanol, which stained nuclei and mucin granules in mucous/goblet cells, likely due to their polyanionic character. Control sections were processed with normal anti-rabbit or anti-mouse IgG.

In other experiments, cells were briefly washed with PBS containing calcium and magnesium (1 mM each) and fixed in 2% (w/v) freshly prepared paraformaldehyde in PBS for 20 min on ice. Following a 10-min incubation in 0.1% (v/v) Triton X-100 in PBS and an overnight incubation at 4 °C in PBS containing 0.1% (w/v) bovine serum albumin, the cells were sequentially incubated at room temperature with primary and secondary antibodies (30 min each, both diluted in PBS containing 0.1% (w/v) bovine serum albumin). After several washes with PBS, cells were visualized under a confocal microscope within 3 h. Mouse anti-MUC5AC monoclonal antibody 45M1 (40) was obtained from Lab Vision Corp. The epitope recognized by the anti-MUC5AC monoclonal antibody is not located in the CK domain, and therefore, this antibody detects endogenous MUC5AC, but not SHGF-MUC5AC/CK.

To visualize the ultrastructure of cells under an electron microscope, cells were briefly washed in 1% (v/v) osmium tetroxide in phosphate-buffered saline, dehydrated in 100% ethanol, and embedded in Epon resin following published procedures (41). Sections (90 nm thick) were selected for evaluation.

Live Cell Imaging—Cells in CO\textsubscript{2}-deficient medium plus 1% (v/v) fetal bovine serum, 5 mM l-glutamine, and 20 mM HEPE\textsubscript{S} (pH 7.8) (see Figs. 2–5) or in Hanks’ balanced salt solution plus 1% fetal bovine serum, 5 mM l-glutamine, essential and nonessential amino acids, and 20 mM HEPE\textsubscript{S} (pH 7.8) were observed using a Zeiss LSM 510 laser scanning microscope.
confocal microscope (University of North Carolina M. Hooker Microscopy Facility) at 37 °C on the microscope stage using 488-nm laser excitation for GFP.

**Analysis of Fluorescence Recovery after Photobleaching (FRAP)—** Live cells were imaged at 37 °C on the temperature-controlled stage of the Zeiss LSM 510 laser scanning confocal microscope using the 488-nm line of a 30-milliwatt argon laser and an oil immersion objective (×63, 1.4 numerical aperture). SHGFP-MUC5AC/CK fluorescence in a defined region of the mucin granule or the endoplasmic reticulum was photobleached for seven iterations for a total time period of ~0.07 s at 80% laser power/100% transmission. In the case of the granules, the bleached region was a circular spot with the width of the laser beam (i.e. radius (r) of ~0.26 μm). The fluorescence intensity (F) in the bleached area was monitored as a function of time after bleaching by scanning at 80% laser power/0.5% transmission every 0.3–0.7 s. As judged by bleaching entire granules, SHGFP-MUC5AC/CK photobleaching under these conditions was irreversible. Data sets with incomplete recoveries or in which the focal plane shifted were discarded by visual inspection of the recorded images. To assess differences in SHGFP-MUC5AC/CK mobility in the mucin granules under different conditions, we determined the characteristic diffusion times or recovery half-times ({t_θ}), i.e. the time required for F to recover to 50% of the plateau fluorescence intensity (#F_p#, from the recovery curves of normalized (fractional) fluorescence intensities versus correction times (42)). Thus, the data were fitted to a one-phase exponential curve: \( F(t) = F_0 \times (1 - e^{-t/t_\theta}) \) and \( t_\theta = \ln(2)/k \), where k is the rate constant. Although the differences in \( t_\theta \) obtained were minimal, an exponential fitted the data better than a hyperbolic equation or the expression of Feder et al. (43).

Differences in SHGFP-MUC5AC/CK mobility between the granule and endoplasmic reticulum were assessed by determining the apparent or effective diffusion coefficients (\( D_{app} \)) (40), defined as the mean squared displacement of SHGFP-MUC5AC/CK over time. \( D_{app} \) integrates the absolute diffusion coefficient, which is largely due to the viscosity of the compartment, and effects caused by interactions and collisions with other proteins components. Because these organelles have very different geometries, they did not comply with the assumptions of a single other proteins components. Because these organelles have very different geometries, they did not comply with the assumptions of a single

**RESULTS**

The CK Domains in MUC5AC and MUC5B Form Disulfide-linked Homodimers—To visualize mucin granules in live cells, we employed GFP technology in HT29–18N2 cells, a human colon cell line known to differentiate into mucous/goblet cells when grown in a defined serum-free culture medium (37). A priori, a fusion protein between GFP and a protein domain in the mucin polypeptide had a reasonable chance to interact with endogenous mucin and to be transported eventually into the mucin granule. We chose the CK-like domain because it is a short domain (~100 amino acid residues) present at the C termini of all human and animal gel-forming mucins known (6, 50). Expression studies have suggested that the CK domains in porcine submaxillary mucin (porcine MUC19) (14, 15), rat MUC2 (16), and MUC2 (17) are involved in the formation of disulfide-linked dimers early in the process of mucin multimerization (6). The high degree of protein conservation among gel-forming mucins strongly suggested that the CK domains in MUC5AC and MUC5B are also dimerization domains. To test this possibility, COS-7 cells were transfected with expression plasmids encoding fusion proteins containing the signal peptide of the murine Ig µ-chain, followed by six consecutive histidine residues and the MUC5AC or MUC5B CK domain.

In this study, control cells were transfected with the parental vector pSecTag2A, which lacks mucin-coding sequences. 24 h after transfection, the cells were incubated for 4 h with [35S]cysteine/methionine, and the mucin CK domains were isolated from the medium by adsorption to and elution from a metal affinity adsorbent. The eluates were analyzed by reducing SDS gel electrophoresis and autoradiography (Fig. 2). Although no specific proteins were detected in the medium from untransfected cells (lane 1) or cells transfected with the parental plasmid (lane 2), two major and closely migrating protein bands were detected in the medium of cells expressing MUC5AC/CK (lane 3) or MUC5B/CK (lane 4). A third, less abundant protein with a higher electrophoretic mobility was judged to be a proteolyzed CK domain based on its apparent molecular mass. In both cases, the smaller of the two major purified proteins had a molecular mass of ~20 kDa, which was consistent with the expected molecular mass deduced from the corresponding cDNA sequences of SHGFP-MUC5AC/CK and SHGFP-MUC5B/CK. The larger proteins were judged to correspond to N-glycosylated species based on their sensitivity to tunicamycin, an inhibitor of protein N-glycosylation, which allowed detection of only the smaller species (data not shown). The respective CK domains in MUC5AC and MUC5B have a conserved Asn-X-(Ser/Thr) peptide motif. It is therefore not surprising that they were secreted from COS-7 cells as a mix-

**Fig. 2. Expression and secretion of the MUC5AC and MUC5B CK domains.** Untransfected COS-7 cells (lanes 1 and 5) and cells transfected with the expression plasmids pSecTag2 (lanes 2 and 6), pMUC5AC/CK (lanes 3 and 7), and pMUC5B/CK (lanes 4 and 8) were metabolically labeled with [35S]-labeled amino acids 24 h post-transfection. Proteins were purified from the culture medium with TALON-IMAC (idented methyl affinity chromatography) beads and separated by reducing SDS gel electrophoresis with (lanes 1–4) or without (lanes 5–8) prior reduction of the proteins with 2-mercaptoethanol. The gels were dried, and bands were detected by fluorography. The molecular weights (MW) of the standards are in thousands.
ture of unglycosylated and N-glycosylated species. Previous studies have demonstrated N-glycosylation of the CK domain in porcine MUC19/porcine submaxillary mucin (14, 15). Overexpression of the domains likely indicated that a significant portion of the recombinant proteins were not N-glycosylated, as reported in previous experiments with recombinant porcine MUC19/porcine submaxillary mucin (14).

Under nonreducing conditions, no bands were observed in the medium of untransfected cells (Fig. 2, lane 5) or cells transfected with the parental plasmid (lane 6), whereas a broad band centered at ∼40 kDa was purified from the medium of cells expressing MUC5AC/CK (lane 7) or MUC5B/CK (lane 8), indicating that these recombinant proteins formed disulfide-linked dimers. These results suggest that the MUC5AC and MUC5B CK domains, like the corresponding domains in porcine MUC19/porcine submaxillary mucin (15), rat MUC2 (16), and MUC2 (17), are N-glycosylated domains involved in the formation of disulfide-linked dimers during the assembly of the multimeric species of these mucins (6). The experiments in this study were focused on the CK domain of MUC5AC, and studies on the MUC5B CK domain are currently in progress.

SHGFP-MUC5AC/CK Is Expressed and Co-localized with Native Mucins in Mucous/Goblet Cells—pSHGFP-MUC5AC/CK directs the synthesis of a fusion protein between GFP and the MUC5AC CK domain with a signal peptide and six histidines at the N terminus. C-terminal (rather than N-terminal) GFP fusion proteins were used for two major reasons. First, the CK domain is found only at the C termini of mucins and other proteins (6). Second, studies by Forstner and co-workers (51) suggest that the most C-terminal amino acid residues of the rat MUC2 CK domain are important for dimerization. When expressed in non-mucin-producing cell lines (including COS-7, Chinese hamster ovary, Madin-Darby canine kidney, and 3T3) or in undifferentiated HT29–18N2 cells stably expressing SHGFP-MUC5AC/CK (HT29-SHGFP-MUC5AC/CK cells), SHGFP-MUC5AC/CK was predominantly distributed in a perinuclear region and in a reticular network coming off the nuclear envelope, characteristic of the Golgi complex and the endoplasmic reticulum, respectively. Consistent with this conclusion, a fusion protein between red fluorescent protein and the galactosyltransferase trans-Golgi retention signal co-localized with SHGFP-MUC5AC/CK in the perinuclear region. Preliminary studies indicated that, in non-mucin-producing cells, SHGFP-MUC5AC/CK had an intracellular distribution typical of a secreted protein synthesized in the endoplasmic reticulum and transported through the Golgi complex prior to its secretion. Indeed, SHGFP-MUC5AC/CK could be detected in the culture medium of transfected cells by Western blotting with anti-His antibody or after TALON-IMAC (identvalidated methal affinity chromatography) absorption of 35S-labeled proteins secreted in the medium (data not shown).

HT29–18N2 is a human mucin-secreting colon adenocarcinoma cell line derived from HT29 cells that exhibits an undifferentiated phenotype in the presence of serum. However, when these cells are cultured at high cell densities in a defined serum-free medium, a fraction of the cells undergo a differentiation process into goblet-like cells within the next 1–2 weeks (37). These cells synthesize MUC2 and MUC5AC, which are found in large mucin granules. We generated HT29–18N2 cells stably expressing SHGFP-MUC5AC/CK by retroviral transduction and subsequent selection of positive cells in selective culture medium. Both polyclonal and monoclonal cell lines expressing the recombinant mucin were screened for GFP expression, isolated, and expanded. With respect to the experiments presented in this study, polyclonal and monoclonal cells produced the same results.

HT29-SHGFP-MUC5AC/CK cells chemically fixed and processed for electron microscopy revealed that mucous cells were not present after 3 days in serum-free medium (Fig. 3A, panel a), whereas they were abundant 8 days later (panel b). These cells characteristically appeared with much of their cytoplasm filled with large granules in one pole and the nucleus in the opposite side (panel b; also see below). Mucous cells also exhibited the characteristic goblet morphology on hematoxylin/eosin-stained sections of cells previously embedded in paraffin.

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2 J. Perez-Vilar, manuscript in preparation.
ular, Golgi complex, or endosomes/lysosomes. Mucin granules did not co-localize with markers for the endoplasmic reticulum. These granules were investigated. It was evident that, in live cells, mucin granules and from culture to culture, but values of up to 30% were not constant with these observations, live cell imaging of differentiated HT29-SHGFP-MUC5AC/CK cells showed that SHGFP-MUC5AC/CK was found in the mucin granules. Consistent with this conclusion, expression of MUC5B, MUC2, and TFF3 (trefoil factor-3) was detected by reverse transcription-PCR (data not shown).

**SHGFP-MUC5AC/CK Is Preferentially Located in Mucous Granules of Live Mucous/Goblet Cells**—The cytochemical characterization of chemically fixed cells strongly suggested that SHGFP-MUC5AC/CK was located in the mucin granules. Consistent with these observations, live cell imaging of differentiated cultures showed that SHGFP-MUC5AC/CK was found mainly in cells with large vesicular structures (Fig. 3, A and B) that had diameters in the same range (0.5–2.0 μm) as the mucin granules observed in chemically fixed cells. These granules did not co-localize with markers for the endoplasmic reticulum, Golgi complex, or endosomes/lysosomes. Mucin granules occupied one side of the cell, whereas the nucleus was in the opposite pole (Fig. 4C), as was also evident in fixed goblet cells visualized under an electron microscope (see above). The percentages of mucous cells varied with the age of the culture and from culture to culture, but values of up to 30% were not uncommon. The underlying reason for this variability was not investigated. It was evident that, in live cells, mucin granules were separated by cytoplasmic space (Fig. 3, C and D), whereas in fixed cells, the intergranular space was often not present (Fig. 3, A, B, and E), suggesting that fixation and subsequent sample processing altered the morphology of the goblet cell, likely inducing mucin swelling and apposition. FRAP analysis also indicated that the granules were not physically connected (data not shown).

**ATP Triggers the Discharge of SHGFP-MUC5AC/CK Accumulated in Mucin Granules**—The defining feature of mucous/goblet cells is the regulated secretion of gel-forming mucins accumulated in the mucin granules. Thus, the presence of mucous secretagogues induces a rapid exocytosis of the granules and, eventually, secretion of large amounts of mucins. Consequently, it was important to establish whether the steady-state distribution of SHGFP-MUC5AC/CK in the granules reflected a true accumulation rather than a steady but transient distribution (for instance, fast processing in and transport out of the endoplasmic reticulum/Golgi complex and slower transport out of the granule). For these experiments, differentiated cells were incubated in the presence of 100 μg/ml brefeldin A, a compound that disorganizes the Golgi complex (52). As shown in Fig. 5A, inhibition of protein synthesis did not result in disappearance of SHGFP-MUC5AC/CK from the granules. Similarly, brefeldin A did not affect intraluminal SHGFP-MUC5AC/CK fluorescence, although the endoplasmic reticulum in many of these cells had expanded subregions and, in general, increased SHGFP-MUC5AC/CK fluorescence throughout the organelle (Fig. 5B). These results are consistent with long-term accumulation of SHGFP-MUC5AC/CK in the mucin granules, as expected for a regulated secretory granule.
We then determined whether HT29-SHGFP-MUC5AC/CK cells and ultimately their mucin granules are responsive to the presence of mucous secretagogues. It has been reported that mucous cells secrete mucins in response to ATP, a well-characterized purinergic mucous secretagogue (53). The optimal concentration of ATP varies among cell types, and in the case of HT29 cells, ATP in the millimolar range is optimal to rapidly induce granule exocytosis (54). Therefore, we visualized live HT29-SHGFP-MUC5AC/CK cells before and after addition of 3 mM ATP. The cells were kept at 37 °C on the confocal microscope stage in a buffered HCO₃⁻-free physiological medium and studied by time-lapse imaging. SHGFP-MUC5AC/CK fluorescence within both the granule and endoplasmic reticulum was recorded. However, to visualize the fluorescent signal in the latter organelle, the fluorescence intensity in the granules was saturated. Fig. 5C shows how a cluster of resting mucous/goblet cells with abundant fluorescent mucin granules (panel a) lost most of its fluorescent signal within 5 min of ATP addition (panel b), even when the initial intragranular fluorescent signal was saturated. Although intragranular fluorescence intensity followed inverse exponential kinetics ($R^2 = 0.99$) with a characteristic decay rate of $-0.37$ absorbance units/s, the fluorescence intensity in the endoplasmic reticulum linearly decreased with a characteristic rate of $-0.08$ absorbance units/s (panel c), which was comparable with the fluorescence decay in control cells similarly imaged. These results indicate that SHGFP-MUC5AC/CK stored in mucin granules was discharged upon ATP addition directly from the mucin granules. Consistent with this conclusion, it has been found that mucin exocytosis in HT29 cells is maximal within 5 min after ATP addition (54).

**SHGFP-MUC5AC/CK Is Not Covalently Linked to Endogenous MUC5AC**—In mucin-producing cells, extracellular gel-forming mucins can be found soluble in the culture medium or in the mucus adherent to the cell surface, likely representing freshly secreted polymeric mucins not fully hydrated. To determine whether SHGFP-MUC5AC/CK was present in the adherent MUC5AC-containing surface mucus, HT29-SHGFP-MUC5AC/CK cells grown on Transwell filters were fixed with paraformaldehyde and stained with anti-MUC5AC monoclonal antibody as described for Fig. 3D, but without prior permeabilization with Triton X-100. Fig. 6A shows $xz$ images of HT29-SHGFP-MUC5AC/CK cells demonstrating that SHGFP-MUC5AC/CK (panels a and c) and MUC5AC (panels b and c) did not co-localize in the surface mucus. Only anti-MUC5AC monoclonal antibody stained the cell-associated mucus, whereas the SHGFP-MUC5AC/CK signal was confined to the cells. This observation suggests that SHGFP-MUC5AC/CK was not covalently linked to endogenous MUC5AC, and hence, it was released into the surrounding culture medium upon secretion.

The following observations are consistent with the above conclusion. (a) Discharge of intragranular SHGFP-MUC5AC/CK upon ATP addition did not result in localization of the protein to the cell surface, but rapid disappearance into the culture medium (Fig. 5C).² (b) SHGFP-MUC5AC/CK could be visualized outside the cells only in closed intercellular spaces (Fig. 6B, panel a), known as mucous lakes (55). Mucous lakes, which very likely were formed by the secretory activity of mucous/goblet cells enclosing them (panel b), were readily identified in fixed cells visualized under an electron microscope (panel c). The “lake” mucous matrix resembled the intragranular matrix in that both were composed of a network of fibrous material (panel c, inset), likely representing mucin polymers and aggregates. Indeed, the presence of MUC5AC in mucous lakes was corroborated by immunofluorescence of fixed cells using anti-MUC5AC antibody (data not shown). Throughout

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**Fig. 5.** ATP-regulated discharge of intragranular SHGFP-MUC5AC/CK in live mucous/goblet cells. A, two representative live mucous/goblet cells visualized under a confocal microscope after 4 h in cycloheximide-containing culture medium. Scale bar = 3 μm. B, a representative live mucous/goblet cell observed under a confocal microscope after 4 h in brefeldin A-containing culture medium. To visualize SHGFP-MUC5AC/CK fluorescence in the endoplasmic reticulum, the mucin granule signal was saturated. The inset shows a mucous cell with an unsaturated intragranular fluorescent signal. Scale bar = 2 μm. C, a cluster of live mucous/goblet cells visualized under a confocal microscope before (panel a) and after (panel b) addition of ATP. Total fluorescence intensities immediately following ATP addition were simultaneously measured in the endoplasmic reticulum and mucin granules (panel c). The asterisks indicate the goblet cell in which fluorescence intensities in the endoplasmic reticulum and mucin granules were followed over time after ATP addition. Scale bars = 3 μm. a.u., absorbance units.
**FIG. 6.** SHGFP-MUC5AC/CK is not covalently associated with endogenous mucins. A, differentiated cells grown on Transwell filters for 8 days were fixed with paraformaldehyde and immunostained with anti-MUC5AC monoclonal antibody/rhodamine-conjugated anti-mouse IgG without prior permeabilization with Triton X-100. xz scans under a confocal microscope permitted the visualization of SHGFP-MUC5AC/CK in the cells (green signal in panels a and c) and MUC5AC in the cell-associated mucus (red signal in panels b and c). Scale bar = 5 μm. B, shown is an SHGFP-MUC5AC/CK-containing mucous lake in live cultures of differentiated HT29-SHGFP-MUC5AC/CK cells (panels a and b) as observed
this work, we will refer to mucus/mucins in mucous gels as extracellular mucus/mucins.

To determine whether SHGFP-MUC5AC/CK immobilized in the extracellular mucous matrix or, as expected from the above results, freely diffused through it, mucous lakes were analyzed by FRAP. As shown in Fig. 6C (panel a), after bleaching a 1-μm width strip, a significant and instant decrease in the fluorescence intensity over the entire mucous lake region, and not only in the bleached region, was observed. The same result occurred when the bleaching was done in a larger or smaller region. These results are consistent with (a) very rapid equilibration of unbleached SHGFP-MUC5AC/CK with bleached SHGFP-MUC5AC/CK during the time scale of the experiment (42) and (b) the presence of limiting amounts of SHGFP-MUC5AC/CK in the mucous lakes. Indeed, inspection of the changes in fluorescence intensity over time showed that the pre-bleaching intensity dropped to half its value and failed to recover (panel b). Although quantitative estimations of diffusion were not possible under these conditions (42), these results suggest that the fusion protein diffused very rapidly through the extracellular mucous/mucin matrix. The results support the conclusion that SHGFP-MUC5AC/CK was not covalently linked to the mucin polymers/aggregates in mucous lakes.

The above results strongly suggested that SHGFP-MUC5AC/CK and endogenous mucins (MUC5AC) were not covalently linked in the mucin granules and that, upon granule exocytosis, SHGFP-MUC5AC/CK diffused into the culture medium. This conclusion was further supported by the biochemical detection of MUC5AC and SHGFP-MUC5AC/CK in the culture medium and cell extracts of differentiated HT29-SHGFP-MUC5AC/CK cells, respectively. Protein secreted into culture medium and cell extracts of differentiated HT29-MUC5AC/CK cells, respectively. SHGFP-MUC5AC/CK protein was mobile, we would expect a fraction of intragranular SHGFP-MUC5AC/CK fluorescence outside the bleached spot was photobleached, resulting in underestimated $M_f$ values. As shown by Angelides et al. (48) and assuming that all of the fluorescent protein was mobile, such an underestimated would be significant only when the area of the circular system is less than 4 times the area of the bleached spot. In our FRAP analysis, only granules with diameters between 1.1 and 1.8 μm, i.e. corresponding to areas 4.2–10.3 times bigger than the area of the bleached spot, were examined. Hence, assuming that all of the intraluminal SHGFP-MUC5AC/CK protein was mobile, we would expect $M_f$ values between 70 and 90% (48). However, the experimental $M_f$ values obtained ranged in most cases from 16 to 57% (Fig. 7C) and therefore could not be explained by the recovery of an entirely mobile SHGFP-MUC5AC/CK pool in a constrained system, but rather reflected the existence in the granules of a significant immobile fraction of the fluorescent protein. Such a conclusion is supported by the following observations: (a) the persistence in some granules of a bleached area after the fluorescence intensity reached a plateau (Fig. 7A), which indicated that unbleached SHGFP-MUC5AC/CK outside that area was not limiting; (b) the lack of a statistical correlation between $M_f$ values and the respective granule diameters (see below); and (c) the substantial increase in intragranular SHGFP-MUC5AC/CK $M_f$ values when cells were incubated with bafilomycin (see below).

The fluorescence recovery curves reasonably followed a one-phase exponential equation (Fig. 7D), which permitted an estimate of the SHGFP-MUC5AC/CK $t_{1/2}$, the characteristic diffusion time or recovery half-time. $t_{1/2}$ values ranged from $0.9$ to $5$ s. In most cases, the $t_{1/2}$ values were between 1 and 2 s, with a mean value of $1.3$ s (Fig. 7E). The wide range of intragranular SHGFP-MUC5AC/CK $M_f$ and $t_{1/2}$ values under a confocal microscope. In most cases, mucous lakes were enclosed by goblet cells (panel b). In chemically fixed cells processed for the electron microscope, mucous lakes were readily identified (panel c) and contained an intraluminal fibrous material similar to the intragranular matrix (panel c, inset). Scale bar = 2 μm. C, a representative mucous lake was bleached (white stripes), and the recovery of the fluorescence was recorded over time. Panel a, pre-bleaching (left), immediately post-bleaching (center), and 14-s post-bleaching (right) images; panel b, the corresponding bleaching/recovery curve of a representative analysis. Note the overall loss of fluorescence in the mucous lake immediately after the bleaching pulse. Scale bars = 5 μm. a.u., absorbance units. D, secreted (M) and cellular (C) proteins of differentiated cells were separated on agarose gels, transferred to nitrocellulose paper, and detected with anti-MUC5AC monoclonal antibody/HRP-conjugated anti-rabbit IgG (panel a) or anti-His tag antibody/HRP-conjugated anti-mouse IgG (panel b) with (Reduced) or without (Unreduced) prior reduction with 2-mercaptoethanol. Positive bands were detected by chemiluminescence.
prompted us to test whether these parameters correlated with one another or with the mucin granule diameter, which also exhibited a wide range of values. Both Pearson’s test and Spearman’s non-parametric test did not support the existence of a significant correlation between any of these parameters, although it must be considered that only granules/H11022 min diameter were analyzed. Collectively, these results show that intragranular SHGFP-MUC5AC/CK molecules were distributed between a mobile phase, where they could slowly diffuse, and an immobile phase.

**SHGFP-MUC5AC/CK Mobile Fraction and Mobility in the Endoplasmic Reticulum Are Higher than in the Mucin Granule**—It was important to determine whether the SHGFP-MUC5AC/CK highly immobile fraction and the low mobility within the granule were the result of the intragranular environment or, alternatively, an intrinsic property of the recombinant protein. To distinguish between these possibilities, we carried out FRAP analysis in the endoplasmic reticulum. Because of the heterogeneous nature and irregular geometry of this organelle, we used strip photobleaching, which has been shown to provide far more consistent results than spot photobleaching for analyzing endoplasmic reticulum protein diffusion (42, 49, 56). As shown in Fig. 8A, unbleached SHGFP-MUC5AC/CK apparently equilibrated with the bleached protein within 1 min of bleaching. Moreover, in contrast to the situation in the granules, a large portion of the pre-bleaching mean fluorescence intensity recovered after the bleaching pulse (Fig. 8B), suggesting that a large fraction of SHGFP-MUC5AC/CK in this organelle was mobile. Thus, the corresponding SHGFP-MUC5AC/CK mean Mf was considerably higher (~86%) than in the mucin granules (Fig. 8C), indicating that the majority of the SHGFP-MUC5AC/CK molecules were indeed mobile while in the lumen of the endoplasmic reticulum.

SHGFP-MUC5AC/CK background-corrected fluorescence recovery curves followed a hyperbolic (rather than a one-phase exponential) equation (Fig. 8C), with t1/2 = 3 s. Interestingly, this value was of the same order of magnitude as the corresponding parameter in the mucin granules. However, because the area bleached in the endoplasmic reticulum (~2 μm² assuming that ~50% of the bleached area was occupied by elements of the endoplasmic reticulum was considerably larger than in the mucin granules (~0.238 μm²), then SHGFP-MUC5AC/CK moved substantially faster in the endoplasmic reticulum. Consistent with this initial assessment, the SHGFP-MUC5AC/CK Ds value within the endoplasmic reticulum was almost 100-fold larger than in the mucin granule, with mean ± S.E. of 1.42 ± 0.17 μm²/s (n = 34) and 0.014 ± 0.003 μm²/s (n = 20), respectively. Moreover, because of the low Mf values in the granules, intragranular SHGFP-MUC5AC/CK Ds values were very likely overestimations (48), and therefore, the differences in SHGFP-MUC5AC/CK mobility between this organelle and the endoplasmic reticulum might be even larger. These results indi-
cated that the intragranular SHGFP-MUC5AC/CK low mobility and the high immobile fraction were not likely caused by SHGFP-MUC5AC/CK intrinsic properties, but reflect a response to the intraluminal environment of this organelle.

The vacuolar H^+ -ATPase Inhibitor Bafilomycin A₁ Increases SHGFP-MUC5AC/CK Mobile Fractions in Mucin Granules—Evidence obtained in different cell systems suggests there is a negative pH gradient from the endoplasmic reticulum to the Golgi complex to secretory granules (57). Indeed, using pH-sensitive fluorescent compounds, Verdugo et al. (32) suggested the existence of acidic pH in the mucin granule. The activity of the vacuolar H^+ -ATPase in the membrane of secretory granules might explain, at least in part, this gradient (26). In view of these data, intragranular pH could be one of the factors contributing to SHGFP-MUC5AC/CK fractionation between an immobile phase and a slow mobile fraction. To test this hypothesis, cells were incubated for up to 4 h with bafilomycin A₁, a specific inhibitor of the vacuolar H^+ -ATPase (58). At the end of the treatment, FRAP analysis was carried out as described above for control cells. Although mucous/goblet cells with abundant granules were present in bafilomycin-treated cultures, many of the granules exhibited a heterogeneous distribution of the SHGFP-MUC5AC/CK fluorescence (Fig. 9A). After bleaching only granules with homogeneous fluorescence (Fig. 9B), a prerequisite for FRAP analysis, normalized photobleaching curves (Fig. 9C) indicated that the percent of the plateau fluorescence intensity relative to the pre-bleaching intensity was larger than in control cells (Fig. 7B). This curve profile suggests that a higher proportion of intragranular SHGFP-MUC5AC/CK was mobile under conditions of vacuolar H^+ -ATPase inhibition. Indeed, as shown in Fig. 9D, the differences between the intragranular SHGFP-MUC5AC/CK mean Mf values in control and bafilomycin-treated cells were statistically significant (p < 0.001). As was the case in control cells, the normalized photobleaching-corrected FRAP recovery curves (Fig. 9E) followed a one-phase exponential expression with an intragranular mean t½ value (~1.7 s) statistically indistinguishable (p = 0.834) from the corresponding parameter in control cells (Fig. 9F), suggesting that bafilomycin did not alter SHGFP-MUC5AC/CK mobility. These results indicate that intragranular SHGFP-MUC5AC/CK immobilization was reversible and dependent on the activity of the vacuolar H^+ -ATPase, suggesting that intraluminal pH was a critical factor for intragranular organization.

DISCUSSION

We have generated a mucous/goblet cell line stably expressing a fluorescent mucin domain accumulates in mucin granules. The fusion protein SHGFP-MUC5AC/CK consists of an N-terminal signal peptide, followed by GFP, six consecutive histidines, and the CK domain of MUC5AC. The CK domain is found in the C termini of all gel-forming mucins, from fishes to humans, and it is involved in mucin dimerization, which is the first step in the formation of mucin covalent oligomers/multimers (6). Consistent with this notion, the MUC5AC and MUC5B CK domains were secreted from transfected cells as disulfide-linked dimers (Fig. 2). Interestingly, whereas SHGFP-MUC5AC/CK was secreted and could be detected free in the extracellular mucus, it was not covalently linked to endogenous MUC5AC (Fig. 5). These results suggest a role in dimerization for protein sequences N-terminal to the CK domain. This possibility is also supported by expression studies in LS174T cells showing that GFP fused to the entire C-terminal region of MUC2 forms homodimers and also heterodimers with endoge-
nous MUC2 (17). However, the fact that MUC6, one of the two major secreted mucins in the gastric mucosa, has only a C-terminal CK domain and not the additional domains present in the C-terminal region in MUC2, MUC5AC, MUC5B, and MUC19, indicates that, if such a role indeed exists, it is not an absolute requirement for dimerization.

Several criteria indicated that HT29-SHGFP-MUC5AC/CK cells, like the parental cell line HT29–18N2 (37), underwent an in vitro mucous cell differentiation process when cultured at high density in a defined serum-free medium (Figs. 2 and 3). Thus, up to 30% of the cells developed a mature mucous/goblet cell phenotype, including biogenesis of abundant periodic acid-Schiff-reactive mucin granules. The granules stored large quantities of SHGFP-MUC5AC/CK, as judged by their intragranular fluorescence intensity, together with endogenous MUC5AC. Moreover, intragranular SHGFP-MUC5AC/CK was essentially mobile (Fig. 8). Its intraluminal mean $D_{\text{eff}}$ ($\approx 1.4 \, \mu m^2/s$) was consistently smaller than the reported coefficient for ssGFP-KDEL ($\approx 9–10 \, \mu m^2/s$) (42, 59), but larger than the elastase-GFP $D_{\text{eff}}$ ($\approx 0.6 \, \mu m^2/s$) (60). Although the Stokes-Einstein expression (61) indicates that diffusion is inversely related to the viscosity of the solution, other factors, such as interactions or collisions with mobile or immobile intraluminal components (62), affect protein mobility within cell compartments. Because the ssGFP-KDEL mean $D_{\text{eff}}$ in the endoplasmic reticulum lumen of HT20–18N2 cells ($\approx 9 \, \mu m^2/s$) (data not shown) does not differ from the values reported in other cell types (59), then interactions of the CK domain with other proteins (e.g., MUC5AC precursors) might explain the relatively low mobility of SHGFP-MUC5AC/CK. As for other cell types, the endoplasmic reticulum in mucous/goblet cells expanded in the presence of brefeldin A (Fig. 5). This response likely reflects the increased accumulation of mucin and other secretory product precursors of those forms retro-transported from the Golgi complex (54). Interestingly, preliminary studies
FIG. 10. Intraluminal organization of mucin granules. A, the results reported in this study suggest the existence in the granule of a mobile compartment in which secretory products very slowly diffuse and an immobile matrix in which the secretory products are immobilized by noncovalent interactions. The spatial relationship between these two intraluminal subcompartments could be envisioned by two simple but antagonistic models. In the core model, the matrix forms an inaccessible, likely dehydrated condensed core, where immobilized secretory products are entrapped during the process of matrix condensation. The mobile compartment with the mobile fraction would be positioned around the core, and therefore, exchange of mobile and immobile products would be limited with the interface between both subcompartments. In the meshwork model, the immobile, likely condensed matrix forms a three-dimensional network embedded in the mobile fraction, which has access to every region of the network. Therefore, exchange of mobile and immobile species could take place throughout the entire matrix network. These models predicted different FRAP outcomes, as shown by the schematics representing hypothetical granules prior to bleaching (p), immediately after bleaching (b), and at equilibrium post-bleaching (pb). Note that the core model predicts the absence of fluorescence recovering in the bleached area. Our FRAP analysis showed that 1) immobile and mobile SHGFP-MUC5AC/CK proteins were homogeneously distributed in the granule, 2) SHGFP-MUC5AC/CK fluorescence recovered in the bleached area, and 3) SHGFP-MUC5AC/CK redistribution after the bleaching was homogeneous. As indicated under “Discussion,” these results favor a three-dimensional meshwork model. B, shown is an xy confocal image of mucin granules with fluorescence-free cores in a live mucous/goblet cell scanned as described for Fig. 4. Scale bar = 0.5 μm. C, shown are representative FRAP xy images of a core mucin granule (upper panels) showing pre-bleaching (left), immediately post-bleaching (center), and 24-s post-bleaching (right) images and the corresponding normalized bleaching curve (lower panel). Scale bars = 0.5 μm. D, shown is a schematic representing the organization of granules with fluorescence-free cores following the meshwork model for intragranular organization. The expected FRAP pre-, immediately post-, and post-bleaching images are indicated.
suggest that SHGFP-MUC5AC/CK mobility in the endoplasmic reticulum of brefeldin A-treated cells did not differ compared with control cells. If confirmed, these results will further suggest that SHGFP-MUC5AC/CK mobility is governed by interactions of the CK domain with other components of the endoplasmic reticulum.

The intragranular SHGFP-MUC5AC/CK $D_{eff}$ was 2 orders of magnitude smaller than in the endoplasmic reticulum, suggesting that the intragranular medium was especially viscous; the fusion protein collided and/or interacted with other luminal compounds. Moreover, even assuming large intragranular concentrations of Ca$^{2+}$ and other cations, hinderance due to charge interactions involving the polyanionic mucin matrix and SHGFP-MUC5AC/CK cannot be ruled out. Indeed, the complexity of the intragranular environment is easily revealed by plotting the recovery radial intensities as a function of granule diameter at each post-bleaching time point. None of these curves could be fitted to a gaussian expression, indicating that intragranular SHGFP-MUC5AC/CK movement cannot be explained by simple diffusion (data not shown). In any case, similar $D_{eff}$ values for intragranular SHGFP-MUC5AC/CK are found only in slowly diffusing membrane receptors (44). In contrast to the situation in the endoplasmic reticulum, a significant fraction of intragranular SHGFP-MUC5AC/CK was immobile during the time scale of the experiments. It is generally assumed that the granule intraluminal matrix is mainly constituted by condensed mucin networks (29, 30), likely bound to Ca$^{2+}$, although the exact packing mechanism is unknown. In any case, a long-term strong interaction between SHGFP-MUC5AC/CK and the condensed mucin matrix could explain our results, suggesting that a similar mechanism might permit intragranular accumulation of non-mucin secretory proteins (e.g. lysozyme and TFF3) in mucous cells.

Intrgranular SHGFP-MUC5AC/CK immobilization could be reversed by inhibiting the activity of the vacuolar H$^+$/ATPase, as judged by the increase in its mean $M_r$ value (Fig. 9). Although studies on the localization of vacuolar H$^+$/ATPase in mucous/goblet cells are not yet available, the current evidence suggests that this protein resides in the secretory granule membrane, where its activity is critical for maintaining an acidic intraluminal pH (26, 63, 64). Hence, by inhibiting the vacuolar H$^+$/ATPase, the intragranular pH very likely increased, and ionic and/or hydrophobic interactions important for SHGFP-MUC5AC/CK immobilization and also matrix organization (e.g. condensation/decondensation of mucin oligomers/multimers) were eventually lost. For instance, the theoretical and empirical $M_r$ values for SHGFP-MUC5AC/CK and GFP (65) suggest that the fusion protein is negatively charged at the pH of the endoplasmic reticulum lumen, but likely unchanged while in the granule. Thus, charge neutralization might permit protein-protein hydrophobic interactions and ultimately SHGFP-MUC5AC/CK immobilization.

Intragranular SHGFP-MUC5AC/CK fluorescence appeared to be homogeneously distributed in most of the granules in control cells and, after a bleaching pulse, did not show a preferential redistribution to certain areas. Interestingly, in bafilomycin-treated cells, intragranular SHGFP-MUC5AC/CK distribution was often irregular (Fig. 9A), likely reflecting a structural disorganization of the intragranular matrix. These observations support the existence of a pH-dependent tri-dimensional meshwork-type (rather than a compact and inaccessile) intragranular matrix. This matrix would be likely embedded in a mobile phase in which secretory products could very slowly diffuse (Fig. 10A). The matrix network might create a steric hindrance that contributes to the slow intragranular diffusion of proteins in the mobile fraction. A similar two-phase model is also suggested by studies on the mechanism of intragranular Ca$^{2+}$/K$^+$ ion exchange (66, 67).

Our results do not rule out the formation of granule cores, which were inaccessible to the SHGFP-MUC5AC/CK mobile fraction. Indeed, a small percentage of untreated mucous/goblet cells (~5%; n = 104) had granules with fluorescence-free centrally located cores (Fig. 10B). It is possible that, in these cores, immobilized SHGFP-MUC5AC/CK had lost its emission or, alternatively, that the fusion protein never had access to the interior. In any case, FRAP analysis of the core granules did not indicate heterogeneous redistribution of SHGFP-MUC5AC/CK fluorescence (Fig. 10C), consistent with the existence of a three-dimensional network, likely a pH-dependent matrix in the rest of the granule (Fig. 10D). The observation that bafilomycin only increased the intraluminal SHGFP-MUC5AC/CK $M_r$ value up to ~20% over untreated cells is consistent with the existence of other factors affecting granule matrix organization and protein accumulation. Whatever these additional factors may be, their effects were rapidly neutralized during regulated secretion, as suggested by the rapid disappearance of intragranular SHGFP-MUC5AC/CK fluorescence upon ATP addition (Fig. 5). Indeed, once SHGFP-MUC5AC/CK was secreted and reached the extracellular space (i.e. the mucous lakes), there was a sharp increase in its mobility (Fig. 6). These results are consistent with the existence of an intragranular condensed mucin matrix and its subsequent expansion upon exocytosis, as proposed in the ion exchange-triggered phase transition model of granule exocytosis (29, 30, 68, 69).

In summary, our results suggest that the mucin granule lumen is compartmentalized into a mobile fraction, where secretory products are able to diffuse, albeit very slowly, and into a pH-dependent immobile matrix, where secretory proteins are retained by a pH-dependent mechanism. The incorporation of secretory products from a mobile into an immobile osmotically inert phase would permit the packing of large amounts of secretory products. In addition, mucin granule compartmentalization may have important functions beyond its role in the mechanism of mucin/protein packaging. Thus, intraluminal Ca$^{2+}$ in the mobile fraction might be critical for the regulation of granule exocytosis (67). Alternatively, a mobile phase might make it possible for certain late pH-dependent mucin modifications, including disulfide bonding through D domains (18, 19), proteolytic processing (20), and even terminal glycosylation, to continue in the granule.

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