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Background: Imaging colonic mucus on living cells, tissues, and organs is required for live microscopy.
Results: We have identified, synthesized, and validated a new human colonic mucus bacterial marker (MUB_{70}).
Conclusion: This non-toxic marker is used to image the secreted colonic mucus.
Significance: Beyond imaging applications, Cy5-MUB_{70} might be used for diagnostic and prognosis applications on colonic mucinous carcinoma.

Imaging living cells and organs requires innovative, specific, efficient, and well tolerated fluorescent markers targeting cellular components. Such tools will allow proceeding to the dynamic analysis of cells and the adaptation of tissues to environmental cues. In this study, we have identified and synthesized a novel non-toxic fluorescent marker allowing a specific fluorescent staining of the human colonic mucus. Our strategy to identify a molecule able to specifically bind to the human colonic mucus was on the basis of the mucus adhesion properties of commensal bacteria. We identified and characterized the mucus-binding property of a 70-amino acid domain (MUB_{70}) expressed on the surface of *Lactobacillus* strains. The chemical synthesis of MUB_{70} was achieved using the human commensal bacterium *Lactobacillus reuteri* AF120104 protein as a template. The synthesized Cy5-conjugated MUB_{70} marker specifically stained the colonic mucus on fixed human, rabbit, and guinea pig tissues. Interestingly, murine tissue was not stained, suggesting significant differences in the composition of the murine colonic mucus. In addition, this marker stained the mucus of living cultured human colonic cells (HT29-MTX) and human colonic tissue explants. Using a biotinylated derivative of MUB_{70}, we demonstrated that this peptide binds specifically to Muc2, the most abundant secreted mucin, through its glycosylated moieties. Hence, Cy5-MUB_{70} is a novel and specific fluorescent marker for mammalian colonic mucus. It may be used for live imaging analysis but also, as demonstrated in this study, as a marker for the diagnosis and the prognosis of colonic mucinous carcinomas.

The gastrointestinal mucus layer establishes a physical barrier between the luminal content and the epithelial surface and provides efficient protection against luminal aggressions (1). It is continuously removed (enzymatic destruction and mechanical shearing, *i.e.* peristalsis) and renewed through the secretory activity of epithelial goblet cells (2). In addition to its role as a physical barrier, it may also allow oxygen diffusion from the intestinal epithelium into the lumen (3), although no precise quantification has yet been achieved. This intestinal oxygen diffusion has been shown to play a critical role in the *Shigella* virulence modulation in the vicinity of the intestinal epithelial barrier, possibly controlling the virulence of other pathogens and restricting the replicative niche of anaerobic bacteria to the intestinal lumen (4). *In vivo* observations are a prerequisite for oxygen detection in this environment, yet studying this largely unexplored microenvironment at the epithelial interface using live imaging techniques (two-photon microscopy, fluorescent life-time imaging and high-resolution microscopy (photoactivated localization *microscopy* and stochastic optical reconstruction *microscopy*)). These techniques have been limited because of the lack of appropriate tools. They require the development of specific, non-toxic, and non-destructive colonic mucus fluorescent markers.

The colonic mucus is composed of two distinct layers: a firmly adherent layer bound to the epithelial surface and a more fluid loosely adherent one. The latter is likely the result of bacterial degradation and proteolysis (5). It is composed of 95% water and 5% mucin molecules, salts, immunoglobulins (IgA and IgG), and trefoil peptides (6). Among the secreted mucins, the main gel-forming molecules are Muc2, Muc5ac, Muc5b,

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[S] This article contains supplemental Figs. S1–S7, Table S1, and methods.
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and Muc6 (expressed from chromosome 11p15.5) (7). Muc2 is the predominant mucin in the colonic mucus layer. It is highly glycosylated, resulting in a relative resistance to proteolysis in the lumen, partially because of the release of bacterial mucinases (8–11). Muc2 shows differential glycosylation profiles in the small intestine (ileum) and in the large intestine (colon) being enriched in either sialylated or sulfated oligosaccharide species, respectively (9, 12). The mucus production and composition modulations are commonly observed in the colonic mucinous carcinoma but also in the major inflammatory bowel diseases like Crohn's disease (13, 14) and ulcerative colitis (15).

Our strategy to identify a molecule able to specifically bind to the human colonic mucus was on the basis of the mucus adhesion properties of commensal bacteria. These microorganisms, such as *Lactobacillus* spp., express cell surface proteins named mucus binding proteins (MucBP,[2] Protein Families database (PFAM) PF06458) that are involved in their intestinal mucus adhesion property. As an example, in the human intestine, *Lactobacillus reuteri* has been identified as an inhabitant of the ileum and colon loosely adherent mucus layer (16). The MucBP protein family is characterized by the presence of well conserved mucin binding domains (MucBD) expressed as repeats in many cell surface MucBP of *L. reuteri* (17–19). In addition, it has been suggested that the *L. reuteri* MucBD-associated domain (MUBAD) may also play a role in the function of MucBP, although this property was not confirmed (18).

In this study, we characterized a novel mucus binding property of the *L. reuteri* MUBAD domain. We demonstrated that MUBAD was able to oligomerize and specifically bind to Muc2 through its carbohydrate moiety. MUBAD is therefore a novel MucBD of 70 amino acids in length, hereafter named MUB$_{70}$. The chemical synthesis of a fluorescent conjugated Cy5-MUB$_{70}$ marker provided access to a specific marker of the colonic mucus that can be used as a marker for colon live imaging applications. In addition, as Muc2 expression is modulated in mucinous carcinomas, further applications of MUB$_{70}$ in the diagnosis and the prognosis of these diseases are anticipated, as demonstrated on tissues from patients diagnosed with colonic mucinous carcinomas.

EXPERIMENTAL PROCEDURES

MUB$_{70}$ Chemical Synthesis—The synthesis was carried out on an ABI 433 synthesizer (Applied Biosystems, Foster City, CA) equipped with a conductivity flow cell to monitor Fmoc deprotection, from a Fmoc protected phenylalanine linked to polystyrene-*p*-hydroxy-benzyl resin (capacity 0.52 mmol/g, Rapp Polymere GmbH). Fmoc amino acids, Dmb, and pseudoproline dipeptides were activated with 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate/*N*,*N*-diisopropyléthylamine and single-coupled with an 8-fold molar excess regarding the resin. After a classical resin cleavage protocol, the dimeric form of MUB$_{70}$ was purified using a three-step Reversed-phase high-performance liquid chromatography method. Recovery of the MUB$_{70}$ monomeric form was completed during biotin or Cy5 conjugation. Peptide masses were confirmed using electrospray ionization mass spectrometry. More detailed information as well as the analytical gel filtration technique is provided in the supplemental methods.

Biochemical Characterization and Biological Properties

Colonic Tissue Collection—Ex vivo human colon samples were obtained from Dr. E. Labruyère (Institut Pasteur), and tissue processing was performed as described previously (20) and stored in serum-free RMPI media (the surgical procedure is described in the supplemental methods). Human mucinous carcinoma formamide fixed samples were obtained from Dr. T. Lazure (Hôpitaux Universitaires Paris-Sud, Kremlin-Bicêtre) and Pr. I. Sobhani (Hôpital Henri Mondor, Créteil). Rabbit colon and ileum samples were collected on naïve New Zealand White rabbits weighing 2.5–3 kg and fixed in paraformaldehyde 3%. The same procedure was applied on intestine samples collected on guinea pigs (Charles River Laboratories, Inc.) and C57/B6 mice (Janvier).

Cell Culture—HeLa cells were grown in DMEM supplemented with 10% FCS. HT-29 MTX colonic epithelial cells (kindly provided by Dr. Lessufleur) (21) were grown to confluency in 24-well tissue culture plates in RPMI medium supplemented with 10% FCS and 1% essential amino acids. Mucus production in HT-29 MTX cells was observed after 21 days.

MUB$_{70}$ Marker—For staining living HT-29 MTX cells, human colon *ex vivo* model and fixed colon Cy5-MUB$_{70}$ was incubated (1 μg/ml) in a serum-starved culture medium (DMEM and RPMI, respectively) for 2 h at 37 °C prior to observation.

Pull-down Assay—Pull-down assays were performed in the presence of 600 μg of biot-MUB$_{70}$ bound to 500 μl of Avidin-agarose beads (Thermo Scientific) in a phosphate buffer (pH 8) for 1 h at 4 °C. After three washes, 10 mg of soluble human colonic mucus extract were incubated with the loaded beads for 2 h at 4 °C. After three washes, beads were boiled in the presence of 1× Laemmli buffer. As a negative control, Avidin-agarose beads were loaded with 15 μg of biotin (Sigma-Aldrich) and processed using the same procedure. Experiments were performed on two independent occasions.

Dot Blot Assay—Soluble mucus components used in pull-down assays (input and output) were transferred to nitrocellulose membranes (Invitrogen) that were blocked in PBS/5% milk and further incubated with the primary antibodies diluted in PBS/1% milk/0.01% Tween 20 (Sigma-Aldrich) overnight. Membranes were washed in PBS three times and then incubated with secondary antibodies for 1 h before washing. Antibody binding was detected with chemiluminescence (ECL kit, GE Healthcare).

Tissue Immunostaining—Following PFA 4% or Carnoy fixation, as indicated, samples were washed in PBS, incubated at 4 °C in PBS containing 12% sucrose for 90 min, then in PBS with 18% sucrose overnight, and frozen in optimum cutting temperature (OCT) formulation (Sakura) on dry ice. 7-μm sections were obtained using a cryostat CM-3050 (Leica).

Fluorescence Microscopy—Fluorescently labeled tissues and cells were observed using a wide-field epifluorescent micro-

[2] The abbreviations used are: MucBP, mucus binding protein(s); MucBD, mucus binding domain(s); MUBAD, mucin binding domain-associated domain; MTX, methotrexate.

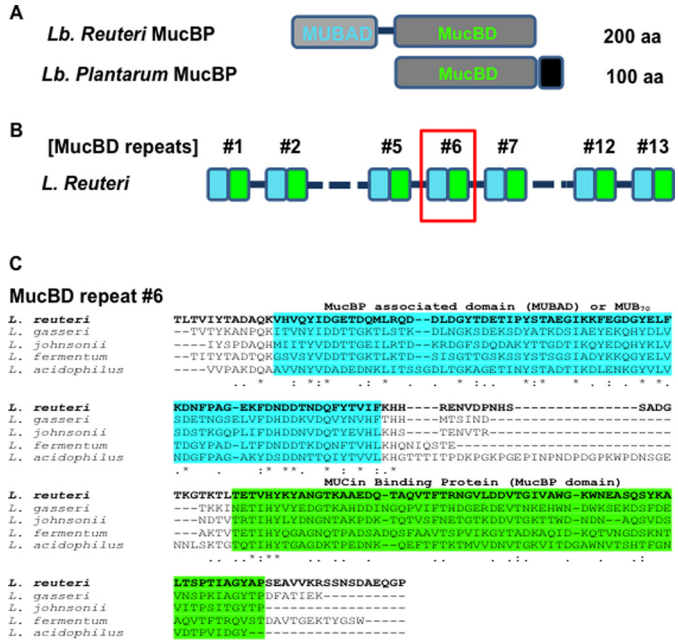

FIGURE 1. **MUB$_{70}$ identification in *L. reuteri*.** *A*, MucBP diversity illustration comparing *L. reuteri* (GenBank™ AF120104) AND *L. plantarum* (lp_1229). *aa*, amino acids. *B*, representation of MUB$_{70}$/MucBD 15 repeats of *L. reuteri* AF120104. *C*, sequences comparison between the *L. reuteri* AF120104 protein sequence and homologous proteins in *L. gasseri* (ZP_07711585), *L. johnsonii* (ZP_04008294.1), *L. fermentum* (YP_001843489.1), and *L. acidophilus* (YP_004034456.1) are performed using Clustalw software. The MUB$_{70}$ sequence and conserved amino acid are highlighted in *blue*. The MucBD (PFAM 06458) sequence and conserved amino acids are highlighted in *green*.

scope (Zeiss Definite Focus), a laser-scanning confocal microscope (Leica tryptocasein soy medium SP5), or a two-photon confocal microscope (Zeiss LSM710) (supplemental methods), as indicated. Image analysis was performed using the Axovision, ImageJ, and Zen 2008 SP 1.1 (Zeiss) and Imaris software, as indicated.

RESULTS

Identification of MUBAD or MUB$_{70}$ in L. reuteri AF120104 Protein Sequence—MUB$_{70}$ was initially identified in MucBP associated with the well characterized MucBD (PFAM 06458). However, MUB$_{70}$ was described as being present in some but not all proteins of this family (18), reflecting the diversity of MucBP sequences and sizes (Fig. 1*A*). Shorter MucBP do not contain MUB$_{70}$ (*i.e.* the *Lactobacillus plantarum* lp-1229 sequence) (22) (Fig. 1*A*). The MUBAD sequence could not be associated with any homologous domain referenced in the PFAM database, and, thus, its function remained unknown (18). MUBAD was named MUB$_{70}$, as its minimum conserved sequence among *Lactobacillus* strains is 70 amino acids. MUB$_{70}$ is repeated from 1 to 18 times in different *L. reuteri* cell surface proteins (18). In the *L. reuteri* AF120104 cell surface precursor protein, MUB$_{70}$ homologous sequences are repeated 13 times (17) (Fig. 1*B*). The sequence comparison showed that repeats 6, 7, and 9 are identical and are the most conserved sequences, among others (supplemental Table S1). The MUB$_{70}$ repeat 6 sequence possesses 23% identity with AF120104 homologous proteins MUB$_{70}$ domains in other *Lactobacillus* strains (*Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus fermentum*, and *Lactobacillus acidophilus*) (Fig. 1*C*). This peptide was selected as a template for the chemical synthesis.

Synthesis of the Cy5-MUB$_{70}$ and Biot-MUB$_{70}$—Despite considerable advances in the field, the straight chemical synthesis of long peptide chains remains a difficult task. Considering solid phase peptide synthesis, most of the deprotection and coupling difficulties are related to inter- or intramolecular hydrogen bonds occurring over the synthesis. N-alkylated amino-acids such as Dmb/Hmb (23) or pseudoproline (24) have been developed to overcome the resulting aggregation propensity of the protected peptide chain anchored to the resin. Here, the presence of hydroxyl amino acids in the MUB$_{70}$ sequence provided the opportunity to introduce several properly spaced pseudoproline dipeptides. Taking advantage of the absence of cysteine in the MUB$_{70}$ sequence, a single cysteine was incorporated at the N terminus to allow N-terminal specific labeling. Using a classical Fmoc/tBu methodology (25), a first synthesis (Strategy 1, Fig. 2*A*) was achieved at a 100 micromolar scale from a polystyrene-based resin. TFA-mediated cleavage of the peptide resin followed by LC-MS analysis of the crude product (66%) in acid conditions indicated the presence of the target peptide as a major peak (around 8% by area integration) in a quite complex chromatogram (supplemental Fig. S1*A*). Moreover, MS analysis of the identified peak revealed the presence of a complex mixture of similar peptides with a mass deviation of −18 or +67, reflecting the presence of aspartimide and piperidide byproducts in a highly significant amount. Aspartimide formation (26) and subsequent base-catalyzed ring opening during Fmoc solid phase peptide synthesis has been described to be strongly dependent on the previous coupled amino acid (27) in relation with the global mixing time of the Asp-containing peptide resin in the Fmoc deprotection solution (28). Along this line, the MUB$_{70}$ sequence accumulates eight highly sensitive occurrences (three Asp-Gly, two Asp-Asn, two Asp-Asp, one Asp-Thr), among which the Asp-Gly sequences are particularly prone to aspartimide formation. Therefore, in a second attempt to synthesize MUB$_{70}$, a systematic protection of each glycine amide moiety occurring before an Asp derivative was achieved by coupling Fmoc-Asp(OtBu)-(Dmb)Gly-OH dipeptides (29), namely in positions 29, 50, and 63, in reference to the C terminus (Fig. 2*A*, *Strategy 2*). Satisfactorily, the aggregation of the peptide chain was diminished, resulting in deprotection and coupling efficiency improvement. Altogether, the yield of the crude product was similar (65%) to that of the first synthesis, but the target peptide peak area integration was increased from 8% to 25% (supplemental Fig. S1*B*), and a significant lowering of aspartimide side reactions was observed by LC-MS.

A first, the RP-MPLC purification protocol was applied in an acidic environment (pH 2) to maintain the peptide in its reduced form. The remaining aspartimide and piperidide side products that tend to coelute in this condition (supplemental Fig. S1*C*) were shown to be well separated when analyzed by RP-HPLC in neutral conditions using 50 mM ammonium acetate (pH 6.5) as a buffer (data not shown). Despite the presence of 2.5 equivalents of tris(2-carboxyethyl)phosphine as a reductive agent into the loaded mixture, scaling up this protocol through a second RP-MPLC purification step revealed the high propensity of MUB$_{70}$ to dimerize in these conditions. More-

A

Synthesis strategy	Operating sequences
1	H-CVHVQYIDGETDQMLRQDDLDGYTDETIPYSTAEGIKKFEGDGYELFKDNFPAGEKFDNDTNDQFYTVI-OH
2	H-CVHVQYIDGETDQMLRQDDLDGYTDETIPYSTAEGIKKFEGDGYELFKDNFPAGEKFDNDDTNDQFYTVI-OH

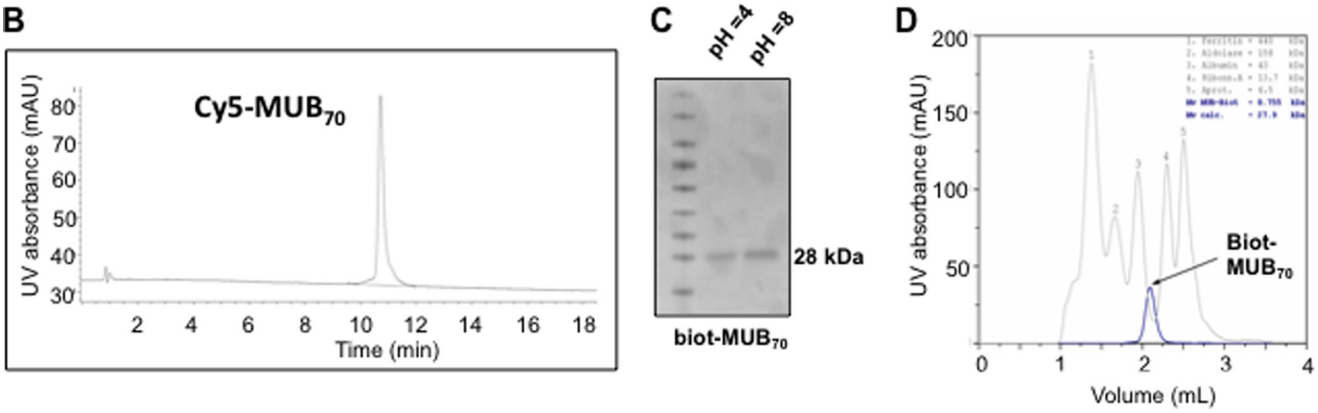

FIGURE 2. **MUB$_{70}$ chemical synthesis and biochemical analysis.** *A*, MUB$_{70}$ synthesis strategies description. Operating sequences for designed syntheses 1 and 2, where secondary amino acid surrogates are colored in *red* (pseudoproline dipeptides) or *green* (Dmb dipeptides). Proline residues are colored in *blue*. *B*, Cy5-MUB$_{70}$ RP-HPLC profile. *C*, SDS-PAGE visualization of the multimeric form of biot-MUB$_{70}$ performed after incubation of the peptide in Tris 25 mM buffer at pH 4 and pH 8. *D*, characterization of biot-MUB$_{70}$ by gel filtration chromatography on Superdex 200 5/150 GL column. The elution profile of biot-MUB$_{70}$ is shown at 280 nm (milliabsorption units).

over, oxidation of the sulfhydryl moiety occurred along the run, up to completion before lyophilization. RP-HPLC analysis at pH 2 of the resulting partially purified material showed a significant shift between both dimer- and monomer-associated truncated peptides retention times (supplemental Fig. S1*D*). Consequently, a last RP-MPLC purification step was run according to the first protocol to yield the MUB$_{70}$ dimeric form with 90% purity (supplemental Fig. S1*E*).

To summarize, the improvement of the synthesis by the incorporation of Dmb and pseudoproline dipeptides and the introduction of a three-steps purification process were combined to isolate the target peptide as a covalent dimer with an overall yield of 2%. Monomer recovery and simultaneous conjugation of biotin or fluorophore (Fig. 2*B*) via the maleimide precursors are described in the supplemental methods.

Biochemical Properties of MUB$_{70}$—MUB$_{70}$ is predicted to be a negatively charged peptide at a pH higher than 4 (net charge is −12.9 at pH 7) (supplemental Fig. S2*A*). No specific hydrophobic domain was predicted through a Kyte-Doolittle analysis of MUB$_{70}$ (supplemental Fig. S2*B*). This result is consistent with its high solubility in a phosphate buffer at pH 8 (see "Experimental Procedures"). The theoretical molecular mass of a biotinylated MUB$_{70}$ (biot-MUB$_{70}$) is 8.8 kDa. However, when migrating on a SDS-PAGE gel, the apparent molecular mass is around 28 kDa, and this property is independent from the pH, which seems to indicate a stable oligomerization of biot-MUB$_{70}$ (Fig. 2*C*). To confirm the multimeric organization of the MUB$_{70}$ peptide, an analytical gel filtration was performed on the biot-MUB$_{70}$-conjugated peptide, allowing the determination of its quaternary structure. The elution profile was recorded at 280 nm. At 0.1 and 1 mg/ml, biot-MUB$_{70}$ gave a single peak at an elution volume of 2.1 ml (Fig. 2*D* and supplemental Fig. S2, *C* and *D*). The molecular mass was determined to be 27.9 kDa, proposing that biot-MUB$_{70}$, with a theoretical mass of 8.8 kDa, exists as trimer in a phosphate buffer.

Cell Toxicity of MUB$_{70}$—As Cy5-MUB$_{70}$ is dedicated to be used on living cells and organs, its cell toxicity has been evaluated. Cell viability of differentiated HT-29 MTX and HeLa cells was assessed after incubation with Cy5-MUB$_{70}$ using a SYTOX Green assay (see supplemental methods). Incubating Cy5-MUB$_{70}$ (1 μg/ml) for up to 10 h in a serum-starved medium does not significantly affect the cell viability (Student's t test, nonsignificant, $p > 0.05$, $n = 3$) (supplemental Fig. S3*A*). This data were consistent with the absence of an intracellular fluorescent signal (Fig. 3*A*).

Specific Staining of Human, Rabbit, and Guinea Pig Colonic Mucus Using Cy5-MUB$_{70}$—Cy5-MUB$_{70}$ was incubated on living differentiated HT-29 MTX human epithelial colonic cells, which have the property to constitutively produce a mucus layer after differentiation (see "Experimental Procedures"). As observed using a live epifluorescent microscope, Cy5-MUB$_{70}$ binds to the cell surface mucus layer. A z projection observation allowed the visualization of fluorescent mucus patches, typical of mucus aggregates produced by differentiated HT-29 MTX cells (21), as the cells remained unstained (Fig. 3*A*). This observation was confirmed by incubating Cy5-MUB$_{70}$ on human colon explants. As shown in Fig. 3*B*, the mucus layer, observed using a two-photon microscope, is stained heterogeneously on the whole width as the epithelium and the lamina propria remain unstained. The proportion of mucus stained by Cy5-

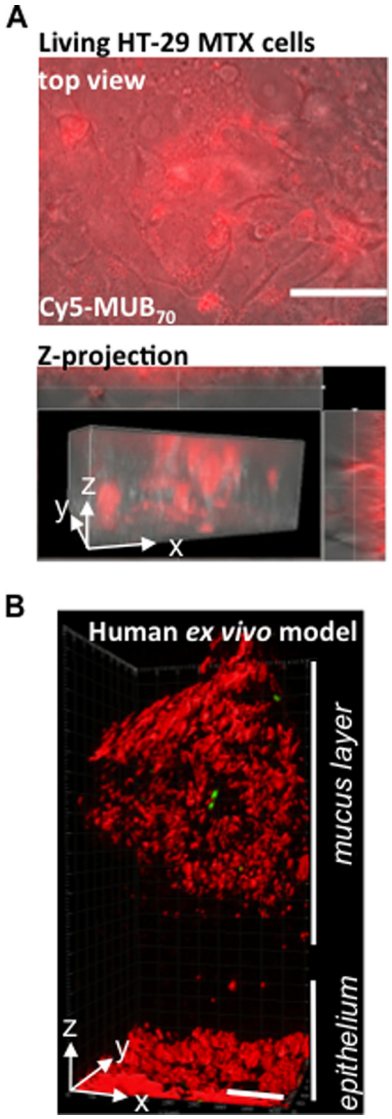

FIGURE 3. **Cy5-MUB$_{70}$ colonic mucus binding property.** *A*, HT-29 MTX living cells were incubated for 2 h with Cy5-MUB$_{70}$ in a serum-free media. The resulting fluorescent signal (*red*) was visualized at the surface of the cell layer using an epifluorescent microscope. Z projection, performed using ImageJ software, allowed three-dimensional localization of the Cy5-MUB$_{70}$ fluorescence signal in the mucus layer. *Scale bar* = 10 μm. *B*, multiphoton excitation imaging of the binding of Cy5-MUB$_{70}$ to the human colonic mucus. Three-dimensional reconstruction (isosurface representation) shows the colonic epithelium covered by the mucus layer (up to 1000 μm) after 90 min of incubation with Cy5-MUB$_{70}$. Human tissue autofluorescence is detected in the same *red* channel as Cy5. *Scale bar* = 100 μm.

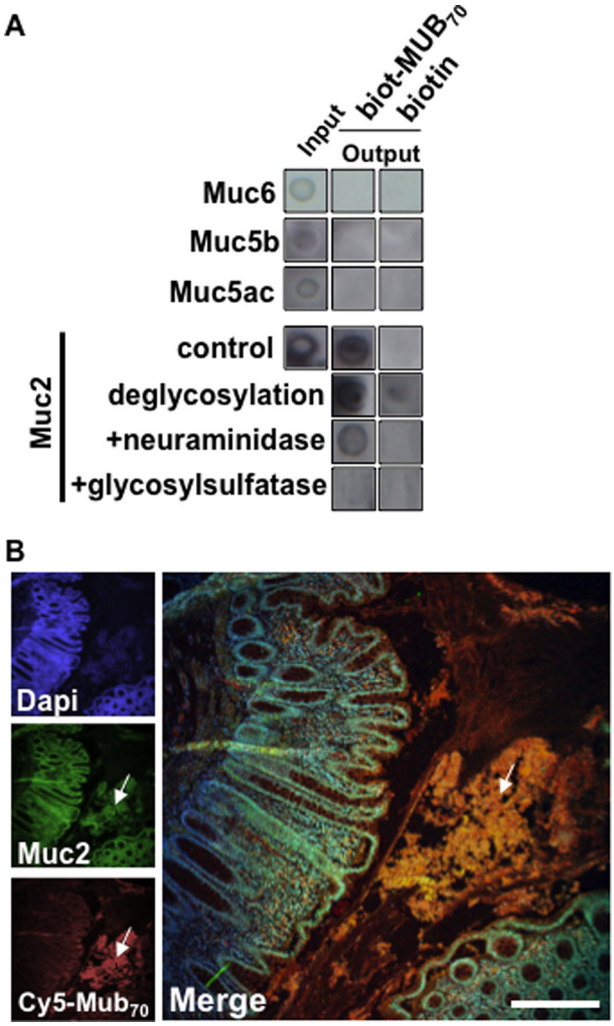

FIGURE 4. **Cy5-MUB$_{70}$ is specifically binding to the glycosylated moiety of Muc2 secreted in the colon mucus layer.** *A*, immunodetection of Muc2, Muc5ac, Muc5b, and Muc6 (dot blot analysis) on human mucus extracts eluted after a pull-down assay performed with biot-MUB$_{70}$ on avidin-conjugated beads (see "Experimental Procedures"). Biotin is used as a negative control. Immunodetection of Muc2 (dot blot analysis) on deglycosylated mucus extracts eluted after a pull-down assay performed with biot-MUB$_{70}$ on avidin-conjugated beads. Non-deglycosylated mucus extract is used as a positive control. *B*, colocalization of Muc2 (*green*) and Cy5-MUB (*red*) observed on fixed (PFA 4%) human colonic luminal mucus (*white arrows*). Observations are performed using a confocal microscope. *Scale bar* = 40 μm.

MUB$_{70}$ might depend on the Cy5-MUB$_{70}$ solution concentration and on the thickness of the mucus layer, as thinner layers could be fully stained (supplemental Fig. S3*C*). Optimal staining is observed after 90 min onto a 1-mm thick mucus layer (supplemental Fig. S3*D*).

Different animal models were used to confirm this result. Rabbit, guinea pig, and mouse colon were tested. Interestingly, the colonic mucus staining using Cy5-MUB$_{70}$ was confirmed on the rabbit and the guinea pig models (supplemental Fig. S4, *A* and *B*). However, the mouse colonic mucus was not stained applying the same procedure (supplemental Fig. S4*C*), which indicates major differences in its composition compared with human colonic mucus. The specific colonic mucus binding property of Cy5-MUB$_{70}$ was confirmed on the rabbit model, as negative results were obtained on ileal mucus samples (supplemental Fig. S3*D*). These results rule out the possibility of an aspecific trapping of Cy5-MUB$_{70}$ in the mucus layers. As a control, the Cy5 fluorophore does not have the property to bind to the purified human mucus (supplemental Fig. S5). As a conclusion, these results suggest that Cy5-MUB$_{70}$ interacts with a specific colonic mucus-secreted component present in human, rabbit, and guinea pig but not in mouse.

Biot-MUB$_{70}$ Specifically Binds to the Glycosylated Moiety of Muc2 from Colonic Mucus—To identify a MUB$_{70}$ ligand present in the soluble extracts of human colonic mucus, biot-MUB$_{70}$, a biotinylated form of MUB$_{70}$, was synthesized (see supplemental methods) to perform pull-down assays. Biot-MUB$_{70}$ was incubated with avidin beads and further with

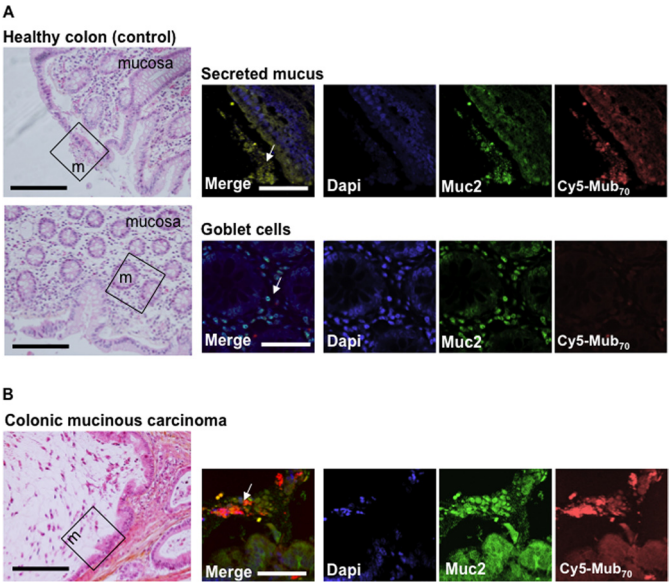

FIGURE 5. **Human colonic mucinous carcinoma staining using Cy5-MUB$_{70}$.** *A*, on healthy colon tissues, the colonic mucus-secreted fraction was detected using an α-Muc2 polyclonal antibody (*green*) and Cy5-MUB$_{70}$ (*red*) (*white arrows*). Nuclei are stained with DAPI (*blue*). Cy5-MUB$_{70}$ (*red*) does not allow goblet cell detection (*lower panel*, *white arrow*). *B*, on pathogenic tissues originating from patients diagnosed with a colonic mucinous carcinoma, the mucus production within the colonic mucosa was performed using an α-Muc2 polyclonal antibody (*green*) and Cy5-MUB$_{70}$ (*red*) (*white arrows*). Nuclei are stained with DAPI (*blue*). On each sample, *red* and *green* signals colocalize. Each observation was performed on five independent samples using a confocal microscope. Colonic mucosa (*mu*) and detected mucus (*m*) are indicated in the *left panels*, corresponding to a hematoxylin-eosin staining of each tissue. For hematoxylin-eosin staining, *scale bars* = 200 μm. For immunofluorescence staining, *scale bars* = 80 μm.

human colonic mucus extracts (produced *in vitro* from differentiated HT-29 MTX cells, see supplemental methods). Biotin was used as a negative control. A dot blot assay was performed, focusing on secreted mucins, which are the major components of the colonic mucus. Muc2 was immunodetected in the eluted fraction but not in the negative control. Muc5ac, Muc5b, or Muc6, all detected in the input, were absent from the eluted fraction (Fig. 4*A*). This result was confirmed by immunofluorescence colocalization detection of Muc2 and Cy5-MUB$_{70}$ on fixed healthy human colon samples (Fig. 4*B*), on human mucus purified from *ex vivo* samples (supplemental Fig. S6*A*), and on fixed rabbit colon samples (supplemental Fig. S6, *A* and *B*). Processing a chemical deglycosylation step on the soluble mucus extract (see supplemental methods) prior to the pull-down assay abolished the interaction between Muc2 and biot-MUB$_{70}$ (Fig. 4*A*). We could specifically demonstrate that enzymatic desulfatation (glycosylsulfatase) but not desialylation (neuramidase) (see supplemental methods) lead to a loss of this interaction (Fig. 4*A*), highlighting the role of the sulfate groups found specifically in the human colonic mucus (12) in the binding of the MUB$_{70}$ peptide. This result could explain the differential staining observed on the rabbit ileal *versus* the colonic mucus (see above and supplemental Fig. S3*B*) as Muc2, expressed and secreted in both organs, possesses differential sulfatation patterns in these organs (9, 12). As a conclusion, MUB$_{70}$ binds to the sulfated Muc2 oligosaccharides in the colonic mucus, thus exploiting its specific sulfatation profile.

Cy5-MUB$_{70}$ Is a New Specific Marker for Colonic Mucinous Carcinomas—As colonic mucinous carcinomas are characterized by abnormal overproduction of Muc2 in the colon mucosa (30), we hypothesized that Cy5-MUB$_{70}$ could be used as a novel fluorescent marker for the diagnosis of this pathology. We demonstrated, on five different samples collected from patients diagnosed with colonic mucinous carcinomas, that a specific staining was observed within the mucus accumulation areas (a representative sample is shown in Fig. 5*B*). As shown previously on a healthy colon (Fig. 3*B*), the luminal mucus secreted fraction is detected by Cy5-MUB$_{70}$ (Fig. 5*A*, *top panel*). As a control, no mucosal staining was observed in the colonic mucosa in healthy colon tissues originating from the same patients (Fig. 5*A*, *lower panel*). Interestingly, we demonstrate that goblet cells are not recognized by Cy5-MUB$_{70}$. This observation might be the consequence of a higher level of mucus compaction, resulting in a lower accessibility for MUB$_{70}$ to bind Muc2. In colonic mucinous carcinomas (Fig. 5*B* and supplemental Fig. S7, *A* and *B*), the fluorescent signal observed with Cy5-MUB$_{70}$ (*red*) colocalizes with the presence of Muc2 (*green*) in the pathologic extensive mucus accumulation observed within the colonic mucosa and associated with tumor cells. Cy5-MUB$_{70}$ has been validated as a potent innovative diagnostic tool for colonic mucinous carcinoma detection and might be optimized with alternative markers (*e.g.* biotin) for practical clinical applications.

DISCUSSION

In summary, *L. reuteri* MUBAD or MUB$_{70}$ is a new MucBD. A chemically synthesized MUB$_{70}$ is a novel specific colonic mucus marker interacting with the sulfated moiety of Muc2 oligosaccharides, known as the main component of this epithelium surface protective layer. The MUB$_{70}$ trimerization property is anticipated to contribute to its interaction property with Muc2, as observed in the human and rabbit colonic mucus but not in the mouse model. To confirm this hypothesis, further structural analyses are required. This approach would be complementary to the MucBP full-length structure determination in which no oligomerization property was described (19). We have demonstrated that MUB$_{70}$ interacts specifically with the sulfated moiety of Muc2, specifically abundant in the colonic mucus. Hence, conjugating MUB$_{70}$ with a fluorescent dye (*i.e.* Cy5) provides a physiological marker, allowing the direct observation of the colonic mucus in *in vitro* and e*x vivo* live imaging approaches beyond classical immunofluorescence techniques. Interestingly, MUB$_{70}$ is not toxic for living cells as it has no cell penetration property, allowing its specific localization in the mucus layer located on the epithelium surface. In addition to the colonic mucinous carcinoma (30), Muc2 expression is up-regulated in mucinous carcinomas affecting various organs (31), including the lung (32), the stomach (33, 34), the breast (35), the prostate (36), and the bile ducts (37). Hence, targeting Muc2 with MUB$_{70}$, as observed on human colonic mucinous carcinomas, is anticipated to provide promising innovative approaches to develop new prognosis and diagnostic tools on various mucinous carcinomas. Further investigations will be required to define the specificity and the sensitivity of this new marker on the different types of mucinous carcinomas.

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