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Selective Induction of Mucin-3 by Hypoxia in Intestinal Epithelia

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Abstract Epithelial cells line mucosal surfaces (e.g., lung, intestine) and critically function as a semipermeable barrier to the outside world. Mucosal organs are highly vascular with extensive metabolic demands, and for this reason, are particularly susceptible to diminished blood flow and resultant tissue hypoxia. Here, we pursue the hypothesis that intestinal barrier function is regulated in a protective manner by hypoxia responsive genes. We demonstrate by PCR confirmation of microarray data and by avidin blotting of immunoprecipitated human Mucin 3 (MUC3), that surface MUC3 expression is induced in T84 intestinal epithelial cells following exposure to hypoxia. MUC3 RNA is minimally detectable while surface protein expression is absent under baseline normoxic conditions. There is a robust induction in both the mRNA (first evident by 8 h) and protein expression, first observed and maximally expressed following 24 h hypoxia. This is followed by a subsequent decline in protein expression, which remains well above baseline at 48 h of hypoxia. Further, we demonstrate that this induction of MUC3 protein is associated with a transient increase in the barrier restorative peptide, intestinal trefoil factor (ITF). ITF not only colocalizes with MUC3, by confocal microscopy, to the apical surface of T84 cells following exposure to hypoxia, but is also found, by co-immunoprecipitation, to be physically associated with MUC3, following 24 h of hypoxia. In exploration of the mechanism of hypoxic regulation of mucin 3 expression, we demonstrated by luciferase assay that the full-length promoter for mouse Mucin 3 (Muc3) is hypoxia-responsive with a 5.08 ± 1.76-fold induction following 24 h of hypoxia. Furthermore, analysis of both the human (MUC3A) and mouse (Muc3) promoters revealed potential HIF-1 binding sites which were shown by chromatin immunoprecipitation to bind the pivotal hypoxia-regulating transcription factor HIF-1α. Taken together, these studies implicate the HIF-1α mediated hypoxic induced expression of mucin 3 and associated ITF in the maintenance of intestinal barrier function under hypoxic conditions. J. Cell. Biochem. 99: 1616–1627, 2006.

Key words: hypoxia; barrier; transcription; inflammation

Mucosal tissues, such as the bladder, lung, or intestine, provide a physical barrier between biologic compartments, preventing the free mixing of luminal antigenic material with the lamina propria which houses the mucosal immune system. For this purpose, epithelia are crucially positioned for electrolyte and fluid homeostasis as well as nutrient uptake and detoxification in that they mediate vectorial and selective transport of ions, water, and macromolecules between blood and the external environment [Tsukita et al., 2001]. The establishment and maintenance of a selectively permeable barrier occurs through interactions of the extracellular domains of multiple transmembrane adhesion domains between adjacent cells (adherens junction, tight junction, gap junction) or between the mucosal layer and extracellular matrix components. These interactions not only determine the physical integrity of the tissue as a whole, but furthermore,

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establish the physical organization of lipids and proteins within the plasma membrane in a polarized fashion. In intact tissues, epithelia overlying the mucosa are supported by a rich and extensive underlying vasculature. As such, even small perturbations in blood flow can result in relatively large decreases in oxygen delivery (hypoxia) to the supporting epithelium [Karhausen et al., 2003]. Recent work has focused on understanding the interplay between hypoxia and epithelial function as it relates to mucosal inflammation [Taylor et al., 2000; Furuta et al., 2001; Karhausen et al., 2004; Louis et al., 2005]. Progress in the understanding of the underlying disease pathophysiology depends on a more complete understanding of the cell biology related to molecular signaling mechanisms and the complexity of cellular oxygen sensing.

Mucins are high molecular weight, highly glycosylated epithelial glycoproteins characterized by large number of O-linked glycosaccharides to threonine/serine/proline-rich tandem repeat domains [Gum, 1995]. Mucins can be classified in one of two forms as either membrane associated or secreted gel-forming proteins [Rose, 1992; Carlstedt et al., 1995]. They form a protective layer lining luminal spaces along the surface of the epithelium, providing a physical barrier to bacterial invasion [Mack et al., 1999, 2003] as well as a source of lubrication and a barrier against mechanical trauma [Rose, 1992; Gum, 1995]. Profiles of mucin expression are relatively tissue specific [Audie et al., 1993; Gum, 1995; Van Klinken et al., 1997] with the primary mucins of the colorectal tract consisting of the secreted MUC2 and the membrane bound MUC1 and MUC4, as well as MUC3 [Ogata et al., 1992; Audie et al., 1993; Shirazi et al., 2000]. MUC3 which has been shown to consist of multiple splice variants including both transmembrane and multiple potential secreted forms [Crawley et al., 1999; Williams et al., 1999].

MUC3 expression has proved to be complex with the gene encoding 11 exons and at least as many as 3 potential splice variants [Crawley et al., 1999]. Additionally, total MUC3 protein appears to encompass the products of two tandem genes MUC3A and MUC3B [Pratt et al., 2000]. A number of studies have suggested that MUC3 contributes to the protective aspects of the intestinal epithelium, and decreased expression has been reported in both healthy and affected patients with Crohn's disease [Buisine et al., 1999]. Furthermore, it has been demonstrated that specific allelic variants of MUC3A have distinct and increased associations with both ulcerative colitis [Kyo et al., 1999, 2001] and Crohn's disease [Kyo et al., 2001]. However, rather little is known about the regulation of MUC3, and whether regulated expression provides a physiologic role.

While the complexity of the human gene has complicated studies of human MUC3, both the human MUC3A promoter [Gum et al., 2003] and mouse Muc3 promoter [Shekels and Ho, 2003] have recently been cloned and characterized. The mouse Muc3 promoter has further been shown to be responsive to inflammatory cytokine stimulation indicating a potential role for mouse Muc3 in intestinal mucosal defense [Shekels and Ho, 2003]. Based on these previous studies, and prompted by results from microarray analysis in epithelia, we pursued the hypothesis that MUC3 is selectively responsive to hypoxia through transcription-dependent pathways.

MATERIALS AND METHODS

Epithelial Cell Culture

T84 epithelial cells were used throughout these studies, and cultured as previously described [Colgan et al., 1996]. Culture medium was supplemented with heat-inactivated calf serum, penicillin, streptomycin, HEPES, heparin, L-glutamine. Hela cells and Mode-K murine intestinal epithelial cells were both cultured as previously described [Comerford et al., 2002].

Analysis of Messenger RNA Levels by PCR

The transcriptional profile of T84 epithelial cells subjected to control (normoxia, pO_2 147 torr) or hypoxia (pO_2 20 torr for 6 or 18 h hypoxia) was assessed from total RNA using quantitative genechip expression arrays (Affymetrix, Inc., Santa Clara, CA) as described previously [Furuta et al., 2001]. RT-PCR was utilized to verify epithelial MUC3 mRNA regulation. Briefly, 1 μg of DNAse I treated (GenHunter, Inc., Nashville, TN) total RNA was reverse transcribed into cDNA using a reaction cocktail consisting of 4 μl of 25 mM $MgCl_2$, 2 μl of 10× reverse transcriptase buffer, 2 μl of 10 mM of each dNTP, 0.5 μl of RNase in ribonuclease

inhibitor (20 U total), 15 U of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of Oligo (dT)15 primer in a total volume of 20 μl. Thermostable *Tfl* DNA polymerase (Promega, Inc., Madison, WI) from *Thermus flavus* was used for second strand cDNA synthesis and DNA amplification. The MUC3 PCR reaction contained 1 μM each of the sense primer (5′-CAC CAA GGT TCA GCA GAT CCG C-3′) and the antisense primer (5′-GTT CAT CAA TGG GCG ACC GG-3′),10 μl of 5× PCR buffer, 1 mM $MgSO_4$, 0.2 mM dNTP and 5 U of *Tfl* enzyme mix in a total volume of 50 μl. Each primer set was amplified using 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 5 min. The PCR transcripts were visualized on a 1.5% agarose gel containing 5 μg/ml of ethidium bromide. As a control for amount of starting template, parallel DNA amplification was performed using 1 μl of starting cDNA template and 1 μM each of human β-actin sense primer (5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′) and antisense primer (5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′), yielding a 661 bp product.

MUC3 Immunoprecipitation/ITF Detection

Confluent T84 epithelial cells exposed to indicated experimental conditions were surface labeled with biotin, lysed, and cell debris was removed by centrifugation as described previously [Louis et al., 2005]. Lysates were precleared with 50 μl pre-equilibrated protein-G sepharose (Pharmacia, Uppsala Sweden). Immunoprecipitation of MUC3 was performed with mouse anti MUC3 mAb Clone M3.1 (Neomarkers, Inc., Fremont, CA) followed by addition of 50 μl pre-equilibrated protein-G sepharose and overnight incubation. Washed immunoprecipitates were boiled in non-reducing sample buffer (2.5% SDS, 0.38 M Tris pH 6.8, 20% glycerol, and 0.1% bromophenol blue), resolved by SDS–PAGE, electroblotted to nitrocellulose, and blocked overnight in blocking buffer. Biotinylated proteins were labeled with streptavidin-peroxidase and visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Alternatively, MUC3 immunoprecipitates were washed under low stringency conditions with lysis buffer alone, resolved by SDS–PAGE, and probed for ITF using a rabbit polyclonal anti human ITF antibody [Ogata and Podolsky, 1997].

Immunofluorescent Staining of Epithelial Monolayers

T84 cells were grown to confluence on membrane permeable filters and processed for immunofluorescence as described previously [Lawrence et al., 2003]. Following exposure to hypoxia for 24 h, inserts were fixed for 10 min at RT in 1% paraformaldehyde in cacodylate buffer (0.1 M sodium cacodylate; pH 7.4, 0.72% sucrose). After washing once with PBS, the cells were stained for 1 h at room temperature with the monoclonal anti-MUC3 antibody (100 μg/ml), and rabbit polyclonal anti-ITF (1:100). After washing twice in PBS, the monolayers were incubated with either goat anti-mouse Oregon Green (1 μg/ml) or goat anti-rabbit Texas Red (1 μg/ml). Fluorescent secondary antibodies were purchased from Molecular Probes (Eugene, OR). Stained inserts were carefully excised and mounted in polyvinyl alcohol mounting media. BioRad Laser Sharp imaging software (BioRad, Hercules, CA) was used for confocal imaging and processing.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using Mode-K cells for HIF-1α binding to the mouse Muc3 promoter and Hela cells, for HIF-1α binding to the MUC3 human promoter as described previously [Kong et al., 2004; Louis et al., 2005]. For these experiments, confluent cells were subjected to normoxia or hypoxia for 18 h. Briefly, 2×10^7 cells were fixed with 1% paraformaldehyde for 10 min. Crosslinking was stopped by the addition of 125 mM glycine, and then, chromatin derived from isolated nuclei was sheared using a F550 micro-tip cell sonicator (Fisher Scientific, Morris Plains, NJ). After centrifugation, supernatants containing sheared chromatin were incubated for 4 h either with 5 μg of anti-HIF-1α antibody/ml of lysate (Novus Biologics, Littleton, CO for murine HIF-1α/ModeK cells or BD Transduction Laboratories, San Diego, CA for human HIF-1α/Hela cells) or control IgG. Protein G sepharose was added and the incubation continued overnight at 4°C. Immune complexes were washed extensively and then eluted from the protein G sepharose. The supernatants were transferred to a new tube, 1 μg/μl of RNAse added and incubated for 5 h at 67°C. Samples were then frozen at −80°C

and 60 μg/μl proteinase K added and incubated for 2 h at 45°C. Next, samples were diluted with TE containing 10 μg of tRNA followed by one extraction with phenol/chloroform and one extraction with chloroform. DNA was precipitated from the samples, washed, dried and resuspended in 30 μl of autoclaved water. DNA was diluted 10-fold and then 1 μl of sample was used for each PCR reaction. The sequences of the mouse Muc3 promoter-specific primers spanning the putative HIF-1 binding region were: sense, 5′-CTC ACC TCA ACT CAC ACA CTT C-3′ and antisense, 5′-GCA AGA ATA TCA CCA CTC CTC AA-3′. The size of the amplified product resulting from the use of this primer pair was 122 bp. The sequence of the human MUC3 promoter-specific primers spanning the putative HIF-1 binding region were: sense, 5′-GGT CAA ACA ATG TGC TGG GTC-3′ and antisense, 5′-CAA GGC TGA GGG GTG TGG AT-3′. The size of the amplified product resulting from the use of this primer pair was 240 bp.

MUC3 Reporter Assays

Mode-K cells were used here to assess inducibility of the mouse Muc3 promoter by hypoxia. Plasmids expressing sequence corresponding to full-length murine Muc3 (bp −1214 to −1) have been previously characterized [Shekels and Ho, 2003]. All constructs were co-transfected with Renilla luciferase vector using standard methods of overnight transfection utilizing Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). In subsets of experiments, cells were transfected with a promoterless vector (pGL3-Basic, Promega Corp., Madison, WI) to control for background luciferase activity. As a positive control for hypoxia, cells were transfected with a PGL3-based HRE plasmid containing four tandem HIF-1 enhancer sequences from the 3′-region of the erythropoietin gene [Sheta et al., 2001]. After transfection, cells were subjected to hypoxia or normoxia for 24 h. Luciferase activity was assessed (Turner Designs, Sunnyvale, CA) utilizing a luciferase assay kit (Stratagene). All luciferase activity was normalized with respect to a constitutively expressed Renilla reporter gene.

MUC3 Immunostaining

Parafin embedded tissue specimen sections from colonoscopic biopsy from human patients with *clostridium difficile* and ulcerative colitis, generously provided by Dr. Paul Beck, were deparafinized using serial incubations with xylene, EtOH, and hydrogen peroxide. Then antigen retrieval was performed using a 1:10 dilution of Dako antigen retrieval solution, pH 9. Sections were then blocked using blocking solution and the biotin/avidin blocking kit from BioGenex. Sections were then probed with mouse monoclonal antihuman MUC3 antibody-2 (clone 1147/B7, Labvision, Neomarkers, Fremont, CA), treated with supersensitive link and detected by addition of HRP conjugated goat anti mouse IgG antibody. HRP-conjugated bound antibody was then detected using DAKO fast-red substrate system and then stained slides were counterstained with hematoxylin (Gill's formula from Vector Laboratories, Burlingame, CA) and mounted with Accergel.

Data Analysis

MUC3 promoter activities in normoxia and hypoxia were compared by Student's *t*-test, where appropriate. Values are expressed as the mean and SEM from at least three separate experiments.

RESULTS

Hypoxia Induces MUC3 mRNA and Protein

We have previously demonstrated that intestinal epithelial cells are uniquely resistant to changes in barrier function elicited by hypoxia [Furuta et al., 2001]. A transcriptional profiling approach, similar to that done previously [Furuta et al., 2001], was utilized to identify potential hypoxia-regulated genes, which might influence barrier in model epithelia (T84 cells). Microarray analysis [Lockhart et al., 1996] identified a qualitative induction of MUC3 following epithelial subjection to hypoxia. Among the family of mucin glycoproteins, this induction by hypoxia was selective for MUC3 (Fig. 1A). Indeed, other mucin transcripts were not significantly changed by hypoxia (e.g., MUC1 and MUC2, Fig. 1A). Based on this analysis, we pursued MUC3 as a potential protective mechanism in hypoxia. RT-PCR analysis was employed to verify microarray results (Fig. 1B), and revealed prominent induction of MUC3 mRNA expression as early as 8 h following introduction to hypoxia (4.2-fold increase of

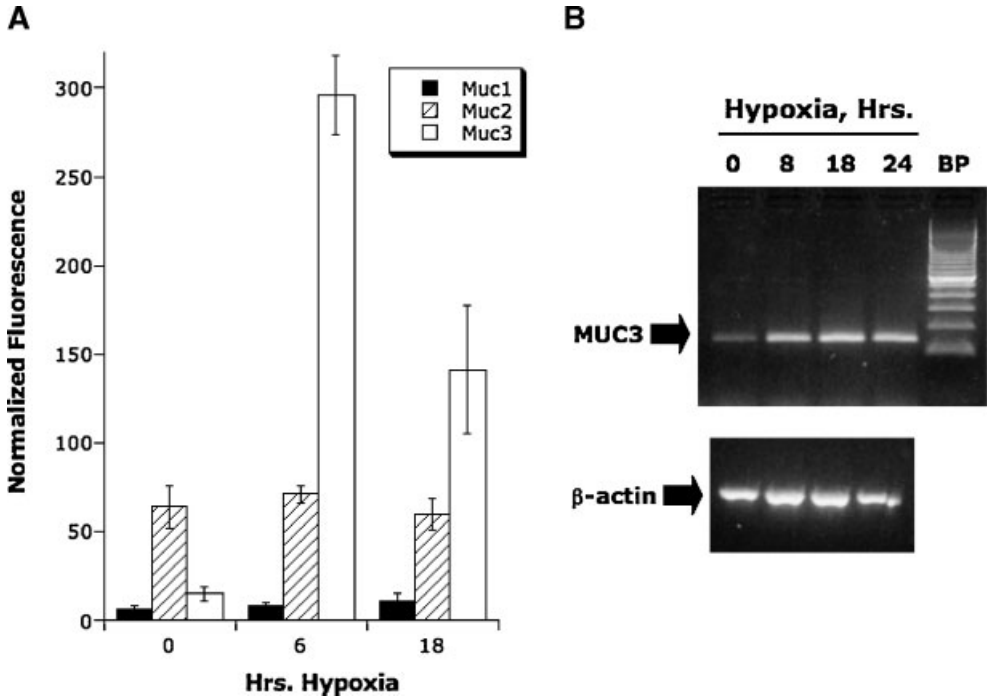

Fig. 1. Influence of hypoxia on intestinal mucin expression and in cultured intestinal epithelial cells. **A**: Cultured T84 cell monolayers grown to confluence were exposed to hypoxia for 0, 6, and 18 h. Relative MUC3 mRNA levels were assessed by affymetrix gene chip with expression levels depicted as normalized fluorescence. **B**: Induction of MUC3 mRNA was confirmed by RT-PCR from T84 cells exposed to hypoxic conditions for 8, 24, or 48 h relative to normoxic controls. As shown in the lower panel, RT-PCR for b-actin was used to control for amount of starting template. Results depict representative blots from 3 different experiments.

integrated band density in cells exposed to hypoxia compared to normoxia). Such induction was maintained through 24 h time periods of hypoxia.

We next assessed whether MUC3 surface protein was induced by hypoxia. Mucins exist predominantly on the apical membrane surface, and as such membrane mucins should be readily labeled with biotin and detectable by avidin blots of immunoprecipitations. As shown in Figure 2A, immunoprecipitation of surface biotinylated protein and avidin blot from epithelial cells subjected to hypoxia (range 0–48 h) revealed a prominent increase in expression of a heavily glycosylated ~300–350 kDa protein consistent with Mucin-3, with maximal protein levels observed by 24 h. Interestingly, such expression diminished with increased exposure to hypoxia (i.e., 48 h). This decrease in MUC3 at 48 h was not associated with cytotoxicity (data not shown), and at present, we do not know the nature of such decrements in surface MUC3 expression. Nonetheless, these observations confirm our mRNA findings and implicate a transcriptionally-mediated induction of surface Mucin-3 by hypoxia.

Association of Surface Mucin-3 with Intestinal Trefoil Factor

Previous studies have suggested that various mucins associate with intestinal trefoil factor (ITF) in the human intestine [Thim, 1997; Williams and Wright, 1997]. In addition, we have previously demonstrated that ITF is a hypoxia-inducible, barrier protective molecule expressed on the surface of intestinal epithelial cells and characterized the kinetics of induction of ITF expression in intestinal epithelial cells, in vitro [Furuta et al., 2001]. This work was also remarkable for an associated augmented release of soluble ITF from the cell surface into the media, first noted following 48 h of hypoxia [Furuta et al., 2001]. Prior work in our lab employed mice with constitutive expression of HIF-1 under normoxic conditions, due to absence of a functional HIF-1 regulating von Hippel Lindau tumor suppressor protein, to further demonstrate that the hypoxic induction of ITF protein is HIF-1 dependent [Karhausen et al., 2004].

Based on these findings, we examined whether MUC3 and ITF associate in a

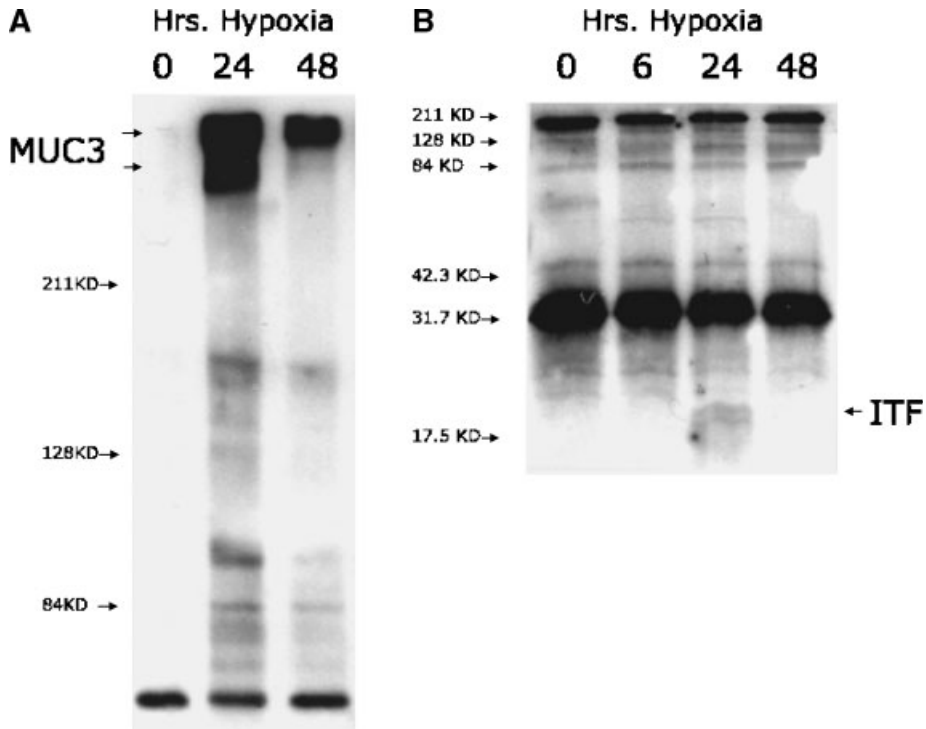

Fig. 2. Hypoxic induction of MUC3 protein and associated ITF in T84 monolayers. **A**: T84 intestinal epithelial cells were grown to confluence and exposed to hypoxia for 0, 24, and 48 h. Cells were surface biotinylated then MUC3 was isolated by immunoprecipitation with high stringency washing and then detected by avidin blotting. **B**: Alternatively antibody-MUC3 immunoprecipitates from T84 cells similarly exposed to varying periods of hypoxia were washed under low stringency conditions allowing preservation of noncovalent interactions. Isolated proteins were then resolved by SDS-PAGE and blotted for ITF using a rabbit polyclonal anti-ITF antibody. Results depict representative blots from 3 separate experiments.

hypoxia-dependent manner. As shown in Figure 2B, probing of MUC3 immunoprecipitates with anti-ITF resulted in detectable expression of ITF at 24 h of hypoxia. Similar to our findings addressing MUC3 expression at 48 h, ITF was undetectable, on the cell surface, at 48 h of hypoxia. We next immunolocalized MUC3 alone, and in association with ITF, following epithelial subjection to hypoxia (24 h). For these purposes, we utilized confocal microscopy (Fig. 3). In normoxic cells (Fig. 3A), MUC3 (shown in green) was prominently expressed on the apical aspect of polarized epithelia, with some diffuse staining along the basal and lateral epithelial aspects. The pattern of ITF expression (shown in red) paralleled that of MUC3, with prominent apical localization. Merged images (Fig. 3B) showed significant co-localization between MUC3 and ITF after 24 h of hypoxia, with x-z images (Fig. 3C) demonstrating increased colocalization more particularly on the apical aspect of the epithelium.

Functional Hypoxia-Response of Murine Muc3 Promoter

Using a previously characterized luciferase reporter construct of the murine Muc3 promoter [Shekels and Ho, 2003], we extended these studies to determine whether the mouse Muc3 promoter is hypoxia-inducible. For these purposes, we used a wild-type luciferase reporter construct to transfect ModeK mouse intestinal epithelial cells. ModeK cells were used for analysis of mouse Muc3 promoter activity both for their ease of transfection relative to T84 cells and in order to optimize expression of the mouse promoter and minimize potential species-specific differences in promoter regulation. As shown in Figure 4, cells transiently transfected with the full-length mouse Muc3 promoter (including nucleotides -1214 to -1) exposed to hypoxia (24 h) showed a 5.09 ± 1.76-fold increase in luciferase activity over normoxia controls ($P < 0.01$).

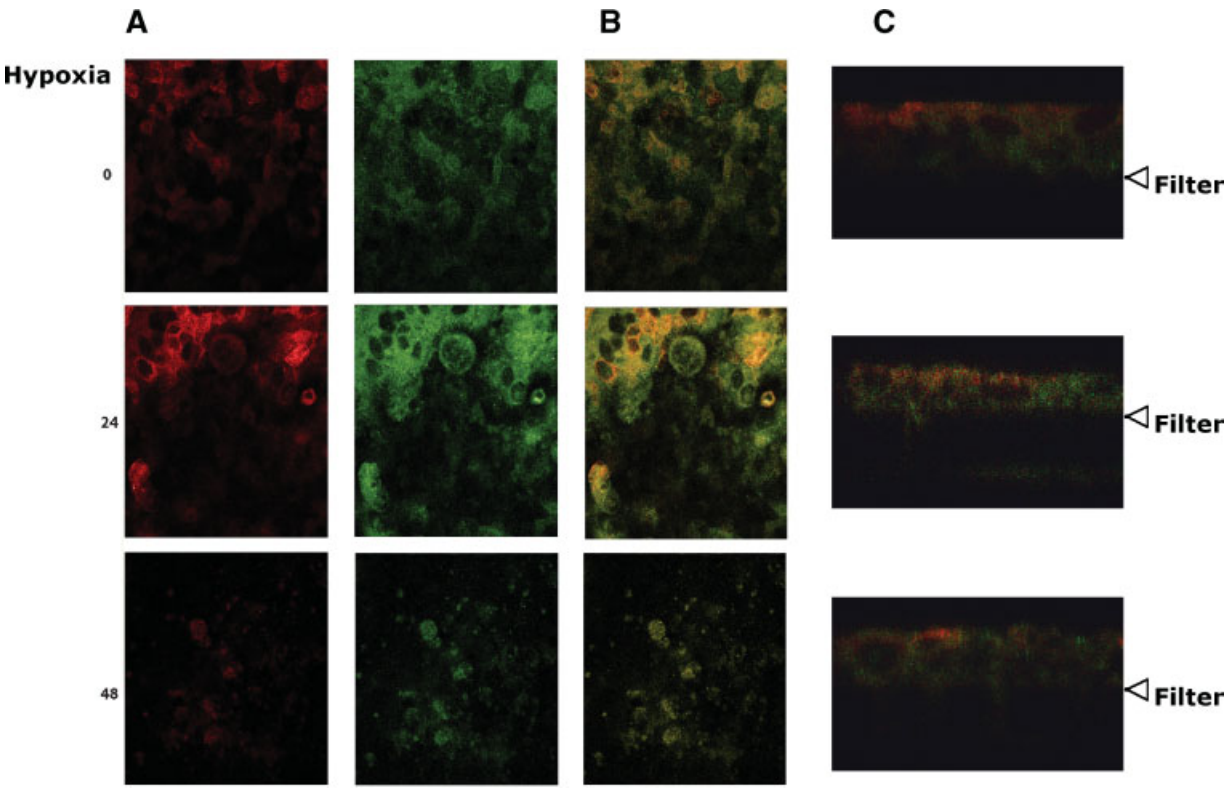

Fig. 3. Colocalization of MUC3 and ITF at the apical surface of cultured intestinal epithelial cells following hypoxic stimulation. T84 intestinal epithelial monolayers were grown as confluent monolayers on polycarbonate supports, fixed, and stained with mouse anti-human MUC3 and rabbit anti-human ITF, followed by detection with species-specific fluorescent secondary antibodies. Confocal microscopy was used to detect the presence of MUC3 (green) or ITF (red) in cells incubated for either 0, 24, or 48 h of hypoxia, as indicated. Areas of colocalization of MUC3 and ITF appear yellow. En face images of the apical surface of each monolayer are shown in (**A**) with colocalization in (**B**) while (**C**) depicts representative merged x-z images from near the apical surface of the epithelial monolayers.

Association of HIF-1α in MUC3 Induction

In an attempt to gain specific insight into the mechanisms of MUC3 induction, we began examining induction pathways from hypoxia response genes. In the course of our experiments, we identified two regions with clusters of previously unappreciated putative HIF-1 binding sites in the mouse MUC3 gene promoter (DNA consensus at positions −385 to −381, −362 to −358, −215 to −211, −166 to −162, −108 to −104, and −55 to −51 relative to the major transcription start site [Shekels and Ho, 2003]). Therefore, we determined whether these regions of the MUC3 promoter bind HIF-1α. For these purposes, we utilized chromatin immunoprecipitation (ChIP) to analyze HIF-1α binding in live cells. As shown in Figure 5A, ChIP analysis of nuclei derived from ModeK cells revealed a prominent band of 122 bp in hypoxic, but not normoxic samples. No bands were evident in control IgG immunoprecipitates, and input samples (pre-immunoprecipitation) revealed the predictable 122 bp band under conditions of both hypoxia and normoxia. Such results indicate that hypoxia induces HIF-1α binding to the distal 122 bp region of the mouse Muc3 promoter extending from −445 to −324 bp. Further ChIP analysis failed to demonstrate HIF-1α binding to the more proximal 260 bp region extending from −295 to −36 bp of the promoter (data not shown).

Inspection of the human MUC3 promoter revealed similar potential binding sites for HIF-1α, with a potential site located from −662 bp to −658 bp and another more proximal potential binding site located from −67 bp to −63 bp. As shown in Figure 5B, ChIP analysis of nuclei from hypoxia-exposed Hela cells revealed a 240 bp band under conditions of hypoxia, but not normoxia, representing HIF-1α binding to the region of the MUC3 promoter extending

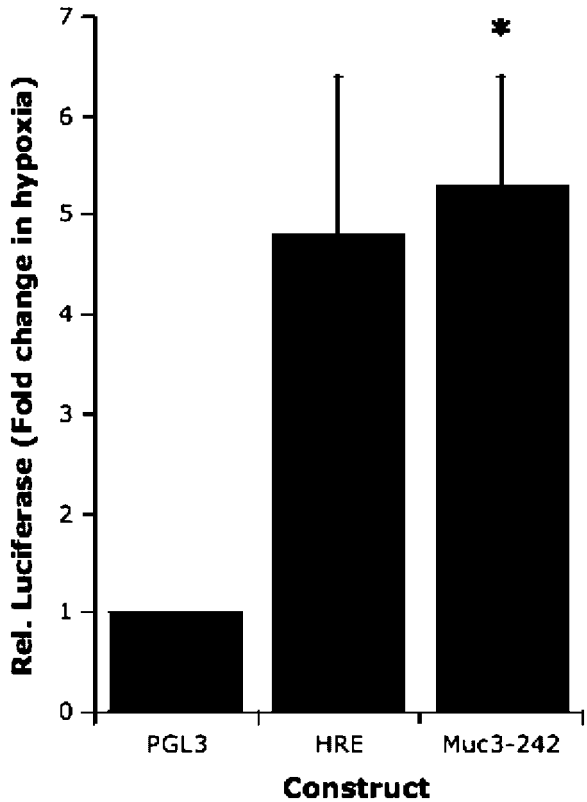

Fig. 4. Influence of hypoxia on the mouse Muc3 promoter. Mode-K mouse intestinal epithelial cells were transfected either with a 1214 bp luciferase reporter construct of the mouse Muc3 promoter (MUC 242), promoterless empty vector (PGL3), or an HRE plasmid containing 4 tandem hypoxia-responsive elements as a positive control. For each experiment, cells were co-transfected with renilla luciferase vector as a control for transfection efficiency. Cells were incubated for 48 h under either normoxia or hypoxic conditions and then assayed for firefly and renilla luciferase. Results are normalized according to renilla luciferase and depicted as fold change in hypoxia relative to normoxic controls, presented as mean ± SE, where * indicates significance between individual plasmids and empty PGL3 plasmid ($P < 0.01$).

from −873 bp to −634 bp. Further ChIP analysis failed to demonstrate HIF-1α binding to the region of the promoter encompassing the proximal putative HIF site (data not shown). Taken together, these ChIP analyses and reporter construct data provide strong evidence for a functional hypoxia response element (HRE), mediated by HIF-1, within the mouse Muc3 promoter. Additionally, as described above, ChIP analysis of the human MUC3 promoter supports a role for HIF-1 in the hypoxia-induced expression of the human MUC3 protein.

Expression of MUC3 in Inflamed Human Colon

Previous work in mouse models of colitis has demonstrated that inflamed intestinal epithelium is hypoxic [Karhausen et al., 2004] and that HIF-1 activation is, in turn, protective with respect to preservation of barrier function and severity of disease [Karhausen et al., 2004]. In light of this association between intestinal inflammation and hypoxia and our finding that MUC3 is induced by hypoxia in a HIF-1-dependent manner, we examined the expression of MUC3A in biopsy sections from patients with ulcerative colitis and colitis associated with C. difficile infection. Immunohistochemical staining for mucin 3A (Fig. 6) was more prominent in regions of the apical colonic epithelium of patients with ulcerative colitis (Fig. 6B), relative to unaffected controls (Fig. 6A). There was a similar, although less prominent, increase in apical epithelial MUC3 staining in sections from patients with C. difficile colitis.

DISCUSSION

These studies provide a molecular link between hypoxia and intestinal epithelial barrier protection. Here, we identify a previously unappreciated HIF-1α regulated pathway for induction of MUC3.

The hypoxia-dependent induction of MUC3 represents a novel, innate protective mechanism that may guard the immunologic components of the lamina propria from exposure to pathogenic luminal bacteria, antigens, and toxins during episodes of diminished oxygen delivery. Accumulating evidence suggests a variety of physiological roles for mucins [Rose, 1992; Mack et al., 1999, 2003; Wright, 2001], however, molecular mechanisms regulating mucin expression are just beginning to be studied in detail. Here, we demonstrate that MUC3 expression is regulated by HIF-1, a member of the Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors [Kewley et al., 2004]. HIF-1 exists as an αβ heterodimer, the activation of which is dependent upon stabilization of an O_2-dependent degradation domain of the α subunit by the ubiquitin-proteasome pathway [Semenza, 2001]. Of note on this accord, we did not precisely map the MUC3 HRE. Attempts to define this site in exact terms were hampered by the complexity of the flanking region around the HIF-1 consensus sequence (data not shown). For example, the immediate region of the HIF-1 consensus site also contains potential

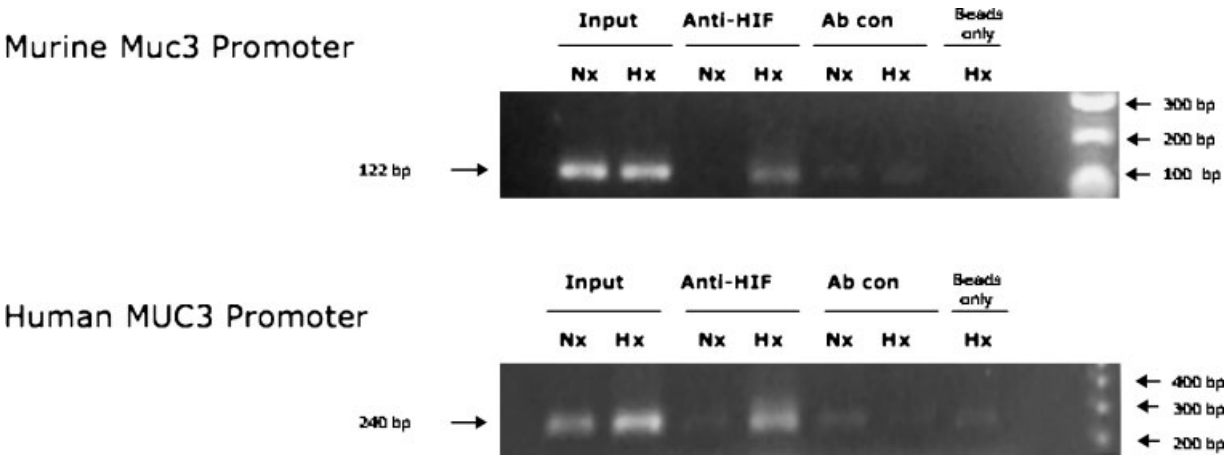

Fig. 5. Binding of HIF-1a to human and murine mucin 3 promoters. Chromatin immunoprecipitation was used to examine HIF-1a binding to the mouse Muc3 promoter in Mode-K cells (**panel A**) and the human MUC3 promoter in Hela cells (**panel B**) grown under normoxia or 18 h of hypoxic conditions. Reaction controls included PCR performed using cell-type specific whole cell genomic DNA (input), sample precipitated with isotype matched control antibody, and samples precipitated with protein G sepharose beads alone.

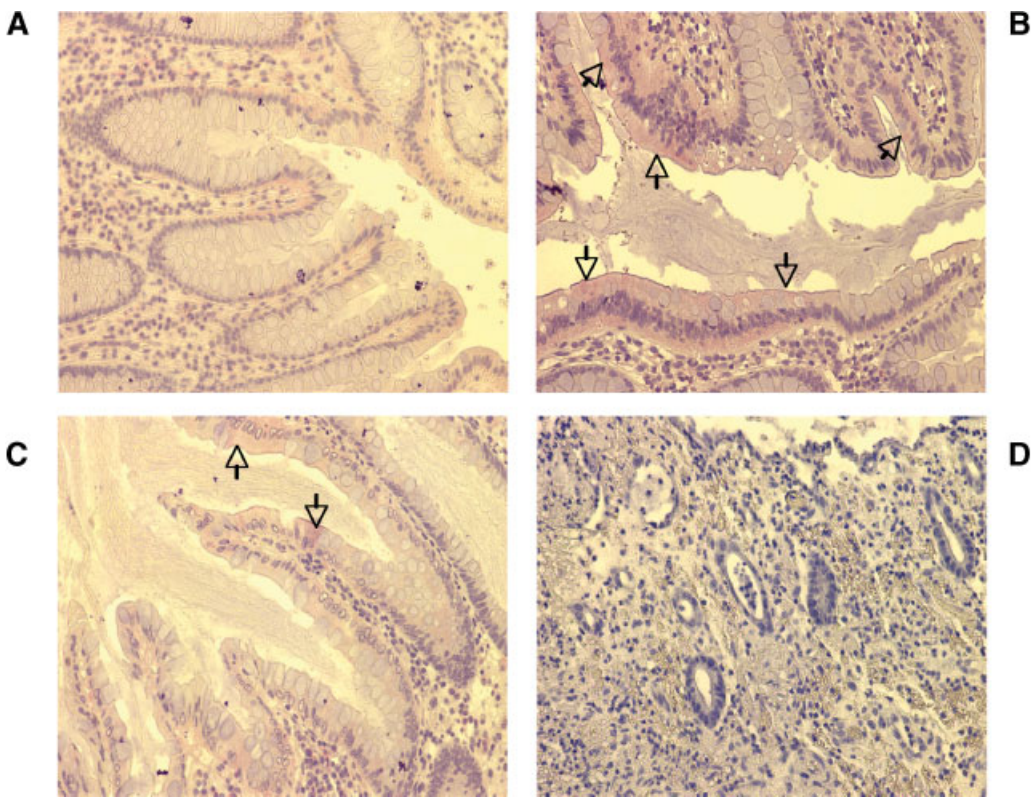

Fig. 6. MUC3 expression in inflamed human colon. Paraffin embedded biopsy sections obtained from normal colon (**A**) as well as from patients with ulcerative colitis (**B**) and C. difficile colitis (**C**) were stained for MUC3A expression (pink) and counterstained with hematoxylin (blue). Arrows indicate patchy areas of pink apical epithelial staining. Background staining is demonstrated with a disease control section from a patient with C. difficile colitis stained without primary antibody (**D**).

consensus binding sites for the transcription factors c-rel, NFKB, the glucocorticoid receptor, the estrogen receptor, and CREB. Important in this regard, some of these transcription factors, such as NFKB [Wu et al., 2004; Witt et al., 2005], its isoform c-rel [Leeper-Woodford and Detmer, 1999], and CREB [Taylor, 2004] have been implicated in either induction or repression of genes in hypoxia. Therefore, it is possible that regulation of the *MUC3* gene at this site could involve an interplay between positive and negative regulatory signals, and given this complexity, more work will be necessary to define the exact details of this HRE.

As part of our analysis, we showed a physical association between MUC3 and ITF. ITF is a small, protease-resistant peptide that is abundant throughout the mammalian intestinal tract [Williams and Wright, 1997; Taupin and Podolsky, 2003]. Increased ITF expression has been observed in proximity to sites of injury in the gastrointestinal tract, including peptic ulcers [Cook et al., 1997] and active inflammatory bowel disease [Wright et al., 1993]. ITF can promote epithelial barrier function, protecting against injury and facilitating repair after damage occurs irrespective of the nature of the initial wound. However, specific transcriptional pathways for ITF induction are not well understood [Itoh et al., 1999; Taupin and Podolsky, 2003]. ITF has also been associated with various mucin molecules [Wiede et al., 1999], and it has been suggested that trefoil peptides may act, in part, by stabilizing the protective mucous gel layer overlying the epithelium [Wiede et al., 1999; Wright, 2001; Thim et al., 2002]. Given these findings, it will be important to further examine the functional implications of this association between MUC3 and ITF. Efforts to examine this phenomenon have thus far been hindered by technical issues related to the nontransfectable nature of intestinal epithelial cells, which are functionally mature with respect to the achievement of intact barrier function, in vitro.

However, this evidence of ITF association with MUC3, provides a novel link between the barrier restorative peptide, ITF and a specific mucin whose altered expression has also been linked both to inflammatory bowel disease and eventual malignant transformation. We further demonstrate altered expression of MUC3A in affected colonic tissue from patients with ulcerative colitis. Of note, our results are in contrast with previous reports of decreased total MUC3 expression in patients with Crohn's disease. This may be related to staining specifically for MUC3A rather than total MUC3 or, less likely, related to intrinsic differences between Crohn's disease and ulcerative colitis. Our results suggest that the MUC3/ITF association may represent a potential novel therapeutic approach for a variety of diseases associated with mucosal hypoxia and altered epithelial barrier function, including inflammatory bowel disease [Wakefield et al., 1989], necrotizing enterocolitis [Hsueh et al., 2003], and ischemic colitis [Okuda et al., 2005]. Efforts to better understand MUC3 signaling pathways and to identify other hypoxia-elicited protective elements could provide future focus for development of novel treatments.

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