

HIF-dependent induction of apical CD55 coordinates epithelial clearance of neutrophils

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ABSTRACT Sites of inflammation are associated with dramatic shifts in tissue metabolism. Inflammation can result in significant tissue hypoxia, with resultant induction of hypoxia-responsive genes. Given this association, we hypothesized that neutrophil (PMN) ligands expressed on epithelial cells may be regulated by hypoxia. Initial studies confirmed earlier results that epithelial hypoxia enhances PMN transepithelial migration and promotes apical clearance of PMN from the epithelial surface. A screen of known PMN ligands revealed a surprisingly stable expression pattern in hypoxia. However, this screen identified one gene, CD55, as a highly hypoxia-inducible molecule expressed on the apical membrane of mucosal epithelia. Subsequent studies verified the induction of CD55 mRNA and protein expression by hypoxia. Overexpression of CD55 by transfection in nonhypoxic epithelia resulted in a similar pattern of apical PMN clearance, and peptide mimetics corresponding to the PMN binding site on DAF blocked such apical clearance of PMN. Studies directed at understanding molecular pathways of hypoxia inducibility revealed that a ~200 bp region of the CD55 gene conferred hypoxia inducibility for CD55. These studies identified a functional binding site for the transcriptional regulator hypoxia-inducible factor (HIF). Taken together, these results identify HIF-dependent induction of epithelial CD55 in the resolution of ongoing inflammation through clearance of apical PMN.—Louis, N. A., Hamilton, K. E., Kong, T., Colgan, S. P. HIF-dependent induction of apical CD55 coordinates epithelial clearance of neutrophils. *FASEB J.* 19, 950–959 (2005)

Key Words: inflammation • adhesion • mucosa • leukocyte • resolution

Ongoing inflammatory responses are characterized by dramatic shifts in tissue metabolism. These changes include lactate accumulation with resultant metabolic acidosis and diminished availability of oxygen (hypoxia) (1–3). Such shifts in tissue metabolism result at least in part from profound recruitment of inflammatory cell types, particularly myeloid cells such as neutrophils (PMN) and monocytes (4). The vast majority of inflammatory cells are recruited to, as opposed to resident at, inflammatory lesions (5). It is important to

understand the interactions between microenvironmental metabolic changes (e.g., hypoxia) as they relate to recruitment signals and molecular mechanisms of leukocyte recruitment during inflammation (4). Little is known about how hypoxia directly influences mucosal inflammation at the molecular level. Some studies have implicated hypoxia in inflammatory diseases such as colitis (6–9); recent studies in murine models identified the epithelium as the central target of hypoxia during ongoing inflammation (10).

Transepithelial migration of PMN is a morphological marker of active mucosal inflammation and occurs in inflammatory bowel disease (IBD), ischemic colitis, and infectious enterocolitis (11). Before movement through the interstitium to the epithelium, PMN first escape the vasculature through a distinct, well-characterized set of interactions with the endothelium. Less is known about leukocyte interactions with epithelial cells. The current paradigm for transepithelial migration of PMN across epithelial monolayers envisions a process consisting of sequential molecularly defined events, including initial CD11b/18-mediated interactions of PMN with members of the junctional adhesion molecule (JAM) family of proteins (12). Subsequent CD47-mediated orchestration of PMN movement through the paracellular space is now well accepted, involving CD47 interactions with signal regulatory protein- α (SIRP α) (13). Recent studies implicate decay accelerating factor (DAF, CD55) in the terminal stages of epithelial-PMN interactions at the apical surface (14). These studies and others (15) have revealed that CD55 functions as an anti-adhesive ligand promoting the release of PMN from the epithelial surface as a terminal step in this pathway. Based on these studies, we defined the influence of epithelial hypoxia on PMN trafficking in an established in vitro model (16).

Initial studies of epithelial hypoxia identified a transcription-dependent induction of PMN transmigration. Subsequent profiling of potential hypoxia-inducible PMN ligands identified CD55 as a central determinant for enhanced rates of PMN transmigration. Additional studies probing molecular details of CD55 induction by

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doi: 10.1096/fj.04-3251com

hypoxia identified a previously unappreciated pathway involving transcriptional regulation by hypoxia-inducible factor-1 (HIF-1). Such studies provide new insight into contributions of hypoxia to inflammatory disease processes and suggest that hypoxia-inducible CD55 enhances PMN clearance, herein defined as liberation of PMN from the apical epithelial surface.

MATERIALS AND METHODS

Cell culture

Caco-2 cells and T84 cells were grown on permeable 5 cm² or 0.33 cm² polycarbonate ring supports, 5 mm pore size (Costar Corp., Cambridge, MA, USA), using described techniques (16). Cells were plated as inverts as described (16). T84 cells for transmigration assay were grown on collagen-coated supports and monolayers were used 5–7 days after plating when high resistance monolayers had been established (based on transepithelial resistances of > 800 ohm*cm²). Extensive experience with the in vitro hypoxia model has demonstrated that confluent T84 monolayers remain intact and viable beyond 48 h in hypoxia (16–20). Such viability studies are based on measurement of transepithelial resistance (TER), a sensitive measurement of monolayer integrity and epithelial viability (21). TER was screened for surveillance monolayers from each passage prior to transmigration and used only if TER remained > 800 ohm*cm². HeLa cells used for promoter transfection were plated on 24-well plates, grown to confluence, and treated as described (22). Where indicated, human umbilical vein endothelial cells (HUVEC) were cultured as described (23, 24).

Differential biotinylation of apical or basolateral surface proteins

Differentiation between apical and basolateral expression of CD55 was performed as described (14, 20). Briefly, confluent T84 cell monolayers were exposed to 48 h of hypoxic or normoxic conditions. Monolayers were then washed once with HBSS with 10 mM HEPES, pH 7.4, and exposed at the apical, basolateral, or both surfaces to 1 mM sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL, USA) in HBSS for 10 min at 4°C. The monolayers were repeatedly washed with HBSS containing 150 mM NH₄Cl to quench residual biotin. After centrifugation to remove cellular debris, 10 µg/mL OE1 antibody was added to immunoprecipitate CD55. Biotinylated surface CD55 was resolved by SDS-PAGE and detected by avidin-horseradish peroxidase (Pierce Chemical).

Real-time PCR

mRNA expression was quantified by real-time PCR (iCycler; Bio-Rad Laboratories Inc., Hercules, CA, USA), as described (25). Primer sets containing SYBR Green I (sense, antisense, and transcript size, respectively, were: JAM-B (5'-CCA ACA AGT AGT CAC AGC AG-3', 5'-CAC TCC GAC CCA GTT TCT TC-3', 107 bp), CD55 (5'-TGA ATC GAG ATG TCC ATA GTC AA-3', 5'-CGG ATC ATT TAT TTC TAT TTA GGA A-3', 140bp), Occludin (5'-GGA GGC TGG TAG ATC ATC AC-3', 5'-TGG GTT CCT GCA ATC AAG-3', 147 bp), CD47 (5'-GAA GTG ATG GAC TCC GAT TTG-3', 5'-AAG GGT CTC ATA GGT GAC AAC, 135 bp), ICAM-1 (5'-CCA AGG TAT TGG AGG ACT CC-3', 5'-CAA GAC TGC AGT GAA CCA TG-3', 135 bp) JAM-A (5'-CCA CAA CAA GAG CTC CCA TT-3',

5'-ACT GGG GTC CTT CCA TCT CT-3', 141 bp), and for JAM-C (5'-AAG TCT GTA GCA TCT GTG TAC AG-3', 5'-AGA GAA GTG AAA GTA GAG TCT GG-3', 167 bp). A β-actin primer set (5'-GGT GGC TTT TAG GAT GGC AAG-3', 5'-ACT GGA ACG GTG AAG GTG ACA G-3', 161 bp) was used to control for starting cDNA template concentrations. Transcript levels and fold change in mRNA were determined as described (26).

CD55 protein detection

Indicated cells were grown to confluence on 5 cm² polycarbonate supports and treated as described. Monolayers were then washed and lysed with nonreducing Laemmli sample buffer. Proteins were resolved by electrophoresis on a 10% polyacrylamide gel, then transferred to nitrocellulose membranes. A characterized mouse monoclonal antibody (clone OE1) was selected from a panel of antibodies generated to epithelial plasma membranes according to its ability to specifically regulate neutrophil transmigration at the apical membrane surface. The antibody was demonstrated to specifically bind to and block CD55 function within the short consensus repeat-3 region (14). Membranes were probed for CD55 using and developed by enhanced chemiluminescence as described (14). Representative blots were then probed for β-actin expression as a control for protein load using a mouse monoclonal anti-human β-actin antibody (Abcam Inc., Cambridge, MA, USA).

Transmigration/adhesion assays

PMN were freshly isolated from whole blood obtained by venipuncture from healthy human donors and anticoagulated with acid citrate dextrose as described (27), with the following exception: PMN sedimentation was achieved with a dual gradient consisting of histopaque and 2% gelatin. Whole blood was layered onto Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA), and plasma and mononuclear cells were removed by aspiration from the buffy coat after centrifugation (400 g, 25 min) at room temperature. RBCs were sedimented using 2% gelatin and residual RBCs were removed by lysis in ice-cold NH₄Cl buffer. PMN were then resuspended to a final concentration of 2.5 × 10⁷ in HBSS with 10 mM HEPES, pH 7.4, without Ca²⁺ or Mg²⁺ (Sigma-Aldrich). PMN were used within 2 h after isolation.

PMN transmigration assays were performed as described (14). All experiments were performed in the physiologically relevant basolateral-to-apical direction. Briefly, 10⁶ PMNs were added to the upper chambers of transwell inverts and stimulated to migrate in response to an *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) gradient. PMN transmigration was performed at 37°C for the periods indicated. Transmigrated PMNs were quantified by assay for the azurophilic enzyme myeloperoxidase (14). To block the CD55 neutrophil binding site, posthypoxic T84 monolayers were preincubated at the apical surface for 15 min with a characterized consensus sequence peptide for the epithelial CD55-neutrophil binding site or scrambled peptide control (14), then assayed for PMN transmigration, as described above.

DAF consensus sequence peptide

A consensus sequence peptide for the epithelial DAF-OE1 binding epitope (amino acid sequence EVEHWYRSG) was identified by phage panning of a random 9-mer LL9 phage library for affinity binding to the OE1 antibody, as described (14). Briefly, phage expressing peptide sequences recognized by the OE1 antibody were selected, amplified, then subjected

to sequence analysis to determine a consensus sequence. The resultant consensus peptide was synthesized, along with a scrambled control (amino acid sequence SPLAQAVRSSSR) and assayed for the ability to bind OE1 as well as affect PMN transmigration (14). PMNs were preincubated with varying concentrations of peptide, then applied to the basolateral surface of T84 monolayers after 48 h of hypoxia; transmigration and apical accumulation were assessed relative to control monolayers, as described above.

CD55 promoter constructs

PCR primers were designed to recognize an 820 bp region of the CD55 promoter based on the human CD55 promoter (28). An 842 bp segment of DNA was isolated and cloned into the PGL3Basic luciferase expression vector (Promega, Madison, WI, USA). Homology to the published sequence was determined by sequencing by the BWH core sequencing facility.

Truncations of the CD55 promoter were generated using a combination of restriction enzyme digestion and site-directed mutagenesis (QuikChange XL site-directed mutagenesis kit, Stratagene, San Diego, CA, USA) to introduce unique restriction sites. Luciferase construct truncations including sequentially decreasing continuous segments of the promoter region upstream of the transcription initiation site spanning positions -352 to +88, -200 to +88, -157 to +88, -106 to +88, and -60 to +88 (termed -352, -200, -157, -106, and -60, respectively) were generated and tested.

Binding site mutants were generated by PCR using site-directed mutagenesis (in -106 plasmid) with the following primer sets: for Sp1 mutant (5'-CAG AGC CCC AGC CCA GAC CCA AAC CAA AGC ACT C-3', 5'-ATG AGT GCT TTG GTT TGG GTC TGG GCT GGG GCT CTG-3'); CRE/Ap1 mutant (5'-GTC CCA CCC TTG GTG TAC CAG AGC CCC AGC CCA G-3', 5'-CTG GGC TGG GGC TCT GGT ACA CCA AGG GTG GGA C-3'); USF mutant (5'-CTG GTA TTG CGG AGC CAC AAC GCT TCT GAC TTA CTG CAA CTC-3', 5'-GAG TTG CAG TAA GTC AGA AGC GTT GTG GCT CCG CAA TAC CAG-3'); and HIF-1 mutant (5'-AGC CCA GAC CCC GCC CAA ATA GAT CAT TTA ACT GGT ATT GCG-3', 5'-CGC AAT ACC AGT TAA ATG ATC TAT TTG GGC GGC GTC TGG GCT-3'). All mutations were screened by sequencing, then transfected into HeLa cells, subjected to hypoxia, and assayed as described above. CD55 promoter constructs were cotransfected with a Renilla luciferase vector into HeLa cells using polyfect (Qiagen, Valencia, CA, USA). As a 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 (29). Caco-2 cells overexpressing CD55 were generated by transient transfection with a plasmid containing the full-length CD55 gene in a pcDNA3 vector (kindly provided by Dr. J. K. Atkinson, Washington University School of Medicine, St. Louis, MO, USA) (30). In each case, the cells were incubated overnight, then exposed to hypoxia and harvested for luciferase or assayed for PMN transmigration, as indicated.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as described (31) with minor modifications. HeLa cells were grown to confluence, fixed with 1% formalin, and chromatin was isolated and sheared. Sheared chromatin was incubated with an anti-HIF-1 α antibody (Transduction Laboratories, Lexington, KY, USA) and immune complexes were precipitated with protein G Sepharose beads. Cross-linking was then reversed and DNA was purified using a PCR purification kit (Qiagen).

HIF binding to the CD55 promoter DNA was quantified by standard PCR using primers (sense, 5'-CCA GCG ATC TCC TCC TCC T-3' and antisense 5'-CCG AGT CGC ATC TAC GCC C-3') designed to amplify a 256 bp region of the CD55 promoter extending from -210 bp to +46 bp with respect to the initiation start site. Chromatin incubated with beads without antibody was used to control for nonspecific binding of DNA.

Data analysis

PMN transmigration and luciferase reporter data were compared by 2-factor ANOVA or Student's *t* test, where appropriate. Values are expressed as the mean and SE from at least three separate experiments.

RESULTS

Influence of hypoxia on PMN TM dynamics

Initially, we confirmed earlier findings indicating that pre-exposure of epithelia (T84 cells) to hypoxia enhances physiologically directed (i.e., basolateral-to-apical) PMN TM (16). Here we used a recently described kinetic model (32) to examine the dynamics of PMN TM over 90 min. As shown in Fig. 1, epithelial pre-exposure to hypoxia (pO₂ 20 torr, 48 h) resulted in a significant shift in the kinetics of fMLP-stimulated PMN TM ($P < 0.01$ by ANOVA). These studies indicated that at as early as 30 min, significantly more PMN had migrated across posthypoxic epithelial monolayers ($P < 0.05$). Such enhanced TM was evident at each point over the 90 min period. Calculated rates of PMN TM revealed a nearly 2-fold increase across posthypoxic epithelia (rates of 10.4×10^3 vs. 20.0×10^3 for epithelia pre-exposed to normoxia and hypoxia, respectively, $P < 0.0001$).

In parallel studies we addressed whether hypoxia might regulate the rate of PMN detachment from the apical epithelial membrane. The rationale for these studies stems from recent work indicating that PMN ligands expressed on the apical surface of epithelia (e.g., CD55) control PMN displacement from apical membrane surface and, as such, serve as an anti-adhesive terminal step in the PMN TM. As shown in Fig. 1B, increased rates of PMN TM (Fig. 1A) were attributable in great part to increased disengagement of PMN from the apical surface of posthypoxic epithelia. Indeed, early assessment of apical PMN revealed a nearly 3-fold increase in apical PMN ($P < 0.025$). Apical PMN numbers remained elevated relative to normoxic controls for 60 min and rapidly declined thereafter as a result of apical disengagement. Such studies indicate that hypoxia significantly influences the dynamics of PMN trafficking through epithelial monolayers.

Identification of hypoxia-inducible CD55

To gain initial insight into mechanisms of increased PMN TM by hypoxia, we determined whether enhanced PMN TM required new transcriptional activity.

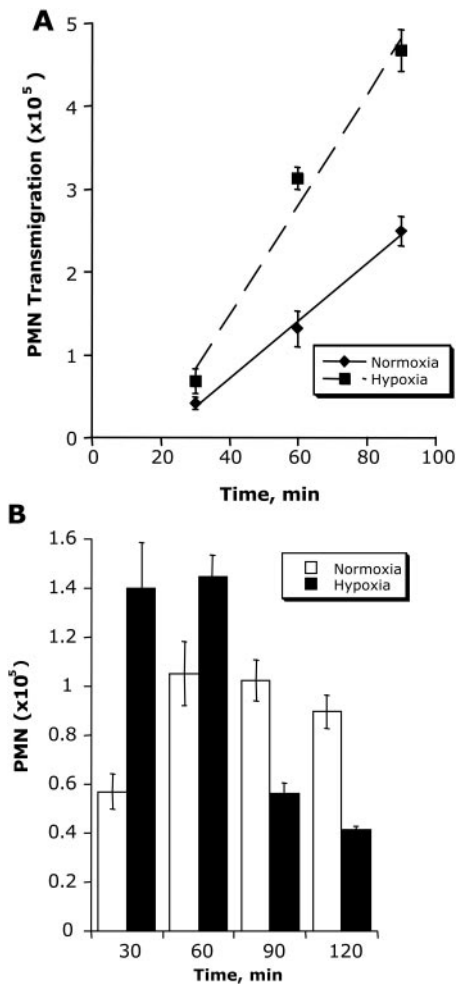


Figure 1. Influence of hypoxia on kinetics of neutrophil transmigration. *A*) Monolayers were exposed to hypoxia for 48 h, then assayed for neutrophil transmigration. The number of PMN completely traversing the monolayer was assayed at each time point. Results are depicted as the number of transmigrated neutrophils vs. time after 48 h pre-exposure to hypoxia (■) relative to normoxic controls (◆). A best linear fit was determined for the data from normoxic (—) and hypoxic (---) monolayers, and rates of transmigration were determined by the slope of the line corrected for monolayer surface area. *B*) The number of PMN accumulating at the apical surface of posthypoxic T84 monolayers (■) is depicted relative to normoxic controls (□). Data are presented as a mean + SE for 9 monolayers per condition from 3 experiments.

To do this, T84 cells were subjected to hypoxia in the presence or absence of the transcriptional inhibitor 5,6-dichlorobenzimidazole riboside (DRB, 10 μ M) and assessed for changes in PMN TM. These experiments revealed an $81 \pm 7\%$ decrease in hypoxia-regulated PMN TM in the presence of DRB ($P < 0.01$). These findings were not a result of epithelial toxicity as measured by epithelial permeability (electrical resistance, data not shown) and LDH release (data not shown). Consequently, such increases in TM by hypoxia require new mRNA synthesis.

Based on these findings of increased PMN TM kinetics and the necessity for new epithelial mRNA synthesis,

we profiled the influence of hypoxia on epithelial mRNA expression of a panel of molecules shown to either modulate PMN transmigration such as occludin (33) or function as PMN ligands, including CD47 (34), intercellular adhesion molecule-1 (ICAM-1) (35), and members of the JAM family of proteins (12). As shown in **Table 1**, we used real-time PCR to profile mRNA derived from T84 cells exposed to various time points of hypoxia. As can be seen, mRNA expression patterns of PMN ligands in response to hypoxia were surprisingly stable. No differences in expression were observed for ICAM-1, occludin, JAM-A, JAM-B, or JAM-C after 2, 6, or 18 h hypoxia. A small decrease ($\sim 30\%$) in CD47 expression was noted, although we had shown that CD47 is unlikely to be important for regulation of hypoxia-induced PMN TM (16). The exceptional observation was CD55 (**Table 1**, **Fig. 2A**), whereby CD55 mRNA was rapidly (within 2 h) induced by hypoxia (maximal 6.2 ± 0.7 -fold increase over normoxia, $P < 0.001$) and returned to baseline within 18 h. We verified these results in a nonepithelial primary cell (HUVEC) and revealed that hypoxia (12 h period) induced CD55 mRNA by 3.4 ± 0.6 -fold ($P < 0.025$ compared with normoxia). We recently showed that CD55 represents an apically expressed PMN ligand that functions in a novel fashion to promote the detachment of PMN from the luminal surface (i.e., an "anti-adhesive" ligand) (14).

We next verified these findings of CD55 mRNA induction by hypoxia at the protein level. CD55 protein expression was confirmed in T84 and Caco-2 cells (**Fig. 2B**). Increased levels of CD55 protein (determined by Western blot) were evident by 24 h hypoxia in T84 cells and remained elevated relative to controls after 48 h exposure. Caco-2 cells showed a similar response relative to T84 cells with induction in CD55 protein after 24 h hypoxia and persisting for up to 48 h hypoxic exposure. Such findings are consistent with work in HUVEC indicating induction of CD55 cell surface protein by hypoxia (23, 24). Differential biotinylation studies (**Fig. 2C**) further confirmed that baseline CD55 expression as well as hypoxia-induced CD55 was limited to the apical membrane (i.e., no change in polarity). Such findings identify the apically expressed epithelial

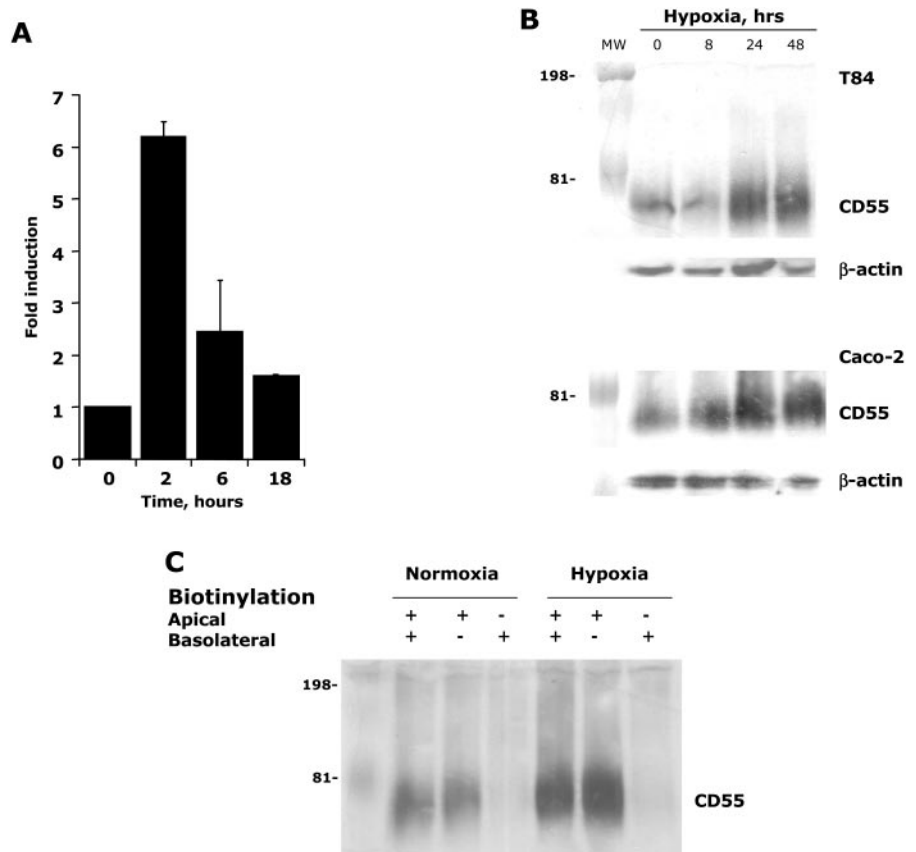
TABLE 1. Influence of hypoxia on epithelial PMN ligand expression^a

Epithelial gene	Time in hypoxia (h)		
	2 h	6 h	18 h
CD55	$6.2 \pm 0.30^*$	$2.4 \pm 0.99^*$	$1.6 \pm 0.04^*$
CD47	$0.7 \pm 0.06^*$	$0.6 \pm 0.09^*$	$0.7 \pm 0.07^*$
ICAM-1	1.0 ± 0.01	1.0 ± 0.01	1.0 ± 0.01
Occludin	1.1 ± 0.15	1.0 ± 0.26	1.1 ± 0.05
JAM-A	1.2 ± 0.12	1.2 ± 0.05	1.0 ± 0.18
JAM-B	0.9 ± 0.16	1.2 ± 0.12	1.0 ± 0.22
JAM-C	0.9 ± 0.08	0.9 ± 0.09	0.9 ± 0.08

^a Results presented as fold change in mRNA (\pm SEM) relative to normoxia, each normalized to β -actin. * $P < 0.025$ compared to normoxia controls.

Figure 2. Influence of hypoxia on CD55 mRNA and protein expression in cultured intestinal epithelial cells.

A) Cultured T84 cell monolayers grown to confluence were exposed to hypoxia for 0, 2, 6, or 18 h of hypoxia. Relative CD55 mRNA levels were assessed by real-time PCR with fold changes determined by normalization for background fluorescence and β -actin expression relative to normoxic controls. **B)** Monolayers of T84 cells (top panel) or Caco-2 cells (bottom panel) were grown to confluence and exposed to 0, 8, 24, or 48 h of hypoxia. Representative Western blots depict CD55 expression after hypoxic exposure for T84 (top) or Caco-2 cells (bottom). Results depict representative blots from 3 separate experiments for each cell line. These same blots were probed for β -actin expression as control for protein loading for each blot. **C)** Confluent monolayers of T84 cells were exposed to 48 h of normoxia or hypoxia and biotinylated at the apical, basolateral, or both surfaces. Cells were lysed and CD55 was then isolated by immunoprecipitation and detected by avidin blotting. Representative avidin blot depicts apical vs. basolateral CD55 for both conditions.



anti-adhesive molecule CD55 as a dominantly expressed PMN ligand rapidly inducible by hypoxia.

Role of hypoxia-inducible CD55 in PMN TM

Based on the above results, we hypothesized that CD55 induction by hypoxia functionally explains increased PMN TM after hypoxia. Two approaches were used to test this hypothesis. First, we forced overexpression of CD55 in intestinal epithelial cells under normoxic conditions and examined the influence on PMN TM. As shown in **Fig. 3A**, transient transfection of full-length CD55 cDNA in Caco2 cells resulted in a nearly 5-fold increase in CD55 expression, comparable to that seen with 48 h hypoxia (see **Fig. 2B**). Using these cells, we then examined whether PMN TM in normoxia might be different from mock-transfected cells. As shown in **Fig. 3B**, overexpression of CD55 resulted in a 3.7 ± 0.4 -fold increase in PMN TM and a significant decrease in apical PMN (Fig. 3C). Calculated rates of PMN TM revealed a nearly 1.7-fold increase across cells transfected with hCD55 (rates of 10.5×10^3 PMN/min/cm² vs. 17.7 for mock and hCD55 transfected, respectively, $P < 0.01$ by ANOVA). This pattern is precisely the pattern we observe after epithelial exposure to hypoxia (see **Fig. 1**), suggesting that increased surface CD55, independent of hypoxia, significantly regulates the dynamics of PMN TM.

Second, we used a characterized blocking peptide (linear sequence EVEHWYRSG) directed against the

PMN binding site of epithelial CD55 (14) and examined the relative influence of this peptide on PMN TM after epithelial exposure to hypoxia. As shown in **Fig. 3D**, this synthetic peptide blocked PMN TM and accumulation of PMN on the apical surface of epithelia to a greater extent on posthypoxic epithelia ($P < 0.025$ by ANOVA). As before (14), parallel experiments performed with a scrambled peptide control revealed no change in PMN TM or PMN accumulation (data not shown). Such data support our findings that hypoxia-induced CD55 expression is functionally important for PMN TM.

Role of HIF-1 in CD55 induction

We next gained insight into mechanisms of CD55 induction by hypoxia. Studies using the transcriptional inhibitor DRB (see above) indicate the likelihood that CD55 induction by hypoxia requires new mRNA synthesis; therefore, we examined the influence of hypoxia on CD55 promoter activity. The CD55 promoter had been cloned and characterized (28); based on this work, we cloned an 842 bp sequence into a PGL3 luciferase reporter vector (**Fig. 4A**). Sequence analysis of this 842 bp construct showed > 99% homology to the CD55 promoter (positions -733 to +88 relative to the major transcription start site). Minor differences from the published sequence consisted of 21 bp insert occurring between -535 and -534 bp and a single bp deletion at position +2. As shown in **Fig. 4B**, transient

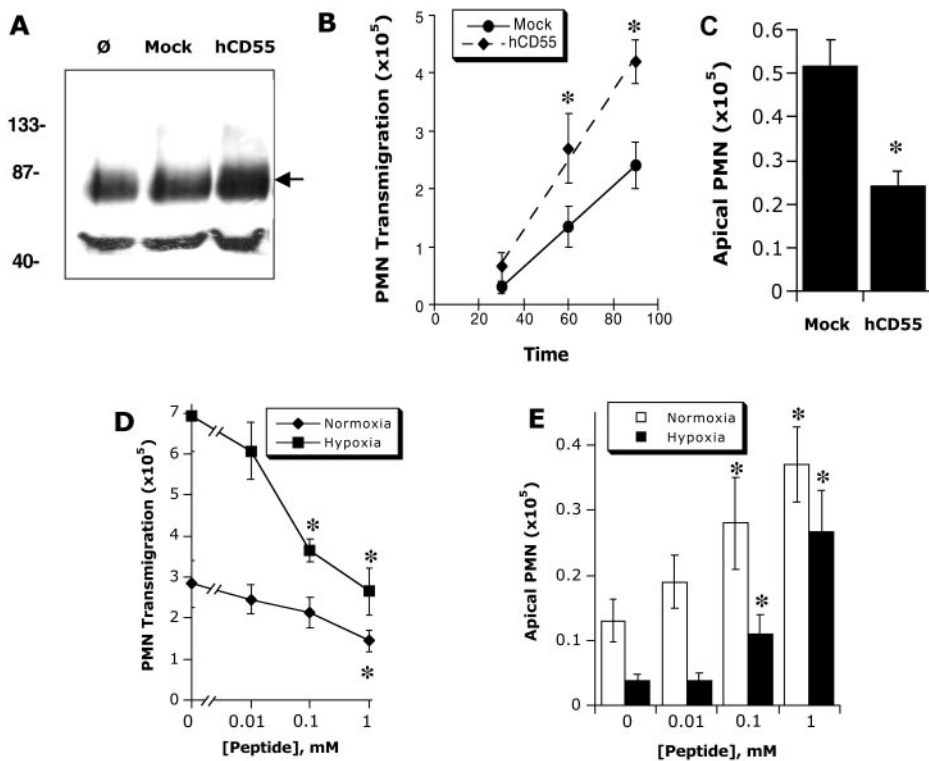


Figure 3. Influence of CD55 on PMN transmigration. *A)* Caco-2 cells transfected with wild type human CD55 (hCD55) gene were assayed for CD55 expression relative to nontransfected or mock transfected controls, as shown by representative Western blot. The same blots were probed for β -actin expression as a control for protein loading. *B)* Caco2 cells overexpressing CD55 (—) were assayed for PMN transmigration relative to mock transfected controls (---). Data was analyzed for linear fit and rates of transmigration were determined as the slope of the best fit line, corrected for monolayer surface area. Results for each time point represent mean + SE ($n=3$). *C)* In the same set of experiments, apical accumulation of PMN after 90 min transmigration was determined for hCD55 overexpressors relative to mock transfected controls. To determine the influence of CD55 blockade, posthypoxic T84 monolayers (■) were treated with the CD55 consensus sequence peptide and assayed for PMN transmigration

(*D*) and apical accumulation of PMN (*E*) relative to normoxic controls (□). Data depicts mean + SE from 9–12 monolayers in each condition from 3 experiments.

transfection of HeLa cells with this wild-type CD55 promoter construct (–733) followed by exposure to hypoxia revealed significant hypoxia inducibility (2.9 ± 0.6 -fold increase over normoxia, $P < 0.025$).

In an attempt to further define the contribution of regions in the 5' flanking region of the CD55 promoter to hypoxia inducibility, the full-length promoter (–733) was truncated using a combination of site-directed mutagenesis and serial restriction enzyme digestion (Fig. 4A; see Materials and Methods). Successive truncations each maintained activity well above the empty PGL3 vector, although increasing truncation at the 5' end resulted in a progressive decrease in baseline activity (e.g., in normoxia, construct –60 contained $20 \pm 6\%$ activity relative to the wild-type –733 construct). Subsequent examination of these CD55 promoter truncations in hypoxia revealed a progressive increase in hypoxia inducibility, with increasing truncation of the wild-type construct (Fig. 4B). Hypoxia inducibility was particularly prominent in the region spanning positions –200 to +88 (compare Fig. 4A, B)

We next determined whether this region of the CD55 promoter binds HIF-1 α . We used ChIP to analyze HIF-1 α binding in live cells. As shown in Fig. 5A, ChIP analysis of nuclei derived from HeLa cells revealed a dominant band of 256 bp in hypoxic, but not normoxic, samples. No bands were evident in beads-only controls, and input samples (preimmunoprecipitation) revealed the predictable 256 bp band under conditions of hypoxia and normoxia. Such results indicate that hypoxia induces HIF-1 α binding to the proximal –210

bp relative to the primary site of transcription initiation for CD55.

In the course of our experiments, we identified three potential HIF-1 binding sites within the CD55 gene promoter (depicted as HRE in Fig. 4A). For each HRE, DNA consensus motifs were 5'-CACGTG-3', 5'-CGCAC-3' and 5'-AGCAC-3' located at positions –370 bp to –365 bp, –129 to –125, and –40 bp to –36 bp, respectively, relative to the major transcription start site (28). Given that all truncations retained hypoxia inducibility, we examined whether the potential HIF-1 binding site located from –40 bp to –36 bp, present in all truncations, conferred hypoxia inducibility. An HIF-1 α binding site mutation (–106 Δ HIF) was introduced in the –106 construct; as shown in Fig. 5A, hypoxia inducibility was significantly decreased in the –106 Δ HIF construct, suggesting that the HIF-1 site at positions –40 bp to –36 bp of the promoter is needed at least to some extent for hypoxia-inducible activity.

Additional analysis of the full-length CD55 promoter construct using sequence software (e.g., MatInspector) revealed putative binding sites for other hypoxia-responsive transcription factors, including AP1/CREB (36) and USF (37). The individual contributions of each putative transcription factor binding site were analyzed by site-directed mutagenesis using the –106 truncation. Although both mutations resulted in decreased baseline activity, neither the AP1 nor USF mutant demonstrated significant changes in hypoxia inducibility compared with wild-type constructs (data not shown.) Taken together, these results provide

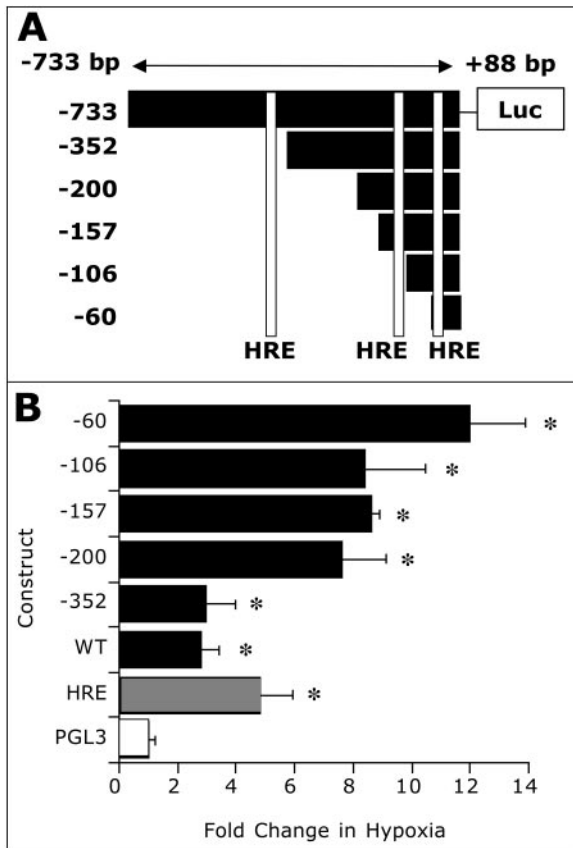


Figure 4. Influence of hypoxia on CD55 promoter activity. *A*) Serial truncations of the CD55 promoter reporter were generated. Potential HIF sites located at -370 bp, -229 bp, and -40 bp relative to the transcription start site are indicated (HRE). *B*) HeLa cells were cotransfected with promoter constructs and Renilla reporter plasmids and assayed for firefly and Renilla luciferase activity, respectively, after exposure to hypoxia for 48 h. Results depict the fold change in relative luminescence in hypoxia relative to normoxic controls. An HRE plasmid containing 4 tandem hypoxia-responsive elements is shown as a positive control (gray bar). Data shown are representative experiments from $n = 4$, normalized for background vector (empty PGL3, white bars) and Renilla luciferase to control for transfection efficiency, and presented as mean \pm SD, where * indicates significance between individual plasmids and empty PGL3 plasmid ($P < 0.025$).

strong evidence for a functional hypoxia inducible activity, mediated by HIF-1, in the proximal region the CD55 promoter.

DISCUSSION

Transepithelial migration of PMN represents an important component of the innate immune response and is a pathological hallmark of active mucosal inflammation characteristic of such diverse diseases as IBD, periodontitis, cystitis, and ischemia-reperfusion (11). It is now appreciated that as part of the ongoing inflammatory response, hypoxia may be a contributing factor to the

overall tissue phenotype (10, 38). Important in this regard, a primary mucosal target for hypoxia is the epithelium (10). We report here that HIF-1-dependent induction of CD55 by hypoxia represents an apical anti-adhesive pathway for clearance of PMN after transmigration.

Studies *in vitro* and *in vivo* have implicated both soluble and cell surface ligands in regulation of PMN transepithelial migration by hypoxia (16). Soluble ligands include primarily chemokines such as IL-8 (16) (and its murine homologue macrophage inflammatory protein-2) (39). These same studies also implicated a cell surface protein(s) in the dynamic regulation of PMN TM (16), and thereby provided our rationale to identify hypoxia-regulated PMN ligands that may contribute to the dynamics of PMN-epithelial interactions. Somewhat surprisingly, this screen revealed that none of the known epithelial adhesion molecules were regulated by hypoxia, including occludin, ICAM-1, or members of the JAM family of proteins. While epithelial CD47 mRNA levels were consistently decreased $\sim 30\%$,

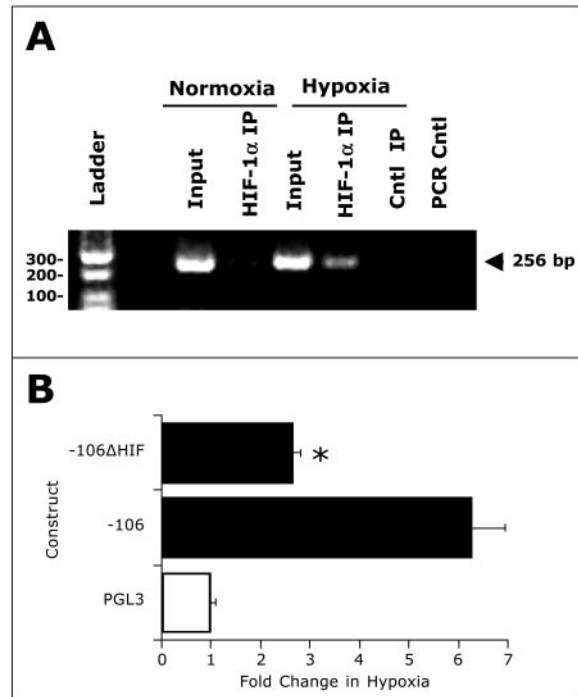


Figure 5. Identification of HRE in the CD55 promoter. *A*) Chromatin immunoprecipitation was used to examine HIF-1 α binding to the CD55 promoter in normoxic and hypoxic HeLa cells. Reaction controls included PCR performed using whole HeLa cell genomic DNA (input) and samples precipitated by protein G Sepharose beads alone. *B*) The putative HIF site located at -40 bp was eliminated in the -106 construct by site-directed mutagenesis. The resulting construct (-106Δ HIF) was cotransfected along with Renilla luciferase vector into HeLa cells followed by 48 h of hypoxia. Data shown are representative experiments from $n = 4$, are normalized for background vector (empty PGL3, white bars) and Renilla luciferase to control for transfection efficiency, and presented as mean \pm SD, where * indicates significance between -106 and -106Δ HIF ($P < 0.025$).

we have shown that functional surface protein is not influenced by hypoxia (16). Taken together, these data indicate that adhesion molecules per se are less likely to be the limited factor for PMN trafficking under such conditions. Rather, these studies implicated the anti-adhesive molecule CD55 as the critical control point for dynamic regulation of PMN TM. Such events at the apical surface likely reflect the terminal steps of transmigration (14). Our results suggest that this terminal step is controlled simply by the total amount of accessible CD55 on the apical membrane surface. Indeed, forced overexpression of CD55 in normoxia recapitulated our observations in hypoxia, and synthetic peptides directed against the PMN binding site on CD55 (14) effectively blocked this response in hypoxia. It is possible that this terminal step could be more complicated. For instance, others have shown that a close extracellular spatial interaction exists between CD55 and ICAM-1 and that such an association may limit accessibility to the functional short consensus repeat-3 (SCR-3) domain of CD55 (40). Is it possible, therefore, that the dynamics of PMN adhesion (i.e., ICAM-1) and anti-adhesion (i.e., CD55) at the apical surface are controlled by the relative amounts of individual proteins expressed on this membrane domain. Under our defined conditions of hypoxia, the predominant phenotype is one of anti-adhesion (i.e., expression of CD55 >> ICAM-1). More studies are needed to determine whether this phenotype is different with other inflammatory stimuli.

At tissue and cellular levels, hypoxia induces an array of genes pivotal to survival in low oxygen states. As a global regulator of oxygen homeostasis, the $\alpha\beta$ heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) facilitates both oxygen delivery and adaptation to oxygen deprivation (41, 42). Genes induced by HIF-1 include those necessary for cell, tissue, and whole animal adaptive responses to hypoxia as well as enzymes involved in anaerobic metabolism, angiogenesis, and vasodilation. More recent studies have indicated that HIF regulates the expression of a cluster of genes important as endogenous regulators of inflammation (4). This gene cluster encodes a large number of proteins expressed on the apical epithelial membrane surface, including the intestinal trefoil factor (19), P-glycoprotein (22, 43), ecto-5'-nucleotidase (20, 25), and the adenosine A₂B receptor (25). The present findings of HIF-1-dependent CD55 expression confirm an additional member of this "apical cluster" group.

It is likely that hypoxia-induced CD55 represents a protective mechanism for mucosal inflammation. CD55 has historically been viewed as a regulator of innate immunity through its regulation of the complement pathway (44). CD55 is expressed in most tissues exposed to serum components, and CD55 expression has been shown, in human endothelial systems, to be augmented in response to numerous inflammatory stimuli, including cytokines (45, 46) and conditions found within the hypoxic microenvironment (e.g., VEGF) (47). Whereas baseline expression of CD55 in

epithelium is sporadic, marked increases in expression are seen in areas of intestinal metaplasia or inflammation (48, 49), particularly in patients with ulcerative colitis (50). Rodent models have provided significant insight into the role of CD55 in hypoxia and mucosal inflammation in vivo. For example, Oshima et al. reported the up-regulation of CD55 expression in guinea pig gastric epithelium after ischemia/reperfusion injury (51). Similarly, Yamada et al. recently demonstrated a crucial role for CD55 in inflammatory damage associated with renal ischemia/reperfusion (52). Moreover, some evidence suggests that mucosal inflammation, as modeled by colitis, may critically involve CD55. For instance, Lin et al. revealed that the course of colitic disease is significantly worsened in *cd55*^{-/-} mice, reflected by a large increase in PMN accumulation and profound epithelial destruction (53). Although these observations could reflect aberrant complement activation related to the loss of CD55, it is also possible that loss of CD55-dependent PMN clearance could result in enhanced intestinal epithelial damage with concomitant loss of barrier. Consistent with our finding of HIF-dependent CD55 induction in the present work, we recently demonstrated that epithelial HIF is protective during experimental colitis (10). These studies used transgenic models of intestinal epithelial loss and gain of HIF function, and revealed profound epithelial hypoxia during colitis and that the magnitude of colitic disease was inversely related to the level of intestinal epithelial HIF expression. Though we did not address CD55 expression in these studies, a predominant phenotype was loss of barrier function in *hif-1 α* null epithelia. Additional studies are needed to distinguish the relative contributions of complement activation and PMN clearance during the course of colitis and other mucosal diseases. FJ

The authors wish to thank J. K. Atkinson (Washington University) for plasmid containing full-length CD55 and D. Theodorescu (University of Virginia) for PGL3-HRE plasmid. The authors also acknowledge technical assistance from Dionne Daniels and Richard Cotta. This work was supported by National Institutes of Health grants DK62007, DK50189, DE161191 and by the Marjorie J. LeMay, M.D., Fellowship in Medicine from Harvard Medical School.

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Received for publication October 25, 2004.

Accepted for publication January 27, 2005.