

Identification of Pur α as a New Hypoxia Response Factor Responsible for Coordinated Induction of the β_2 Integrin Family¹

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Central to the process of inflammation are hypoxic conditions that lead to the binding of circulating leukocytes to the endothelium. We have previously shown that such binding is mediated by monocytes being able to directly sense hypoxic conditions and respond by inducing their surface expression of the β_2 integrin family of adhesion molecules. In this study, we show that coordinated induction of the β_2 integrins during direct hypoxia-sensing occurs through transcriptional activation of each of the genes by which they are encoded. Certain of the molecular mechanisms that mediate this activation in transcription are dependent upon hypoxia-inducible factor-1 (HIF-1), whereas others are HIF-1 independent. In search of these HIF-1-independent mechanisms, we identified Pur α as a new hypoxia-response factor. Binding of Pur α to the HIF-1-independent β_2 integrin promoters is induced by hypoxia and mutagenesis of these Pur α -binding sites almost completely abolishes the ability of the promoters to respond to hypoxic conditions. Additional studies using siRNA directed against Pur α also revealed a loss in the hypoxic response of the β_2 integrin promoters. Taken together, our findings demonstrate that hypoxia induces a coordinated up-regulation in β_2 integrin expression that is dependent upon transcriptional mechanisms mediated by HIF-1 and Pur α . *The Journal of Immunology*, 2007, 179: 1934–1941.

Acute and chronic inflammatory responses are characterized by significant shifts in tissue metabolism. As part of these changes, oxygen demand often exceeds supply and can result in overall oxygen deficits (hypoxia) (1–5). Central to the inflammatory process is the recruitment of large numbers of myeloid cells, such as neutrophils and monocytes (6). The vast majority of inflammatory cells are recruited to, as opposed to being resident at, inflammatory lesions. Use of existing oxygen by infiltrating leukocytes perpetuates the state of oxygen deficit experienced by the surrounding tissue (7, 8). Consequently, a thorough understanding of the molecular events that lead to myeloid recruitment at the sites of inflammation is an important step toward developing effective therapies.

It is now appreciated that adhesion-based interactions coordinated by leukocyte β_2 integrins are the primary means by which myeloid cells interact with other cell types, including endothelial and epithelial cells (9, 10). The β_2 integrins are a family of four glycoprotein heterodimers composed of a unique α -subunit, encoded by the CD11a, CD11b, CD11c, or CD11d gene, nonco-

valently associated with a common β -subunit encoded by the CD18 gene (11–14). Expression of the different subunits of the β_2 integrins is coordinately regulated during leukocyte differentiation and during activation. Such coordinated regulation occurs at the level of gene transcription (15).

We have previously shown that the hypoxia associated with inflammation dramatically induces transcription of the CD18 gene (16). The molecular mechanisms that mediate this induction involve the heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1)³ (17, 18). Although this finding provided an initial insight into how hypoxia regulates β_2 integrin expression, the means by which hypoxia influences coordinated regulation of the different members of the β_2 integrin family remained unknown. In the current study, we aimed to identify these molecular mechanisms.

Initial observations revealed that hypoxia induces transcription, not only of the CD18 gene, but also each CD11 gene that encodes an α -subunit of the β_2 integrin family. Transcriptional induction of the CD11a and CD11d genes was found to be mediated by HIF-1-dependent mechanisms. However, induction of the CD11b and CD11c genes is mediated by mechanisms that are independent of HIF-1. Further studies revealed that hypoxia induces transcription of the CD11b and CD11c genes by mechanisms involving the ssDNA-binding protein Pur α . This finding identifies Pur α for the first time as a hypoxia-response factor. Furthermore, our results demonstrate that Pur α acts together with HIF-1 to drive coordinated induction of the β_2 integrin family in response to hypoxia.

Materials and Methods

Cell culture

The human promonocytic cell line U937 and human microvascular endothelial cells were obtained and cultured as previously described (16, 19).

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³ Abbreviations used in this paper: HIF-1, hypoxia-inducible factor-1; ChIP, chromatin immunoprecipitation.

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U937 cells were exposed to hypoxia by replacing the growth medium with fresh medium equilibrated with a hypoxic gas mixture and incubated in a hypoxic chamber (Coy Laboratory Products). Measured pO_2 was 20 mm Hg with the balance made up of nitrogen, carbon dioxide (ambient 5% CO_2), and water vapor (16). Where indicated, human microvascular endothelial cells monolayers were activated by the addition of 100 ng/ml LPS (List Biological Laboratories). Leukocyte-endothelial adhesion assays were performed as previously described (16, 20).

Immunoprecipitation and Western blotting

U937 cells were exposed to experimental conditions and washed with HBSS, and then their surface proteins were labeled with biotin as described previously (16, 21). Cells were lysed and debris was removed by centrifugation, and then lysates were precleared with protein-G Sepharose (Pharmacia Biotech) (16). CD18 immunoprecipitation was performed with 10 μ g/ml of the mAb IB4 followed by incubation with protein-G Sepharose (16). Washed immunoprecipitates were boiled in nonreducing sample buffer, separated by SDS-PAGE under nonreducing conditions, and transferred to nitrocellulose (16). Biotinylated proteins were labeled with streptavidin-peroxidase and visualized by ECL (Amersham Biosciences). Densitometric analysis of resulting blots ($n = 4$ per experiment) was performed using Image J software (National Institutes of Health, Bethesda, MD).

In subsets of experiments, individual CD11 integrin chains were immunoblotted following U937 cell exposure to indicated periods of hypoxia. Anti-CD11a (clone MHM.24) was obtained from the Developmental Studies Hybridoma Bank, anti-CD11b polyclonal Ab was a gift from C. Parks (Emory University, Atlanta, GA), anti-CD11c polyclonal Ab was obtained from Santa Cruz Biotechnology, and anti-CD11d was a gift from D. Stanton (ICOS, Bothell, WA).

Quantitative polymerase chain reactions

Messenger RNA was quantified by real-time PCR as described previously (22). The primer sets consisted of 1 μ M of sense primer and 1 μ M of antisense primer each containing SYBR Green I (Molecular Probes). Primer sets (sense sequence, antisense sequence, and product size, respectively) for the following genes were used: CD11a (5'-AACTGGACTCAGGATGCCC-3', 5'-CAAGGAAGGAACCAAGAGAGG-3', 239bp), CD11b (5'-TCTCAGAGTCTTCTGTAAACAG-3', 5'-AGCTGAGGGGGCTGGTGG-3', 299bp), CD11c (5'-GAGAAATGATCCCTCTTTGCC-3', 5'-GTCCTTTTGGGGAACACAGC-3', 230bp), CD11d (5'-TATCATGGATTCAACCTGG-3', 5'-CGGGCCACAGGCCAGGAG-3', 283bp), and human β -actin (5'-GGTGGCTTTTAGGATGGCAAG-3', 5'-ACTGGAACGGTGAAGGTGACAG-3', 162bp). The analysis of β -actin was used to control for variations in the quantity of starting template. Transcript levels and fold changes in mRNA were determined as described previously (23).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using U937 cells subjected to normoxia or hypoxia and the mAb 9C12 that specifically interacts with Pur α or control IgG (16, 24). Primer sets for the following genes were used in the PCR phase of the assays (sense sequence, antisense sequence, and product size, respectively): CD11a (5'-AGTGAGAAACCATGACAGCAGTG-3', 5'-GCGTCAGGAGGCCCGTGGG-3', 289bp), CD11b (5'-GGCTAAGTCTATTCAGCTTGTTCA-3', 5'-GGAACCACAAGGAAGCCA CCAA-3', 275bp), CD11c (5'-TGCATCCATCTAAGCAAAGGGCA-3', 5'-GAGGAGTGCTGCCCTGGTCC-3', 278bp), CD11d (5'-CGGGCTCCCTGGGTACCAA-3', 5'-CAGAAGGTGCTCACCCCTCCCA-3', 250bp) and CD18 negative control (5'-TGCAACCCACCACTTCTCTCA-3', 5'-ACCCTCGGTGTGCTGGAGTC-3', 166bp).

Transfection assays

U937 cells were used to assess the induction of the β_2 integrin gene promoters by hypoxia. These cells were transfected by electroporation with a mixture of a plasmid that constitutively expresses Renilla luciferase (Invitrogen Life Technologies) and a construct generated by cloning one of the β_2 integrin gene promoters into the plasmid vector pATLuc such that it is immediately upstream of a firefly luciferase reporter. The CD11a promoter spanned nucleotides -525 to +103 relative to the major site of transcription initiation, the CD11b promoter spanned nucleotides -242 to +71, the CD11c promoter spanned nucleotides -128 to +36, the CD11d promoter spanned nucleotides -419 to +60, and the CD18 promoter spanned nucleotides -79 to +19 (15, 19, 25-27). Background firefly luciferase activity was assessed by transfecting U937 cells with the Renilla expression plasmid mixed with the parental plasmid pATLuc that lacks a

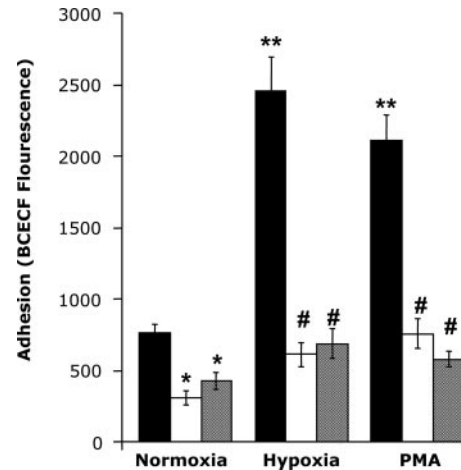


FIGURE 1. Leukocyte hypoxia enhances CD11-dependent adhesion to endothelia. The promyelocytic cell line U937 was exposed to normoxia (pO_2 147 torr) or hypoxia (pO_2 20 torr) for 24 h, labeled with the fluorescent marker BCECF, and then assessed for adhesion to LPS-activated endothelia. Adhesion assays were performed in the presence or absence of functionally inhibitory Abs directed against β_1 integrins (filled histograms), CD11a (open histograms), or CD11c (shaded histograms). Results are presented as the mean \pm SEM of three independent experiments. Significant differences compared with when the anti- β_1 Ab is used are indicated at the levels of *, $p < 0.05$ and #, $p < 0.01$. Significant increases compared with normoxia are indicated at the level of **, $p < 0.01$.

functional β_2 integrin promoter (27). After transfection, cells were subjected to hypoxia or normoxia and lysed, and luciferase activity was assessed using a dual luciferase assay kit (Stratagene) and a Turner Designs luminometer. All firefly luciferase activity was normalized with respect to the constitutively expressed Renilla luciferase reporter gene. In subsets of experiments, Pur α binding site mutations were introduced into the CD11b and CD11c promoters by site-directed mutagenesis (28). Specifically, within the CD11b gene, the sequence 5'-GGCAGGCTG-3' spanning nucleotides -114 to -106 relative to major site of transcription initiation was mutated to the sequence 5'-GGCAtCTG-3' (28). The mutated nucleotides are indicated in lower case type. Within the CD11c gene the sequence 5'-CTTCCTCC-3' spanning nucleotides -40 to -30 relative to major site of transcription initiation was mutated to the sequence 5'-aTTaaTTa aaa-3' (19). All mutations were confirmed by DNA sequencing. The ability of the mutated CD11b and CD11c promoters to respond to hypoxia was assessed as described above.

Inhibition of HIF-1 α expression

HIF-1 α depletion was accomplished by using phosphorothioate derivatives of antisense (5'-GCCGCGCCCTCCAT-3') or control sense (5'-ATGGAGGGCGCCGGC-3') oligonucleotides as described previously (16). Western blot analysis for HIF-1 α was performed as described previously (29). HIF-1 β repression was accomplished using commercial siRNA provided by Dharmacon.

Inhibition of Pur α expression

Pur α depletion was accomplished by using siRNA directed toward the Pur α mRNA sequence 5'-CCGCAAGTACTACATGGATCT-3'. As a control for these experiments, a mismatched siRNA was used with the sequence 5'-CCGCAAGTATACgTGGATCT-3'. The two mismatches to the Pur α mRNA sequence in this control siRNA are indicated in lowercase type. Both the test and control siRNA were expressed from the plasmid vector pSUPER provided by Dr. R. Agami of the Netherlands Cancer Institute (Amsterdam, The Netherlands) (30).

Expression of recombinant Pur α

The Pur α expression construct, pHAPur1, was provided by E. Johnson (Mount Sinai School of Medicine, New York) and the empty vector control, pHA, produced by religation following liberation of the Pur α sequence by RsrII and EcoRI digestion (24).

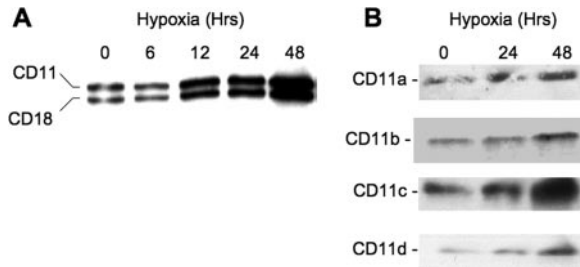


FIGURE 2. Hypoxia induces surface expression of β_2 integrin protein. *A*, U937 cells were exposed to hypoxia (pO_2 20 torr) for the indicated periods of time. Cells were washed, and surface proteins were biotinylated and then lysed. CD18 was immunoprecipitated and resolved by SDS-PAGE, and the resultant Western blot was probed with avidin peroxidase. As indicated, the CD18 Ab immunoprecipitates heterodimeric complexes of CD11 and CD18. A representative experiment of four is shown. *B*, CD18 immunoprecipitations were immunoblotted with Abs directed against CD11a, CD11b, CD11c, or CD11d.

Results

Leukocyte hypoxia induces increased adhesion to endothelia

Leukocyte β_2 integrins are coordinately expressed as heterodimers composed of a common CD18 subunit linked to a subunit of CD11a, CD11b, CD11c, or CD11d (11–14). In previous studies, we found that expression of the β_2 integrin CD18 subunit is induced by hypoxia (16). Furthermore, we demonstrated that this induced expression contributes in a significant way to increased leukocyte binding to endothelium (16). However, CD18 induction would have no functional meaning without a concomitant induction of at least one of the β_2 integrin CD11 subunits. Consequently, we hypothesized that CD11 subunits are hypoxia inducible. To address this hypothesis, U937 cells were subjected to normobaric hypoxia for 24 h and then assessed for their ability to adhere to LPS-activated endothelial cells in the presence of inhibitory Abs directed against CD11a or CD11c. As shown in Fig. 1,

the component of increased leukocyte adhesion attributable to hypoxia (3.1 ± 0.6 -fold increase, $p < 0.01$) was nearly completely inhibited by Abs directed against either CD11a or CD11c, but was unaffected by an Ab directed against β_1 integrins. These results indicate that hypoxia-induced leukocyte adhesion to activated endothelial cells is dependent upon at least two of the β_2 integrin α -subunits.

Hypoxia induces surface expression of CD11 protein

Because hypoxia induces CD11-dependent adhesion, we reasoned that this might be mediated by increased expression of CD11 protein. Initially, we examined induction of the sum total of all five β_2 integrin molecules. This was achieved by subjecting U937 cells to a range of hypoxic periods (6–48 h), labeling surface proteins with biotin, and then immunoprecipitating CD18 from the lysates. Immunoprecipitates were resolved by SDS-PAGE and Western blots were probed with avidin-peroxidase. As can be seen in Fig. 2*A*, this protocol precipitates β_2 integrin heterodimeric complexes of CD11 and CD18. Consistent with our previous work, the common CD18 subunit is significantly induced by hypoxia (16), with induction observed as early as 12 h of hypoxia (2.7 ± 0.6 -fold by densitometry, $p < 0.05$), and maximally at 48 h (9.7 ± 2.3 -fold by densitometry, $p < 0.01$). In parallel, hypoxia-inducible CD11 protein is also observed, with maximal induction observed at 48 h of hypoxia (8.7 ± 1.9 -fold by densitometry, $p < 0.01$). We next used immunoprecipitation and Western blot analysis to assess the expression of the individual members of the CD11 family (Fig. 2, *B* and *C*). These analyses revealed increased expression of each member of the CD11 family upon hypoxia exposure (for CD11a, CD11b, CD11c, and CD11d; 3.1 ± 0.4 , 3.6 ± 1.1 , 5.4 ± 1.3 , and 5.5 ± 0.8 -fold increase by densitometry, respectively, all $p < 0.025$). Consequently, these results support our hypothesis that all the proteins known to be formed into β_2 integrin heterodimers are coordinately induced by hypoxia.

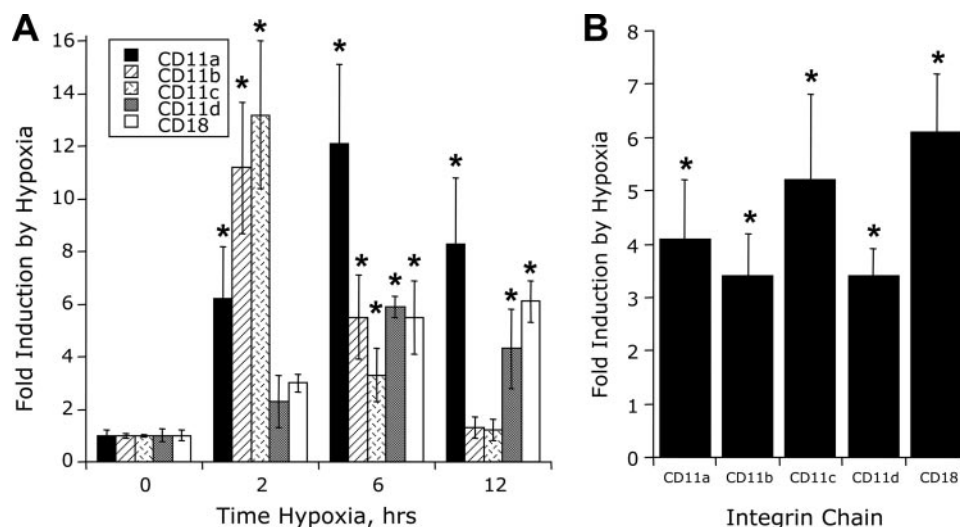


FIGURE 3. Hypoxia induces expression of β_2 integrin mRNA in U937 and PBMC. *A*, U937 cells were exposed to hypoxia (pO_2 20 torr) for 0, 2, 6, or 12 h. The levels of mRNA encoding CD11a (filled histograms), CD11b (open histograms), CD11c (shaded histograms), and CD11d (stippled histograms) were then determined by real-time PCR relative to β -actin. Results are presented as the mean \pm SEM of three independent experiments. Significant increases compared with normoxia are indicated at the level of *, $p < 0.01$. *B*, PBMC were isolated and exposed to hypoxia (pO_2 20 torr, 8 h). Total RNA was isolated and the levels of mRNA encoding CD11a, CD11b, CD11c, CD11d, and CD18 were then determined by real-time PCR relative to β -actin. Results are presented as the mean fold change in hypoxia \pm SEM of three independent experiments. Significant increases compared with normoxia are indicated at the level of *, $p < 0.01$.

Hypoxia induces expression of CD11 mRNA

Our previous studies demonstrated that the increase in CD18 protein induced by hypoxia is reflected as an increase in the steady-state levels of CD18 mRNA (16). Consequently, we determined whether increased mRNA levels also underlie the induction of CD11 proteins elicited by hypoxia. Using real-time PCR, we observed that the steady-state level of each CD11 mRNA was increased in U937 cells exposed to hypoxia (Fig. 3A). For these purposes, CD18 was used as a positive control (16). Interestingly, both the time-course and magnitude of mRNA induction varied depending on the protein encoded. Maximal induction of both CD11b and CD11c mRNA occurred within 2 h of hypoxia exposure and then rapidly subsided. However, maximal induction of CD11a and CD11d occurred after 6 h, then subsided slowly. In addition, the maximal level to which hypoxia induced CD11d mRNA was approximately half of that to which it induced CD11a, CD11b, and CD11c mRNA.

To rule out that this response represents an aberrancy in a leukemic cell line (U937 cells), anticoagulated blood from normal human volunteers was exposed to hypoxia (pO_2 20 torr) for 8 h, PBMC were isolated, and assessed for CD11a-d expression by real-time PCR. As shown in Fig. 3B, these studies revealed a significant, hypoxia-inducible increase in all CD11 genes (4.1 ± 1.1 , 3.4 ± 0.8 , 5.2 ± 1.6 , and 3.4 ± 0.5 for CD11a, b, c and d, respectively; all $p < 0.025$). Consistent with previous studies (16), CD18 served as a positive control for these experiments (6.1 ± 1.1 -fold increase over normoxia, $p < 0.025$). Such results indicate that CD11 induction by hypoxia extends to cell types other than cultured leukocyte cell lines and into ex vivo tissue (whole human blood).

Transcriptional induction of the β_2 integrin gene promoters

We next determined whether hypoxia-inducible CD11 reflects an increase in transcription, as had been found previously with CD18 (16). As shown in Fig. 4A, U937 cells transiently transfected with the wild-type CD11a promoter (nucleotides -525 to $+103$) showed a 57 ± 7 -fold increase in activity ($p < 0.01$, with mean normoxia luciferase values of 155 ± 35 counts) when subjected to 24 h of hypoxia. The CD11b promoter (nucleotides -242 to $+71$) showed a 43 ± 8 -fold increase ($p < 0.01$, with mean normoxia luciferase values of 486 ± 68 counts), the CD11c promoter (nucleotides -128 to $+36$) showed a 31 ± 5 -fold increase ($p < 0.01$, with mean normoxia luciferase values of 446 ± 49 counts), the CD11d promoter (nucleotides -419 to $+60$) exhibited a 16 ± 4 -fold increase ($p < 0.01$, with mean normoxia luciferase values of 87 ± 7 counts) and, as previously shown, the CD18 promoter (nucleotides -79 to $+19$) exhibited a greater than 20-fold increase (16). These results demonstrate that transcription driven by the isolated promoter region of each β_2 integrin gene is induced by hypoxia. Thus, transcriptional activation of the β_2 integrin genes appears to help mediate increased leukocyte adhesion during conditions of hypoxia.

HIF-1-dependent and independent regulation of CD11 gene promoters

Each β_2 integrin promoter is activated by hypoxia (Fig. 2A). Our previous work demonstrated HIF-1 α -dependent regulation of CD18 (16). Furthermore, chromatin immunoprecipitation analysis directly demonstrated HIF-1 binding to the endogenous CD18 gene (16). To determine whether CD11 promoters are controlled by HIF-dependent mechanisms, we first screened for expression of the various α -chains of HIF (HIF-1, 2, and 3) by Western blot. These studies revealed that U937 cells express only HIF-1, but not

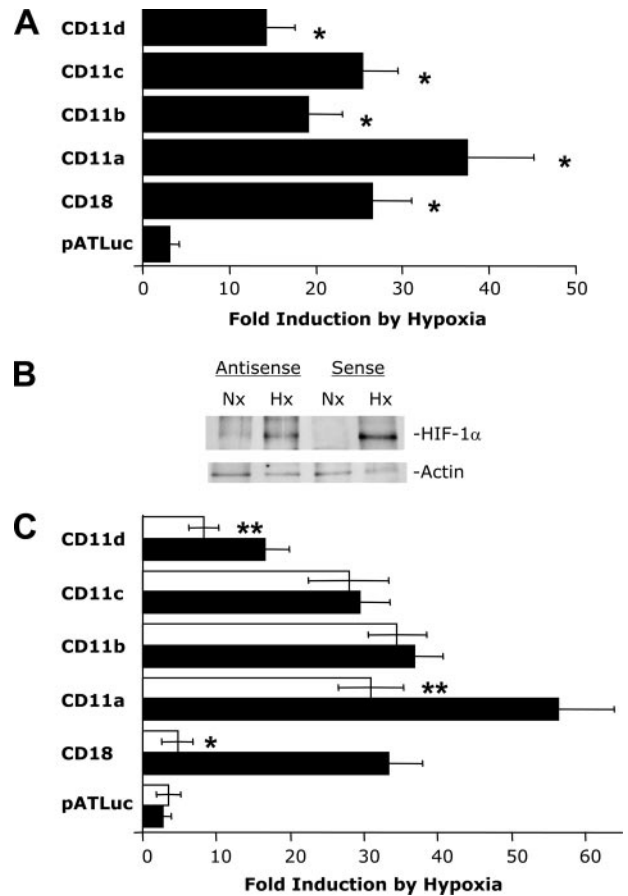


FIGURE 4. Hypoxia induces β_2 integrin gene expression by both HIF-1-dependent and HIF-1-independent mechanisms (A). U937 cells were transfected with firefly luciferase reporter constructs containing the CD11a, CD11b, CD11c, CD11d, or CD18 gene promoters. Parallel transfections were performed using pATLuc that represents the parent of these constructs and is empty of β_2 integrin gene sequences. All transfection reactions contained the Renilla luciferase plasmid pRL-CMV. Transfected cells were exposed for 24 h to normoxia or hypoxia, and changes in the activity of pRL-CMV were taken as reflecting variations in transfection efficiency. Following correction for these variations, the level of firefly luciferase activity directed by the β_2 integrin constructs above that conferred by pATLuc under parallel conditions was determined. The fold increase in expression caused by hypoxia was then calculated. B, U937 cells were loaded with HIF-1 α antisense or sense control oligonucleotides, as indicated, exposed to normoxia (Nx) or hypoxia (Hx), and examined for expression of HIF-1 α by Western blot. C, U937 cells were loaded with HIF-1 α antisense oligonucleotides (open histograms) or HIF-1 α sense oligonucleotides (filled histograms). Loaded cells were then transfected with luciferase β_2 integrin promoter constructs in parallel with the parental vector pATLuc that is empty of β_2 integrin sequences. Cells were then exposed to hypoxia for 24 h and luciferase activity was determined. After correction for variations in pRL-CMV expression, the level of firefly luciferase activity directed by the β_2 integrin constructs above that conferred by pATLuc under parallel conditions was determined. The fold induction by hypoxia was then calculated. All histograms represent the mean \pm SEM of three independent experiments, *, $p < 0.01$ and **, $p < 0.05$.

HIF-2 or HIF-3 (data not shown). Based on this evidence, we blocked expression of HIF-1 α with antisense oligonucleotides (16) (Fig. 2B; $72 \pm 6\%$ decrease in protein, $p < 0.025$) and examined CD11a-d inducibility by hypoxia. HIF-1 α antisense treatment resulted in $49 \pm 6\%$, $44 \pm 7\%$, and $88 \pm 9\%$ reduction in hypoxia-inducibility, respectively, for CD11a, CD11d, and CD18 promoters (Fig. 2C). Neither CD11b or CD11c promoter activity was

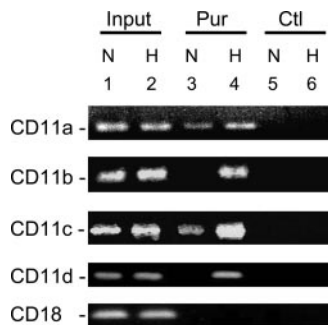


FIGURE 5. Pur α binding to the CD11 gene promoters in vivo. Chromatin immunoprecipitation was performed to examine Pur α binding to the CD11 gene promoters in normoxic (N) and hypoxic (H) U937 cells. *Lane 1*, normoxia input genomic DNA; *Lane 2*, hypoxia input genomic DNA; *Lane 3*, normoxia Pur α immunoprecipitation; *Lane 4*, hypoxia Pur α immunoprecipitation; *Lane 5*, normoxia IgG control immunoprecipitation; *Lane 6*, hypoxia IgG control immunoprecipitation. The proximal CD18 gene promoter was used as a negative control. A representative example of three independent experiments is shown.

significantly influenced by the HIF-1 α antisense oligonucleotide, suggesting that both HIF-1-dependent and HIF-1-independent mechanisms regulate hypoxic induction of the β_2 integrins.

Additional evidence of a role for HIF-1 was provided by targeted repression of HIF-1 β . For these purposes, we used siRNA directed against HIF-1 β and screened CD11a-d and CD18 promoters. This siRNA approach effectively decreased the expression of HIF-1 β by $82 \pm 7\%$ by real-time PCR. Similar to our findings with HIF-1 α antisense, HIF-1 β siRNA blocked induction of CD11a, CD11d, and CD18 ($58 \pm 8\%$, $63 \pm 10\%$, and $78 \pm 11\%$ reduction in hypoxia-inducibility, respectively, all $p < 0.025$). CD11b and CD11c promoter activity were not significantly influenced by the HIF-1 β siRNA ($p =$ not significant). Moreover, the combination of HIF-1 α antisense and HIF-1 β siRNA to maximally block the HIF pathway did not influence this induction pattern (data not shown).

The transcription factor Pur α exhibits increased binding to the β_2 integrin gene promoters during hypoxia

Our use of HIF-1 α antisense oligonucleotides and HIF-1 β siRNA indicate that hypoxia induces CD11b and CD11c gene expression by mechanisms that are, for the most part, independent of HIF-1 (Fig. 4B). Important in this regard, our own analysis of the individual CD11 gene promoters revealed no obvious consensus motifs for HIF (S. Colgan, unpublished observations). Consequently, in the next phase of our analysis, we sought to identify the HIF-1-independent mechanisms. We reasoned that coordinated induction of the β_2 integrin genes would likely involve common transcription factors. Therefore, we analyzed the β_2 integrin gene promoters for shared consensus transcription factor binding sites. This analysis revealed that all the CD11 gene promoters contain close repeats of the sequence GGN, in which N is not G. Such repeats represent the recognition element of the transcription factor Pur α (31, 32). Our own analysis has revealed that the CD18 gene promoter contains no binding site for Pur α (data not shown). ChIP analyses of each of the CD11 gene promoters demonstrated that binding of Pur α is indeed a common feature of the CD11 genes (Fig. 5). Furthermore, this analysis revealed that Pur α binding to the CD11 gene promoters is strongly induced by hypoxia (Fig. 5). For these purposes, the proximal region of the CD18 promoter served as a negative control.

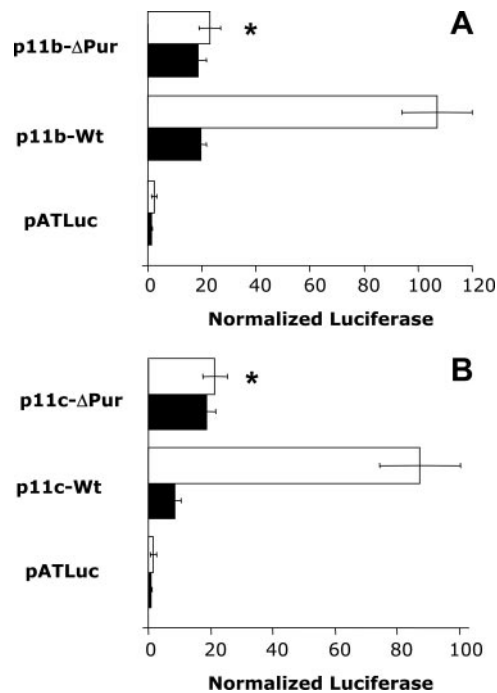


FIGURE 6. Binding of Pur α to the CD11b and CD11c gene promoters is necessary for their induction by hypoxia. The wild-type CD11b and CD11c promoters were introduced into the firefly luciferase reporter plasmid pATLuc to generate the constructs p11b-Wt and p11c-Wt, respectively. Point mutations that abolish Pur α binding were introduced into the wild-type sequences to generate the constructs p11b- Δ Pur and p11c- Δ Pur. U937 cells were transfected in parallel with p11b-Wt, p11b- Δ Pur, and pATLuc (A) or p11c-Wt, p11c- Δ Pur, and pATLuc (B). Each firefly luciferase plasmid was mixed with the Renilla luciferase plasmid pRL-CMV. Transfected cells were subjected to normoxia (filled histograms) or hypoxia (open histograms) for 24 h and assessed for firefly luciferase activity relative to Renilla luciferase. The relative level of firefly luciferase activity directed by p11b-Wt, p11c-Wt, p11b- Δ Pur, and p11c- Δ Pur above that conferred by the parent plasmid pATLuc was then calculated. Each histogram represents the mean \pm SEM of three independent experiments.

Role of Pur α in HIF-1-independent induction of the CD11b and CD11c genes

We next addressed the functional significance of Pur α to the HIF-1-independent induction of CD11. Given the possible interactions of HIF-1 α and Pur α on the CD11a and CD11d promoters (Figs. 4 and 5, respectively), we focused on the CD11b and CD11c promoters. First, as shown in Fig. 6, mutagenesis of Pur α binding sites in the CD11b (Fig. 6A) and CD11c (Fig. 6B) promoters almost completely abolishes their induction by hypoxia. Importantly, the mutations did not adversely influence the basal activity of any of these promoters. These results suggest that Pur α binding sites contribute significantly to hypoxia induction that is HIF-1 independent. As a second approach, we knocked down expression of Pur α in U937 cells by siRNA. As shown in Fig. 7A, this strategy successfully decreased Pur α expression by $>85\%$ (based on densitometric determination). In addition, and consistent with our findings using ChIP (Fig. 5), this analysis revealed that hypoxia induces Pur α mRNA (Fig. 7A). Examination of CD11b and CD11c promoter activity in U937 cells treated with Pur α siRNA showed significantly decreased hypoxia-induction (Fig. 7, B and C), confirming the role of Pur α as a hypoxia response factor.

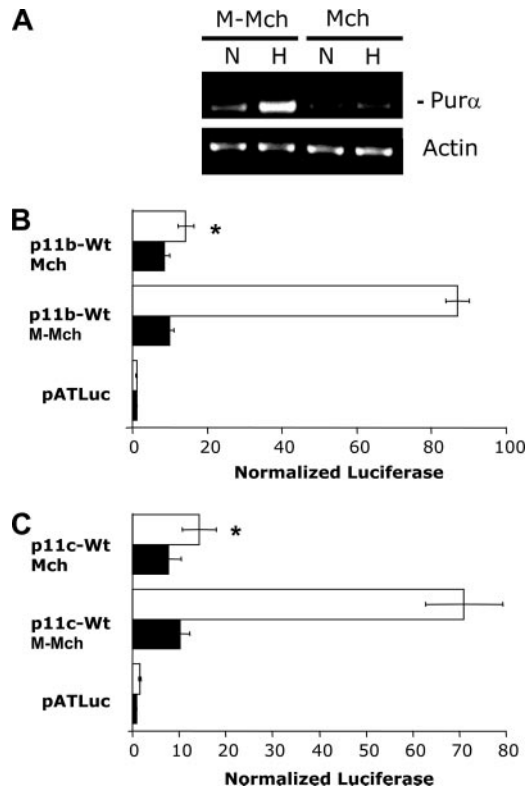


FIGURE 7. Pur α mediates hypoxia induction of the CD11b and CD11c promoters. *A*, U937 cells were transfected with a pSUPER plasmid that either expresses a siRNA directed against Pur α (Mch) or pSUPER expressing a mismatch control (M-Mch). Cells were then subjected to normoxia (N) or hypoxia (H) for 24 h and real-time PCR analysis used to determine the expression levels of Pur α and β -actin mRNA. *B*, U937 cells were transfected in parallel with p11b-Wt and pATLuc each mixed with the Renilla luciferase plasmid pRL-CMV. Transfection of p11b-Wt was performed in the presence of a pSUPER plasmid that either expresses a siRNA directed against Pur α (Mch) or pSUPER expressing a mismatch control (M-Mch). Transfected cells were subjected to normoxia (filled histograms) or hypoxia (open histograms) for 24 h and luciferase activities determined. After correction for variations in pRL-CMV expression, the level of firefly luciferase activity directed by pATLuc and p11b-Wt was determined. Each histogram represents the mean \pm SEM of three independent experiments. *C*, U937 transfections performed as described in *B* but using p11c-Wt rather than p11b-Wt.

Overexpression of Pur α recapitulates CD11b and CD11c promoter activity in hypoxia

Mutatagenesis and siRNA analysis suggested that Pur α binding is necessary for hypoxia induction of the CD11b and CD11c gene promoters (Figs. 6 and 7). We next sought to determine whether expression of Pur α was also sufficient to activate these promoters. This was achieved by cotransfecting the CD11b and CD11c reporter constructs with the plasmid pHAPur1 in which Pur α is constitutively expressed from the CMV promoter (24). As shown in Fig. 8A, transfection of U937 cells with pHAPur1 resulted in significant increases in Pur α expression in normoxia but not in hypoxia. Consistent with this engineered pattern of Pur α expression, CD11b and CD11c promoter activity was significantly increased under normoxic conditions but not under conditions of hypoxia (Fig. 8, B and C). The levels to which pHAPur1 induced the CD11 promoters under normoxic conditions were approximately equivalent to those reached by the promoters under hypoxic conditions in the absence of Pur α overexpression. Taken together with our siRNA and mutagenesis results (Figs. 6 and 7), these observations

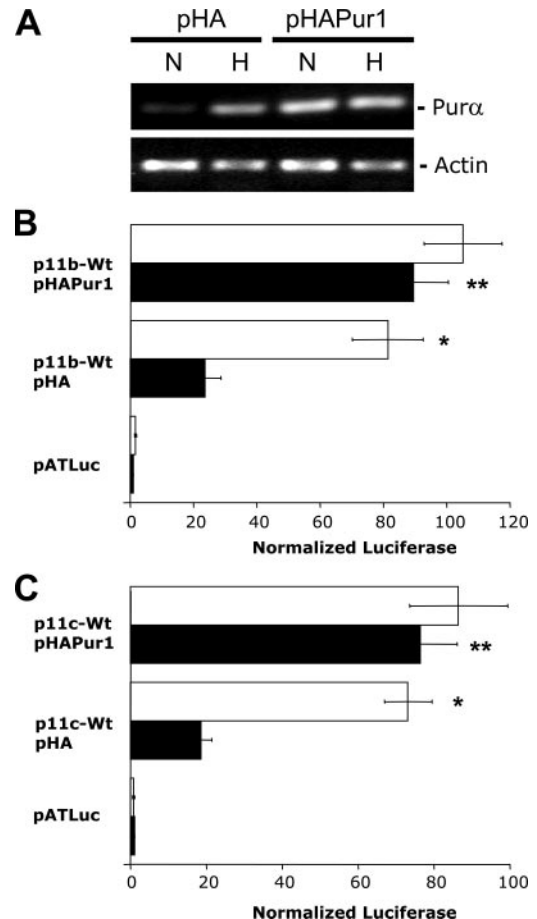


FIGURE 8. Expression of recombinant Pur α is sufficient to recapitulate hypoxic induction of the CD11b and CD11c gene promoters. *A*, U937 cells were transfected with a plasmid that either constitutively expresses Pur α (pHAPur1) or is empty of Pur α cDNA (pHA). Cells were then subjected to normoxia (N) or hypoxia (H) for 24 h and real time PCR analysis used to determine the expression levels of Pur α and β -actin mRNA. *B*, U937 cells were transfected in parallel with p11b-Wt and pATLuc each mixed with the Renilla luciferase plasmid pRL-CMV. Transfection of p11b-Wt was performed in the presence of either pHAPur1 that constitutively expresses Pur α or its empty equivalent pHA. Transfected cells were subjected to hypoxia for 24 h and firefly and Renilla luciferase assays performed. The levels of Renilla luciferase activity were taken as reflective of transfection efficiency and used to correct the firefly luciferase assay results. Each histogram represents the mean \pm SEM of three independent experiments. *C*, U937 transfections performed as described in *B* but using p11c-Wt rather than p11b-Wt.

indicate that Pur α is both necessary and sufficient to effect HIF-1-independent induction of the β_2 integrins in response to hypoxia.

Discussion

It is recently appreciated that as part of the ongoing inflammatory response, hypoxia may be a contributing factor to the overall tissue phenotype (33, 34). Shifts in energy metabolism associated with either localized or systemic hypoxia represents both a cause and an effect of inflammation. Hypoxia promotes inflammatory lesions through mechanisms involving increased monocyte binding to the vascular endothelium (6), and the vast majority of inflammatory cells are recruited to, as opposed to being resident at, inflammatory lesions. This induced binding is dependent upon the increased function of adhesion molecules expressed on both the leukocyte surface and the surface of endothelium (35–37). A critical group of

leukocyte adhesion molecules is the β_2 integrin family. This family comprises four heterodimers, composed of a common β subunit encoded by the CD18 gene linked with one of four possible α subunits encoded by the CD11a, CD11b, CD11c, and CD11d genes (11–14). In this study, we demonstrate that the α subunits of β_2 integrin family are coordinately induced by hypoxia and identify, for the first time, that Pur α represents a common transcriptional regulator of this response.

Previous work has shown that endothelial cells contribute to inflammation by directly sensing hypoxic conditions and responding through induction of the selectin and ICAM-1 genes (35, 36). These studies suggested that in hypoxia, endothelial cells communicate with leukocytes primarily through the release of chemokines/cytokines and the functional activation of leukocyte-adhesive properties (35, 36). However, it is recently appreciated that monocytes have the capacity to transcriptionally induce adhesion molecules (e.g., CD18) independent of the hypoxic response of the endothelium. Central to this mechanism is leukocyte HIF-1 α (16). Because β_2 integrins are expressed on the monocyte surface as heterodimers, induction of the CD18 gene would have no functional consequence without concomitant induction of at least one of the CD11 genes. Initially, we profiled induction of the CD11a, CD11b, CD11c, and CD11d genes and demonstrated that each is induced at the mRNA and protein level following subjection to hypoxia. Notable were the differences in kinetics of this response for the various integrins. For example, induction of CD11b and CD11c is more rapid and transient than induction of CD11a and CD11d. Our previous studies have shown that the hypoxic induction of the CD18 gene is mediated by its interaction with HIF-1. Using antisense oligonucleotides and siRNA, we demonstrate in this study that HIF-1 contributes, albeit to a lesser extent than CD18, to induction of the CD11a and CD11d genes. However, this approach (HIF-1 antisense/siRNA) did not significantly influence hypoxia inducibility of CD11b or CD11c genes. Consequently, β_2 integrin induction by hypoxia appears to be mediated by both HIF-1-independent and HIF-1-dependent mechanisms. That said, we have no evidence that either CD11a or CD11d are directly regulated by HIF-1. For example, we did not identify an obvious consensus binding sequence for HIF-1 α , and the degree of transcriptional inhibition using HIF-1 antisense approaches was significantly less than CD18, which bears a classic HIF response element (16). Such findings implicate a more indirect role for HIF in CD11a and CD11d regulation. From this standpoint, a number of transcription factors have been implicated in the control of β_2 integrin expression, including AP-1, MS-2, Sp1, and members of the Ets family (19, 27, 28, 38–42), and it is appreciated that HIF cooperates with a number of other transcriptional regulators (1, 43).

It is striking that the CD11a, CD11d, and CD18 genes that are dependent upon HIF-1 are all induced to maximal levels after the CD11b and CD11c genes that are HIF-1-independent. We reasoned that such HIF-1-independent mechanisms likely involved a transcription factor that binds both genes such that their induction can be coordinated. In this study, we identified this factor as Pur α . This is the first description of Pur α mediating a hypoxia response. However, previously a role for Pur α in inflammation had been implied by its control of TGF- β 1, CD11c, and CD43 expression and its increase in infiltrating eosinophils and activated endothelium during allergic reactions (19, 44, 45). Calcium mobilization is characteristic of the cellular activation that occurs during inflammation. In this regard, it is of note that the DNA binding activity of Pur α is increased by interaction with the calcium binding protein calmodulin (46).

Although Pur α has the capacity to bind dsDNA, its ability to bind ssDNA is at least 10-fold greater (31). The binding sites for Pur α within the CD11b and CD11c genes are associated with repeat sequences with the potential to form slippage structures with single-stranded loops. An induction in the availability of such loops might contribute to the dramatic induction of Pur α binding to the CD11b and CD11c promoters observed by chromatin immunoprecipitation. Indeed, it is possible that Pur α could itself contribute to the generation of single-stranded conformations because with isolated plasmids it can effect DNA unwinding (47).

Taken together, our results indicate that hypoxia coordinately induces β_2 integrins by mechanisms which involve both HIF-1 α and Pur α . Our results indicate that indirect and direct oxygen sensing are linked at the molecular level. Specifically, while in this study we demonstrate that Pur α is required for direct hypoxia induction of the CD11c gene, previously we have reported it is also required for CD11c induction by phorbol ester (19). Taking phorbol ester as a mimic of a cytokine response, our data implicate Pur α in both direct and indirect hypoxia sensing during inflammation.

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Disclosures

The authors have no financial conflict of interest.

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