Intrinsic regulation of matrix metalloproteinase-2 revealed by in vivo transcriptional analysis in ischemia

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Matrix metalloproteinase-2 (MMP-2) plays an essential role in angiogenesis and arteriogenesis, two processes critical to restoration of tissue perfusion after ischemia. MMP-2 expression is increased in tissue ischemia, but the responsible mechanisms remain unknown. We studied the transcriptional activation of the MMP-2 gene in a model of hindlimb ischemia by using various MMP-2-lacZ reporter mice and chromatin immunoprecipitation. MMP-2 activity and mRNA were increased after hindlimb ischemia. Mice with targeted deletion of MMP-2 had impaired restoration of perfusion and a high incidence of limb gangrene, indicating that MMP-2 plays a critical role in ischemia-induced revascularization. Ischemia induced the expression and binding of c-Fos, c-Jun, JunB, FosB, and Fra2 to a noncanonical activating protein-1 (AP-1) site present in the MMP-2 promoter and decreased binding of the transcriptional repressor JunD. Ischemia also activated the expression and binding of p53 to an adjacent enhancer site (RE-1) and increased expression and binding of nuclear factor of activated T-cells-c2 to consensus sequences within the first intron. Deletion of either the 5′ AP-1/RE-1 region of the promoter or substitution of the first intron abolished ischemia-induced MMP-2 transcription in vivo. Thus, AP-1 transcription factors and intronic activation by nuclear factor of activated T-cells-c2 act in concert to drive ischemia-induced MMP-2 transcription. These findings define a critical role for MMP-2 in ischemia-induced revascularization and identify both previously uncharacterized regulatory elements within the MMP-2 gene and the cognate transcription factors required for MMP-2 activation in vivo after tissue ischemia.

angiogenesis | gelatinase | gene expression | skeletal muscle | transcription factor

Ischemia secondary to arterial ischemia remains a major source of morbidity and mortality. Despite their importance, the cellular and molecular mechanisms that drive transcription of the genes essential for reversal of ischemia in vivo remain largely undefined. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes important in tissue remodeling and repair. MMP-2 has been implicated in the collateral arterial enlargement that occurs in response to tissue ischemia (1), and its expression is essential for retinal and tumor-induced angiogenesis (2, 3). MMP-2 (gelatinase A) can cleave most extracellular matrix proteins, including collagen, and can proteolytically activate other proenzymes, such as MMP-9.

The lack of MMP-14 (membrane-type-1-MMP), the cell surface protease that cleaves proMMP-2 to its active form, abolishes postnatal angiogenesis (4), and targeted deletion of MMP-9 impairs revascularization after hindlimb ischemia (5). Experimental hindlimb ischemia increases expression of MMP-2, MMP-9, and MMP-14 (6). Despite the potential importance of MMP-2 in ischemia-induced angiogenesis and arteriogenesis, the role of MMP-2 in this process and the transcriptional regulatory mechanisms that regulate MMP-2 in tissue ischemia remain undefined.

In vitro analysis of transcriptional induction of MMP-2 has shown widely varying requirements for transcription factors and promoter sequence requirements among cultured cell types. Binding of the transcription factors YB-1, AP-2, and p53 to an enhancer element designated RE-1, located at −1,322 bp relative to the translation start site in the rat MMP-2 promoter, mediates transcription in mesangial (7) and tumor cells (8). In contrast, the proximal Sp-1 and AP-2 sites at −90 to −50 bp are sufficient for constitutive MMP-2 transcription in astrocytoma cell lines (9). We recently demonstrated that activation and binding of activating protein-1 (AP-1) family transcription factors, specifically Fra1-JunB and FosB-JunB heterodimers, to a noncanonical AP-1 site (located at −1,392 bp relative to the start site of translation) are essential for hypoxia-induced MMP-2 transcription in cardiac fibroblasts (10).

Despite these widely varying in vitro findings, there are no reports describing MMP-2 transcription in any in vivo context. The purpose of this study was to determine the promoter elements and transcription factors critical for ischemia-induced MMP-2 expression in vivo. We demonstrate that the lack of MMP-2 impairs ischemia-induced revascularization and identify AP-1 transcription factors, p53, and nuclear factor of activated T cells (NFAT) c2 as critical to ischemia-induced MMP-2 transcription.

Methods

Transgenic MMP-2 Reporter Strains. Three transgenic reporter strains were generated to define the transcriptional role of MMP-2 promoter segments. The F8 mouse strain carries ~5 Kb of the rat MMP-2 gene coupled to a β-galactosidase reporter gene (Fig. 2). The MMP-2 promoter fragment extends from −1,686 bp (relative to the translation start site) to the middle of the second exon to include potential regulatory effects of exon and intron sequences. To determine the significance of the 5′ AP-1 and RE-1 sites, the F8-del strain, similar to F8 but lacking the 5′ end of the promoter (−1,686 to −1,241 bp) was created. To determine the role of the first intron of MMP-2 on gene expression, a third strain (F8-HGH) was created by replacing the MMP-2 first intron and second exon with the heterologous human growth hormone (HGH) intron. The detailed cloning, screening, and characterization of these lines is provided in the Supporting Methods, which is published as supporting information on the PNAS web site.

Hindlimb Ischemia and Blood Flow Determination. Hindlimb ischemia was induced by unilateral femoral artery excision (11)
under ketamine/xylazine anesthesia in 6- to 8-week-old CD-1 mice (Charles River Laboratories), transgenic reporter mice, mice with targeted deletion of MMP-2 (12) (C57Bl6 background, kindly provided by Timothy Baxter, University of Nebraska, Lincoln), and C57Bl6 control mice. All procedures were approved by the Institutional Animal Care and Use Committee. At selected time points, blood flow was determined in both limbs by using a blood perfusion monitor (Vasamedics BPM3045) as described in refs. 13 and 14.

**Zymography and β-Galactosidase Assay.** Surgically excised gastrocnemius muscle was homogenized for zymography in M-Per lysis buffer (Pierce) or reporter lysis buffer for β-galactosidase assay (Promega). β-galactosidase assay, zymography, and densitometry were done as described in refs. 8 and 10.

**Chromatin Immunoprecipitation.** Ischemic and nonischemic (contralateral) gastrocnemius muscles from CD-1 mice were minced in cross-linking buffer A (PBS-calcium magnesium free/1 mM EDTA/0.5% Nonidet P-40/0.25% Triton X-100/10% glycerol), cross-linked with 1% formaldehyde, homogenized with a Polytron (Brinkmann Instruments), shaken for 30 min and further homogenized with a Dounce homogenizer. Chromatin immunoprecipitation was performed as described in ref. 15 with antibodies (gel shift antibodies, Santa Cruz Biotechnology) against FosB, JunB, c-Fos, c-Jun, Fra1, Fra2, JunD, AP-2α, YB-1, p53, and NFATc2. PCR primer sequences and conditions are listed in *Supporting Methods*. Pilot experiments were done to ensure that the number of cycles was within the range of exponential amplification for each transcription factor. Positive controls (mouse tail DNA and input chromatin before immunoprecipitation) and negative controls (mock immunoprecipitation) and negative controls (mock immunoprecipitation without primary antibody) were run in each experiment and all experiments were performed three to five times with samples from separate animals. To confirm transcription factor binding to the AP-1 and RE-1 sites, competitive oligonucleotide binding studies were performed (see *Supporting Methods*; see also Figs. 8 and 9, which are published as supporting information on the PNAS web site).

**Quantitative Real-Time RT-PCR.** Total RNA was extracted by homogenizing whole gastrocnemius muscles in TRI Reagent ( Molecular Research Center, Inc.). Five micrograms of total RNA was transcribed by using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen). Using SYBR Green Detection and an Applied Biosystems Prism 7900HT detection system, transcribed cDNA was amplified for 40 cycles for 15 s at 95°C and 60 s at 59.5°C. PCR primer sequences are listed in *Supporting Methods*. MMP-2 mRNA was normalized to its respective GAPDH mRNA.

**Immunohistochemistry.** Five-micrometer sections were cut of paraffin-embedded muscle after fixation in 4% buffered paraformaldehyde. Sections were stained with a MOM Kit (Vector Laboratories) with the primary antibodies (1:250 dilution) for 4–8 h, followed by biotinylated secondary antibodies [goat-anti-mouse (Zymed) and rabbit-anti-goat and goat-anti-rabbit (Chemicon)] (1:500 dilution) for 1–2 h. Primary antibodies included a monoclonal antibody to β-galactosidase (CR7001M, Cortex Biochem), antibodies to transcription factors (listed above) and control mouse IgG.

**Statistical Analysis.** Chromatin immunoprecipitation and immunohistochemical staining experiments were performed at least three times with samples from different animals with similar results. For blood flow and β-galactosidase assays, data were compared with ANOVA or paired t test (ischemic and contralateral limb values from each animal) as appropriate. Differences were considered significant at *P* < 0.05.

**Results**

**Ischemia Induces MMP-2 Activity, mRNA, and Transcription in Skeletal Muscle.** Femoral artery excision caused a 2-fold decrease in blood flow after 3 days (Fig. 1A) and increased MMP-2 activity by zymography (Fig. 1C) and MMP-2 mRNA by quantitative real-time PCR (Fig. 1B) in skeletal muscle. Mice lacking MMP-2 had impaired restoration of blood flow after hindlimb ischemia compared with C57Bl6 mice (Fig. 1D) and a high incidence (40%) of gangrene and limb autoamputation, which was not seen in any C57Bl6 animals. Hindlimb ischemia in the F8 transgenic reporter mouse demonstrated that ischemia-induced MMP-2 transcription peaked on day 3 (Fig. 2C). The degree of ischemia was relatively constant (Fig. 2B) over the time period studied (6 h to 7 days) as we have described in refs. 13 and 16. Expression of the β-galactosidase reporter gene was seen in a variety of cell types, with prominent expression in the endothelium, smooth muscle of arterioles, inflammatory cells, and skeletal myocytes (Fig. 2D, see also Fig. 10, which is published as supporting information on the PNAS web site).

**Ischemia-Induced MMP-2 Transcription Requires Two Distinct Regions of the MMP-2 Gene.** To determine which regions of the MMP-2 gene are critical for ischemia-induced transcription, we then
Ischemia Induces Expression and Binding of AP-1 Transcription Factors and p53 to the 5′ Aspect of the MMP-2 Promoter. The AP-1 and RE-1 sites (−1,370 bp and −1,346 bp in the mouse MMP-2 promoter) were studied with chromatin immunoprecipitation in ischemic and nonischemic limbs. Ischemia induced the binding of FosB, JunB, c-Fos, c-Jun, and Fra2 to the AP-1 site and concurrently decreased basal JunD binding (Fig. 4). Immunohistochemistry demonstrated increased expression of FosB, JunB, c-Fos, c-Jun, Fra2, and JunD in ischemic muscle (Fig. 5; see also Fig. 11, which is published as supporting information on the PNAS web site). FosB and c-Fos staining were more prominent in the vascular and inflammatory cells, whereas c-Jun, JunB, Fra2, and JunD were more uniformly expressed in skeletal myocytes. There was no binding of YB-1 to the RE-1 site in ischemic or nonischemic muscle by chromatin immunoprecipitation, a marked increase in p53 binding with ischemia.
and no change in basal occupancy by AP-2 (Fig. 6A). Immunohistochemistry demonstrated increased p53 and AP-2 expression in ischemic muscle with expression in skeletal myocytes (Fig. 6B). No change in expression of YB-1 was found with ischemia (data not shown). Ischemia-induced AP-1 and p53 binding to the specific 5’ AP-1 site and decreased JunD binding. Results shown are representative of 3–6 similar experiments per transcription factor.

**Ischemia Induces NFATc2 Expression and Binding to the First Intron of the MMP-2 Gene.** To identify ischemia-induced transcription factor interactions that would account for the requirement of the first MMP-2 intron, we studied the sequence of the first intron for potential transcription factor binding sites with TESS, (Transcription Element Search Software) (15) with a high stringency algorithm (log-likelihood score >14). Numerous potential binding sites for AP-2 and NFATc2 were identified (Fig. 7A), and one FosB site with a log-likelihood score of 12. Chromatin immunoprecipitation with three primer sets to span these sites (Fig. 7A) demonstrated ischemia-induced NFATc2 binding only to a cluster of sites in the 3’ aspect of the intron (Fig. 7D), no basal or ischemia-induced binding of AP-2 (Fig. 7B), and no change in basal FosB occupancy with ischemia (Fig. 7C). Immunohistochemistry demonstrated increased NFATc2 protein in ischemic myocytes (Fig. 7E).

**Discussion**

We identify both the 5’ AP-1/RE-1 region and the first intron as critical for ischemia-induced MMP-2 expression. In contrast to the previously well characterized 5’ AP-1/RE-1 region (7, 8, 10), there are no studies of transcriptional regulation in the MMP-2 intron, and we consequently identified ischemia-induced expression of NFATc2 and its binding to the 3’ aspect of the intron. Our findings collectively suggest that NFATc2 mediates MMP-2 transactivation via intronic regulation of the MMP-2 promoter. There are no prior reports of intronic regulation of an MMP gene or of an NFAT transcription factor that regulates MMP-2 expression. NFAT regulation was initially described in T cell activation (18); however, recent reports describe NFAT family members as regulating skeletal muscle development (19), regeneration (20), and cardiac ischemia-reperfusion injury (21). Our laboratory recently reported that NFATc1 regulates MMP-14 (membrane-type 1-MMP) transcription in mesangial cells (15). Thus, significant roles for various NFAT family members in regulating MMP transcription may be emerging.
Intronic regulation by NFAT family members has been described for other genes, including NFATc3 for CD21 (22) and multiple NFAT family members for the T cell receptor gene (23).

The finding that the AP-1/RE-1 region (contained within −1,686 to −1,241 bp in the rat MMP-2 gene) is essential for ischemia-induced MMP-2 expression is consistent with induction of AP-1 factors in hypoxia in vitro and ischemia in vivo. Hypoxia induces transcriptional activation of the canonical AP-1 components c-Fos and c-Jun in vitro (24), as does experimental cerebral ischemia (25) and ischemia in human brain tissue (26). Nonischemic stimuli can trigger AP-1 transcription factor expression, e.g., moderate exercise in human skeletal muscle increases c-Fos, c-Jun, Fra-1, and JunB (27). Similarly, the increased occupancy of the RE-1 site by p53 in ischemic muscle (Fig. 6) defines an in vivo transcriptional role for the previously described activation of p53 by hypoxia in vitro (28) and by ischemia-reperfusion of skeletal muscle in vivo (29).

Ischemia increases expression of transcription factors (e.g., AP-2) that does not always correlate with increased binding to the MMP-2 promoter (Fig. 6 A and B). These discrepancies emphasize the value of chromatin immunoprecipitation to define actual occupancy by a transcription factor of a specific site on the chromatin of the promoter of interest. Chromatin immunoprecipitation for foxA transcription factors has shown similar disparities between protein expression and actual promoter occupancy (30). As chromatin immunoprecipitation requires PCR amplions of ~600 bp generated from precipitated chromatin fragments of 1,000–1,500 bp, positive results may be due to transcription factor binding anywhere in the 1,500-bp fragment. We therefore confirmed ischemia-induced specific binding of c-fos and p53 to the AP-1 and RE-1 sites, respectively, by competitive oligonucleotide binding studies (Figs. 8 and 9). Ischemia-induced binding of AP-1 factors and p53 to the 5′ region of MMP-2 were partially suggested by our prior in vitro studies of hypoxia-induced JunB-Fra1 and JunB-FosB binding to the AP-1 site (10) and p53 binding to the RE-1 site in conjunction with YB-1 and AP-1 (7, 8, 31). The functional importance of these sites is confirmed by the loss of ischemia-induced MMP-2 transcription upon deletion of this region (Fig. 3).

The decreased binding of JunD at the AP-1 site noted in ischemia (Fig. 4) is consistent with JunD acting as a repressor that becomes displaced by other AP-1 family members to allow transcriptional activation (32). JunD expression is decreased in end-stage human cardiomyopathy (33), a condition in which MMP-2 expression is elevated (34). Ischemia-induced displacement of basal JunD occupancy from the AP-1 site by other AP-1 proteins (Fig. 4) is an in vivo demonstration of the JunD/AP-1 transcriptional repression/transactivation balance previously well characterized only in vitro (32).

Induction of MMP-2 transcription has been detected in endothelial cells, vascular smooth muscle cells, and skeletal myocytes (Fig. 2D). MMP-2 is essential for vascular smooth muscle cell migration in vivo (35) and endothelial cell migration during angiogenesis (2, 3). MMP-2 is also up-regulated in skeletal myocytes after nonischemic cardiotoxin injury (36). We have shown that MMP-2 can serve as a growth factor in vitro (37). The stimuli for transcriptional activation of MMP-2 may be different (i.e., hypoxia vs. cell injury) in various cell types in ischemic tissue. Accordingly, MMP-2 may serve different functions in tissue repair and revascularization in various cell types as well. This concept is supported by the distinct cellular patterns of expression of the various AP-1 family transcription factors members noted in ischemic muscle tissue (Fig. 5). These findings suggest that cell-type specific transcriptional mechanisms activate MMP-2 expression.

There are limitations of this study to note. The high incidence of gangrene after induction of hindlimb ischemia in MMP-2 (−/−) mice precluded arteriography and capillary counts at later time points to define how the lack of MMP-2 impairs ischemia-induced revascularization and exacerbates hindlimb ischemia. Milder models of ischemia or nonhypoxic inflammatory cell models (cardiotoxin injection) may be required to elucidate these mechanisms. We used three primer sets within the first intron to localize ischemia-induced NFATc2 binding to the 3′ aspect of the intron (Fig. 7); finer analysis of these interactions awaits development of an in vitro model exhibiting intronic regulation similar to that noted in vivo with ischemia.

This study shows a role for novel transcription factors (NFATc2), and how previously undefined regulatory regions (intron 1) mediate ischemia-induced MMP-2 transcription in vivo.
These findings were not predicted by prior in vitro studies of MMP-2 transcription from our laboratory (7, 8, 10, 31) or others (9) and demonstrate the importance of in vivo transcriptional analysis to define novel mechanisms of gene regulation in clinically relevant conditions such as tissue ischemia. Most cultured cell lines constitutively produce MMP-2, whereas MMP-2 is transcriptionally induced after injury or ischemia in vivo. This discrepancy may account for the failure of transcriptional studies in cell culture to define the regulatory elements we identify in vivo. Given the importance of MMP-2 in a wide range of inflammatory, neoplastic, and fibrotic disorders (38), this experimental approach, and our findings regarding MMP-2 transcription in tissue ischemia, may be a model for understanding the transcription of MMP-2 in other in vivo models of development and clinical disease.

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