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NF-κB binds to a polymorphic repressor element in the MMP-3 promoter

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Abstract

A 5T/6T polymorphic site in the matrix metalloproteinase-3 (MMP-3) promoter has been identified as a repressor element involved in inhibiting induction of MMP-3 transcription by interleukin 1; and the 6T allele has been associated with decreased expression of MMP-3 as compared to the 5T allele. Zinc-binding protein-89 (ZBP-89) was cloned from a yeast one-hybrid assay via its ability to interact with this site, but when the protein was over-expressed, it resulted in activation of the MMP-3 promoter rather than repression. Here we show that in nuclear extracts isolated from human gingival fibroblasts stimulated with IL-1, this site is bound by p50 and p65 components of NF-κB in addition to ZBP-89, and that recombinant p50 binds preferentially to the 6T binding site. These results are consistent with a role for NF-κB in limiting the cytokine induced expression of MMP-3.
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Keywords: Stromelysin-1; ZBP-89; Gene expression regulation; NF-κB

Stromelysin-1 (MMP-3) is a metalloproteinase with broad substrate specificity, capable of degrading proteoglycan, laminin, fibronectin, and the non-fibrillar collagens [1]. Perhaps equally important, it is also capable of activating other pro-MMPs, including MMP-1, -8, -9, and -13 [2–6], of inactivating plasminogen activator inhibitor I [7], and of cleaving FasL [8] and E-cadherin [9]. MMP-3 is produced by synovial and gingival fibroblasts, chondrocytes, macrophages, neutrophils, and endothelial cells in response to inflammatory cytokines [e.g., interleukin-1 (IL-1) and tumor necrosis factor (TNF)] and mitogens. Its role in tissue destruction associated with chronic inflammation in rheumatoid arthritis and periodontitis is well established. In addition, it also plays an important role in normal tissue remodeling as well as in other pathological processes such as cancer [10–12] and atherosclerosis [13–15].

In a previous study of the MMP-3 promoter region, the SIRE site (stromelysin IL-1 responsive element; $^{-1614}$G(T)TTTTTCCCCCCATCAAAG^{-1595}), was identified as a site of IL-1 induced DNA binding [16]. Transient transfection experiments using a reporter construct in which the SIRE site was mutated in the context of a 2.3 kb MMP-3 promoter fragment showed that the mutant construct has a twofold greater IL-1 induction as compared to the wild type. These results indicated that proteins binding to the SIRE site act as a repressor of cytokine induced expression of MMP-3 [16].

This same site has also been identified as the site of a common 5T/6T polymorphism, and there is evidence that the repressor binds with higher affinity to the 6T site than to the 5T [17,18]. A 5T reporter construct was twice as transcriptionally active as a 6T construct in transient transfection experiments [18], and tissue levels of MMP-3 protein are determined by the polymorphism as expected from in vitro studies [19,20]. In addition, there are now many studies that find an association of the polymorphism with either susceptibility to or severity of various diseases and conditions [21–26]. These studies suggest that both low and high levels of MMP-3 can have important pathological consequences. For example, in cardiovascular disease homozygosity for the high-expressing 5T allele is associated with increased risk for myocardial infarction and aneurysm [27–30], while the low-expressing 6T allele is associated with stenosis and

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more rapid progression of atherosclerosis [17,31–34]. Based on these studies it is clear that levels of MMP-3 must be tightly controlled to maintain health, and that the SIRE site plays an important role in this fine-tuning control.

Recently, zinc binding protein 89 (ZBP-89) was cloned from a yeast one-hybrid system through its ability to bind to this site [35]. However, it seemed to function as an activator rather than a repressor of MMP-3 transcription in over-expression studies, and its binding was dependent on Cs in the site rather than the Ts as expected for the repressor [35]. Binding of endogenous ZBP-89 to the SIRE site was not shown.

In the experiments reported here, we confirm that ZBP-89 is expressed in HGF, and that it is included in IL-1 induced binding to the SIRE site in nuclear extracts isolated from these cells. In addition, we show that these complexes also contain the well-known transcription factor, NF-κB, and that recombinant NF-κB p50 binds preferentially to the 6T SIRE site. These results are consistent with a role for NF-κB in fine-tuning expression of MMP-3 in response to inflammatory cytokines.

Materials and methods

Cell culture. Human gingival tissue samples from patients undergoing periodontal surgery were obtained from Howard M. Sobel, D.D.S. and Kevan S. Green, D.M.D. of Sobel Periodontal Associates, P.C. The tissue was processed by enzymatic dispersion to produce primary cultures [36,37]. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (penicillin, streptomycin, and amphotericin; Gibco-BRL). Cells between passages 3 and 5 were used for experiments. Cells were serum deprived for 16 h in serum free EMEM supplemented with 10% insulin, transferrin, and sodium selenite (Sigma Chemical) prior to the addition of 100 ng IL-1β/ml (DuPont-Merck) or 10 ng TNFα/ml (Sigma Chemical) or 10 ng/ml IL-4 (Sigma Chemical) or 10 ng LPS/ml (Calbiochem).

Northern blot analysis. Total RNA was isolated according to the acid–phenol method of Chomczynski and Sacchi [38], and 15 μg was run on 1% agarose–formaldehyde gels. A 900 bp cDNA fragment of ZBP-89 was cloned by PCR from HGF, inserted in the pGEM-T Easy vector (Promega), and fully sequenced. Probes were made by random priming (Stratagene, La Jolla, CA) of the ZBP-89 cDNA and of a cDNA corresponding to glyceraldehyde-3-phosphate dehydrogenase (a gift of R. Newton, Wilmington, DE).

Nuclear extract isolation and EMSA Nuclear extracts were isolated according to the method of Schreiber et al. [39] and quantitated in mini-Bradford assays (Pierce). Synthetic oligonucleotides corresponding to the 5T or 6T SIRE site (5′ ACAAGACATGG(T)TTTTTCCC CCCATCAAAG 3′; Invitrogen) were annealed and treated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. A consensus NF-κB binding site was obtained from Santa Cruz Biotechnology and labeled in the same way. An oligonucleotide probe corresponding to a ZBP-89 binding site from the ornithine decarboxylase promoter was also used in competition experiments (5′ GGCCGATGCGCCCCTC CCCGCGCCGATC 3′, [40]). Binding reactions contained 5 μg protein, 20 mM Hepes–OH, pH 7, 50 mM NaCl, 0.2 M EDTA, 5% glycerol, 4 μg poly(dI–dC), and 10,000 cpm probe in a total volume of 20 μl.

Western blotting. Twenty micrograms nuclear extract was separated on 10% SDS–polyacrylamide gels. Proteins were transferred to Immobilon-P Transfer Membrane (Millipore) and the membrane was blocked overnight with Super Block blocking buffer (Pierce). The blot was then incubated with anti-ZBP-89 mouse monoclonal antibody (BD Transduction) diluted 1:1000 in Super Block for 1 h at room temperature. After washing three times in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween 20), the blots were incubated with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Pierce) for 1 h. After three more washes in TBST, the blot was incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to X-ray film.

Results

ZBP-89 is expressed in HGF and binds to the SIRE site

Zinc binding protein-89 was cloned from a cDNA library through its ability to bind to the SIRE site in a yeast one-hybrid system, and the in vitro translated protein was shown to bind to the site in EMSA [35]. In order to begin to determine the role of endogenous ZBP-89 in regulation of MMP-3 expression in HGF, Northern blot analysis was done to determine whether or not ZBP-89 is expressed in these cells, and if so, whether its levels are regulated by inflammatory cytokines. Results of these experiments (Fig. 1) showed that ZBP-89 mRNA and protein are expressed in HGF, but levels do not change appreciably in response to IL-1β, IL-4, TNFα or LPS.

Supershift analysis showed that ZBP-89 is contained in IL-1 induced complexes binding to the SIRE site in HGF nuclear extracts (Fig. 2). We previously reported that there are two major complexes binding to the SIRE site in gingival and synovial fibroblasts. Each of these

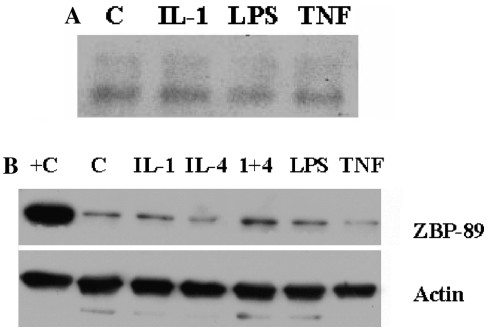

Fig. 1. ZBP-89 is expressed in HGF. (A) Total RNA was isolated from untreated HGF and from cells treated for 12 h with IL-1β (100 ng/ml), LPS (10 ng/ml) or TNFα (10 ng/ml). Northern blots were hybridized to a ^{32}P-labeled cDNA probe corresponding to human ZBP-89. (B) Nuclear extract isolated from rat kidney (as a positive control) and nuclear extract isolated from untreated HGF and cells treated for 12 h with IL-1 (100 ng/ml), IL-4 (10 ng/ml), IL-1 and IL-4, LPS (10 ng/ml) or TNFα (10 ng/ml) were separated on 10% SDS–PAGE and transferred to a nylon membrane. Proteins were detected with anti-ZBP-89 and anti-actin antibodies followed by chemiluminescence. +C, positive control, rat kidney nuclear extract. C, control, no cytokines.

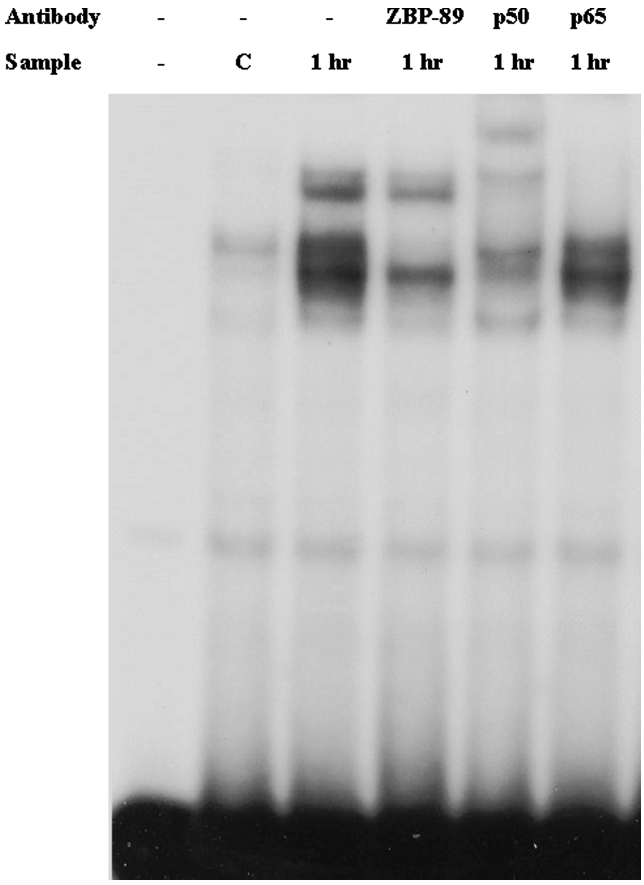

Fig. 2. IL-1 induced binding to the SIRE site contains NF-κB p50 and p65 in addition to ZBP-89. Nuclear extract isolated from HGF cultures 1 h after addition of IL-1 (100 ng/ml) was incubated with a ^{32}P-labeled oligonucleotide corresponding to the 6T SIRE site. Antisera against NF-κB p50, p65, and ZBP-89 were added as indicated. P, probe alone, no extract.

two complexes can be further resolved and shown to contain two complexes. The ZBP-89 antisera preferentially inhibited the upper complex of Complex 2. It did not affect binding of the lower portion of Complex 2 and did not affect Complex 1 at all. These results indicate that although ZBP-89 is present in HGF nuclear extracts and capable of binding to the SIRE site, it is not the only protein capable of binding to this site.

IL-1 induced binding to the SIRE site contains NF-κB p50 and p65

A search of the TRANSFAC database [41] showed that the SIRE binding site has some similarity to the consensus binding site for dorsal, a *Drosophila* homologue of human NF-κB. Since IL-1 is known to induce NF-κB in these cells, antibodies specific for p50 and p65 components of NF-κB were used in supershift experiments. Results, shown in Fig. 2, demonstrated that NF-κB is in fact involved in binding to the SIRE site. Both p50- and p65-specific antisera resulted in supershifts, but

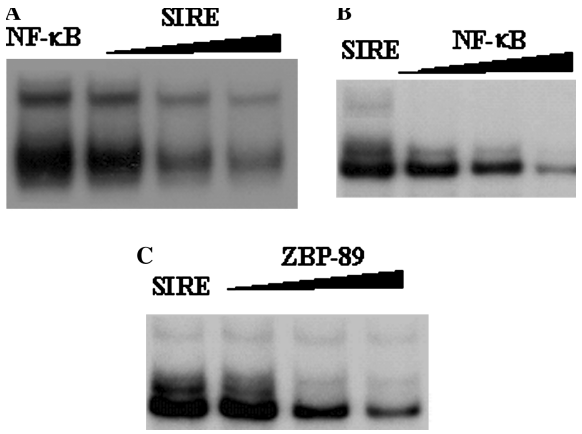

Fig. 3. Cross-competition of NF-κB consensus and ZBP-89 binding sites with the 6T SIRE site. Nuclear extract isolated from HGF cultures 1 h after addition of IL-1 (100 ng/ml) was incubated with the indicated ^{32}P-labeled oligonucleotide probes in the presence and absence of increasing amounts of the indicated unlabeled competitor oligonucleotide binding sites.

a c-Rel antibody had no effect (not shown). Both bands of Complex 1 were shifted by antisera directed against p65, while the p50 antibody shifted the lower portion of Complex 1 and the lower portion of Complex 2. Although the possibility of alternative dimers cannot be ruled out, these results are consistent with the conclusion that Complex 1 contains primarily p50/p65 heterodimers, with the upper, lighter band perhaps consisting of p65/p65 homodimers, and that Complex 2 contains both p50/p50 homodimers and ZBP-89 binding independently. In addition, competition EMSA experiments (Fig. 3) showed that the SIRE binding site can compete for both complexes binding to an NF-κB consensus probe, although only partially. The unlabeled NF-κB consensus site was able to compete easily for binding of Complex 1 to the SIRE site probe, but less well for Complex 2. An unlabeled ZBP-89 binding site competed for the upper portion of Complex 2.

Recombinant NF-κB p50 binds preferentially to the 6T SIRE site

The fact that binding of ZBP-89 to the SIRE site was shown to be dependent on the C's in the binding site rather than the T's [35] suggested strongly that it could not be responsible for the decreased transcription of the 6T promoter construct as compared to the 5T [18]. Recombinant NF-κB p50 was used in EMSA to determine whether it could differentiate between 5T and 6T SIRE site probes. As shown in Fig. 4, recombinant p50 resulted in more binding to the 6T probe as compared to the 5T. Furthermore, competition with unlabeled 5T and 6T probes showed that the 6T competitor was more effective than the 5T competitor, suggesting that p50 homodimers bind preferentially to the 6T site. Taken

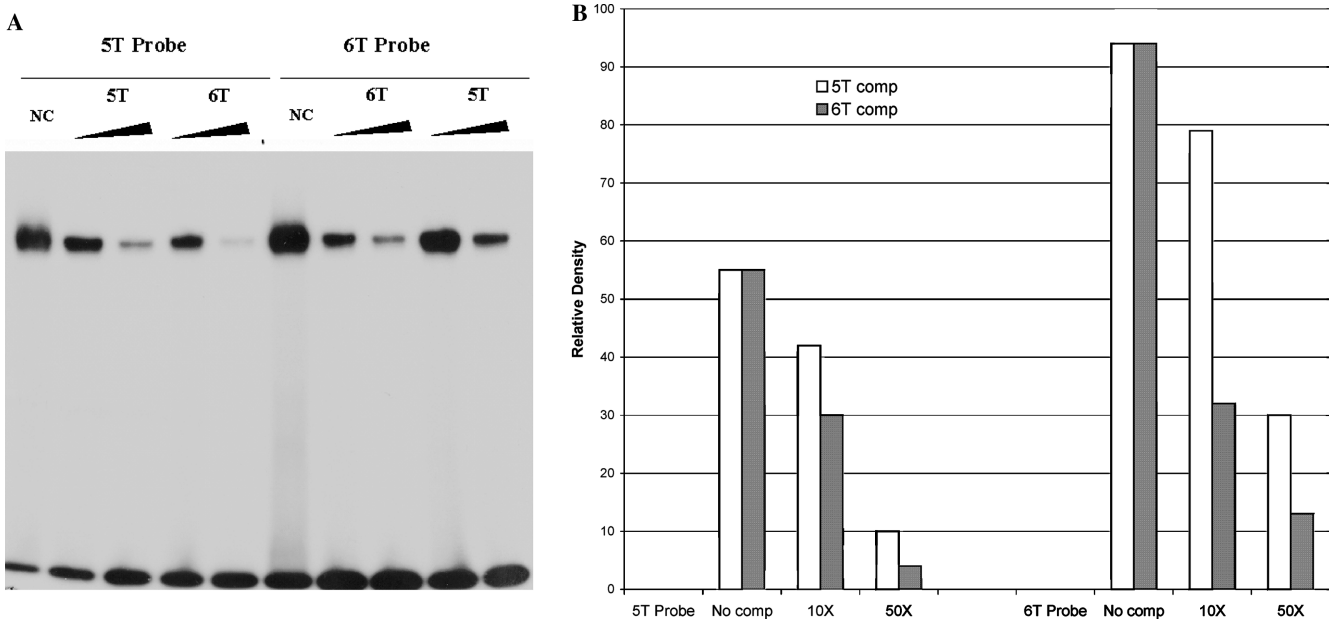

Fig. 4. Recombinant NF-κB p50 binds preferentially to the 6T SIRE site. (A) Nuclear extract isolated from HGF cultures one hour after addition of IL-1 (100 ng/ml) were incubated with a ^{32}P-labeled probe corresponding to the 5T or 6T SIRE site, as indicated. Increasing amounts of unlabeled 5T or 6T SIRE site oligonucleotides were added in competition. NC, no competition. (B) Autoradiographs were quantitated by scanning densitometry and expressed as relative density for comparison.

together, these results are consistent with a role for NF-κB as an IL-1 induced repressor of cytokine-induced expression of MMP-3 in HGF.

Discussion

Previous studies identified the SIRE site as a repressor of MMP-3 transcription [16,18]. Binding to this repressor element is induced by IL-1 and TNF, and mutation of two C's in the site eliminates binding and increases IL-1 induced transcription from the MMP-3 promoter [16]. Ye et al. [17] also identified the same region as the site of a common 5T/6T polymorphism and showed increased transcription from a 5T promoter construct as compared to a 6T [18]. These results suggested that the repressor binds with higher affinity to the 6T site than the 5T. They later cloned ZBP-89 in a yeast one-hybrid assay through its ability to bind to the SIRE site [35]. However, binding of the recombinant protein was dependent on C's in the site rather than the T's, and the over-expressed protein activated transcription from the MMP-3 promoter rather than repressing it. Thus, it was unlikely that ZBP-89 could by itself be responsible for the repressor function of the SIRE site.

Here we show that ZBP-89 mRNA and protein are in fact expressed in HGF (Fig. 1), and that ZBP-89 does bind to the SIRE site in HGF nuclear extracts (Fig. 2). However, results of supershift experiments made it clear that ZBP-89 alone is not responsible for IL-1 induced binding, since the ZBP-89 antisera affected only one of the four sub-complexes. A search of the TRANSFAC database [41] suggested that this site might be bound by some form of NF-κB, and supershift experiments confirmed that IL-1-induced binding to the SIRE site contains not only ZBP-89, but also p50 and p65 components of NF-κB (Fig. 2). Based on the fact that the ZBP-89 antibody did not shift any complexes containing p50 or p65, and vice versa, it seems likely that binding of ZBP-89 and NF-κB to the SIRE site are mutually exclusive, competitive events. Consistent with this, co-immunoprecipitation studies failed to find any evidence of physical interaction between ZBP-89 and p50 or p65 (not shown).

As noted previously, the SIRE site has also been identified as the site of a common genetic polymorphism [17]. Based on data from transient transfection experiments, one would expect that an individual homozygous for the 5T allele would express more MMP-3 than a heterozygous individual or one homozygous for the 6T allele, all other things being equal. Given the importance of this protein in both normal and pathological tissue remodeling, one might also expect that these differences would be reflected in altered susceptibility to and/or severity of certain diseases. In fact, there are numerous studies that show association of the 5T/6T polymorphism to various diseases and conditions [21–26], and most of these correlations are consistent with the hypothesis that the 5T/5T genotype would result in higher levels of MMP-3. Thus, for example, the 5T/5T

genotype (higher expression of MMP-3) is associated with increased risk for aneurysms and myocardial infarction [27–30], while the 6T/6T genotype (lower expression) is associated with increased progression of atherosclerosis and stenosis [17,31–34]. Moreover, genotype at the SIRE site has been correlated with MMP-3 protein levels in tissue samples [19,20]. There is therefore ample evidence to suggest that this element and its polymorphism are physiologically important, and that it does act in vivo as a repressor element.

The present data do not allow any conclusion to be drawn as to which of the complex(es) binding to the SIRE site actually act as repressor(s) or how this function is accomplished. The fact that ZBP-89 over-expression resulted in activation of MMP-3 expression in transient transfection experiments [35] does not necessarily exclude it from consideration as a repressor in vivo. ZBP-89 has been reported to act as either an activator or a repressor of transcription, depending on context. When it is a repressor, it normally acts by competing with members of the Sp1 family for the same binding site [40,42–45], although it has also been shown to have a repressor domain [44]. It is therefore possible that ZBP-89 acts as a repressor by competing with NF-κB for binding to the SIRE site. However, as mentioned before, ZBP-89 binding was shown to be dependent on the C's in the site, but not the T's [35]. Since the 6T site has been shown to be a better repressor than the 5T [16,18–20], it is likely that the true repressor would bind preferentially to the 6T site. NF-κB is therefore a more attractive candidate for the repressor, since recombinant p50 does bind preferentially to the 6T site.

It was somewhat surprising to identify NF-κB as a repressor of cytokine-induced expression of MMP-3, since previous reports on MMP-3 regulation have suggested that NF-κB activates its expression [46–50]. However, none of these reports showed a direct role for NF-κB binding to the MMP-3 promoter and there are no canonical NF-κB sites in the promoter sequence. Thus it is possible that NF-κB acts as an activator of MMP-3 transcription, either through another non-canonical site or perhaps indirectly by activating other transcription factor(s), while at the same time acting as a repressor via the SIRE site.

Although NF-κB is most often an activator of transcription in response to inflammatory stimuli, there are a growing number of examples of NF-κB acting as a repressor. Many of these examples involve p50/p50 homodimers [51–59]. Since p50 does not have an activation domain [60,61], this inactive dimer can repress transcription by competing with p50/p65 heterodimers for binding to the same site. Thus, variations in the relative abundance of NF-κB dimers during cell differentiation or aging, or in different physiological conditions, can have important effects on gene regulation [51,53,54,57,58]. LPS tolerance is a well-studied example of this [55,56,59,62]. In addition, a polymorphism has also been identified in the TNFα promoter that reduces binding of p50 homodimers and thereby increases the enhancer function of the site, increasing transcription of the gene [52]. This polymorphism has also been associated with accelerated erosive rheumatoid arthritis [63]. It must also be noted however that NF-κB p50 homodimers have also been shown to be capable of activating gene expression by recruiting Bcl-3 [64–66], and that p65-containing dimers can also act as repressors by recruiting co-repressors or histone deacetylases [67,68]. Further experiments are needed to differentiate among these possibilities.

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