A Polymorphism of the Human Matrix γ-Carboxyglutamic Acid Protein Promoter Alters Binding of an Activating Protein-1 Complex and Is Associated with Altered Transcription and Serum Levels

Matrix γ-carboxyglutamic acid protein (MGP) is a mineral-binding extracellular matrix protein synthesized by vascular smooth muscle cells (VSMCs) and chondrocytes that is thought to be a key regulator of tissue calcification. In this study, we identified four polymorphisms in the promoter region of the human tissue calcification. In this study, we identified four polymorphisms in the promoter region of the human MGP gene. Transfection studies showed that the G→T and T→C polymorphisms have an important impact on in vitro promoter activity when transiently transfected into VSMCs. We found that one of these polymorphisms (T→C) is significantly correlated with serum MGP levels in human subjects. Promoter deletion analysis showed that this polymorphism lies in a region of the promoter critical for transcription in VSMCs. This region contains a potential activating protein-1 (AP-1) binding element located between −142 and −136. We have demonstrated that the T→C polymorphism results in altered binding of an AP-1 complex to this region. The −138T allelic variant binds AP-1 complexes consisting primarily of c-Jun, JunB and its partners Fra-1 and Fra-2 in rat VSMC. Furthermore, the −138T variant form of the promoter was induced following phorbol 12-myristate 13-acetate treatment, while the −138C variant was refractive to phorbol 12-myristate 13-acetate treatment, confirming that AP-1 factors preferentially bind to the −138T variant. This study therefore suggests that a common polymorphism of the MGP promoter influences binding of the AP-1 complex, which may lead to altered transcription and serum levels. This could have important implications for diseases such as atherosclerosis and aortic valve stenosis, since it strongly suggests a genetic basis for regulation of tissue calcification.

EXPERIMENTAL PROCEDURES

Subjects—DNA for the initial polymorphism identification was extracted from 40 subjects. Samples for the MGP assay and subsequent genotyping were obtained from 156 healthy subjects (55–65 years old) in Maastricht as described previously (12).

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Afsin Farzaneh-Far§§, John D. Davies§§, Levienja A. Braam, Henri M. Spronik, Diane Proudfoot‡, Shiu-Wan Chan‡, Kevin M. O'Shaughnessy**, Peter L. Weissberg‡, Cees Vermeer, and Catherine M. Shanahan‡

From the 2Division of Cardiovascular Medicine and 2Clinical Pharmacology Unit, University of Cambridge, Addenbrooke’s Hospital (ACCI level 6), Hills Road, Cambridge CB2 2QQ, United Kingdom and the 2Department of Biochemistry and Cardiovascular Research Institute, University of Maastricht, 6200 MD, Maastricht, The Netherlands

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† To whom correspondence should be addressed: Division of Cardiovascular Medicine, University of Cambridge, Addenbrooke’s Hospital (ACCI level 6), Hills Road, Cambridge CB22QQ, UK. Tel.: 44 1223 331504; Fax: 44 1223 331504; E-mail: af24@cam.ac.uk.

‡ These authors have contributed equally to this work.

¶ These authors have contributed equally to this work.

§ These authors have contributed equally to this work.

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1 The abbreviations used are: MGP, matrix Gla protein; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; VSMC, vascular smooth muscle cell; Gla, γ-carboxyglutamic acid; SSCP, single-stranded conformational polymorphism; PCR, polymerase chain reaction; ANOVA, analysis of variance; C/EBP, CCAAT/enhancer-binding protein; AP, activating protein.
**Single-stranded Conformational Polymorphism (SSCP) Analysis**—15 overlapping PCR primer pairs were used to amplify 3.3-kilobase pair DNA upstream of the MGP gene in 40 unrelated individuals. 5 μl of PCR product was added to 5 μl of loading dye (98% formamide, 0.2% bromphenol blue, 0.25% xylene cyanol, 10 mM EDTA), denatured by heating to 94 °C for 5 min, and then cooled to 90 °C to preserve single-stranded structure. The denatured samples were loaded onto 6–8% acrylamide gels and electrophoresed at 5-watt constant power overnight. The gel reactions were carried out either at 4 °C or 20 °C. Fragments were visualized by silver staining. Whenever a persisting deviant pattern was observed, the samples were sequenced in order to identify the sequence polymorphism involved.

**Genotyping—**Genotyping for the G–7A polymorphism was performed by using a mismatch PCR fragment amplified with the forward primer (5'-CAGTGATGGCGCCAAAATTCTTCGCA-3') and the reverse primer (5'-TATTGCCATGGGAGAGGTCTCC-3'), followed by digestion with the restriction enzyme NcoI. The T–138C polymorphism was genotyped using a mismatch PCR fragment amplified with the forward primer (5'-AAGCTAGTGGCCAAAATTCTTCGCA-3') and the reverse primer (5'-GAACATGCTGGGAACTTTCCTCCAC-3'), followed by digestion with the restriction enzyme BsiSI. The PCRs were performed in a total volume of 25 μl of a buffer solution containing the following: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.0 mM MgCl₂, 0.25 mM dNTP, 10 units of Taq DNA polymerase, and 0.25 μM forward and reverse primers. For the G–7A polymorphism, the reaction was run with a 3-min denaturation at 94 °C followed by 30 cycles of 94 °C for 30 s, 64 °C for 60 s, and 72 °C for 60 s. For the T–138C polymorphism, the reaction was run with a 3-min denaturation at 94 °C followed by 30 cycles of 94 °C for 30 s, 57 °C for 60 s, and 72 °C for 60 s.

**Reporter Plasmids—**Reporter plasmids were a kind gift from Dr. R. Schule (University of Freiburg, Germany). They consisted of progressive MGP promoter deletions (pCMV-138C) and the reverse primer (5'-TAGACGCTGAGGAGGGGACAC-3'), followed by digestion with the restriction enzyme NcoI. The T–138C polymorphism was genotyped using a mismatch PCR fragment amplified with the forward primer (5'-AAGCTAGTGGCCAAAATTCTTCGCA-3') and the reverse primer (5'-GAACATGCTGGGAACTTTCCTCCAC-3'), followed by digestion with the restriction enzyme BsiSI. The PCRs were performed in a total volume of 25 μl of a buffer solution containing the following: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.0 mM MgCl₂, 0.25 mM dNTP, 10 units of Taq DNA polymerase, and 0.25 μM forward and reverse primers. For the G–7A polymorphism, the reaction was run with a 3-min denaturation at 94 °C followed by 30 cycles of 94 °C for 30 s, 64 °C for 60 s, and 72 °C for 60 s.

**Reporter Plasmids—**Reporter plasmids were a kind gift from Dr. R. Schule (University of Freiburg, Germany). They consisted of progressive MGP promoter deletions (−3570, −530, −270, and −102) inserted into the pGL2-Basic luciferase reporter plasmid (Promega). MGP promoter constructs specific for the −7G and −138T mutations were generated with the QuickChange site-directed mutagenesis kit (Stratagene). Mutated constructs were sequenced to confirm successful site-directed mutagenesis. Plasmid DNA was prepared using an endotoxin-free Maxi Prep kit (Qiagen). The sense sequences for the single-stranded conformational polymorphism (SSCP) Analysis—15 overlapping PCR primer pairs were used to amplify 3.3-kilobase pair DNA upstream of the MGP gene in 40 unrelated individuals. 5 μl of PCR product was added to 5 μl of loading dye (98% formamide, 0.2% bromphenol blue, 0.25% xylene cyanol, 10 mM EDTA), denatured by heating to 94 °C for 5 min, and then cooled to 90 °C to preserve single-stranded structure. The denatured samples were loaded onto 6–8% acrylamide gels and electrophoresed at 5-watt constant power overnight. The gel reactions were carried out either at 4 °C or 20 °C. Fragments were visualized by silver staining. Whenever a persisting deviant pattern was observed, the samples were sequenced in order to identify the sequence polymorphism involved.

**Cell Culture, PMA Treatment, and Transient Transfection Assay—**VSMCs were derived from adult Wistar rat aortas by enzyme dispersion and grown for 48 h after transfection before being harvested for analysis of luciferase activity. Briefly, the cells were washed three times with cold PBS with 0.05% Tween 20 for 15 min each. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit Ig antibodies (NA934; Amersham Biosciences) and an exposure was made on Kodak XMR film.

**RESULTS**

**Identification of Four Common Polymorphisms in the Promoter Region of MGP—SSCP analysis of 15 overlapping PCR fragments upstream of the MGP gene in a cohort of 40 individuals revealed the presence of four deviant bands (Fig. 1a). Subsequent sequencing of these bands confirmed four polymorphic sites at nucleotide positions −7 (G or A), −128 (T or C), −514 (C or T), and −2247 (G or A). Fig. 2 shows the approximate localization of these polymorphisms on a schematic diagram of the MGP gene.

The Region of the Promoter in the Vicinity of the T–138C Polymorphism Is Essential for Transcription in VSMCs—Progressive MGP promoter deletion constructs transiently transfected into rat VSMCs revealed a major loss of transcription following deletion of the −270 to −102 region (Fig. 3). This suggests that the sequence between −270 and −102 is critical.
For MGP transcription in VSMCs. Interestingly, the T–138C polymorphism lies in this region, suggesting that it may have significant effects on MGP expression.

Promoter Polymorphisms Are Associated with Modulated Gene Expression in Vitro—The influence of the two most common polymorphisms on gene expression was examined by using reporter gene constructs transiently transfected into VSMCs. These demonstrated independent impact of both common polymorphisms on transcriptional activity of the MGP gene (Fig. 4). The −7A variant had −1.5-fold higher activity than the −7G variant (p < 0.001), whereas the −138C variant had −4-fold higher activity than the −138T variant (p < 0.001).

The T–138C Polymorphism Is Associated with Variations in Serum MGP Levels—To test whether the MGP promoter polymorphisms had any effects on MGP levels in vivo, restriction fragment length polymorphism analysis was performed for the T–138C and G–7A polymorphisms on a sample of 156 healthy subjects in whom serum MGP had been assayed (Fig. 1b). The T–514C and A–2447G polymorphisms were not investigated in this respect, since they were much less common (incidence of the rarer allele was <5%). The population frequencies of the G–7A and T–138C polymorphisms were not significantly different from the distribution expected from Hardy-Weinberg equilibrium (p > 0.5 and p > 0.1, respectively). There were highly significant variations in serum levels of MGP as a function of the T–138C polymorphism (ANOVA, p < 0.0001; Kruskal-Wallis test, p < 0.0001) but not the G–7A polymorphism (ANOVA, p = 0.67; Kruskal-Wallis test, p = 0.759) (Table I and Fig. 5). Thus, the CC variant at −138 was associated with higher mean serum levels of MGP (124.6 units/ml) than subjects with the TT variant (96.4 units/ml). A gene dose effect is also evident, with the CT heterozygotes having intermediate values (101.9 units/ml).

The T–138C Polymorphism Leads to Altered Binding Affinity for VSMC Nuclear Proteins—To investigate the possibility that the T–138C polymorphism alters binding of nuclear pro-

![Fig. 1. a, the CC variant of the T–138C polymorphism appeared as a deviant band on SSCP analysis. Persisting deviant patterns on SSCP were sequenced to identify the polymorphism causing the shift. b, genotyping of the T–138C polymorphism using mismatch PCR followed by digestion with the restriction enzyme BsrSI. The presence of a T nucleotide at position −138 produced a BsrSI restriction endonuclease site giving fragments of 118 and 24 bp. The presence of a C nucleotide at position −138 did not produce a restriction endonuclease site for BsrSI, leaving an uncut fragment of 142 bp.](image)

![Fig. 2. The structure of the human MGP gene 5’-flank (3.3 kilobase pairs) showing putative transcription factor binding sites. ETS, Ets transcription factor family; AP-1, activating protein-1; RAR/RXR, retinoid A and X receptor; NF-Y, NF-Y/CCAAT-binding factor; CRE, cAMP-responsive element; AP-2, activating protein-2; VDR, vitamin D receptor. The region in the vicinity of the T–138C polymorphism is shown in greater detail. The underlined sequence represents the oligonucleotides used for the EMSAs. The location of the T–138C polymorphism is marked out in boldface type.](image)

![Fig. 3. Progressive MGP promoter deletion constructs transiently transfected into rat VSMCs demonstrate a major loss of transcription following deletion of the −270 to −102 region of the promoter (p < 0.0001). VSMCs were exposed for 2 h to 4 μg of MGP promoter-luciferase and 0.1 μg of Bux-β-galactosidase constructs in the presence of 20 μl of Superfect solution and 1.2 ml of medium 199. Transfected cells were grown for 48 h after transfection before being harvested for analysis of luciferase activity. Bars represent relative luciferase activity (mean ± S.D.). Luciferase expression was normalized against β-galactosidase activity to account for variation in transfection efficiency. All experiments were conducted in sextuplicate in four independent transfection experiments.](image)
The population frequencies of the G−7A and T−138C polymorphisms were not significantly different from the distribution expected from Hardy-Weinberg equilibrium (p > 0.1).

Table I

| Genotype | n   | Mean MGP (S.D.) |
|----------|-----|----------------|
| −7 AA    | 21  | 97.4 (17.3)    |
| −7 GA    | 70  | 99.1 (19.1)    |
| −7 GG    | 65  | 101.3 (20.9)   |
| −138 CC  | 10  | 124.6 (10.9)   |
| −138 CT  | 54  | 101.9 (17.2)   |
| −138 TT  | 92  | 95.9 (19.5)    |

The T−138C Polymorphism Leads to Altered Responses of the MGP Promoter to PMA—Since AP-1 binding sites mediate the effects of phorbol esters, we evaluated the effects of the T−138C polymorphism on PMA stimulation of the −270 MGP promoter construct (Fig. 10). 2 h of treatment with 100 nM PMA resulted in a statistically significant 1.6-fold increase in transcription of the −138T variant of the promoter (p = 0.008) transiently transfected into rat VSMCs. However, treatment with 100 nM PMA had no significant effect on transcription of the −138C variant of the promoter (p = 0.9).

The AP-1 Complex Binding in the Region of the T−138C Polymorphism Contains pc-Jun, JunB, Fra-1, Fra-2, and FoxB—The components of the AP-1 complex binding to the −138T oligonucleotide were initially investigated by performing supershifts using antibodies reactive to c-Myb, pc-Jun, pan-Fos, pan-Jun, ATF-2, pan-CREM, and pan-C/EBP (Fig. 11). The pan-Jun antibody blocked the appearance of complex A, and anti-pc-Jun caused a strong clear supershift. The pan-Fos antibody also reduced the intensity of the shift, while those antibodies recognizing other factors had no effect (Fig. 11).

Antibodies recognizing other components of AP-1 were used to further probe the identity of the constituent proteins in the rat VSMC complex A (Fig. 12). A blockshift was observed with the c-Jun polyclonal antibody, and the presence of c-Jun was confirmed by a strong supershift seen with the anti pc-Jun monoclonal antibody. Antibodies to JunB caused a supershift indicating its presence within the complex. Of the Fos family antibodies, FosB caused a weak supershift, whereas Fra-1 and Fra-2 blocked the lower and upper components of complex A, respectively (Fig. 12). The addition of both Fra-1 and Fra-2 antibodies resulted in an additive effect with a very extensive block shift (Fig. 12). A similar pattern of antibody reactivity and AP-1 binding was observed when labeled consensus TRE was used with rat nuclear extract (data not shown).

pc-Jun, JunB, Fra-1, and Fra-2 Are Detected by Western Blotting in Rat VSMC, and the Levels of pc-Jun Increase following PMA Treatment—Western blotting of rat VSMC with antibodies recognizing pc-Jun, JunB, Fra-1, and Fra-2 confirmed the presence of these proteins (Fig. 13). Furthermore, the level of pc-Jun
protein increased following treatment with 100 nM PMA, when compared with ethanol vehicle alone (Fig. 13).

**DISCUSSION**

The present study has identified four novel polymorphisms in the promoter region of the human MGP gene. Transfection studies showed that the two most common of these have an important impact on in vitro promoter activity in rat VSMCs. Using MGP promoter deletion constructs, a region of the promoter critical for transcription was identified as being between -270 and -102. Interestingly, this key region contained the T-138C polymorphism. The importance of this region is consistent with the finding that the T-138C polymorphism has the greatest effect on in vitro transcription in VSMCs. Furthermore, complex A formation is preferentially reduced by competition with cold -138T (lanes 9–11), compared with competition with cold -138C (lanes 12–14). Similarly, complex B is preferentially reduced by competition with cold -138C (lanes 5–7) compared with competition with cold -138T (lanes 2–4).

The differing constitutive expression of the T-138T and T-138C luciferase constructs as well as the different serum levels associated with the T-138C polymorphic variants appears to be paralleled by altered ability to bind nuclear factors (Fig. 6). Nuclear complex A binds preferentially to the T-138C variant, whereas nuclear complex B binds preferentially to the T-138T variant.
The T→138C polymorphism occurs in a region (−142 to −136) with partial homology to a TRE. The consensus TRE sequence TGA(C/G)TCA is known to bind the AP-1 transcription factor complex in many genes and is defined by its ability to mediate phorbol ester-dependent induction of transcription (17). AP-1 is a collective term for a range of nuclear factors that bind the TRE as dimers. Members of the Jun gene family (c-Jun, JunB, and JunD) bind as homo- or heterodimers, while members from the Fos gene family (c-Fos, FosB, Fra-1, and Fra-2) form heterodimers with Jun (18). In addition, certain members of the ATF, CREB/CREM, Maf, Nrl, and JBP1/2 transcription factors can form leucine zipper dimers with Jun and/or Fos and bind a more diverse range of cis elements (18, 19).

The MGP promoter sequence in the region of the −138T polymorphic variant (TGACTGT) has a 5-nucleotide identity with the TRE binding site. Gel mobility shift assay using nuclear extract from 3T3, 3T6, and VSMC cells. The extracts were incubated with γ-32P-labeled double-stranded oligonucleotides corresponding to the −138T allele, −138C allele, and consensus TRE binding site (MGP-TRE). Incubation of the −138T and consensus TRE oligonucleotides with nuclear extract from different cell types produced protein-DNA complex with the same mobility shift as the −138T oligonucleotide. In contrast, the −138C labeled oligonucleotide does not bind, or only weakly binds, the nuclear proteins comprising complex A.

Gel mobility shift assay using γ-32P-labeled double-stranded oligonucleotides corresponding to the −138T allele, −138C allele, and MGP-mutTRE(C) allele. MGP-TRE(T) is identical with −138T except for the substitution of the TRE consensus binding sequence (−TGAC(T/A)TCA−) in place of the MGP putative AP-1 binding site (TGACTGTT-). MGP-mutTRE(C) is identical with MGP-TRE(T) except for substitution of a T for a C in a position corresponding to the T−138C polymorphic site (−TGACCCCA−). Labeled DNA probe was added to each reaction mixture containing nuclear extract from rat VSMC cells. The consensus MGP-TRE(T) oligonucleotide forms a more intense complex in the same region as complex A when compared with MGP-TRE(C) and −138T labeled oligonucleotides. The binding in this intense complex is significantly reduced by mutation of T in the TRE consensus oligonucleotide (−TGAC(T/A)TCA−) to C (−TGACCCCA−). These experiments strongly suggest that AP-1 complex proteins are components of complex A in VSMCs and that the T in position −138 is critical for binding of the factors within complex A.

Effects of the T→138C polymorphism on phorbol ester stimulation of the −270 MGP promoter construct. Treatment with 100 nM PMA resulted in a statistically significant 1.6-fold increase in transcription of the −138T MGP promoter construct (p = 0.008) transiently transfected into rat VSMCs. Similar PMA treatment of cell transfected with the −138C MGP promoter construct had no significant effect on transcription (p = 0.9). Ethanol (0.1%) was the vehicle for PMA. Interestingly, we note once again that in the ethanol vehicle treated cells the −138C polymorphism is more active than that for −138T. PMA, phorbol 12-myristate 13-acetate; Veh, ethanol vehicle.

Supershift assays were performed using antibodies to phosphorylated c-Jun p39 (pc-Jun), pan-Jun, pan-Fos, ATF-2, pan-CREM, pan-C/EBPβ, or c-Myb. The antibodies were added to the nuclear extract mixture for 10 min prior to the addition of the γ-32P-labeled double-stranded probe and then incubated for 30 min at room temperature before being loaded onto the gel. The antibody recognizing pc-Jun supershifted complex A (lane 2), and the anti-pan-Jun antibody blocked complex A formation (lane 4). In addition, anti-pan-Fos antibody also blocked complex A formation (lane 5). In contrast, antibodies against ATF-2 (lane 6), pan-CREM (lane 6), c-Myb (lane 1), or pan-C/EBP (lane 7) caused no supershift or blockshift.

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Nuclear factor binding was significantly reduced by mutation of T in (TGACTCA) to C (TGACCCA). This site corresponds to the T–138C polymorphism in the MGP promoter (Fig. 9). As mentioned above, the −138T variant appears to be a stronger binder of the AP-1 complex than the −138C variant. It therefore seems that the mutation of a T to a C nucleotide in the −138 position results in significant loss of AP-1 binding affinity. These experiments strongly suggest that AP-1 complex proteins bind the MGP promoter in the vicinity of the T–138C polymorphism and that the T in position −138 is critical for effective binding.

Antibodies to various AP-1 component proteins were used in gel mobility shift assays to determine the composition of the nuclear complexes binding in the region of the −138T variant of the polymorphism (Figs. 11 and 12). Antibodies against c-Jun, pc-Jun, and JunB blocked or supershifted complex A bound to −138T, indicating that these proteins were present. The polyclonal antibodies to Fra-1 and Fra-2 blocked the upper and lower regions of complex A, respectively. When both Fra-1 and Fra-2 antibodies were added in combination, this resulted in a considerable reduction of complex A formation (Fig. 12). Furthermore, FosB antibodies caused a weak but detectable supershift. Moreover, Western blot analysis confirmed the presence of Fra-1, Fra-2, pc-Jun, and JunB in rat VSMC (Fig. 13). Therefore, the AP-1 complex binding in the −138T region of the MGP promoter in rat VSMCs contains pc-Jun, JunB, Fra-1, and Fra-2 proteins. The levels of FosB are probably low but need to be further quantified. Interestingly, although AP-1 complexes are usually involved in trans-activation processes, the presence of JunB, Fra-1, and Fra-2 in AP-1 complexes has been associated with repression as well as activation (20–24).

The ability of AP-1 complexes containing Fra-1 and Fra-2 to act as repressors/weak activators is in part thought to be due to the absence of activation domains in these transcription factors. (25, 26). This may well account for the lower transcription levels of the −138T variant when compared with the −138C form in normal rat VSMCs.

AP-1 binding sites mediate phorbol ester-dependent induction of transcription in many genes (17). We therefore investigated the functional effects of the T–138C polymorphism by examining the effects of PMA on the −138T and −138C MGP promoter variants (Fig. 10). Incubation of VSMCs for 2 h with 100 nM PMA resulted in induction of transcription of the −138T variant of the MGP promoter but not the −138C variant. This indicates that the T–138C polymorphism has significant functional effects on MGP transcription by causing altered responsiveness of the promoter to phorbol ester. The mechanism of this activation may be due to an increase in the levels of phosphorylated c-Jun (pc-Jun), since Western blotting showed that the levels of pc-Jun increases as a result of PMA treatment (Fig. 13). Therefore, the T–138C polymorphism appears to alter the ability of the promoter to respond to certain secondary messenger pathways by altering AP-1 binding.

The ability of the MGP promoter to respond to extracellular calcium is clearly important for its function as a regulator of extracellular calcification. We have recently shown that MGP transcription is regulated in VSMCs by a G protein-coupled cation-sensing mechanism that is functionally related to the cell surface calcium-sensing receptor (10). We demonstrated that increases in extracellular calcium concentration in the range between 2.2 and 6.0 mM result in induction of MGP transcription. Interestingly, Ng et al. (27) recently identified an AP-1 binding extracellular calcium-responsive element in the promoter of the keratinocyte gene Involucrin. Using supershift EMSAs, they showed that JunD, Fra-1, and Fra-2 were the major factors that bound this AP-1 element. Furthermore, they

![Fig. 12. Gel mobility shift assay using 32P-labeled double-stranded oligonucleotides corresponding to the −138T allele. A range of Jun and Fos antibodies were tested for reactivity against AP-1 in rat VSMC nuclear extract. The JunB antibodies were reactive, causing a supershift (lane 4). The JunD antibody was not obviously reactive (lane 5). The c-Jun polyclonal antibody was weakly reactive causing a block shift (lane 3). However, the clear reactivity of the pc-Jun monoclonal antibody in lane 2 confirms the presence of c-Jun. Of the Fos antibodies tested, those recognizing FRA-1 and Fra-2 were the most reactive, blocking the lower and upper component of the AP1 shift, respectively (lanes 9 and 10). When both Fra-1 and Fra-2 antibodies were combined, the shift was almost totally blocked (lane 7). FosB antibody binding caused a weak supershift (lane 8). Antibodies recognizing ATF2 (lane 11) and c-Myc (lane 12) are nonreactive negative controls. The reactivity of the pan-Jun antibody is very obvious (lane 1). This is in contrast to the low, but discernable, levels of interference noted for the pan-Fos (lane 6).](http://www.jbc.org/)

![Fig. 13. Western blot analysis of AP-1 factors in rat VSMCs. 30 μg of whole cell protein lysate derived from cells cultured for 24 h either in the presence of 100 nM PMA or with ethanol vehicle (0.1%) were run on a 12% SDS-polyacrylamide gel electrophoresis gel and blotted with a clonal antibody recognizing Fra-1, Fra-2, JunB, and pc-Jun, indicating the presence of these proteins in rat VSMCs. PMA treatment resulted in increased levels of pc-Jun.](http://www.jbc.org/)

with the consensus TRE sequence (TGACTCA). We performed cold competition experiments demonstrating that increasing concentrations of cold consensus TRE oligonucleotides were able to compete effectively with the −138T radiolabeled oligonucleotide for the binding complex A in VSMC nuclear extracts (Fig. 7). In fact, cold consensus TRE was a better competitor for the radiolabeled −138T oligonucleotide than cold −138T, showing it to be a stronger binding site. In contrast, the −138C oligonucleotide competed very poorly, indicating weak or no binding to complex A (Fig. 7). When control AP-1 containing nuclear extracts derived from ST3 and ST6 cells were analyzed for factor binding to both the consensus TRE site and −138T labeled oligonucleotides, the protein complexes observed were of identical mobility in the gel (Fig. 8). However, the −138C variant did not exhibit similar binding intensity patterns, indicating that it bound AP-1 very poorly or not at all (Fig. 8).

Furthermore, we have shown that the T nucleotide at position 5 of the consensus TRE site is necessary for strong binding.
showed changes in the levels of these AP-1 components following changes in extracellular calcium concentration. The current study has demonstrated the involvement of c-Jun, JunB, Fra-1, and Fra-2 in VSMC MGP transcriptional regulation. It therefore remains to be seen if the effects of extracellular calcium on MGP transcription are mediated via these transcription factors acting through the AP-1 site in the vicinity of the T–138C polymorphism.

Other workers have studied the MGP promoter and characterized response elements close to where the T–138C polymorphism. C/EBP and retinoic acid response elements exist immediately downstream and an ETS site immediately upstream of the T–138C polymorphism. We have confirmed that the MGP promoter is repressed by retinoic acid as shown by others and that the polymorphism has no effect on this response (29). The transcription factor C/EBPβ has previously been shown to be a strong stimulator of MGP transcription acting through the −138 to −102 region of the promoter (28). We have shown in this paper that C/EBP does not bind in the region of the polymorphism (Fig. 11). This may be because the labeled oligonucleotide used lacked the CCAAT site immediately downstream (Fig. 2). The presence of these response elements so close to the T–138C polymorphism, however, could be of functional significance due to differential protein–protein interactions resulting from the presence or absence of the AP-1 complex and need to be investigated further.

The main sources, pharmacodynamics, and pharmacokinetics of circulating MGP are currently unknown. Data from rats and mice suggest that MGP is mainly produced in vascular smooth muscle cells and developing cartilage (7, 30). Therefore, it seems reasonable to assume that VSMCs are a major source for the circulating serum MGP that we measured in our adult population. Since MGP is an important regulator of tissue and vascular calcification, our study suggests that there may be a genetic basis to tissue and vascular calcification in vivo. It is interesting to hypothesize that these promoter polymorphisms may affect an individual’s susceptibility to vascular or valvular calcification. It is possible that the −138C variant provides protection against tissue calcification in VSMC by resulting in a functional protein variant that we are currently investigating the effects of the T–138C polymorphism on in vivo vascular and coronary artery calcification as measured by electron beam CT. Furthermore, since calcification occurs as part of the atherosclerotic process, it will be of interest for future studies to look for any associations of the T–138C polymorphism with atherosclerosis.

A recent study by Herrmann et al. (31) analyzed the effects of MGP polymorphisms on vascular calcification and myocardial infarction. They found that the −7A allele was more frequent in patients with myocardial infarction and among individuals with femoral calcification. In addition, they performed transient transfection experiments with MGP promoter constructs showing that the −138T variant had a 20% greater activity than the −138C variant and no effect of the G−7A polymorphism. The disparity with our results may be related to their use of rat VSMC lines in contrast to our primary adult rat VSMCs. It is known that the composition of the AP-1 complex varies between cell types. Since the AP-1 binding site in the MGP promoter is important for transcription, the presence of different AP-1 complexes would be expected to result in altered transcription. It would therefore be of interest to determine if the AP-1 composition reported here for primary adult rat VCs is maintained in the cell lines used by Herrmann et al.

The correlation of polymorphisms with clinical phenotype described by Herrmann et al. (31) were weak or limited to subgroups of patients. Moreover, calcification was measured using ultrasound of the carotid and femoral arteries. This is a relatively crude measure of calcification (32) and may explain why the observed correlation with the −7A allele was only found in femoral calcification and not in carotid calcification. The ideal way to measure calcification in such a study would be by electron beam CT. Not only does this provide an accurate quantitative measure of calcification, but it is also capable of measuring calcification in the coronary arteries directly.

This study has demonstrated that a novel promoter polymorphism of MGP alters binding of an AP-1 transcription factor complex and is associated with altered serum levels in man. We have identified the components of the AP-1 complex in rat vascular smooth muscle cells as consisting primarily of c-Jun, JunB, Fra-1, and Fra-2. This has important implications for understanding the mechanisms underlying conditions that involve vascular calcification, such as atherosclerosis and aortic valve stenosis.

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A Polymorphism of the Human Matrix γ-Carboxyglutamic Acid Protein Promoter Alters Binding of an Activating Protein-1 Complex and Is Associated with Altered Transcription and Serum Levels

Afshin Farzaneh-Far, John D. Davies, Levienja A. Braam, Henri M. Spronk, Diane Proudfoot, Shiu-Wan Chan, Kevin M. O'Shaughnessy, Peter L. Weissberg, Cees Vermeer and Catherine M. Shanahan

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