Characterization and Functional Analyses of the Human G Protein–Coupled Receptor Kinase 4 Gene Promoter

Sandra Hasenkamp, Ralph Telgmann, Jan A. Staessen, Claudia Hagedorn, Corinna Dördelmann, Martin Bek, Stefan-Martin Brand-Herrmann, Eva Brand

Abstract—The G protein–coupled receptor kinase 4 is involved in renal sodium handling and blood pressure regulation. Missense variants have already been tested functionally and are associated with hypertension, but no data on promoter analyses are yet available. We scanned 94 hypertensive white subjects for genetic variation and performed promoter reporter gene analyses in HEK293T, COS7, and SaOs-2 cells. Transient transfections with various full lengths and wild-type deletion constructs revealed that 1851 bp of the flanking region and 275 bp of the 5′-untranslated region were sufficient for transcriptional activities and composed a powerful cis-active element in the distal 293 bp. The −1702T and +2T alleles resulted in drastic general reductions of promoter function, whereas an activity increasing effect of +268C was cell type specific. Electrophoretic mobility-shift assay, supershift, and cotransfection analyses of transcription factor binding sites predicted in silico (Alibaba2.1/Transfac7) resulted in allele-specific binding patterns of nuclear proteins and identified the participation of CCAAT/enhancer-binding protein transcription factor family members. The G protein–coupled receptor kinase 4 core promoter resides in the first 1851 bp upstream of its transcription start site. The 4 identified genetic variants within this region exert allele-specific impact on both cell type- and stimulation-dependent transcription and may affect the expression balance of renal G protein–coupled receptor kinase 4. (Hypertension. 2008;52:737-746.)

Key Words: GRK4 ■ genetic variants ■ functional promoter analyses ■ deletion constructs ■ regulatory regions

Renal sodium reabsorption represents one of the key mechanisms by which the kidney regulates blood pressure. In renal proximal tubule cells, G protein–coupled dopamine 1–like receptors (DRD1 and DRD5) modulate, on stimulation by dopamine, renal sodium handling by influencing the activities of the Na+/K+–ATPase, the Na+/HCO3−-cotransporter, the Na+/phosphate-cotransporter, and the Na+/H+–exchanger. These inhibitory actions of D1-like receptors are predominantly transduced by elevation of intracellular cAMP and subsequent activation of protein kinase A, which, in turn, reduces the activity of the basolateral Na+/K+–ATPase by phosphorylation. Thus, dopamine, via D1-like receptors, displays potent antihypertensive actions through natriuretic and diuretic effects. A family of G protein–coupled receptor kinases (GRKs; serine/threonine protein kinases) is involved in the intracellular regulation of these G protein–coupled receptors. Phosphorylation of the activated G protein–coupled receptor by GRKs leads to desensitization and thereby regulates their signal-transducing performance via G proteins. The GRK family contains 7 members, which are divided into 3 subfamilies: the rhodopsin kinase subfamily (GRK1 and GRK7), the β-adrenergic receptor kinase subfamily (GRK2 and GRK3), and the GRK4 subfamily (GRK4, GRK5, and GRK6). GRKs are ubiquitously expressed, but GRK1 and GRK7 are found almost exclusively in the retina. GRK4 is highly expressed in testis and to lower levels in other tissues, including kidney, brain, and bone. Within the GRK4 transcripts, Premont et al. identified 4 splice variants, which contain all exons (GRK4α), lack exon 2 (GRK4β), lack exon 15 (GRK4γ), or lack both (GRK4δ). The GRK4 gene locus is embedded in a gene cluster region on chromosome 4p16, including genes encoding dopamine receptor (DRD) type 5 and α-adducin, and has been associated with hypertension.

In the present analysis, we scanned 94 hypertensive white subjects for genetic variation in the 5′-flanking and entire coding region of GRK4. Because the molecular basis of GRK4 transcription control is unknown, we characterized its regulatory region by performing detailed functional analyses with deletion constructs of the 5′-flanking region. We also assessed allele-specific transcriptional activities and differential DNA-protein interactions in the human embryonic kidney.

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cell line HEK293T, African monkey fibroblast-like kidney cell line COS7, and osteoblast-like osteosarcoma cell line SaOs-2 under relevant kinetic stimulatory regimes.

Methods

Screening of the GRK4 Gene

Genomic DNA of 94 hypertensive individuals was screened for genetic variants by PCR/single strand conformation polymorphism (SSCP) sequencing. Positions of the detected variants were assigned corresponding to the transcription start site (TSS) defined in Accession No. NM_182982. For details, see http://hyper.ahajournals.org.

In Silico Analyses of Putative Transcription Factor Binding Site

A portion of 60 bp (30 bp flanking either side of the respective genetic variant) was subjected to computer-aided analyses using the Alibaba2.1 net-based transcription factor binding site (TFBS) search tool (http://www.gene-regulation.com/pub/databases.html) and the Transfac 7.0 database. For sequence analyses, all of the parameter settings were individually tested, and the recommended settings were found most efficient. For G+2T and C-1702T, the setting of “pairsim to known sites” was set to 36, and the “minimum matrix conservation” was set to 70%.

Construction of Reporter Gene Vectors and Site-Directed Mutagenesis

Promoter constructs were generated by PCR for the full-length and deletion constructs. For primer positions and cloning details see at http://hyper.ahajournals.org. Constructs were linked to the luciferase gene in the promoterless vector pGL3basic (Promega). Genetic variants C-1702T (H5), G-1436C (H4), G+2T (H3), and G+268C (H2) were introduced by QuikChange site-directed mutagenesis (Stratagene).

Table. Identified Genetic Variants Within the Human GRK4 Gene (NM_182982)

<table>
<thead>
<tr>
<th>No.</th>
<th>Position With Respect to the TSS</th>
<th>Localization</th>
<th>Nucleotide Exchange</th>
<th>AS Exchange</th>
<th>rs No.</th>
<th>Vector Construct</th>
<th>Allele Frequency</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1702*</td>
<td>5'-flanking region</td>
<td>C&gt;T</td>
<td>*</td>
<td>H5</td>
<td>0.995/0.005</td>
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</tr>
<tr>
<td>2</td>
<td>−1436*</td>
<td>5'-flanking region</td>
<td>G&gt;C</td>
<td>*</td>
<td>H4</td>
<td>0.995/0.005</td>
<td></td>
</tr>
<tr>
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<td>−955</td>
<td>5'-flanking region</td>
<td>T&gt;A</td>
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<td></td>
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<tr>
<td>4</td>
<td>−943*</td>
<td>5' -flanking region</td>
<td>Del A</td>
<td>*</td>
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<td></td>
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<tr>
<td>5</td>
<td>+2</td>
<td>5'-UTR</td>
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<td>G&gt;C</td>
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<td>H2</td>
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<tr>
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<td>*</td>
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<td>−17*</td>
<td>Intron 15</td>
<td>G=A</td>
<td>*</td>
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</table>

Four of the detected variants are located within the defined 5'-flanking region and 3 within the 5'-UTR, and 8 variants were found in exon-flanking intron regions and 5 within the coding region. Six of these variants have not been published before.

"* Data show newly identified variants.

Identification of Genetic Variants in the Human GRK4 Gene

We detected 20 genetic variants, 4 of which were located within the defined 5'-flanking region, 3 within the 5'-

Transient Transfection Assays

HEK293T, COS7, and SaOs-2 cells were transfected with Lipo- vectamine 2000 (Invitrogen), luciferase activity was determined using a Sirius single-tube luminometer (Berthold Detection Systems). For details on stimulation assays and cotransfections, please see http://hyper.ahajournals.org. Transfection experiments were repeated ≥3 times in triplicate. Significance was calculated by unpaired, 2-tailed t test (95% CI); the significance levels were set at P<0.05.

Electrophoretic Mobility Shift Assay and Supershift Assay

Nuclear proteins from HEK293T and COS7 cells were extracted by a modified procedure of the protocol published by Schreiber et al. For the expanded Methods section see http://hyper.ahajournals.org.

For supershift assays, the electrophoretic mobility shift assay (EMSA) protocol was extended by 15 minutes of incubation with 2 μg of specific antibodies against members of CCAAT/enhancer-binding protein (C/EBP) family: anti-C/EBPα (14AA), anti-C/EBPβ (C-19), and anti-C/EBPδ (C-22) antibodies (Santa Cruz Biotechnology).

Isolation of Total RNA and Generation of cDNA for Detecting Endogenous GRK4 Expression

Total RNA was extracted by TRizol reagent (Invitrogen). Generation of cDNA was performed with a First Strand cDNA Synthesis kit (Fermentas) and amplified with specific primers. Intactness and purity of cDNA were routinely controlled by diagnostic PCR for human ribosomal protein 27.

Results

Identification of Genetic Variants in the Human GRK4 Gene

We detected 20 genetic variants, 4 of which were located within the defined 5'-flanking region, 3 within the 5'-
 untranslated region (UTR), 8 in exon-flanking intron regions, and 5 within coding regions (the Table). The variants C-1702T, G-1436C, G+2T, G+268C (intron 1), G+54A (intron 12), and G-17A (intron 15) have not yet been published. To identify regulatory sequences and to characterize the variant GRK4 promoter, we exclusively analyzed the variants in the 5'-flanking region (Figure 1A). T-955A and 943delA were flanking a poly-A stretch (n 12) and were, thus, excluded from further experiments.

We assessed endogenous expression of GRK4, DRD1, and C/EBP family members (α, β, and δ) in our cell lines under basal (without stimulation) and stimulatory conditions (10^{-8} mol/L PMA/cAMP) by diagnostic PCR (Figure 1B). The moderate expressions of GRK4 in SaOs-2 (data not shown) were confirmed by gradient and nested PCR (Figure 1B, black box).

Transcriptional Activity Is Cell-Type Specific and Altered by the Presence of Genetic Variants

Transient transfection experiments revealed that 1851 bp of the 5'-flanking region of the GRK4 gene were sufficient for high transcriptional activity in all of the cell lines tested (H1 in Figure 2A through 2C). Molecular variants H2-H5 were used in the context of the full-length promoter construct to investigate their effect on transcriptional activity. In HEK293T cells, the proximal 268C (H2) allele showed slightly lower transcriptional activity compared with the wild-type H1, but this difference was not statistically significant (P=0.3019). Transcriptional activity of the distal 1436C (H4) allele was significantly reduced (P=0.0061), whereas more drastic effects were observed for the +2T (H3) and -1702T (H5) alleles, showing almost abrogated transcriptional activities (both P<0.0001 compared with the wild
These same results were obtained in COS7 cells (Figure 2B), except that the transcriptional activity of the H2 construct was significantly more active compared with the wild type (P < 0.0037). In nonrenal SaOs-2 cells, similar results as in COS7 cells were obtained (Figure 2C).

The amount of transcriptional activities in relation to the wild-type p1851-GRK4-H1 is depicted in Figure 2D, with transcriptional activity of H1 set to 100%. In HEK293T cells, H2 showed slightly lower activities (94%), whereas in COS7 and SaOs-2 cells, the activities were higher (155% and 124%, respectively).

G + 268C Variant Displays Time- and Stimulatory-Dependent Usage of the Promoter in PMA Kinetic Analyses

Kinetic stimulatory influences of PMA on GRK4 transcription were tested over a period of 24 hours by transient transfections in renal cell lines. HEK293T cells, stimulated with 10^{-8} M PMA, showed increased transcriptional activities, except for H3 and H5, which were rather silent and responded to stimulation only with a slight activity increase after 24 hours (Figure 3A). Wild-type H1 and construct H2 (+268C) showed the highest transcription levels with slight increases for H2 after 6 hours, which was repeatedly observed but failed to reach significance. In contrast, the stimulation consequences in COS7 cells differed noticeably (Figure 3B). After a slight transient increase (2 hours; except H2), transcriptional activities decreased, observed for all of the constructs over the 24-hour period. The H3 and H5 constructs again responded rather unaffected to stimulation, whereas H2 showed higher transcription efficiencies compared with wild type. This effect, repeatedly observed at nonsignificant levels in embryonic HEK293T cells, was robust in fully developed COS7 cells.

Genetic Variants Alter the Interactions of the GRK4 Promoter With Nuclear Proteins From Renal Cell Lines Allele Specifically and Cell-Type Dependently

In EMSA experiments, nuclear proteins from renal HEK293T and COS7 cells interacted allele specifically, resulting in different protein binding patterns. In HEK293T, but not in COS7, the +268C allele was bound by an additional protein (Figure 4A,
top left asterisk arrow), which was in accordance with the differences in transfection experiments. Indeed, an in silico analysis for putative transcription factor (TF) bindings indicated nuclear respiratory factor-1 as specific for the +268C allele.

A loss of protein binding occurred with the +2T allele in HEK293T (Figure 4B, top left asterisk arrow) but not in COS7, indicating cell- and allele-specific differences, although reporter assays led to similar results in both cell lines. In silico analysis of this region predicted an additional putative binding site for Oct-1 in the presence of the T allele.

For G-1436C, we identified different binding patterns in both cell lines with a C allele–specific competition in HEK293T cells (Figure 4C, left asterisk arrow). In COS7 cells, we identified 2 specific bindings with specific competition patterns for both alleles (Figure 4C, right arrows), and, congruently, loss of the Oct-1 site was predicted in silico. These complex results indicate that GRK4 promoter portions are bound by several factors and that the presence of different alleles alters the nuclear protein interactions, dependent on the cell type.

Transcriptional Abrogation by Truncation of Distal Promoter Portions From the Full Length Construct

We demonstrated, across all of the cell lines tested, that a portion of 1851 bp of the human GRK4 promoter region is strongly transcriptionally active. In HEK293T, truncated constructs did not attain efficiency of the full-length construct p1851-GRK4-H1 and showed lower activities (Figure 5A). The 293-nucleotide shorter p1558-GRK4-H1 construct showed a strong reduction of transcriptional activity but still displayed residual activity compared with the shuttle vector (P<0.0001). Removal of an additional 429 bp in p1129-GRK4-H1 led to a further significant reduction in performance compared with p1558-GRK4-H1 (P=0.0015); and for p813-GRK4-H1, transcription was completely abrogated compared with p1851-GRK4-H1 (P<0.0001). The p72-GRK4-H1 construct showed similar reductions in transcriptional activity as p1558-GRK4-H1. Only p180-GRK4-H1 and p6-GRK4-H1 were able to partly restore the activity of the full-length construct to 61% and 45%, respectively (Figure 5D).

In COS7 cells, the activities of all of the deletion constructs were similar to HEK293T (Figure 5B). Only the p180-GRK4-H1 construct was able to reconstitute the transcriptional activity, whereas the truncations in all of the other constructs led to strong reductions of transcriptional activities, even in the p6-GRK4-H1 construct. In SaOs-2 cells (Figure 5C), the transcriptional activity of deletion constructs was comparable to that found in renal cells. The amount of transcriptional activities in relation to the full-length construct (p1851-GRK4-H1 was set 100% transcriptional activity) is presented in Figure 5D. Irrespective of differentiation and cell type, regulatory regions seemed to be identically distributed in all of the tested cell lines.

Specific Interactions of the Distal Promoter Region With Nuclear Proteins Are Responsible for Differences in Transcriptional Activities

The p1558-GRK4-H1 deletion construct, which lacks the first 293 bp of the full-length construct, showed prominent decreases in transcriptional activity in all of the cell lines (Figure 5A through 5C). Overlapping fragments (A, B, and C) covering this section were designed with fragment B bearing the C-1702T transition (Figure 4E). EMSA experiments using these fragments with HEK293T nuclear extracts showed a specific DNA binding, exclusively within the distal fragment A. The overlapping sequences of these fragments revealed that binding was restricted to the most distal 60 bp of the full-length construct, analyses with COS7 nuclear extracts revealing the same results (data not shown). Because a specific shift was lacking in fragment B, we reanalyzed the C-1702T site with specific oligos. The resulting allele-specific protein bindings were identical in both cell lines (Figure 4D, top asterisk arrows), congruent with the transfection results, presented by the H5 construct (Figure 2A through 2D). In good accordance with that, the further Alibaba2.1 analysis of the sequence surrounding the variant revealed a
consensus half-site for a glucocorticoid receptor (GR) as an additional TF specific to the T allele.

In silico analyses of the distal promoter sequence of fragment A (60 bp) predicted a clustered site, containing 3 sites for the C/EBP transcription factor family (Figure 4E). Other factors within this cluster are Oct-1 and neural retina leucine zipper protein.

**Cotransfections Support the Differentiation-Dependent Usage of the GRK4 Promoter**

To verify the results of the net-based analyses of the distal GRK4 promoter region and to characterize potential TFs, supershift experiments and cotransfections were carried out, targeting C/EBP family members. Although supershift analyses failed to show specific binding of C/EBPα, C/EBPβ, or C/EBPδ in HEK293T or COS7 cells (data not shown), cotransfection experiments in HEK293T (Figure 6A) exposed either p1851-GRK4-H1 or p1558-GRK4-H1 to overexpressed (exogenous) C/EBPα, C/EBPβ, or C/EBPδ. The transactivators C/EBPα and C/EBPδ led to a significant activation of p1851-GRK4-H1, with C/EBPδ being the more potent factor (P<0.0001) and p1558-GRK4-H1 being similarly activated but to a lesser extent compared with p1851-GRK4-H1. Overexpression of C/EBPβ resulted in a modest activation only of p1851-GRK4-H1. In COS7 cells (Figure 6B), opposite results were observed. A significant reduction of p1851-GRK4-H1 with the most potent factor C/EBPδ was shown. The reduction of p1558-GRK4-H1 was much stronger and led to almost complete abrogation in transcription with C/EBPδ. The overexpression of C/EBPβ failed to reach significance for p1558-GRK4-H1.

**Discussion**

In the present study, we (1) identified 20 genetic variants within the 5′-flanking and coding regions of the human GRK4 gene, 6 of which were newly detected, C-1702T, G-1436C, −943delA, C+8A (intron 1), G+54A (intron 12), and G-17A (intron 15), (2) and identified relevant functional sequences for transcriptional regulation (3) and analyzed the transcriptional consequences of 5′-flanking variants. GRK4 plays an important role in the renal dopaminergic system. Phosphorylation of the activated DRD1 results in its desen-
Figure 5. Identification of regulatory sequences within the GRK4 promoter. Distal truncations of the GRK4 promoter led to differently reduced transcriptional efficiencies (depicted as relative light units [RLUs]) in HEK293T (A), COS7 (B), and SaOs-2 cells (C). Relations of deletion to full-length constructs are shown by setting the full-length activity as 100% (D). Significances refer to the full-length p1851-GRK4–H1 construct in all of the figures (*P<0.05; **P<0.01; ***P<0.001).
sitization and inactivation, which leads to impaired urinary sodium excretion.\(^4\) Felder et al\(^4\) reported that the nonsynonymous variant GRK4\(^{-}/H9253^A142V\) was associated with human essential hypertension, resulting in an increase in GRK4 kinase activity, leading to an increased DRD1 receptor phosphorylation. According to these findings, Sanada et al\(^3\) reported that renal GRK4 suppression lowered blood pressure in spontaneously hypertensive rats. Indeed, the human chromosome 4p16 locus, in which the GRK4 and \(^{-}/H9251\)-adducin genes reside, has convincingly been linked to essential hypertension.\(^7\) To the best of our knowledge, no studies have yet characterized the molecular basis of GRK4 promoter regulation. Therefore, we focused on promoter analyses in the context of a 2125 bp GRK4 promoter portion and, in addition, a series of truncated promoter constructs was generated for both the identification and characterization of regulatory elements. The GRK4 2125 bp promoter portion, containing 1851 bp of the 5'-flanking region and 275 bp of the 5'-UTR, was identified as highly sufficiently active across tested cell lines, including the embryonic HEK293T, the differentiated COS7, and the nonrenal SaOs-2.

Transcriptional activities measured by reporter gene assays underlie 2 main regulating features: the transcription effects depending on the assembly of the initiation complex at the TSS and the variances in transcript stability.\(^14,15\) Cis-regulatory elements are responsible for directing the transcription in combination with the core promoter to define specific expression patterns.\(^16\) Genetic variants within these regulatory regions may alter gene transcription and expression patterns by affecting the consensus sites of TF and influencing DNA binding affinity.

Our observed differentiation- and tissue-independent promoter activity is congruent with the reported expression of GRK4. Its physiological impact might, therefore, critically depend on its protein level and kinase activity. Cell stimulation induces several signal transduction pathways, which, in

![Figure 6](http://hyper.ahajournals.org/)

Figure 6. Cotransfections reveal a participation of C/EBP family members on GRK4 transcription. In HEK293T cells (A), the cotransfected C/EPBs (-α, -β, and -δ) showed activating effects on transcription levels, with highest levels through C/EBPδ. Conversely, in COS7 cells (B), coexpressions revealed transcription repressions, with almost abrogated transcription by C/EBPδ. Significances are referred to the particular construct (ns indicates not significant; *P<0.05; **P<0.01, ***P<0.001).
turn, activate further processes within the cell, among others the appropriation/activation of TF. Stimulation with the phorbolester PMA leads to an activation of the protein kinase C, which regulates GRK4 via phosphorylation. GRK2 phosphorylation by protein kinase C leads to an increase in kinase activity, whereas a kinase inhibition by protein kinase C phosphorylation is reported for GRK5, belonging to the same subfamily as GRK4. Our results on PMA stimulation in COS7 cells suggest the occurrence of a feedback mechanism between the amount of phosphorylated GRK4 protein and its gene transcription. Indeed, after a slight and short-timed activation of 2 hours, the transcriptional activities were downregulated, whereas the increasing levels observed in HEK293T may be a differentiation-dependent consequence. Moreover, our stimulation kinetics revealed that carrying the +268C allele enables factors to interfere with GRK4 transcription and expression, which probably do not interact with the wild-type sequence, and might result in reduced kinase levels and activities as exerted by the wild-type promoter in vivo. EMSA experiments revealed a C allele–specific shift in COS7 cells, indicating an additional protein interaction. In silico analysis predicted putative overlapping binding sites for Krox-20, Sp1, and, in case of the C allele, the nuclear respiratory factor-1. Krox-20 is a segment-specific mammalian TF in drosophila with the early growth response factor 2 as the human homolog, and Sp1 is a known transactivating factor in TATA-less promoters. Nuclear respiratory factor-1, a factor binding predominantly to antioxidant response elements of genes in mitochondria, is an unlikely candidate for renal GRK4 transcription. Our EMSA experiments provided cell- and allele-specific binding patterns for G+2T, which is located adjacent to the declared TSS. This sequence portion was bound by 2 different proteins or protein complexes in both HEK293T and COS7 cells. Appropriation of the wild-type sequence resulted in formation of a third protein complex exclusively with HEK293T nuclear extracts. This additional shift was not observed for the variant sequence in HEK293T or COS7 cells and could not be predicted by Alibaba2.1 analysis. A putative consensus site for the Ying-Yang factor was predicted for either allele. This ubiquitous, conserved, and multifunctional zinc-finger TF is able to bind to initiator elements and can directly interact with TFIIIB and RNA-polymerase II. Dependent on interactions with other factors, Ying-Yang factor can repress or activate transcription, a mechanism that may be proposed for the GRK4 promoter, including the potential participation of Oct-1 in the presence of the T allele. With respect to the G-1436C site, EMSA experiments revealed distinct binding patterns for renal cell lines. Alibaba2.1 predicted a loss of TFBS for Oct-1 (acting as a positive or negative regulator of gene transcription and DNA replication) for the C allele–carrying promoter. The putative loss of an Oct-1 TFBS may explain the slight reductions in transcriptional activities for the −1436C allele.

Promoters lacking the TATA box often contain a pyrimidine-rich initiator-site and downstream promoter element sequences at the beginning and within exon 1 as essential for the assembly machinery and transcription by presenting the core promoter. The core promoter is a minimum region required for docking the transcription machinery and initiation of basal transcription. The relatively high transcription efficiencies of the truncated promoter construct p6-GRK4-H1, which contains only the 5′-UTR, strongly indicated the potency of this minimal promoter in all 3 of the cell systems, showing that the 5′-UTR alone is sufficient for basal transcriptional activity. A drastic transcriptional abrogation provided by the most distal deletion construct indicated that this portion contains enhancer elements, being important for GRK4 transcription. In silico programs revealed several TFBSs, especially for this portion, and a cluster of the C/EBP TF family was predicted, whose members are involved in cell differentiation and functions of different tissues, acting through homodimerization or heterodimerization. Although all 6 of the C/EBP isoforms (α through θ) contain a transactivation domain, these basic region leucine zipper proteins can have inhibitory functions as well, depending on dimerization, leading to a reduced DNA-binding affinity of bound proteins. The possibility of being expressed either as a transcriptional activator (liver-enriched activatory protein) or as a transcriptional repressor (liver-enriched inhibitory protein) is specific for C/EBPβ. EMSA experiments of the first truncated portion showed a specific protein binding site for the distal 60 bp, confirming the in silico analysis. Participation of C/EBP family members was unambiguously demonstrated in cotransfections, showing that each C/EBP protein (α, β, or δ) regulates GRK4 promoter activity differentially and that the C/EBPα, β, or δ factor binding differs between the 2 renal cell lines in opposite directions. The cell type–specific participation of C/EBP family members indicated that the GRK4 promoter usage depends on the differentiation state of the given cell line and the appropriation of its proteins being able to take part in transcription processes. Within the sensitivity limitations of the supershift analyses, we were unable to identify an interaction of a specific factor of the C/EBP family. However, in cotransfection experiments, we identified C/EBPβ as the most potent activator in HEK293T and inhibitor in COS7 cells. The importance of this promoter portion for GRK4 transcription was further substantiated by specific EMSA analyses of the distal C-1702T variant, which was not observed within the complex fragment B containing this variant. This apparent contradiction between the functionality of the C-1702T variant in reporter gene assays and the missing binding of nuclear proteins in fragment B may be due to limitations of the EMSA method. Functional assays with p1851-GRK4-H5 indicated that the binding of the distal 60 bp of fragment A is a prerequisite for recruiting further factors to the −1702T allele–carrying promoter. The binding pattern of the specific EMSA analysis for this variant underlines the decreased transfection results in both cell lines. Inserting the T allele, a putative TFBS for a GR half-site was predicted in silico analysis. These hexanucleotide halves (TGTYCT) are normally arranged as inverted repeats for a head-to-head binding of receptor dimers. The methyl group of
thymin at position 3 is able to interact hydrophobically with the receptor and is present in the −1702T allele. After ligand (eg, glucocorticoid) activation in cytoplasm, the monomeric GRs is able to translocate to the nucleus, where the receptor dimerization occurs only after DNA binding to glucocorticoid response elements. The resulting transcriptional modulation can either be active or repressive for the target gene. Le et al.25 reported that GR interacts with other TFs, such as C/EBPβ, Ying-Yang, and Oct-1, through adjacent binding sites. Indeed, the in silico analysis predicted a C/EBP family member at this consensus site in case of the variant GRK4 promoter, resulting in decreased transcription.

In both analyzed cases, the deletion and the variance of this distal promoter portion, drastically reduced transcription efficiencies were observed, indicating this sequence as pivotal for GRK4 transcription.

In conclusion, we functionally characterized the GRK4 promoter and performed differential allelic analyses on the basis of newly identified promoter variations. Although +268C enhanced GRK4 transcriptional activities, −1702T, −1436C, and +2T displayed a transcriptionally decreasing effect. The identified regulatory regions in the GRK4 promoter were independent of cell type and differentiation state, whereas DNA-protein interactions involving C/EBP family members were differentiation dependent.

Perspectives

Additional experiments should help unravel more specifically the complex DNA-protein and protein-protein interaction patterns at the GRK4 promoter to understand its interindividually different transcriptional and expression capacities. These mechanisms suggest a molecular basis for and play a role in renal sodium handling and blood pressure regulation by affecting the expression balance of renal GRK4.

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Disclosures

None.

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Characterization and Functional Analyses of the Human G Protein–Coupled Receptor Kinase 4 Gene Promoter

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CHARACTERIZATION AND FUNCTIONAL ANALYSES OF THE HUMAN
G PROTEIN-COUPLED RECEPTOR KINASE 4 GENE (GRK4) PROMOTER

Hasenkamp et al, GRK4 and promoter analyses

Sandra Hasenkamp; Ralph Telgmann; Jan A. Staessen; Claudia Hagedorn; Corinna Dördelmann;
Martin Bek; Stefan-Martin Brand-Herrmann; Eva Brand

_Hypertension_

_Expanded Method Section_
Supplementary Methods

Screening of the GRK4 gene

Genomic DNA was extracted from white blood cells using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Genetic variants were detected by PCR/single strand conformation polymorphism (SSCP) analysis as previously described. Genetic variants were detected by PCR/single strand conformation polymorphism (SSCP) analysis as previously described. Genomic DNA was extracted from white blood cells using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Genetic variants were detected by PCR/single strand conformation polymorphism (SSCP) analysis as previously described.\(^1\)

Oligonucleotide primers were designed to amplify 26 overlapping fragments (7764 bp in total) covering 1851 bp of the 5'-flanking region, the entire 5'-UTR, exons 1-16 including exon-intron boundaries, the 3'-UTR, and the initial 3'-flanking region (primer sequences and exact conditions available upon request). Amplicons presenting different migration patterns were sequenced using an automated ABI 3730 fluorescence sequencer (PE Applied Biosystems, Foster City, USA). Positions of the detected variants were assigned corresponding to the transcription start site (TSS) defined in Accession number NM_182982.

In silico analyses of putative transcription factor binding sites (TFBS)

A portion of 60 bp (30 bp flanking either side of each genetic variant) was subjected to computer-aided analyses using the Alibaba2.1 net-based TFBS search tool (http://www.gene-regulation.com/pub/databases.html)\(^2\) and the Transfac 7.0 database. For sequence analyses all parameter settings were individually tested and recommended settings found most efficient. For the G+2T and the C-1702T variants, the setting of ‘pairsim to known sites’ was set to 36 and the ‘minimum matrix conservation’ was set to 70%.
Construction of reporter gene vectors and site-directed mutagenesis

Promoter constructs were generated by PCR using genomic DNA from a volunteer (wild type sequence) as template. For the full length construct, primers were located at position -1851 bp upstream of the TSS (NM_182982) linked to a KpnI restriction site and at position +275 bp, linked to a MluI site, providing a total length of 2125 bp. For the deletion constructs, sense primers were positioned at -1558 bp, -1129 bp, -813 bp, -180 bp and +72 bp. One additional construct was generated, containing the complete 5’-UTR (p6-GRK4-H1), with primers located at -6 bp, and +478 bp. Constructs were linked to the luciferase gene in the promoterless vector pGL3basic (Promega, Mannheim, Germany).

Genetic variants C-1702T, G-1436C, G+2T, and G+268C were introduced by QuikChange site-directed mutagenesis (Stratagene, Amsterdam, Netherlands). Presence and correctness of all constructs and appropriate nucleotides were confirmed by automated sequencing.

Cell culture and transient transfection assays

HEK293T, COS7 and SaOs-2 cells were maintained in Dulbecco’s Modified Eagle Medium with 10% fetal calf serum (iron-supplemented for HEK293T, 477 μg/100mL; CellConcepts, Umkirch, Germany), L-Glutamine (2 mM/mL), 100 U/mL penicillin and 100 ng/mL streptomycin. Cells were plated in 24-well plates and transfected the next day with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). Luciferase activity was determined using a Sirius single-tube luminometer (Berthold detection systems, Pforzheim, Germany). In case, cells were stimulated with 10^-8 M PMA (Fluka Riedel-de Haën, Seelze, Germany) for 2, 6, 12, and 24 hrs prior to harvesting. Transfections of deletion constructs were conducted with equimolar amounts of
reporter vectors, using the inert vector p0GH (Nichols Institute, San Juan Capistrano, USA) for adjustment of DNA content. For cotransfection, expression vectors for C/EBPα, C/EBPβ, and C/EBPδ (kind gift from Dr. Birgit Gellersen Endokrinologikum, Hamburg, Germany) were used in a 1:3 ratio. Transfection experiments were repeated at least three times in triplicates. Significance was calculated by unpaired, two-tailed t test [C.I. 95%]; the significance levels were set at \( P<0.05 \).

**Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay**

Nuclear proteins from HEK293T and COS7 cells were extracted by a modified procedure of the protocol published by Schreiber et al.\(^3\) Intactness of nuclear protein extracts was ascertained by PAGE and Coomassie blue staining. Protein content was measured using the BCA protein quantification kit (Pierce, Rockford, USA). Single-stranded oligonucleotides (30 bp) synthesized at a coupling efficiency of >98.5% and purified by HPLC (IBA, Göttingen, Germany) were labeled using the Biotin 3’ End DNA Labeling Kit (Perbio, Bonn, Germany), annealed over night, and quality controlled by PAGE.

EMSAs were performed using 5 \( \mu \)g nuclear protein extracts, mixed with 500 ng pre-sheared poly dI•dC (Amersham, Braunschweig, Germany) and unlabeled oligonucleotide (4 pmol) as competitor. DNA was cross-linked to PVDF membrane by UV-light (312 nm). Bands were visualized by Chemoluminescent Nucleic Acid Detection Kit (Perbio). Membrane was exposed to CL-X Posure Film (Perbio). For Supershift assays, the EMSA protocol was extended by 15 min incubation with 2 \( \mu \)g of specific anti-C/EBPα (14AA), anti-C/EBPβ (C-19), and anti-C/EBPδ (C-22) antibodies (St. Cruz Biotech, Heidelberg, Germany).
Isolation of total RNA and generation of cDNA for detecting endogenous GRK4 expression

For expression analyses, total RNA of stimulated (PMA/cAMP) and unstimulated cells (without, w/o) was extracted by Trizol Reagent (Invitrogen). Generation of cDNA was performed with First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) and amplified with specific primers. Intactness and purity of cDNA was routinely controlled by diagnostic PCR for human Ribosomal Protein 27 (hRP27). In SaOs-2 cells, gradient PCR was used to detect at least very low signals, thus GRK4 expression had to be confirmed by nested PCR.

References

