Functional Polymorphism of the Anpep Gene Increases Promoter Activity in the Dahl Salt-Resistant Rat


Abstract—We have reported that aminopeptidase N/CD13, which metabolizes angiotensin III to angiotensin IV, exhibits greater renal tubular expression in the Dahl salt-resistant (SR/Jr) rat than its salt-sensitive (SS/Jr) counterpart. In this work, aminopeptidase N (Anpep) genes from SS/Jr and SR/Jr strains were compared. The coding regions contained only silent single nucleotide polymorphisms between strains. The 5′ flanking regions also contained multiple single nucleotide polymorphisms, which were analyzed by electrophoretic mobility-shift assay using renal epithelial cell (HK-2) nuclear extracts and oligonucleotides corresponding with single nucleotide polymorphism-containing regions. A unique single nucleotide polymorphism 4 nucleotides upstream of a putative CCAAT/enhancer binding protein motif (nucleotides −2256 to −2267) in the 5′ flanking region of the SR/Jr Anpep gene was associated with DNA-protein complex formation, whereas the corresponding sequences in SS rats were not. A chimeric reporter gene containing ~4.4 Kb of Anpep 5′ flank from the Dahl SR/Jr rat exhibited 2.5- to 3-fold greater expression in HK-2 cells than the corresponding construct derived from the SS strain (P<0.05). Replacing the CCAAT/enhancer binding protein cis-acting element from the SS rat with that from the SR strain increased reporter gene expression by 2.5-fold (P<0.05) and abolished this difference. CCAAT/enhancer binding protein association was confirmed by chromatin immunoprecipitation and correlated with expression, suggesting selection for a functional CCAAT/enhancer binding protein polymorphism in the 5′ flank of Anpep in the Dahl SR/Jr rat. These results highlight a possible association of the Anpep gene with hypertension in Dahl rat and raise the prospect that increased Anpep may play a mechanistic role in adaptation to high salt. (Hypertension. 2007;49:467-472.)

Key Words: CD13 | renal salt-handling | Dahl rat | salt-sensitive hypertension | polymorphism

Aminopeptidase N (Anpep; EC 3.4.11.2; also known as CD13, gp150, microsomal aminopeptidase, and aminopeptidase M), is a homodimeric, membrane-bound, zinc-dependent aminopeptidase that preferentially releases neutral amino acids from the amino terminus of oligopeptides and has specificity similar to that of cytosolic leucine aminopeptidase (reviewed in References 1,2). Anpep belongs to the M1 family of the MA family of peptidases, also known as gliuzincins, and includes membrane-bound type II glycoproteins. It has been cloned from 6 different mammalian species.3,4 It is widely distributed in tissues and, in the kidney, concentrated in the brush border membrane of proximal tubule cells.5–7 Anpep has been implicated in the regulation of enkephalins and pain, angiogenesis, tumor metastasis and invasion, inflammation, secretion, and apoptosis (reviewed in Reference 2).

Among Anpep substrates, which include neuropeptides (Met and Leu enkephalins, neurokinin A, Met-lys-bradykinin, and edorphins), vasoactive peptides (kallidin, somatostatin, and angiotensins), and chemotactic peptides (monocyte chemotactic protein/MCP-1 and N-formyl-methionine leucine phenylalanine/f-MLP), is the heptapeptide Ang III, which is metabolized to the natriuretic heptapeptide angiotensin IV (Val-Tyr-Ile-His-Pro-Phe) by deletion of the NH2-terminal arginine (reviewed in References 7,8). Unlike Ang II or Ang III, angiotensin IV is a natriuretic peptide capable of inhibiting renal Na uptake via blockade of ouabain-sensitive Na-K ATPase activity, increasing cortical blood flow, and reducing blood pressure in the rat.9–11

The expression of the Anpep gene, which consists of 20 exons, is regulated by alternate tissue-specific promoters, that is, an epithelial-specific proximal promoter and distal myeloid-specific promoter 8 kb upstream.12 Both promoters drive the expression of transcripts encoding identical cognate proteins. Differences in 5′ untranslated sequences derived from alternate noncoding first exons account for the reported
size differences between myeloid-specific (≈3.7 kb) and epithelial-specific (≈3.4 kb) transcript sizes. The immediate 5′ flank of the human Anpep gene contains a TATA-like element (ATATAA) 22 bases upstream of the transcriptional start site (+1) that is conserved with the orthologous porcine gene. The proximal epithelial-specific promoter is also characterized by conserved binding sites for several trans-acting factors, including hepatocyte nuclear factor 1 and Sp1, which are thought to play important roles in Anpep proximal promoter activity.\(^1,14\) An enhancer region controlling proximal promoter function has also been identified within ≈2.7 kb of the start site, which contains canonical binding sites for members of the winged helix Ets family, including CCAAT/enhancer binding protein (C/EBP).\(^7\)

From the rat sequencing data, it can be deduced that the Anpep gene lies within quantitative trait loci (QTLs) for blood pressure identified in both Dahl salt-sensitive (SS/Jr)-Lewis and Dahl SS/Jr–salt-resistant (SR/Jr) crosses.\(^15,16\) available online at http://hyper.ahajournals.org). We have reported that Anpep transcript abundance, protein abundance, and activity are greater in the kidneys of Dahl SR/Jr than in SS/Jr rats, raising the possibility that increased Anpep-mediated signaling, perhaps by angiotensin IV, reduces renal tubular Na uptake in adaptation to high salt.\(^15\) Consistent with this is the finding that the nonclipped kidney in the Goldblatt model has a highly significant increase in membrane-bound Anpep activity.\(^17,18\) Based on these results, we have proposed that the Anpep gene may be linked to salt-sensitive hypertension in the Dahl rat. The present study was performed to further examine this possibility by comparing the Anpep gene between Dahl SS/Jr and SR/Jr rat strains.

**Methods**

**Rat Strains and Reagents**

Male Dahl SS (SS/Jr), Dahl SR (SR/Jr), and Lewis rats were purchased from Harlan Sprague–Dawley (Indianapolis). Animal studies were approved by the University of Illinois at Chicago animal care committee. Culture medium (DMEM F12) and T4 polynucleotide kinase were purchased from Gibco BRL. Luciferase reporter and Gal4-binding domain plasmids were purchased from Harlan Sprague–Dawley (Indianapolis). Animal studies were performed by adding 2 g of C/EBPβ antibody (Abcam) 10 minutes before the addition of the labeled probe. Samples were run on a 6% non-denaturing polyacrylamide gel. DNA–protein complexes were visualized by autoradiography. Supershift assay was performed by adding 2 μg of C/EBPβ antibody (Abcam) 10 minutes before adding the labeled probe.

**Cell Culture**

Mycoplasma-free human renal epithelial (HK-2) cells were obtained from the American Type Culture Collection and were maintained in Mycoplasma-free human renal epithelial (HK-2) cells were obtained from the American Type Culture Collection and were maintained in DMEM-F12 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO₂.

**Analysis of Anpep Gene**

Genomic DNA was isolated by phenol–chloroform extraction. The 4.4-kb 5′ flanking regions of the Anpep gene were PCR amplified using express sequence tag-based sequences and cloned into the TA vector (Invitrogen) for automated sequencing. Polymorphisms were confirmed by sequencing both strands of DNA derived from 5 or 6 rats from each strain. DNA sequences were compared using DNA software for Windows (Molecular Biology Insights).

**Transfections and Luciferase Assays**

Anpep promoter/enhancer constructs were generated by inserting 4.4-kb 5′ flanking regions of Dahl SS/Jr and SR/Jr rats as SacI/XhoI fragments into a pGL3-basic promoter-less vector digested with SacI/XhoI. pAnpep SS/SSr construct was generated by replacing the −2232 to −2394 sequence of the SS/Jr rat with that of the SR/Jr rat by digesting the SS/Jr rat promoter sequence with Mscl and Nhel restriction enzymes. To generate a SR/Jr Anpep promoter with mutant C/EBPβ binding site (SR/C/EBP β mutant), the −2252 to −2394 region was amplified using a primer 5′CTGAGCGAAGTGTGAAAGTGA 3′ and then cloned into the Mscl and Nhel site of SR/Jr Anpep promoter plasmid. C/EBPβ expression vector was described previously.\(^19\) C/EBPβ dominant-negative mutant (C′30) was a HindIII and Ascl deletion of wild-type C/EBPβ provided by Dr. Daniel G. Tenen (Harvard Medical School, Boston, MA.).

Transient transfection of HK-2 cells was performed using the Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). Approximately 48 hours after transfection, luciferase reporter activity was measured in fresh whole-cell lysates using a commercially available luciferase assay system (Promega) and TD20/20 luminometer. To control for variations in transfection efficiency, cells were cotransfected with a control β-galactosidase expression vector (pSV-βGal, Pharmacia), and luciferase activity was normalized for β-galactosidase activity, measured by the β-galactosidase assay kit (Promega), in the same samples.

**Electrophoretic Mobility Shift Assays**

Nuclear extracts from HK-2 cells were prepared as described previously.\(^21\) Probes for gel-shift analysis were generated by PCR using specific primers flanking each single nucleotide polymorphism (SNP, details in an online supplement available http://hyper.ahajournals.org), and were end-labeled with [γ-32P] ATP using polynucleotide kinase (Promega). Oligonucleotides corresponding with the −2277 to −2255 sequence, containing a C/EBP-regulated canonical binding site, −2256 to −2266, and the −2272 T SNP (5′ CTGAGT-GAGTTTCGCACATGTA 3′; wild type; WT) and a mutant form containing 4 mutated bp (5′ CTGAGTGAAGTTCGGAAaTGTA 3′), were used as probes to examine fragment 9. Electrophoretic mobility-shift assay (EMSA) was performed by incubating (30 minutes, room temperature) nuclear extract (25 μg) from HK-2 cells in a total volume of 30 μL of EMSA buffer with 32P-labeled double-stranded oligonucleotide (8 fmol). For competition assays, a 100-fold excess of unlabeled oligonucleotide was added for 15 minutes before the addition of the labeled probe. Samples were run on a 6% non-denaturing polyacrylamide gel. DNA–protein complexes were visualized by autoradiography. Supershift assay was performed by adding 2 μg of C/EBPβ antibody (Abcam) 10 minutes before adding the labeled probe.

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation assay was performed using a kit (Upstate). Chromatin fragments were isolated from nuclei by sonication in SDS lysis buffer (1% SDS, 10 mmol/L of EDTA, and 50 mmol/L of Tris; pH 8.1). Fragments were precleared with protein A agarose beads and salmon sperm DNA followed by overnight incubation with continuous shaking at 4°C either in the presence of control normal rabbit IgG or polyclonal C/EBPβ antibody. The beads were washed once each with a low-salt immune complex, high-salt immune complex, and LiCl wash buffer followed by 2 washes with 10 mM Tris/1 mM EDTA (pH 8.0) buffer and incubated at 65°C overnight in a 50-μL buffer consisting of 20 mmol/L of Tris/HC1 (pH 8.0), 100 mmol/L of NaCl, and 10 mg/mL of protease K. The resulting DNA fragments were subjected to real-time PCR using primers flanking the C/EBP putative cis-binding sequence (palindrome) and −2272 T/C SNP in the Anpep promoter region (forward: 5′ GATTGGCAAAGAGGACACG 3′ and reverse: 5′ GGAGGCCATCAGAAGCC 3′) and control primers flanking the PDE4B promoter that do not contain any C/EBP or C/EBP-related transcription factor binding sites (forward: 5′ AGTTTATTAAACCTAGGTCCTTC 3′ and reverse: 5′ CCAATAAACCAGTGCTACGATC 3′). Quantitative real-time PCR analysis for chromatin immunoprecipitation assays and quantitation of C/EBPβ protein binding to the 5′ flanking region was calculated as described previously.\(^22\) ΔCt is derived by subtracting the threshold...
old cycles \((C_I)\) of SS/Jr and SR/Jr rat chromatin immunoprecipitates amplified using control primers from corresponding samples \((C_I)\) using primers flanking the C/EBP\(\alpha\) site and the 2272 T to C polymorphism. \(\Delta C_I\) of 1 is equivalent to a 2-fold change in sensitivity.

Statistical Analysis
Comparisons were made by ANOVA. Data are expressed as mean±SD. Experimental values were significantly different at \(P<0.05\).

Results
Identification of Anpep Gene Polymorphisms
Silent SNPs in Coding Region of Anpep Gene Between SS/Jr and SR/Jr Rats
Anpep cDNA was sequenced in 7 SR/Jr and 8 SS/Jr rats. The sequences corresponded with that in Genbank (Accession No. 205108 M25073) with the exception of silent SNPs (see online Appendix). Two silent SNPs were identified at positions 2306 and 2292, that is, T/C and G/A, respectively.

Anpep Gene Polymorphisms Within the 5' Promoter Region Between SS/Jr and SR/Jr Rats
The 4.4-kb 5' flanking region of the Anpep gene was sequenced from the Dahl SR/Jr and SS/Jr strains. A total of 11 SNPs were identified between SR/Jr and SS/Jr strains (Figure 1).

CEBP\(\alpha\) Associates With 5' Flanking Region of Anpep Gene in SR/Jr but Not SS/Jr Rats
EMSA was performed using 11 PCR-generated fragments, each containing 1 of the 11 SNPs detected between SR/Jr and SS/Jr strains (Figure 1). Inclusion of these PCR-generated DNA fragments with nuclear extract from HK-2 cells revealed complex formation only in fragment 9 from SR/Jr but not from the SS/Jr strain (Figure 2, C2272 + protein and Figure 1. Polymorphisms in Anpep 5' flanking region of Anpep gene; 4.4-kb 5' flanking regions of SS/Jr and SR/Jr were sequenced; 11 SNPs, 4 of which were within or adjacent to cis-elements (colors), were identified.
shifted the complex (Figure 4a, labeled WT probe lanes 3 and C/EBP CA/AG mutations (2263/64 and /H11002 detected with a labeled WT but not mutant probe with cell protein extracts (Figure 4). A DNA–protein complex was GAA) in fragment 9 was examined by EMSA using HK-2

ment 9.

putative

/H11002 upstream (2256 to YAAY (/H11002 contained the putative canonical C/EBP DNA binding site: RTTGCG- /H11002 was incorporated into a separate PCR product (fragments 1 to 11). No differential binding of the probes between rat strains was detected except for fragment 9, which shows an additional DNA–protein complex in SR/Jr but not SS/Jr strains (arrow). (Fragment 9 differs between strains only in the T/C2272 SNP.) The other bands present both in SS/Jr and SR/Jr strains could be nonspecific or because of binding of other transcription factors. See text for details.

Figure 2. EMSA analysis reveals interaction of a protein with fragment 9 in SR/Jr (C-2272) but not SS/Jr (T-2272) rat Anpep. Each SNP was incorporated into a separate PCR product (fragments 1 to 11). No differential binding of the probes between rat strains was detected except for fragment 9, which shows an additional DNA–protein complex in SR/Jr but not SS/Jr strains (arrow). (Fragment 9 differs between strains only in the T/C2272 SNP.) The other bands present both in SS/Jr and SR/Jr strains could be nonspecific or because of binding of other transcription factors. See text for details.

Figure 3. Chromatin immunoprecipitation assay–C/EBPα transcription factor binds to the endogenous kidney Anpep promoter region of SR/Jr rats. Chromatin fragments derived from SS/Jr and SR/Jr rat kidneys were cross-linked and immunoprecipitated with polyclonal antibody against C/EBPα. Immunoprecipitates from each sample were analyzed by real-time PCR using primers spanning fragment 9 and control primers as detailed in the Methods section. The number on the y axis indicates the ΔΔCT (See Methods for details). Numbers on the top of bars of graph indicate the relative expression derived by 2−ΔΔCt. n=5; **P<0.01 vs SS/Jr.

Discussion

The present results identify a functional polymorphism of a C/EBPα cis-element in the promoter of Anpep gene in the Dahl SR/Jr rat. The C/EBP transcription factors are a highly conserved group within the basic leucine zipper family that control genes involved in the control of cellular growth and

Anpep Promoter Activity Is Greater and Stimulated by c/EBPα in the SR/Jr Versus SS/Jr Rat Strain

Anpep promoter activity was measured using luciferase reporter constructs harboring the −4.4-kb 5′ flanking region of Anpep was reduced by ½=50% (P<0.05; Figure 4b) by introducing the same mutations, that is, C/A-2263/64 and A/G-2258/59, indicating coordinate decreases in C/EBPα–DNA association and promoter activity.

Figure 4. EMSA analysis reveals interaction of a protein with fragment 9 in SR/Jr (C-2272) but not SS/Jr (T-2272) rat Anpep. Each SNP was incorporated into a separate PCR product (fragments 1 to 11). No differential binding of the probes between rat strains was detected except for fragment 9, which shows an additional DNA–protein complex in SR/Jr but not SS/Jr strains (arrow). In contrast, 100-fold excess of mutant oligonucleotide did not abolish the binding of c/EBPα protein with WT probe, suggesting the specificity of c/EBPα binding (Figure 4a, right, lane 2).

In parallel experiments, luciferase promoter activity of the 4.4-kb 5′ flanking region of Anpep was reduced by ½=50% (P<0.05; Figure 4b) by introducing the same mutations, that is, C/A-2263/64 and A/G-2258/59, indicating coordinate decreases in C/EBPα–DNA association and promoter activity.

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differentiation, immune and inflammatory responses, and neural function/memory.23 Six C/EBP members (α to δ) have been cloned. Our data show that both C/EBPα and C/EBPβ forms of C/EBP are expressed in HK-2 cells, consistent with previous reports in the kidney.23 The essential C/EBP binding site has been reported to contain a dyad symmetrical repeat 5' - RRTGCGYAY-3', where R is A or G and Y is C or T,24 and its palindrome.24 The present results show that C/EBPα association with fragment 9 requires both TTCAGAA, a segment of the defining dyad repeat of C/EBP cis-elements, and a T SNP 4 bp upstream from this sequence. This is the first report, to our knowledge, of an SNP not within the dyad repeat regulating C/EBPα association. However, SNPs at a wide range of distances, ranging from a few to hundreds of base pairs,25,26 from the consensus binding sequences have been reported to be capable of influencing the DNA–protein association. The mechanisms of these regulations are not often clear, but may involve another protein, for example, enhancer or cofactor, binding to either DNA or C/EBPα.

Promoter activity for the 4.4 kb 5' flanking region of the Anpep gene from SR/Jr rats is greater than that from SS/Jr strain in HK-2 cells, consistent with greater Anpep transcript and protein abundance in the kidneys.4 The difference in promoter activity was present even in the presence of a dominant-negative mutant of C/EBPα, suggesting other important cis-acting elements and/or a role of C/EBPβ. Eleven SNPs are identified in the 4.4 kb 5' flanking region of the Anpep gene. EMSA was used to screen for effects of these SNPs on the DNA–protein interaction. C/EBPα was the only trans-acting factor identified in this manner as physically

Figure 4. EMSA analysis of C/EBPα binding in fragment 9. a, C/EBPα associates with canonical binding site in fragment 9. Nuclear extracts were prepared from HK-2 cells, and EMSA was performed using C/EBPα binding site with wild type (WT), and mutant (M) C/EBPα binding site with 4 nucleotide changes as described in the Methods section. Nuclear extracts were incubated with competitor oligonucleotides (WT and M) and C/EBPα antibody as indicated 10 minutes before adding the labeled probe. The position of the super shifted band is indicated by the arrowhead. b, C/EBPα stimulates Anpep activity of SR/Jr rat in HK-2 cells. pGL3 control vector, SR/Jr Anpep promoter, and SR/Jr Anpep promoter harboring mutant C/EBP binding site (SR/cEBPmutant) were cotransfected with β-galactosidase expression plasmid (internal control), and luciferase assay was carried out as described in the Methods section. n = 6; *P<0.05 vs SR C/EBP mutant; **P<0.01 vs pGL3.

Figure 5. C/EBPα increases promoter activity in SR/Jr but not in the SS/Jr strain. Luciferase reporter activity assay in HK-2 cells for 4.4-kb Anpep regulatory regions of SS/Jr and SR/Jr and control pGL3 vectors cotransfected with C/EBPα (+/C/EBPα) and dominant-negative C/EBPα expression vectors (+/C/EBPαDN) (see Methods). The luciferase activity was normalized with β-galactosidase activity of each sample; n = 6; *P<0.05, **P<0.01 vs SS/Jr and pGL3.

Figure 6. Insertion of fragment 9 from SR/Jr rat into 4.4-kb 5' flanking region of SS/Jr (SS/SR) increases the promoter activity. Control pGL3 and the 4.4-kb 5' flanking regions of SS/Jr, SR/Jr rats, and SS/Jr with insertion of fragment 9 from SR/Jr rat (SS/SR) cloned in front of luciferase reporter gene in pGL3 vector were transiently cotransfected with cytomegalovirus β-galactosidase internal control vector into HK-2 cells. The luciferase activity was normalized with β-galactosidase activity of each sample. n = 6; *P<0.05 vs SS and pGL3.
associating with the functionally significant SNP-containing region. This does not, however, exclude binding by other factors with different binding affinities or in a combinatorial context in vivo.

The results raise the possibility that the Anpep gene is a salt-sensitive hypertension susceptibility gene in the Dahl rat model. The 2272 C SNP is present in the SR/Jr but not SS/Jr rat and can be inferred to map to a previously mapped QTL identified in the Dahl SS/Jr × SR/Jr cross. The 2272 C SNP is also present in the Lewis rat, a less salt-sensitive strain. We have reported previously that Anpep maps to a QTL defined in a Dahl SS/Jr–Lewis cross,15 suggesting that this SNP is important in differentiating the Lewis from Dahl SS/Jr rat strains. Additional strategic congenic and transgenic rat experiments are necessary to confirm a link between adaptation to high salt and the Anpep gene.

Perspectives

The present results identify Anpep as a new candidate gene in the Dahl rat. Although many genes have been tested, the specific genes underlying salt-sensitive hypertension in the Dahl rat remain elusive. Whether Anpep is truly important will require complementary strategies. Transgenic, congenic, and consomic strains are likely to be especially useful in the further judgment of Anpep.

A critical component to evaluating Anpep as a candidate gene is demonstrating a functional or physiological mechanism that links Anpep to the regulation of renal salt handling. In this regard, Anpep is especially intriguing, because it has been linked to RAS by metabolizing angiotensin III to angiotensin IV, a “diuretic” peptide. This raises the spectra of another level of complexity in the regulation of RAS. Significantly, it may lead to the discovery of a physiological role for angiotensin IV, which has been reported to be, as opposed to angiotensin II, a diuretic peptide, which reduces Na+ uptake in tubule cells. Time will tell whether this Anpep “pans out” as a cause of salt-sensitive hypertension in the Dahl rat and/or plays a role in human forms of hypertension.

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Disclosures

None.

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