**HCaRG, a Novel Calcium-regulated Gene Coding for a Nuclear Protein, Is Potentially Involved in the Regulation of Cell Proliferation**

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Since a negative calcium balance is present in spontaneously hypertensive rats, we searched for the gene(s) involved in this dysregulation. A cDNA library was constructed from the spontaneously hypertensive rat parathyroid gland, which is a key regulator of serum-ionized calcium. From seven overlapping DNA fragments, a 1100-base pair novel cDNA containing an open reading frame of 224 codons was reconstituted. This novel gene, named HCaRG (hypertension-related, calcium-regulated gene), was negatively regulated by extracellular calcium concentration, and its basal mRNA levels were higher in hypertensive animals. The deduced protein showed no transmembrane domain, 67% α-helix content, a mutated calcium-binding site (EF-hand motif), four putative "leucine zipper" motifs, and a nuclear receptor-binding domain. At the subcellular level, HCaRG had a nuclear localization. We cloned the human homolog of this gene. Sequence comparison revealed 80% homology between rats and humans at the nucleotide and amino acid sequences. Tissue distribution showed a preponderance in the heart, stomach, jejunal, kidney (tubular fraction), liver, and adrenal gland (mainly in the medulla). HCaRG mRNA was significantly more expressed in adult than in fetal organs, and its levels were decreased in tumors and cancerous cell lines. We observed that after 60 min ischemia followed by reperfusion, HCaRG mRNA declined rapidly in contrast with an increase in c-myC mRNA. Its levels then rose steadily to exceed base line at 48 h of reperfusion. HEK293 cells stably transfected with HCaRG exhibited much lower proliferation, as shown by cell count and [3H]thymidine incorporation. Taken together, our results suggest that HCaRG is a nuclear protein potentially involved in the control of cell proliferation.

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Ionized calcium concentration in plasma is maintained within a very narrow range. The major players maintaining extracellular calcium homeostasis are calciotropic hormones, parathyroid hormone (PTH),1, 1,25-dihydroxyvitamin D, calcitonin, and calcium itself. Indeed, extracellular calcium regulates its own concentration as an extracellular messenger by acting on calcium receptors or calcium sensors. The calcium-sensing receptor is linked to several intracellular second messenger systems via guanylyl nucleotide-regulating G proteins and activates phosphoinositide-specific phospholipase C, leading to accumulation of inositol 1,4,5-trisphosphate and diacylglycerol (1–5).

Cells of the parathyroid gland possess such a calcium sensor (6). Even slight reductions in extracellular ionized calcium concentration (on the order of 1–2% or less) elicit prompt increases in the rate of PTH secretion and mRNA levels. Historically, research on the parathyroid gland has focused on the chemistry, regulation, synthesis, and secretion of PTH. There is growing interest in other calcium-regulating proteins of this gland that are also negatively regulated by extracellular calcium, such as chromogranin A and secretory protein-I (7), as well as a hypertensive factor of parathyroid origin (PHF) (8, 9).

Arterial hypertension is associated with numerous disturbances of calcium metabolism manifested not only in humans but also in genetic as well as acquired models of hypertension (10–14). Disturbances in renal and intestinal handling of calcium in hypertension have been reported by several investigators (15). Urinary calcium has generally been shown to be increased (so-called urinary leak) and intestinal calcium absorption diminished in genetically hypertensive or spontaneously hypertensive rats (SHR) (15, 16). Cytoplasmic free calcium concentration has most often been found to be elevated in circulating platelets, lymphocytes, erythrocytes, and vascular smooth muscle cells (VSMC) from hypertensive animals and humans (for a review, see Ref. 17). In SHR as well as in low renin hypertensive patients, there seems to be an inverse relationship between extracellular and intracellular calcium (18). It has been hypothesized that certain genetic abnormalities

1 The abbreviations used are: PTH, parathyroid hormone; PTC, parathyroid cell(s); BN, Brown-Norway rats; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; nCARE, negative calcium-responsive element; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PHF, parathyroid hypertensive factor; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SHR, spontaneously hypertensive rat(s); SSC, standard sodium citrate; VSMC, vascular smooth muscle cells; WKY, Wistar-Kyoto rat(s); bp, base pair(s).
might be responsible for the link between some forms of hypertension, calcium homeostasis, and the parathyroid gland. To identify new genes that might be abnormally regulated by extracellular calcium in the parathyroid gland of genetically hypertensive rats, we prepared a cDNA library from the parathyroids of SHR. In this study, we describe the isolation and characterization of a novel gene, designated HCaRG (for hypertension-related, calcium-regulated gene), negatively regulated by extracellular calcium with higher mRNA levels in SHR. HCaRG is a nuclear protein with putative "leucine zipper" motifs and is potentially involved in the regulation of cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**Parathyroid cells (PTC) were isolated from SHR and Wistar-Kyoto rats (WKY). Primary cultures were passaged in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, as described previously (9). They were then maintained in Ham’s F-12 medium containing a low (0.3 mM) or normal (2.0 mM) total calcium concentration for 2 or 48 h. COS-7 or HEK293 cells were cultured in DMEM containing 10% fetal calf serum. All cell types were maintained in 5% CO2 at 37 °C.

**Radiolabeling—**SHR were anesthetized lightly with flurane, and the right kidney was removed through a mid-abdominal incision. The rats were kept at ambient temperature during the surgery. Their core temperature, monitored by radio-telemetry, was 38 °C. The left kidney was subjected to warm transient ischemia by occlusion of the left renal artery and vein with a microclip, as described previously (19). The skin incision was closed during the 60-min renal ischemia period. It was then reopened, and the clip was removed. The wound was closed with a 2"0" suture. The rats had access to water immediately after surgery.

**SHR Parathyroid cDNA Library—**Parathyroid glands were removed from 100 12-week-old SHR and frozen immediately in liquid nitrogen. The glands were added to a guanidinium thiocyanate solution and homogenized. Poly(A) RNA was isolated on an oligo(dT) column. The cDNA library was constructed with poly(A) RNA as template and the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). A summary of the cloning of Human HCaRG—A 439-bp DNA fragment of rat HCaRG was 32P-labeled and served as a probe for screening a human VSMC cDNA library. DNA from positive phages was purified, and the fragments were cloned in pBluescript. All fragments were sequenced. We obtained a 1355-bp fragment containing the coding region of HCaRG.

**Ex vivo Thrombosis—**To determine the subcellular localization of HCaRG protein in mammalian cells, the coding region of HCaRG was fused to green fluorescent protein (GFP) cDNA and was transfected into the cells. Briefly, the entire coding region of HCaRG was amplified by PCR with the primers ATG TCT GTG GGT GCC GCA CCT CTT TCT GG and GCA CGA GCC ACA GCC AGC TAC CCA TTG GTC TTT GCA GCT CCA TAC TTG CAC CAT CCC and TAA TAC GAC TCA TTA GGA GAC, gel-purified, and fused in frame to GFP in pEGFP-C1 (CLONTECH, Palo Alto, CA) through a blunt HindIII site. pEGFP-HCaRG was then sequenced. Similarly, the coding sequence of HCaRG was fused in frame to glutathione S-transferase (GST) in pGEX-IX (Amersham Pharmacia Biotech) through a blunt EcoRI site.

**Cloning of Human HCaRG—**Four mixtures of degenerate oligonucleotide primers were initially designed according to the putative amino acid sequence of PHF with the following degenerate sequence: 5’-TA/TAC/TG/GTT/GTC GT/GCC CAC TTT CG-3’. From initial RACE experiments (described below), one unique sequence primer TAC TCC GTG TCC CAC TTT CG was selected for its ability to generate reverse transcription (RT)-PCR DNA fragments from PTC total RNA and used subsequently as candidate primer for 3’-RACE. In brief, for 3’-RACE, total RNA from PTC was reverse-transcribed with a hybrid primer consisting of oligo(dT) (17-mer) extended by a unique 17-base oligonucleotide (adapter). PCR amplification was subsequently performed with the adapter, which bound to cDNA at its 3’-ends, and the candidate primer mentioned above (21). For 5’-RACE, RT was undertaken with an internal primer derived from the sequence of the cDNA fragment generated by 3’-RACE. A 4a homopolymer tail was then appended to the first strand reaction products using terminal deoxynucleotidyl transferase. Finally, PCR amplification was accomplished with the hybrid primer previously and a second internal primer upstream to the first one (21).

**Subcloning—**The DNA fragments generated from the RACE experiments were separated by electrophoresis, isolated from agarose gel, and extracted by the phenol-chloroform method (20). pSP72 plasmid (Promega) was digested at the Smal site and ligated to blunt DNA fragments from T4 DNA ligase. The transformed DH5α bacteria were grown, and recombinant bacteria were selected by PCR. Similarly, HCaRG was subcloned in pcDNA1Neo (Invitrogen, Carlsbad, CA).

To determine the subcellular localization of HCaRG protein in mammalian cells, the coding region of HCaRG was fused to green fluorescent protein (GFP) cDNA and was transfected into the cells. Briefly, the entire coding region of HCaRG was amplified by PCR with the primers.

**Northern Blot Hybridization—**Northern blot hybridization was performed with Sequenase version 2.0 (U.S. Biochemical Corp.). 5 μg of recombinant plasmid template were denatured, annealed with T7 or SP6 primers, and labeled with [32P]dATP by extension, using the chain termination method of Sanger according to the manufacturer’s protocol.
rinsed successively in glycerine buffer plus PBS and then dehydrated in ethanol. Actual prehybridization was done with 50% formamide, 0.2% SDS, 0.1% Sarcosyl, 5× standard sodium citrate (SSC: NaCl (0.15 M), sodium citrate (0.015 M), pH 7.0), and 2% blocking reagent (Roche Molecular Biochemicals) for 1 h at 60 °C. Hybridization was performed by adding 50% formamide to 50 μl of 4× SSC and 50% formamide per section. The slides were incubated overnight at 60 °C in a humidified chamber. During hybridization, a coverslip was placed over the tissue section. After hybridization, it was removed, and the sections were rinsed with 4× SSC and then washed with 4× SSC for 15 min and in 2× SSC for 15 min at room temperature. Finally, the sections were washed with 0.1× SSC for 30 min at 55 °C and rinsed with buffers 1 and 2 of the DIG Luminescent Detection Kit (Roche Molecular Biochemicals). They were then incubated with anti-DIG alkaline phosphatase antibody (1:500) in buffer 2 for 40 min and washed twice in buffer 1 for 15 min and in buffer 3 for 2 min. Incubation in the color solution nitro blue tetrazolium/5-bromo-4-chloro-3-indol phosphate (NBT/BCIP-phos) was carried out for 45 min, after which the slides were washed in distilled water and dry-mounted with Gelto.

In Vitro Transfection—The full length of the HCaRG coding sequence was synthesized by RT-PCR with specific primers and inserted downstream of the T7 promoter into the pSp72 vector. In vitro transcription and translation were performed using a TNT-T7-coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine. A plasmid containing the luciferase gene supplied by the manufacturer was used as a control. The synthesized proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis in the absence or presence of β-mercaptoethanol. Radioactive protein bands were detected by scanning with a PhosphorImager.

Antibody Production—E. coli cells transformed with pGEX-3X were grown in LB medium containing 50 μg/ml ampicillin at 37 °C until A600 = 0.5. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cells were cultured for 2 h. Purification of glutathione S-transferase-HCaRG was performed according to the manufacturer’s protocol. Polyclonal antiserum with antibodies recognizing HCaRG were produced by immunization of rabbits with glutathione S-transferase-HCaRG protein.

Immunocytochemical Reaction at the Electron Microscopic Level—Rat tissues (liver, anterior pituitary, spleen, heart, and adrenal gland) were quickly removed and fixed in 4% paraformaldehyde with 0.05% glutaraldehyde in phosphate buffer solution for 90 min. A part of the specimens was dehydrated before embedding in Lowicryl K4M with the AFS system (23). Sections were mounted on 224-codons preceded by two in-frame stop codons and followed by the acid sequence of PHF (24)) and a hybrid oligo(dT) primer, 3′-RACE experiments, performed on total RNA extracted from SHR PTC cultured in low calcium medium, generated one major 700-bp fragment that was digested and cloned in the BamHI site of pSPT7. Since a BamHI site was present in the 700-bp fragment, a recombinant plasmid containing a 300-bp insert was isolated and sequenced. This fragment was used to screen the PTC library and to generate new oligonucleotide primers to extend the cDNA toward the 5′- and 3′-ends by RACE. From seven overlapping DNA fragments isolated in the above experiments and from SHR PTC cDNA library screening, a 1100-bp cDNA was reconstituted (Fig. 1A). The rat 1100-bp reconstituted cDNA sequence contained an open reading frame of 322 amino acids and was determined by Northern hybridization.

Stable Transfection—HEK293 cells were plated in a 100-mm plate at a density of 0.5 × 10⁶ cells/plate. They were transfected with the control plasmid pcDNA1/Neo (Invitrogen) or with the plasmid containing rat HCaRG using a standard calcium phosphate coprecipitation method. The cells were then cultured in 100-mm plates containing 200 μg/ml of G418 (Geneticin, Life Technologies, Inc.) for 2 weeks, the clones were picked, and the level of ectopic HCaRG expression was determined by Northern hybridization.

Cell Counting and [3H]Thymidine Incorporation—The rate of stable clone cell proliferation was measured by counting the number of cells after plating. Cells were cultivated at a density of 0.1 × 10⁶ cells/well in 24-well plates with triplicate plates for each clone line. Every 24 h, the cells were trypsinized and counted in a hemocytometer. HEK293 cells that stably expressed either Neo control plasmid or HCaRG were used for the estimation of DNA synthesis by [3H]thymidine incorporation. The clones were trypsinized at 90% confluency, counted in a standard hemocytometer, and inoculated at an identical initial cell density of 40,000 cells/ml in DMEM containing 10% fetal bovine serum and G418 at 400 μg/ml. All cells were inoculated in poly-L-lysine-pretreated 24-well plates in a volume of 1 ml/well (40,000 cells/well). They were allowed to attach and grow for a period of 24–48 h. The growth media were then replaced by DMEM containing 0.2% fetal bovine serum and G418 (400 μg/ml) for a period of 48 h to synchronize the cells. After the synchronization period, the cells were supplied with fresh medium containing 10% fetal bovine serum and allowed to grow for another 48 h. [3H]Thymidine, 1 μCi/ml (ICN) was added to the cells for the last 4 h of the 48-h growth period. At the end of incubation, the medium was removed, and the monolayers were washed twice with PBS. The cells were then fixed with ethanol/acetic acid (3:1, v/v), and DNA was digested/extracted with 0.5 N perchoric acid at 80–90 °C for 20 min.

RESULTS
Isolation of a Novel cDNA Whose Expression Is Negatively Regulated by Extracellular Calcium in the SHR Parathyroid Gland—Using sense candidate primers (from a putative amino acid sequence of PHF (24)) and a hybrid oligo(dT) primer, 3′-RACE experiments, performed on total RNA extracted from SHR PTC cultured in low calcium medium, generated one major 700-bp fragment that was digested and cloned in the BamHI site of pSPT7. Since a BamHI site was present in the 700-bp fragment, a recombinant plasmid containing a 300-bp insert was isolated and sequenced. This fragment was used to screen the PTC library and to generate new oligonucleotide primers to extend the cDNA toward the 5′- and 3′-ends by RACE. From seven overlapping DNA fragments isolated in the above experiments and from SHR PTC cDNA library screening, a 1100-bp cDNA was reconstituted (Fig. 1A). The rat 1100-bp reconstituted cDNA sequence contained an open reading frame of 224 codons preceded by two in-frame stop codons and followed by the most frequent variant of the poly(A) tail (Fig. 1B). A 342-bp intron was localized at position −52 from the translation initiation site.

Poly(A) RNA was isolated as described and analyzed by Northern hybridization with the 32P-labeled 300-bp fragment (Fig. 2A). Two bands were detected with this probe, with approximate lengths of 1.2 and 1.4 kilobase pairs. These results suggest either the existence of two genes or differential splicing. Furthermore, they indicate that the reconstituted 1100-bp cDNA is almost full-length cDNA, estimated at 1.2 kilobase pairs by the major band in the Northern hybridization experiments.

Regulation of the expression of this novel gene was investigated by competitive RT-PCR assay in PTC from WKY and SHR. Cells between 5 and 12 passages were tested in these studies. In WKY PTC, lowering of ambient calcium from 2.0 to 0.3 mM induced a rapid 2-fold increase in the mRNA levels of this novel gene at 2 h, which lasted up to 48 h (Fig. 2B). This calcium regulation was detected in WKY PTC up to about 12 passages but disappeared in long term cultures. Lowering of calcium concentrations in the cell medium also increased the
mRNA levels of this novel gene in SHR PTC but to a lesser extent than in WKY cells (data not shown). We then compared its mRNA levels between two normotensive rat strains (Brown Norway, BN, or WKY) and hypertensive animals (SHR). We observed that the mRNA levels of this novel gene were significantly higher in PTC derived from SHR (Fig. 2C, left panel) compared with normotensive WKY at normal calcium. Similarly, when we extracted RNA (Fig. 2C, right panel) or proteins (Fig. 2D) directly from the kidneys, we found significantly higher levels of this novel gene in hypertensive rats. These results clearly show that this novel gene is negatively regulated by extracellular calcium concentrations and that its levels are significantly higher in genetically hypertensive rats compared with two normotensive strains. We therefore named this gene \( \text{HCaRG} \) (hypertension-related, calcium-regulated gene).

### Sequence and Structure of \( \text{HCaRG} \) cDNA

The deduced protein contained 224 amino acids with a calculated molecular mass of 22,456 Da. The estimated pI of the protein was 6.0. It comprised no known membrane-spanning motif but had an estimated 67% \( \alpha \)-helix content. The absence of a putative signal peptide sequence suggested an intracellular protein. There were two cysteines in the sequence, indicating possible intracellular localization.

**Fig. 1.** cDNA cloning of \( \text{HCaRG} \). A, reconstitution scheme of \( \text{HCaRG} \) cDNA. Overlapping fragments leading to the reconstitution of rat \( \text{HCaRG} \) 1100-bp cDNA are shown. cDNA fragments were initially obtained using 5' RACE and 3' RACE strategies as well as by screening a SHR parathyroid cDNA library. The first cDNA fragment was by 3'-RACE (3r 290). This initial fragment served to screen the SHR parathyroid cDNA library. Fragments \( \text{HCaRG}_{2c-t3} + 2c-t7 \), \( \text{HCaRG}_{825} \), \( \text{HCaRG}_{10-ic} \), and \( \text{HCaRG}_{10–174} \) were isolated from the cDNA library. Fragments 5r 285 and 5r 260 were obtained by 5'-RACE. This reconstitution was confirmed by sequencing an 860-bp PCR product with nested primers in 5r 260 and \( \text{HCaRG}_{825} \) and containing the complete open reading frame. B, nucleotide and deduced amino acid sequences of \( \text{HCaRG} \). The translation initiation start site codon is at position 1, and the termination codon is at position 675. The deduced amino acids are indicated below the nucleotide sequence. The localization of a 482-bp intron is indicated at position 52 by a triangle.
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**Fig. 2. Identification of a novel gene negatively regulated by extracellular calcium.** A, Northern blot analysis of poly(A) RNA isolated from PTC. HCaRG mRNA appears as a doublet of approximately 1.2 and 1.4 kilobase pairs. The positions of ribosomal RNAs and glyceraldehyde-3-phosphate dehydrogenase transcript are indicated. B, PTC extracted from normotensive rats (WKY) (from passages 8–10) were incubated in low (0.3 mM) or normal (2 mM) calcium-containing medium for 2 and 48 h. Total RNA was extracted and analyzed by RT-PCR as described under "Experimental Procedures." Incubation of PTC for 2 h in 0.3 mM (L) calcium significantly increased HCaRG mRNA compared with 2 mM (N) calcium; this increase lasted up to 48 h. C, significantly higher basal HCaRG levels were found in PTC from hypertensive rats compared with the normotensive rat strain WKY (left panel). This was confirmed with RNA (right panel) and proteins (D) extracted directly from the kidneys of SHR and BN.Lx, another normotensive strain. The figure represents the mean ± S.E. of two independent experiments performed in duplicate. **, p < 0.02; *, p < 0.05 as evaluated by the unpaired t test.

intermolecular disulfide bridges (Cys$^{64}$–Cys$^{219}$). The protein had several putative phosphorylation sites for protein kinase C and protein kinase A and one potential Asn-glycosylation site (Asn$^{76}$). To confirm that HCaRG mRNA encodes a peptide of expected size, the HCaRG cDNA inserted into pSP72 was incubated in vitro in a coupled transcription/translation labeling system. It was transcribed by T7 RNA polymerase and translated in rabbit reticulocyte lysate. As shown in Fig. 3 (lane 4), HCaRG mRNA directed the synthesis of a peptide with a molecular mass of 27 kDa, which closely corresponded to the molecular mass calculated from the amino acid sequence. Polyacrylamide gel electrophoresis analysis of the reaction product in the absence of the reducing agent β-mercaptoethanol showed bands of 27 and 43 kDa (Fig. 3, lane 5). These results suggest possible intramolecular or intermolecular disulfide bridges and the formation of homodimers or heterodimers with other protein(s) present in the lysate.

**Fig. 3. In vitro translation of HCaRG cDNA.** cDNA was cloned into pSP72 vector and used for coupled transcription/translation in the presence of [35S]methionine. Lane 1, molecular weight markers; lane 2, translation products of the control luciferase gene; lane 3, translation products without the insert; lane 4, translation product from HCaRG cDNA; lane 5, translation products of HCaRG cDNA. The proteins were separated by 15% polyacrylamide gel electrophoresis in the presence (lanes 1–4) or absence (lane 5) of β-mercaptoethanol. Translation/translation of HCaRG cDNA yields a protein of 27 kDa (lane 4). In the absence of β-mercaptoethanol, a product of 43 kDa was also observed (lane 5), suggesting intramolecular or intermolecular disulfide bridges and the formation of homodimers or heterodimers with other protein(s) present in the lysate.

**Cloning of Human HCaRG—**We then used a 439-bp cDNA fragment of rat HCaRG (+1 to +440) in Fig. 1) to screen a human VSMC cDNA library. We identified several positive clones that were purified, subcloned in pBluescript vector, and sequenced. We obtained a 1355-bp sequence containing full-length human cDNA, while all other clones contained only partial sequences. A recent sequence search in GenBank$^TM$ revealed a region with complete DNA sequence homology within three cosmids containing the zinc finger protein 7 gene (accession numbers AF124523, AF146367, and AF118808). Although the nucleotide sequence of human HCaRG could be found in these cosmids, we are the first to assign an expressed gene sequence to this DNA region.

Sequence comparison between human HCaRG and rat HCaRG showed 80% identity at the nucleotide level (data not presented) and, similarly, 80% homology at the amino acid level (Fig. 4). Analysis of protein structure with the PROSEARCH data base revealed four overlapping putative leucine zipper consensus motifs (Fig. 4, underlined). Further analysis revealed homology to the EF-hand calcium-binding motif (eight of the 10 most conserved amino acids) (Fig. 4, dashed box). We also identified a nuclear receptor-binding motif (Fig. 4, boldface and italic type). All of these motifs were conserved in the rat and human amino acid sequence.

**Subcellular Localization of HCaRG—**We expressed GFP-HCaRG in COS-7 cells. Fluorescence study showed that GFP-HCaRG localized in the nucleus, while cytoplasmic fluorescence was very faint (Fig. 5B). GFP, on the other hand, had a very diffuse localization (Fig. 5A). This result was confirmed by immunofluorescence using antibodies specific to HCaRG (Fig. 5C) and by electron microscopy (Fig. 5D). Electron microscopy was also performed on different tissues. In all tissues studied, HCaRG was found in the nucleus with some labeling in protein synthesis sites.

**HCaRG Expression in Various Human Tissues—**A human MTP$^{28}$ array was hybridized with human $^{32}$P-labeled HCaRG cDNA as a probe. The array contained 76 poly(A) RNAs from various adult tissues, cell lines, fetal tissues, and cancerous cell
Sequence comparison between human HCaRG and rat HCaRG. The deduced amino acid sequences of rat HCaRG (rHCaRG) and of human HCaRG (hHCaRG) are aligned. Identical amino acids are boxed, while homologous amino acids are shaded. We calculated 80% homology between these two sequences. Analysis revealed homology to the EF-hand motif, with eight out of the 10 most conserved amino acids (dashed box). Further analysis using the PROSEARCH data base revealed four overlapping putative leucine zipper consensus motifs (underlined). We also identified a nuclear receptor-binding domain (boldface and italic type).

In Situ Hybridization of HCaRG mRNA in the Kidney and Adrenal Gland—HCaRG expression was determined in SHR tissues by in situ hybridization. The labeled antisense riboprobe hybridized to the medulla and zona fasciculata of the adrenal cortex (Fig. 7). In the kidney, labeling was almost exclusively located in the cortex and concentrated in the tubular component, contrasting with the virtual absence of the signal in glomeruli (Fig. 7). In these organs, the signal was clearly greater in hypertensive rats compared with their normotensive controls. The sense probe was used as a negative control and appropriately revealed a low signal under our hybridization conditions, demonstrating the specificity of the reaction (Fig. 7, lower panels).

HCaRG mRNA Levels after Ischemia-Reperfusion—The process of kidney injury and repair recapitulates many aspects of development. It involves dedifferentiation and regeneration of epithelial cells, followed by differentiation (25–27). Since we observed that HCaRG mRNA levels are lower in fetal than in adult organs, we evaluated HCaRG expression after unilateral renal ischemia in uninephrectomized rats (19), since contralateral nephrectomy has been shown to stimulate cell regeneration (28–31). We noted that HCaRG mRNA declined rapidly to its lowest levels at 3 and 6 h of reperfusion (Fig. 8A). These values then increased steadily to higher than base line at 48 h of reperfusion. This was observed in both the kidney medulla (Fig. 8A) and cortex (Fig. 8B). In contrast to the decline in HCaRG mRNA levels, the proto-oncogene c-myc expression, which is correlated with hyperplastic response in mammalian cells, was rapidly increased following renal ischemia and reperfusion (31). c-myc mRNA levels were low in control kidneys and increased dramatically in the postischemic kidney at 3 h of reperfusion, at a time when HCaRG mRNA levels were already reduced (Fig. 8, A and C).

Overexpression of HCaRG Inhibits Cell Proliferation—HEK293 cells were stably transfected with either plasmid alone or with plasmid containing rat HCaRG. After transfection, several clones were examined for the determination of rat HCaRG mRNA levels. Four clones (HCaRG clones 1, 5, 8, and 9) expressed variable amounts of rat HCaRG mRNA, as detected by Northern blots, while no HCaRG mRNA levels were

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found in clones transfected with the plasmid alone (Fig. 9). Clones expressing the highest levels of HCaRG (clones 8 and 9) were selected for cell proliferation studies. For these studies, cells that were transfected with the vector alone or polyclonal HCaRG-transfected cells served as controls. The proliferation rates of the HCaRG-transfected cell lines and vector control cells were examined under normal growth conditions (10% fetal calf serum and G-418) by counting cell numbers every day for a period of 8 days after plating. Cell lines transfected with the vector alone (Neo clones 1 and 6) showed a similar growth rate as nontransfected cells (not presented). Clones 8 and 9 expressing high levels of rat HCaRG revealed a much lower proliferation rate than vector control cells, while polyclonal cells expressing intermediate values of HCaRG fell in between (Fig. 10A). Consistent with a lower proliferation rate, stable HCaRG transfection clones 8 and 9 showed much lower [3H]thymidine incorporation than clones transfected with the empty vector (Fig. 10B).

FIG. 6. Tissue distribution of HCaRG mRNA. A, comparison of HCaRG expression in fetal (gray bars) versus adult (black bars) human organs. HCaRG mRNA is expressed less in all fetal tissues compared, particularly in the heart, kidney, and liver. B, Northern blot containing 2 μg of poly(A)+ RNA from fetal and adult human hearts. HCaRG is more expressed in all regions of the adult heart (left (L) and right (R)). C, comparison of HCaRG expression in adult human organs versus cancerous cell lines. HCaRG mRNA is expressed less in most cancerous cell lines compared. Lymphocyte is shown as follows: normal lymphocyte (●); Burkitt’s lymphoma Raji (■); Burkitt’s lymphoma Daudi (□). Leukocyte is shown as follows: normal (●); leukemia HL-60 (▲); leukemia K-562 (■); leukemia MOLT-4 (□). Rectum is shown as follows: normal (●); colorectal adenocarcinoma SW480 (□). Lung is shown as follows: normal (●); lung carcinoma A549 (□). D, Northern blot containing 20 μg of total RNA isolated from three different human tumors (T) and normal tissue (N) excised at the same operational site. HCaRG expression is decreased in brain, kidney, and liver tumors. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIG. 7. In situ hybridization of HCaRG mRNA in the kidney and adrenal. In situ hybridization of HCaRG mRNA in the rat adrenal shows specific detection in the zona fasciculata and medulla. Specific hybridization in the kidney is restricted to proximal tubules, contrasting with its virtual absence in the glomeruli (G). Upper panels, antisense probe; lower panels, sense probe.

FIG. 8. Kidney cortex and Adrenal. Antisense sense

DISCUSSION

The cloning of a novel extracellular calcium-responsive gene (HCaRG) in the rat parathyroid gland from SHR is described here. HCaRG mRNA and protein levels were higher in cultured PTC and in several organs of SHR, compared with their normotensive counterparts. They were negatively regulated by extracellular calcium; i.e. lowering extracellular calcium led to increases in HCaRG mRNA. The identification of an extracellular calcium-sensing receptor from the parathyroid gland has provided novel insights into the mechanisms of direct action of extracellular calcium on several cell types. The calcium sensor has also been localized in the cerebral cortex and cerebellum, in the tubular region of the kidney cortex, the thyroid, adrenal medulla, lung, and blood vessels (1, 32, 33). As shown here, HCaRG mRNA levels are also detected in several of these tissues. The calcium receptor is a member of the superfamily of G protein-coupled receptors activating phospholipase C (34, 35). In the parathyroid gland, it is the key mediator of inhibition of PTH expression by high calcium (36). The calcium sensor has been shown, in the kidney, to be directly related to inhibition of tubular reabsorption of calcium and magnesium in the thick
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FIG. 8. Analysis of kidney mRNA of HCaRG and c-myc mRNA content after ischemia and various periods of reperfusion. A, dot blot of total RNA taken from the medulla of kidneys that underwent 60-min ischemia and reperfusion for various time periods (solid lines) or from contralateral control kidneys (dotted lines). HCaRG mRNA declined rapidly to its lowest levels at 3 and 6 h of reperfusion. It then increased steadily to exceed base line at 48 h of reperfusion. In contrast, c-myc mRNA levels rose dramatically by 12 h and returned below HCaRG mRNA levels at 48 h of reperfusion. B, representative Northern blots of HCaRG and c-myc mRNA from the cortex of kidneys that underwent 60-min ischemia and 3, 6, 12, 24, or 48 h (HCaRG) or 12 or 24 h (c-myc) of reperfusion (I/R) or from contralateral control kidneys (C).

HCaRG is not only expressed in the parathyroid gland but also in most organs tested, although at highly variable levels. Elevated HCaRG levels have been noted consistently in the tissues of genetically hypertensive animals, suggesting abnormalities of HCaRG regulation in several organs of SHR that could be due to either 1) decreased extracellular calcium levels, 2) an abnormal response to extracellular calcium, 3) abnormal transcription/stability of HCaRG mRNA in hypertensive rats, or 4) a combination of these. A state of negative calcium balance has been described in SHR that could support the first possibility. On the other hand, 2-fold higher HCaRG mRNA levels were observed in PTC from SHR than from WKY at normal calcium concentration (Fig. 2C). Thus, the modest reduction of calcemia in hypertension will not be the sole explanation of increased levels, suggesting increased expression or decreased degradation of this gene product in hypertension.

No homologous protein sequence to the HCaRG open reading frame was found in the SWISSPROTEIN data base. The HCaRG coding sequence contains one consensus motif known as the EF-hand or helix-loop-helix calcium motif (Fig. 4, dashed box). This motif generally consists of a 12-residue, calcium-binding loop flanked by two α-helices. Eight of the 10 most conserved amino acids are present in HCaRG. The nCARE core is present in an Alu repeat in 111 copies in the human genome, suggesting the possibility that other genes may possess functional nCARE (38). With the properties described in the present study, HCaRG may be one of them.

FIG. 9. Characterization of stable cell lines. A, HEK293 cells transfected with pcDNA1/Neo or pcDNA1/Neo rat HCaRG were examined for expression of rat HCaRG by Northern blot using rat HCaRG as a probe. Rat HCaRG was undetectable in cells transfected with the empty vector, while different levels of expression were observed in cells transfected with the vector expressing HCaRG. B, the levels of ectopic expression were determined by densitometric measurement and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
and others (43–45) have demonstrated that dimerization of single helix-loop-helix structures controls calcium affinity and that even homodimers can bind two calcium molecules with positive cooperativity (40). Hydrophobic interactions at the interface between calcium-binding sites appear to stabilize the calcium domains. Our in vitro translation studies showed the appearance of a protein band of about 43 kDa under nonreducing conditions. HCaRG protein might form reductant-sensitive, noncovalent homodimers compatible with its putative high α-helix content, but the existence of a functional calcium domain in HCaRG protein remains to be established. Several characteristics of HCaRG are similar to those of S100A2 protein, a calcium-binding protein of the EF-hand type that is preferentially expressed in the nucleus of normal cells but down-regulated in tumors (44). As with HCaRG, S100A2 expression is down-regulated by calcium (46, 47).

We also cloned the human homolog of HCaRG from a VSMC cDNA library, using a 437-bp fragment of rat HCaRG as a probe. The coding sequence was found to be 80% homologous to the rat sequence and to contain the putative EF-hand domain. A restriction fragment length polymorphism permitted us to localize the HCaRG locus on chromosome 7 of rats. The gene was assigned within a 4.4-centimorgan region on the long arm of chromosome 7 between Mit 3 and Mit 4 markers. By analogy, we suggested the assignment of HCaRG on human chromosome 8q21–24. In a recent search of GenBank™, homologies were found with three chromosome 8 clones containing zinc finger protein 7. It was therefore possible to localize HCaRG on chromosome 8q24.3, confirming our initial assignment. This region contains loci involved in several bone diseases, including osteopetrosis and multiple exostosis, and several human neoplasms (48, 49).

Many DNA-binding proteins utilize zinc-containing motifs to bind DNA. Other classes of DNA-binding proteins have a DNA recognition domain at their N terminus that dimerizes to form a two-chain coiled-coil of α-helices, also known as a leucine zipper. We identified four overlapping leucine zipper regions conserved in the rat and human sequence, and the high α-helix content of HCaRG makes it a possible DNA-binding protein. We are currently investigating this possibility. It has been shown that nuclear receptors require the ligand-dependent recruitment of co-activator proteins to effectively stimulate gene transcription (50). The nuclear receptor interaction domain of these factors is highly conserved and contains the consensus sequence LXXLL. This motif is sufficient for ligand-dependent interaction with nuclear receptors (51). We have identified one of these motifs in HCaRG. Nuclear localization of HCaRG protein makes this gene a potential transcription regulator.

Recently, a new transcription factor from the rat kidney (Kid-1) was identified (52–55). It was reported that Kid-1 mRNA levels declined after renal injury secondary to ischemia (55). Similarly, decreased HCaRG mRNA levels are seen when epithelial cells are dedifferentiated and proliferate (following ischemia and reperfusion). In the model of unilateral ischemic injury, it was shown that contralateral uninephrectomy attenuates apoptotic cell death and stimulates tubular cell regeneration (28–31). We demonstrate here that HCaRG mRNA levels decreased 3 and 6 h after ischemia in contrast to c-myc expression which is correlated with hyperplastic responses (31). We also observed that its levels are lower in all fetal organs tested when compared with adult organs and lower in tumors and the cancerous cell lines tested. It is possible that the gene product may exert a negative effect on growth. This was confirmed by the stable expression of HCaRG in HEK293 cells. We found that HCaRG overexpression had a profound inhibiting effect on HEK293 cell proliferation. This was shown not only by lower cell number but also by lower DNA synthesis, suggesting that the effect seen was not due to a death-promoting effect of HCaRG.

In conclusion, we have cloned the cDNA of a novel gene that is regulated negatively by extracellular calcium and presents greater expression in several organs of the genetically hypertensive rat model, which is known to demonstrate negative calcium balance. HCaRG mRNA levels are rapidly regulated by calcium, perhaps via the action of calcium receptor signaling. Comparison of HCaRG mRNA levels in fetal organs with those in adult organs and normal and tumor cells showed that HCaRG is more expressed in all adult normal tissues tested. We also report that HCaRG mRNA levels are modulated during ischemia-reperfusion injury, which mimics kidney ontogeny. Furthermore, its nuclear localization, identified motifs, and patterns of expression make this gene a potential regulator of cellular proliferation.

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