Cloning and characterization of the human soluble adenylyl cyclase

Weidong Geng, Zenglu Wang, Jianning Zhang, Berenice Y. Reed, Charles Y. C. Pak, and Orson W. Moe

Center for Mineral Metabolism and Clinical Research and Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas

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Geng, Weidong, Zenglu Wang, Jianning Zhang, Berenice Y. Reed, Charles Y. C. Pak, and Orson W. Moe. Cloning and characterization of the human soluble adenylyl cyclase. Am J Physiol Cell Physiol 288: C1305–C1316, 2005. First published January 19, 2005; doi:10.1152/ajpcell.00584.2004.—We identified the human ortholog of soluble adenylyl cyclase (hsAC) in a locus linked to familial absorptive hypercalciuria and cloned it from a human cDNA library. hsAC transcripts were expressed in multiple tissues using RT-PCR and RNA blotting. RNA blot analysis revealed a predominant 5.1-kb band in a multiple human tissue blot, but three splice transcript variants were detected using RT-PCR and confirmed by performing sequence analysis. Immunoblot analysis showed 190- and 80-kDa bands in multiple human cell lines from gut, renal, and bone origins in both cytosol and membrane fractions, including Caco-2 colorectal adenocarcinomas, HEK-293 cells, HOS cells, and primary human osteoblasts, as well as in vitro induced osteoclast-like cells. The specificity of the antiserum was verified by peptide blocking and reduction using sequence-specific small interfering RNA. Confocal immunofluorescence cytochemistry localized hsAC primarily in cytoplasm, but some labeling was observed in the nucleus and the plasma membrane. Cytoplasmic hsAC colocalized with microtubules but not with microfilaments. To test the function of hsAC, four constructs containing catalytic domains I and II (aa 1–802), catalytic domain II (aa 231–802), noncatalytic domain (aa 648–1,610), and full-length protein (aa 1–1,610) were expressed in Sf9 insect cells. Only catalytic domains I and II or full-length proteins showed adenylyl cyclase activity. Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\) all increased adenylyl cyclase activity in a dose-dependent manner. While hsAC had a minimal response to HCO\(_3\)- in the absence of divalent cations, HCO\(_3\)- robustly stimulated Mg\(^{2+}\)-bound hsAC but inhibited Mn\(^{2+}\)-bound hsAC in a dose-dependent manner. In summary, hsAC is a divalent cation and HCO\(_3\)- sensor, and its HCO\(_3\)- sensitivity is modulated by divalent cations.

bicarbonate sensor; calcium homeostasis; hypercalciuria

USING GENOME-WIDE LINKAGE analysis of three kindreds, our group mapped a locus and cloned a novel gene linked to absorptive hypercalciuria (AH), a syndrome of intestinal Ca\(^{2+}\) hyperabsorption, hypercalciuric Ca\(^{2+}\) nephrolithiasis, and low bone mineral density (26, 27). The predicted amino acid sequence of one gene in this locus is homologous to rat soluble adenylyl cyclase (sAC) (4). The human ortholog of sAC (hsAC) has 33 exons and spans 104 kb, and the full-length cDNA of the intestine has 5,085 nucleotides (GenBank accession no. AF331033). The polypeptide sequence predicts two adenylyl cyclase catalytic sites. hsAC is highly polymorphic, with at least 17 sites of allelic sequence variation. Although some of these sequence variations can be found in apparently healthy individuals, seven of them occur with higher frequency in patients with AH than in healthy individuals and can potentially be pathogenic mutations. The functional significance of these base changes is not yet defined. We first proceeded to characterize the wild-type hsAC protein.

cAMP is a second messenger that transduces signals to intracellular effectors. Classically, cAMP comes from G protein-coupled transmembrane adenylyl cyclase (tmAC) (22, 33–35). Buck and coworkers (4, 9, 23, 37) purified, cloned, and characterized sAC from rat testis. sAC is distinct from the traditional G protein-regulated tmACs. sAC is soluble and is located in the cytoplasm and the intracellular organelles (40), enabling cAMP to be generated directly inside the cell and compartmentalized in the vicinity of its targets (4, 39). sAC does not respond to the heterotrimeric G protein regulators and forskolin, but rather is stimulated by HCO\(_3\)- ion. It is considered a HCO\(_3\)- sensor in the rat and plays the essential role in mammalian sperm biology (9, 30, 37, 41). Homozygous sAC-deficient mice are infertile because of a severe sperm motility defect, but this phenotype can be rescued by provision of cell-permeant cAMP (12). Besides the testis and sperm cells, sAC is also found in the kidney, the choroid plexus (9), and multiple cell lines (39). The broad expression pattern of sAC suggests that sAC may subserve a multitude of functions throughout the body, including Ca\(^{2+}\) homeostasis. The human ortholog has not been characterized extensively to date.

MATERIALS AND METHODS

Cell culture. Human embryonic kidney (HEK)-293 cells derived from human kidney, Caco-2 cells derived from human colorectal adenocarcinoma, HOS cells derived from human osteogenic sarcoma, NRK cells derived from normal rat kidney, OKP cells derived from opossum kidney, LLCPK cells derived from pig kidney, and RAW264.7 cells derived from mouse monocytes were obtained from the American Type Culture Collection (Manassas, VA). Cells were seeded onto 100-mm culture dishes with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified 5% CO\(_2\) in air. Culture medium was replaced every 48 h. Human primary osteoblasts were purchased from Cambrex Bioproducts (Walkersville, MD) and cultured in the osteoblast growth medium (Cambrex Bioproducts). Osteoclast-like cells were induced from murine monocyte RAW264.7 cells with 50 ng/ml receptor activator of NF-κB ligand (RANKL) and 5 ng/ml monocyte colony-stimulating factor (M-CSF) (15, 28, 29, 31, 38). Osteoclasts were fixed and stained for tartrate-resistant acid phosphatase (Kamiya Biomedical, Seattle, WA) after 7–10 days as a marker for differentiation. To examine functional resorptive activity, induced osteoclasts were plated on culture plates coated with apatite [Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)]; 6H\(_2\)O; Kamiya Biomedical], and resorption pits were detected by staining the apatite with silver nitrate (Sigma, St. Louis, MO). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: O. W. Moe, Center for Mineral Metabolism and Clinical Research, Univ. of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-8885 (e-mail: Orson.Moe@UTSouthwestern.edu).

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negatively stained resorption pits were visualized under a digitized microscope.

Cloning of hsAC and hsAC mRNA analysis. hsAC cDNA was PCR amplified from a human testis marathon ready cDNA library (Clontech, Palo Alto, CA) using forward primer 5′-GAACATGAA-CACCTCCAAAAAGAAGAAT-3′ and reverse primer 5′-GAATGAT-TGTCCACGTTATTAGC-3′ derived from human. The PCR product was TA cloned (Invitrogen, Carlsbad, CA) and sequenced. The putative amino acid sequence of hsAC was analyzed for functional domains using the SMART program (http://smart.embl-heidelberg.de/). hsAC mRNA was analyzed using RT-PCR. Primers spanning exons 3–6 of hsAC were as follows: forward, 5′-CAGCAGTGTCATGTACATGGA-3′, and reverse, 5′-AGCTCCCCAGCAGTGTGA-3′. For HOS and Caco-2 cDNA, total RNA was isolated from HOS and Caco-2 cells using TRIzol reagent (Sigma), and RT-PCR was performed using the Gold RNA PCR kit (PE Biosystems, Foster City, CA) and Expand High Fidelity PCR system (Roche Diagnostics, Indianapolis, IN). PCR products were TA cloned and sequenced. Human testis, kidney, and small intestine cDNA libraries were purchased from Clontech.

RNA blots were performed using a single-stranded riboprobe hybridized against a multiple tissue human polyA mRNA blot (Clontech) according to the manufacturer’s recommendations. Riboprobes were used because uniformly 32P-dCTP-labeled (specific activity 107 cpm/μg), double-stranded hsAC cDNA probes failed to yield satisfactory hybridization signals. To generate the cRNA probe, hsAC (nt 181–2,161) was subcloned into pBluescript SK− (Stratagene, La Jolla, CA), sequenced, and used as a template. [32P]UTP-labeled (PerkinElmer, Wellesley, MA) antisense hsAC cRNA was synthesized using Strip-EZ RNA stripable probe synthesis kit (Ambion, Austin, TX). The filters were stripped and reprobed with β-actin for internal control of loading.

hsAC immunoblotting. A peptide (SLEGDALLA) corresponding to the extreme NH2 terminus of hsAC (variant VI) was selected based on its antigenicity and lack of homology with other protein sequences using a BLAST homology search. The location of the immunogenic peptide is shown in Fig. 1D. Rabbits were immunized with this peptide to generate anti-hsAC serum (Invitrogen). For immunoblots, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and we collected them by scraping them from the culture wells. Harvested cells were suspended in 1 ml of lysis buffer (10 mmol/l Tris, pH 7.4, 1 mmol/l EDTA, 1% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin, and 0.5 mmol/l phenylmethylsulfonyl fluoride) on ice and disrupted by two 10-s bursts from a Tekmar sonic disruptor (Fisher Scientific, Hampton, NH) at power setting 60. Protein concentrations were determined using a bicinechonic acid protein assay (Pierce, Rockford, IL). Equal amounts of sample proteins were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membrane and the membrane was incubated for 1 h in blocking solution (10% nonfat dry milk in PBS, pH 7.4). Subsequently, anti-hsAC serum were added for 3 h, after which the blot was washed several times in PBS containing Tween 20 and then incubated 1 h with a 1:5,000 dilution of goat anti-rabbit IgG horse-radish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ) in PBS containing 5% nonfat dry milk. Reactive bands were visualized using enhanced chemiluminescence (Amersham). For competitive immunoblotting experiments, 2 μl of anti-hsAC serum was incubated with 670 μg of either the immunogenic peptide or BSA overnight at 4°C before immunoblotting.

Crude membrane and cytosolic fractions were prepared from multiple cell lines. Cells were scraped and disrupted by performing sonication twice for 10 s each time in 50 mM Tris-HCl (pH 7.2) and 150 mM NaCl (pH 7.2) with complete protease inhibitor cocktail (Roche Diagnostics), followed by centrifugation at 1,000 g for 10 min to remove nuclei and debris. Cell lysates were centrifuged at 100,000 g for 1 h, and the crude membrane pellet was suspended by sonication it two times for 2 s each time in lysis buffer.

siRNA for knockdown hsAC. The 21-nucleotide small interfering RNA (siRNA) targeting hsAC was custom synthesized (Ambion, Austin, TX) to obtain the following sequences: sense, 5′-AUGUAGCGUGGAGAUCUUAAU-3′, and antisense, 5′-AUGGAUCUCAG-GCUACUUAAU-3′. Oligonucleotides were annealed according to the manufacturer’s instructions. Approximately 24 h before transfection, HOS cells were plated in six-well culture plates at an appropriate cell density so that they were ~70% confluent the next day. For the complex formation and transfection, 6 μl of the siPORT Amine transfection reagent (Ambion, Austin, TX) were mixed with 200 μl of serum-free medium and incubated at room temperature for 20 min, and then siRNA (100 nM) was added to the complex and incubated at room temperature for an additional 20 min. The mixture was applied to HOS cells, and cells were incubated with the mixture for 5 h and then switched to regular cell culture medium (DMEM with 10% FBS). Cell culture medium was replaced every 2 days, and HOS cells were harvested every 24 h up to 5 days for immunoblot analysis.

Confluent human osteoblasts from second passages, which were primary osteoblasts were grown to 20% confluence on glass-bottomed culture dishes. Cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, quenched with 100 mM glycine for 5 min at room temperature, permeabilized in 0.1–0.3% Triton X-100 for 5 min on ice, blocked with PBS-10% milk for 30 min at room temperature, incubated in anti-hsAC for 4°C overnight, and then incubated in FITC-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 30 min at room temperature. For colocalization with microfilaments, total actin was stained with rhodamine-phalloidin (Sigma) for 30 min. For colocalization with microtubules, cells were incubated in anti-β-tubulin for 4°C overnight and then in Alexa Fluor 633 F(ab′)2 fragment of goat anti-mouse IgG (Molecular Probes) for 30 min at room temperature. Confocal immunofluorescence microscopy was performed with a Zeiss LSM 410 confocal microscope using a krypton/argon laser. Images were analyzed using LSM 5 Image Browser software.

Expression of hsAC in Sf9 cells. Human sAC was expressed using the Bacto-to-Bac Baculovirus Expression System in Spodoptera fragi-perda (Sf9) cells (Invitrogen). Briefly, either full-length or the indicated putative functional domains were subcloned into pFastBac HT plasmid (Invitrogen). This involved PCR amplification of specific domains using Pfu DNA polymerase, followed by double digestion of plasmid and PCR fragments with EcoRI and SalI and ligation with T4 DNA ligase. All constructs were partially sequenced in both directions to confirm that the inserts were in frame with the His epitope. Sf9 cells grown at a density of ~2 × 106 cells/ml were infected with baculovirus. After infection, Sf9 cells were collected every 24 h for up to 5 days. Recombinant hsAC was confirmed on the basis of immunoblotting, confocal immunocytochemistry, and enzymatic activity. For recombinant hsAC expression in Sf9 cells, actin was stained with Oregon Green 488 phalloidin (Molecular Probes) and hsAC was detected using anti-hsAC serum.

Adenyl cyclase assay. Adenyl cyclase activity was measured as described by Alvarez and Daniels (1, 2). An assay was performed in 200 μl of total reaction volume using ~800 μg of Sf9 cell lysate in the presence of 50 mM Tris-HCl, pH 7.5, substrate [32P]ATP (0.1 μCi/assay), 2 mM cAMP, 0.1 mM GTP, 20 mM phosphoenolpyruvate, 3 U of pyruvate kinase, and 2 mM ATP. The appropriate concentrations of MgCl2, MnCl2, and/or HCO3− were added. The assay was started by the addition of cell lysate, and the mix was incubated for 15 min at 37°C and stopped by the addition of 2.2 M HCl containing [3H]cAMP, heating the solution to 95°C for 4 min, and then chilling it on ice. [32P]cAMP generated by the reaction and [3H]cAMP were recovered with the use of an acidic alumina column (ICN Biomedical, Irvine, CA). [3H]cAMP and [32P]cAMP content were determined by performing liquid scintillation spectrometry using dual isotope windows for [3H] and [32P]. [3H]cAMP was used to

AJP-Cell Physiol • VOL 288 • JUNE 2005 • www.ajpcell.org
Fig. 1. Human soluble adenylyl cyclase (hsAC) transcripts in human tissues and cells. A, top: RNA blot (1 μg of human polyA+ RNA per lane) probed with 32P-labeled, single-stranded RNA (hsAC nt 181–2,161) revealing a 5.1-kb band (hsAC); bottom: 32P-labeled DNA β-actin probe. B: PCR amplification of hsAC cDNA using a pair of primers that span exons 3–6. Three PCR products from human testis, kidney, and small intestine cDNA libraries are arbitrarily labeled variants 1, 2, and 3. C: PCR products variants 1, 2, and 3 (V1, V2, and V3) were individually cloned and sequenced from cDNA libraries of human testis, kidney, and small intestine and reverse-transcribed RNA from human osteosarcoma (HOS) and Caco-2 cells. With the use of similar primer pairs, PCR reactions revealed the three isolated clones from each tissue or cells. D: sequence of the three variant cDNA transcripts V1, V2, and V3. V1 had 37 extra 5′-nucleotides to exon 5 derived from the intron sequence. V2 was a full-length cDNA. V3 was missing the entire exon 5. Exons are indicated by brackets. Boldfaced ATG codons indicate putative potential ATGs. Asterisk indicates the single nucleotide that resulted in a new ATG in V1. The location of the immunogenic peptide used for antiserum production is underlined.
calculate the efficiency of cAMP recovery. To assess the dose-response relationship, hsAC activity was assayed as a function of varying Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ concentrations. For HCO$_3^-$ sensitivity, hsAC was assayed as a function of varying HCO$_3^-$ in the presence of either 5 mM MgCl$_2$ or 5 mM MnCl$_2$. Curve fitting was performed using SigmaPlot software (SPSS, Chicago, IL).

With regard to the HCO$_3^-$ dose-response relationship in the presence of Mn$^{2+}$, one needs to consider that the addition of HCO$_3^-$ might decrease free ionic Mn$^{2+}$ concentration in the assay solution because of the formation of MnHCO$_3^-$ complex and subsequent lowering of the stimulatory effect of Mn$^{2+}$. To compensate for this complication, we increased the total Mn$^{2+}$ concentration in the assay medium as we increased the HCO$_3^-$ concentration so that free ionic Mn$^{2+}$ stayed constant. We performed separate experiments and empirically measured the association constant of MnHCO$_3^-$ in vitro on the basis of the reduction of free HCO$_3^-$ by increasing Mn$^{2+}$ at three pH levels (pH 7.1–7.7) and obtained a value of $5 \times 10^{-8}$. We used this value to clamp the free Mn$^{2+}$ constant while varying HCO$_3^-$.

Ca$^{2+}$ measurement. Ionic Ca$^{2+}$ in the cyclase assay reaction was determined using Nova 8 (Nova Biomedical, Waltham, MA) for the range from 0.2 to 6 mM. For the lower range of 0–1,000 nM, ionic Ca$^{2+}$ was measured fluorometrically with Photomultiplier Detection System 814 (Photon Technology International, Lawrenceville, NJ) using 10 μM fura-FF (Molecular Probes) as the fluorescence indicator ($\lambda_{\text{exitation}}$, 340/380 nm; $\lambda_{\text{emission}}$, 510 nm) (14). After we determined ionic Ca$^{2+}$ in the cell lysate using fura-FF ratiometric fluorometry, we manipulated Ca$^{2+}$ in either direction by adding either EGTA or CaCl$_2$. The final ionic Ca$^{2+}$ concentration in each reaction was determined for each sample.

Statistics. Data are presented as means ± SE. Comparisons were performed using a paired Student’s t-test. $P < 0.05$ was considered statistically significant in all analyses.

RESULTS

hsAC transcripts in human tissues and cell lines. Using a RNA probe corresponding to the hsAC nucleotide 181–2,161 (aa 1–659) against a human multiple tissue blot containing 1 μg of polyA$^+$ RNA, we detected a predominant 5.1-kb transcript in brain, heart, skeletal muscle, thymus, kidney, liver, placenta, lung, and peripheral blood leukocyte (Fig. 1A). A faint doublet was discernible in the kidney, liver, and heart, which may suggest multiple transcripts. Although transcripts appear to be ubiquitous using RT-PCR, labeling on RNA blots for small intestine and colon were visible only after prolonged exposure. Different regions of the hsAC cDNA were examined using nested primers. With the use of a pair of primers spanning exons 3–6 of hsAC, PCR reactions revealed three bands in commercially purchased cDNA from normal human tissue, testis, kidney, and small intestine, and in cDNA we performed reverse transcription from HOS and Caco-2 cells (Fig. 1B). PCR products from each tissue or cell were subcloned and sequenced for each clone (Fig. 1C). Sequence analysis revealed that the top band had an extra 37 5’-nucleotides to exon 5. The bottom band had no exon 5 sequence at all (Fig. 1D). On the basis of the primary cDNA sequence, the presence of an extra 37 nucleotides before exon 5 and the omission of the entire exon 5 both result in new start codons in the hsAC sequence. We arbitrarily named these three variants V1, V2, and V3 (Fig. 1, B and C). None of these splice variants led to frame shift beyond exon 6, giving rise to identical predicted COOH termini. In V1, the extra 37 nucleotides had an extra C (indicated by asterisks in Fig. 1D) that resulted in a new ATG start codon. No splice variations were detected in the remaining exons of hsAC.

hsAC expression and cellular distribution. We next analyzed the expression of the hsAC protein. The specificity of the anti-hsAC serum was authenticated on the basis of competitive inhibition by the immunogenic peptide and siRNA knockdown (Fig. 2). No HEK-293 and Caco-2 cell signals were detected with competitive inhibition by preincubating the immune serum with the immunogenic peptide (Fig. 2A). The introduction of 100 nM hsAC siRNA caused ~73% decrease in hsAC protein expression in HOS cells by day 5 after transfection (Fig. 2B).

Immunoblot analysis of kidney-derived (HEK-293, OKP, NRK, and LLCPK1), gut-derived (Caco-2), and bone-derived (primary osteoblast, HOS) cells revealed two bands at ~190 kDa and ~80 kDa (Figs. 2 and 3), with the 80-kDa band being dominant. Both the 190- and 80-kDa bands were present in the

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**A**

![Image](http://ajpcell.physiology.org/)

Fig. 2. Specificity of anti-hsAC serum. A: immunoblotting of whole cell lysates from human embryonic kidney (HEK)-293 and Caco-2 cells using anti-hsAC serum (1:5,000 dilution). Two bands at ~190 and ~80 kDa were detected. The right side shows preincubation of anti-hsAC immune serum with the peptide epitope. The same experiment was repeated three times with the same results. B: HOS cells were transfected with 100 nM hsAC-specific small interference RNA (siRNA) for 5 h, and cells were harvested every 24 h up to 5 days. Whole cell lysate (40 μg) was immunoblotted using anti-hsAC and then stripped and reprobed with anti-β-actin for internal control of equal sample loading. Three independent experiments produced similar results.
pellet and the supernatant after 100,000 g centrifugation of HOS, Caco-2, and HEK-293 cells. The 190-kDa band signal was relatively weak in HOS and HEK-293 cells. Immunoblot analysis of kidney cell lines from pig, rat, and opossum revealed a diverse expression pattern (Fig. 3C). In LLCPK cells, a 135-kDa band was detected in the cytosol and an 80-kDa band was observed in the membrane fraction. In NRK cells, a 128-kDa band was detected in the membrane fraction and a 70-kDa band was detected in both the cytosol and the membrane fraction. In OKP cells, 190- and 85-kDa bands were detected only in the cytosol fraction.

To further define the intracellular distribution of hsAC, we performed immunofluorescence microscopy of primary cultured human osteoblasts (Fig. 4A) and in vitro induced osteoclast-like cells (Fig. 4B). In human osteoblasts, hsAC was located primarily within the cells. About 30% of the cells demonstrated strong fluorescence at the plasma membrane. About 50% of the cells showed fluorescence inside the nucleus. No signal was detected in preimmune serum or in the IgG control. All staining was reduced >80% by the immunogenic peptide. Inside the cell, hsAC exhibited a fibrillar distribution. Colocalization with actin or tubulin showed that these fibrils were associated not with the microfilament but rather with the microtubules (Fig. 4B). Osteoclast-like cells were induced from RAW264.7 cells in the presence of RANKL and M-CSF. Multinuclear cells typically form from days 5 to 7 after induction and are functionally absorptive as evidenced by resorptive pit formation on the apatite-coated culture plates. hsAC was localized in the cytosol in osteoclasts and highly concentrated in the perinuclear region (Fig. 4C).

Four expression plasmids were constructed to produce recombinant NH2-terminal hexahistidine-tagged hsAC in Sf9 cells. Each construct contained either the full-length hsAC or various predicted functional domains of cyclase (Fig. 5). hsAC1–802 spans catalytic domains I and II. hsAC231–802 includes only catalytic domain II. hsAC648–1610 includes the entire COOH terminus lacking both catalytic domains. hsAC1–1610 is the full-length hsAC polypeptide. After plasmid transfection, Sf9 cells expressed recombinant hsAC on day 2, peaked on day 3 or 4, and then declined on day 5 (data...
not shown). A similar expression time course also was observed in immunoblot analysis using anti-hexahistidine antibodies (data not shown). The various constructs had different distribution patterns in Sf9 cells. The full-length and noncatalytic domains were located close to the plasma membrane and exhibited more of a “ring” pattern. In addition to the ring pattern, the second catalytic or both catalytic domains were located inside the cytosol. This suggests that in Sf9 cells, hsAC
adenylyl cyclase activity (Fig. 6). The addition of Mg$^{2+}$ and Mn$^{2+}$ significantly increased cyclase activity 2.7- or 78.5-fold, respectively (Fig. 6). In contrast, Mg$^{2+}$ and Mn$^{2+}$ significantly decreased the Mn$^{2+}$-bound cyclase activity 2.4- or 2.1-fold for the catalytic or full-length hsAC, respectively. In all of the assay conditions, no statistically significant difference was found for the full-length and catalytic domains of hsAC. It is noteworthy that there was some endogenous adenylyl cyclase activity in Sf9 cells that could be stimulated by both Mg$^{2+}$ and Mn$^{2+}$ (Fig. 6).

Next, we investigated the dose-response relationship of the Mg$^{2+}$, Mn$^{2+}$, and HCO$_3^-$ (Fig. 7). Only the catalytic domain (hsAC$^{1-802}$) and the full-length (hsAC$^{1-1610}$) constructs were all four constructs in the absence of the divalent cation, HCO$_3^-$ had no effect on adenylyl cyclase activity. However, in the presence of Mg$^{2+}$ and Mn$^{2+}$, the enzyme was sensitive to HCO$_3^-$. HCO$_3^-$ significantly increased the Mg$^{2+}$-bound cyclase activity 7.2-fold for the catalytic domains (hsAC$^{1-802}$) and 6.6-fold for full-length hsAC (hsAC$^{1-1610}$) (Fig. 6). In contrast, HCO$_3^-$ significantly decreased the Mn$^{2+}$-bound cyclase activity 2.4- or 2.1-fold for the catalytic or full-length hsAC, respectively. In all of the assay conditions, no statistically significant difference was found for the full-length and catalytic domains of hsAC. It is noteworthy that there was some endogenous adenylyl cyclase activity in Sf9 cells that could be stimulated by both Mg$^{2+}$ and Mn$^{2+}$ (Fig. 6).

All Sf9 cells were transfected with pFasHT plasmids containing catalytic domains I and II, catalytic domain II, noncatalytic domain, full-length hsAC, or empty plasmid (Control). Cells were harvested every 24 h up to 5 days, fixed, permeabilized, and double-stained with Oregon Green 488 phalloidin (green), followed by anti-hsAC (red). xy, x-y section; z, z section. Images show representative cells collected on day 3 posttransfection. Three independent experiments showed similar results.

hsAC: regulation by divalent cations and HCO$_3^-$. We next examined adenylyl cyclase activity of recombinant hsAC in Sf9 cells. Whole Sf9 cell lysate from day 3 postinfection was used because of the higher hsAC antigenic expression. Compared with Sf9 cells alone, both full-length hsAC$^{1-1610}$ and the double-catalytic domain hsAC$^{1-802}$ constructs demonstrated adenylyl cyclase activity (Fig. 6). The addition of Mg$^{2+}$ or Mn$^{2+}$ significantly increased cyclase activity 2.7- or 78.5-fold, respectively (Fig. 6). No significant cyclase activity was detected in the second catalytic domain (hsAC$^{231-802}$) or in the noncatalytic domain of hsAC (hsAC$^{648-1610}$). This indicates that both catalytic domains are needed for cyclase activity. In all Sf9 constructs in the absence of the divalent cation, HCO$_3^-$ had no effect on adenylyl cyclase activity. However, in the presence of Mg$^{2+}$ and Mn$^{2+}$, the enzyme was sensitive to HCO$_3^-$. HCO$_3^-$ significantly increased the Mg$^{2+}$-bound cyclase activity 7.2-fold for the catalytic domains (hsAC$^{1-802}$) and 6.6-fold for full-length hsAC (hsAC$^{1-1610}$) (Fig. 6). In contrast, HCO$_3^-$ significantly decreased the Mn$^{2+}$-bound cyclase activity 2.4- or 2.1-fold for the catalytic or full-length hsAC, respectively. In all of the assay conditions, no statistically significant difference was found for the full-length and catalytic domains of hsAC. It is noteworthy that there was some endogenous adenylyl cyclase activity in Sf9 cells that could be stimulated by both Mg$^{2+}$ and Mn$^{2+}$ (Fig. 6).

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used in these experiments. No statistically significant differences in the dose responses were observed between the full-length (hsAC1–1610) and catalytic domains (hsAC1–802) (data not shown) of hsAC. Both Mg$^{2+}$ and Mn$^{2+}$ increased cyclase activity in a dose-dependent manner (Fig. 7). Mg$^{2+}$ stimulation fitted well with first-order kinetics, with $K_m$ of 2.41 mM (Fig. 7A). Mn$^{2+}$ also rapidly increased cyclase activity from 1 to 5 mM, peaking at 20 mM but with a 20-fold higher $V_{max}$. The Mn$^{2+}$ dose-response curve displayed a sigmoid shape (Fig. 7B), with half-maximal activation at 3.9 mM and a Hill coefficient of 2.37.

As expected on the basis of the data shown in Fig. 6, the HCO$_3^-$ dose response was opposite, depending on the ambient divalent cation for both the catalytic and full-length hsAC. HCO$_3^-$ robustly increased the Mg$^{2+}$-bound adenyl cyclase (Fig. 7C) but inhibited the Mn$^{2+}$-bound adenyl cyclase (Fig. 7D). The half-maximal effect for both the stimulation and inhibition are shown at comparable HCO$_3^-$ concentrations (8.7 mM for Mg$^{2+}$ vs. 3.9 mM for Mn$^{2+}$). Because HCO$_3^-$ addition may complex Mn$^{2+}$ and decrease ionic Mn$^{2+}$ stimulation, we conducted the experiment shown in Fig. 7D with increasing total Mn$^{2+}$ as we increased the HCO$_3^-$ clamping free Mn$^{2+}$ constant.

In addition to Mg$^{2+}$ and Mn$^{2+}$, we studied the effect of Ca$^{2+}$ on cyclase activity. For catalytic domain and full-length hsAC in the absence of Mg$^{2+}$ and HCO$_3^-$, Ca$^{2+}$ weakly stimulates cyclase activity (data not shown). To better visualize the dose response of Ca$^{2+}$, we stimulated the basal level of cyclase activity by adding a small amount of Mg$^{2+}$ and HCO$_3^-$ in the Ca$^{2+}$ study as previously described by other investigators (17, 23). With the presence of 2 mM Mg$^{2+}$ and 20 mM HCO$_3^-$, Ca$^{2+}$-stimulated cyclase activity in a dose-dependent manner from 0.12 to 1.31 mM (Fig. 8A). At more physiological cytosolic concentrations from 2 to 1,200 nM ionized Ca$^{2+}$, cyclase activity also increased in a dose-dependent manner (Fig. 8B). No significant differences between the catalytic and full length hsAC were observed at either higher or physiological concentrations of ionic Ca$^{2+}$ (Fig. 8, A and B).

**DISCUSSION**

HCO$_3^-$-regulated processes are pervasive in biology, and sAC may be the link between ambient HCO$_3^-$ concentration and known cell signaling molecules. Because of the importance of HCO$_3^-$ concentration in osteoblast and osteoclast function (3, 6, 7), and because sequence variation in hsAC is associated with lower bone density and increased intestinal Ca$^{2+}$ absorption (24, 26), we were interested in the role of hsAC in human Ca$^{2+}$ homeostasis.

In this study, we have demonstrated that hsAC is a functional adenyl cyclase that responds to divalent ions and HCO$_3^-$ ion regulation. Transcripts are ubiquitous as shown by RT-PCR and RNA blots of organs of Ca$^{2+}$ homeostasis. hsAC is of low abundance in human tissue and is detectable only with a single-stranded riboprobe on a 1-μg polyA$^+$ RNA gel. The signal in the colon and small bowel is visible only after extremely long exposure. In contrast to its rodent ortholog, hsAC appears to be more complex in that it has multiple transcriptors (17, 23). With the presence of 2 mM Mg$^{2+}$ and 20 mM HCO$_3^-$, Ca$^{2+}$-stimulated cyclase activity in a dose-dependent manner from 0.12 to 1.31 mM (Fig. 8A). At more physiological cytosolic concentrations from 2 to 1,200 nM ionized Ca$^{2+}$, cyclase activity also increased in a dose-dependent manner (Fig. 8B). No significant differences between the catalytic and full length hsAC were observed at either higher or physiological concentrations of ionic Ca$^{2+}$ (Fig. 8, A and B).

Discussed here are the multiple splice sites and their potential regulation by HCO$_3^-$ and cAMP, as well as the functional impact of these variations on the cyclase activity. The results of these studies suggest that the regulation of hsAC by HCO$_3^-$ and cAMP may be important in the context of Ca$^{2+}$ homeostasis.
A specific anti-hsAC antiserum labeled hsAC antigen in HEK-293, Caco-2, HOS, and primary cultured human osteoblast cells. Two bands of /H11011190- and /H1101180-kDa mass are detected in human kidney, small intestine, and bone cell lines, with the 80-kDa band being the predominant one. If the transcript variants do give rise to different polypeptides, because of the locale of the epitope, one would expect this antiserum to label definitively the V1 variant and perhaps the V2 variant (Fig. 1D). The relationship between the three transcripts and protein bands is not clear. The /H11011190-kDa band is compatible with the full-length translation open-reading frame. The /H1101180-kDa band may be a posttranslationally processed protein containing the NH2-terminal epitope. Multiple putative proteolytic cleavage sites are predicted from the primary amino acid sequence of hsAC, the rodent sAC is thought to be processed by cleavage, and the cleaved COOH terminus has been postulated to be an autoinhibitory domain (18).

hsAC is detected in both cytosolic and membrane fractions of the cell lysate after 100,000 g centrifugation. The relative distribution differs slightly from one cell line to another, but the presence in both pellet and supernatant is consistent, except in OKP cells, in which very little or no hsAC antigen is present in the membrane pellet. Although the primary sequence predicts possible putative transmembrane regions, native hsAC is unlikely to be a true transmembrane protein, because it can be released easily into the supernatant by weak nonionic detergents. In immunocytochemistry, hsAC has different distribution in different cells. We examined native hsAC in two human bone cells: osteoblasts and osteoclasts. Native hsAC in primary cultured human osteoblasts shows intracellular, plasma membrane, and nuclear localization patterns. The intracellular pattern is definitely fibrillar, and by colocalization, the enzyme is associated with microfibrils rather than microfilaments. hsAC is present in osteoclasts as well as in its uninduced progenitor RAW cells. The pattern is distinct from osteoblasts in that it has a more diffuse nonfibrillar intracellular staining with accentuation in the perinuclear region. We next examined the expression pattern of recombinant hsAC consisting of different regions of the protein in Sf9 cells. Intracellular staining was evident in all constructs. The full-length (hsAC1–1610) and noncatalytic domain (hsAC648–1610) proteins demonstrated a ring pattern denoting plasma membrane association. hsAC harboring catalytic domains I and II (hsAC1–802) or catalytic domain II alone (hsAC231–802) showed primarily intracellular localization.
dent experiments were performed.

... represents the average of a triplicate result in one experiment. Two independent expression experiments were performed.

B

Cyclase Activity (pmol/mg/mn) [Ca^2+] in mM (with 2 mM Mg^2+ & 20 mM HCO_3^-)

\( [\text{Ca}^{2+}] \text{ in mM (with 2 mM Mg}^{2+} \text{ & 20 mM HCO}_3^{-}] \)

\( \text{Cyclase Activity (pmol/mg/mn)} \)

Fig. 8. Dose-response curves showing the effect of Ca^{2+} on cyclase activity of recombinant hsAC in Sf9 cell lysate. Whole lysates of Sf9 cells expressing the recombinant full-length (■) or catalytic (○) hsAC were assayed for adenyl cyclase activity in millimolar concentrations (A) and the physiological intracellular concentrations (B) of Ca^{2+} with 2 mM Mg^{2+} and 20 mM HCO_3^-.

Staining in Sf9 cells. These results suggest that there may be signals between aa 802 and aa 1,610 that confer membrane association.

A series of seminal papers demonstrated that the rat sAC is a soluble adenyl cyclase that responds to HCO_3^- stimulation and thus is considered the HCO_3^- sensor (9, 17, 30, 37, 41). hsAC is also an adenyl cyclase, but it is distinct from the rat ortholog. When expressed in Sf9 cells, full-length hsAC as well as hsAC containing the catalytic domains yielded adenyl cyclase activity. We specifically addressed the second catalytic domain because the putative protein translated from the V3 transcript should lack the first catalytic domain. No cyclase activity was evident in the second catalytic domain or in the noncatalytic domain of hsAC. This suggests that both catalytic domains are necessary for a functional adenyl cyclase. If the V3 transcript indeed translates to polypeptide in native tissue, it may perform functions other than those of adenyl cyclase. No difference is found between the double-catalytic domain (hsAC1–802) and full-length hsAC (hsAC1–1610) when expressed in insect cells. This finding appears to be distinct from the rat sAC, in which the catalytic domain has 10 times the cyclase activity of the full-length domain. It is most likely that the catalytic domain of hsAC is proteolytically cleaved in Sf9 cells to a form that resembles hsAC1–802. One cannot detect a 180-kDa band in immunoblots of Sf9 lysates from cells infected with the catalytic domain (hsAC1–802) or the full-length hsAC (hsAC1–1610). This complete processing of the full-length protein may explain the lack of difference in cyclase activity between hsAC1–802 and hsAC1–1610.

We have shown that three divalent cations activate hsAC, with Mg^{2+} being the most potent, followed by Mg^{2+} and Ca^{2+}. This finding was previously described for rat sAC (16, 17, 23). The finding that only the catalytic region of hsAC is sufficient for these interactions has been described in the rat protein (36). The range of concentrations tested for Mg^{2+} and Ca^{2+} are both within the expected normal intracellular range, so hsACs could very well be Mg^{2+} and Ca^{2+} sensors. It is interesting that Mg^{2+} and Mn^{2+} have different kinetics, suggesting that they may bind to different sites on the protein. Although Mn^{2+} has been shown to activate the rat sAC, no kinetic studies have been performed (23). Although the overall homology of the protein is low, catalytic domains I and II of hsAC resemble the catalytic site of the tmAC. The mechanism of generating cAMP by tmAC has been studied extensively (10, 11, 19, 36). The enzymatic core (catalytic domains I and II) uses a two-metal-ion catalytic mechanism in which, upon metal binding, the enzyme undergoes a conformational transformation. It is proposed that Mg^{2+} and Mn^{2+} bind to two distinct sites on tmAC (36); both of which lead to activation of the enzyme.

In addition to HCO_3^- sensitivity in the recombinant state, the role of sAC as a HCO_3^- sensor previously was elegantly shown in intact mammalian cells (25). Our present study has shown that the cyclase activity of hsAC is also HCO_3^- sensitive. However, HCO_3^- has divergent effects on hsAC, depending on the identity of the divalent cation. In the presence of 5 mM Mg^{2+} (−KM of Mg^{2+}), HCO_3^- stimulated adenyl cyclase activity, >20-fold, with a KM of 7.8 mM. This clearly enables hsAC to function as a HCO_3^- sensor in the intact cell. Surprisingly, in the presence of 5 mM Mn^{2+}, HCO_3^- inhibited cyclase activity with a virtually identical KM (3.9 mM). This effect is not due to complexing of Mn^{2+} by HCO_3^−, because we clamped the free Mn^{2+} concentration constant. The physiological significance of this finding is unclear, because millimolar quantities of Mn^{2+} are not usually encountered in the cytoplasm. However, this finding may uncover interesting mechanisms of HCO_3^- interaction with the protein. In the tmAC, Zn^{2+} and Mg^{2+} appear to bind to identical sites (36), but while Mg^{2+} stimulates the enzyme, Zn^{2+} inhibits it. The interaction between HCO_3^- and hsAC may be quite different, depending on whether hsAC binds to Mg^{2+} or Mn^{2+}. Cann et al. (8) demonstrated that a point mutation in the active site (Lys646) of a class III adenyl cyclase reduced catalytic activity by 95% and also reduced HCO_3^- activation, and they proposed that the HCO_3^- binding site might be situated in the vicinity of that residue.
Many biological processes are modulated by ambient HCO₃⁻ concentrations. In bone, high HCO₃⁻ and alkaline pH inhibit bone resorption, and low HCO₃⁻ and acidic pH stimulate it (5, 6). Low HCO₃⁻ stimulates and high HCO₃⁻ inhibits osteoclastic activity (3, 13, 20, 21). Metabolic alkalosis stimulates osteoblasts (6). It is thought that systemic pH and HCO₃⁻ alter bone mineral content by direct physiochemical means or biologically via osteoblasts and osteoclasts, but the signaling pathway between HCO₃⁻ and bone cells remains unclear. In the kidney, there are multiple HCO₃⁻-sensitive processes. One important one is the stimulation of Ca²⁺ absorption in the distal nephron by luminal HCO₃⁻ (32). hsAC is expressed in this part of the nephron and can potentially be the mediator of this response. Although the precise function of hsAC in bone and kidney remains to be determined, the clinical association of hsAC to kidney stones and low bone density (26, 27) and the presence of hsAC in both kidney and bone cells suggest that hsAC may provide a link for HCO₃⁻ sensing.

In conclusion, hsAC is a human ortholog of the rat sAC that is expressed in multiple tissues and cells involved in Ca²⁺ homeostasis. hsAC has a multiply spliced transcript with an unknown biological significance at present but has potential roles in mediating HCO₃⁻-regulated cell function in bone and kidney cells. The hsAC protein exists freely as a cytosol as well as in membrane-associated forms. The cyclase catalytic activity as well as divalent ion and HCO₃⁻ sensitivity are fully preserved in the catalytic NH₂-terminal half of the protein.

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