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The β_2 Subunit of Soluble Guanylyl Cyclase Contains a Human-Specific Frameshift and Is Expressed in Gastric Carcinoma

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Soluble or nitric oxide (NO) stimulated guanylyl cyclases are obligate heme-containing heterodimers (α/β). We report the full-length cDNA of the human ortholog of the rat β_2-subunit from human kidney. A database search yielded matches of the 3′ non-coding sequence with previously unassigned expressed sequence tags from kidney and stomach signet ring cell carcinoma. PCR comparison of cDNA from stomach signet ring cell carcinoma and normal stomach tissue demonstrated β_2 subunit expression in cancer but not in normal tissue. On the cDNA level a frameshift deletion of one nucleotide was present in the novel human sequence which was confirmed on the genomic DNA level. In four closely related nonhuman primate species the frameshift deletion was absent while analysis of genomic DNA from different ethnic backgrounds revealed the uniform presence of the frameshift deletion in the human population.

Key Words: **soluble guanylyl cyclase; nitric oxide; cyclic GMP; primates; evolution.**

Soluble guanylyl cyclases (sGCs) play a critical role in the cardiovascular system (1). In vasodilation, sGC activated by nitric oxide (NO) derived from the endothelium induces relaxation of vascular smooth muscle (1). NO-activation of sGC in the kidney leads to increased sodium excretion (2). Both vasodilation and increased renal sodium excretion leads to a lowering of systemic blood pressure. sGCs are heme-containing heterodimers composed of α and β subunits that are activated by NO and CO (1, 3, 4). Both subunits possess a catalytic domain in the C-terminal position, the primary structure of which is conserved in the membrane form of guanylyl cyclase and adenylyl cyclase (5). Four subunits have been cloned to date (β_1, α_1, β_2 and α_2). The prototypical α_1/β_1 isoform has a wide tissue distribution and has been purified from native tissue and characterized extensively by several groups. This enzyme contains a prosthetic heme group that mediates activation by NO, leading to a more than 200-fold increase in formation of cGMP from GTP. The corresponding human enzyme has recently been thoroughly characterized after overexpression in Sf9 cells (6). The natural occurrence of a second NO-sensitive guanylyl cyclase isoform has been demonstrated in human placenta (7). This α_2/β_1 heterodimeric enzyme also contains a prosthetic heme group and displays similar pharmacological properties as the α_1/β_1 isoform (7). The β_2 subunit is predominantly expressed in rat kidney (8) in principal cells of the collecting duct where NO has been shown to inhibit sodium reabsorption (9). So far, an enzyme isoforms containing the β_2 subunit has not been purified from native tissues. However, Gupta *et al.* have presented evidence for the formation of a functional NO-sensitive α_1/β_2 heterodimer *in vitro* (10). Gupta *et al.* also presented evidence for the downregulation of the β_2 subunit in the kidney of rats with salt sensitive hypertension (10) and cosegregation of the respective gene with blood pressure in these rat crosses (11).

We have recently cloned a partial cDNA of the human ortholog of the β_2-subunit from human heart and have mapped the corresponding gene by fluorescence *in situ* hybridization and linkage analysis to chromosomal band 13q14.3 between markers D13S168 and D13S155 (12). This gene localization was confirmed by fluorescence *in situ* hybridization using a different probe (13). Here, we report the full-length sequence of the human β_2 cDNA and identify tumor-specific expression of the subunit in gastric carcinoma and a human-specific frame shift present in the general human population.

MATERIALS AND METHODS

Isolation of full-length β_2 cDNA. For 5′ and 3′ RACE we used Marathon-Ready kidney cDNA (Clontech) with the Advantage cDNA PCR Kit (Clontech) according to the manufacturer's protocol. Two

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nested gene-specific primers were used for 5′ RACE (P41, 5′-TCTGCGGATGCTGAAAATGTTGA-3′; P44, 5′-AACACAACCATGCTCCTTCTTCCCT-3′ and 3′ RACE (P40, 5′-AGGGAAGAAGGAGCATGTTGTGTT-3′; P45 5′-TCAACATTTTCAGCATCCGCAGA-3′). Reactions were run in a thermal cycler with the following parameters in a 50 μl reaction volume: 94°C 1 min, 5 cycles 94°C 1 min and 72°C 3 min, 5 cycles 94°C 1 min and 70°C 3 min and 25 cycles 94°C 1 min and 68°C 3 min. 5′ and 3′ RACE products were cloned into the vector pCRII using the TA-cloning kit (Invitrogen) and sequenced by Abi Prism Dye Terminator Cycle Sequencing (Perkin Elmer). Two gene specific primers from the 5′ and 3′ untranslated region of the amplified β_2-subunit cDNA ends were designed (P78, 5′-GCGGCCGCTTGGTGCTGCATCTCAATC-3′ and P75, 5′-GGGAAAATTAAGCCCAGGGGTTCAT-3′). Using these primers the following protocol was run with the following thermal profile in a 25 μl reaction volumes (94°C 1 min, 35 cycles 94°C 20 s, 60°C 15 s and 68°C 180 s). A 2755 bp PCR product was subcloned into pCR2.1 using the TA-cloning kit (Invitrogen) and sequenced. This full-length clone was designated clone H.

Isolation of genomic DNA fragments. For isolation of the respective exon from the human genomic PAC clone (LLNLP704E17877Q3), PAC DNA was prepared using the Maxi DNA preparation kit (Qiagen). LB-broth containing kanamycin (50 mg/l) was inoculated and grown to an optical density of 0.15 at 578 nm. IPTG was added in a final concentration of 1 mM and bacteria were grown to an optical density of 0.9 at 578 nm and were harvested by centrifugation. Subsequently the standard manufacturer's protocol was used with the modification that buffer QF was preheated to 65°C before elution over tip 500 columns. Approximately 100 μg were obtained from a 500 ml culture. This PAC-DNA was digested with various restriction enzymes, run on an agarose gel, photographed and blotted on a nirocellulose membrane. Primer P90 (5′-GAAGCCATCCTGAAACTCTTTGGAGAATAC-3′) and P91 (5′-CTCCCAGTGTCCGTAGCATCCTGTCATAGC-3′) were endlabelled using 50 μCi of [γ-^{32}P]ATP and T4 Polynukleotidkinase (Promega) according to the manufacturer's protocol. Nitrocellulose membranes with PAC DNA cut with various restriction enzymes were hybridized using ExpressHyb (Clontech) according to the manufacturer's protocol and exposed to phosphoimager scans. A 269 bp band from combined restriction with NcoI and PstI hybridized with both labelled primers, with longer fragments hybridizing after NcoI or PstI restriction alone. The 269 bp fragment was cloned NcoI/PstI into the vector pGEM5ZF+ and sequenced. By an analogous strategy a 1000 bp XmnI/PstI fragment from the PAC clone that hybridized with primer P90 was isolated to identify the intron/exon boundary five prime to the frame shift deletion. This fragment was cloned into the EcoRV/PstI cut pBluescript vector and sequenced.

Preparation of genomic DNA. Genomic DNA was prepared from 300 mg tissue (human heart or macaca mulatta kidney). The tissue was minced in liquid nitrogen and was additionally homogenized with a Polytron in 1.5 ml lysis-buffer (10 mM EDTA, 10 mM Tris–HCl, 150 mM NaCl, 0.2% SDS, pH 7.4). Proteinase K was added to a final concentration of 100 μg/ml and this homogenate was incubated for 48 h at 37°C. From this homogenate genomic DNA was isolated by standard phenol/chloroform extraction and sodium acetate precipitation. The yield was approximately 500 μg/300 mg of tissue. Genomic DNA was isolated from 2 ml of citrate-blood by adding 4 ml red-blood-cell buffer (10 mM $KHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA, pH 7.4). After lysis for two times 15 min on ice and centrifugation at 400*g* at 10°C for 10 min the lymphocyte pellet was washed two times with phosphate buffered saline. Lymphocytes were then resuspended in 1 ml lysis-buffer and proteinase K digestion and phenol chloroform extraction of genomic DNA was performed as described above.

PCR-analysis. PCR-analysis of human and nonhuman genomic DNA was performed using primers P92 (5′-AGCCATCCTGAAACTCTTTGGAGA-3′) and P93 (5′-GGCCCAGGACTGCCACAGC-3′) in a 25 μl reaction volumes using Taq Polymerase High Fidelity (Gibco) according to the manufacturers recommendations using the following thermal profile (95°C 1 min, 36 cycles 95°C 20 s, 60°C 20 s and 72°C 20 s and a final extension step at 72°C for 4 min). 10 μl were digested with Eco57I or XmnI in a 20 μl volume and incubated for 1 h at 37°C and were run on a 1% agarose gel in comparison with 10 μl of undigested PCR-product. PCR products were 213 bp for nonhuman primates and 209 bp for humans. Digested fragments of the human PCR products were 58 bp and 151 bp (Eco57I) and 27 bp and 182 bp (XmnI). PCR analysis of the Human Stomach 3 Matched cDNA Pair (Clontech) was done according to the manufacturer's suggestions. For analysis of the human β_2 subunit primers P78 (5′-GCGGCCGCTTGGTGCTGCATCTCAATC-3′) and P79 (5′-GTACATGGAATTCAGCACGTTCACTATTTG-3′) were used under the following cycling conditions: 95°C 1 min, 38 cycles 95°C 20 s, 58°C 20 s and 68°C 90 s and a final extension step at 68°C for 4 min (1546 bp PCR-product). For analysis of the human β_1 subunit primers P8 (5′-TAAGAGCCCTGGAAGATGAAAAGA-3′) and P7 (5′-TGGGGTAATGGACAAGGACAAA-3′) the same cycling conditions were used except an elevated annealing temperature of 60°C (846 bp PCR-product).

Southern blot analysis. For southern blots using human and primate genomic DNA the 1000 bp XmnI/PstI genomic fragment described above (40 ng) was labelled with ^{32}P (50 μCi [α-^{32}P]dCTP) using the Megaprime labelling system (Amersham) according to the manufacturers protocol. Genomic human or primate DNA (40 μg) was digested with 120 units XmnI (New England Biolabs) in a volume of 300 μl for 48 h. An 0.8% agarose gel was run overnight at 30 V and photographed. The agarose gel was incubated for 15 min in an 0.25 M HCl solution, then transferred to a denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 40 min and finally washed two times for 20 min in neutralization solution (1.5 M NaCl, 1 mM EDTA, 0.5 M Tris/HCl (pH 7.2)). The gel was then blotted on a nylon membrane (Immobilon Ny$^+$, Millipore) and crosslinked using the UV-Stratalinker 2400 (Stratagene). The blots were prehybridized in Express Hyb solution (Clontech) for 30 min at 60°C. After boiling of the probe for 5 min at 95°C it was added to the Express Hyb solution and the blots were hybridized at 60°C in a rotating hybridization oven. The blots were then washed four times with 2 × SSC with 0.1% SDS (see (14)) at room temperature for 15 min and two times for 30 min at 50°C in 0.2 × SSC with 0.1% SDS. The blots were then exposed on a phosphoimager plate for 48 h and analyzed by phosphoimager scan.

RESULTS

Using 5′ and 3′ RACE PCR a 2755 bp PCR product representing the full length sequence of the human β_2 subunit cDNA was isolated from human kidney (submitted to GenBank under Accession No. AF218382). A blast search using this sequence identified matches in the 3′ untranslated region of the human β_2 subunit with three previously unassigned ESTs from human kidney (GenBank AI822009, AI792818, and AA917369). Another previously unassigned human EST from a poorly differentiated gastric adenocarcinoma with signet ring cell features was identified as a fragment of the 3′ untranslated region of the human β_2 subunit (GenBank AI247180). To determine whether this reflects the general expression of this subunit in the stomach or whether the expression is tumor specific, we compared expression of the novel human subunit in normal human stomach versus gastric carcinoma cDNA. As shown in Fig. 1, an amplification

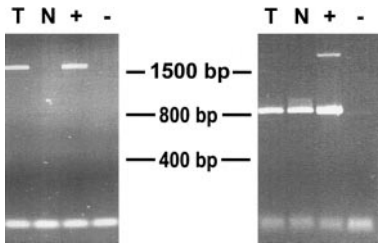

FIG. 1. Analysis of cDNA from a stomach signet ring cell carcinoma and normal tissue from a 71 year old male Caucasian. T, tumor; N, normal tissue; +, positive control (human kidney cDNA); −, negative control. Left: PCR using primers specific for the human β_2 subunit. The 1546 bp product was detectable in tumor tissue from stomach but not in the respective normal stomach tissue. The lower bands represent primer dimer artifacts. Right: PCR using primers specific for the human β_1 subunit. The 846 bp product was detectable in tumor and normal stomach tissue.

product of 1546 bp representing the human β_2 subunit was expressed in gastric signet ring cell carcinoma cDNA, but not in the normal stomach cDNA from the same patient. As control we run the same reactions with primers specific for the human β_1 subunit, which was present both in tumor and in normal stomach cDNA (see Fig. 1).

Analysis of the new human β_2 subunit nucleotide sequence revealed an open reading frame of 1854 bp (from position 281 to 2134). The deduced amino acid sequence displayed a similarity of 58% with the rat β_2 subunit and 37% with the rat β_1 subunit. In comparison with the published rat β_2 subunit this amino acid sequence was N-terminally shorter by 17 amino acids (Fig. 2) (8). However, in another reading frame before the open reading frame an amino acid sequence highly homologous to the rat β_2 subunit was apparent (see Fig. 2). Further inspection of this amino acid sequence revealed a 74 amino acid sequence (deduced from nucleotides 54–278) showing 33% amino acid similarity with the first 77 amino acids of the rat β_1 subunit. In particular, this sequences started with the motif "MYGF" conserved in β subunits from drosophila (15), tobacco hornworm (16) or medaka fish (17) (Fig. 2).

To rule out the possibility that both amino acid sequences were artifactually separated due to a frameshift deletion of one base pair introduced by the taq polymerase or reverse transcriptase, we isolated the critical exon on the genomic level. A human genomic PAC clone (LLNLP704E17877Q3) had previously been confirmed to contain exons of the human β_2 subunit cDNA by direct dye terminator cycle sequencing using internal primers (12). The exon containing the critical region was isolated from this genomic PAC clone as described under Methods. The resulting sequence confirmed the cDNA nucleotide sequence on the genomic level (submitted under GenBank Accession No. AF218383).

To determine how far the apparent discrepancy between the novel human sequence and the rat ortholog reaches back in evolution, we cloned and sequenced the respective exon in four of the closest relatives of humans by PCR analysis of genomic DNA (see Materials and Methods). In the Chimpanzee (Pan troglodytes, submitted to GenBank AF218386), Gorilla (Gorilla gorilla, AF218385), Gibbon (Hylobates lar, AF218387) and Rhesus monkey (Macaca mulatta, AF218384) the reading frame in the respective exon was intact thus joining both observed amino acid sequences with homology to β subunits of sGC. Similar to the rat sequence one extra phenylalanin codon was apparent in the nonhuman primate sequences.

This evolutionary dissimilarity between humans and nonhuman primates raised the possibility that the observed frameshift is not uniformly present in the general human population. To develop a rapid means for analysis of genomic DNA we used the same PCR primers as for the amplification of the respective sequences from nonhuman primates (see Materials and Methods) and analyzed the PCR-products by sequencing or by restriction analysis. As shown in Fig. 3 the isolated human sequence isolated from PAC clone LLNLP704E17877Q3 contains an Eco57I restriction site that is critically dependent on the missing nucleotide splitting the potential coding sequences. This Eco57I site was absent in the investigated nonhuman primates. Likewise, an XmnI restriction site dependent on the extra phenylalanin codon in the human sequence was absent in the investigated nonhuman

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HS•β2     MYGFINTCLQSLVIEKFGEETWEKLKTSAEVQDA--FMTYTMYDDVITIKLIQEACNILGVS
HS β2
RN β2
RN β1     MYGFVNHALELLVIRNYGPEVWEDIKKEAQLDEEGQFLVRIIYDDSKTYDLVAAASKVLNLN

HS•β2     MEAILKLFGEYF-QF.
HS β2                  KMSGYDRMLRTLGGNLMEFIENLDALHSYLALSYQFMNAPSFRVER
RN β2     MEAILKLFGEYFFKFCKMSGYDRMLRTLGGNLTEFIENLDALHSYLALSYQFMNAPSFRVEE
RN β1     AGEILQMFGKMFFVFCQESGYDTILRVLGSNVREFLQNLDALHDHLATIYPGMRAPSFRCTD
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FIG. 2. Amino acid alignment of the novel human β_2 subunit sequence with the rat β_2 and rat β_1 subunit sequence. The first line represents the deduced human amino acid sequence from the 5′ untranslated region (HS*β_2, the stop codon is represented by a dot), the second line (HS β_2) represents the main reading frame starting from position 281 plus the lysine (K) before the start methionine (M). For comparison the rat β_2 subunit (RN β_2, Swissprot P22717) and the rat β_1 subunit (RN β_1, Swissprot P20595) is shown.

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RN β2      G  E  Y  F  F  K  F  C  K  M
RN β2      GGCGAATACTTCTTTAAGTTCTGTAAGATG
MM β2      GGAGAATACTTCTTTCAATTCTATAAGATG
PT β2      GGAGAATACTTCTTTCAATTCTGTAAGATG
GG β2      GGAGAATACTTCTTTCAATTCTGTAAGATG
HL β2      GGAGAATACTTCTTTCAATTCTGTAAGATG
HS β2      GGAGAATAC---TTTCAATTCTG-AAGATG
              GAAnnn---nTTC    CTG-AAG
               XmnI             Eco57I
```

FIG. 3. Restriction sites specific for the missing phenylalanine (XmnI) and the frameshift (Eco57I) in the human β_2 subunit sequence. A comparison of the rat amino acid (first line) and the respective rat (RN) and primate nucleotide sequences is shown: rhesus monkey (MM), chimpanzee (PT), gorilla (GG), gibbon (HL), and human (HS). Recognition sites for the resctriction enzymes are shown below the human sequence.

primate sequences. Genomic DNA from different ethnic backgrounds was kindly provided by Svante Pääbo (MPI für evolutionäre Anthropologie, Leipzig) and additional human genomic DNA samples were taken from a local collection of human heart tissues. PCR amplification and restriction analysis revealed the previously identified frameshift in all screened individuals. These included approximately 100 German (genomic DNA derived from heart tissue), 2 Indonesian, 1 Asian Indian, 1 Iranian, 1 Thai, 1 Filipino, 2 Japanese, 2 Chinese, 4 Australian Aboriginee, and 6 native African individuals. In all of these cases the Eco57I restriction site was present indicating the presence of the frameshift in all screened individuals (Fig. 4). Similar results were obtained using the restriction enzyme XmnI demonstrating the absence of the phenylalanine codon in all screened humans (data not shown). To confirm these data, we performed genomic southern blots after restriction of genomic DNA with XmnI. From the analysis of human genomic DNA from the previously identified PAC clone, we expected a fragment of 2400 bp for the alleles containing an XmnI restriction site and 7100 bp for alleles lacking this site. As control we included genomic DNA from the rhesus monkey (macaca mulatta). A unique band at 2400 bp was visible in human genomic DNA, while the expected

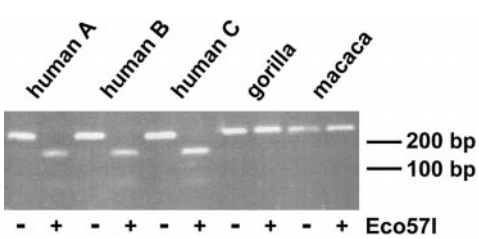

FIG. 4. PCR and restriction analysis of the critical exon of the β_2 subunit of genomic DNA from different humans and nonhuman primates. 10 μl were either digested with Eco57I (+) or run directly on a 1% agarose gel as comparison (−). Expected PCR product lengths were 213 bp for nonhuman primates and 209 bp for humans. Expected Eco57I-digested fragments of the human PCR products were 58 bp and 151 bp.

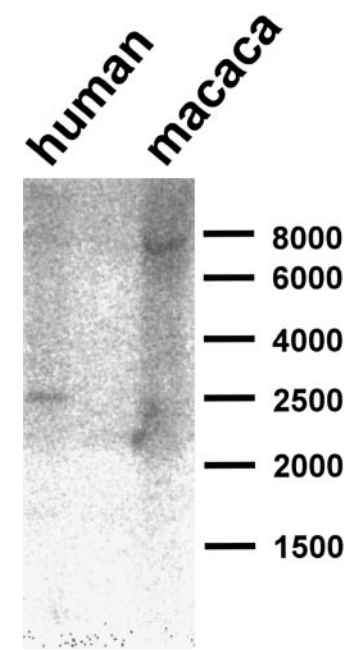

FIG. 5. Southern blot analysis after restriction of genomic DNA with XmnI. The blot was exposed on a phosphoimager plate for 48 h and analyzed by phosphoimager scan. A fragment of 2400 bp for the human specific allele and a fragment of 7100 bp for alleles lacking the frameshift site (*Macaca mulatta*) is visible.

band at 7100 bp was detected for the rhesus monkey DNA (Fig. 5). Analyis of human DNA samples using this approach confirmed the previous data, indicating the uniform presence of one human specific allele in the general population.

DISCUSSION

Humans are evolutionarily related to the African great apes, the chimpanzee (*Pan troglodytes*), and the gorilla (*Gorilla gorilla*) (18–23). The nucleotide sequence identity in comparison with humans has recently been determined to be 99.2% (chimpanzee) and 99.1% (gorilla) across a non-coding 3.4-kb intron sequence (24). Given this over 99% identity on the DNA level, it is proposed that only a few changes in gene expression or function are responsible for the differences between humans and these closely related primates (21). Such differences might also explain variations in susceptibility and biological response to diseases such as cancer (25, 26).

We have cloned and sequenced the full length sequence of the human β_2 subunit from human kidney. Comparison of the deduced amino acid sequence with the rat ortholog demonstrated an amino acid sequence similarity of 78%. Compared with the sequence similarity of the respective β_1 subunit, α_1 subunit and α_2 subunit sequences of 99, 88, and 90% (rat α_2 sequence, own unpublished data) this indi-

cates that the evolutionary pressure to conserve the amino acid sequence is less for the β_2 subunit sequence than for any of the other subunits. This could indicate that the role of the β_2 subunit could also be functionally more diverse in different species than the other subunits. Compared to the published rat β_2 subunit the human ortholog is truncated by 17 amino acids. Compared with the rat β_1 subunit the novel sequence is truncated by 77 amino acids (see Fig. 2). Deletion mutants of the β_1 subunits lacking only 64 N-terminal amino acids have been shown to be NO-insensitive (27, 28). The functional properties of the β_2 subunit have remained elusive. After the cloning of the novel subunit (8) coexpression experiments with the other subunits have consistently failed to produce catalytic activity in different laboratories (see (3)). In 1997, Gupta and co-workers presented data showing dimerization of a β_2/green fluorescent protein fusion protein and the α_1 subunit and NO-sensitive guanylyl cyclase activity after coexpression of these subunits in COS cells (10). Based on expression experiments in A7R5 smooth muscle cells an inhibition of α_1/β_1 catalyzed activity is postulated by the authors (10). The novel finding of catalytic activity after coexpression of the α_1 and β_2 subunit by Gupta and co-workers could be explained by the use of a β_2/green fluorescent protein fusion protein (10). Possibly the N-terminally attached green fluorescent protein can substitute for the lacking N-terminal heme binding domain as compared with the rat β_1 subunit (8). However, the use of such a fusion construct does not allow to compare the functional characteristics of the N-terminally extended β_2 subunit with the human sequence. We are at present not able to functionally express the rat β_2 subunit or the human β_2 subunit with any of the other known subunits in the Baculovirus Sf9 expression system. Whether this reflects our failure to choose the appropriate experimental conditions or whether the rat and human β_2 subunits are non-functional or have a function other than guanylyl cyclase activity remains unclear at present.

The cloning of the novel human β_2 subunit and the assignment of three EST's from human kidney is consistent with the predominant expression of the rat β_2 subunit in kidney (8). The assignment of an EST from gastric carcinoma and the finding that the human β_2 subunit is expressed in gastric tumor but not in normal gastric tissue raises the possibility that this subunit might have a role in tumorigenesis or cell growth. In addition, its application as a diagnostic marker for gastric cancer seems conceivable. Recently, the membrane bound receptor guanlylyl cyclase C has been characterized as a tumor marker for colon carcinomas (29, 30). Further experiments will be directed towards expression screening in various human tumor tissues and the validation of the novel human β_2 subunit as a potential diagnostic marker.

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