Alternative splicing for the α_1 subunit of soluble guanylate cyclase

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Soluble guanylate cyclase (sGC), the receptor for nitric oxide, is a heterodimer consisting of α and β subunits. We investigated the mRNA species for the α_1 subunit in human brain, heart, artery and immortalized B-lymphocytes. Three mRNA species were identified in these tissues. The major mRNA species contained the full expression sequence of the α_1 subunit. Two other types of mRNA were detected in which 5′ sequences were deleted by splicing (506–590 and 412–590). Each of these deletions included the predicted translation start site, indicating that translation of these two alternatively spliced RNA species does not result in the production of full-length α_1 subunits. The relative amounts of the two mRNA species with deletions of the translation start site differed significantly between cell lines of immortalized B-lymphocytes from different individuals. sGC enzymic activity was significantly decreased in cellular extracts from cell lines with high proportions of mRNA species containing the deletion 506–590 when compared with extracts from cell lines that contained mostly mRNA without this deletion.

Key words: hypertension, nitric oxide, signalling pathway.

INTRODUCTION

Nitric oxide (NO), a short-lived free radical, has been suggested to have important roles in cardiovascular regulation, immune response and neuronal long-term potentiation (reviewed in [1,2]). Ablation of the gene for the endothelial type of nitric oxide synthase is associated with the development of hypertension in rodents [3], suggesting that NO is necessary for the maintenance of normal blood pressure. cGMP, the second messenger of NO, is formed after the activation of soluble guanylate cyclase (sGC). The pharmacological blockade of sGC results in blunted NO-mediated vascular relaxation [4,5], suggesting that the vascular effects of NO require functional sGC activity. The vasoactive effects of NO might also be mediated independently of sGC because the inhibition of sGC does not completely abolish acetylcholine-induced vasorelaxation [4,5].

Soluble GC is a heterodimer consisting of α and β chains. The terminology for the subunits of sGC has recently been revised [6]. Human vascular tissues express mRNA for the α_1 and β_1 subunits [6], formerly known as α_3 and β_3 subunits [7,8], but not for the α_2 subunit, which has previously been cloned from human fetal brain [9]. Alternatively spliced transcripts have been reported for the human α_2 [10] and β_1 [11] subunits resulting in amino acid insertions or deletions respectively. We studied transcripts for the α_1 subunit of sGC in human tissues. We found that mRNA for the α_1 subunit undergoes alternative splicing, resulting in several mRNA species. Two of these alternatively spliced mRNA species are predicted to result in the formation of N-terminally truncated protein for the α_1 subunit.

EXPERIMENTAL

Source of tissues

Epstein–Barr-virus-transformed B-cell lines were generated as described previously [12] from peripheral blood of six male and two female patients presenting to the Hypertension Clinic. The age of the donors was between 39 and 84 years. Splenic artery was salvaged from a splenectomy specimen that was removed from a patient with abdominal trauma. Normal cortical human brain tissue was obtained from a patient who died of breast cancer. Fresh heart tissue was obtained from the explanted heart of a patient who underwent heart transplantation for congestive heart failure. The procedures that were followed during the research studies were approved by the local Institutional Review Board, and informed consent was obtained from all blood donors.

Table 1 Nucleotide sequences and annealing temperatures (T_m) for the oligonucleotides used for the PCR amplification of the α_1 subunit of sGC

Product (nt)	T_m (°C)	5′ Primer sequence	3′ Primer sequence
225–2984	52	5′-AGAGACCAGGACTCAGTTC-3′	5′-TGACGACAACTATCACAAACAC-3′
225–695	55	5′-AGAGACCAGGACTCAGTTC-3′	5′-TTTCTTGTATGTTCTTCTCAGG-3′
200–695	55	5′-CCTGGAGGAGCTTAGAGAC-3′	5′-TTTCTTGTATGTTCTTCTCAGG-3′
232–695	55	5′-GATCTCACCATGTGCGGATTTG-3′	5′-TTTCTTGTATGTTCTTCTCAGG-3′
602–1032	54	5′-AACGAGTCTTCAGAGGAGG-3′	5′-AGAAGGGTACTGAAGCTGT-3′
919–1656	52	5′-CAAAGAATCTCTTGGTGAAG-3′	5′-GCACTGGATTCAACAATGTAG-3′
1563–2334	53	5′-TTGTACGAGTGAGGAGATG-3′	5′-GCCAGAGTGACATTGTTTC-3′
2220–2984	52	5′-CCCATGGAGAACCTATCAAG-3′	5′-TGACGACAACTATCACAAACAC-3′

Abbreviation used: sGC, soluble guanylate cyclase.

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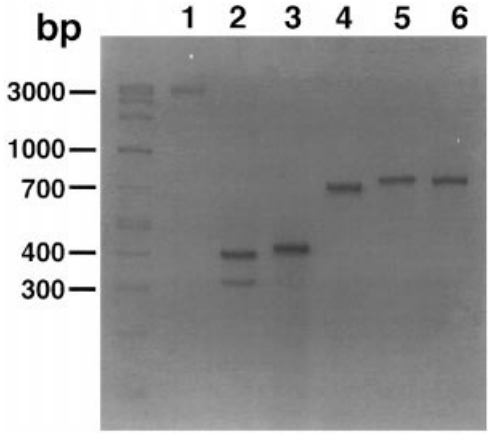

Figure 1 Analysis of cDNA containing the complete coding sequence for the α_1 subunit of sGC

Arterial mRNA was reverse-transcribed with poly(dT) primer. Next, cDNA was amplified by PCR between nt 200 and 2984 of the α_1 subunit (lane 1), as described in the Experimental section. Nested PCR was performed with different primer pairs, resulting in the amplification of the regions nt 200–695 (lane 2), nt 602–1032 (lane 3), nt 919–1656 (lane 4), nt 1563–2334 (lane 5) and nt 2220–2984 (lane 6). Nested PCR of the nucleotide regions described above did not result in visible DNA products when primers were omitted from the initial PCR reaction (results not shown). The amplified cDNA products were separated by agarose-gel electrophoresis and stained with ethidium bromide. Molecular mass markers are shown at the left.

Reverse transcription and PCR

RNA was isolated with a commercial reagent (Stat-60; Tel-Test, Friendswood, TX, U.S.A.). Total RNA was quantified by UV spectrophotometry. Messenger RNA was reverse-transcribed with reverse transcriptase (Superscript; Gibco, Gaithersburg, MD, U.S.A.) and a 15-mer oligodeoxythymidine primer. PCR primers for the α_1 subunit were designed (Table 1) on the basis of the previously published cDNA sequence [7] with minor corrections as described by Zabel [6] and Gansemann (GenBank accession number HSU 58855). The cDNA was amplified during 30–40 cycles on a thermal cycler (RoboCycler; Stratagene, La Jolla, CA, U.S.A.) by using *Taq* or *Vent* DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.). The annealing temperatures (T_m) are indicated in Table 1 for each primer pair. For amplification of the complete expression sequence from cDNA, nested PCR was performed with primer sets resulting in the amplification of bases 25–2984 followed by the amplification of nt 200–2984. Amplified products were revealed by agarose-gel electrophoresis followed by staining with ethidium bromide. The nucleotide sequence of the 400–800 bp-containing PCR products was determined by automated sequencing of both DNA strands (ABI Prism 373; PE Biosystems, Foster City, CA, U.S.A.).

Competitive PCR

Competitive PCR for the α_1 subunit was performed with primer pairs resulting in the amplification of bases 602–1032 for the α_1 subunit of sGC, bases 262–470 for the β_1 subunit of sGC [8] and bases 113–652 for β-actin, as described previously [13]. We generated competitive DNA for the α_1 subunit (300 bp), the β_1 subunit (500 bp) and β-actin (250 bp) by PCR under conditions of low stringency as described previously [14]. Amplified DNA products that were distinguishable from cDNA by their size differences were cloned and amplified in TA cloning vectors (Invitrogen, Carlsbad, CA, U.S.A.). The nucleotide sequence of the cloned DNA was unrelated to the expression sequence of sGC subunits with the exception of primer-specific sequences. Competitive DNA was quantified by spectrophotometry, diluted and added to cDNA samples. To determine the concentration of the PCR products, amplified competitor DNA and cDNA were separated by gel electrophoresis and stained with ethidium bromide. The gel was exposed to Polaroid 55 positive/negative film (Cambridge, MA, U.S.A.). Negative transparencies were scanned with a GS-670 imaging densitometer and analysed with Molecular Analyst software (Bio-Rad, Hercules, CA, U.S.A.). Concentrations were calculated on the basis of the densities and sizes of the amplified bands for competitor and cDNA. The linearity of the assay was confirmed by measuring sGC mRNA that had been diluted over a range of four magnitudes. The intra-assay coefficient of variation for the competitive PCR assay was 20%.

RNase protection assay

A bacterial clone of cDNA for the α_1 subunit was provided by Dr G. Guellaen (Unité INSERM 99, Creteil, France). A fragment containing nt 1–695 was obtained by digestion with the restriction enzymes *Eco*R1 and *Xmn*1. The isolated fragment was cloned into pGEM-3Z. A second template for spliced cDNA was generated by PCR. We amplified nt 232–694 from myocardial cDNA with *Pfu* polymerase (Promega, Madison, WI, U.S.A.). The 285 bp PCR product containing deletions 318–411 and 506–590 was isolated by gel electrophoresis and cloned into pGEM-3Z that had been cut with *Hin*cII and *Sma*I. The vectors containing the two inserts were linearized by cuts at the polylinker site that was located 5′ to the insert. The probes for the α_1 subunit were generated with T7 RNA polymerase from *Escherichia coli* (Promega). The RNase protection assay was performed as described previously [15]. In brief, total RNA was hybridized with ^{32}P-labelled cRNA overnight and digested with ribonucleases A and T1. The RNA sample was then applied to a 7% (w/v) polyacrylamide gel containing 8 M urea. After electrophoresis, the protected portions of the probe were localized by autoradiography.

Measurement of sGC enzymic activity

cGMP was determined in cellular extracts of B-cell lines. Cellular extracts were derived as described previously [10]. In brief, cells were suspended in extraction buffer containing 50 mM triethanolamine hydrochloride, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin. After several passes through a 22-gauge needle, the suspension was centrifuged at 30000 ***g*** for 30 min and the supernatant was saved for further enzymic studies. Cyclase activity was assayed at 37 °C for 10 min in 50 mM triethanolamine hydrochloride (pH 7.4)/3 mM $MgCl_2$/100 μM GTP/0.5 mM isobutylmethylxanthine/100 μM 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine ('PAPA NONOate') (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) in the presence of 1 mg/ml protein extract. The concentration of newly formed cGMP was directly proportional to the length of incubation between 0 and 10 min. cGMP was measured with a commercially available ELISA kit in accordance with the manufacturer's instructions (Cayman Chemical Company). No cross-reactivity was observed with 1 μM cAMP or 100 μM GTP.

RESULTS

Identification of splice variants by reverse-transcriptase-mediated PCR

To detect splice variants of the α_1 subunit, we applied a

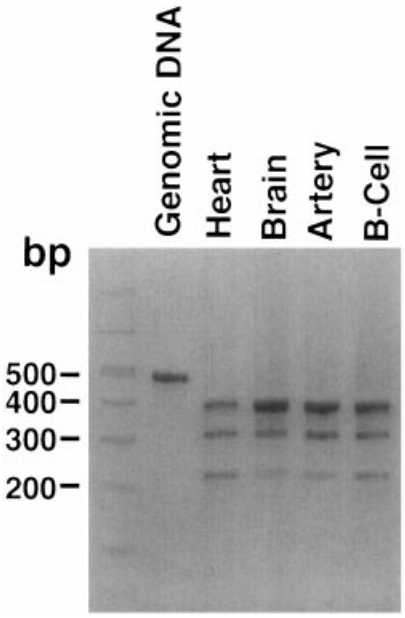

Figure 2 Comparison of genomic DNA and cDNA for the α_1 subunit of sGC

Genomic DNA was isolated from human heart. mRNA was isolated from human heart, brain, artery and immortalized B-cells. The cDNA was generated by reverse transcription of mRNA with oligo(dT) primers. The region nt 200–695 from genomic DNA and cDNA was amplified by PCR. The DNA products were separated by electrophoresis on 2% (w/v) agarose gel and stained with ethidium bromide. Molecular mass markers are shown at the left.

combination of reverse transcription and PCR. Initially, nt 200–2984 of the α_1 subunit were amplified from arterial cDNA by PCR. The observed size of the amplified cDNA product was between 2.5 and 3 kb (Figure 1). Secondly, nested PCR was performed with primer pairs, resulting in the amplification of overlapping nucleotide sequences of cDNA containing between 400 and 700 bp covering the whole coding sequence of the α_1 subunit as well as adjacent 5′ and 3′ untranslated regions. Separation of the amplified PCR products with the nucleotide sequences 602–1032, 919–1656, 1563–2334 and 2220–2984 by agarose-gel electrophoresis showed that only one cDNA species was amplified with each of the primer pairs (Figure 1). Sequencing of these PCR products indicated that the nucleotide sequence of the amplified DNA was identical with that of the α_1 subunit that had been previously reported by Zabel [6] and Ganseman. In contrast with the PCR products from the region nt 602–2984 of the α_1 subunit, amplification of cDNA in the region nt 200–695 resulted in the detection of two PCR products with molecular masses that were smaller (Figure 1) than predicted from the previously published cDNA sequence from human brain [7]. Furthermore, three DNA products with sizes smaller than predicted were detected after the direct amplification of myocardial cDNA with primer pairs covering nt 200–695 (Figure 2). Sequencing of these PCR products revealed that the following nucleotide sequences were missing in comparison with the previously described cDNA: nt 318–411 resulting in a 402 bp PCR product, nt 318–411 and 506–590 resulting in a 317 bp fragment, and nt 318–590 resulting in a 223 bp product. All three cDNA products were also observed in human brain, splenic artery and immortalized B-lymphocytes (Figure 2), indicating that the identified cDNA species were not expressed in an organ-specific fashion.

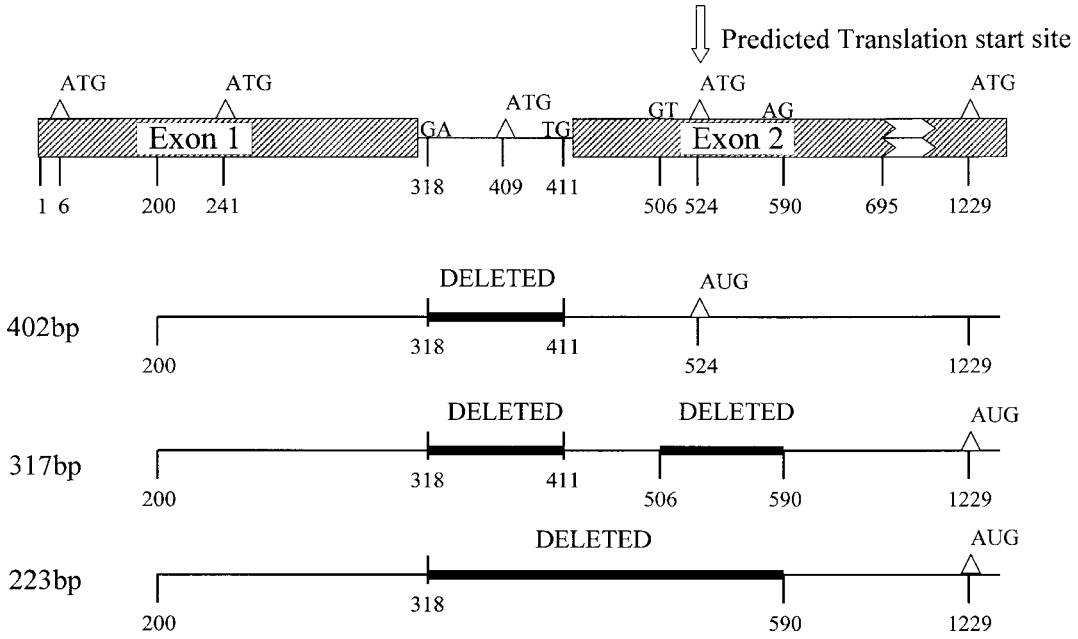

Figure 3 Proposed genomic organization for the 5′ untranslated region of the α_1 subunit of sGC

The 5′ untranslated region is divided into two exons by an intron spanning nt 318–411. Of the putative translation start sites at positions 6, 241, 409 and 524, only the last is located in the correct reading frame; three mRNA species are generated from this region. The major mRNA species contains both exons and has nt 318–411 deleted by splicing. Alternative splicing results in two other mRNA species with additional deletions of nt 506–590 or nt 412–590. Splicing of the region nt 506–590 results in the removal of the predicted translation start site at position 524. The next potential translation start site within the correct reading frame is located at position 1229. Initiation of translation from this alternative translation start site would result in a protein that did not contain the 235 N-terminal amino acids. The numbers at the left are the sizes of reverse-transcriptase-mediated PCR productslk with primers spanning nt 200–695.

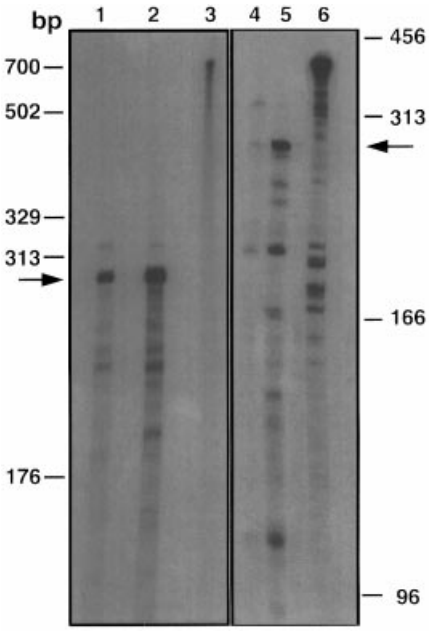

Figure 4 Identification of mRNA species for the α_1 subunit of sGC by RNase protection assay

The RNase protection assay was performed with RNA from human heart and splenic artery. In lanes 1–3, 5 μg of total RNA from human heart and 25 μg of RNA from splenic artery were hybridized with a probe that was complementary to nt 1–695 of the α_1 subunit, as described in [7]. After digestion with ribonucleases A and T1, the samples was applied to 7% (w/v) polyacrylamide gel containing 8 M urea (lane 1, heart; lane 2, artery). Undigested probe containing the 695 nt of the α_1 subunit was run on lane 3. In lanes 4–6, a different probe was used that contained 285 bp complementary to nt 232–695 with the deletions 318–411 and 506–590. After incubation with RNase, myocardial RNA, RNA from artery and undigested probe were applied to lanes 4, 5 and 6 respectively. The arrows indicate major fragments of protected probe. The positions of Molecular mass markers are shown at each side.

In contrast with amplified cDNA, PCR of the α_1 subunit sequence nt 200–695 from myocardial genomic DNA resulted in a PCR product consistent with the predicted size of 496 bp (Figure 2). Sequencing of the DNA that was amplified from genomic DNA showed that the amplified genomic DNA was identical with the previously described cDNA [7] except for the insertion of a C at position 366. Furthermore, the ends of the missing sequence nt 318–411 contained the splice donor and acceptor sites AG and GT respectively. A possible branch site consisting of the nucleotides GCGCCAC was found 35 nt from the putative splice acceptor site. These results suggest that nt 318–411 consist of an intron that is removed by splicing in all of the observed mRNA species (Figure 3). Nucleotides 318–411 are located in the 5′ untranslated region of nascent mRNA (Figure 3). Splicing of these nucleotides results in the removal of a potential AUG translation start site at nt 409. Initiation of protein synthesis at this alternative start site would not result in the formation of the α_1 subunit owing to an incorrect reading frame.

Similarly to the splice sequence nt 318–411, nt 506–590 contained the splice donor and acceptor sites GT and AG. A possible branch site consisting of the nucleotides AGATCAC was identified at position 552. In contrast with the splicing of nt 318–411, deletion of nt 506–590 results in the removal of the predicted translation start site at position 524. The next potential AUG translation start site within the correct reading frame is located at nt 1229. In contrast with the predicted translation start site at position 524, the AUG at nt 1229 is not associated with the consensus sequence CACC, which has previously been shown to result in an increased efficiency of translation. Therefore the initiation of translation at nt 1229 seems to be less likely. Furthermore, the start of translation at position 1229 or at another AUG site downstream of nt 1229 would result in the formation of an α_1 subunit from which the 235 N-terminal residues would be missing and which would probably be non-functional.

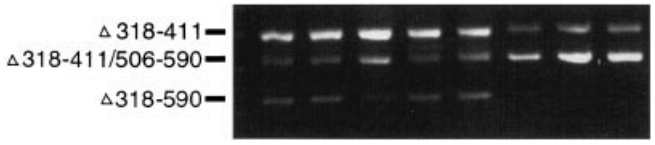

Figure 5 mRNA composition of the α_1 subunit of sGC in several immortalized B-cell lines

mRNA from eight B-cell lines was reverse-transcribed with poly(dT). The cDNA in the region nt 200–695 of the α_1 subunit was amplified by PCR. DNA products were separated by gel electrophoresis and stained with ethidium bromide. Cell lines with high contents of cDNA without the deletion nt 506–590 migrating at 402 bp were applied to the five lanes on the left. In contrast, cDNA from cell lines with high proportions of mRNA containing the deletion nt 506–590 migrated at 317 bp, as shown in the three lanes on the right.

Identification of mRNA species by RNase protection assay

To confirm that the amplified cDNA products were due to splicing of mRNA, we analysed mRNA with an RNase protection assay with a probe complementary to the initial 696 bases of pre-spliced RNA for the α_1 subunit. No protected band was detected in the 700 bp region (Figure 4, lanes 1–3). These results indicated that all of the investigated mRNA species for the α_1 subunit had undergone some splicing within the region nt 1–696. We detected a band of strong intensity that was migrating slightly below the 300 bp marker (Figure 4, lanes 1–3, arrow). These results are consistent with the protection of bases 412–696 by mRNA containing the deletion 318–411. Another band of lower intensity migrated between 313 and 329 bp, which could have resulted from the protection of the first 317 bp of the probe. The decreased intensity of the band migrating between 313 and 329 bp suggests that most of the transcripts do not contain the complete sequence nt 1–312. Results obtained by RNase protection assay with probe containing the sequence nt 25–312 indicated that there were several transcription start sites in the region nt 25–300 (D. Ritter, unpublished work).

To confirm the presence of the splice variant with the deletion of nt 506–590, we performed another RNase protection assay with a probe consisting of 285 bp of complementary RNA containing the sequence nt 232–695 with the deletions nt 318–411 and nt 506–590. A band of strong intensity was found to migrate sligthly faster than the 313 bp marker, confirming the presence of mRNA with these two deletions (Figure 4, lanes 4–6, arrow).

Differential distribution of splice variants for the α_1 subunit

To investigate whether the proportions of splice variants for α_1 subunit differed between individuals, we evaluated mRNA for the α_1 subunit in immortalized B-cell lines from eight individuals. Five of the cell lines predominantly expressed mRNA containing nt 506–590. In contrast with these five B-cell lines, three cell lines from other individuals contained mostly mRNA with the deletion 506–590 (Figure 5). We examined whether these three cell lines

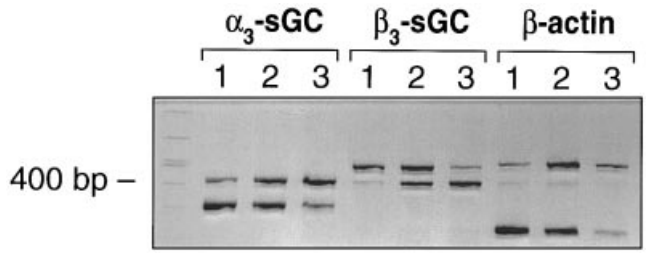

Figure 6 Competitive PCR for the α_1 and β_1 subunits of sGC and β-actin

mRNA species were quantified by competitive PCR of cDNA in the presence of decreasing concentrations of competitor. The amplified cDNA species and competitors migrated at approx. 400 and 300 bp for the α_1 subunit, 400 and 500 bp for the β_1 subunits, and 500 and 250 bp for β-actin. The following amounts of competitor were applied: α_1 subunit, 10^{-20} mol (lane 1) to 10^{-22} mol (lane 3); β_1 subunit, 3×10^{-19} mol (lane 1) to 3×10^{-21} mol (lane 3); β-actin, 5×10^{-19} mol (lane 1) to 5×10^{-21} mol (lane 3). Bands stained with ethidium bromide were scanned by imaging densitometry. Concentrations of cDNA were calculated on the basis of measured band densities as described in the Experimental section.

Table 2 Expression of α and β subunits for sGC and sGC enzymic activities in eight B-cell lines

Results are expressed as means ± S.E.M. $^*P < 0.05$ and $^{**}P < 0.001$, Student's t test.

Predominant splice species ...	nt 506–590 deleted ($n = 3$)	nt 506–590 present ($n = 5$)
Percentage with 506–590 deletion:	70 ± 2**	19 ± 3
cDNA for α_1-subunit (fmol/g of RNA)	5 ± 2	6 ± 2
cDNA for β_1-subunit (fmol/g of RNA)	276 ± 41	344 ± 66
cDNA for β-actin (fmol/g of RNA)	340 ± 28	288 ± 83
Maximal enzymic activity (fmol of cGMP/min per mg of protein)	49 ± 4*	391 ± 102

contained structural variations in their nucleotide sequence for the α_1 subunit of sGC. The sequence nt 506–590 from these three cell lines was identical with that from other individuals. Several single nucleotide polymorphisms were identified in the coding sequence downstream from position 590 (results not shown). However, none of the observed polymorphisms were found exclusively in these three cell lines, suggesting that the identified polymorphisms are unrelated to the expression of splice variants. Furthermore, alternative splicing was not related to the abundance of mRNA for the α_1 subunit because mRNA concentrations were similar in these eight B-cell lines (Table 1, Figure 6).

sGC mRNA and enzymic activity in B-cell lines

Of the three observed mRNA species for the α_1 subunit, only the mRNA containing nt 506–590 encoded full-length α_1 subunit. We investigated the level of sGC enzymic activity in the eight cell lines. Despite similar mRNA concentrations for the subunits of sGC, sGC enzymic activity was significantly lower in cell lines that contained the splice variant with the deletion 506–590 as the predominant mRNA (Table 2). These results demonstrate that the enzymic activity of sGC is associated inversely with the relative proportion of the splice variant with the deletion nt 506–590.

DISCUSSION

Heterogeneity of mRNA for the subunits of sGC has been previously described. Chhajlani et al. [11] identified a variant cDNA for the human β subunit of sGC with a 100 nt deletion from the coding sequence. Additionally, Behrends et al. [10] found a variant of the α_2 subunit that contained additional nucleotides in the proposed catalytic domain of sGC. This variant was enzymically inactive and functioned as a dominant-negative protein when co-expressed with wild-type subunits of sGC inSf9 cells. To our knowledge, no information exists about the abundance of these splice variants in human or other mammalian tissue. Therefore the effects of these variants on sGC enzymic activity are not known when they are expressed at physiological concentrations.

We have investigated the mRNA species for the α_1 subunit, which is the most abundant α subunit in adult human tissues including vascular tissue [8]. First, we found that a portion of the previously published cDNA sequence for the α_1 subunit [7] probably contained an intron. All of the mRNA species from several human tissues had 94 nt deleted from the 5′ untranslated region of pre-spliced mRNA.

Furthermore, we identified two mRNA variants that had various portions of their untranslated and translated sequences deleted by splicing (nt 412–590, nt 506–590), including the predicted translation start site at position 524. These two forms of alternatively spliced mRNA, which were found in all of the investigated tissues, should not result in the formation of the complete α_1 subunit for sGC. It is unlikely that the generation of N-terminally truncated α_1 subunit from these two mRNA variants would be functional. Other investigators have shown by expression experiments *in vitro* with an N-terminally truncated α_2 subunit and a wild-type β subunit that the intact α subunit is necessary for normal sGC enzymic activity [16,17].

Next we measured the relative amounts of the various RNA species for α_1 subunit in B-cell lines from different individuals. Cell lines from most individuals predominantly expressed mRNA containing the translation start site. We identified three cell lines that expressed mostly the splice variant with the deletion nt 506–590. The enzymic activity of sGC was significantly decreased in these cell lines, providing evidence that splice variants containing the deletion nt 506–590 were not contributing to the observed enzymic activity of sGC in these cell lines.

sGC has an important function as the intracellular receptor of NO. Ablation of the gene for particulate guanylate cyclase, the receptor of atrial natriuretic peptide [18], or deletion of the gene for endothelial nitric oxide synthase [3] resulted in the development of hypertension in a mouse model. The impaired production of NO has been associated with essential hypertension in humans [19]. Furthermore, the response to NO has been found to be blunted in ethnic subgroups with a high prevalence of hypertension [20,21] and in some patients with hypercoagulable state [22]. A decreased abundance of intact α_1 subunits for sGC due to the aberrant splicing of mRNA might contribute to the development of hypertension or other cardiovascular diseases. Further studies are warranted that will address the role of splice variants for the α_1 subunit of sGC in human vascular disease.

In summary, three species of mRNA were identified for the α_1 subunit of sGC in human brain, heart, vascular tissue and immortalized B-cells. Only one of the three mRNA species contained the predicted translation start site that is necessary for the formation of the functional α_1 subunit. The relative abundance of the mRNA species that contained the translation start site was correlated with sGC enzymic activity.

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