Human soluble guanylate cyclase: functional expression and revised isoenzyme family

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Soluble guanylate cyclase (sGC), a heterodimeric (α/β) haem protein that converts GTP to the second messenger cGMP, functions as the receptor for nitric oxide (NO) and nitrovasodilator drugs. Three distinct cDNA species of each subunit ($\alpha1$ – $\alpha3$, $\beta1$ – $\beta3$) have been reported from various species. From human sources, none of these have been expressed as functionally active enzyme. Here we describe the expression of human α/β heterodimeric sGC in Sf9 cells yielding active recombinant enzyme that was stimulated by the nitrovasodilator sodium nitroprusside or the NO-independent activator 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1). At the protein level, both α and β subunits were detected in human tissues,

suggesting co-expression also *in vivo*. Moreover, resequencing of the human cDNA clones [originally termed $\alpha3$ and $\beta3$; Giuli, Scholl, Bulle and Guellaen (1992) FEBS Lett. **304**, 83–88] revealed several sequencing errors in human $\alpha3$; correction of these eliminated major regions of divergence from rat and bovine $\alpha1$. As human $\beta3$ also displays more than 98 % similarity to rat and bovine $\beta1$ at the amino acid level, $\alpha3$ and $\beta3$ represent the human homologues of rat and bovine $\alpha1$ and $\beta1$, and the isoenzyme family is decreased to two isoforms for each subunit ($\alpha1$, $\alpha2$; $\beta1$, $\beta2$). Having access to the human key enzyme of NO signalling will now permit the study of novel sGC-modulating compounds with therapeutic potential.

INTRODUCTION

Guanylate cyclases catalyse the conversion of GTP to cGMP in response to various extracellular stimuli, thereby providing an important second messenger for the regulation of protein kinases, phosphodiesterases and ion channels [1,2]. Soluble guanylate cyclase (sGC) is the main receptor for vasodilating nitric oxide (NO)-releasing compounds (nitrovasodilators), which have been used for over 100 years in the treatment of coronary heart disease and angina pectoris. Since the discovery of endogenous, L-arginine-derived NO as an intercellular second messenger [3,4], it became clear that sGC is the physiological receptor for this important signalling molecule [5,6] and presumably the most relevant molecular target for NO-releasing drugs in human cardiovascular therapy [7].

sGC has been purified from various tissues as a heterodimeric haem protein composed of a larger α and a smaller β subunit [8–10]. One [11] or two [12] prosthetic haem groups are essential for stimulation of sGC by NO, and function as its molecular receptor [13–15]. Corresponding α and β cDNA clones have been isolated from rat and bovine lung and brain cDNA libraries and have been designated $\alpha1$ and $\beta1$ [16–19]. Moreover the existence of additional isoforms has been reported: (1) $\alpha2$ cDNA was isolated from human fetal brain [20], (2) $\alpha2i$, an alternatively spliced variant of $\alpha2$, has been detected in several cell lines and tissues at the mRNA level [21], and (3) a $\beta2$ cDNA clone was isolated from rat kidney [22]. Because heterodimerization of α and β subunits is essential for basal and stimulated enzyme activity [17,23], the existence of multiple α and β isoforms implies regulatory mechanisms for sGC activity in living cells via

alternative heterodimerization. In fact, the human $\alpha2$ isoform is active when co-expressed with bovine $\beta1$ in mammalian cells [20], whereas $\alpha2i$ is inactive under the same conditions [21]. In a mammalian expression system, rat $\alpha1$ and $\beta2$ form an active heterodimer; however, this is less sensitive than the $\alpha1/\beta1$ heterodimer to NO [24].

However, human sGC has neither been purified from native tissue sources nor been expressed as functional recombinant enzyme (Table 1). Moreover, no human homologues of rat and bovine $\alpha1/\beta1$ and rat $\beta2$ have been detected, and the physiological complementary subunit(s) of the human $\alpha2$ and $\alpha2i$ isoforms are unknown. Instead, additional cDNA clones coding for further sGC isoforms were isolated from an adult human brain library and termed $\alpha3$ and $\beta3$ [25]. Thus the sGC family seems to comprise at least six isoenzymes ($\alpha1$ – $\alpha3$, $\beta1$ – $\beta3$). In the present study we expressed and characterized for the first time a

Table 1 Present terminology of human NO receptor cyclase cDNA species

Isoform	Subunit ... α	β
1	Not detected	Not detected
2	cDNA; active when co-expressed with bovine $\beta1$ *	Not detected
3	cDNA†	cDNA†

* Reference [20].

† Reference [25].

Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; rhsGC, recombinant human sGC; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; KRB, Krebs/Ringer buffer.

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The nucleotide sequence data reported for the human soluble guanylate cyclase $\alpha1$ subunit (new terminology) will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession no. Y15723.

functional heterodimeric human sGC, composed of $\alpha 1$ and $\beta 1$, as an NO receptor, and revised the isoenzyme family of sGC α and β subunits.

EXPERIMENTAL

Reagents

All chemicals were obtained from Sigma (Deisenhofen, Germany) unless stated otherwise. [α - 32 P]GTP (1.48×10^{13} Bq/mmol) was obtained from Amersham. For the sGC assay, sodium nitroprusside (SNP) and 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) were prepared as 10-fold concentrated stock solutions in 1 mM sodium acetate, pH 5.0, and 10% (v/v) DMSO respectively. The saturated CO solution (1 mM at 20 °C) was prepared by bubbling water with CO gas (99.97% purity; Linde, Unterschleissheim, Germany) for 30 min. For cGMP determination in Sf9 cells, SNP and YC-1 were prepared as 100-fold concentrated stock solutions in 40 mM sodium acetate buffer, pH 5.0, and DMSO respectively. sGC $\alpha 3$ and $\beta 3$ cDNA species were provided by G. Guellaen [25].

Baculovirus construction

cDNA species, originally designated $\alpha 3$ and $\beta 3$ cDNA species [25] but later renamed $\alpha 1$ and $\beta 1$ (see the Results section), comprising the complete coding and 270 bp ($\alpha 1$) and 500 bp ($\beta 1$) of 3' non-coding sequences, but lacking 5' untranslated regions, were cloned into the pVL1393 baculovirus transfer vector (Pharmingen, San Diego, CA, U.S.A.). For this purpose, *Bam*HI sites (underlined in the sequences below) were introduced by PCR immediately upstream of the translational start sites. Fragments were amplified with the primer pairs for $\alpha 1$ (nucleotide numbering in accordance with the corrected $\alpha 1$ cDNA sequence; see the Results section), 5'-AAGGATCCATGTTCTGCACG-AAGCTC-3' (nt 524–541) and 5'-ATTATGGAAGCAGGGA-GG-3' (nt 1249–1232), and for $\beta 1$, 5'-AAAAGGATCCATGTACGGATTTGTGAAT-3' (nt 89–106) and 5'-ATGCGTGA-TTCCTGGGTACC-3' (nt 711–692). Products were cut with *Bam*HI/*Bsa*AI ($\alpha 1$) or *Bam*HI/*Kpn*I ($\beta 1$) and ligated with *Bsa*AI/*Eco*RI (1193–3015, $\alpha 1$) or *Kpn*I/*Eco*RI (692–2444, $\beta 1$) cDNA fragments to the *Bam*HI/*Eco*RI-cleaved vector. Recombinant baculoviruses, obtained by co-transfection of transfer vectors and BaculoGold DNA (Pharmingen), were plaque-purified from the culture medium. For preparation of high-titre virus stocks, Sf9 cells were spinner-cultured to a density of 0.5×10^6 cells/ml, infected at a multiplicity of infection of 0.1 plaque-forming unit per cell, and harvested 6 days after infection.

Sf9 cell culture and production of recombinant human sGC (rhsGC)

Sf9 cells were cultured in IPL-41 medium supplemented with 10% (v/v) fetal calf serum, 4% (w/v) tryptose phosphate broth, 1% (v/v) Pluronic F68, 0.5% Amphotericin B (all Gibco BRL), and 80 μ g/ml gentamycin sulphate (Merck) at 27 °C. Monolayer cultures (2.5×10^6 cells per Petri dish, 90 mm in diameter) or spinner cultures (2×10^6 cells/ml) were infected with hsGC $\alpha 1$ and/or hsGC $\beta 1$ viruses at a multiplicity of infection of 1–2 plaque-forming units per cell and cultured at 27 °C. Cells were harvested 72 h after infection, when protein production, basal and NO-stimulated enzyme activity were maximal (results not shown). All subsequent procedures were performed at 4 °C. Cells were homogenized by sonication in lysis buffer [25 mM triethanolamine (pH 7.8)/50 mM NaCl/1 mM EDTA/5 mM dithiothreitol/1 μ M leupeptin/0.5 mg/l soybean trypsin inhibitor]. Crude supernatant and particulate fractions were separated

by centrifugation (20000 g) for 15 min at 4 °C; the pellet was resuspended in an equal volume of lysis buffer. Aliquots were subjected to SDS/PAGE [26] followed by Coomassie Blue protein staining or protein immunoblot (see below). For storage, fractions were brought to a final concentration of 50% (v/v) glycerol and kept at –20 °C. Protein concentrations were determined by the method of Bradford [27].

sGC activity assay

sGC activity in crude soluble fractions of Sf9 cells was measured at 37 °C for 10 min by the conversion of [α - 32 P]GTP to [32 P]cGMP as described [28], in a total volume of 100 μ l, containing 50 mM triethanolamine/HCl, pH 7.4, 3 mM MgCl₂, 3 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM cGMP, 5 mM creatine phosphate, 0.25 mg/ml creatine kinase and 500 μ M GTP. Reactions were started by the simultaneous addition of the enzyme-containing Sf9 fraction and the sGC activators SNP, CO or YC-1. [32 P]cGMP was separated and measured as described [28].

cGMP formation in intact Sf9 cells

Sf9 monolayer cultures (see above) were co-infected with hsGC $\alpha 1$ and hsGC $\beta 1$ viruses and cultured for 48 h. Medium was replaced by 10 ml Krebs/Ringer buffer [KRB: 119 mM NaCl/4.74 mM KCl/2.54 mM CaCl₂/1.19 mM MgSO₄/1.19 mM KH₂PO₄/25 mM NaHCO₃/0.1% BSA/10 mM Hepes (pH 7.4)] containing 1 mM IBMX, and cells were incubated for 1 h at 27 °C. For activation of sGC, stock solutions of SNP or YC-1 were added to the KRB. After 2 min at room temperature, cells were washed with ice-cold KRB and harvested. The cGMP content of ethanol-extracted cells was determined with a radioimmunoassay kit (Biotrend, Köln, Germany), in accordance with the manufacturer's protocol.

DNA sequencing and amino acid sequence analysis

PCR products and the original sGC cDNA clones were sequenced in an ABI200 DNA sequencer (Applied Biosystems). The deduced amino acid sequences were aligned by using the Clustal method (Lasergene Multiple Sequence Alignment; DNASTAR, Madison, WI, U.S.A.).

Generation of antisera

Primary antisera were generated by the immunization of rabbits against unique synthetic peptides corresponding to human $\alpha 1$ (Phe-Thr-Pro-Arg-Ser-Arg-Glu-Glu-Leu-Pro-Pro-Asn-Phe-Pro, residues 634–647) and $\beta 1$ (Lys-Gly-Lys-Lys-Glu-Pro-Met-Gln-Val-Trp-Phe-Leu-Ser-Arg-Lys-Asn-Thr-Gly-Thr-Glu-Glu-Thr, residues 593–614) sequences, coupled to keyhole limpet haemocyanin via an additional C-terminal ($\alpha 1$) or N-terminal ($\beta 1$) cysteine residue. Antisera were affinity-purified against the respective peptides coupled to epoxy-activated Sepharose (Pharmacia, Freiburg, Germany) in accordance with the manufacturer's protocol.

Immunodetection of sGC in human tissues

Human lung tissue was obtained from the tumour-free area of lung resections; human cortex and cerebellum were from a normal autopsy procedure. All tissues were immediately frozen in liquid nitrogen and kept at –70 °C. For protein extraction, the frozen tissue was crushed in a liquid-nitrogen-cooled stainless steel mortar. The resulting powder was resuspended in $2 \times$ concentrated Laemmli buffer [26] preheated to 95 °C, incubated

for 10 min at 95 °C, centrifuged for 20 min at 20000 *g*, and the supernatant was subjected to SDS/PAGE [8 % or 10 % (w/v) gel] [26]. Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham) in 48 mM Tris base/39 mM glycine/0.04 % SDS/10 % (v/v) methanol at 1 mA/cm² for 45 min by semi-dry blotting. Equal protein transfer in each lane was verified by reversible protein staining with 0.1 % Ponceau S in 5 % (v/v) acetic acid. Blots were developed with the enhanced chemiluminescence detection system (Amersham) in accordance with the manufacturer's protocol, with either crude antisera (diluted 1:2000, for Sf9 cellular extracts) or affinity-purified antibodies (diluted 1:1000, for tissue extracts), and, as secondary antibody, goat horseradish peroxidase-conjugated anti-rabbit antiserum (DAKO, Glostrup, Denmark). For re-analysis of membranes with a different primary antibody, membranes were rinsed extensively with Tris-buffered saline [10 mM Tris/HCl (pH 8)/50 mM NaCl] containing 0.05 % (v/v) Tween 20.

Statistics

Results are expressed as means $\pm$ S.E.M. for at least $n = 3$ experiments. Values of $P < 0.05$ (analysis of variance) were considered statistically significant.

RESULTS

sGC $\alpha 3$ and $\beta 3$ are the human homologues of rat and bovine sGC $\alpha 1$ and $\beta 1$

In a homology-based screening for human sGC subunits, Giuili et al. [25] isolated $\alpha 3$ and $\beta 3$ cDNA clones from a human brain

cDNA library derived from the frontal lobe. Whereas human $\beta 3$ is almost identical with bovine and rat $\beta 1$ at the amino acid level, human $\alpha 3$ contains two main areas of divergence (designated S1 and S2; see Figure 1A) from rat and bovine $\alpha 1$. Because $\alpha 3$ and $\beta 3$ have not been studied at the protein level, it is unknown whether they are physiologically co-expressed and constitute active heterodimeric enzyme. To address this question we performed PCR experiments to study sGC expression in human tissues (results not shown). However, we were unable to amplify $\alpha 3$ -specific fragments from different human cDNA libraries. All fragments obtained with α -specific primers showed consistently identical discrepancies from the published $\alpha 3$ sequence (results not shown). Therefore either $\alpha 3$ was not expressed and a novel subunit had been detected, or the $\alpha 3$ sequence is genetically polymorphic, or the published sequence contained sequencing errors. Accordingly we re-sequenced the original $\alpha 3$ and $\beta 3$ cDNA clones, kindly provided by the authors, and found 19 incorrect sites within the $\alpha 3$ sequence. The reported $\beta 3$ cDNA sequence was correct. Sequence correction of the $\alpha 3$ subunit led to several frame shifts in S1 and S2, resulting in predicted peptide sequences highly similar to the corresponding sequences in rat and bovine $\alpha 1$ (Figure 1B). In addition, a further small stretch of divergence (residues 526–532 of $\alpha 3$, corresponding to residues 529–535 in $\alpha 1$ terminology; see below) disappeared after correction, and an additional glutamic residue was inserted (between Glu-319 and Tyr-320 of $\alpha 3$, corresponding to Glu-322 in $\alpha 1$ terminology; see below), which is also found in rat and bovine $\alpha 1$ (results not shown). A small N-terminal stretch of decreased similarity between human $\alpha 3$ and rat/bovine $\alpha 1$ (residues 27–60) is also less conserved between rat and bovine $\alpha 1$ (results not

Figure 1 sGC $\alpha 3$ is the human homologue of rat and bovine sGC $\alpha 1$

(A) Domain organization of α isoforms (reviewed in [53]). A putative C-terminal catalytic cyclase domain (hatched) is shared by the sGC $\alpha 1$ and $\beta 1$ subunits, particulate guanylate cyclases and adenylylate cyclase [54]. A central sGC homology region is common to sGC $\alpha 1$ and $\beta 1$ subunits, whereas the N-termini are unique and essential for NO activation [29]. S1 (residues 121–187) and S2 (residues 670–717) are amino acid sequences unique to $\alpha 3$, with no similarity with the $\alpha 1$ isoforms given in [25]. (B) Correction of $\alpha 3$ cDNA sequence eliminates S1 and S2 regions of divergence. Alignments of deduced amino acid sequences in human $\alpha 3$ S1 (top) and S2 (bottom) with the corresponding sequences after cDNA sequence correction (now termed $\alpha 1$), and with rat [17] and bovine $\alpha 1$ [19] are shown. Conserved amino acids are shaded.

Figure 2 Expression and subcellular distribution of rhsGC $\alpha 1$ and $\beta 1$ subunits in Sf9 cells

(A) Sf9 monolayer cultures were either non-infected controls (Co) or infected with hsGC $\alpha 1$ or hsGC $\beta 1$ baculoviruses, and then cultured for 72 h. The 20 000 *g* soluble (S) and particulate (P) fractions of the cell homogenates were analysed by SDS/PAGE [10% (w/v) gel] and Coomassie Blue protein staining. Large amounts of the recombinant polypeptides were found in the pellet fraction. (B) Sf9 monolayer cultures were either non-infected controls (Co) or co-infected with hsGC $\alpha 1$ and hsGC $\beta 1$ baculoviruses. S and P fractions [see (A)] were analysed by protein immunoblotting. Proteins were detected first with anti-hsGC $\beta 1$ polyclonal antiserum (lanes 1–4); subsequently the same blot was re-analysed with anti-hsGC $\alpha 1$ polyclonal antiserum without stripping the $\beta 1$ -specific antibody (lanes 5–8). The asterisk indicates a degradation product of $\alpha 1$.

Figure 3 Effects of SNP and YC-1 on rhsGC in intact Sf9 cells

Sf9 monolayers co-infected with hsGC $\alpha 1$ and hsGC $\beta 1$ baculoviruses were cultured for 48 h and treated for 2 min with 10, 100 or 1000 μ M SNP (columns 2–4), 10 or 100 μ M YC-1 (columns 5 and 6) or 10 or 100 μ M YC-1 in the presence of 100 μ M SNP (columns 7 and 8), or were left untreated (column 1) in the presence of the phosphodiesterase inhibitor IBMX. The total content of cGMP was determined by radioimmunoassay. Results are means $\pm$ S.E.M. for a representative experiment performed in triplicate.

shown). Taken together, correction of the erroneous $\alpha 3$ cDNA sequence eliminated all coherent stretches of divergence at the amino acid level between human $\alpha 3$ and rat/bovine $\alpha 1$. Therefore we suggest a novel isoform classification for $\alpha 3$ as the human homologue of rat and bovine $\alpha 1$. Likewise, $\beta 3$ displays 98.5 %

Table 2 rhsGC activity in Sf9 cellular extracts

Sf9 spinner cultures co-infected with hsGC $\alpha 1$ and hsGC $\beta 1$ baculoviruses were harvested 72 h after infection, and specific sGC activity (pmol of cGMP/mg per min) in the 20 000 *g* soluble fraction was measured in the absence or presence of 100 μ M SNP or 100 μ M YC-1. Expts. 1–3 were performed with independent sGC preparations. Results are means $\pm$ S.E.M. for two or three independent experiments performed in duplicate (Expt. 1), or of a representative experiment performed in triplicate (Expts. 2 and 3).

Expt.	Conditions	rhsGC activity	Stimulation factor
1	Basal	1487 $\pm$ 139	
	Basal + 100 μ M SNP	7966 $\pm$ 1314	5.4
	Basal + 100 μ M YC-1	5522 $\pm$ 1251	3.7
2	Basal	607 $\pm$ 50	
	Basal + 100 μ M SNP	6069 $\pm$ 1077	10.0
3	Basal	1361 $\pm$ 255	
	Basal + 100 μ M SNP	29746 $\pm$ 8747	21.8

and 99 % similarity to rat /bovine $\beta 1$, and therefore represents its human homologue. hsGC $\alpha 1$ and hsGC $\beta 1$ (human soluble GC $\alpha 1$ and $\beta 1$) is suggested as the new nomenclature for the former $\alpha 3$ and $\beta 3$ subunits.

Characterization of rhsGC

To examine whether the human $\alpha 1$ and $\beta 1$ subunits constitute enzymically active heterodimeric sGC, we expressed both subunits in Sf9 insect cells by using the baculovirus expression system. As revealed by SDS/PAGE of fractionated Sf9 cell extracts (Figure 2A), recombinant $\alpha 1$ and $\beta 1$ proteins migrated with apparent molecular masses of 79.5 and 68.5 kDa respectively, which is close to the molecular masses predicted from the respective cDNA species (77.5 and 70.5 kDa). The identities of the respective bands were confirmed by protein immunoblot with subunit-specific antisera raised against peptide sequences near the C-termini of hsGC $\alpha 1$ and $\beta 1$ (Figure 2B).

Basal and SNP-stimulated rhsGC activity was detected in intact Sf9 cells as well as in Sf9 cellular extracts. In intact cells (Figure 3), cGMP levels were elevated 200-fold (20 pmol cGMP/10⁶ cells) compared with non-infected Sf9 cells (0.1 pmol cGMP/10⁶ cells; results not shown), reflecting high basal rhsGC activity. cGMP levels further increased up to 60-fold in response to SNP (1 mM) in a concentration-dependent manner. In soluble fractions of Sf9 cells (Table 2), basal rhsGC activity (up to 1500 pmol cGMP/mg per min) was similar to that of bovine sGC expressed in a similar system [29], and sensitive to SNP. The stimulation factor in response to 100 μ M SNP varied between independent rhsGC preparations, ranging from 5-fold to 20-fold stimulation. This variation seemed to be due both to different basal activities (compare Expt. 1 with Expt. 2 in Table 2) and to variability in sensitivity to NO (compare Expt. 1 with Expt. 3). The observed responsiveness to NO is comparable to that of crude soluble rat platelet fractions (6.5-fold) [30], recombinant rat (3.3-fold) [31] or purified bovine sGC (10-fold) [32]. However, it was significantly lower than observed with some purified bovine sGC preparations (175-fold) [10].

The insoluble particulate fraction of Sf9 cells contained no measurable basal and only weak NO-stimulated sGC activity (results not shown), although large amounts of recombinant $\alpha 1$ and $\beta 1$ were found there, independently of whether the subunits were expressed separately (Figure 2A) or together (Figure 2B). This suggests that, under the current conditions, the bulk of recombinant protein in cellular extracts does not contribute to

Figure 4 Effect of YC-1 on NO- and CO-stimulated rhsGC in Sf9 cellular extracts

Sf9 spinner cultures co-infected with hsgc α 1 and hsgc β 1 baculoviruses were harvested 72 h after infection. sGC activity of the soluble fraction was assayed in the absence (control) or presence of 100 μ M YC-1, 100 μ M SNP, 100 μ M SNP plus 100 μ M YC-1, 200 μ M CO, or 200 μ M CO plus 100 μ M YC-1. Results are means $\pm$ S.E.M. for at least four independent experiments. *Significant difference ($P < 0.05$) from control reaction; **significant difference ($P < 0.05$) from 100 μ M YC-1.

the measured sGC activity. This could be due to misfolding of the protein in insect cells or lack of essential modifications, or could reflect a loss of haem because of suboptimal lysis conditions.

NO-induced sGC activation causes enzyme desensitization or, with nitrovasodilators, tolerance. With YC-1 there is now a

promising lead compound to influence sGC activity independently of NO. YC-1 was first described as a specific NO-independent activator of sGC in rabbit platelets [30,33], and also potentiates the activation of purified bovine sGC by NO [32,34]. Here we investigated whether human α 1/ β 1 heterodimer is also a target of YC-1. Indeed, in Sf9 cellular extracts, rhsGC activity was stimulated up to 3.7-fold by 100 μ M YC-1 (Table 2). Similar stimulation factors have been reported for rat and human platelets [30,33], which might also contain α 1/ β 1 heterodimers, and purified bovine sGC [32,34]. The YC-1 effect was concentration-dependent with an EC_{50} of 20 μ M (results not shown). Moreover YC-1 also stimulated NO-activated rhsGC (Figure 4). It is noteworthy that, in intact cells, 10 μ M YC-1 was sufficient to reach maximum effects on basal and NO-stimulated sGC activity (Figure 3), compared with 100 μ M in cellular extracts. Thus the activation of sGC by YC-1 *in vivo* might involve metabolic potentiation. It has been reported that CO, a putative physiological activator of sGC [35], and YC-1 also act synergistically on sGC [34]. However, CO (200 μ M) had no effect on rhsGC, and the activation by the combination of 200 μ M CO and 100 μ M YC-1 was not significantly more effective than by YC-1 alone (Figure 4).

Detection of sGC α 1 and β 1 in human tissues

To investigate the distribution of α 1 and β 1 in human tissues, we performed protein immunoblots with antibodies against synthetic α 1 and β 1 peptides (Figure 5). sGC immunoreactive polypeptides were detected only in brain and lung. Neither α 1 nor β 1 polypeptides were found in kidney, liver and pancreas under our conditions (results not shown). The detected polypeptides comigrated with the respective subunits expressed in insect cells, indicating no major modification differences between the native and recombinant enzyme subunits. Subunit α 1 was found mainly in cortex and lung; a small amount was also detected in cerebellum (Figure 5A, filled arrowhead). Some faster-migrating faint bands detected in cortex and cerebellum competed fully with the corresponding peptide and might represent degradation

Figure 5 Immunodetection of sGC subunits in human tissues

SDS extracts of rhsGC α 1-overexpressing (A) or β 1-overexpressing (B) Sf9 cells, human cortex, human cerebellum or human lung were subjected to SDS/PAGE [8% (w/v) gel] followed by protein immunoblotting. Blots were developed with affinity-purified anti-hsGC α 1 (A) or anti-hsGC β 1 (B) polyclonal antibodies in the absence or presence of the respective peptide. Filled arrowhead, α 1; open arrowhead, β 1; arrow, cross-reactivity of anti-hsGC α 1.

Table 3 Revised terminology of NO receptor cyclase cDNA species and/or proteins detected in human tissues

Isoform	Subunit ... α	β
1	cDNA, protein; active when co-expressed with human $\beta 1$	cDNA, protein; active when co-expressed with human $\alpha 1$
2	cDNA; active when co-expressed with bovine $\beta 1^*$	Not detected

* Reference [25].

products of $\alpha 1$. Interestingly, a high-molecular-mass protein (more than 150 kDa) was specifically detected by anti-hsGC $\alpha 1$ in human cortex (arrow in Figure 5A). It is as yet unknown whether this polypeptide has an accidental overlap with the hsGC $\alpha 1$ peptide sequence, or whether it represents an sGC-related protein. Specific signals for $\beta 1$ were detected in human cortex, cerebellum and lung (Figure 5B, open arrowhead). Taken together, these results indicate that both human $\alpha 1$ and $\beta 1$ proteins are expressed *in vivo*, occur together in the same organs and therefore might constitute the active NO receptor in humans.

DISCUSSION

The NO-cGMP signalling pathway, with sGC as the central enzyme, has an important role in maintaining blood pressure, inhibiting platelet adhesion and aggregation, modulating neuronal transmission and many other physiological processes [36]. Therefore sGC is an important target protein for therapeutic drugs such as nitrovasodilators [37,38] or new antithrombotic agents [39]. In this paper we present for the first time the expression and initial characterization of an active rhsGC and the detection of the corresponding sGC polypeptides in human tissues. Furthermore we found that the sGC subunits previously described as $\alpha 3$ and $\beta 3$ in fact represent the human homologues of rat and bovine $\alpha 1$ and $\beta 1$. Taken together with rhsGC activity and human sGC expression *in vivo*, this implies that the human $\alpha 1/\beta 1$ heterodimer is also the functional homologue of rat or bovine sGC. The resulting new terminology of the revised isoenzyme family is depicted in Table 3.

The enzyme activity of expressed rhsGC is sensitive to SNP and the NO-independent activator YC-1, as reported for purified bovine sGC [32,34]. However, the responsiveness of rhsGC to NO seemed to decline on the lysis of cells, as intracellular cGMP levels of rhsGC-expressing cells increased markedly in response to SNP, whereas the stimulation factors observed in cellular extracts were only moderate (up to 20-fold). This suggests that either NO sensitivity declined and/or basal rhsGC activity increased on extract preparation, which also seemed to contribute to the observed variation in stimulation factors. Loss of NO sensitivity could be due to loss of haem, which has been proposed to increase basal sGC activity as well. [40]. Alternatively, an increase in basal activity could be due to autoactivation of sGC, the exact mechanism of which is unknown but seems to involve redox processes [41,42].

Previous studies have shown that transcription of sGC subunits and presence of mRNA is regulated in a manner specific to cell type [43–45] and development [46], and influenced by extracellular and intracellular messenger molecules [47,48]. In contrast, it was reported that rat $\alpha 1$ and $\beta 1$ as well as human $\alpha 2$ mRNA species are widely expressed in different tissues [17,21]. Here we show that large amounts of $\alpha 1$ and $\beta 1$ subunits were restricted to only a few of the human tissues so far analysed. Human sGC was

found in cortex and cerebellum, consistent with the role of NO and possibly CO in neurotransmission [35,49], and also in lung, a prominent source of native sGC in other species [8,10]. Interestingly, the relative concentrations of $\alpha 1$ were high in lung and low in cerebellum, whereas much more $\beta 1$ was found in cerebellum than in lung, suggesting that complementary sGC subunits are not necessarily expressed in a 1:1 ratio. However, the physiological significance of this finding remains to be elucidated.

It would be of great therapeutic interest to have a non-NO-releasing activator of sGC, because all known nitrovasodilators are prone to tolerance and cross-tolerance to other nitrovasodilators [50,51]. YC-1 is a good candidate for a lead substance for such drugs. Here we show that YC-1 is also an activator of human sGC and increased the effectiveness of submaximally stimulating concentrations of NO. Therefore YC-1 or related substances might be able to potentiate the effects of NO-releasing nitrovasodilators or, more importantly, of endogenous endothelium-derived NO. At present there is also discussion of whether CO is a physiological ligand of sGC [13,15,52]. Because YC-1 was reported to potentiate the activation of sGC by CO, it was proposed that a putative endogenous YC-1-like molecule or sGC binding protein might enhance weak effects of CO *in vivo* [34]. However, we were unable to stimulate rhsGC by CO, and activation by the combination of YC-1 and CO was not significantly more effective than by YC-1 alone. This might be explained by the overall lower responsiveness of the rhsGC preparation used here, compared with the bovine enzyme analysed by Friebe et al. [34].

Importantly, our findings show that the pharmacological principle of YC-1 is fully transferable to human sGC. However, the mechanism of action of YC-1 is not understood. Although YC-1 seems to act directly on sGC, we found that in rhsGC-expressing Sf9 cells, YC-1 was 10-fold more potent than in cellular extracts, suggesting that additional factors or metabolic events might be involved when YC-1 elevates the cGMP concentration in living cells.

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