Volume 292, number 1,2, 217–222 FEBS 10353 November 1991

ADONIS 001457939101059V

Molecular cloning and expression of a new α-subunit of soluble guanylyl cyclase

Interchangeability of the α-subunits of the enzyme

Christian Harteneck, Barbara Wedel, Doris Koesling, Jürgen Malkewitz, Eycke Böhme and Günter Schultz

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67–73, D-1000 Berlin 33, Germany

Received 5 September 1991; revised version received 17 September 1991

A cDNA coding for a new subunit of soluble guanylyl cyclase with a calculated molecular mass of 81.7 kDa was cloned and sequenced. On the basis of sequence homology, the new subunit appears to be an isoform of the α_1-subunit and was designated α_2 as the new subunit is very similar to the α_1-subunit in the middle and C-terminal part; it is quite diverse in the N-terminal part. Preceding experiments had shown that coexpression of the α_1- and β_1-subunits is necessary to obtain a catalytically active guanylyl cyclase in COS cells [(1990) FEBS Lett. 272, 221–223]. The finding that the α_2-subunit was able to replace the α_1- but not the β_1-subunit in expression experiments demonstrates the interchangeability of the α-subunit isoforms of soluble guanylyl cyclase.

Soluble guanylyl cyclase; cDNA; Amino acid sequence; Expression

1. INTRODUCTION

Guanylyl cyclases exist in membrane-bound and cytosolic forms. Membrane-bound guanylyl cyclases consist of one polypeptide chain with a single transmembrane domain and belong to the group of receptor-linked enzymes. The isoforms of the membrane-bound enzyme differ in their N-terminal extracellular receptor domains, which are coupled to a common intracellular catalytic domain [1,2]. In contrast to the membrane-bound enzymes, cytosolic guanylyl cyclase has been purified as a heterodimer consisting of 73 kDa (α_1) and 70 kDa (β_1) subunits, which both have been cloned and sequenced [3–6]. The amino acid sequences revealed homologies between the subunits including the putative catalytic domain in the C-terminal region, which is also shared with the membrane-bound guanylyl cyclases and adenylyl cyclases [2]. Expression experiments revealed that coexpression of both subunits is required for the formation of an active enzyme [7,8]. Another subunit of soluble guanylyl cyclase was recently cloned and sequenced, which shows high homologies towards the β_1-subunit and was therefore designated β_2 [9]. As this subunit has not been coexpressed with any other guanylyl cyclase subunit, it is not known whether this subunit can form a catalytically active heterodimer with either the α_1- or the β_1-subunit or whether another not yet identified subunit is necessary for the assembly of a functional enzyme. Here we report on a new soluble guanylyl cyclase subunit, whose cDNA was amplified by the polymerase chain reaction (PCR) and subsequently isolated from an human fetal brain library.

Correspondence address: D. Koesling, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, D-1000 Berlin 33, Germany.

2. MATERIALS AND METHODS

Degenerated oligonucleotides – (antisense: CGGTCGACTTC/TTCIGAC/TATC/TGTIGGITTC/TAC/AIGC, sense: CGAAGCTTCCA/GAAIAG/AG/ACAG/ATAICG/AIGGCAT) – corresponding to highly conserved amino acid sequences within the putative catalytic domain of the guanylyl cyclases, with restriction endonuclease recognition sites added to the 5'-end to aid subcloning amplified products, were used as primers in the PCR to amplify cDNAs coding for guanylyl cyclases.

RNA (5 μg) which had been isolated from oposum kidney cells as described by Turpen and Griffith [10] was used to synthesize cDNA. The PCR was performed in the presence of 120 pmol of each primer and 5 units of Taq-Polymerase (Serva, Heidelberg, Germany) in 30 cycles (92°C, 90 s; 55°C, 120 s; 72°C, 180 s) in a thermocycler. The PCR fragments were incubated with the large fragment of DNA polymerase I (Klenow) to fill possible recessed 3'-termini and digested with *Sal*I and *Hin*dIII recognizing sequences introduced by the primers, separated on 1% agarose gels, subcloned in M13 and sequenced by the dideoxy chain termination method using Sequenase (USB, Bad Homburg, Germany). A cDNA library from human fetal brain constructed in lambda ZAP by Stratagene was screened with the subcloned PCR product coding for the new soluble guanylyl cyclase subunit as a probe. Labelling of the fragment was performed by random oligonucleotide priming and extension of double-stranded DNA [11]. The procedures for screening were carried out as described by Maniatis et al. [12], and hybridization was performed at 65°C. Membranes were washed at 65°C with 2 × SSC (1 × SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0) containing 0.1% SDS (w/v), if the cDNA used as a

probe was derived from a species other than the library; otherwise, the membranes were washed with 0.1 × SSC containing 0.1% SDS (w/v).

For Northern blots, total RNA was isolated as described by Cathala et al. [13]. Total RNA (10 μg) was glyoxylated, separated on agarose gels and blotted on GeneScreen nylon membranes. Hybridization in the presence of formamide at 42°C was carried out with a single-stranded probe derived from a 2.4 kb cDNA insert cloned in M13. Washing of Northern blots (60°C) was performed according to the GeneScreen instruction manual.

For expression, the full length clone HR0 was excised from lambda ZAP II by *Not*I and *Xho*I and subcloned in *Sal*I and *Eag*I-cut pBR 322. The resulting construct was digested with *Sac*II to achieve elimination of two ATGs (see Results) on two small *Sac*II fragments in the 5'-non-coding region. After separation on an agarose gel, the vector and the fragment containing the coding region were eluted and religated. The resulting clone was digested with *Hin*dIII and *Xba*I, the insert was isolated and subcloned in *Hin*dIII- and *Xba*I-cut expression vector pCMV. For transfection, about 4×10^6 COS cells (African green monkey kidney cells transformed by an origin-defective simian virus 40 mutant) were seeded on 150 cm^2 plates. On the next day, the cells were transfected with 14 μg of plasmid per plate by the DEAE-dextran method [14]. After 72 h, the cells were washed with phosphate-buffered saline solution and scraped off the plates in a 50 mM triethanolamine-HCl buffer, pH 7.4, containing 1 mM dithiothreitol. After passing the cells 10 times through a 22-gauge needle, the suspensions were centrifuged for 20 min at 200 000 × g_{av}.

Guanylyl cyclase activity of the obtained cytosolic fraction (30–50 μg of protein per assay tube) was determined by incubation for 15 min at 37°C in the presence of 50 mM triethanolamine-HCl buffer, pH 7.4, containing 3 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic GMP, 5 mM creatine phosphate, 4.6 U/tube creatine phosphokinase, 0.05 mM [α-^{32}P]GTP (about 0.5 μCi) and 3 mM $MnCl_2$, with or without 0.1 mM sodium nitroprusside (SNP), in a total volume of 0.1 ml, as described previously [15].

For immunoblotting, cytosolic COS fractions were subjected to 10% SDS polyacrylamide gels containing 4 M urea and transferred electrophoretically to a nitrocellulose membrane in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol at 250 mA for one hour. Processing of the filters and treatment with antibodies was performed as previously described [16] except that the ECL Western blotting detection system according to the recommendation of Amersham was used for detection. The antibody used is directed against a peptide (VYKVETVGDKYMTVSGLP) of the β_1-subunit; this sequence is highly conserved among the subunits of soluble guanylyl cyclase. Coupling of the peptides and immunization was performed as described [16].

3. RESULTS

New members of the guanylyl cyclase family were searched for, using the PCR to amplify template cDNA prepared from opossum kidney cells. Sequence analysis of the 300 bp long PCR products revealed clones corresponding to the sequences of GC-B and GC-C and clones containing a sequence similar to the α_1-subunit of soluble guanylyl cyclase. The insert of the new clone was used as a probe to screen a human fetal brain cDNA library. Besides several clones containing only parts of the sequence or introns, one clone (HR04; 2953 bp) with an open reading frame was found, which encoded a protein with a calculated molecular mass of 81.7 kDa. The nucleotide and deduced amino acid sequences of HR04 are presented in Fig. 1. The potential initiation codon at bp 391 is preceded by the sequence CGGCAGC, which is in reasonable agreement with Kozak's rules for a consensus sequence for eukaryotic initiation sites [17]. Two other ATGs (bp 84 and 211) further upstream in the 5'-non-coding region are in other reading frames and are followed by stopcodons. The open reading frame stops at bp 2587, and there are two polyadenylation sites (bp 2761 and 2876) in the 3'-non-coding region. Blot hybridization analysis of total RNA derived from the human cell lines A431 (epithelial tumor), HEL (erythroleukemia) and SH-SY5Y (neuroblastoma) with the cDNA coding for the α_2-subunit exhibited a hybridizable RNA with an estimated size of 3.6 kb (data not shown). Comparison of the deduced amino acid sequence of HR0 with the sequences of the other subunits of soluble guanylyl cyclase (Fig. 2) reveals that the new subunit most closely resembles the α_1-subunit as 48% of the amino acids are shared with the α_1-subunit. The protein encoded by HR0 was, therefore, designated the α_2-subunit. The relative amount of identical amino acids between the α_1- and α_2-subunits ranges from 27% identities in the N-terminal region to about 87% in the central part of the sequences and in the C-terminal domain, the latter domain being conserved between all guanylyl cyclases. There are almost no identities, however, in the last 30 C-terminal amino acids of the α_1- and α_2-subunits. To ensure that the differences between the α_1- and α_2-subunits are not due to the different species from which the respective cDNAs were obtained, a cDNA encoding for a part of the α_2-subunit was also isolated from bovine brain (data not shown). The overall homologies of the α_2-subunit to the β-subunits are considerably lower than to the α_1-subunit, as only 30% and 23% of identical amino acids are shared with the β_1- and β_2-subunits, respectively.

In expression experiments, COS cells were transfected with combinations of the available cDNAs coding for the various subunits of soluble guanylyl cyclase, i.e. α_1, α_2 and β_1, in the expression vector pCMV, to study their ability to form catalytically active dimers of the enzyme. Immunoblotting experiments were performed with cytosolic fractions of the transfected cells to verify the expression of the subunits. As shown in Fig. 3, an antibody raised against a peptide with the sequence of the β_1-subunit, whose sequence is conserved between all guanylyl cyclases, recognized proteins with appropriate molecular masses (approximately 70 kDa, β_1; 73 kDa, α_1; 79 kDa, α_2) in the cytosols of the cells which had been transfected with the respective cDNAs. The subunits were not detected when the peptide was present during incubation with the antibody, whereas additional faint bands remained unaffected under these conditions indicating unspecific binding. The less intensive recognition of the α_1- and α_2-subunits (in comparison with the β_1-subunit) can be explained by the differences between the sequence of the peptide used for immunisation and the sequences of α_1- and α_2-subunits.

In parallel, basal guanylyl cyclase activity and stimu-

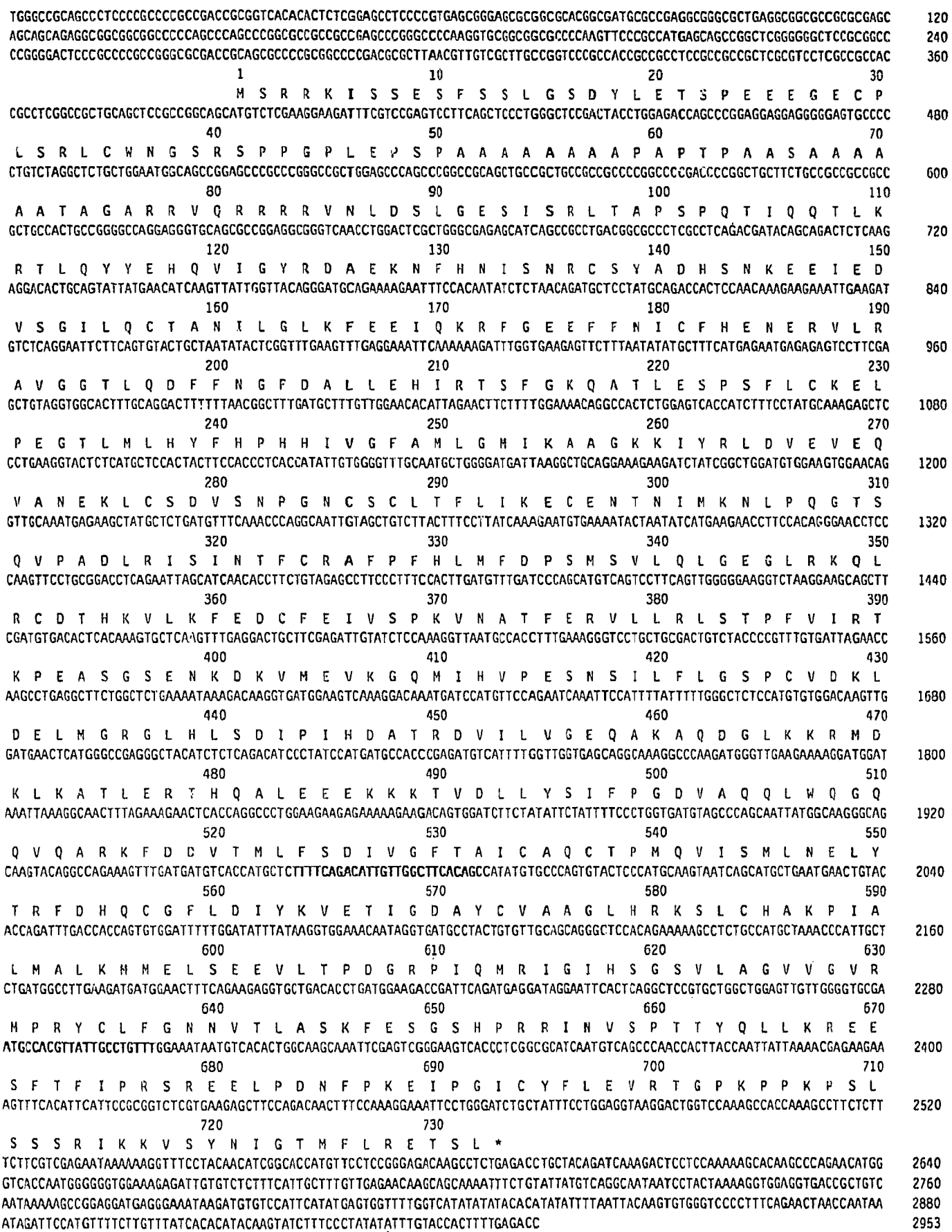

Fig. 1. Complete nucleotide and deduced amino acid sequences of the α_2-subunit of soluble guanylyl cyclase. The nucleotides used in the PCR are indicated by bold letters.

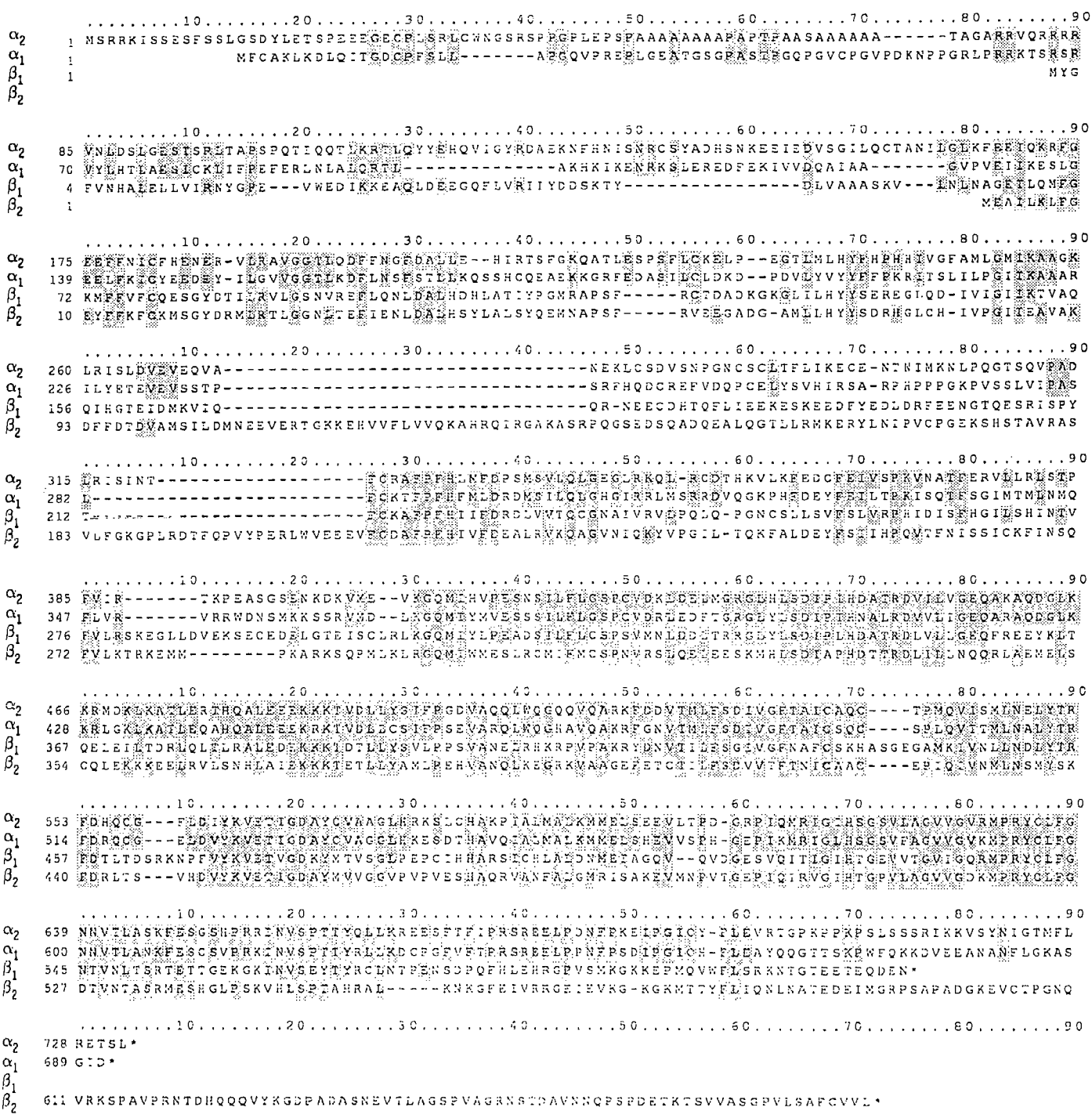

Fig. 2. Alignment of amino acid sequences of four subunits of soluble guanylyl cyclase. The sequences shown are the new α_2-subunit of human soluble guanylyl cyclase, the α_1-subunit of bovine soluble guanylyl cyclase [5], the β_1-subunit of bovine soluble guanylyl cyclase [3] and the β_2-subunit of rat soluble guanylyl cyclase [9]. Identities of amino acids with the α_2-subunit are shadowed.

lation by SNP was determined in cytosolic fractions of the transfected cells. In the cytosol of untreated COS cells and in the cytosol of COS cells that had been transfected with the vector without an insert, cyclase activity was undetectable (Fig. 4). Expression of the α_2-subunit alone did not result in an increase in cGMP formation, confirming that an assembly of a heterodimer is required to yield a catalytically active enzyme [7,8]. Coexpression of the α_2-subunit with the β_1-subunit, but not with the α_1-subunit, led to the formation of a functionally active guanylyl cyclase with specific activities between 20 and 100 pmol of cGMP·min^{-1}·mg^{-1}. SNP enhanced GC activity to 300 and 800 pmol of cGMP·min^{-1}·mg^{-1}, corresponding to an

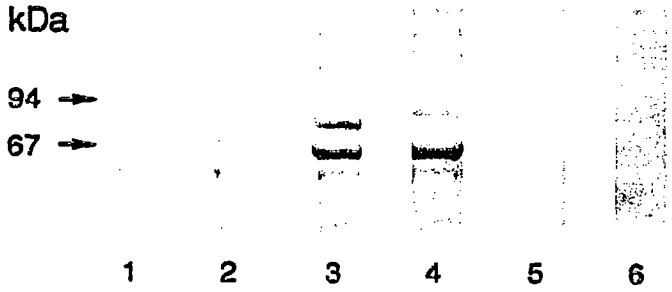

Fig. 3. Immunoblotting analysis of 100 000 × g supernatants of transfected COS-7 cells. Cytosolic proteins of COS cells (100 μg) transfected with 1:1 mixture of the expression vector pCMV containing the coding region of the β_1-subunit and pCMV with the coding region of the α_1-subunit (lanes 3 and 5) and of COS cells transfected with a 1:1 mixture of pCMV containing the cDNA of the β_1-subunit and pCMV with the α_2-subunit (lanes 4 and 6) were electrophoresed on a 10% SDS polyacrylamide gel containing 4 M urea. In lanes 1 and 2, cytosol of non-transfected COS cells and of COS cells which were transfected with the vector without an insert, respectively, were applied. The proteins were transferred to nitrocellulose membranes and incubated with a peptide antibody against the β_1-subunit, which crossreacts with the α_1- and α_2-subunits. The antibody incubation of stripes 5 and 6 was performed in the presence of the peptide.

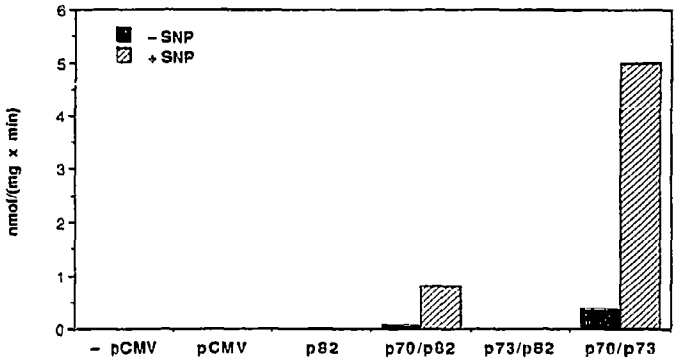

Fig. 4. Guanylyl cyclase activity of expressed subunits. Activity of soluble guanylyl cyclase was determined in the cytosol of non-transfected cells (- pCMV) and in the cytosols of COS cells transfected with unmodified expression vector (pCMV) and with the vector containing the cDNA coding for the α_2-subunit (p82). For expression of the heterodimers, COS cells were transfected with a 1:1 mixture of the vectors containing the cDNA coding for the β_1-subunit and for the α_2-subunit (p70/p82), with a 1:1 mixture of vectors containing the cDNA coding for the α_1-subunit and for the α_2-subunit (p73/p82), and with a 1:1 mixture of vectors containing the cDNA coding for the β_1-subunit and for the α_1-subunit (p70/p73). Enzyme activities were determined with (0.1 mM) and without sodium nitroprusside (SNP) three days after treatment of the cells with or without the expression vectors. Data are shown for one representative experiment out of three similar experiments, performed in triplicates.

about 10-fold increase in enzyme activity. Control experiments with coexpressed α_1- and β_1-subunits yielded an enzyme exhibiting 3–6 times higher basal and SNP-stimulated activities. The lower activity of the $\alpha_2\beta_1$ heterodimer was not caused by a relative lack of α_2- subunit as doubling the amount of cDNA coding for the α_2-subunit used for transfection led only to a slight increase in enzyme activity (data not shown).

4. DISCUSSION

Yuen et al. [9] identified an isoform of soluble guanylyl cyclase subunits which, on the basis of sequence homology, was designated β_2. Here we report on another subunit of soluble guanylyl cyclase with a calculated molecular mass of 81.7 kDa, which shows the highest degree of homology towards the α_1-subunit and was, therefore, designated α_2. Expression experiments showed that this subunit indeed represents an isoform of the α-subunit as coexpression of α_2 and β_1 but not of α_2 and α_1 leading to the assembly of a catalytically active enzyme. These results confirm the necessity of the coexpression of an α- and β_1-subunit for a functional soluble guanylyl cyclase and beyond that demonstrate the interchangeability of the isoforms of the α-subunits. Nothing is known so far about different biological functions of the isoforms. Besides the lower basal and stimulated activity of the $\alpha_2\beta_1$-heterodimer with possibly higher activity of the $\alpha_2\beta_2$-heterodimer, we were not able to detect differences in regulations, at least with regard to activation by NO-containing compounds. The existence of another potent stimulator of soluble GC cannot be excluded but does not seem very likely as both heterodimers are activated by NO, which appears to be a universal signal molecule found in more and more tissues [18]. The α_1-subunit may be expressed under conditions with a relatively high demand for cGMP, whereas the α_2- subunit is expressed to form a less active guanylyl cyclase. Further experiments have to show if the expression of the isoforms of the subunits is cell type-specific or can be differentially regulated within one cell in response to exogenous conditions.

Acknowledgements: We thank Drs. Frederic Libert and Gilbert Vassart for helpful suggestions regarding the PCR and for supply of oligonucleotides. This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

REFERENCES

[1] Chinkers, M. and Garbers, D.L. (1991) Annu. Rev. Biochem. 60, 553–575.
[2] Koesling, D., Böhme, E. and Schultz, G. (1991) FASEB (in press).
[3] Koesling, D., Herz, J., Gausepohl, H., Niroomand, F., Hinsch, K-D., Mülsch, H., Böhme, E., Schultz, G. and Frank, R. (1988) FEBS Lett. 239, 29–34.
[4] Nakane, M., Saheki, S., Kuno, T., Ishii, K. and Murad, F. (1988) Biochem. Biophys. Res. Commun. 157, 1139–1147.
[5] Koesling, D., Harteneck, Ch., Humbert, P. Bosserhoff, A., Frank, R., Schultz, G. and Böhme, E. (1990) FEBS Lett. 266, 128–132.
[6] Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W. and Murad, F. (1990) J. Biol. Chem. 265, 16841–16845.
[7] Harteneck, Ch., Koesling, D., Söling, A., Schultz, G. and Böhme, E. (1990) FEBS Lett. 272, 221–223.
[8] Buechler, W.A., Nakane, N. and Murad, F. (1991) Biochem. Biophys. Res. Commun. 174, 351–357.
[9] Yuen, P.S.T., Potter, L.R. and Garbers, D.L. (1990) Biochemistry 29, 10872–10878.
[10] Turpen, T.H. and Griffith, O.M. (1986) BioTechniques 4, 14–15.
[11] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.

[12] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Publications, Cold Spring Harbor, New York.
[13] Cathalia, G., Savouret, J.-F., Mendez, B., West, B.L., Karin, M., Martial, J.A. and Baxter, J.G. (1983) DNA 2, 329–335.
[14] Cullen, B.R. (1987) Methods Enzymol. 152, 684–704.
[15] Schultz, G. and Böhme, E. (1984) in: Methods of Enzymatic Analysis, 3rd edn., vol. 4 (Bergmeyer, H.U., Bergmeyer, J. and Graßl, M. eds.) pp. 379–389, Verlag-Chemie, Weinheim.
[16] Hinsch, K.-D., Tychowiecka, I., Gausepohl, H., Frank, R., Rosenthal, W. and Schultz, G. (1989) Biochim. Biophys. Acta 1013, 60–67.
[17] Kozak, M. (1989) J. Cell Biol. 108, 229–241.
[18] Moncada, S., Palmer, M.J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–134.