Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 351-357

Expression of soluble guanylate cyclase activity requires both enzyme subunits

Wolfgang A. Buechler *#[1], Masaki Nakane * and Ferid Murad *

*Pharmaceutical Discovery, Abbott Laboratories,
Abbott Park, Illinois 60064

#Department of Pharmacology
Northwestern University Medical School
Chicago, Illinois 60611

Received November 27, 1990

Summary. - Soluble guanylate cyclase purified from rat lung exists as a heterodimer of two subunits (70 kDa and 82 kDa). Recent cloning and sequencing of both subunit entities have revealed their primary structures. Transient expression in COS-7 cells by transfection with expression vectors containing the coding regions of the 70 kDa or the 82 kDa subunit cDNA showed no guanylate cyclase activity when cells were transfected with either subunit cDNA alone. However, a marked enzymatic activity was found after transfection with both subunits that was activated by sodium nitroprusside. The combination of separately expressed guanylate cyclase subunits could not reconstitute enzymatic activity *in vitro*. Furthermore, cotransfection with antisense oligonucleotides against the 70 kDa subunit or the 82 kDa subunit mRNA inhibited the guanylate cyclase activity. These data indicate that both the 70 kDa and the 82 kDa subunits must be present and interactive with each other in order to see basal guanylate cyclase activity and activation with sodium nitroprusside. © 1991 Academic Press, Inc.

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing); EC 4.6.1.2) catalyzes the formation of guanosine 3',5' - cyclic monophosphate (cGMP) from GTP. In a variety of tissues and cell systems, cGMP has been established as an intracellular signal molecule. For example cGMP plays an important role in vascular smooth muscle relaxation by endothelium-dependent and independent vasodilators (1). Also, during the retinal

[1]To whom correspondence should be addressed at Dept. 47R, AP9A, #119, Abbott Laboratories, Abbott Park, Il 60064, Fax: 708-688-7135.

Abbreviations:
sodium nitroprusside, SNP; complementary DNA, cDNA; guanosine 3',5' - cyclic monophosphate, cGMP.

0006-291X/91 $1.50
Copyright © 1991 by Academic Press, Inc.
All rights of reproduction in any form reserved.

phototransduction process, cGMP directly effects sodium channel opening in the plasma membrane (2).

To date, four different isoenzymes of guanylate cyclase have been characterized showing that the enzyme exists in a soluble and several particulate isoforms (3,4). The membrane isoenzyme form exists as single polypeptide chain with extracellular receptor and intracellular catalytic domains and can be activated by atrial natriuretic peptides (5-6). On the other hand, the soluble guanylate cyclase is a heterodimer of 70 kDa and 82 kDa subunits (7-8) and can be activated by nitric oxide (NO) or other NO-generating substances (9-11).

Recently, we and others have confirmed on a molecular level that the isoenzymes are structurally different proteins (12-14). In addition, DNA sequence and predicted amino acid sequence comparisons between the 70 kDa and the 82 kDa subunits have shown a high degree of homology (45% identical) in the carboxy-terminal portion of the proteins (15). Considerable homology was found within the carboxy-terminus of the two soluble guanylate cyclase subunit proteins when compared with the cytoplasmic, catalytic domain of the particulate enzyme from rat brain (16,17) and also with predicted catalytic domains of bovine brain adenylate cyclase (18). Although both soluble guanylate cyclase subunits are now characterized with regard to their primary molecular structure, it still remains unclear how both subunits interact to permit cGMP synthesis and nitrovasodilator activation.

We describe here the transient expression of soluble guanylate cyclase subunits in COS-7 cells and demonstrate that both subunits must be coexpressed and interact *in vivo* to allow the catalytic and regulatory activity of the enzyme.

Materials and Methods

Materials. - Restriction endonucleases were from Boehringer Mannheim (Indianapolis, IN). T4 DNA ligase, Lipofectin transfection reagent and Opti-MEM I medium were from BRL (Gaithersburg, MD). The mammalian expression vector pcDL-SRα was kindly provided from Dr. Takebe (AIDS Research Center, National Institute of Health, Tokyo, Japan). COS-7 cells were from American Type Culture Collection (Rockville, MD).

Subcloning of soluble guanylate cyclase cDNAs. - Based on the unique EcoRI-site of the parental expression vector (pcDL-SRα / EcoRI 478) that contains the SV40 early promoter-enhancer and parts of the long terminal repeat (LTR) of human T-cell leukemia virus type 1 (HTLV-1) (19), we subcloned the entire 1875- nucleotide coding region for the 70 kDa subunit or the 2070-nucleotide coding region for the 82 kDa subunit generated from rat lung soluble guanylate cyclase in both orientations (either of them in order and reverse direction).

Cell culture and expression in COS-7 cells. - 2 x 10^6 COS-7 cells/well were grown in Dulbecco's Modified Eagles Medium (DMEM) to 80% confluency in 6-well plates (Costar). On the day of transfection the medium was changed to Opti-MEM I with the reduced serum content to increase the transfection efficiency. Cells were then transfected for 6 hrs with a mixture of Lipofectin (20) and the plasmid DNAs (3 µg/well). After changing the medium to DMEM (+10% fetal calf serum), the cells were

further incubated for 64 hrs of culture. The cells were washed twice with cold phosphate-buffered saline solution and scraped off in 200 µl of 50 mM triethanolamine-HCl buffer pH 7.6. After sonication of the cells (on ice, 30 sec), the suspension was centrifuged for 20 min (4°C) at 100 000 x g. The supernatant fraction was removed, kept on ice and assayed for guanylate cyclase activity.

Cotransfection of antisense oligonucleotides and soluble guanylate cyclase subunit cDNAs. - According to the consensus sequence for the translational start site (ATG, Kozak's rule (21)) we synthesized the following oligonucleotides (21-mer). For inhibition of the 70 kDa and the 82 kDa mRNA translations, the antisense oligonucleotides: 5'-TAC ATG CCA AAA CAC TTG GTA-3', and 5'-TAC AAG ACG TCC TTC AAG TTT-3' respectively, were made. Together with the expression vectors that contain the cDNAs of soluble guanylate cyclase subunits, the antisense oligonucleotides were transfected to cells using Lipofectin.

Assay of guanylate cyclase activity. - The total protein amount in the supernatant fractions was measured according to Bradford (22). All enzymatic assays were performed using 30 µg of supernatant protein. Each sample was preincubated for 10 min at 37°C with or without 0.1 mM sodium nitroprusside (SNP) in a final volume of 0.1 ml of 50 mM Tris- HCl buffer pH 7.6 containing a final concentration of 1.25 mM 3-isobutyl-1-methyl-xanthine, 7.5 mM creatine phosphate and 5 units creatine phosphokinase. Guanylate cyclase assays were started by addition of 4 mM $MgCl_2$ and 1 mM GTP. After 10 min incubation at 37°C, the reaction was terminated by addition of 0.9 ml of 50 mM ice-cold sodium acetate buffer (pH 4.0), and heated at 90°C for 3 min. The amount of cGMP formed was determined by radioimmunoassay, as described previously (23).

Results and Discussion

Expression of soluble guanylate cyclase activity in COS-7 cells needs both subunits (the 70 kDa and the 82 kDa). - Complementary DNAs, containing the entire coding region of either the 70 kDa or the 82 kDa subunit of soluble guanylate cyclase from rat lung were subcloned in a mammalian expression vector pcDL-SRα in the sense or antisense orientation. With transient expression in COS-7 cells we could clearly detect guanylate cyclase activity only in cells which were transfected with both subunit cDNAs (Fig.1). Compared with cell extracts from non-transfected cells or from cells transfected either with the 70- or the 82 kDa subunit cDNAs alone (in sense orientation), a 13 to 15-fold increase of guanylate cyclase activity with 0.1 mM sodium nitroprusside was observed when we transfected the cells with both cDNAs together. Maximal expression of guanylate cyclase activity during the transfection of COS-7 cells could be detected 64 hrs after the transfection and this was clearly dependent on the amount of the DNA transfected (data not shown). Coexpression of both subunit cDNAs in reverse orientation did not show increased enzymatic activity. When both subunits were co-expressed, the increased activity of guanylate cyclase with sodium nitroprusside was consistently demonstrated, although the low basal activities (without SNP) were somewhat variable between experiments. While we don't have an explanation for this variabilty, stimulation of enzyme activity by SNP also ranged from 3-fold to 15-fold between

experiments. We also tested the expression levels of the 70 kDa and the 82 kDa subunit protein by Western Blot analysis and found that the amount of protein expressed is the same when we transfected cells with either subunit cDNA alone or both together (data not shown).

Combined cytosols from separate transfected soluble guanylate cyclase subunits lack the ability to reconstitute full enzyme activity. - To investigate whether a functionally active guanylate cyclase can be reconstituted *in vitro*, we have combined cytosols from COS-7 cells, that have been separately transfected either with the 70 - or the 82 kDa subunit cDNAs. We changed the mixing ratio between cytosols of the expressed 70- and the 82 kDa proteins (the total protein amount was kept constant with 30 µg) from separately transfected cells and compared the enzyme activity with cytosols from cells either transfected alone (70 or 82) or with both cDNAs (Fig.2). Our results with various combinations of these cytosolic fractions showed no significant reconstitution of guanylate cyclase activity (± SNP treatment). These results indicate that the single guanylate cyclase subunits

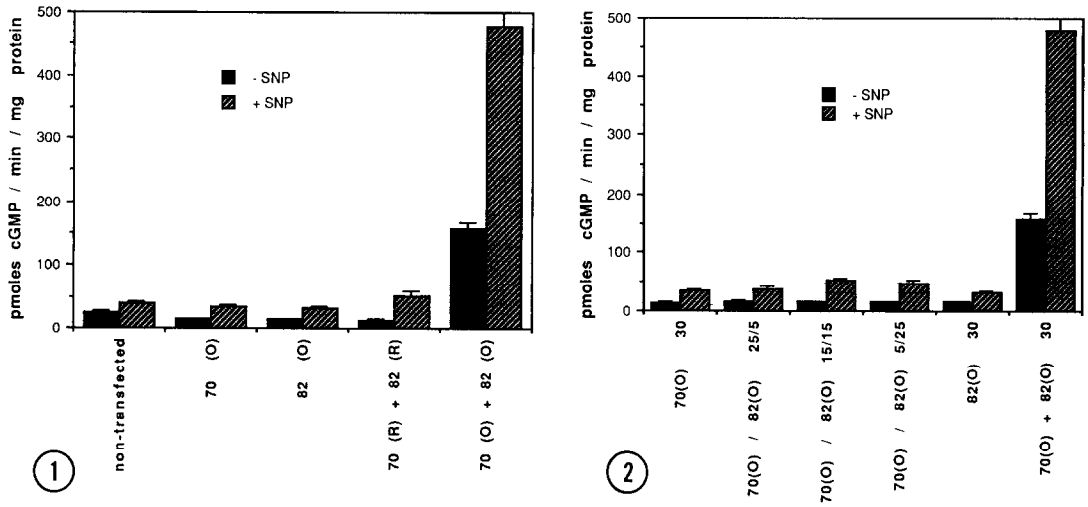

Fig. 1
Transient expression of cDNAs to the 70 kDa and 82 kDa subunits of soluble guanylate cyclase in COS-7 culture. Transfections were performed with the respective cDNAs either alone or together, as indicated. After 64 hrs in culture, the cells were harvested to measure guanylate cyclase activity as described in Materials and Methods. An effect of the orientation of the cDNAs (O=order, R=reverse) on enzyme activity (±SNP) was also tested. Data are shown as means ±SD of triplicates of one representative experiment .

Fig. 2
Effect of combination of cytosolic fractions from separately transfected COS-7 cells with the 70 kDa or the 82 kDa cDNA alone. Soluble guanylate cyclase activity was determined without and with 0.1 mM SNP stimulation and compared with supernatant fractions from cells transfected with both cDNAs together (right column). The total protein amount tested for enzymatic activity was kept constant (30 µg) and is designated. The data shown are means ± SD from triplicate culture plates.

expressed *in vivo* cannot become a functionally active holoenzyme, when combined *in vitro* .

Antisense oligonucleotides directed against the mRNAs for the 70 and the 82 kDa subunits inhibit soluble guanylate cyclase activity. We co-transfected COS-7 cells with both cDNAs to the 70 and- 82 kDa subunits plus the antisense oligonucleotides made against the translation start sites of the 70 and- 82 kDa mRNAs (Fig.3). Because of the instability of oligonucleotides during cell culture (24), we used relatively high concentrations (5 μM) of antisense oligonucleotides in these experiments. We were able to see considerable inhibition (between 65% and 88%) of the guanylate cyclase activity (± SNP) from supernatant fractions of transfected cells when antisense oligonucleotides to either or both the 70 kDa or 82 kDa subunits were used. These results indicate that suppressing the translation of either subunit alone was sufficient for loss of enzymatic activity. Since the co-transfection of both subunit cDNAs together with sense oligonucleotides had no influence on guanylate cyclase activity (data not shown), we conclude from these results that both subunit proteins must be co-expressed in the same cell to allow catalytic activity of the holoenzyme.

In the present study, we demonstrated that the expression of a functionally active guanylate cyclase in transiently transfected COS-7 cells requires the co-expression of both subunits (the 70 kDa and the 82 kDa) for basal catalytic activity and activation by nitrovasodilators. Several lines of evidence support this conclusion. (i) We were able to

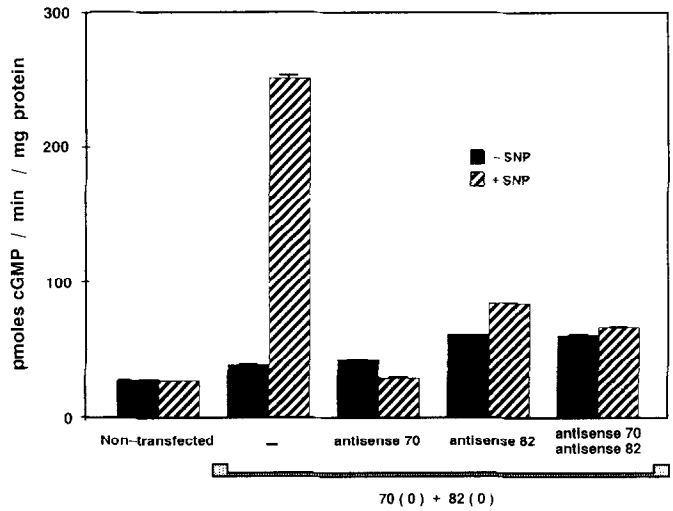

Fig. 3
Effect of antisense oligonucleotides directed against the 70 kDa and the 82 kDa messenger RNAs on the level of transiently expressed soluble guanylate cyclase. After cotransfection of antisense oligonucleotides (5 μM) together with both soluble guanylate cyclase cDNAs, the guanylate cyclase activity (±SNP activation) was measured as described in Materials and Methods. The results shown are means ± SD of triplicates of one representative experiment.

detect significant increases (15-fold) of guanylate cyclase activity (± SNP activation), only when the cells were transfected with both subunits together in a correct (order) orientation. In contrast, transfection of cells with either subunit alone (in order direction) did not result in elevated enzymatic activity. (ii) Cotransfection of an antisense oligonucleotide against the 70 kDa subunit or the 82 kDa subunit mRNA together with both subunit cDNAs resulted in a marked loss of enzymatic activity. These data confirm our recent results on stable expression of soluble guanylate cyclase in L-cells (14) suggesting that both subunits of the heterodimer are required for catalytic activity and activation by nitric oxide. While this manuscript was in preparation, *Harteneck et al.* (25) reported about a 50-fold increase in enzyme activity by sodium nitroprusside for transfected soluble guanylate cyclase from bovine lung. The greater degree of SNP-activation is probably related to the higher amount of cDNA transfected (14 µg) in their experiments. Since we originally found that the carboxy-terminal portion of the 82 kDa subunit protein is highly homologous with that of the 70 kDa subunit, and both proteins also share significant homology with the catalytic region of particulate guanylate cyclase from rat brain and that of adenylate cyclase from bovine brain (14,15), a simplified model for the catalytic activity of guanylate cyclases would involve a cooperative interaction of two catalytic domains located on different subunits. With regard to adenylate cyclase the requirement for two interactive catalytic sites may be met with a monomeric model (18).

Acknowledgments: The authors gratefully thank Ms. Ruth Huang and Ms. Kathy Kohlhaas for excellent technical assistance. These studies were supported in part by grants from the National Institutes of Health (HL 28474 and DK 30787).

References

1. Rapoport, R. M. and Murad, F. (1983) J. Cyclic Nucleotide and Protein Phosphorylation Res. **9**, 281-296
2. Fesenko, E.E., Kolesnikov, S.S. and Lyubarsky, A.L. (1985) Nature **313**, 310-313
3. Garbers, D.L. (1989) J. Biol. Chem. **264**, 9103-9106
4. Murad, F. (1989) in: Molecular Mechanisms of Hormone Action, 40th Colloquium Mosbach, FRG, 186-194
5. Kuno, T., Andresen, J.W., Kamisaki, Y., Waldman, S.A., Chang, L.Y., Saheki, S., Leitman, D.C., Nakane, M. and Murad, F. (1986) J. Biol. Chem. **261**, 5817-5823
6. Leitman, D.C., Andresen, J.W., Catalano, R.M., Waldman, S.A., Tuan, J.J., and Murad, F. (1988) J. Biol. Chem. **263**, 3720-3728
7. Nakane, M., Saheki,S., Kuno, T., Ishii, K., and Murad, F. (1988) Biochem. Biophys. Res. Commun. **157**, 1139-1147
8. Kamisaki, Y., Saheki, S., Nakane, M., Palmieri, J.A., Kuno, T., Chang, B.Y., Waldman, S.A., and Murad, F. (1986) J. Biol. Chem. **261**, 7236-7241

9. Arnold, W. P., Mittal, C.K., Katsuki, S., and Murad, F. (1977)) Proc. Natl. Acad. Sci. USA **74**, 3203-3207
10. Murad, F., Mittal, C. K., Arnold, W.P., Katsuki, S. and Kimura, H. (1978) Adv. in Cyclic Nucleotide Res. **9**, 145-158
11. Lewicki, J.A., Brandwein, H.J., Mittal, C.K., Arnold, W.P., and Murad, F. (1982) J. Cyclic Nucleotide Res. **8**, 17-25
12. Chinkers, M., Garbers, D.L., Chang, M-S., Lowe, D.G., Chin, H., Goeddel, D.V., and Schulz, S. (1989) Nature **338**, 78-83
13. Kösling, D., Harteneck, C., Humbert, P., Bosserhoff, A., Frank, R., Schultz, G., and Böhme, E. (1990) FEBS Lett. **266**, 128-132
14. Nakane, M., Arai, K., Saheki, S., Kuno, T., Büchler, W., and Murad, F. (1990) J. Biol. Chem. **265**, 16841-16845
15. Nakane, M. (1989) 7th International Conference on Cyclic Nucleotides, Calcium, and Protein Phosphorylation, Abstract Form p. 121
16. Thorpe, D. S. and Morkin, E. (1990) J. Biol. Chem. **265**, 14717-14720
17. Chinkers, M., Garbers, D.L., Chang, M.-S., Lowe, D.G., Chin, H., Goeddel, D.V. and Schulz, S. (1989) Nature **338**, 78-83
18. Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.-J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R., and Gilman, A.G. (1989) Science **244**, 1558-1564
19. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokoya, K., Arai, K., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. **8**, 466-472
20. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chang, W.H., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) Proc. Nat. Acad. Sci. USA **84**, 7413-7417
21. Kozak, M. (1984) Nucleic Acids Res. **12**, 857-872
22. Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254
23. Kimura, H., Mittal, C.K. and Murad, F. (1975) J. Biol. Chem. **250**, 8016-8022
24. Holt, J.T., Redner, R.L., and Nienhuis, A.W. (1988) Mol. Cell. Biol. **8**, 963-973
25. Harteneck, C., Kösling, D., Söling, A., Schultz, G. and Böhme, E. (1990) FEBS Lett. **272**, 221-223