Expression of soluble guanylyl cyclase

Catalytic activity requires two enzyme subunits

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Purified soluble guanylyl cyclase consists of two subunits (70 and 73 kDa) whose primary structures were recently determined. The availability of cDNA clones coding for either subunit allowed to study the question of the functional roles of the two subunits in expression experiments. Enzyme subunits were expressed in COS-7 cells by transfection with expression vectors containing the coding region for the 70 of 73 kDa subunit of the enzyme. No significant elevation in the activity of soluble guanylyl cyclase was observed in cells transfected with cDNA coding for one of the subunits. In contrast, transfection of cells with cDNAs coding for both subunits resulted in a marked increase in activity of soluble guanylyl cyclase. Enzyme activity was stimulated about 50-fold by sodium nitroprusside. The results indicate that formation of cyclic GMP by soluble guanylyl cyclase requires both 70 and 73 kDa subunits.

Soluble guanylyl cyclase; Expression; COS cell

1. INTRODUCTION

Guanylyl cyclases (GTP pyrophosphate-lyase (cyclizing); EC 4.6.1.2) are enzymes catalyzing the formation of cyclic GMP from GTP. The cyclic nucleotide acts as an intracellular signal molecule in the regulation of various cellular functions, e.g. the tonus of vascular smooth muscle and retinal phototransduction [1-3]. At least two different forms of guanylyl cyclases exist, a plasma membrane-bound and a cytosolic enzyme form, differing in structure, regulation and other properties [2,3]. The plasma membrane-bound forms of guanylyl cyclase, which are regulated by peptide hormones such as sea urchin egg peptides and atrionatriuretic peptides, exist as single polypeptide chains with extracellular receptor and intracellular catalytic domains [4]. The soluble enzyme form is a heterodimer containing a heme-moiety [5] and is regulated by nitric oxide (NO) and NO-containing compounds [1-3]. The enzyme purified by immunoaffinity procedures from bovine and rat lung consists of two subunits of 70 and 73 kDa [6] and 70 and 82 kDa [7], respectively. The amino acid sequence of the 70 kDa subunit was determined two years ago [8,9] and was shown to contain a C-terminal region (about 250 amino acids) which is highly homologous with a cytoplasmic part of plasma membrane-bound guanylyl cyclases and the two hydrophilic domains of an adenylyl cyclase from rat brain [10,11]. The 73 kDa subunit of the soluble guanylyl cyclase was recently cloned and sequenced; the calculated M_r of the deduced amino acid sequence is 77.5 kDa [11]. Sequence comparison of this subunit with the 70 kDa one revealed strong similarities between both subunits with about 32% identical amino acids. Like the 70 kDa subunit, the 73 kDa subunit shares homologies in a region of about 250 amino acids with the plasma membrane-bound guanylyl cyclases and with the two hydrophilic domains of the adenylyl cyclase from rat brain [11]. Although the primary structure of the enzyme is known, the functional relationship between the two subunits remains unclear.

Here we report on the expression of soluble guanylyl cyclase subunits in COS-7 cells and demonstrate that the expression of neither the 70 kDa nor the 73 kDa subunit is sufficient for enzymatic activity, whereas expression of both subunits yields a catalytically active enzyme which is stimulated by sodium nitroprusside.

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2. MATERIALS AND METHODS

A chimeric construction containing the coding sequence for the 70 kDa subunit of soluble guanylyl cyclase was ligated from three different clones, N, 1L and 2 L [8], containing parts of the coding region, via internal restriction sites for *Nco*I and *Hin*cII. The 5'-*Bam*HI-*Nco*I-fragment (881 bp) of clone N, the *Nco*I-*Hin*cII-fragment (544 bp) of clone 1l and the *Hin*cII-*Eco*RI-fragment (793) of clone 2L were stepwise ligated. The resulting 2.2 kb fragment was cut with *Eco*RI, the restriction site was filled, using the large fragment of DNA polymerase I (Klenow). After a restriction cut with *Hin*dIII, the fragment was ligated in the *Sma*I- and *Hin*dIII-digested expression vector pCMV [12].

The insert (3.2 kb) of clone K0 1 coding for the 73 kDa subunit of soluble guanylyl cyclase [10] was cut with *Eco*RI, the recessed 3'-termini was filled, using the large fragment of DNA polymerase I

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(Klenow), and the insert was ligated in the *Sma*I-digested expression vector pCMV.

About 4×10^6 COS-7 cells (African green monkey kidney cells transformed by an origin-defective simian virus 40 mutant) were seeded on 150 cm^2 plates the day before transfection. The cells were transfected with 14 μg of plasmid per plate by the DEAE-dextran method [13]. 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 $\times$ g_{av}.

Guanylyl cyclase activity of the obtained cytosolic fraction (80–200 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 [γ-^{32}P]GTP (about 0.5 μCi) and 3 mM $MnCl_2$, with or without 0.1 mM sodium nitroprusside, in a total volume of 0.1 ml, as described previously [14].

For immunoblotting, cytosolic COS-7 fractions were subjected to 10% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine, 0.02% (m/v) SDS and 20% (v/v) methanol at 250 mA for one hour. Processing of the filters and detection with antibodies was performed as previously described [15]. The 70 kDa subunit was detected by an antibody directed against a C-terminal peptide of this subunit [6], the 73 kDa subunit by an antibody directed against the C-terminal sequence, KKDVEEANANFLGKASGID (one letter code). Coupling of the peptides and immunization was as in [15]. Guanylyl cyclase applied in immunoblots was purified from bovine lung to apparent homogeneity as described [6].

3. RESULTS AND DISCUSSION

The availability of cDNA clones coding for both subunits of soluble guanylyl cyclase allowed one to study the guanylyl cyclase activity of either one of the subunits and of their combination in expression experiments. COS-7 cells were transfected with expression vectors pCMV containing the coding region of the 70 kDa subunit (psGC-70) or of the 73 kDa subunit (psGC-73) as well as with the vectors psGC-70 *plus* psGC-73. In control experiments, cells were transfected with the vector pCMV without any insert. Three days after the transfections, expression of soluble guanylyl cyclase subunits was studied by immunoblotting of the cytosolic proteins obtained from the transfected cells (Fig. 1). In cytosols of COS-7 cells transfected with psGC-70 or with psGC-70 *plus* psGC-73, a 70 kDa protein was detected by the antibody raised against the C-terminal peptide of the 70 kDa subunit, whereas in cytosols of cells transfected with psGC-73 or with psGC-70 *plus* psGC-73 a protein was detected with an M_r slightly smaller than that of the 73 kDa subunit of guanylyl cyclase purified from bovine lung; this difference may be due to different posttranslational modifications or proteolysis after cell disintegration.

In order to examine whether expressed guanylyl cyclase subunits exhibit catalytic activity and whether stimulation by NO-containing compounds is detectable, enzyme activity was determined in the cytosols of COS-7 cell transfected as described above. In untreated COS-7 cell cytosols, guanylyl cyclase activity was undetectable (Table I). After transfection of COS-7 cells with psGC-70, psGC-73 or with unmodified pCMV, no significant increase in the activity of soluble guanylyl cyclase was observed. With sodium nitroprusside, very low activity (2–5 pmol $\cdot$ $mg^{-1} \cdot min^{-1}$) was observed after transfection with psGC-70 or psGC-73. Combination of cytosolic proteins of COS-7 cells transfected with psGC-70 or psGC-73 yielded the same low increase in soluble guanylyl cyclase activity. In contrast, transfection of COS-7 cells with psGC-70 *plus* psGC-73 resulted in the expression of soluble guanylyl cyclase with high catalytic activity. In the presence of Mn^{2+}, basal guanylyl cyclase activity amounted to 20–70 pmol cGMP $\cdot$ $min^{-1} \cdot mg^{-1}$. Sodium nitroprusside enhanced guanylyl cyclase activity to 1000–3000 pmol cGMP $\cdot$ $min^{-1} \cdot mg^{-1}$, corresponding to an about 50-fold increase in enzyme activity. Basal and stimulated activity of soluble guanylyl cyclase of COS-7 cells transfected with psGC-70 and psGC-73 was dependent on the amount of plasmid used for transfection (data not shown).

These results demonstrate that both 70 and 73 kDa subunits are necessary for the catalytic function of soluble guanylyl cyclase stimulated by NO-containing

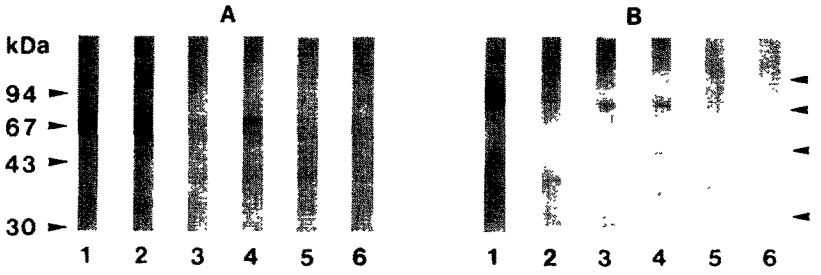

Fig. 1. Immunoblotting analysis of 100 000 $\times$ *g* supernatants of transfected COS-7 cells. Purified soluble guanylyl cyclase (1 μg, lanes 1), cytosolic proteins (100 μg) of untreated COS cells (lanes 6) and of COS cells transfected with the expression vector pCMV containing the coding region of the 70 kDa subunit (lanes 2) or of the 73 kDa subunit of soluble GC (lanes 3), of COS cells transfected with a combination of both plasmids (lanes 4) or transfected with pCMV without insert (lanes 5) were electrophoresed by 10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and incubated with peptide-antibodies against the 70 kDa subunit (panel A) and peptide-antibodies against the 73 kDa subunit (panel B).

Table I

Activity of guanylyl cyclase expressed in COS-7 cells

COS-7 cells transfected with	guanylyl cyclase activity (pmol cGMP·min^{-1}·mg^{-1})	
	−SNP	+SNP
none	nd	nd
pCMV	nd	nd
psGC-70	nd	2.8
psGC-73	nd	2.5
psGC-70 psGC-73*	nd	3.0
psGC-70 + psGC-73	22.8	1001

Activity of soluble guanylyl cyclase was determined in the cytosols of COS-7 cells transfected with the unmodified expression vector pCMV, vectors containing the cDNA of the subunits of 70 kDa (psGC-70) or 73 kDa (psGC-73) or a combination of psGC-70 and psGC-73, and in cytosols of non-transfected cells. Enzyme activity was determined with 0.1 mM and without sodium nitroprusside (SNP) 3 days after treatment of the cells with or without the expression vectors. Data are shown for a representative experiment out of 3 similar experiments, performed in triplicate.

*Combination of the cytosolic proteins of COS-7 cells transfected with the vectors psGC-70 or psGC-73; nd = activity not detectable

substances. The expression of a functionally active guanylyl cyclase in transfected COS-7 cells required the coexpression of both subunits in the same cell as the combination of the cytosolic proteins of COS-7 cells transfected with psGC-70 or psGC-73 did not result in a significant increase in soluble guanylyl cyclase activity. These data and the high structural homology between the two subunits suggest that both subunits are involved in the catalytic function. The present findings support the assumption that two of the putative catalytic domains of cyclases are required for the formation of a cyclic nucleotide as also suggested by the presence of two putative catalytic domains in the adenylyl cyclase molecule.

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