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The incorporation of a purified, membrane-bound form of guanylate cyclase into phospholipid vesicles and erythrocytes

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The purified membrane-bound form of guanylate cyclase was incorporated into artificial unilamellar phospholipid vesicles. The rate and extent of enzyme incorporation into the vesicles was dependent upon the phospholipid concentration and the time period of incubation. The enzyme was incorporated at a significantly faster rate after removal of carbohydrate with endoglycosidase H. The incorporation of the enzyme led to a 10-fold decrease in the apparent maximal velocity and a 2-fold increase in the apparent Michaelis constant for MnGTP. Extraction of liposomes containing guanylate cyclase with 0.2% Lubrol PX resulted in the recovery of 85% of the original amount of added activity, suggesting that the decrease in maximal velocity was not due to enzyme denaturation. Phosphatidylcholine liposomes differentially effected the activity of the membrane-form of guanylate cyclase, dependent on the nature of the fatty acid present on the phospholipid. Specific activities ranged between 458 nmol/min per mg and 2.6 μmol/min per mg, dependent upon the fatty acids present. Liposomes containing the membrane-bound form of guanylate cyclase were subsequently fused with erythrocytes using poly(ethylene glycol) 4000 in attempts to introduce the enzyme into intact cells. The enzyme was successfully introduced into the erythrocytes; greater than 90% of the enzyme activity was subsequently shown to be associated with erythrocyte membranes. Cyclic GMP concentrations of erythrocytes increased from essentially nondetectable to 4 pmol/10^9 cells after introduction of the enzyme. These results demonstrate that guanylate cyclase can be incorporated into liposomes in an active state and that such liposomes can be used to introduce the enzyme into cells where it can subsequently function to generate cyclic GMP.

Introduction

Guanylate cyclase activity is distributed in both the soluble and particulate fractions of many tissue homogenates, although in some tissues (invertebrate spermatozoa, intestinal mucosa) it appears to exist only as the membrane-bound form [1,2]. The soluble form of the enzyme has been purified from various tissue sources [3–5] and appears to contain heme [6], while the form associated with the membrane has been purified to apparent homogeneity only from sea urchin spermatozoa; it appears to be a glycoprotein [7]. The membrane form of guanylate cyclase has been previously shown to be stimulated by agents which perturb the phospholipid environment such as mellitin, gramicidin S and alamethicin [8,9], as well as by the polyene antibiotic filipin [10] and various fatty acids [11] and lipids [12]. The activity of the soluble form of the enzyme has been shown to be stimulated by nitric oxide [13–15], various nitroso-agents [16–18], and fatty acids [19–20]. Despite the many reports on guanylate cyclase, however, the actual mechanism(s) by which cyclic

GMP metabolism is regulated remains unclear.

The incorporation of the purified membrane form of guanylate cyclase into liposomes could provide a method by which to introduce the enzyme into various cell types by fusion procedures. Such methodology might be of significant importance since it could allow the introduction of guanylate cyclase into a new environment where mechanisms of regulation might be uncovered. Here, we report the stable introduction of the membrane-bound form of guanylate cyclase into defined phospholipid vesicles. Enzyme activity was retained, although altered, by incorporation into the vesicles; these liposomes could then be fused with erythrocytes. The introduction of enzyme into the erythrocyte resulted in the formation of cyclic GMP by these cells, suggestive of a functional reconstitution.

Experimental procedures

Materials. DEAE-Sephacel, Blue Sepharose and CNBr-activated Sepharose were obtained from Pharmacia. Endoglycosidase H was from Miles Laboratories. [α-^{32}P]GTP was purchased from Amersham and phospholipids from Sigma. RPMI-1640 was from Gibco Laboratories.

Guanylate cyclase assay. Guanylate cyclase activity was determined as previously described [7,21]. The reaction mixture (10 min at 30°C) typically contained 0.2 mM GTP, 10 mM $MnCl_2$ and 25 mM triethanolamine. Formed cyclic [^{32}P]GMP was purified by zinc carbonate coprecipitation of 5′-nucleotides and subsequent chromatography of the supernatant fluid on neutral alumina.

The membrane-bound form of guanylate cyclase from sea urchin spermatozoa was purified as previously reported [7]. In summary, Lubrol PX extracts from 50 g sperm particles were applied to a GTP-Sepharose column and enzyme, eluted with 0.5 mM EDTA, was then added to a DEAE-Sepharose column. Enzyme was eluted with buffer containing 500 mM NaCl, concentrated, and dialyzed against a buffer containing 25 mM triethanolamine, 10 mM dithiothreitol, 0.1% Lubrol PX and 50% glycerol. Final purification was by preparative gel electrophoresis. Basal activity after elution from preparative electrophoresis was 10–15 μmol cyclic GMP formed/min per mg protein in the presence of Mn^{2+} (30°C). Treatment of enzyme with endoglycosidase H to remove carbohydrate was as previously described [7].

Preparation of liposomes. Small unilamellar liposomes were prepared essentially as previously described [22]. Briefly, phospholipids were dried under a stream of N_2. The phospholipid was dispersed by vigorous agitation in buffer containing 50 mM Tris-HCl (pH 7.6), 0.5% dimethylsulfoxide, 0.2 mg/ml phenylmethylsulfonyl fluoride, 2 mM dithiothreitol and 150 mM NaCl and sonicated under a stream of N_2 at 4°C for 2–5 min with a Branson Sonifier until no further increase in turbidity was observed. In experiments using mixtures of phospholipids, the same protocol for forming liposomes was used with the desired ratios of various phospholipids. Enzyme protein was then mixed with phospholipid vesicles for various periods of time and liposomes were collected by centrifugation at 300 000 × *g* for 30 min and resuspended to a volume of between 2–5 ml. Liposomes or supernatant fluid obtained after the 300 000 × *g* centrifugation were added directly to a guanylate cyclase assay mixture and activity was determined. The calculated amount of enzyme incorporated into the liposomes was based on the amount of protein found in the centrifuged liposomes and in the supernatant fluid. For the incorporation of guanylate cyclase into erythrocytes, the membrane-bound form of guanylate cyclase was encapsulated into multilamellar vesicles by the previously described procedure [23]. Multilamellar vesicles were prepared essentially the same as the small unilamellar vesicles except that enzyme was present during the resuspension step; the sonication step was omitted.

Incorporation of guanylate cyclase into erythrocytes. Human erythrocytes were gently washed three times and resuspended to the desired cell concentration in serum-free RPMI-1640. 1 ml of the cell suspension was added to the previously pelleted liposomes containing the encapsulated, purified membrane-bound form of guanylate cyclase and centrifuged at 7500 × *g* at 4°C. The supernatant fluid was removed and 250 μl of 35% poly(ethylene glycol) in serum-free RPMI-1640 was added to the pellet and mixed for 1 min. After 1 min, 10 ml serum-free RPMI-1640 were added

with gentle mixing. The fused erythrocytes/liposomes were collected by centrifugation at 1000 $\times g$ for 10 min and subsequently washed three times with serum-free RPMI-1640. The erythrocytes were then assayed for guanylate cyclase activity or cyclic GMP concentrations. The final erythrocyte/liposome pellet was homogenized in a buffer containing 50 mM triethanolamine, 5 mM dithiothreitol and assayed for guanylate cyclase activity in the soluble and particulate fractions.

Cyclic GMP determination. Erythrocytes, which had been fused with liposomes containing guanylate cyclase were washed three times in serum-free RPMI-1640 and were then incubated at 30°C for various periods of time immediately after washing. The time of the incubation was considered to be the time after the addition of the erythrocyte mixture to a 30°C bath. The reactions were stopped by the addition of 1 ml 0.5 M perchloric acid and the acidified erythrocyte suspensions were then frozen and thawed five times prior to purification of cyclic GMP. Purification and radioimmunoassay of the cyclic GMP was as described previously [24,25].

Other methods. Protein was determined by the method of Lowry et al. [26]. Kinetic constants were determined from slope and intercept replots as suggested by Cleland [27].

Results

Incorporation of guanylate cyclase into liposomes

The amount of membrane and endoglycosidase H-treated membrane guanylate cyclase found in phosphatidylcholine liposomes was determined by the measurement of enzymatic activity and protein in the 300 000 $\times g$ pellet and supernatant fractions. Approx. 50 μg enzyme protein in 500 μl buffer were incubated with 500 μl liposome mixture for 3 h. Incorporation of enzyme activity into the liposomes was maximal at a phospholipid concentration of 7–10 mg/ml for the unmodified forms of the enzyme, while the endoglycosidase H-treated enzyme was incorporated to a greater extent at lower phospholipid concentrations (Fig. 1). Assay of guanylate cyclase activity in liposomes under isotonic and hypotonic conditions shows essentially no difference in activity, suggesting that the active site is located on the external surface.

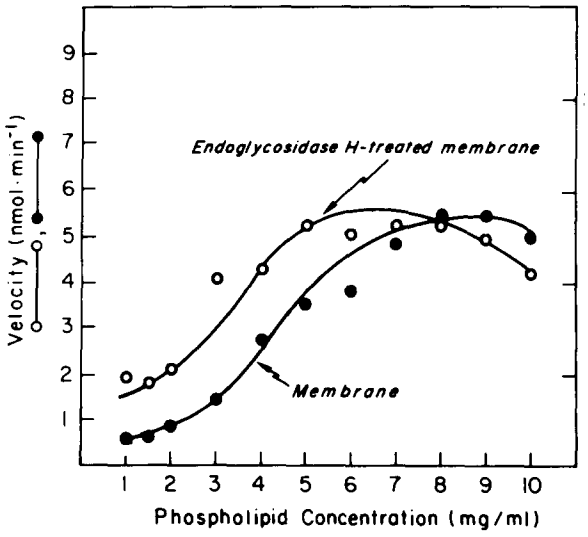

Fig. 1. The effect of phosphatidylcholine concentration on the incorporation of membrane (●———●) or endoglycosidase H-treated membrane forms of guanylate cyclase (○———○) into liposomes. Liposomes were incubated with the appropriate enzyme for 3 h and liposomes collected by centrifugation at 300 000 $\times g$ for 30 min. Liposomes were resuspended in 2–5 ml of a solution containing 50 mM Tris, 5 mM dithiothreitol and were assayed as in Experimental procedures.

The introduction of guanylate cyclase into liposomes was dependent upon the incubation time (Fig. 2). Endoglycosidase H-treated enzyme was incorporated very rapidly into the liposomes with maximal introduction within 30 min. In contrast, the detergent-solubilized, native form of the enzyme was maximally incorporated after 2 h of incubation.

Effect of liposome incorporation on enzyme activity

Prior to liposome incorporation, guanylate cyclase exhibited linear kinetics on double-reciprocal plots as a function of MnGTP. After liposome incorporation, the membrane form of the enzyme continued to exhibit linear kinetics on double-reciprocal plots, but there was a marked reduction (approx. 10-fold) in the apparent Vmax (Fig. 3). Endoglycosidase H-treated enzyme showed approximately the same reduction in the apparent Vmax after liposome incorporation (not shown). Extraction of liposomes containing guanylate cyclase with 0.2% Lubrol WX resulted in the recovery of 85% of the initially incorporated activity,

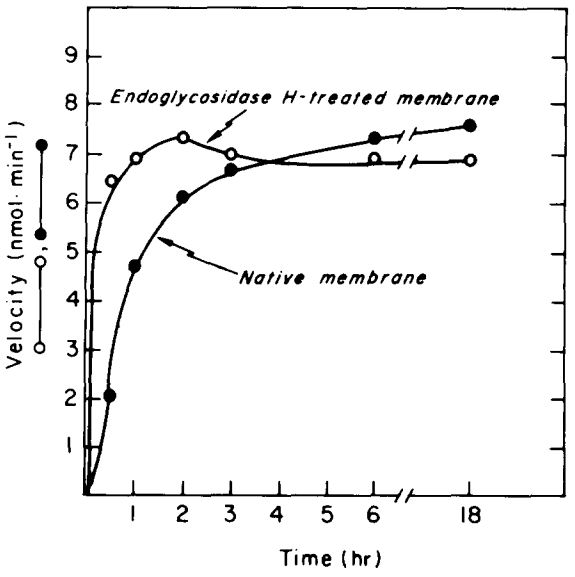

Fig. 2. Time-course of guanylate cyclase incorporation into phosphatidylcholine vesicles. Phosphatidylcholine vesicles (8 mg/ml) were prepared and incubated with the membrane (●——●) or endoglycosidase H-treated membrane (○——○) forms for the indicated time. After the incubation, liposomes were collected by centrifugation and assayed as described in Experimental procedures.

suggestive that the loss in enzyme activity after liposome incorporation was not due to a denaturation of enzyme during introduction into the liposomes.

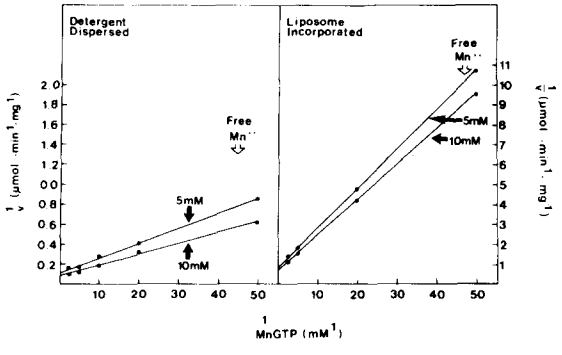

Fig. 3. Reciprocal velocity as a function of reciprocal MnGTP concentration. The detergent-solubilized and liposome-incorporated membrane forms of guanylate cyclase were assayed for activity as described in Experimental procedures. GTP concentrations were varied between 20 and 400 μM, while Mn^{2+} was adjusted to keep it at the indicated free metal ion concentration.

Effect of other phospholipids

The addition of phosphatidylethanolamine or phosphatidylglycerol to phosphatidylcholine liposomes caused a reduction in the activity of the liposome-incorporated membrane-bound form of guanylate cyclase (Fig. 4). Guanylate cyclase was maximally inhibited (85%) by liposomes containing 40 mol% phosphatidylglycerol. Phosphatidylethanolamine also inhibited the activity of the enzyme but to a lesser extent. Phosphatidylserine and cholesterol had essentially no effect on the activity. Additionally, the activity of the enzyme was not affected by detergent-solubilized phosphatidylcholine/phosphatidylglycerol or phosphatidylcholine/phosphatidylethanolamine-containing liposomes.

Effect of fatty acid chain-length on enzyme activity

To determine if fatty acid chain-length had an effect on enzyme activity, guanylate cyclase was

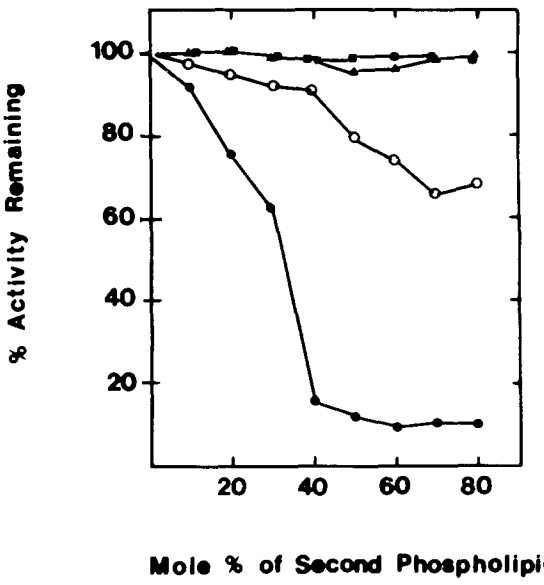

Fig. 4. The effect of additional phospholipids on the activity of the membrane form of guanylate cyclase. Egg yolk phosphatidylcholine vesicles containing the indicated mole percent of the additional phospholipid were prepared and incubated with the membrane form of guanylate cyclase for 6 h then collected and assayed as previously described. The compositions of vesicles were: ○——○, phosphatidylcholine and phosphatidylethanolamine; ●——●, phosphatidylcholine and phosphatidylglycerol; ■——■, phosphatidylcholine and phosphatidylserine; ▲——▲, phosphatidylcholine and cholesterol.

incorporated into phosphatidylcholine liposomes with defined fatty acid chain-lengths (Table I). Enzyme activity was not related to chain-length, although activity was higher with certain fatty acids. The enzyme activity showed considerable variation, dependent upon the fatty acids present with specific activities ranging from 458 nmol/min per mg protein to 2.6 μmol/min per mg protein.

Effect of monospecific guanylate cyclase antisera

The incubation of liposomes containing guanylate cyclase with monospecific antiserum resulted in a concentration-dependent inhibition of enzyme activity (Fig. 5). At high concentrations of antiserum, the enzyme was inhibited greater than 90%. Solubilization of antiserum-treated liposomes, which had been washed extensively to remove nonspecifically adsorbed antibody, with buffer containing 0.5% Lubrol PX did not result in the liberation of any additional activity. This suggests that the enzyme has its active site oriented towards the external surface and that the reduction in total enzyme activity after liposome incorporation is not due to a differential orientation of the enzyme in the liposomes.

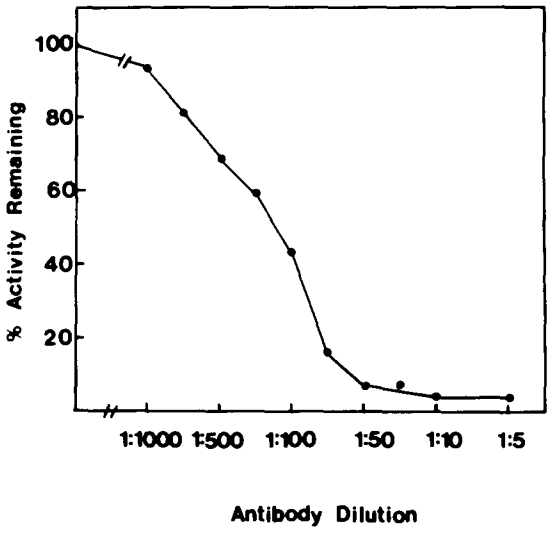

Fig. 5. The effect of various concentrations of monospecific antibody on the activity of liposome-incorporated membrane guanylate cyclase (●———●). Monospecific antibody directed against the membrane form of guanylate cyclase was incubated with the liposome-incorporated enzyme overnight at 4°C. The liposomes were washed five times with buffer containing 25 mM triethanolamine, 5 mM dithiothreitol, 150 mM NaCl and were then solubilized in washing buffer containing 0.2% Lubrol PX. Aliquots of solubilized liposomes were added into a guanylate cyclase assay.

TABLE I

RELATIVE ACTIVITY OF THE MEMBRANE FORM OF GUANYLATE CYCLASE INCORPORATED INTO PHOSPHATIDYLCHOLINE VESICLES CONTAINING DEFINED FATTY ACIDS

Percent activity is the enzyme activity relative to the activity in egg yolk phosphatidylcholine vesicles. The specific activity of the membrane form of guanylate cyclase in egg yolk phosphatidylcholine vesicles was 986 nmol/min per mg. All phospholipids were at a final concentration of 10 mg/ml. Enzyme was incubated with the respective liposome for 16 h prior to assay.

Fatty acid	Activity (%)
Dilauryl	262
Dimyristoyl	46
Dipalmitoyl	61
1-Oleoyl-2-palmitoyl	268
Diheptadecanoyl	77
Distearoyl	62
Dioleoyl	58

Introduction of guanylate cyclase into erythrocyte membranes

Fusion of multilamellar vesicles containing purified, membrane-bound guanylate cyclase with erythrocytes resulted in the stable incorporation of enzyme activity into erythrocytes. After fusion of the multilamellar vesicles containing the membrane form of guanylate cyclase with erythrocytes, enzyme activity was shown to be associated only with the particulate fraction (more than 99%) of an erythrocyte homogenate; this activity could be subsequently solubilized with buffer containing 1% Lubrox PX. The enzyme continued to exhibit linear kinetics on double-reciprocal plots as a function of MnGTP after introduction into the erythrocytes. A ratio of 0.5 mg liposome phospholipid to 10^8 erythrocytes (presumably resulting in maximal liposome-erythrocyte fusion) resulted in the maximal reconstituted activity. Approx. 20% of the total activity added could be recovered in the erythrocytes under these conditions. Higher

concentrations of liposome phospholipid resulted in less total activity being incorporated. This is possibly a result of increased liposome-liposome fusion rather than liposome-erythrocyte fusion. To determine if erythrocyte-incorporated guanylate cyclase was reconstituted in a functional sense, cyclic GMP concentrations were determined. Erythrocytes containing the liposome-incorporated guanylate cyclase showed a time-dependent increase in cyclic GMP concentrations, whereas control (liposome fusion without guanylate cyclase) erythrocytes showed no increase in cyclic GMP. Some cyclic GMP was already formed prior to the warming of the erythrocytes. Cyclic GMP concentrations reached a maximal concentration of 4.0 pmol cyclic GMP/10^9 cells, 6–8 min after the beginning of the incubation at 30°C (Fig. 6) and decreased to a basal level of approx. 1.5 pmol/10^9 cells by 10 min after the initiation of the incubation. Cyclic GMP in control erythrocytes was less than 0.2 pmol/10^9 cells at all time-points.

The addition of liposomes containing the membrane form of guanylate cyclase to erythrocytes in the absence of poly(ethylene glycol) 4000 did not result in the incorporation of any detectable enzyme activity and no elevations of cyclic GMP concentrations were observed (not shown).

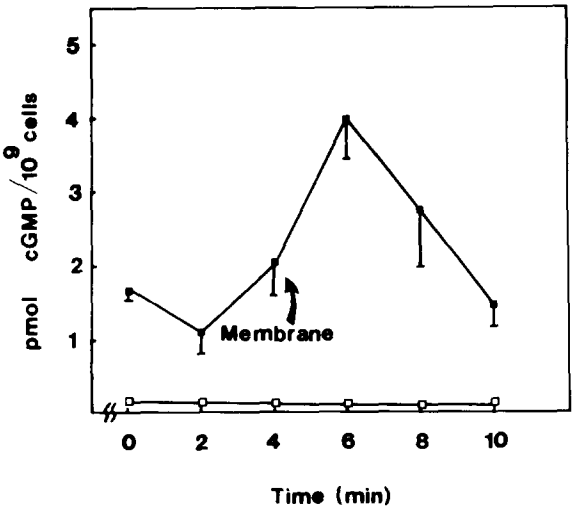

Fig. 6. Time-course of cyclic GMP elevations in erytrhocytes. The membrane-bound form of guanylate cyclase (total activity of approx. 25 nmol cyclic GMP formed per min) was encapsulated into multilamellar vesicles and fused with human erythrocytes as described in the Experimental procedures. The time points represent the time after warming the erythrocytes to 30°C.

Discussion

This paper describes the incorporation of the membrane-bound form of guanylate cyclase into artificial phospholipid vesicles and erythrocytes. The introduction of the membrane form of guanylate cyclase into liposomes resulted in a reduction in the maximal velocity and a change in the apparent Michaelis constant for the enzyme. In crude preparations, the membrane form of guanylate cyclase exhibits positive cooperativity with respect to MnGTP and is activated 4–12-fold by detergent dispersion [28–30]; however, upon purification the sea urchin sperm enzyme loses its positive cooperativity and shows typical Michaelis-Menten kinetics with respect to MnGTP [31]. With respect to detergent activation, the liposome-incorporated, membrane form of guanylate cyclase resembles the normal membrane-bound form of guanylate cyclase where detergent increases activity by 5–12-fold.

The hydrophobic sites do not appear to be located at the active site of the enzyme since activity is not eliminated but only reduced after liposome incorporation. Also, extraction of liposomes with detergent results in the recovery of essentially all of the initial enzyme activity. This would suggest that the lipid environment places a steric restraint on the enzyme; this restraint is then removed after detergent dispersion resulting in an 'activated' enzyme..

The reduction in total activity, however, possibly could be a result of differential orientation of the enzyme in the liposome such that the active site is obscured. Incubation of the liposomal membrane form of guanylate cyclase with monospecific antiserum, however, resulted in the inactivation of the enzyme and no recovery of additional activity upon detergent treatment of the washed liposomes. This suggests that the observed change in activity is not a function of differential orientation but rather due to a steric effect or a conformational change in the enzyme.

In liposomes of different phospholipid compositions it was not apparent whether the reduction in enzyme activity was a function of the polar

headgroup or of the fatty acid chain-length. In experiments using phosphatidylcholine vesicles with defined fatty acids, no clear pattern of inhibition as a function of chain-length was apparent. This is different than the pattern of inhibition observed using soluble guanylate cyclase immobilized on various alkyl-agarose gels [32].

Liposomes have been used to introduce a variety of biologically active molecules into cells [33–38]. The fusion of liposomes containing encapsulated guanylate cyclase with human erythrocytes resulted in the stable incorporation of enzyme activity into the particulate fraction of an erythrocyte homogenate. Since cyclic GMP was also elevated, it may now be possible to reconstitute the enzyme into cells which contain only low amounts of guanylate cyclase or to incorporate the membrane-bound form into cells which contain predominantly the soluble form of the enzyme and determine the effects of such incorporation on cyclic GMP concentrations and subsequent cell function.

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