The Guanylyl Cyclase-A Receptor Transduces an Atrial Natriuretic Peptide/ATP Activation Signal in the Absence of Other Proteins*

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Attempts to activate partially purified preparations of the guanylyl cyclase-A (GC-A) receptor with atrial natriuretic peptide (ANP) have previously failed, leading to speculation that essential cofactors are lost during purification procedures. The receptor was modified to contain the FLAG epitope (DYKDDDDK), expressed in SF9 cells, and purified to apparent homogeneity (4.3 μmol cyclic GMP formed/min/mg protein; 5.8 nmol 125I-ANP binding site/mg protein) by a combination of immunoaffinity, Q-Sepharose FF, and wheat germ agglutinin batch chromatography. High initial protein/detergent ratios, the presence of glycerol (40%), and the inclusion of protein phosphatase inhibitors in all buffers resulted in the purification of a receptor that continued to transduce the ANP/ATP activation signal. Both native and purified GC-A contained a single class of high affinity ANP binding sites (Kd = 60 pm) and an equivalent EC50 for ATP (0.3 μM). Positive cooperativity as a function of MnGTP was retained during purification. Thus, GC-A is capable of transducing a ligand binding signal in the absence of other proteins.

The atrial natriuretic peptide receptor (NPR-A) coupled to guanylyl cyclase activity (GC-A)1 is a prototype for a family of membrane-bound guanylyl cyclases currently numbering 6 in mammals (A–F). The B and C cyclases appear to be receptors for C-type natriuretic peptide and heat-stable enterotoxins/guanylin, respectively, while D, E, and F remain orphan receptors (1–4).

Members of the family contain a single apparent transmembrane domain that separates an amino-terminal extracellular, ligand binding domain from an intracellular region that contains both a cyclase catalytic domain and a protein kinase homology domain (KHD) (1). The function of the KHD is not fully understood, but since ATP is required for transduction of the ANP binding signal to activation of the cyclase catalytic activity, and since various mutations within the KHD interrupt activation of GC-A is somewhat analogous to the guanine nucleotide-dependent activation of adenyl cyclase by hormones, non-hydrolyzable analogues of GTP activate adenyl cyclase in the absence of hormone, and sub-micromolar as opposed to millimolar concentrations of nucleotide are effective (10).

During previous attempts to purify GC-A, the ANP/ATP activation signal has vanished, even though ANP has continued to bind to the receptor (11, 12). Thus, it was suggested that additional factors (10) specifically ATP binding proteins (8), are required for transduction of a hormone binding signal to activation of the cyclase catalytic domain. Now we show, however, that after purification of detergent-solubilized GC-A to apparent homogeneity, the cyclase retains ability to be activated by ANP/ATP.

EXPERIMENTAL PROCEDURES

Materials—SF9 cells (13) were obtained from the American Type Culture Collection. Baculovirus was from Pharmingen. cDNA encoding FLAG-GC-A was kindly provided by Dr. Michael Chinkers (Vollum Institute, Oregon Health Sciences Center). Lumbilin 129A (from ICI) was a gift from Dr. Elliott Ross (University of Texas Southwestern Medical Center at Dallas). Polyoxyethylene-10-lauryl ether was from Sigma, M1 and M2 immunoaffinity columns were from IBI, wheat germ agglutinin-agarose was from Vector, and Q-Sepharose FF was from Pharmacia. 125I-ANP, [α-32P]GTP, and [1H]cGMP were from DuPont NEN, and rat ANP (99–126) was from Peninsula. Reagents used for radioimmunoassay of cGMP have been previously described (14). Expression of FLAG-GC-A in SF9 cells—The baculovirus expression vector PVL-FLAG-GC-A was cotransfected with BaculoGold virus into SF9 cells, and the recombinant virus was then cloned according to Kitts et al. (15). SF9 cells infected with the recombinant virus expressed ~0.7–1 pmol of 125I-ANP binding site/mg of particulate protein, while no significant binding was detected in uninfected cells.

Solubilization of FLAG-GC-A—48 h post-infection with the recombinant virus, SF9 cells expressing FLAG-GC-A were pelleted and frozen in liquid nitrogen. The frozen pellets were thawed in the presence of a solution containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 40% glycerol, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM β-glycerol phosphate, and 5 mM sodium azide (buffer A). The thawed cells were broken in a Dounce homogenizer, and crude particulate fractions were obtained and washed by centrifugation (30,000 × g) in the same buffer. The particulate fraction was resuspended in buffer A at a concentration of 20–30 mg of protein/ml. FLAG-GC-A was then solubilized by rocking the solution for 60 min at 4 °C in buffer A containing 1% Lubrol 129A or polyoxyethylene-10-lauryl ether. After 60 min, the detergent extract was diluted with buffer A to 0.5% of either non-ionic detergent and centrifuged at 100,000 × g. The supernatant fluid was obtained and diluted to 0.1% detergent with buffer A. Approximately 20–40% of the 125I-ANP binding sites detected in the particulate fraction were solubilized. Higher recoveries (up to 70–80% of 125I-ANP binding sites could be obtained at higher detergent:protein ratios; however, enzyme solubilized under these conditions lost ANP and ATP sensitivity (not shown).

Purification of FLAG-GC-A—All purification procedures were performed at 4 °C. Detergent extracts (200 ml) were rocked with 6 ml of M2 immunoaffinity resin for 2–3 h and then washed batchwise by centrif-
with 100 H2O was added in the initial wash of the Dowex. Adenylyl cyclase assays (17), with the modification that only 0.2 ml of detergent, 0.25 mM ATP, 150 mM NaCl, and 0.025% phosphatidylcholine (10 times, 30 ml) with buffer B (buffer A containing 0.05% [125I]NaI was removed by a G-25 spin column. The sample was then electrophoresed using an 8% separating gel by the method of Laemmli (18). The gel was stained with silver (19), vacuum-dried, and subjected to autoradiography with HYPER-FILM-MP (Amersham).

After removing the supernatant fluid from the IODOBEAD, free [125I]ANP was bound (125I-ANP binding) to the membranes and purified FLAG-GC-A. The amount of [32P]cGMP was determined as described previously for guanylyl cyclase activity (14). The values in the table are from one experiment representative of three such experiments.

### TABLE I

| Total protein | Specific activitya | Kd | Recovery | Purification | Specific activityb | Recovery | Purification |
|--------------|-------------------|----|----------|-------------|-------------------|----------|-------------|
| Membranes   | 485 mg            | 0.69 | 113 | 100 | 1 | 0.22 | 100 | 1 |
| Lubrol 12A9 extract | 35 mg | 3.9 | 64 | 141.3 | 6.3 | 2.2 | 70.9 | 10 |
| Purified FLAG-GC-A | 94 ng | 5784 | 66 | 0.16 | 8407 | 4259 | 0.4 | 19380 |

a pmol of 125I-ANP bound/mg of protein as determined by the Scatchard analysis.

b nmol cyclic GMP formed/min/mg protein with MnGTP as substrate in the presence of 0.1% Triton X-100.

### FIG. 1

SDS-polyacrylamide gel electrophoresis of FLAG-GC-A. Left panel, the membrane fraction (469 ng of protein), Lubrol 12A9 extract (713 ng of protein), or purified FLAG-GC-A (2.5 ng of protein) were electrophoresed and stained as described under “Experimental Procedures.” Right panel, 125I-Nal was incubated with buffer or with purified FLAG-GC-A (0.6 ng) in the presence of IODOBEAD. The labeled samples were electrophoresed and subjected to autoradiographic analysis as described under “Experimental Procedures.” Molecular weight markers are myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), and ovalbumin (45,000).

### RESULTS AND DISCUSSION

#### Expression of Recombinant FLAG-GC-A in Sf9 Cells

To facilitate rapid purification of the receptor, the FLAG epitope...
(DYKDDDDK) was introduced just to the carboxyl side of the signal peptide, thereby representing the amino terminus of the processed cyclase. The insertion of the FLAG epitope did not appear to alter biological activity of the enzymes since the number of $^{125}$I-ANP binding sites/mg protein or the relative cyclase activation in response to ANP/ATP in Sf9 membrane preparations was equivalent whether wild-type or FLAG-GC-A was expressed (data not shown).

ANP/caged ATP maximally activated FLAG-GC-A at 48 h following infection with recombinant baculovirus, at which time 0.6–1.0 pmol of $^{125}$I-ANP binding sites/mg protein of the crude particulate fraction were obtained (Table I). The described protocol to grow Sf9 cells (2% fetal bovine serum in IPL-41 in suspension), 0.7–1.0 nmol of $^{125}$I-ANP binding sites/liter of culture were routinely obtained.

Solubilization of Epitope-tagged GC-A—Initial experiments using many types of detergents such as Triton X-100 and deoxycholate to solubilize GC-A resulted in complete losses of ANP/ATP sensitivity (not shown). Of the various detergents tested, Lubrol 12A9 or polyoxyethylene-10-lauryl ether resulted in the highest ANP/ATP-responsive cyclase. However, high detergent/protein ratios during the initial solubilization decreased responsiveness to ANP/ATP. The ANP/ATP response of GC-A also was very sensitive to the concentration of glycerol, and the response to ANP/ATP was largely attenuated when solubilization buffers contained 20% glycerol but was retained in 40% glycerol. For the highest recovery of ANP/ATP-responsive cyclase, FLAG-GC-A was routinely solubilized with 1% detergent at protein concentrations of greater than 20 mg/ml and 40% glycerol. Under these conditions, 41% of the initial $^{125}$I-ANP binding activity and 7% of the particulate protein were solubilized (Table I). The $K_d$ for $^{125}$I-ANP binding for the solubilized and the membrane-bound receptor was the same under these conditions (Table I).

Purification of Ligand-sensitive FLAG-GC-A—The main objective of this study was to determine whether or not GC-A, serving as a prototype for the membrane guanylyl cyclases, could signal in the absence of other protein factors. Batch procedures using peptide competition (M2 immunoaffinity resin), N-acetylglucosamine competition (wheat germ agglutinin-agarose), and EDTA chelation (M1 immunoaffinity resin) were used to minimize the time required for purification. The capacity of the M2 immunoaffinity resin in buffer B restricted the amount of $^{125}$I-ANP binding sites applied to 40 pmol/ml resin. Typically, 40–50%, 60%, and 15–20% of FLAG-GC-A was recovered at the M2 immunoaffinity, wheat germ agglutinin-agarose, and M1 immunoaffinity steps, respectively. The final yield was typically about 0.2%.

Purity of FLAG-GC-A—The silver-stained FLAG-GC-A recovered after the above purification steps was the only major...
A band detected (Fig. 1), and densitometry of the silver-stained gel suggests that it is greater than 95% pure. The purity also was examined by the use of nonspecific radioiodination. FLAG-GC-A was the only major iodinated species, although longer exposures revealed a minor contaminant at about 70 kDa, a protein not detected by silver staining. The specific activity of the receptor preparation of 5.8 nmol of $^{125}$I-ANP bound/mg protein is comparable with previously purified ANP receptor (6–7 nmol/mg protein), suggestive of a highly purified cyclase preparation (12, 22).

**Ligand Binding Characteristics of FLAG-GC-A**—With either the crude detergent extract or the purified preparation of cyclase, a single class of high affinity $^{125}$I-ANP binding sites ($K_d = 65$ pm) was obtained (Fig. 2).

**Ligand/ATP Responsiveness of FLAG-GC-A**—In crude particulate fractions, ANP or caged ATP activated the cyclase by 1.3- to 1.7-fold, respectively, when added alone, but when added together, the cyclase was activated approximately 16-fold. The requirement for both ANP and the nucleotide, ATP, for maximum stimulation of GC-A has been previously reported (5, 8, 9). After detergent extraction, ANP and ATP continued to stimulate minimally when added alone, but together stimulated the cyclase about 9-fold. However, the detergent-solubilized GC-A is highly labile. Considerable ANP/ATP stimulation (50%) was lost upon storage at 4 °C for 24 h, and only about 20% remained after incubation at 25 °C for 60 min.²

Following an approximate 1300-fold purification of GC-A, the receptor remained responsive to ATP/ANP (Fig. 3). About 10% or 30% activations of FLAG-GC-A were obtained when ANP or caged ATP were added alone, respectively. When an equivalent number of $^{125}$I-ANP binding sites (0.5–1 fmol ANP binding sites) were added per assay, the amount of cGMP produced upon incubation with ANP/ATP was approximately the same for the detergent extract or the purified enzyme preparation (see also Fig. 4, A and B). Therefore, the maximal responsiveness to ANP/ATP did not change significantly during the purification procedure. The basal activities were 2–3-fold higher in the purified preparation, and consequently the -fold stimulation by ANP/ATP decreased from approximately 8-fold in the crude detergent extract to 3–4-fold in the purified GC-A preparation. The guanylyl cyclase reaction was linear for at least 5 min, and as shown in Fig. 5, ANP/ATP appears to alter $V_{max}$ as a function of MgGTP. ATP at millimolar concentrations is required for maximal stimulation by ANP (Fig. 5).

2 S. K.-F. W, C.-P. Ma, D. C. Foster, A.-Y. Chen, and D. L. Garbers, unpublished results.
guanylyl cyclases normally display positive cooperative kinetics as a function of MnGTP. FLAG-GC-A also yielded positive kinase substrates such as histone IIIs and VIIs, we have not activity (27) suggests that protein kinase activity may be ab-
without the loss of protein kinase activity observed in the c-Kit mu-
tated to an Asn (26). An Asn rather than an Asp is also
molecule. This is in contrast to the G protein-coupled receptor
other protein cofactors. In this unique signaling system, which utilizes ATP as the signal
nase activators such as histone IIIs and VIIs, we have not observed ATPase activities from 150 ng of our purified
guanylyl cyclase.

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Figure 6. Kinetics of guanylyl cyclase activation by MnGTP. Guan-
ylyl cyclase activities were determined using equivalent amounts of
125I-ANP binding sites (0.1 fmol/assay) of crude detergent extract ((C) (35 ng of protein) or purified FLAG-GC-A (●) (0.024 ng of protein) with the indicated concentrations of MnGTP. The experiment was performed for 10 min at 37°C in the presence of a constant concentration (1.2 mM) free Mn2+. Results are mean ± S.E. of duplicate assays. Inset, double reciprocal plot. Velocities are expressed as the pmol of cyclic GMP formed per 10 min.

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the catalytic pocket of protein kinases (28). GC-A represents a prototype of the particulate guanylyl cyclase family where there are at least six subtypes identified so far (1–4). The results from this study initially show that the ligand/ATP are able to activate this class of particulate receptor guanylyl cyclases independent of other protein cofactors. In this unique signaling system, which utilizes ATP as the signal transduction molecule, the domains for ligand binding, ATP regulation, and the effector guanylyl cyclase are located in one molecule. This is in contrast to the G protein-coupled receptor system, where the components for ligand binding, signal transducing, and effector are located in separate protein molecules. The mechanism of activation of GC-A is not yet understood, but the ANP/ATP activation is known to be regulated by phosphorylation (29–31) and oligomerization (32, 33). Although additional protein factors therefore are not required for signal transduction, it also seems likely that modulating factors will be discovered based on precedence with other cell surface receptors.
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