Recombinant Expression of a Secreted Form of the Atrial Natriuretic Peptide Clearance Receptor*

J. Gordon Porter†, Robert M. Scarborough, Yu Wang, Dale Schenk, Glenn A. McEnroe, Ling-Ling Kang, and John A. Lewicki

From California Biotechnology, Incorporated, Mountain View, California 94043

A general structure for the atrial natriuretic peptide clearance receptor (ANP C-receptor) has been proposed based on hydropathicity analysis of the deduced amino acid sequence of this membrane protein (Fuller, F., Porter, J. G., Arfsten, A., Miller, J., Schilling, J., Scarborough, R. M., Lewicki, J. A., and Schenk, D. B. (1988) J. Biol. Chem. 263, 9395–9401). The ANP C-receptor is believed to possess a large amino-terminal extracellular domain (436 amino acids), a single hydrophobic transmembrane anchor (23 amino acids), and a short cytoplasmic tail (37 amino acids). As a means of testing the structure and proposed cellular orientation of this protein, we have employed the technique of in vitro mutagenesis to prepare a receptor mutant (anc) lacking the transmembrane and cytoplasmic domains. Expression of this mutant in mammalian cells using a vaccinia virus vector results in secretion of a truncated soluble form of the ANP C-receptor which binds native ANP and synthetic ANP analogs with a specificity similar to that of the native ANP C-receptor. In contrast to the native ANP C-receptor that exists predominantly as a homodimer on the cell surface, the secreted receptor exists as a monomeric species. The results are consistent with the proposed structure of this receptor with the amino-terminal domain containing the ANP-binding site oriented extracellular to the plasma membrane. In addition, these data demonstrate that the receptor does not require association with the plasma membrane or its native dimeric configuration in order to bind ANP ligands with high affinity and specificity.

Atrial natriuretic peptide (ANP)† is a circulating hormone secreted from cardiac atria in response to atrial stretch (1). ANP has been shown to bind to receptors in the periphery and brain (2–7) resulting in a number of diverse biological actions. These effects include natriuresis, diuresis, and the inhibition of secretion of aldosterone and renin (8–11). Two distinct ANP receptor subtypes have been identified and characterized in various tissues and cell types. The ANP B-receptor is a 120,000-dalton subunit protein that is characterized by its rigorous binding specificity for native ANPs. This receptor has been shown to possess intrinsic ANP-dependent guanylate cyclase activity that may mediate many of the biological effects of ANP (12–19). The ANP B-receptor has been purified by several laboratories (15–18), and the amino acid sequence of the protein has recently been deduced from analysis of cloned DNA sequences that encode the receptor (19).

A second receptor, termed the ANP C-receptor, is distinct from the B-receptor in terms of its structure, immunoreactivity, physiological role, and ligand-binding specificity (16, 20–23). The ANP C-receptor exhibits a high affinity for native ANP and is also able to bind various truncated and internal ring-contracted analogs of ANP with high affinity (21–23). The ANP C-receptor represents the major ANP-binding site in cultured vascular cells and in the kidney (5, 22). Although this receptor is not involved in stimulation of particulate guanylate cyclase and does not appear to mediate directly any of the known biological effects of ANP, it has been shown to mediate the metabolic clearance and degradation of this hormone (21, 22).

The purification, cloning, and recombinant expression of the ANP C-receptor has been reported recently, and this has allowed a more detailed understanding of the structural and functional properties of this protein (20, 24, 25). The ANP C-receptor consists of a single ~60,000-dalton subunit that appears to exist predominantly as a homodimer on the cell surface. From hydropathicity analysis of the deduced amino acid sequence, the receptor subunit is shown to have two extended hydrophobic regions. One, at the amino terminus, has the characteristics of a signal sequence and may be involved in translocation of the protein to the cell surface. The second region is located near the carboxyl terminus and is proposed to represent a transmembrane domain. A possible receptor structure thus consists of a large amino-terminal extracellular domain that contains the ANP-binding site, a single transmembrane region, and a short carboxyl-terminal cytoplasmic tail. We have used the technique of in vitro mutagenesis to delete the transmembrane and cytoplasmic domains and have expressed this truncated receptor in mammalian cells. The properties of the resulting soluble and secreted ANP C-receptor mutant are described in this report.

MATERIALS AND METHODS

Preparation of Recombinant Plasmid DNA—The ANP C-receptor cDNA insert ANPRc3/4 was subcloned into the expression vectors pSC11 and pGEM1 using standard recombinant DNA techniques yielding plasmids pSCANPRc3/4 and pGEMANPRc3/4 as described (20, 25). The pGEM1 vector contains the SP6 promoters which allowed the synthesis of receptor-specific RNA using SP6 polymerase (Promega Biotec). In vitro translation of this mRNA was accom-
plished with a cell-free system derived from reticulocytes (Du Pont–New England Nuclear). The pSC11 construct allowed the generation of specific vaccinia recombinants that were used subsequently to infect confluent monolayers of L-M cells as described below.

**Oligonucleotide-directed Mutagenesis—**In vitro mutagenesis was performed by the method of Zoller and Smith (18). Oligonucleotides containing mutated receptor sequences (pSCANPRc3/4) or mutant receptor cDNA sequences (pSC9247) (29) were precipitated with recombinant plasmid DNA containing wild-type receptor cDNA insert (9247). Two oligonucleotides were designed for mutagenesis experiments. One contained a single base change (G-A) and the other a frame-shift mutated receptor cDNA sequence. Sequences of this virus stock were used to infect confluent monolayers of L-M cells as described below.

**Propagation and Isolation of Recombinant Vaccinia Virus—**Vaccinia virus recombinants were isolated and grown as described previously (20). Wild-type vaccinia virus (Wyeth strain) was propagated in CB-1 cells (green monkey kidney) as described (28). CV-1 cells were incubated at 37°C for 2 days and harvested a second time with agarose containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The culture medium was solubilized with NaOH, and counted as described (5). Culture medium from infected cells was also saved and assayed for specific binding of [125I]-ANP. Typically, 0.5 ml of infected cell medium was incubated with [125I]-ANP in the presence of various concentrations of unlabeled ANP for 30 min at 20°C. Culture medium from infected cells was assayed for specific binding of [125I]-ANP. This receptor predicted from the nucleotide sequence of the corresponding cDNA (25). Selection of a peptide region for synthesis was based on hydrophilicity indices determined by the method of Hopp and Woods (32). Peptides (receptor homologous peptides as well as ANP analogs) were synthesized exclusively by the solid phase method using protocols described previously (20). Purified peptides were characterized by amino acid analysis and homogeneity assessed by enzymatic sequencing (27).

**Peptide Synthesis and Conjugation to Carrier—**A peptide (P4) homologous to a specific region of the ANP C-receptor (amino acids 118–136) was synthesized according to the amino acid sequence of this receptor predicted from the nucleotide sequence of the corresponding cDNA (25). Selection of a peptide region for synthesis was based on hydrophilicity indices determined by the method of Hopp and Woods (32). Peptides (receptor homologous peptides as well as ANP analogs) were synthesized exclusively by the solid phase method using protocols described previously (20). Purified peptides were characterized by amino acid analysis and homogeneity assessed by enzymatic sequencing (27).

**Generation of Antisera—**In order to characterize recombinant receptor proteins, we prepared specific antisera against a region of the C-receptor by inoculating rabbits with a peptide (P4) homologous to a distinct region of the deduced amino acid sequence (amino acids 118–136) of the cloned protein. New Zealand white rabbits were immunized by intradermal injection of 1 mg of the corresponding peptide emulsified in complete Freud's adjuvant. Each animal was subsequently boosted three times at 2-week intervals with ~600 μg of immunogen. Serum samples were collected from these animals at 2-week intervals and titered according to their ability to immunoprecipitate recombinant radiolabeled ANP C-receptor. Immunoprecipitation was performed according to the method of Ressler (33) with modifications as described previously (20).

**Endoglycosidase Experiments—**Samples of immunoprecipitated and radiolabeled ANP C-receptor were first solubilized by boiling for 5 min in 35 μl of 0.5% SDS and 1% β-mercaptoethanol. Samples were then diluted into 20 μl of buffer containing 250 mM sodium phosphate, 0.1% SDS, and 2% Nonidet P-40 followed by the addition of 1–2 units of endoglycosidase F Du Pont–New England Nuclear. For endoglycosidase H reactions, immunoprecipitated and solubilized samples were diluted into 10 μl of 100 mM citrate (pH 5.5) followed by the addition of 150 ng of enzyme (Du Pont–New England Nuclear). Incubations were for 12 h at 37°C. Reaction mixtures were then diluted into SDS sample buffer, boiled, and electrophoresed on a 12.5% polyacrylamide gel as described. Results were visualized via autoradiography.

**RESULTS AND DISCUSSION**

**In Vitro Translation of Receptor-specific mRNAs—**A putative schematic representation of the ANP C-receptor is shown in Fig. 1. Based on the hydrophytlicity profile of this protein and analogies with related receptor proteins, we have hypothesized that the ANP C-receptor is comprised of a large extracellular domain (436 amino acids), a single-transmembrane domain (126 amino acids), and a cytoplasmic tail (37 amino acids) (25). As a means of examining this structure further, we have used the technique of in vitro mutagenesis to introduce an in-frame stop codon into the cDNA just upstream from the putative hydrophobic transmembrane domain (amino acids 437–459) (Fig. 1). Subcloning of native and mutant cDNAs into the SP6 transcription vector pGEM1 allowed the synthesis of specific messenger RNAs corresponding to the truncated mutans as well as full length mRNA.
The proposed structure of the receptor is represented in schematic form. The striped area represents the signal sequence. The stippled area represents the proposed transmembrane domain extending from amino acids 437 through 459 and is followed by the cytoplasmic domain (stippled). The DNA sequence used to design the mutagenic oligonucleotide is also represented, in which the codon corresponding to Glu$^{437}$ (GAA) is changed to a stop signal $\text{UAA}$. Also represented are potential sites of receptor glycosylation.

**Fig. 1.** Site-specific mutagenesis of ANP C-receptor cDNA. The proposed structure of the receptor is represented in schematic form. The striped area represents the signal sequence. The stippled area begins at the amino terminus of the mature protein. The black area represents the proposed transmembrane domain extending from amino acids 437 through 459 and is followed by the cytoplasmic domain (stippled). The DNA sequence used to design the mutagenic oligonucleotide is also represented, in which the codon corresponding to Glu$^{437}$ (GAA) is changed to a stop signal $\text{UAA}$ by a G–T transition at nucleotide 1894. Also represented are potential sites of receptor glycosylation.

**Fig. 2.** In vitro translation of ANP C-receptor-specific mRNA. Mutant and native receptor-specific mRNAs were synthesized using the viral SP6 polymerase and translated in vitro using a cell-free reticulocyte lysate (see "Materials and Methods"). Radiolabeled protein products were separated by SDS-polyacrylamide gel electrophoresis and the result visualized by autoradiography. The migration of molecular weight standards is indicated.

Recombinant ANP C-receptor. In vitro translation of the full length receptor mRNA resulted in a ~56,000-dalton primary translation product as predicted by the amino acid sequence. In contrast, translation of the mRNA derived from the mutant cDNA clone resulted in a truncated receptor protein of ~50,000 daltons consistent with removal of 58 amino acids at the carboxyl terminus (Fig. 2).

**Expression of Native and Mutant Receptor in L-M Fibroblasts**—Infection of mouse fibroblasts (L-M cells) with recombinant vaccinia virus containing the native ANP C-receptor cDNA resulted in the expression of a membrane-bound receptor as measured by the specific saturable binding of $^{125}$I-ANP to confluent cell monolayers (20). As shown in Fig. 3A, unlabeled ANP (102–126) competed for binding to these cells with a $K_i$ of 2.2 nM. No specific ANP binding could be detected in the medium of these cells. In addition, $^{125}$I-ANP did not bind to L-M cells infected with control vaccinia virus.

In contrast to cells infected with recombinant virus encoding native receptor, cells infected with vaccinia virus containing mutant receptor sequences (anc-) exhibited no specific binding to infected monolayers. However, specific binding of $^{125}$I-ANP was observed in culture medium from these infected cells, demonstrating that this mutant receptor is a secreted protein (Fig. 3B). Binding of $^{125}$I-ANP to this secreted receptor was saturable (not shown), and competitive binding experiments yielded a $K_i$ for ANP (102–126) of 2.3 nM, a value nearly identical to that seen for the native membrane-associated receptor (5). Data presented in Fig. 3C show that the mutant-secreted receptor possesses the ability to bind actively a ring-contracted ANP analog [(desQSGLGL)ANP (102–121)NH$_2$] as well as truncated linear ANP analogs (not shown) which are specific for the ANP C-receptor (22, 34). This secreted ANP receptor thus retains the affinity and specificity for ANP and C-receptor-specific ANP analogs attributed previously to this receptor in its native environment on the surface of cultured smooth muscle cells.

**Immunoprecipitation of the Secreted ANP C-receptor**—Immunoprecipitation experiments were performed using the P4 antisera described under "Materials and Methods." $K_i$ values were determined as described by Cheng and Prusoff (43).
methionine-labeled L–M cells infected with recombinant vaccinia virus bearing either the intact or truncated (anc–) receptor coding sequences. Immunoprecipitation of whole cell lysates prepared from cells infected with the native receptor construct yielded a 65,000-dalton protein upon electrophoresis. Very little immunoreactive protein was observed in the culture medium (Fig. 4). Conversely, L–M cells infected with anc– display two immunoreactive proteins in the 55,000–60,000-dalton range in the culture medium whereas only a trace amount of a 55,000-dalton protein is evident in the cell lysate itself. The appearance of receptor proteins in the culture medium of cells infected with anc– is consistent with secreted ANP-binding activity.

Experiments were performed to resolve the observed molecular heterogeneity of the secreted ANP C-receptor proteins. Potential N-linked glycosylation of the C-receptor has been suggested by the presence of canonical glycosylation signals in the receptor sequence (25). This contention is supported by the observation that purified ANP C-receptor exhibits an apparent molecular mass of 65,000 upon SDS electrophoresis, which is larger than the value predicted from the amino acid sequence (55,700) and the molecular mass observed upon SDS-gel electrophoresis of the in vitro translation product (56,000 daltons) (24). As shown in Fig. 5, it is evident that the 35S-labeled receptor has an altered electrophoretic mobility upon treatment with endoglycosidase F or H, indicating that the protein has both high mannose and complex sugars attached to it. The mobility of the endoglycosidase F-treated receptor (~56,000 daltons) is similar to that of the in vitro translation product, suggesting that the difference between the observed molecular mass of the native protein and that predicted from the amino acid sequence is due to N-linked glycosylation. The secreted receptor also appears to be glycosylated, since both immunoreactive electrophoretic bands observed in conditioned medium are shifted to lower molecular mass upon treatment with endoglycosidase H. Both immunoprecipitable bands collapse to a single, broad ~50,000-dalton protein band upon treatment with endoglycosidase F. Thus, the observed heterogeneity of the secreted receptor protein upon immunoprecipitation presumably represents alternate glycosylation products of a single secreted protein.

Secreted ANP C-receptor Exists in a Monomeric State—
The ANP C-receptor migrates as a 120,000-dalton homodimer upon SDS-polyacrylamide gel electrophoresis performed under nonreducing conditions (24, 35). However, 125I-ANP cross-linking experiments have suggested that the C-receptor may exist as both monomer and dimer on the cell surface, perhaps in equilibrium (20, 36, 37). Results shown in Fig. 6 support this possibility since 35S-labeled recombinant receptor is seen...
ANP receptor addressed in more detail. The receptor is aberrantly glycosylated but still shows high affinity attachment and maturation of carbohydrate. It is not known if carbohydrate side chains on the extracellular domain are previously to be important in signaling transport of integral membrane and cytoplasmic protein domains have been shown (15, 16, 25, 35) but is here clearly demonstrated. The detection of truncated receptor species increased with extended (>24 h) vaccinia infections (not shown), and truncation of native ANP C-receptor sequences can also result in secretion of a small amount of a truncated receptor protein in addition to a large quantity of membrane-associated receptor. The detection of truncated receptor species increased with extended (>24 h) vaccinia infections (not shown), and its appearance may be the result of proteolysis of membrane-associated C-receptor. Whether release of receptor from the membrane occurs in vivo or is simply an artifact of cell death resulting from extended viral infection has not been determined. However, there are reports of ANP bound to a larger component in serum (38–40), suggesting the existence of naturally circulating ANP-binding proteins that could be derived from the ANP C-receptor.

Glycosylation of the C-receptor has been suggested previously (15, 16, 25, 35) but is here clearly demonstrated. The electrophoretic mobility of the recombinant ANP C-receptor is increased substantially by treatment with endoglycosidase F but only slightly with endoglycosidase H. These differences suggest that the majority of attached carbohydrate is of the N-linked complex type. It is not clear why the truncated mutant receptor is differentially glycosylated, however transmembrane and cytoplasmic protein domains have been shown previously to be important in signaling transport of integral membrane proteins through the endoplasmic reticulum to the cell surface (41, 42). Interference with this transport path may affect proper post-translational modification involving attachment and maturation of carbohydrate. It is not known if carbohydrate side chains on the extracellular domain are necessary or influence ligand-binding properties of the receptor. However, the observation that the secreted ANP C-receptor is aberrantly glycosylated but still shows high affinity for ANP- and C-receptor-specific ANP analogs suggest that specific oligosaccharide structures play only a minor role in ligand-binding affinity and specificity. This point must be addressed in more detail.

The native C-receptor may exist in monomer-dimer equilibrium on the cell surface. However, the data presented here demonstrate that the secreted receptor does not form covalent dimers and yet binds ANP in a fashion similar to native receptor. Based on this observation, it seems likely that monomeric and dimeric forms of the native ANP C-receptor may function equally well in binding ligand on the cell surface and do not represent separate receptor forms as has been suggested previously (36). The fact that the truncated monomeric receptor is readily secreted from cells implies that covalent dimerization of the native protein is not necessary for its transport to the cell surface and may occur only after the receptor has migrated to the extracellular membrane. It is not known if dimerization of this protein is important for other possible receptor functions such as internalization.

Analogous truncations of other cell surface proteins and receptors containing a single transmembrane domain have also led to the secretion of soluble proteins with immunoreactivity or binding activity similar to that of the native membrane protein (44–46). The overall deduced structure of the ANP clearance receptor is also similar to that of other receptors that are involved primarily in ligand sequestration and internalization rather than activation of second messenger systems leading to biological effects. Examples of these are the insulin-like growth factor II/M6P receptor and the low density lipoprotein receptor (47, 48). Both of these receptors contain a single transmembrane domain with a relatively large extracellular ligand-binding domain and are responsible for the internalization of ligand. Other important points that need to be addressed concerning the biological role of this ANP receptor include receptor phosphorylation and cell surface aggregation. We feel that this vaccinia virus expression system will be an efficient tool for examining other ANP C-receptor mutants which will be useful for probing the structure-function relationships of the ANP C-receptor.

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