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Production of an atrial natriuretic peptide variant that is specific for type A receptor

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Receptor-specific variants of atrial natriuretic peptide (ANP) were selected from libraries of filamentous phage particles that displayed single copies of random ANP mutants fused to gene III protein. These ANP variants were differentially selected by binding to immobilized natriuretic peptide receptor A (NPR-A) over competing receptor C (NPR-C) in solution. This method also selected ANP variants with improved secretion expression in *Escherichia coli*. Several of the identified mutations were combined to produce an efficiently expressed ANP analog that was as potent as wild-type ANP in stimulating NPR-A guanylyl cyclase activity but resistant to inactivation mediated by NPR-C. Such NPR-A-selective analogs should be useful for correlating the various activities of ANP to the relevant receptor and may also be more potent therapeutics in the targeting of NPR-A.

Key words: atrial natriuretic peptide/negative selection/phage display/receptor-specific/secretion expression

Introduction

Atrial natriuretic peptide (ANP) is a potent 28 amino acid hypotensive hormone that has both direct and indirect effects on blood pressure, that stimulates salt and water secretion and plays an important role in maintaining cardiovascular homeostasis. The very short half-life of ~3 min for ANP (Crozier *et al.*, 1986; Yandle *et al.*, 1986) permits the rapid modulation of serum ANP concentrations and may be important for its role as a regulatory hormone. However, although this instability is likely to be physiologically significant, the development of ANP as a pharmaceutical may benefit if a longer lasting derivative can be produced.

ANP can bind to either of two cellular natriuretic peptide receptors, NPR-A (Chinkers *et al.*, 1989; Lowe *et al.*, 1989) or NPR-C (Fuller *et al.*, 1988). Both these receptors have homologous extracellular domains of ~440 residues, and a single transmembrane domain. But, while NPR-A possesses both guanylyl cyclase and tyrosine kinase-like domains, NPR-C contains only a small 37 amino acid intracellular domain of unknown function.

The role of these receptors in regulating blood pressure is unclear but NPR-A is thought to be responsible for most of the hormone's biological effects (reviewed by Maack, 1992). Maack and coworkers (1987) have synthesized NPR-C-selective ANP variants and shown that the administration of these analogs causes the elevation of endogenous ANP levels and associated hypotensive effects, presumably by blocking the NPR-C-mediated clearance of ANP (Almeida *et al.*, 1989). Thus NPR-C, together with kidney filtration and proteolysis (Koehn *et al.*, 1987; Olins *et al.*, 1987; Sonnenberg *et al.*, 1988; Tamburini *et al.*, 1989), appears to mediate the rapid clearance of ANP from serum. These studies support the proposal that NPR-A-selective ANP variants that do not recognize NPR-C would have extended serum half-lives and enhanced potency. Additionally, such receptor-selective analogs may help to dissect further the biological roles of these receptors, including recently reported NPR-C effects (Levin, 1993). However, despite the generation of a great number of ANP analogs (Nutt *et al.*, 1989; Minamitake *et al.*, 1990; von Geldern *et al.*, 1992), thus far no such NPR-A-specific ANP derivatives have been reported.

Here, we have applied a method called monovalent phage display (Bass *et al.*, 1990; Lowman *et al.*, 1991) to screen some 10^7 variants of ANP for ones that bind tightly to human NPR-A and not to human NPR-C. After multiple rounds of binding enrichment we isolated a number of ANP variants that bound preferentially to NPR-A. By combining several of these mutations we were able to produce a highly NPR-A-selective ANP variant that does not bind NPR-C. The resistance of this variant to inactivation by cultured cells expressing NPR-C illustrates the potential of enhanced potency for this molecule. Additionally, we have found the selection to be a powerful means of isolating ANP variants that are secreted more efficiently. These studies show the utility of monovalent phage display for isolating receptor-selective hormones and to improve their secretory properties for recombinant expression purposes.

Results and discussion

Recently, the human NPR-A and NPR-C extracellular binding domains have been expressed as IgG fusion proteins that bind ANP with high affinity (Bennett *et al.*, 1991). These reagents were critically important for the manipulations that have allowed us to produce the receptor-selective ANP variants described here. All the references to NPR-A and NPR-C in this paper, that involve *in vitro* manipulations, refer to these IgG receptor fusions. Additionally, the references to ANP refer to the human sequence.

Display of functional ANP on the surface of phagemid

Monovalent display of target proteins by f1 filamentous phage is a proven tool for the selection of hormone variants with greatly enhanced receptor affinities (Lowman and Wells, 1993). We have expanded the utility of this method to enable us to produce receptor-selective variants of ANP. As a first step, to enable positive selection for NPR-A binding and negative selection against NPR-C binding, it was necessary to make ANP phagemid particles that bound to

both receptors. To accomplish this, we fused a synthetic ANP gene to the carboxyl portion of gene III protein (Figure 1A) and produced phagemid that bound tightly to each receptor with an EC_{50} of 180 and 310 pM, respectively (Figure 1B). These values were nearly the same as those measured for displacement of ^{125}I-labeled ANP, by unlabeled native ANP in a similar assay (Figure 2A), and indicated that the gene III fusion did not significantly reduce receptor affinity. Also, isolation of phagemid–receptor complexes, after saturation binding with receptor, showed that only 0.02% of the infective phagemid particles displayed ANP (data not shown). This ensured that the phagemid particles would only display single molecules of ANP and avoid binding artifacts caused by avidity effects (Bass *et al.*, 1990; Cwirla *et al.*, 1990).

Selection of phagemid pools enriched for NPR-A specificity

Seven libraries of variant ANP phagemid were produced from which we could select receptor-specific variants. Each library completely randomized a unique three or four amino acid segment (Figure 3, step 1) and together they mutated the entire ANP molecule except for the C-terminal tyrosine at position 28, and the cystine residues at positions 7 and 23. We decided to omit Tyr28 from our analysis because preliminary deletion studies had suggested that this residue may be important for receptor activation. Prior to the initial negative selection, the libraries were pre-enriched for phagemid that could tightly bind to NPR-A, in order to prevent the loss of rare variants (see Materials and methods). To isolate NPR-A-specific variants the selection strategy shown schematically in steps 2–4 of Figure 3 was then employed.

The binding characteristics of the selected phagemid pools after zero, two, four and seven cycles of enrichment were assessed with measurements of the competitive displacement of each pool from immobilized NPR-A, by either NPR-A or NPR-C. The apparently high affinities observed for NPR-A by the sort 7 pools (red curves in Figure 4A), relative to the low affinities observed for NPR-C by most of these same pools (red curves in Figure 4B), demonstrated that the selected mutations conferred specific binding to NPR-A. Additionally, the progressive increases in maximal binding (see *y*-intercepts), as the sorting proceeded from the starting libraries to the final sort (sort 0, 2, 4 and 7 in black, blue, green and red curves, respectively), showed that the pools were also selected for mutations that increased the percentage of phagemid displaying ANP.

Analysis of individual phagemid selectants

We discovered from our sequence analysis of the selectants that frequent mutations occurred at three positions located outside the randomization windows (see shaded cells, Table I). This was surprising since most of these mutations were likely to have originated from rare polymerase-directed nucleotide misincorporation errors that occurred during the *in vitro* mutagenesis. In fact, T7 DNA polymerase errors have been shown to average only 1 per 100 000 bases (Mattila *et al.*, 1991). The prevalence of these mutations, despite their relative infrequency in the starting libraries,

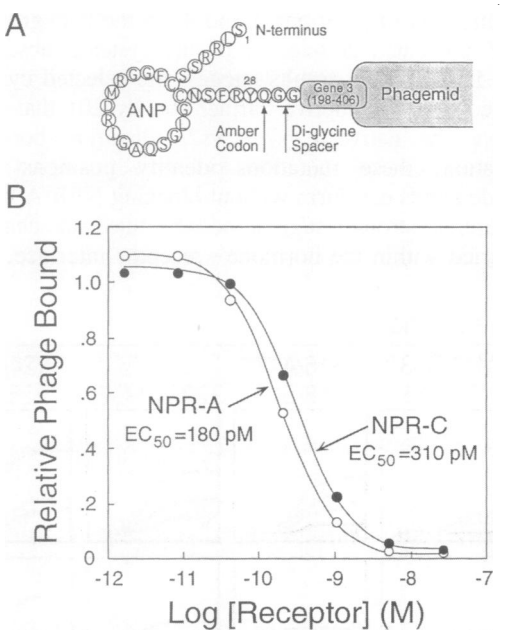


Fig. 1. Receptor binding of ANP display phage. (**A**) Schematic representation of pB1537 phagemid that depicts the surface display of ANP as a fusion protein with a portion of the gene III coat protein. The fusion protein consists of the 28 residue hANP hormone followed by a glutamine (from an amber codon in *SupE E.coli*), two glycines and the C-terminal region of gene III protein (residues 198–406). (**B**) Plot shows ELISA measurements of pB1537 phagemid binding to NPR-A and NPR-C. The EC_{50} values given show the concentration of competing receptor that results in half-maximal binding to the phagemid.

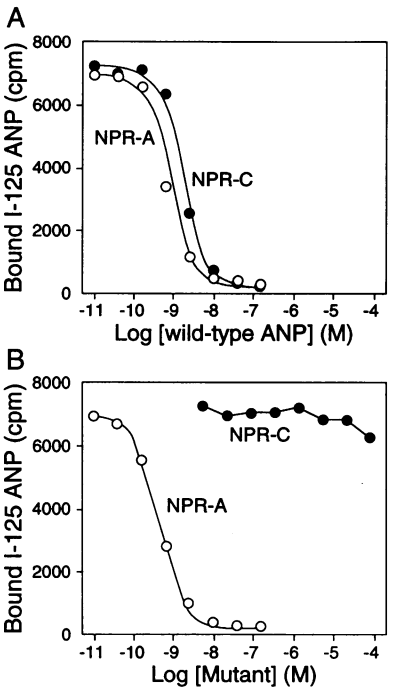


Fig. 2. Binding specificity of wild-type ANP and the ANP hexamutant. The competitive displacement of [^{125}I]ANP from immobilized NPR-A or NPR-C by wild-type ANP (**A**) or the hexamutant containing the mutations R3D, G9T, R11S, M12L, R14S and G16R (**B**) is shown. Conditions used were essentially as described for measuring phagemid affinities except that the radioiodinated ANP tracer replaced the phagemid and serial dilutions of purified hormone were used as the competitor. The EC_{50} values measured for NPR-A and NPR-C binding were 590 pM and 1700 pM for wild-type ANP, and 460 pM and >100 μM for the hexamutant, respectively.

indicated that they must confer a very strong selective advantage.

The substitution G16R in 27 of 64 sequences (Table I) was the most frequently observed misincorporation mutation. Its occurrence within 90% of the unique clones derived from the mutagenesis windows located within the first 19 residues of ANP (Table I) correlated well with the strong NPR-A specificity exhibited by these selected libraries (Figure 4). Binding analysis of phagemid containing only the G16R mutation showed this change contributed dramatic receptor specificity, with NPR-C affinity being reduced 150-fold while NPR-A affinity was unchanged (Table II). In our analysis this mutation confers more NPR-A selectivity than any other single mutation. Interestingly, an ANP-like molecule from snake venom containing Asn16, has recently been shown to have some preference for binding NPR-A (Schweitz *et al.*, 1992). Our results suggest the G16N substitution present in this homolog may contribute to that specificity.

Another prominent characteristic of the selected libraries was the occurrence of cysteines in 33 of the 64 sequences analyzed within the randomization windows (Table I). These mutations occurred singly at specific locations and together were found only at positions 1–6, 19, 22 and 24 (Table I and data not shown). The possibility these cysteines directed the formation of alternative functional ANP disulfide bonds was ruled out since variants that would allow only the non-native disulfide (C7S or C23S) did not bind NPR-A (data not shown). Ultimately, a downstream mutation that removed a gene III cysteine in some variants (e.g. Table I, clone 13), and was correlated with increased display (data not shown), provided the critical clue. We reasoned that since this cysteine (C201) lost a neighboring cysteine (C188), from the ANP–gene III fusion, it may be unpaired and able to disrupt the native ANP disulfide bond. Indeed, subsequent randomization of positions 3 and 4, in the background of C201Y, prevented the selection of such cysteine substitutions (Table IIIA). These results suggest the selected cysteines provided a non-disruptive partner for Cys201 that helped preserve the native Cys7–Cys23 disulfide bond. By implication, these mutations identify positions where disulfide bonds can form without blocking NPR-A binding and therefore strongly suggest the substituted side chains are not buried within the hormone–receptor interface.

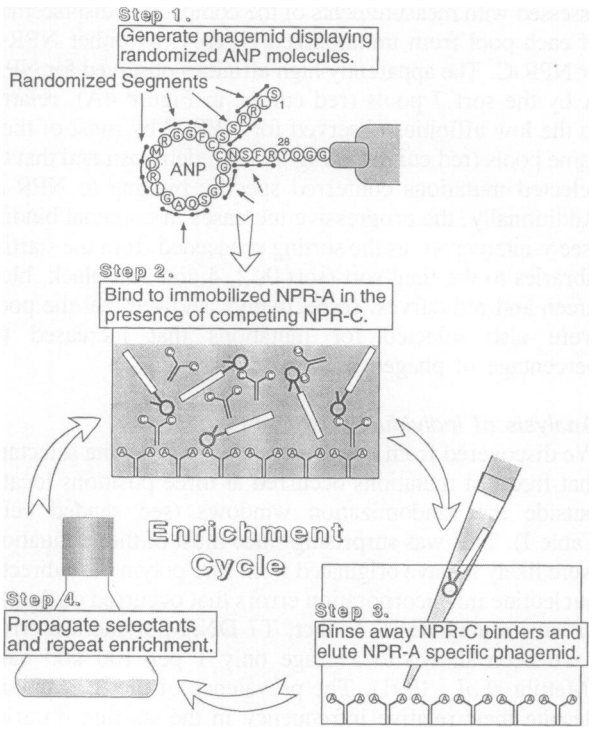


Fig. 3. Strategy for isolation of ANP variants that selectively bind NPR-A. **Step 1:** phagemid libraries that displayed ANP molecules with patches of three or four contiguous residues fully randomized for all 20 amino acids were produced by site-directed mutagenesis as described in Materials and methods. **Step 2:** selections for NPR-A-specific mutants were made by binding libraries to immobilized NPR-A in the presence of competing NPR-C in solution (starting at 4 nM). **Step 3:** after 2 h the bound phage were eluted with 0.2 M glycine (pH 2.0), neutralized and titered by infecting *E.coli* (XL1-blue). **Step 4:** the remaining anti-selected phage were then further propagated for use in the next selection cycle. Phagemid libraries that demonstrated resistance to binding inhibition by NPR-C were anti-selected with higher concentrations of this receptor (up to 200 nM) in subsequent cycles.

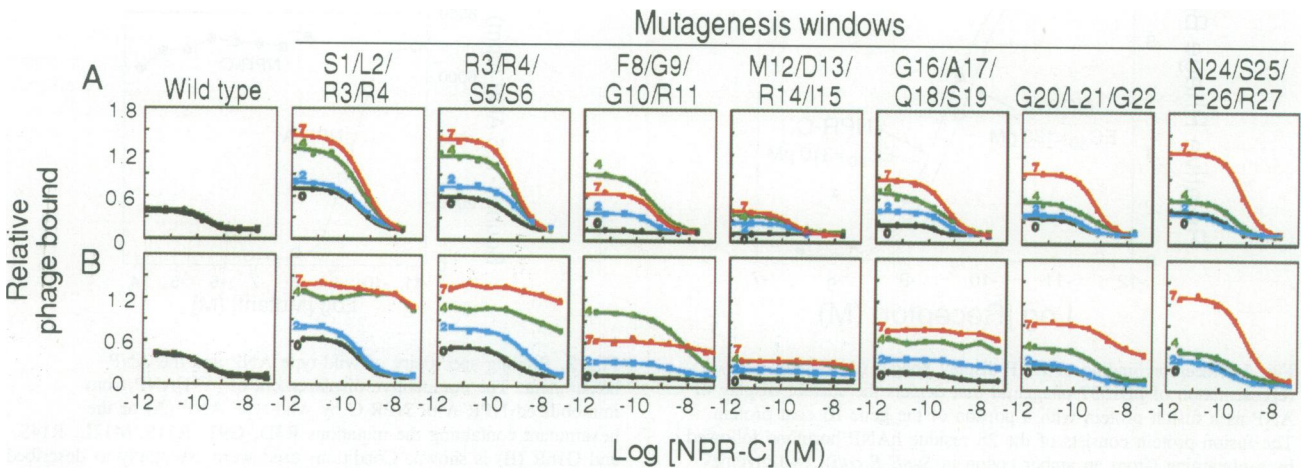
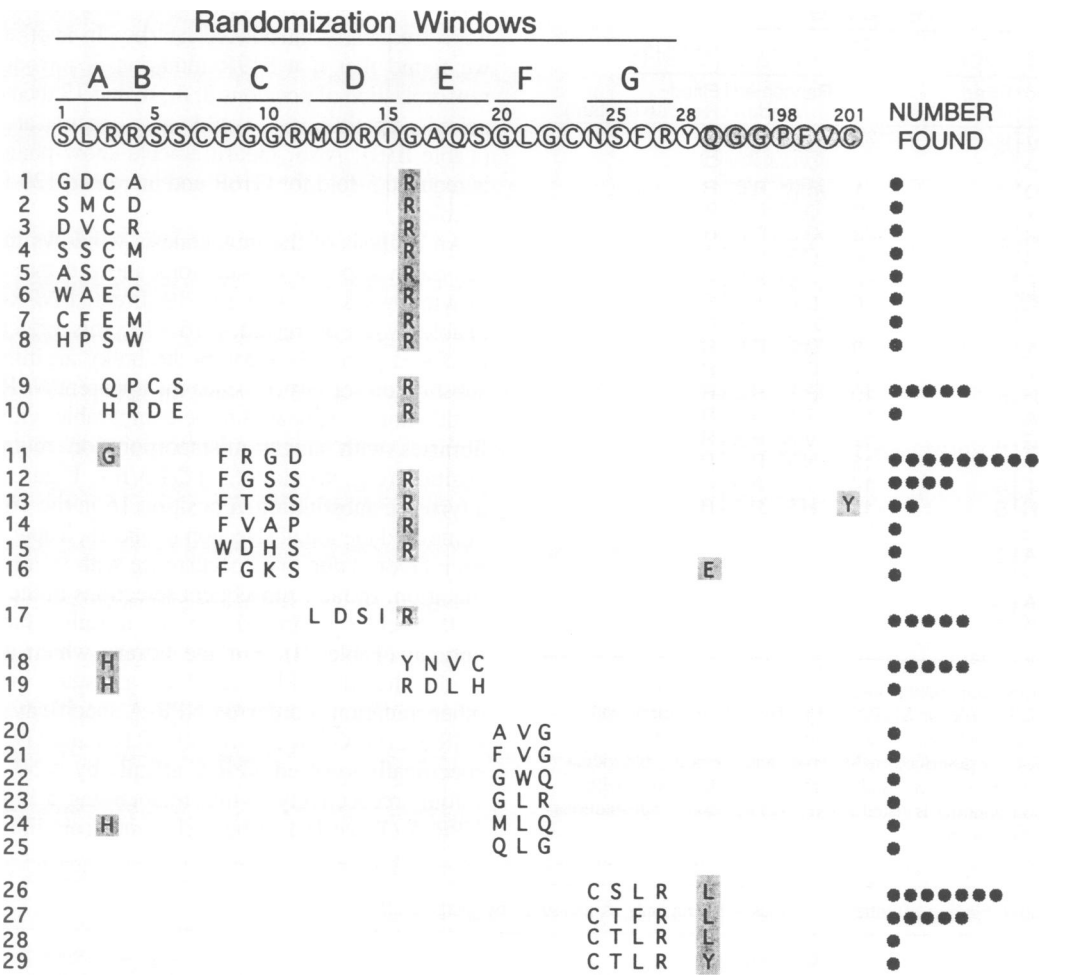


Fig. 4. Binding properties of selected phagemid pools. Displacement plots showing the apparent binding affinity of the seven phagemid library pools to NPR-A (**A**) and NPR-C (**B**) at various points in the selection process. The graphs are labeled with the residues that are randomized in that pool. Within each graph the curves are labeled for the starting library (black), cycle 2 (blue), cycle 4 (green) and cycle 7 (red) phagemid pools. The graph for the wild-type ANP template phage (pB1537) shows the superimposition of three replicate plots.

The remaining misincorporation mutations occurred at Arg3, in 16 of 64 sequences (Table I), and the amber codon at position 29, in 15 of 64 sequences (Table I), and are well correlated with increased ANP display (data not shown). Since amber codons are only partially translated by *SupE* suppressor strains, it is not surprising that mutations to sense codons would increase expression and display of ANP. Such an effect for substitutions of Arg3 is also consistent with the proposal that protein secretion favors a polar distribution of residues flanking the signal sequence with a net negative

Table I. Sequences of selected ANP variants



The amino acid residues found at each randomized position after seven rounds of panning are shown. Residues in shaded cells are mutations that occurred outside the target window as a result of either polymerase-derived nucleotide misincorporations or DNA oligonucleotide synthesis errors. The randomized patches on ANP are designated by the letters A (residues 1–4), B (residues 3–6), C (residues 8–11), D (residues 12–15), E (residues 16–19), F (residues 20–22) and G (residues 24–27). The number of clones found for each sequence is indicated by the number of dots in the right column.

Table II. Receptor binding and phage display measurements of various ANP mutants

Phage	Receptor affinity (EC_{50}) (pM)		Relative affinity (EC_{50} mutant/EC_{50} wild-type)[a]		Relative phage binding[b]	Calculated relative ANP display[c]
	NPR-A	NPR-C	NPR-A	NPR-C		
wt ANP	151	200	1	1	1	1
R3D/R4E	1430	725	9.5	3.6	22	209
V29E	130	215	0.9	1.1	160	120
G16R	85	17 800	0.8	150	0.2	0.2

The relative number of phage displaying ANP[c] was calculated by factoring the relative dilution of the phage stock required for half-maximal binding to the NPR-A-coated plates[b] (values calculated from Figure 5), with its relative affinity for NPR-A receptor as established by phage ELISA[a]. To improve the ELISA signal the phagemid tested had a valine codon in place of the amber codon at position 29 in pB1537.

charge in the immediate N-terminal region of the mature protein (von Heijne, 1986). Indeed, when we randomized the arginine pair at positions 3 and 4 together with residues 16 and 17 (in C201Y backround), acidic substitutions occurred at positions 3 and 4 along with the expected G16R mutation (Table IIIA). Furthermore, measurements have shown that the display of R3D/R4E ANP on phagemid is enhanced 200-fold (Table II and Figure 5) and that the mutation R3D results in a 50-fold increase in the secretion of free ANP from *Escherichia coli* (data not shown). Our results also suggest that ANP expression may be generally hampered by this hormone's very high basicity (pI = 11.9). An examination of the library selectants (Table I) reveals that substitutions at four of the five native arginine residues (R3, R4, R11 and R14) were favored, and that acidic residues were frequently introduced nearby. In another experiment we found that if a S19R mutation is introduced prior to randomization of positions 3, 4, 16 and 17 then counteracting acidic substitutions would also occur at position 17 (Table IIIB). Also, measurements show phagemid display is reduced 5-fold for G16R and increased 120-fold for V29E (Table II).

An analysis of the mutagenesis windows independent of the misincorporation mutations showed that only changes in windows 8−11 and 16−19 directed NPR-A selectivity (Table IV). For residues 16−19, this effect is probably mediated primarily through the bulky arginine or tyrosine substitution for Gly16, since the apparent NPR-C resistance of this library appeared to be comparable with that of other libraries with single misincorporation mutations at this position (e.g. window S1/L2/R3/R4, Figure 4). Although a tyrosine substitution at position 16 in the 16−19 window dominated the initial selection (clone 18, Table I) it may have been favored due to its occurrence with an adjacent cysteine mutation. In fact, subsequent selections in the backround of C201Y clearly showed that the arginine substitution was optimal (Table III). For the library which alters residues 8−11, the native Phe8 residue was usually retained but the other mutations conferred NPR-A specificity. For example G9R/R11D, G10K/R11S, G9T/R11S and G9E/G10R/R11P specifically lowered NPR-C affinity by >50-, 35-, 33- and 7-fold, respectively, while maintaining a high affinity for NPR-A (Table IV). When selectants from libraries mutating

Table III. Selection of acidic residues in ANP

Template

A Wild-Type

	Randomed Window				Fixed Residue (wt)
	3	4	16	17	19
	R	R	G	A	S
1	S	D	R	Q	S
2	T	E	R	S	S
3	T	E	K	H	S
4	N	D	R	G	S
5	D	E	R	T	S
6	H	A	R	E	S
7	E	A	R	T	S
8	H	E	R	A	S
9	A	A	R	T	S
10	E	D	R	H	S
11	D	E	R	A	S
12	E	G	R	G	S
13	D	E	K	T	S
14	T	G	R	T	S
15	S	D	R	N	S
16	L	D	R	H	S
17	D	D	R	A	S
18	A	E	K	H	S
19	G	D	R	A	S
20	Q	E	R	K	S

B Mutant (S19R)

	Randomed Window				Fixed Residue (mutant)
	3	4	16	17	19
	R	R	G	A	R
1	E	E	R	E	R
2	D	E	R	G	R
3	D	L	R	E	R
4	D	E	R	Q	R
5	A	E	R	E	R
6	L	L	I	A	R
7	E	L	R	E	R
8	D	E	R	D	R
9	E	H	R	D	R
10	F	P	R	D	R
11	S	M	R	E	R
12	V	N	R	E	R
13	N	E	R	Q	R
14	L	S	R	A	R
15	H	E	R	E	R

Phagemid libraries randomizing residues 3, 4, 16 and 17 in the backround of C201Y (**A**) or S19R/C201Y (**B**) were produced and subjected to three enrichment cycles using 20, 100 and 200 nM of competing NPR-C as described in Materials and methods. Individual selectants were then sequenced and the residue identity found within the randomization window is listed above. Acidic residue substitutions are highlighted by shading.

Table IV. Relative binding affinities of various ANP mutants as measured by phage ELISA

Mutations	Relative affinity (EC_{50} mutant/EC_{50} wild-type)		Change in receptor preference (NPR-A/NPR-C)
	NPR-A	NPR-C	
wt ANP	1.0	1.0	1.0
ELGR(4)	3.4	2.3	0.7
SDCS(4)	1.0	3.5	3.5
FGKS(11)	2.7	35.0	13.0
FTGS(11)	1.0	33.0	33.0
FRGD(11)	2.5	>140.0	>56.0
FERP(11)	1.0	7.0	7.0
LDSI(15)	8.3	4.8	0.6
RDLH(19)	1.4	>140.0	>100.0
AVG(22)	1.0	2.5	2.5
MLQ(22)	1.8	1.3	0.7
QLG(22)	1.1	1.7	1.5
FVG(22)	1.2	1.2	1.0

The selected residues within each ANP variant listed are identified using the single letter nomenclature for amino acids. Each set of residues is contiguous, listed from the N-terminal side (left) to the C-terminal side (right), and the position of the C-terminal residue is designated in parenthesis. Since the quantities of NPR-C available were limited, some of the weaker affinities could not be precisely measured and are listed as being greater than the value tested. To obtain values for very weak affinities it was necessary to measure the competitive displacement of radioiodinated ANP by purified natriuretic peptide such as shown for Figure 2.

the N-terminal region of ANP (residues 1–4), residues 12–15, residues 20–22 and residues 24–27 were analyzed in the absence of G16R, no significant NPR-A specificity was measured (Table IV and Figure 4).

Generation of an NPR-A agonist that does not bind NPR-C

We combined six of the mutations identified from our selectants to produce an analog that is highly NPR-A-specific and efficiently expressed by *E.coli*. This analog contained five mutations which should either enhance secretion expression or confer NPR-A specificity. These include R3D, which was mentioned earlier to increase secretion 50-fold, and R14S which occurred in the selection (Table I) and should further increase expression by reducing the basicity of ANP. To confer NPR-A specificity the mutations include G9T and R11S which together reduce NPR-C affinity by ~30-fold (Table IV), and G16R which reduces NPR-C affinity by 150-fold (Table II). Additionally, M12L was included since this mutation occurred in the selection (Table I) and oxidation of this methionine is known to effect receptor binding (Koyama *et al.*, 1992). The resulting hexamutant was efficiently secreted from *E.coli* directly into fermentation broths with yields of ~10 mg/l (see Materials and methods). Measurements of the competitive displacement of [^{125}I]ANP showed this analog binds NPR-A with an affinity equivalent to wild-type ANP (EC_{50} of 460 pM versus 590 pM) but binds NPR-C with an affinity that is reduced by >100 000-fold (EC_{50} of >100 μM versus 1700 pM, Figure 2A and B).

We used a cell based assay to determine if the ANP hexamutant could stimulate cellular NPR-A without interacting with cellular NPR-C. In these experiments the dose–response for cGMP production in 293 cells expressing human NPR-A (Lowe and Fendly, 1992) was measured during co-culture with either control 293 cells, or with 293 cells expressing human NPR-C (see Materials and methods). In the presence of the control cells, wild-type ANP gave an EC_{50} of 470 pM for the stimulation of NPR-A cells. However, with a 7-fold excess of NPR-C cells present, giving a NPR-C:NPR-A receptor ratio of ~60, there was a 25-fold reduction in activity (EC_{50} of 12 nM, Figure 6A). This demonstrated that the cellular NPR-C present was able to reduce the effective concentration of wild-type ANP available to NPR-A on cells. By contrast, we found that the receptor-selective ANP hexamutant was equally potent in stimulating NPR-A, whether or not NPR-C cells were present (EC_{50} of 525 and 501 pM, respectively), and therefore completely resistant to the inhibitory effects of NPR-C (Figure 6B).

Conclusions

NPR-A-selective variants, such as the ANP hexamutant, should provide a useful diagnostic tool for assessing the *in vivo* function of both NPR-A and NPR-C, and the efficacy of specific NPR-A targeting. Indeed, analogous engineered receptor specific variants of human growth hormone have already been used to identify the receptor responsible for the priming of neutrophils by human growth hormone (Fu *et al.*, 1992). Also, while published reports have attributed the bulk of *in vivo* ANP turnover to receptor-mediated clearance by NPR-C, the relative contribution of other mechanisms, such as proteolysis, remains unclear. The *in vivo* analysis of an NPR-A-specific ANP should allow us to begin to clarify these and other questions. Recent experiments have shown our ANP hexamutant, enriched for binding the human receptor, is species-specific and does not bind rat NPR-A (data not shown). The development of a similar variant active in an animal model is in progress and should help us to study the biology of these receptors.

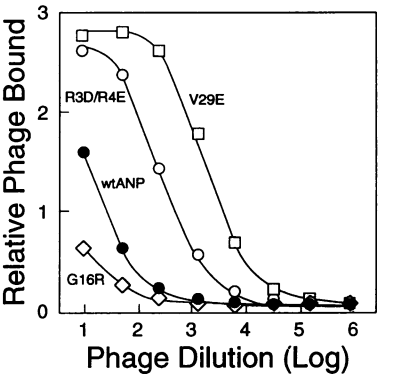


Fig. 5. Binding of phage stocks to immobilized NPR-A. 5-fold serial dilutions of each phage stock were incubated with NPR-A immobilized on microtiter plate wells and the bound phage visualized by ELISA. The relative phage binding was then calculated from the dilution of each stock required to give a half-maximal signal (values in Table II). The procedure was as described for the phage ELISA without the addition of competing receptor (see Materials and methods).

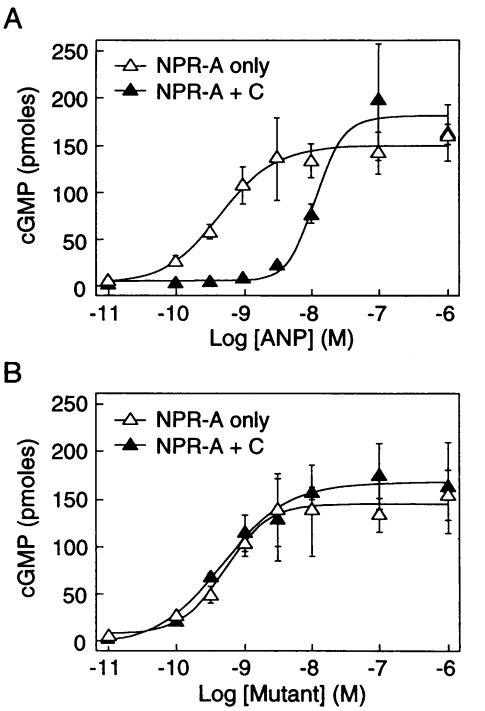


Fig. 6. Resistance of ANP hexamutant to inactivation by cells expressing NPR-C. 293 NPR-A cells were co-cultured with control 293 cells (NPR-A only) or with 293 NPR-C cells (NPR-A + C) and the dose–response stimulation of cGMP production by ANP (**A**) or the NPR-A-specific ANP hexamutant (**B**) measured. Results are expressed as the total amount of cGMP produced (pmol) after stimulation for 10 min at 37°C, and are presented as the mean of three independent stimulations ± standard deviation. The EC_{50} value calculated for wild-type ANP, with and without competing NPR-C cells, was 470 pM and 12 nM, respectively, and that for the ANP hexamutant was 525 pM and 501 pM, respectively.

While we have not yet elucidated the mechanism(s) responsible for the receptor selectivity of the ANP hexamutant, the mutations themselves do suggest possible explanations. Since two of the specificity conferring mutations are bulky substitutions for glycine, these residues may function simply by sterically blocking NPR-C binding. Alternately, if the bound ANP conformations differ for each receptor these glycine mutations may restrict the structure to an NPR-A-selective form by decreasing the flexibility of the backbone. Intriguingly, NMR analysis has shown that the ANP hexamutant has a relatively ordered aqueous structure (Wayne Fairbrother, personal communication) compared with the essentially random form published for the wild-type hormone (Theriault *et al.*, 1987). Further structure and function analysis should allow us to ascertain the mechanism by which this analog derives its selectivity.

In addition to the selection of mutant proteins based on affinity differences, monovalent phage display can be used to identify mutations that increase the proportion of phagemid particles that display fusion protein. These may include selections for mutations through mechanisms such as enhanced secretion, improved protease resistance, or optimized fusions to gene III. An understanding of these selective pressures is necessary for properly interpreting experiments and expands the utility of this technique. In this case, we have not only selected for desirable binding properties but have identified mutations that for the first time allow the direct secretion expression of mature ANP.

Materials and methods

Construction of phagemid displaying ANP

Synthetic oligonucleotides coding for mature ANP (sense strands: 5′-TCTCTGCGTAGATCTAGCTGCTTCGGCGGCCGCATG-3′, 5′-GATCGTATCGGAGCTCAGAGCGGTCTCGGGTGCAA-3′, and 5′-CAGCTTCCGTTACGTAGGCGGGCC-3′, and the complementary sequences: 5′-CAGCTAGATCTACGCAGAGATGCA-3′, 5′-TCTGAG-CTCCGATACGATCCATGCGGCCGCCGAAG-3′ and 5′-CGCCTAC-GTAACGGAAGCTGTTGCACCCGAGACCGC-3′) were assembled and ligated into *Nsi*I- and *Apa*I-digested phagemid vector (Bass *et al.*, 1990). The resulting construction encoded for the secretion of a fusion protein consisting of the 28 residue ANP hormone followed by a glutamine (from an amber codon when using a *SupE*-containing *E.coli* strain), two glycines and the C-terminal region of the gene III protein (residues 198–406). Addition of K07 helper phage (Vieira and Messing, 1987) to *E.coli* cells (XL1-blue; Bullock *et al.*, 1987) harboring the recombinant plasmid produced phagemid that monovalently displayed ANP.

Generation of phagemid that display randomized ANP libraries

Phagemid libraries that displayed ANP molecules with patches of three or four contiguous residues fully randomized for all 20 amino acids were produced by site-directed mutagenesis (Kunkel *et al.*, 1991), using the following oligonucleotides that mutated target codons to NNS sequences (where N represents a mixture of all four bases and S is a mixture of both G and C): 5′-GCTACAAATGCCTATGCANNSNNSNNSNNSTCTA-GCTGCTTCGG-3′ to randomize positions 1–4, 5′-AATGCCTAT-GCATCTCTGNNSNNSNNSNNSTGCTTCGGCGGCCG-3′ to randomize positions 3–6, 5′-CTGCGTAGATCTAGCTGCNNSNNSNNSNN-SATGGATCGTATC-3′ to randomize positions 8–11, 5′-AGCTGCT-TCGGCGGCCGCNNSNNSNNSNNSGGAGCTCAGAGC-3′ to randomize positions 12–15, 5′-GGCCGCATGGATCGTATCNNSNNSNNSNNS-GGTCTCGGGTGC-3′ to randomize positions 16–19, 5′-CGTATCG-GAGCTCAGAGCNNSNNSNNSTGCAACAGCTTCCG-3′ to randomize positions 20–22, and 5′-CAGAGCGGTCTCGGGTGCNNSNNSNN-SNNSTACTAGGGCGGGCC-3′ to randomize positions 24–27.

Each library consisted of at least 5×10^6 independent mutants within the target patch and theoretically exceeds the number of possible sequence combinations (maximum of 32^4 or 1.05×10^6). Stocks of $\sim 10^{14}$ phagemid per ml were made from PEG precipitates of culture broths from XL1-blue cells harboring the plasmid that were superinfected with K07 helper phage.

Selection of receptor-specific ANP variants

Before negatively selecting for NPR-A-specific mutants the libraries were pre-enriched for NPR-A binders. Phagemid stocks (10 μl) were incubated with 50 nM NPR-A in a final volume of 100 μl binding buffer [20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% bovine serum albumin (BSA) and 0.05% Tween 20] for 2 h, and the phage bound to the IgG–receptor fusion isolated by binding to protein A–Sepharose beads. After two cycles of isolation and propagation, the pre-enriched phagemid libraries were panned for NPR-A-specific mutants that bound to immobilized NPR-A in the presence of competing NPR-C in solution. For each library, purified NPR-A (at 1 μg/ml) was coated onto two microtiter plate wells in 50 mM sodium carbonate (pH 9.6) at 4°C overnight. The coating solution was removed and these wells, plus one uncoated well, were blocked with 5% skimmed milk and washed. To one, binding buffer with 4 nM competing NPR-C and 10 μl of library phage stock was added. The other wells served as controls lacking either the competing NPR-C or both the NPR-A coat and the NPR-C. In each case, the final volume was 100 μl. After 2 h the plates were washed and the bound phage eluted with 100 μl 0.2 M glycine (pH 2.0), then neutralized with 13 μl 1 M Tris base and titered by infecting *E.coli* (XL1-blue). The remaining anti-selected phag-mid particles were propagated for use in the next selection cycle. The phagemid titers were used to calculate the reduction in bound phagemid resulting from the addition of competing NPR-C. Phagemid libraries that demonstrated significant resistance to binding inhibition were anti-selected with higher concentrations of NPR-C (up to 200 nM) in subsequent cycles.

ELISA measurement of phagemid affinities for NPR-A and NPR-C

Microtiter plates (Nunc, Maxi-sorp, 96 wells) were coated with purified NPR-A (at 1 μg/ml) in 50 mM sodium carbonate (pH 9.6) at 4°C overnight. The plates were then blocked with 5% skimmed milk and washed with PBS + 0.05% Tween 20. Serial dilutions of competing receptor (NPR-A or NPR-C) and a subsaturating concentration of ANP-phagemid were added to wells in 100 μl of binding buffer. After 2 h the plates were washed, and the bound phagemid stained with a mixture of sheep anti-phage polyclonal antibody and rabbit anti-sheep mAb horseradish peroxidase conjugate, and assayed. Affinities (EC_{50}) were calculated as the concentration of competing receptor that resulted in half-maximal phagemid binding. The ELISA measurements of the selected library pools shown in Figure 4 were also performed as described except that a fixed 1 μl of phage stock was added per microtiter well.

Bacterial expression of ANP hexamutant

Cultures of 16C9 (a non-suppressor strain of *E.coli*; Chang *et al.*, 1987) harboring the expression plasmid were grown for 28 h in an aerated 10 l fermentor at 37°C in medium that initially contained 12 g/l digested casein, 50 mg/l ampicillin, 3 mM glucose, 4 mM isoleucine hydrochloride, 47 mM $(NH_4)_2SO_4$, 10 mM NaH_2PO_4, 18 mM K_2HPO_4, 25 mM KCl, 4 mM trisodium citrate, 12 mM $MgSO_4$, 250 μM $FeCl_3$ and 40 μM each of $ZnSO_4$, $MnSO_4$, $CuSO_4$, $CoCl_2$, H_3BO_3, $NaMoO_4$ and received automated feeds of NH_4OH to keep the pH at 7 and of glucose to maintain a slight excess and avoid anaerobiosis. At harvest the optical density at 550 nm of the culture was ~100.

Over 80% of the recombinant hexamutant (containing the mutations R3D, G9T, R11S, M12L, R14S and G16R) was secreted directly into the fermentation broth and was purified as follows. The cells were removed from 1 l of fermentation culture by centrifugation followed by filtration through a 0.45 μm membrane. The broth was then brought to 0.1% TCA and after 30 min was centrifuged to remove any precipitable material. To the supernatant 0.1 vol. of Nugel P-RP (Separation Industries) was added and gently mixed for 1 h. The reverse phase resin was separated with a sintered glass filter and rinsed extensively with 0.1% TFA. The bound material was then eluted with 50% acetonitrile + 0.1% TFA, concentrated with a Rotovap, and resuspended in 50 ml of 10 mM acetate buffer, pH 4.6. This solution was loaded onto a Mono S column, thoroughly rinsed with 20 mM acetate buffer, pH 4.6, and eluted with a 0–200 mM NaCl gradient in 20 mM acetate buffer, pH 4.6. The eluted fractions assayed to contain ANP activity were then pooled, concentrated by lyophilization, and resuspended in 5 ml of dH_2O. This sample was acidified (to give a final TFA concentration of 0.1%), loaded onto a preparative Vydac C18 column and fractionated over a 0–30% acetonitrile + 0.1% TFA gradient. A large dominant peak that occurred at ~16% acetonitrile was collected. Mass spectroscopy, amino acid analysis and NMR showed this to be the

expected ANP mutant with a purity of >95%. This material was lyophilized for storage.

Measurement of purified ANP affinities for NPR-A and NPR-C

Conditions used were essentially as described for measuring phagemid affinities except that ^{125}I-labeled ANP tracer (Amersham) replaced the phagemid and serial dilutions of purified hormone were used as the competitor. Affinities (EC_{50}) were calculated as the concentration of competing hormone required to displace half of the bound label. Wild-type ANP used in these experiments was obtained commercially (Bachem).

Resistance of ANP hexamutant to inhibition by co-culture with NPR-C cells

NPR-A expressing 293 cells were produced as described in Lowe and Fendly (1992) and have been shown to possess $\sim 3 \times 10^5$ receptors per cell (Jewett *et al.*, 1993). Likewise the human cDNA for NPR-C (Lowe *et al.*, 1990) was expressed, and saturation binding analysis showed these NPR-C expressing 293 cells possessed $\sim 3 \times 10^6$ receptors per cell. Cultures were set up in 12-well tissue culture plates, with 4×10^4 NPR-A cells plus either 2.9×10^5 control or NPR-C cells. The monolayer cultures were treated with serial dilutions of natriuretic peptide in 1 ml of medium [Ham's F12/DMEM (50:50 w/v), 0.1% BSA, 0.1 mM isobutylmethylxanthine and 25 mM HEPES, pH 7.2] for 10 min at 37°C. The reactions were stopped by removing the medium by aspiration and adding 1 ml of ice-cold 10% trichloroacetic acid. The samples were then frozen on dry ice, thawed and centrifuged at 1000 *g* for 10 min, and the supernatant was extracted three times with water-saturated ether. The ether was evaporated from the extracted sample by incubation at 50°C for 20 min, and the cGMP was quantified by radioimmunoassay (BioMedical Technologies).

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