

Characterization of the hormone binding site of natriuretic peptide receptor-C

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Abstract The hormone binding site of rat and human natriuretic peptide clearance receptor (NPR-C), a single transmembrane receptor, has been further refined by mutagenesis. In addition to residue 188 (rat Ala, human Ile), which completely inverts the pharmacology of the rat and human receptors [Engel et al. (1994) *J. Biol. Chem.* 269, 17005–17008], we report a second key residue at position 205 (rat Tyr, human Asn) which modulates affinity to a limited number of ligands. Orthologous mutation of both residues results in tighter binding for human and weaker binding for rat NPR-C. The ligand binding fold of the receptor is formed by at least the first half of the extracellular domain containing two intramolecular disulfide loops, with the two affinity-modulating residues 188 and 205 in the second loop.

Key words: Atrial natriuretic peptide; Natriuretic peptide receptor; Mutagenesis; Receptor/hormone interaction

1. Introduction

The natriuretic peptides (NPs) are vasoactive hormones which play an important role in the regulation of body fluid, electrolyte balance and blood pressure. As antagonists to the renin-angiotensin system, vasopressin, and endothelin, their potential use as drugs for hypertension, acute renal failure and heart failure has been considered. The three members of the family, ANP and BNP which are produced primarily in the heart [1], and CNP which is expressed by endothelial cells [2], share as a common motif a 17-amino acid loop formed by a disulfide bond. Their action is transmitted by two classes of natriuretic peptide receptors (NPRs), the receptor guanylyl cyclases NPR-A and NPR-B [3], and the clearance receptor NPR-C which lacks both the kinase-like and the guanylyl cyclase domains of NPR-A and NPR-B [4]. The physiological role of NPR-C is to mediate the metabolic clearance of the hormones, but it also plays a role in signal transduction by G-protein coupling to mediate phosphoinositide hydrolysis and inhibition of adenylyl cyclase [5,6].

Recently, site-directed mutagenesis in combination with mass spectroscopy helped to elucidate the topology of the extracellular domain of NPR-C [7,8]. The juxtamembrane resi-

dues Cys⁴²⁸ and Cys⁴³¹ (for the splice variant NPR-C6) are involved in NPR-C homodimer formation while the remaining four cysteine residues form two intramolecular loops (Cys⁶³–Cys⁹¹ and Cys¹⁶⁸–Cys²¹⁶). In a previous paper we reported the sequence analysis and functional expression of rat NPR-C, which shows a higher affinity to all natriuretic peptides compared to the human receptor [9]. Orthologous mutagenesis at position 188 for rat and human NPR-C results in a complete reversal of the pharmacology of both receptors [9]. In this paper we report the detection of a second residue at position 205 with a more specific influence on binding to a certain number of naturally occurring and synthetic ligands. Double mutations as well as alanine mutations at positions 188 and 205 further delineate differences in hormone binding between rat and human NPR-C.

2. Materials and methods

2.1. Site-directed mutagenesis and expression of IgG fusion proteins

Site-directed mutagenesis was performed according to Kunkel et al. [10] using a Biorad mutagenesis kit and was verified by sequencing with Sequenase (US Biochemicals, Cleveland, OH). The extracellular domain of rat and human NPR-C was expressed as IgG fusion proteins (C-IgG's) by Chinese hamster ovary cells in serum-free medium as previously described [9,11]. Conditioned media in appropriate dilutions was directly used for the solid phase binding assay.

2.2. Solid phase binding assay

This assay was essentially as previously described [9]. In brief, C-IgG was bound to 96-well plates via Fcγ fragment specific, F(ab')₂ fragment rabbit anti-human IgG (Jackson ImmunoResearch, West Grove, PA). The amount of immobilized C-IgG was the same in each binding experiment so that the total amount of specifically bound tracer was approximately 15% of the total activity. Competition binding was performed by adding 15 pM ¹²⁵I-labeled rANP as tracer and serial dilutions of the competing peptides in PBS, 0.5% BSA, 0.01% Tween-20, pH 7.4. Natriuretic peptide analogs were either purchased from Bachem (Torrance, CA) and Peninsula Laboratories (Belmont, CA), or synthesized by solid phase methods at Genentech. After aliquoting, the amount of peptide in each vial was confirmed by quantitative amino acid analysis.

3. Results

3.1. Identification of the binding-modulating residue 205

Expression of the extracellular domains as IgG fusion proteins provided useful tools to study the pharmacology of rat and human NPR-C in solid phase binding assays using [¹²⁵I]rANP as tracer. Comparison of the observed binding affinities with tissue membrane preparations showed that the IgG constructs as well as the binding assay was consistent with the pharmacology observed for the native full-length receptor [9]. By using rat/human chimeras, we were able to locate the residues modulating the binding behavior in the middle region of the extracellular domain between positions 106 and 266, where

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Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; rANP, rat ANP (28 amino acids); hANP, human ANP (28 amino acids); rBNP, rat BNP (32 amino acids); hBNP, human BNP (32 amino acids); cANF, des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²]ANF_{4–23}-NH₂; NPR, natriuretic peptide receptor; IgG, Immunoglobulin G; C-IgG, NPR-C extracellular domain/IgG Fc fusion protein.

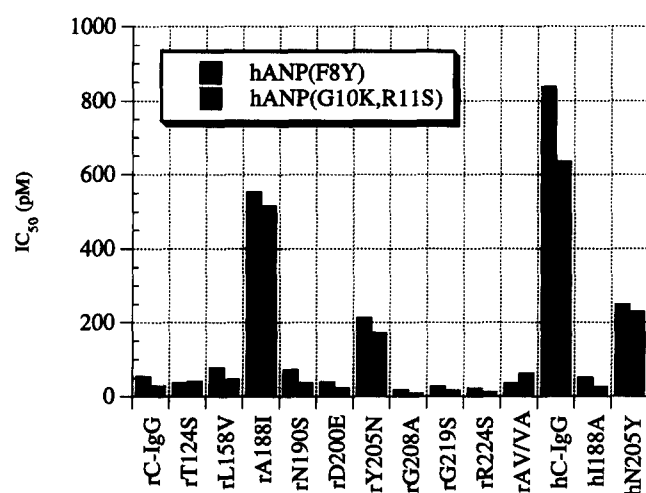


Fig. 1. Modulation of rat and human C-IgG pharmacology by positions 188 and 205. Shown is a representative competition binding experiment in which rat and human C-IgG and a number of orthologous mutants were tested for binding to hANP(F8Y) and hANP(G10K,R11S). The affinity of rY205N and hN205Y is in between the high affinity state of rat C-IgG or hI188A and the low affinity state of human C-IgG and rA188I.

rat and human NPR-C differ by 11 residues. Orthologous site-directed mutagenesis of these residues resulted in the identification of residue 188, Ala in rat and Ile in human, which resulted in a reversal of the hormone binding properties of both receptors [9].

Naturally occurring natriuretic peptides were used to identify position 188 as mediating species specific differences. Testing of synthetic peptide analogs on position 188 mutant receptors confirmed the role of this site in determining the binding properties of rat or human NPR-C. However, in testing synthetic peptides such as hANP(F8Y) [12], hANP(G10K,R11S) [13], hANP(O-Met) [14] and cANF [15] on the full set of mutant receptors we have now identified a second mutant, rY205N, with an altered pharmacology which is in between the high affinity state of rat C-IgG and the lower affinity state of human C-IgG (Fig. 1). Again, rA188I exhibits the lower affinity binding behavior of human C-IgG [9] which holds true for all natu-

rally occurring as well as all truncated or modified versions of the natriuretic peptides tested (exemplified by hANP(F8Y) and hANP(G10K,R11S) in Fig. 1). In contrast, rY205N shows a 4–5-fold loss of affinity towards synthetic peptides such as hANP(F8Y), hANP(G10K,R11S) and hANP(O-Met) whereas all other rat receptor mutants conserve the binding behavior of wild type rat C-IgG (Fig. 1). The orthologous mutation on the human receptor verifies this observation; while hI188A resembles the higher affinity state of the rat C-IgG, hN205Y binds these synthetic analogs tighter than wild type human C-IgG (Fig. 1). Therefore, both residues of the rat receptor, Ala¹⁸⁸ as well as Tyr²⁰⁵ contribute to high affinity binding whereas Ile¹⁸⁸ and Asn²⁰⁵ are responsible for the lower affinity state of the human receptor.

3.2. Double alanine and orthologous mutations

To analyze the impact of both residues 188 and 205 in rat and human C-IgG in concert, we changed both positions to alanine or made the orthologous double mutations (Table 1). Replacement of the rat wild type Tyr²⁰⁵ either by Asn (rY205N) or Ala (rY205A) has the same effect upon binding: Both mutants bind rANP, hANP, hANP(5–27) and CNP tighter than the rat wild type, whereas the affinities for hANP(F8Y), hANP(O-Met) and cANF decrease towards the values measured for human C-IgG (Table 1). hANP(G10K,R11S) and hBNP are bound by rY205N and rY205A in an intermediate affinity state between the natural rat and human receptors. The simultaneous change of both positions 188 and 205 (rA188I,Y205N) disrupts binding of all analogs to the rat receptor, resulting in lower affinity binding.

The analogous substitutions performed on human C-IgG had a less diverse impact on the observed binding affinities (Table 1). As stated above, hN205Y binds all hANP analogs, with the exception of hANP(O-Met), tighter than the wild type receptor. A dramatic increase in affinity is achieved by substituting residue 188 as well. The alanine double mutation (hI188A,N205A) resembles hI188A,N205Y in all cases (Table 1). These double mutants bind all analogs, with the exception of hANP(O-Met) and cANF, even tighter than wild type rat receptor, suggesting that the ligand binding sites of the two receptors have additional differences. This is an interesting observation since the rat receptor binds all analogs with higher

Table 1
IC₅₀ values (pM) of natriuretic peptides on wild type and mutated C-IgG fusion proteins^a

Natriuretic peptides	rANP	hANP	hANP (5-27)	hANP (F8Y)	hANP (G10K,R11S)	hANP (O-Met)	cANF	rBNP	hBNP	CNP
rat C-IgG	6.0 ± 1.0	6.6 ± 2.1	32.4 ± 9.2	49.0 ± 16.1	37.3 ± 17.8	32.8 ± 7.6	368 ± 118	32.7 ± 11.6	28.4 ± 12.8	16.4 ± 3.6
rA188I	9.1 ± 3.8	12.1 ± 5.4	53.8 ± 17.1	324 ± 175	598 ± 173	501 ± 98	1,247 ± 267	75.4 ± 9.5	283 ± 167	18.8 ± 4.3
rY205N	3.7 ± 0.9	3.2 ± 1.4	15.6 ± 4.9	278 ± 108	206 ± 47	501 ± 174	790 ± 263	36.7 ± 9.4	43.5 ± 20.0	11.4 ± 1.1
rY205A	3.3 ± 1.1	3.7 ± 1.1	22.6 ± 4.9	370 ± 139	168 ± 57	857 ± 131	1,196 ± 717	35.6 ± 14.5	55.9 ± 18.2	11.4 ± 1.0
rA188I,Y205N	15.0 ± 2.4	398 ± 59	433 ± 787	>5,000	>3,000	>10,000	not determined	367 ± 269	>6,000	42.8 ± 13.4
human C-IgG	9.5 ± 0.7	13.0 ± 2.2	48.0 ± 12.5	391 ± 175	624 ± 155	456 ± 72	761 ± 260	75.3 ± 21.7	328 ± 118	25.5 ± 6.6
hI188A	5.1 ± 0.7	6.2 ± 1.6	29.1 ± 3.1	47.7 ± 14.3	19.9 ± 6.5	54.9 ± 24.9	381 ± 108	25.4 ± 3.7	18.1 ± 5.9	13.1 ± 1.9
hN205Y	7.5 ± 2.5	10.7 ± 0.7	15.4 ± 2.8	279 ± 81	197 ± 45	1,139 ± 35	753 ± 157	73.9 ± 9.6	345 ± 36	21.9 ± 7.9
hI188A,N205A	4.6 ± 1.4	6.5 ± 3.0	21.4 ± 3.4	28.1 ± 10.9	21.5 ± 7.1	98.2 ± 40.9	389 ± 225	19.4 ± 6.6	10.5 ± 4.4	14.9 ± 5.2
hI188A,N205Y	4.5 ± 3.1	4.2 ± 0.6	23.9 ± 9.0	25.3 ± 5.5	14.6 ± 3.9	41.5 ± 9.5	not determined	21.5 ± 7.1	13.4 ± 6.6	15.1 ± 6.0

^aIC₅₀ values, the doses required to inhibit 50% of [¹²⁵I]rANP binding to the receptors, are depicted as mean ± standard deviation from at least three independent experiments and were determined by a 4 parameter equation, $a + (d - a) / [1 + \exp(b(c - x))]$, using the KaleidaGraph program.

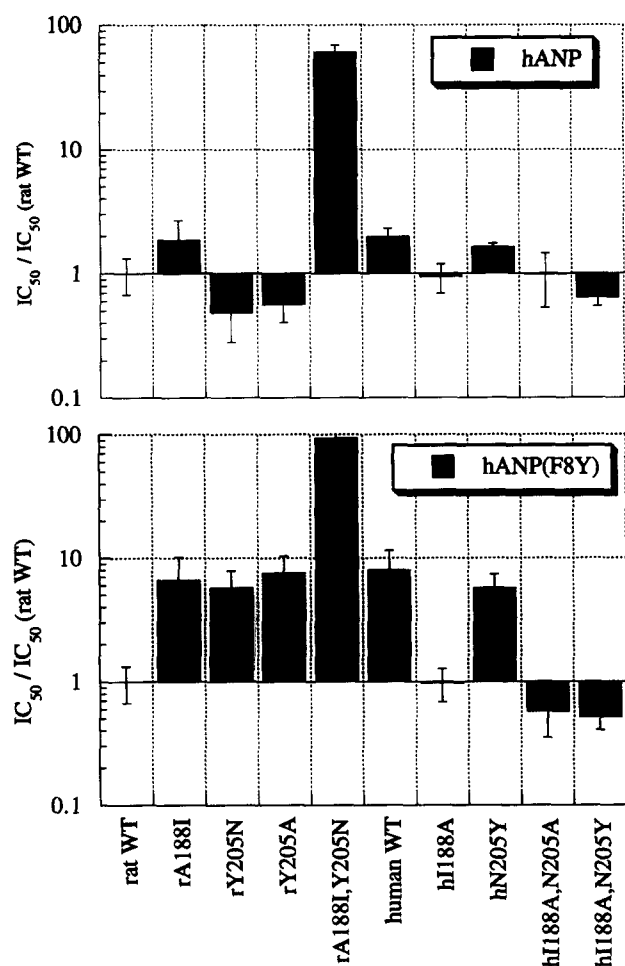


Fig. 2. Binding affinities of rat and human C-IgG mutants relative to rat WT. The binding behaviour of the natriuretic peptides are differently affected by changes in position 205 of the receptor which is exemplified by hANP and hANP(F8Y). Data were taken from Table 1.

affinity than the human receptor and suggests that further work with chimeric and mutant receptors have to be employed to identify additional residues involved in modulating ligand binding.

4. Discussion

In the present study we have refined the molecular basis of the different pharmacology of rat and human NPR-C where the human receptor is more sensitive to changes in ligand structure than the rat receptor. While orthologous mutation at position 188 is sufficient to switch the overall binding behavior of both receptors [9], residue 205, Tyr in rat and Asn in human NPR-C, has a more distinct influence on binding of only a few analogs and was only detected in competition binding assays using synthetic analogs such as hANP(F8Y), hANP(G10K,R11S) and hANP(O-Met).

Based on the modulating effect of residue 205 the hormone analogs presented in Table 1 can be divided into two groups depending on whether their affinity is only slightly or dramatically influenced. The first group is exemplified by hANP in Fig.

2a and comprises in addition rANP, hANP(5–27) and CNP. Even if the observed effects are only on the order of 2–3-fold the reproducibility of the effect suggests that it is significant. Replacement of rat Tyr²⁰⁵ by Asn or Ala slightly increases the binding properties. Simultaneous replacement of Ala¹⁸⁸ by Ile decreases the affinities for these ligands even beyond human wild type so that there is obviously no additivity of both mutational effects. Replacement of the human Asn²⁰⁵ by Tyr and Ala has a similar minor positive effect upon binding. More obvious effects at position 205 are observed in the second group comprising the synthetic analogs and the BNPs (Fig. 2b). Binding of hANP(F8Y), hANP(G10K,R11S), hANP(O-Met) and cANP is reduced in both rat mutants even to the level of the human receptor, resembling rA188I. On the human receptor, again, mutations at position 205 cause only smaller effects compared to those evoked by mutating position 188.

Fig. 3 summarizes our current knowledge of the ligand binding domain of NPR-C which is dictated by the two intramolecular disulfide folds. Based on sequence comparison and mutagenesis, Iwashina et al. [16] identified residues His¹⁰⁴ and Trp¹⁰⁵, which are conserved in all three natriuretic peptide receptors, as essential for ligand binding. This data, together with our results, define only a small number of NPR-C residues that are involved in forming the ligand-receptor complex. The model presented in Fig. 3 does not assign specific contact sites for hormone binding due to the fact that markedly different peptide analogs lead to a similar effect on binding. Steric effects seem to play only a minor role since replacement of the rat (human) wild type Tyr²⁰⁵ (Asn²⁰⁵) either by Asn (Tyr) or Ala has a surprisingly similar effect given the difference in the side chain specific volume of these residues (Tyr: 193.6, Asn: 117.7, Ala: 88.6 Å³). One explanation for our result may be a variable hormone/receptor interface if these side chains are in direct contact with the bound ligand. Alternatively, residues at 188

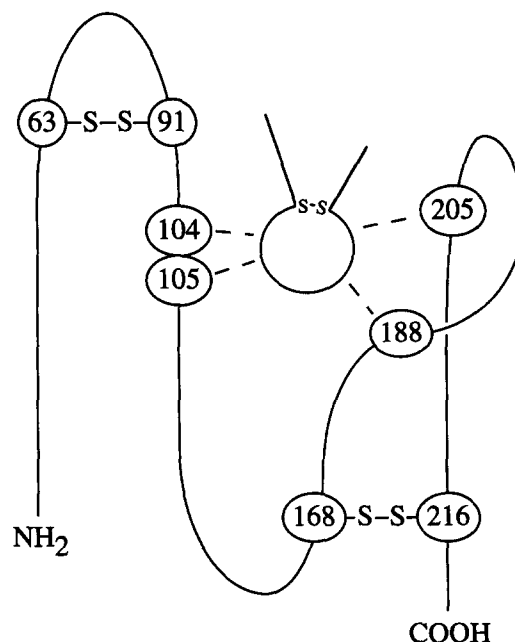


Fig. 3. Structural model of the ligand binding site of NPR-C. Based on the folding pattern dictated by the intramolecular disulfide loops (Cys⁶³–Cys⁹¹ and Cys¹⁶⁸–Cys²¹⁶) and four residues involved in hormone binding: His¹⁰⁴, Trp¹⁰⁵, Ala/Ile¹⁸⁸ and Tyr/Asn²⁰⁵.

and 205 may not be in ligand contact and indirectly influence binding by changing side chain packing in the vicinity of the binding site.

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