Structural analysis of natriuretic peptide receptor-C by truncation and site-directed mutagenesis

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Natriuretic peptide receptor-C (NPR-C) has a unique structure consisting of pre-existing covalent homodimers, but it is not known whether each subunit has ligand-binding activity or whether the dimeric structure is necessary for binding activity. To answer this question, a number of C-terminally truncated mutants were designed, subcloned into the mammalian expression vector pcDNA3 and expressed by transient transfection in COS-1 cells. Truncation at position 461, which eliminates the residue Cys⁴⁶⁹ that is involved in disulphide-linked dimerization,

produced a soluble and monomeric form of NPR-C, as determined by gel filtration on Superose 12. Binding assays of the gel-filtration fractions clearly demonstrated that even monomeric NPR-C contains a high-affinity binding site for natriuretic peptides. Site-directed mutagenesis of the invariant residues (Asp⁴⁰⁷-Arg⁴⁰⁸ and Asp⁴¹¹-Phe⁴¹²) in a region highly conserved among various species established that these invariant residues are essential for ligand-binding activity.

INTRODUCTION

Natriuretic peptides are a family of peptides that affect renal and cardiovascular functions [1]. In mammals, three homologous members of the family have been identified and referred to as atrial natriuretic peptide (ANP), brain natriuretic peptide and C-type natriuretic peptide [2]. These peptides share structural and functional properties; for example, they are characterized by a 17-amino-acid ring structure that is essential for their biological activity. They are involved in the regulation of a variety of physiological processes that are mainly directed towards reducing blood pressure and fluid volume, such as diuresis, natriuresis, vasodilation, steroidogenesis and cell proliferation.

Specific receptors for the natriuretic peptides have been demonstrated in a number of tissues responsible for maintaining fluid and cardiovascular homeostasis [3–6]. Molecular cloning and characterization has established that there are three receptors that can be classified into two categories: (1) natriuretic peptide receptor-A (NPR-A) and -B (NPR-B), which are receptor/guanylate cyclases of about 1000 amino acid residues with intrinsic guanylate cyclase activity in their intracellular domain; they are therefore postulated to mediate the majority of the biological effects of the natriuretic peptides through the generation of cGMP, and (2) natriuretic peptide receptor-C (NPR-C), which is a simple transmembrane protein of about 500 residues with a short cytoplasmic tail, of unknown function. The three receptors can also be distinguished by their ligand specificities: NPR-A shows high and moderate affinity for ANP and brain natriuretic peptide respectively, NPR-B is preferentially activated by C-type natriuretic peptide, and NPR-C binds all three ligands with equally high affinity. Although quite different in their cytoplasmic regions, the two groups of receptors exhibit a considerable degree of similarity in their extracellular domains.

As mentioned above, much is known about the nature and primary structures of the natriuretic peptide receptors, but little

is known about their higher-order structures. A unique structural feature of the receptors is their disulphide-linked homodimeric or oligomeric structures. We have determined, by a combination of site-directed mutagenesis and chemical sequencing, the intramolecular and intermolecular disulphide bonding patterns of bovine NPR-C (bNPR-C), which is known to be a disulphide-linked homodimer. We established that the first four Cys residues are joined sequentially, forming two disulphide loops, and the fifth Cys residue, located in a juxtamembrane extracellular domain, is involved in the interchain disulphide bond (Figure 1) [7]. Our recent cloning of the eel NPR-C has also contributed to identification of the first disulphide loop as a point of contact between the homologous NPR-C subunits [8]. We and others have applied a site-directed mutagenesis approach to determine part of the ligand-binding site [9,10] and the region responsible for conferring ligand specificity [11]. Despite these extensive studies on the structure and function of the natriuretic peptide receptors, the basic question remains of whether the monomeric form of the receptors has ligand-binding activity, or whether they are active only in a dimeric form, forming (like the growth hormone receptor [12–14]) a 2:1 receptor–ligand complex. To address this question and to advance our understanding of the molecular structure and function of the receptors for the natriuretic peptides, we have produced truncation mutants and site-directed mutants of bNPR-C and examined their ligand-binding activity in relation to their ability to form dimers.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA ligation kits were obtained from Takara, Kyoto, Japan; ³²P-labelled nucleotides and ¹²⁵I-ANP (74 TBq/mmol) were from Amersham; rat ANP was from Peptide Institute, Osaka, Japan; Dulbecco's modified Eagle's medium and antibiotics were from Life Technologies, Grand

Abbreviations used: NPR-C, natriuretic peptide receptor-C; ANP, atrial natriuretic peptide; NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; bNPR-C, bovine NPR-C; C-ANF, des-(18–22)-ANP-(4–22)-NH₂; CTLA-4, cytotoxic T-lymphocyte-associated molecule-4.

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Figure 1 Schematic representation of the structures of bNPR-C and its mutants

(a) Structures of deletion mutants. Mutants bNPR-CA477, bNPR-CA461, bNPR-CA400, bNPR-CA378 and bNPR-CA354 were prepared by truncation as shown. MSD, membrane-spanning domain; pre-pro, pre-pro sequence; C, cysteine residue. (b) Amino acid sequence comparisons of members of the NPR family. The sequences of NPR-A (human, rat and murine), NPR-B (human, rat, bovine and eel), NPR-C (human, rat, bovine and eel) and NPR-D (eel) were compared. The percentage identity is shown as the relative number of residues conserved among all species. (c) Structures of mutants generated by site-directed mutagenesis. The amino acids mutagenized to Ala are identified by their residue numbers. Mutants bNPR-C(D407-R408 → A-A) and bNPR-C(D411-F412 → A-A) are denoted as ⁴⁰⁷DR → AA and ⁴¹¹DF → AA respectively.

Island, NY, U.S.A.; fetal bovine serum was from Filtron, Brooklyn, Victoria, Australia; alkaline phosphatase-conjugated goat anti-(rabbit IgG) antiserum was from Tago Inc., Burlingame, CA, U.S.A.; disuccinimidyl suberate was from Pierce, Rockford, IL, U.S.A.; ClearBlot Membrane-P was from Atto, Tokyo, Japan; SequiTherm Long-Read cycle sequencing kit was from Epicentre Technologies Corp., Madison, WI, U.S.A.; Affi-Gel 10 and Econo-Columns were from Bio-Rad, Richmond, CA, U.S.A.; Superose 12 was purchased from Pharmacia, Uppsala, Sweden; pcDNA3 was from Invitrogen, San Diego, CA, U.S.A.

Site-directed mutagenesis

All mutations were introduced into pcDNA3/bNPR-C, a mammalian expression vector (pcDNA3) containing the entire coding sequence of bNPR-C, as described previously [15]. A 1359 bp *XhoI*-*XbaI* restriction fragment was removed from pcDNA3/bNPR-C and replaced with PCR fragments containing the

desired codon changes. The deletion mutants pcDNA3/bNPR-CA477, Δ461, Δ378 and Δ354 were constructed by PCR using the following primers. The upstream PCR primer (5'-ACCCTCGAGGGGGTCCATGA-3') encoded an *XhoI* restriction site (underlined), and the downstream PCR primers: (5'-GGTCTAGACTATTCTTCTAGGCCACCTGAT-3', 5'-GGTCTAGACTACACCATTCTGGTCACGTA-3', 5'-GGTCTAGACTAGACCTGCCAGCAATACC-3', 5'-GGTCTAGACTACTTACTGTAACCAGCTCT-3' and 5'-GGTCTAGATTAAACAACATGTTACGTA-3') encoded an in-frame stop codon (italicized) and a custom *XbaI* restriction site (underlined).

Alteration of Asp⁴⁰⁷-Arg⁴⁰⁸ and Asp⁴¹¹-Phe⁴¹² to alanines was carried out by ligation of PCR products. pcDNA3/bNPR-C(D407-R408 → A-A) was generated by PCR using the following four primers, each having a custom restriction site (underlined): 5'-ACCCTCGAGGGGGTCCATGA-3', 5'-GACGCATATGGGATTCTCTGTG-3', 5'-CCCATATGCGGCTCCGTTGGCATCTATGGA-3' and 5'-GGTCTAGATTAAAGCCACCGCCCGTG-3'. Similarly, pcDNA3/bNPR-C(D411-F412 → A-A) was generated by PCR using the following four primers, each having a custom restriction site (underlined): 5'-ACCCTCGAGGGGGTCCATGA-3', 5'-GGTCTAGATTAAAGCCACCGCCCGTG-3', 5'-GATGCTAGCGTGATCGCCATGACTGAC-3' and 5'-CACGCTAGCAGCCCCATACCGGTCTCCGTT-3'.

All clones were verified by DNA sequence analysis using the Sanger methodology [16], a SequiTherm Long-Read cycle sequencing kit and a LI-COR Model 4000L sequencer (Licor, Lincoln, NE, U.S.A.).

Cell culture and transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in 5% CO₂. Cells were transfected by electroporation with single pulse of 220 V at 960 μF with 20 μg of each receptor cDNA using a Bio-Rad Gene Pulser apparatus, and harvested 48 h after transfection. For immunoblot analysis of soluble and secreted forms of NPR-C (truncation mutants), transfected COS-1 cells were cultured in Dulbecco's modified Eagle's medium without serum (serum-free culture media were used to avoid interference by serum proteins).

Binding assay

Samples (50 μl) were incubated with ¹²⁵I-ANP (5 fmol) in 100 μl of 50 mM sodium phosphate, pH 7.4, 130 mM NaCl, 5 mM EDTA and 0.1% BSA at 30 °C. Non-specific binding was determined by incubating the mixture with non-radioactive ANP (100 pmol). After 30 min, 200 μl of dextran-coated charcoal was added, followed by gentle mixing. The tubes were placed at room temperature for 5 min and then centrifuged at 10000 g for 10 min. The complex of bNPR-C and ¹²⁵I-ANP present in the supernatant was determined using a γ-radiation counter. Specific ANP binding was obtained by subtracting non-specific binding from total binding.

Gel-filtration chromatography

Medium (100 μl) containing bNPR-CA461 or the complex of bNPR-CA461 and ¹²⁵I-ANP was directly applied to a column of Superose 12 (Pharmacia; 1.0 cm × 30 cm) and eluted at 0.2 ml/min. The column was calibrated with β-lactoglobulin (35 kDa), egg albumin (45 kDa), BSA (65 kDa) and IgG (160 kDa). Eluted

samples were assayed for their binding activity in the case of the free receptor, as described above, or for radioactivity in the case of the receptor–radioligand complex.

Affinity labelling of expressed NPR-C and NPR-C mutants

Crude particulate membranes (20 µg of protein) of COS-1 cells or culture medium (20 µl) after transfection with NPR-C and NPR-C mutants were incubated with ^{125}I -ANP (10 fmol) in 100 µl of 50 mM sodium phosphate, pH 7.4, 130 mM NaCl, 5 mM EDTA and 0.1 % BSA at 30 °C for 30 min in the presence or absence of 1 µM unlabelled ANP. The bound ^{125}I -ANP was cross-linked to the receptor by incubation in 1 mM disuccinimidyl suberate for 20 min at 25 °C. The reaction was quenched by the addition of 2 M ammonium acetate and analysed by SDS/PAGE (10 % gel) followed by fluorography.

Immunoblot analysis of expressed NPR-C and NPR-C mutants

Membrane preparations (20 µg of protein) or culture media (20 µl) were loaded on to a separating 10 % polyacrylamide gel in the presence of SDS and separated electrophoretically. Proteins were transferred electrophoretically to ClearBlot membranes at a constant current of 100 mA for 1 h. The membranes were blocked for 1 h at room temperature with 5 % non-fat milk and incubated overnight at 4 °C with anti-Rc70, an antiserum raised against bNPR-C [17,18], at a 1:5000 dilution in 0.1 % Tween 20 as described previously [15]. NPR-C and NPR-C mutants were visualized by incubating the sheets with an alkaline phosphate-conjugated goat antibody against rabbit IgG, and finally with 5-bromo-4-chloro-3-indoyl phosphate (160 µg/ml) and Nitro Blue Tetrazolium (330 µg/ml).

RESULTS

Construction of truncation mutants and determination of their receptor activities

NPR-C is a disulphide-linked homodimer [19–22]; mammalian NPR-Cs are covalently linked through the juxtamembrane fifth cysteine residue [7]. To determine whether one or two NPR-C chains comprise a ligand-binding site, we generated a series of C-terminal truncation mutants by inserting stop codons at residues 478, 462, 401, 379 and 355 using previously cloned bNPR-C (Figure 1a). The mutants were designated by the symbol Δ followed by the residue number of the final amino acid in the truncated protein: bNPR-C Δ 477, bNPR-C Δ 461, bNPR-C Δ 400, bNPR-C Δ 378 and bNPR-C Δ 354, or simply Δ 477, Δ 461, Δ 400, Δ 378 and Δ 354. Since the fifth cysteine residue is located at position 469, the mutants Δ 461, Δ 400, Δ 378 and Δ 354 lack this residue, which is involved in formation of the interchain disulphide bond, and are thus expected to be present as a monomer or a non-covalently associated dimer.

Truncation mutants Δ 477 and Δ 461, which lacked the transmembrane region, were secreted into the culture medium. Competitive binding assays using the culture media and ^{125}I -ANP showed that mutants Δ 477 and Δ 461 remained fully active in binding not only ANP but also C-ANF [des-(18–22)-ANP-(4–22)-NH₂], a NPR-C-specific ligand, as compared with the wild-type receptor, indicating that the transmembrane and cytoplasmic domains are not essential for ligand-binding activity (Figure 2).

The shorter truncation mutants Δ 354, Δ 378 and Δ 400 were all defectively secreted. Neither ^{125}I -ANP-binding activity nor NPR-C immunoreactivity was detected in the culture media of COS-1 cells transfected with these mutant constructs. The causes of

Figure 2 Ligand specificity and Scatchard analysis of bNPR-C Δ 461

bNPR-C and bNPR-C Δ 461 were characterized with respect to their ligand-binding activities by a competitive ^{125}I -ANP binding assay. Both receptors bound ANP and its analogue C-ANF with almost identical affinities; in the figure, only the results obtained with C-ANF are shown. The inset shows Scatchard analysis of C-ANF binding to bNPR-C Δ 461, which gave a linear plot consistent with a single class of binding sites with a K_D of 352 pM. The total binding capacities of bNPR-C and bNPR-C Δ 461 expressed were 1.0×10^{11} and 0.8×10^{11} sites/µg of DNA respectively. Relative numbers of binding sites, obtained by dividing the total numbers by the relative intensities of receptor bands on Western blots, were also within the same range. The amounts of receptor proteins expressed, using the construct described in the Materials and methods section, were between 100 and 150 ng/mg of protein.

Figure 3 Affinity labelling of truncation mutants

Wild-type bNPR-C and mutation products were cross-linked to ^{125}I -ANP and fractionated by SDS/PAGE (10 % gel) followed by fluorography. Mutant bNPR-C Δ 477 migrated as a 130 kDa dimer under non-reducing conditions, whereas mutant bNPR-C Δ 461 migrated as a 63 kDa monomer. Bands that remained in the presence of excess unlabelled ANP represent non-specifically labelled proteins. β -ME, β -mercaptoethanol.

the observed abnormalities in secretion are not clear, but it is interesting to note that the region comprising residues 403–415 that is deleted from the Δ 354, Δ 378 and Δ 400 mutants has an EF-hand-like sequence, as noted by Tuckwell et al. [23]. It has recently been postulated that targeting of secretory proteins to the regulated secretory pathway depends on pH- and Ca^{2+} -mediated aggregation properties of secreted proteins and se-

Figure 4 Gel-filtration analysis of the deletion mutant bNPR-CΔ461

(a) Gel-filtration analysis of the functional size of the deletion mutant bNPR-CΔ461. The graphs show elution profiles of BSA used as a size marker and ^{125}I -ANP-binding activity. The column was eluted at a flow rate of 0.2 ml/min with 10 mM Tris/HCl (pH 7.5). Aliquots (50 μl) of fractions were assayed for their ability to bind ^{125}I -ANP. The column was calibrated with IgG, BSA, egg albumin and β -lactoglobulin, as indicated by arrows 1–4 respectively. (b) Gel filtration of bNPR-CΔ461 complexed with ^{125}I -ANP. Molecular masses (Da) of the calibration markers are given.

cretory vesicle matrix proteins [24]. A typical recent example is the secretion of ANP: Canaff et al. [25] have demonstrated that mutations in the potential Ca^{2+} -binding region of the ANP precursor, which is rich in Asp and Glu residues, resulted in a correlated loss of its secretion and Ca^{2+} -mediated aggregation.

Affinity labelling of mutant receptors

To determine the sizes and subunit structures of the truncation mutants of bNPR-C, we performed affinity labelling. Consistent with the above results from the ^{125}I -ANP-binding assay, active receptors gave discrete bands of the expected size on SDS/PAGE (Figure 3). The mutant receptor Δ461, which lacked the fifth cysteine residue, migrated as a 63 kDa monomer even under non-reducing conditions (lane 3). Mutant Δ477, however, produced two bands, corresponding to the dimer (130 kDa) and monomer (65 kDa), under non-reducing conditions (lane 5), and a single monomer band under reducing conditions (lane 7). This result indicates that this truncated soluble form of the NPR-C (Δ477) retained the ability to form a disulphide-linked homo-

Figure 5 Comparison of the amino acid sequences of the second natriuretic-peptide-binding motifs of natriuretic peptide receptors

Shown are alignments of deduced amino acid sequences of the human NPR-A (hA), rat NPR-A (rA), mouse NPR-A (mA), human NPR-B (hB), rat NPR-B (rB), bNPR-B (bB), eel NPR-B (eB), human NPR-C (hC), rat NPR-C (rC), bovine NPR-C (bC), eel NPR-C (eC) and eel NPR-D (eD). The region shown corresponds to the second highly conserved region indicated in Figure 1(b).

dimer, albeit to a somewhat lower degree, despite the lack of the transmembrane and cytoplasmic domains.

A dimeric structure of NPR-C is not necessary for ligand-binding activity

The above observation that truncation at residue 461 produced a soluble and active form of NPR-C that lacked the ability to form a disulphide-linked homodimer (Figure 3, lane 3) prompted us to examine whether the mutant exists as a monomer or as a non-covalently associated dimer in its native state. Gel filtration of culture media of COS-1 cells transfected with bNPR-CΔ461 clearly showed that the truncation mutant is expressed as a monomer and has full binding activity in its monomeric form (Figures 2 and 4a), providing an answer to the important question of the stoichiometry of the ANP–NPR-C interaction. Preformed complexes of ^{125}I -ANP and the mutant receptor were also eluted as a monomer (Figure 4b), but the size (75 kDa) estimated from the elution position was greater than the expected value of 66 kDa (63 kDa for the receptor and 3 kDa for the ligand). This difference suggests that a conformational change occurs in the receptor on binding of the ligand.

Site-directed mutagenesis of invariant residues in highly conserved regions

Comparison of the NPR-C sequences of various species [8,11,26,27] revealed two highly conserved regions within the extracellular domain: region 1 (residues 123–182) and region 2 (residues 355–419) (Figure 1b). Region 1 contains the NPR motif that we and others have previously identified as being a major site involved in ligand binding [9,10]. Further comparison of the region 2 sequences among various subtypes of the receptor [28–35] allowed us to identify a limited number of residues that are conserved in all known NPR sequences (Figure 5). Primarily for practical reasons we chose, among the invariant residues, those containing a functional side chain as targets for mutagenesis (Figure 1c). Replacement of the conserved pair Asp⁴⁰⁷–Arg⁴⁰⁸ or Asp⁴¹¹–Phe⁴¹² with Ala–Ala resulted in almost complete loss of

Figure 6 Biochemical and immunochemical characterization of receptors derived by site-directed mutagenesis

(a) Affinity labelling of the site-directed mutant receptors. Mutated products and the control NPR-C were cross-linked to ^{125}I -ANP, fractionated by SDS/PAGE (10% gel) and visualized by fluorography. Mutants had little or no ANP-binding activity. (b) Immunoblotting of the site-directed mutant receptors. COS-1 cells were transiently transfected with bNPR-C, bNPR-C(D407-R408 $\rightarrow$ A-A) or bNPR-C(D411-F412 $\rightarrow$ A-A). Detergent extracts from the transfected cells were separated by SDS/PAGE on a 10% gel and then transferred to a nitrocellulose membrane. NPR-C mutants were detected by immunoblotting with anti-NPR-C antibody.

binding activity, as determined by binding assays (results not shown) and by affinity labelling (Figure 6a) using membrane preparations of COS-1 cells transiently transfected with the mutant constructs shown in Figure 1(c). An inability to detect ^{125}I -ANP-binding activity in the mutant receptors was not due to poor expression of the mutants or to improper folding of the mutant chains, since anti-NPR-C immunoblotting demonstrated equivalent levels of expression and formation of disulphide-linked dimers (Figure 6b). These results indicate that the highly conserved regions 1 and 2 comprise the ligand-binding domain.

DISCUSSION

For receptors that have a single transmembrane domain, such as the growth factor receptors and cytokine receptors, ligand-induced receptor dimerization or oligomerization appears to be a general mechanism of receptor activation, which leads to the subsequent recruitment and activation of downstream signalling molecules [36,37]. With the exception of members of the insulin receptor family, which exist as preformed disulphide-linked dimers of paired α and β subunits $[(\alpha\beta)_2]$, the above category of receptors is considered to be in equilibrium between the monomeric and dimeric forms and to undergo, on ligand binding, a transition to yield an active dimer. In most cases the binding stoichiometry of the receptor and ligand is 2:1, as clearly demonstrated for the growth hormone receptor by X-ray crystallography [12,14]. In certain cases, however, formation of a 2:2 receptor-ligand complex has been demonstrated. For example, in the case of the epidermal growth factor receptor, two molecules of ligand bind to two molecules of receptor, inducing their dimeric association [38,39]. In the case of insulin receptor family members, which are similar with respect to the pre-associated covalent nature of the dimeric form to the natriuretic peptide receptor studied here, formation of a 1:1 complex has been assumed, but it is still not clear whether one or two molecules of ligand bind to the dimeric receptor [40]. Clarification of the

details of these interactions is a key to understanding the structural basis for receptor activation. In the present study we analysed the interaction of ANP with its receptor NPR-C, which belongs to the growth factor/cytokine receptor superfamily [7], using C-terminal truncation mutants and site-directed mutagenesis.

The natriuretic peptide receptors are unique in their pre-existing disulphide-linked dimeric (NPR-C) or oligomeric (NPR-A and NPR-B) structures [21,22,41-45]. This feature is in marked contrast to ligand-induced dimerization or oligomerization. We have previously determined the intra- and inter-chain disulphide bonding patterns of NPR-C [7,9], and suggested a similar pattern for NPR-A and NPR-B based on the conservation, among the three receptor subtypes, of the locations of cysteine residues in the extracellular domain. In an effort to characterize further the interaction between the subunits of NPR-C and that of each subunit with the ligand ANP, we prepared C-terminal truncation mutants, analysed their covalent and non-covalent self-association and determined their ligand-binding activities. The results indicated that the monomeric component of the homodimeric NPR-C has by itself full binding activity. This fact, together with the observation with the same mutant receptor that both the ligand-occupied and non-occupied forms were eluted as a monomer on gel filtration (Figure 4), strongly suggests that native disulphide-linked dimeric NPR-C can bind two ANP molecules. As a natural extension of these results, we expected the same binding stoichiometry for NPR-A and NPR-B. However, Rondeau et al. [43] have reported that the stoichiometry of ANP binding to NPR-A is 1:2, i.e. only one ANP molecule per dimeric receptor, based on biochemical and immunochemical estimations. This difference is interesting, and may relate to difference in the functions and signalling mechanisms of these receptor subtypes. Confirmation of the stoichiometries and understanding of the structural basis for this difference must await X-ray crystallographic analysis of the receptor-ligand complexes.

In general structure, there is great similarity between NPR-C and the T-cell receptors [CD28 and the cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4)]. CD28 and CTLA-4 are homologous members of the immunoglobulin superfamily, and interact with B7 molecules on antigen-presenting cells. Both are disulphide-linked homodimers. Our previous determination of the intrachain disulphide bonding pattern of bNPR-C has demonstrated that it also belongs to the immunoglobulin superfamily [7]. Recently Linsley et al. [46] have determined the binding stoichiometry of CTLA-4 and its ligand B7, and showed that the disulphide-linked homodimeric CTLA-4 binds two ligand molecules. Although the functions of the T-cell receptors are quite different from those of NPR-C, these similarities in covalent dimeric nature and binding stoichiometry are worth noting.

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