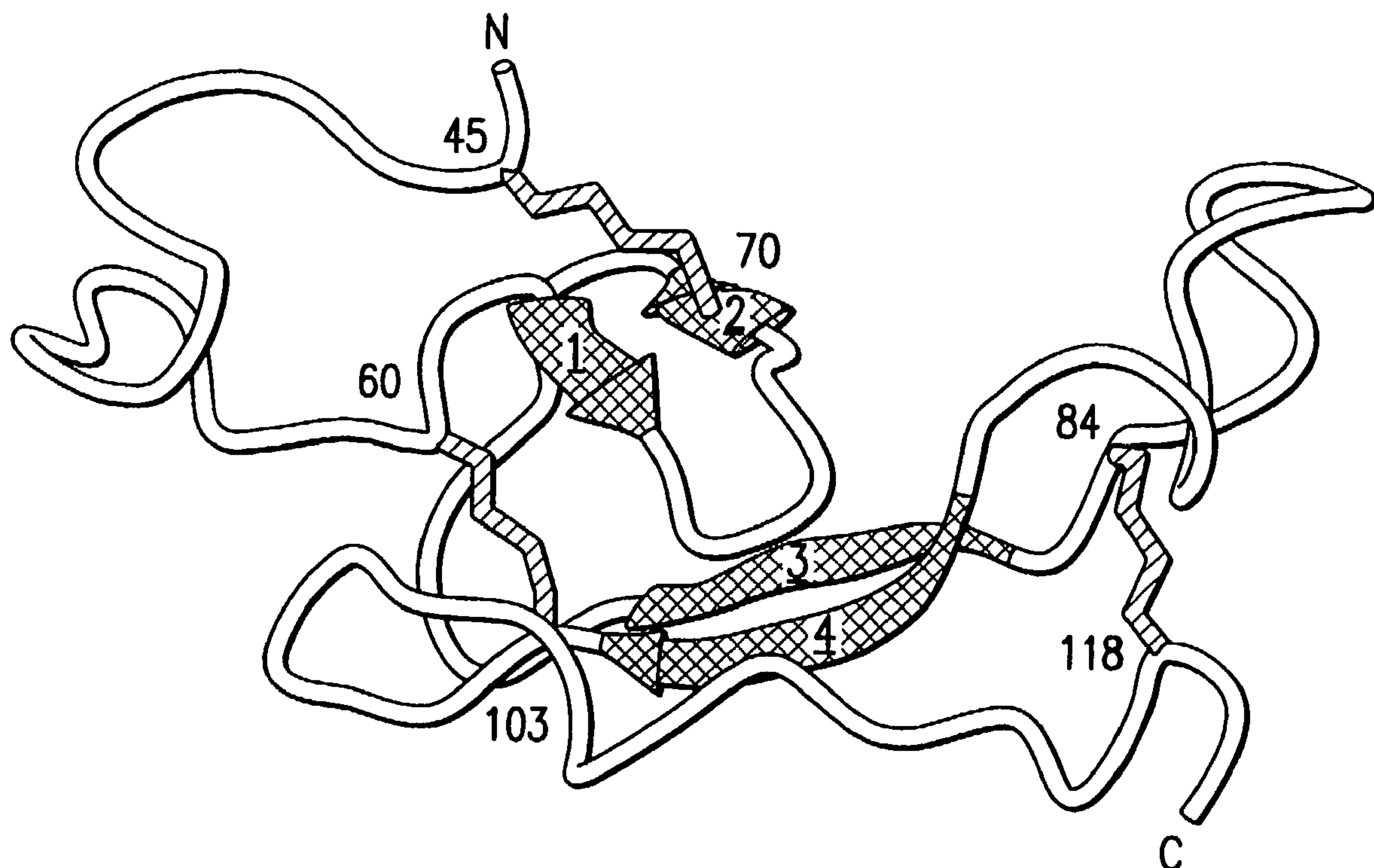




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(54) Titre : MODULATEURS DE RECEPTEURS COUPLES A LA PROTEINE G B1 FONDES SUR UNE STRUCTURE
(54) Title: DETERMINATION OF THE 3D-STRUCTURE OF AN EXTRACELLULAR DOMAIN OF A B1 G-PROTEIN
COUPLED RECEPTOR BY NMR ANALYSIS



(57) **Abrégé/Abstract:**

The present invention relates to a method for identifying modulators of B1 G-protein coupled receptors. The present invention also relates to a method for identifying an antagonist or agonist of the corticotropin-releasing factor receptor 2 (CRFR2). The present invention also relates to a method for improving antagonists or agonists of CRFR2. The present invention also relates to the three-dimensional structure of CRFR2 as representative of the B1 GPCR subfamily and its use as a basis for rational drug design of antagonist or agonists of B1 GPCRs.

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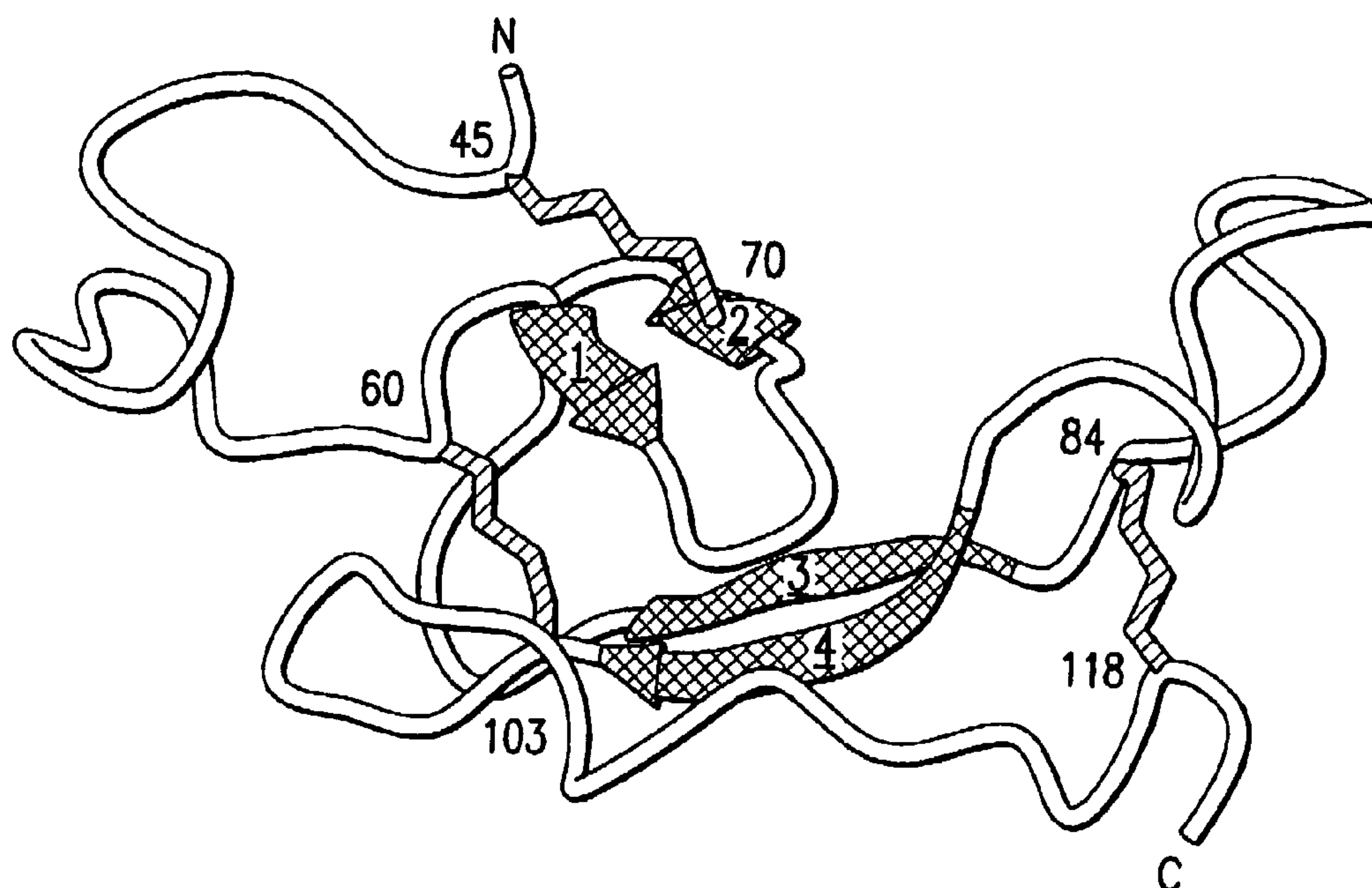
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(54) Title: DETERMINATION OF THE 3D-STRUCTURE OF AN EXTRACELLULAR DOMAIN OF A B1 G-PROTEIN COUPLED RECEPTOR BY NMR ANALYSIS



(57) Abstract: The present invention relates to a method for identifying modulators of B1 G-protein coupled receptors. The present invention also relates to a method for identifying an antagonist or agonist of the corticotropin-releasing factor receptor 2 (CRFR2). The present invention also relates to a method for improving antagonists or agonists of CRFR2. The present invention also relates to the three-dimensional structure of CRFR2 as representative of the B1 GPCR subfamily and its use as a basis for rational drug design of antagonist or agonists of B1 GPCRs.

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DESCRIPTION

STRUCTURE-BASED MODULATORS OF B1 G-PROTEIN COUPLED RECEPTORS

[0001] This application claims priority to U.S. Provisional Patent Application serial number 60/599,936 filed August 9, 2004, which is incorporated herein by reference in its entirety.

[0002] The government owns rights in the present invention pursuant to NIH grant numbers DK26741 and DK059953.

TECHNICAL FIELD

[0003] The field of the invention relates to molecular biology and biophysics, particularly protein structure determination and uses thereof. Certain aspects of the invention relate to rational drug design based on structural determinations of extracellular domains of B1 G-protein coupled receptors (GPCRs), particularly ligand binding domains.

BACKGROUND OF THE INVENTION

[0004] Many medically significant biological processes are mediated by proteins participating in signal transduction pathways involving G-proteins and second messengers such as cAMP. The membrane protein gene superfamily of G-protein coupled receptors (GPCRs) includes a wide range of biologically active receptors, such as hormone, viral, growth factor, and neuro-receptors. GPCRs have been characterized as having seven putative transmembrane (TM) domains (designated TM1, TM2, TM3, TM4, TM5, TM6, and TM7), which are believed to represent transmembrane α -helices connected by extracellular or cytoplasmic loops. Most G-protein coupled receptors have single conserved cysteine residues in each of the first two extracellular loops that form disulfide bonds believed to stabilize the functional protein structure. G-protein coupled receptors may be coupled intracellularly with heterotrimeric G-proteins and various intracellular enzymes, ion channels, and transporters. Different G-protein α -subunits preferentially stimulate particular effectors to modulate various biological functions in a cell.

[0005] One important subfamily of the GPCRs is the corticotropin-releasing factor receptors (CRFR), also known as corticotropin-releasing hormone receptors (CRHR). Corticotropin-releasing factor (CRF) (corticotropin-releasing hormone) is a 41-residue hypothalamic peptide (SEQ ID NO:5) which stimulates the secretion and biosynthesis of pituitary ACTH. Secretion of ACTH leads to increased adrenal glucocorticoid production. CRF

was isolated and characterized based on its role in the hypothalamic-pituitary-adrenal axis (HPA). More recently, however, it has been found to be distributed broadly within the central nervous system (CNS), as well as in extra-neural tissues such as the adrenal glands and testes, where it may also act as a paracrine regulator or neurotransmitter.

[0006] A considerable body of evidence suggests that peptides of the CRF family, *e.g.*, CRF(1), (frog) sauvagine, (fish) urotensin, and the mammalian urocortins 1, 2 and 3 (Ucn 1,2 and 3), play biologically diverse roles by activating CRF receptors. The CRF receptors, encoded by two distinct receptor genes, exist in multiple splice variant forms and display both species and tissue differential expression. Studies with transgenic mice expressing functionally disabled receptors, have underscored the importance of CRFR1 in regulating the hypothalamic-pituitary-adrenal axis in its response to stress. CRFR2 plays an important role in modulating the central nervous system response to stress as well as a unique role in cardiac function and pancreatic hormone release.

[0007] The CRF receptors belong to the B1 subfamily of G-protein coupled receptors (GPCRs). The GPCRs present a large variety of different conformations in their extracellular domains in order to cover their different functions. Indeed, a major ligand-binding site on both CRFR1 and CRFR2 is the N-terminal extracellular domain (ECD₁). The inhibitory binding constants for a bacterially expressed soluble protein fragment, ECD₁-CRFR2 β , (comprising amino acids 39-133 of mCRFR2 β) are: 11.8 (7.4-18.9) nM, 53.7 (18.7-154) nM and 21.1 (15.3-29.0) nM for Ucn 1, Ucn 2, and astressin, respectively. There remains a need for additional compositions and methods for identifying and assessing agonist and antagonist of B1 GPCRs, particular corticotropin releasing factor receptors.

SUMMARY OF THE INVENTION

[0008] Certain aspects of the invention provide methods for identifying modulators (antagonist or agonist) of B1 GPCRs, particularly CRFRs including, but not limited to CRFR1 and CRFR2. Modulators of GPCRs may be used as therapeutic agents for the treatment of a wide range of disorders or illnesses including endocrine, psychiatric, and neurologic disorders or illnesses. One aspect of the invention provides a three-dimensional model (representation) of the extracellular domain (ECD₁) of CRFR2 that can be used as a basis for rational drug design. The method, in preferred aspects, involves analyzing the structure of the extracellular domain of CRFR2 or other B1 GPCRs, designing a candidate modulating agent to fit into or bind the

ligand-interacting or protein-protein interaction sites of the extracellular domain and testing or assessing the candidate modulating agent for activity. Other B1 GPCR family members may be modeled using the atomic coordinates of the ECD1 of CRFR2 appropriately modified in relation to the amino acid sequence of the other B1 GPCR.

[0009] In certain embodiments of the invention, it is contemplated that the methods described herein are applicable to CRFR2, including α, β, γ splice variants, as well as CRFR1 (SEQ ID NO:6, SEQ ID NO:7). In other embodiments of the invention, the methods are applicable to modeling ECD1 of other members of the B1 subfamily of GPCRs. The B1 subfamily of GPCRs includes, but is not limited to receptors for glucagon (SEQ ID NO:8); glucagon like peptide (SEQ ID NO:9); parathyroid hormone (SEQ ID NO:10); secretin (SEQ ID NO:11); calcitonin (SEQ ID NO:12); vasoactive intestinal peptide (SEQ ID NO:13); growth hormone releasing factor (SEQ ID NO:14); pituitary adenylate cyclase-activating polypeptide (SEQ ID NO:15); and glucose-dependent insulintropic polypeptide. It is further contemplated that both transmembrane and soluble forms of the receptors are appropriate for use in the method of the present invention. Certain aspects of the invention contemplate the use of soluble GPCR or their fragments or domains.

[0010] In still further aspects, the structure of the extracellular domain of CRFR2 comprises a three-dimensional nuclear magnetic resonance (NMR) structure as set forth in PDB ID NO: 1U34, which is incorporated herein by reference in its entirety. Also, a set of atomic coordinates representative of a conformer of CRFR2 is provided in Table 3. The result of the NMR analysis of the extracellular domain of CRFR2 is a set of estimates of distances between specific pairs of atoms, called "constraints." The result is an ensemble of models, rather than a single structure. Often the positions of atoms in the different models are averaged, and the average model is then adjusted to obey normal bond distances and angles ("restrained minimization").

[0011] "CRFR2 receptors" as utilized herein refers to receptor proteins that specifically bind corticotropin-releasing factor and other proteins such as urotensin I and urocortin I. U.S. Patent 5,786,203, incorporated herein by reference in its entirety, describes sequences and nucleic acid constructs that may be used in the present invention. In certain embodiments of the invention, it is contemplated that the mouse CRFR2 (GenBank Accession No: NM 009953 SEQ ID NO:1 and NP 034083 SEQ ID NO:2) receptor is used for molecular modeling to design

antagonists or agonists. In certain embodiments, the extracellular domain comprises amino acids 39-133 of SEQ ID NO:2. In other embodiments of the invention, it is contemplated that the human CRFR2 (GenBank Accession No: NM 001883 (SEQ ID NO:3) and NP 001874 (SEQ ID NO:4)) is used for molecular modeling to design antagonists or selective agonists. In further embodiments, the extracellular domain comprises amino acids 8-102 of SEQ ID NO:4. One with skill in the art realizes that the extracellular domain of both human and mouse CRFR2 may be either the entire N-terminal extracellular domain, or functional (ligand-binding) fragments thereof.

[0012] In certain embodiments, it is contemplated that candidate agents are designed to interact with amino acids 67, 68, 69, 90, 91, 92, 93, 102, 103, 112, 113, 114, 115, 116 or combinations thereof, of SEQ ID NO:2 or analogous amino acids of other B1 GPCRs. In specific embodiments, candidate agents are designed to interact with amino acids 67, 92, and 112 of SEQ ID NO:2 or analogous amino acids of other B1 GPCRs. In one embodiment of the present invention, a modulator (antagonist or agonist) binds in proximity to one or more amino acids in the extracellular domain of CRFR2 or in analogous regions of other receptors. In a preferred embodiment of the invention, a modulator (antagonist or agonist) binds in proximity to a short consensus repeat domain of the extracellular domain of a GPCR.

[0013] The present invention also provides methods for improving known modulators of GPCRs by studying or modeling their structure as bound to the extracellular domain of the receptor, and improving their design within the parameters indicated by the structure analysis. By analyzing the structure, it is contemplated that the antagonist or agonist can be modified in order to bind a GPCR with higher affinity. Methods for screening candidate agents include contacting or modeling the agent with the extracellular domain of a GPCR, including but not limited to CRFR2; and analyzing the ability of the candidate agent to bind to the extracellular domain. For example, the binding affinity of candidate agents for the extracellular domain of CRFR2 will be determined. As used herein, "binding affinity" refers to the strength of an interaction between two entities, such as a protein-protein or protein-drug interaction. Binding affinity is referred to as the K_a , or association constant, which describes the likelihood of the two separate entities to be in the bound state. The binding affinity measures the ability of the interaction to minimize the free energy of the system, which comprises the interacting species, as well as the solvent. For example, the unbound and bound portions may be separated from one another through adsorption, precipitation, gel filtration, dialysis, or centrifugation. The

measurement of the concentrations of bound and unbound portions is accomplished, for example, by measuring radioactivity or fluorescence.

[0014] Specific embodiments of the invention include performing computer analysis, *i.e.*, using computer readable medium providing instructions for modeling an agent having the ability to bind the extracellular domain of a GPCR. In one embodiment, the computer readable medium may include computer-instructions for analyzing the coordinate representation of an extracellular domain of, for example, B1 GPCR. The analysis of the extracellular domain enables one with skill in the art to design candidate agents for modulating the activity of the receptor or other protein-protein interactions. An “agent” or “candidate agent” as identified by the methods of the present invention may include, but is not limited to a protein, polypeptide, peptide, peptidomimetic, nucleic acid (including DNA or RNA), small molecule, or compound. In a specific embodiment of the invention, the agent is a peptide or a peptidomimetic. In another specific embodiment, the agent is a drug or a nonpeptide drug. Identification and development of antagonists, agonists, selective antagonists, and selective agonists of CRFR2 are contemplated by the present invention.

[0015] “Peptide,” “polypeptide,” and “protein” may be used interchangeably, and refer to a polymer in which the monomers are amino acids (both traditional and modified) and are joined by amide bonds. However, “peptides” are generally 150 amino acids or less in length, or, in certain embodiments of the invention, are less than 100 amino acids, or less than 75, 70, 65, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10 amino acids in length or any length therebetween. The term “domain” as used herein refers to a subsection of a polypeptide that possesses a unique structural or functional characteristic; typically, this characteristic is similar across a diverse set of polypeptides. The subsection typically comprises contiguous amino acids, although it may also comprise amino acids which act in concert or which are in close spatial proximity, as contrasted with linear proximity, due to protein folding. As used herein, the “extracellular domain” refers to the extracellular domain 1 (ECD₁) of B1 G-coupled proteins receptors, or any functional, ligand-interacting portion or fragment thereof, including substituted or mutated forms of the extracellular domain 1, including fusion proteins. In a preferred embodiment the ECD₁ of CRFR2 is used.

[0016] In specific embodiments, a known modulator is astressin, astressin B, astressin₂-B, or urocortin III. Astressin compounds are CRF analogs with high binding affinity for CRF

receptors. Known CRF receptor modulators that may be improved by the methods of the present invention include, but is not limited to compounds as described in U.S. Patents 6,747,034; 6,664,261; 6,583,143; 6,541,469; 6,531,475; 6,514,982; 6,500,839; and 6,348,466, each of which is incorporated herein by reference in their entirety.

[0017] It is also contemplated that the ECD of GPCR interacts with a second peptide receptor or polypeptide. The interaction could initiate downstream signaling events from one or both receptors. In a particular aspect the second peptide receptor is an EGF-like receptor. Thus, a candidate agent may be designed to modulate other interaction sites on the ECD.

[0018] It is specifically contemplated that any embodiment discussed with respect to a particular method or composition may be implemented with respect to other methods and compositions of the invention.

[0019] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0020] The use of the term “or” in the claims is used in the conjunctive unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

[0021] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. It should be appreciated that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same or similar purposes of the present invention. It should also be realized that such equivalent constructions do not depart from the invention as set forth in the appended claims. It is to be expressly understood that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

[0023] **FIGs. 1A-1B** show the 3D structure of ECD₁-CRFR2 β . FIG. 1A is a ribbon diagram of the lowest energy conformer highlighting the β -sheets and the disulfide bonds. FIG. 1B is a superposition of 20 conformers representing the 3D NMR structure. Only amino acid residues 44-119 are shown. The bundle is obtained by superimposing the backbone C $^{\alpha}$ carbons of residues 58-83 and 99-113. The program MOLMOL was used to generate the figures and in the following figures the conformer with the lowest CYANA target function is used to represent the 3D structure of ECD₁-CRFR2 β .

[0024] **FIGs. 2A-2C** show an image of mapping the conserved amino acids onto the 3D structure of ECD₁-CRFR2 β . FIG. 2A is a stereo view and FIG. 2B is the surface of the 3D structure showing side chains of the conserved amino acids within the B1 family of GPCRs. The salt bridge between Asp65 and Arg101 is labeled by the dashed line. FIG. 2C is a sequence alignment of the ECD₁ of the CRF-R family. FIG. 2D is a sequence alignment of the ECD₁ of the B1 GPCR family. Only a representative set of sequences are shown.

[0025] **FIGs. 3A-3D** depict the identification of the binding site of astressin on the 3D structure of ECD₁-CRFR2 β . FIG. 3A is a 2D [^{15}N , ^1H]-HMQC spectra of ECD₁-CRFR2 β in the absence and presence of astressin. FIG. 3B is a plot of the normalized chemical shift changes $[\Delta(\delta(^1\text{H}))^2 + \Delta(^{15}\text{N})^2/5]^{1/2}$ observed in the complex versus the amino acid sequence. FIG. 3C is a surface representation of ECD₁-CRFR2 β showing the amino acids involved in binding with astressin. FIG. 3D is a competitive displacement by astressin of [^{125}I -DTyr⁰]-astressin bound to membranes from COSM6 cells transiently expressing myc-mCRFR2 β (●); myc-mCRFR2 β (K92Q) (□); myc-mCRFR2 β (R112E) (Δ); myc-mCRFR2 β (I67E)(◇); myc-mCRFR2 β (R112W) (▽).

[0026] **FIGs. 4A-4B** show a two-step model for hormone binding and receptor activation. FIG. 4A is the surface presentation of ECD₁-CRFR2 β and astressin B with the electrostatic potential of both the molecules. The proposed binding interface between ECD₁-CRFR2 β and the ligand is indicated in an opened view. Proposed electrostatic interactions

include Glu39-Arg112 (ECD₁) and Arg35-Glu96 (ECD₁), as well as hydrophobic interactions (Leu37, Ile41 of the ligand with Y115 and P120 of ECD₁). FIG. 4A is a schematic of the hormone binding in the full-length receptor. The peptide hormone structure contains a kink at approximately residue 24. The N-terminal segment is important for receptor activation and signaling. The positively charged surface of the ECD₁ is facing the transmembrane segment. The transmembrane segment of the receptor is modeled using the rhodopsin structure (PDB code 1HZX). Orientation (B) is rotated relative to the standard orientation of (A) by 90 and 180 degrees along the vertical axes and horizontal axes, respectively.

[0027] Color versions of similar drawings can be found in Grace *et al.*, 2004, which is incorporated herein by reference in its entirety.

DETAILED DESCRIPTION OF THE INVENTION

[0028] CRFR2 is a member of the B1 subfamily of G-protein coupled receptors (GPCRs), a class of receptors characterized by seven transmembrane helices. The structure of the N-terminal extracellular portion of CRFR2 may serve as a surrogate for the CRFR2 protein. It is contemplated that the extracellular domain of CRFR2 can be expressed apart from the transmembrane domains in order to more easily elucidate the structure of the ligand-interacting domain of the receptor and its association with other polypeptides, peptides, small molecules, and modifications thereof. The extracellular domain may be obtained by recombinant expression of the portion of the CRFR2 gene that encodes the extracellular domain of the polypeptide, or through *de novo* synthesis.

[0029] Certain aspects of the present invention provide methods of identifying modulators (antagonist or agonist) of B1 GPCRs. The modulators can be used as therapeutic agent for the treatment of a wide range of disorders or illnesses including endocrine, psychiatric, and neurologic disorders or illnesses. The present invention provides a three-dimensional model or representation of the extracellular domain 1 (ECD₁) of an exemplary B1 GPCR, *i.e.*, CRFR2, that is used as a basis for rational drug design. Preferred aspects of the invention involve methods analyzing the structure of the extracellular domain of CRFR2, designing, or modifying an modulating agent to interact with an ECD₁ with favorable molecular interactions, testing or assessing the activity of such an agent, and modeling and designing modulating agents for other B1-GPCRs using the CRFR2 structure or modifications thereof.

[0030] In certain embodiments of the invention, it is contemplated that the methods described herein are applicable to CRFR2, including α, β, γ splice variants, as well as CRFR1 (SEQ ID NO:6, SEQ ID NO:7). In other embodiments of the invention, the methods described herein are applied to the B1 family of GPCRs including, but not limited to receptors for glucagon (SEQ ID NO:8), glucagon like peptide (SEQ ID NO:9), parathyroid hormone (SEQ ID NO:10), secretin (SEQ ID NO:11), calcitonin (SEQ ID NO:12), vasoactive intestinal peptide (SEQ ID NO:13), growth hormone releasing factor (SEQ ID NO:14), pituitary adenylate cyclase-activating polypeptide (SEQ ID NO:15), and glucose-dependent insulintropic polypeptide. Further, both transmembrane and soluble forms of the above receptors are viable targets for use in the methods of the present invention. For example, the soluble forms of CRFR1 and CRFR2 α may be used as representative ECDs or GPCRs.

[0031] In a specific embodiment, the structure of the extracellular domain of CRFR2 comprises a three-dimensional nuclear magnetic resonance (NMR) structure. Solution nuclear magnetic resonance uses an aqueous solution of a purified extracellular domain of CRFR2 polypeptide; while the molecules tumble and vibrate with thermal motion. NMR detects chemical shifts of the atomic nuclei in the polypeptide with nonzero spin. The shifts depend on the electronic environments of the nuclei, namely, the identities and distances of nearby atoms. ^1H is the only naturally occurring atom in proteins observed by NMR. The extracellular domain of CRFR2 is labeled with ^{13}C and ^{15}N . NMR analysis of the extracellular domain of CRFR2 provides estimates of distance between specific pairs of atoms, called "constraints". Constraints obtained are for both bonded and non-bonded atom pairs (through-bond or through-space distances). With a sufficient number of such constraints, the number of configurations consistent with the data becomes finite. The result is an ensemble of models, rather than a single structure. Often the positions are the average position of atoms in the different models, and the average model is adjusted to obey normal bond distances and angles ("restrained minimization"). Structures for the extracellular domain of CRFR2 fitting the NMR data with good stereochemistry are on deposit with the Protein Data Bank in PDB ID 1U34, which is incorporated herein by reference in its entirety. An example of the structure of CRFR2 is the structure defined by the parameters set forth in Table 2, or, alternatively, as set forth in FIG. 1. Furthermore, atomic coordinates of a representative conformer of the structure are in Table 3 below.

[0032] In still further embodiments, a processor may be used to model an extracellular domain of a B1 GPCR and determine agents that may fit and bind to at least one contact point on the GPCR. The processor may be any computer-readable media known in the art. For example, it may be embodied internally or externally on a hard drive, ASIC, CD drive, DVD drive, tape drive, floppy drive, network drive, flash, or the like. Processor can indicate any computing device capable of executing instructions including, without limitation, obtaining the coordinates of the GPCR, determining a binding site on the GPCR, designing an agent that may bind with the binding site of the GPCR, and/or assessing the antagonist or agonist activity of the agent. In one embodiment, the processor is a personal computer (*e.g.*, a desktop or laptop computer operated by a user). In another embodiment, processor may be a personal digital assistant (PDA) or other handheld computing device.

[0033] In some embodiments, the processor may be a networked device and may constitute a terminal device running software from a remote server, wired or wirelessly. Input from a user or other system components may be gathered through one or more known techniques such as a keyboard and/or mouse. Alternatively, the processor may be configured to import data from a server via a wired or wireless network. Output, if necessary, may be achieved through one or more known techniques such as an output file, printer, facsimile, e-mail, web-posting, or the like. Storage may be achieved internally and/or externally and may include, for example, a hard drive, CD drive, DVD drive, tape drive, floppy drive, network drive, flash, or the like. The processor may use any type of monitor or screen known in the art, for displaying information, such as but not limited to, possible agents binding to a contact site of the GPCR. For example, a cathode ray tube (CRT) or liquid crystal display (LCD) can be used. One or more display panels may also constitute a display. In other embodiments, a traditional display may not be required, and the processor may operate through appropriate voice and/or key commands.

[0034] Analysis of the extracellular domain of CRFR2 enables one to design candidate agents or improve known agents for modulating a receptor. An "agent" or "candidate agent" as identified by the methods of the present invention includes, but is not limited to a protein, polypeptide, peptide, peptidomimetic, nucleic acid (including DNA or RNA), molecule, compound or drug. In a specific embodiment of the invention, the agent is a peptide, peptoid, or a peptide mimic. In another embodiment, the agent is a drug or a nonpeptide drug. Antagonists, agonists, selective antagonists, and selective agonists of CRFR2 are agents envisioned by the present invention.

[0035] “CRFR2 receptors” as utilized herein refers to receptor polypeptides or proteins when in native form bind their designated ligands, including, but not limited to corticotropin-releasing factor, urotensin I, and urocortin I. U. S. Patent No. 5,786,203, herein incorporated by reference in its entirety, describes exemplary sequences and nucleic acid constructs for use in the present invention. In certain embodiments of the invention, it is contemplated that the mouse CRFR2 (GenBank Accession No: NM 009953 SEQ ID NO:1 and NP 034083 SEQ ID NO:2) receptor is used for molecular modeling to design antagonists, agonists, selective antagonist, or selective agonist. In certain embodiments the ECD1 comprises amino acids 39-133 of SEQ ID NO:2. In other embodiments of the invention, it is contemplated that the human CRFR2 (GenBank Accession No: NM 001883 SEQ ID NO:3 and NP 001874 SEQ ID NO:4) is used for design of antagonists, agonists, selective antagonist, or selective agonist. In certain embodiments the ECD1 comprises amino acids 8-102 of SEQ ID NO:4. The extracellular domains of both human and mouse CRFR2 may be either the entire N-terminal extracellular domain, functional ligand-binding fragments, or fragments that interact with other cellular components, such as other proteins.

[0036] In still a further embodiment, the extracellular domain of CRFR2 comprises amino acids 39-133 of SEQ ID NO:2. In another embodiment, the extracellular domain of CRFR2 comprises amino acids 8-102 of SEQ ID NO:4. In certain embodiments, it is contemplated that candidate agents are designed to interact with amino acids 67-69, 90-93, 102-103 and/or 112-116, of SEQ ID NO:2 or analogous amino acids in other B1 GPCR ECDs. In specific embodiments, candidate agents are designed to interact with amino acids 67, 92, and 112 of SEQ ID NO:2 or analogous amino acids in other B1 GPCR ECDs. In one embodiment of the present invention, the antagonist or agonist binds in the proximity of one or more amino acids in the ECD of CRFR2. In another embodiment of the invention, the antagonist or agonist binds in the proximity of a short consensus repeat domain of the ECD of CRFR2.

[0037] The present invention also provides for methods to improve known modulators of GPCRs, e.g., CRFR2, by analyzing or studying their structure when bound to the extracellular domain of the receptor, and improving their design within the parameters indicated by the structure analysis. It is contemplated that by analyzing the structure the antagonist or agonist can be modified to bind the receptor with higher affinity.

[0038] Aspects of the invention include methods for screening candidate agents comprising contacting the agent with the extracellular domain of a B1 GPCR, preferably of a CRFR2, and analyzing the ability of the agent to bind to the extracellular domain. For example, it is contemplated that the binding affinity of candidate agents for an extracellular domain will be determined. As used herein, “binding affinity” refers to the strength of an interaction between two entities, such as a protein-protein or protein-drug interaction. Binding affinity may be referred to the K_a , or association constant, which describes the likelihood of the two separate entities to be in the bound state. The binding affinity typically measures the ability of the interaction to minimize the free energy of the system that comprises the interacting species and the solvent. A variety of methods are used to determine the association constant. Typically, two separate entities are mixed, the unbound portion is separated from the bound portion, and concentrations of unbound and bound are measured. One with skill in the art realizes that there are various methods for measuring association constants. For example, the unbound and bound portions may be separated from one another through adsorption, precipitation, gel filtration, dialysis, or centrifugation. The measurement of the concentrations of bound and unbound portions may be accomplished, for example, by measuring radioactivity or fluorescence.

[0039] The terms “peptide,” “polypeptide,” and “protein” may all be used interchangeably, and refer to a polymer in which the monomers are amino acids (both traditional and modified) and are joined together through amide bonds. However, “peptides” are generally 150 amino acids or less in length, or, in certain embodiments of the invention, are less than 100 amino acids, or less than 75, 70, 65, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10 amino acids in length or any length therebetween. The term “domain” as used herein refers to a subsection of a polypeptide that possesses a unique structural and/or functional characteristic; typically, this characteristic is similar across diverse polypeptides. The subsection typically comprises contiguous amino acids, although it may also comprise amino acids which act in concert or which are in close proximity due to folding or other configurations. As used herein, the “extracellular domain” refers to the extracellular domain 1 (ECD₁) of B1 G-coupled proteins receptors, or any functional, ligand-interacting portion or fragment thereof, including substituted or mutated forms of the extracellular domain 1. For example, the ECD₁ of CRFR2 is used in certain methods of the present invention.

[0040] In a still further embodiments, the known modulator is astressin, astressin B, astressin₂-B, urocortin III, or modification thereof. Astressin compounds are CRF analogs with

high binding affinity for CRF receptors. CRF receptor modulators include, but are not limited to compounds as described in U.S. Patent Nos. 6,747,034; 6,664,261; 6,583,143; 6,541,469; 6,531,475; 6,514,982; 6,500,839; and 6,348,466.

[0041] It is also contemplated that the ECD1 domain of GPCR interacts with one or more other receptors, polypeptides, or signal transduction cascades. Thus, the interaction could initiate downstream signaling events from one or more receptors. It is contemplated that the peptide receptor is an EGF-like receptor.

I. CRFR2 ANTAGONISTS AND AGONISTS

[0042] CRFR2 antagonists and agonists identified by the methods of the present invention may be useful in treating physiological conditions or disorders arising from the hypersecretion or hyposecretion of CRF or other natural B1 GPCR ligands. Because CRF is believed to be a pivotal neurotransmitter that activates and coordinates the endocrine, behavioral, and autonomic responses to stress, the CRFR2 antagonists and agonists identified by the methods of the present invention can be used to treat neuropsychiatric disorders. Neuropsychiatric disorders treatable by the CRFR2 antagonists and agonists may include, but are not limited to affective disorders such as depression; anxiety-related disorders such as generalized anxiety disorder, panic disorder, obsessive-compulsive disorder, abnormal aggression; cardiovascular abnormalities such as unstable angina and reactive hypertension; and feeding disorders such as anorexia nervosa, bulimia, and irritable bowel syndrome. CRFR2 antagonists and agonists may also be useful in treating stress-induced immune suppression associated with various diseases states, as well as stroke. Other uses of the CRFR2 antagonists and agonists identified by the method of the present invention include treatment of inflammatory conditions (such as rheumatoid arthritis, uveitis, asthma, inflammatory bowel disease and G.I. motility), Cushing's disease, infantile spasms, epilepsy and other seizures in both infants and adults, and various substance abuse and withdrawal syndromes and conditions (including alcoholism).

II. PROTEIN-STRUCTURE BASED DESIGN OF ANTAGONISTS OR SELECTIVE AGONISTS OF CRFR2

[0043] Molecular modeling may use computers to model the molecular structure. Non-limiting examples of such methods include molecular graphics (*i.e.*, 3-D representations) to computational chemistry (*i.e.*, calculations of the physical and chemical properties). Using

molecular modeling, rational drug design programs can look at a range of molecular structures that may fit into an active site of an enzyme or interact with polypeptide. By using computer programs, for example, a determination can be made as to which compounds actually fit into or bind a given site or potential active. U.S. Patents that provide additional information on molecular modeling include U.S. Patents 6,093,573; 6,080,576; 5,612,894; 5,583,973; 5,030,103; 4,906,122; and 4,812,128, each of which is incorporated herein by reference in its entirety. As used in the methods described herein, the term “computer fitting analysis” or “modeling” refers to a schematic or other work that is prepared using a computer algorithms or computer programs that can process and provide information about protein structure and conformation. A number of such programs and algorithms are readily available and known to those of skill in the art. They can configure a protein sequence into a 3-dimensional molecule and additionally configure it with a ligand or other substrate, such as a particular nucleic acid molecule.

[0044] In the context of the invention, the program or algorithm will configure and improve (in some cases, optimize) an interface, including its amino acid side chains, between the ligand-binding domains of CRFR2 and a ligand, such as a candidate antagonist or agonist. The program or algorithm will also configure and improve or optimize the interface, including its amino acid side chains, between ECD1 of CRFR2 and a ligand. This program or algorithm will allow the detection, identification, and improvement/optimization of contact points between individual protein domains or between protein domains and ligands. A “contact point” refers to the point at which individual protein domains, or protein domain and ligand molecules interact. Such contact points are formed as a result of specific binding between two protein domains or between protein domains and a nucleic acid molecule. Other amino acids within the interface may also be modified to enhance or improve the interaction between protein domains or between protein domains and ligands. Modifications to the interface may result in improved interaction between individual protein domains or between protein domain(s) and ligands present in the complex or may result in improved stability of the protein. In this context, amino acid side chains represent “potential contact points” in the interface that may be modified in various combinations. “Interface” refers to the amino acids between two interacting protein domains or between protein domains and ligands that form contact points, as well as those amino acids that are adjacent to contact points and along the surface between individual protein domains, or between protein domains and ligands.

[0045] An algorithm or program will typically allow the identification of potential contact points, residues that are not properly interacting with the target sequence, residues between two interacting protein domains or between protein domains and ligands inhibiting or reducing. Thus, methods of the invention further include the step of identifying potential contact points between individual protein domains or between protein domains and ligands, and/or identifying amino acids along the interface or in the proximity of the interface that can be modified to improve the interface (that is to modify the interaction as desired by the protein engineer). Computational modeling that occurs in different embodiments of methods of the invention involves modeling of the various entities to show their interactions with one another, such interactions include, but are not limited to interactions between or among ligands, peptides, polypeptides, and single or multiple protein domains.

[0046] Given a B1 GPCR extracellular domain structure, a potential modulator of a B1 GPCR can be identified and analyzed using computer modeling techniques. There are a number of computer programs that can be used to identify potential small molecule and peptide compounds that bind with favorable binding energies. Non limiting examples include: GRID (available from Oxford University, UK), MCSS (available from Accelrys, San Diego, CA), AUTODOCK (available from The Scripps Research Institute, La Jolla, CA), FLEX X (available from Tripos, St. Louis. MO), DOCK (available from University of California, San Francisco), CAVEAT (available from University of California, Berkeley), HOOK (available from Accelrys, San Diego, CA), and 3D database systems such as MACCS-3D (available from MDL Information Systems, San Leandro, CA), UNITY (available from Tripos, St. Louis. MO), and CATALYST (available from Accelrys, San Diego, CA.) Potential inhibitors may also be computationally designed by using such software packages as LUDI (available from Accelrys, San Diego, CA), LEGEND (available from Accelrys, San Diego, CA), and LEAPFROG (Tripos Associates, St. Louis, Mo.). The computer and modeling techniques may be performed on any suitable hardware or computer system, such as, but not limited to, a desktop computer, a personal digital assistant (PDA), a notebook processor, a tablet PC, and the like. This procedure can include computer fitting of potential modulators of B1 GPCRs to ascertain how well the shape and chemical structure of the potential modulator will bind (Bugg *et al.*, 1993; West *et al.*, 1995). Computer readable mediums may also execute instructions to estimate the attraction, repulsion, and steric hindrance of the ECDs with a modulator/inhibitor (*e.g.*, CRFR2 and a potential inhibitor). Generally, the tighter the fit the lower the steric hindrances, the greater the attractive

forces, and the more potent a modulator. Furthermore, the more specificity in the design of a potential drug the more likely that the drug will not interact as well with other proteins. This will minimize potential side effects due to unwanted interactions with other proteins.

[0047] Initially, compounds known to bind CRFR2, for example, astressin, can be systematically modified by computer modeling programs until one or more promising potential analogs are identified. In addition, systematic modification of selected analogs can then be performed by computer modeling programs until one or more potential analogs are identified. It is also contemplated that truncating or varying the length CRFR2 agonists, or functional analogs thereof, will create molecules with CRFR2 antagonistic properties. Truncating a portion of the agonist molecule that inserts itself into the plasma membrane will impair the ability of the molecule to activate downstream signaling events. Amino acids 1-8 are crucial for agonist activity of the CRF peptide (Rivier *et al.*, 1984).

[0048] A potential modulator can be selected from a library of chemicals that are commercially available from most large chemical companies including Merck, GlaxoWellcome, Bristol Meyers Squib, Monsanto/Searle, Eli Lilly, Novartis and Pharmacia UpJohn; or alternatively the potential modulator may be synthesized de novo. As mentioned above, the de novo synthesis of one or even a relatively small group of specific compounds is reasonable in the art of drug design. The potential modulator can be placed into a standard binding assay with a B1 GPCR, or an active fragment thereof, for example, the extracellular domain.

III. SCREENING ASSAYS

[0049] B1 GPCR binding agents or compounds may be tested in biochemical assays to further identify their effectiveness in binding to and modulating B1 GPCR activity. Assays may be conducted in cell free systems, in isolated cells, or in organisms, including animals.

A. *In Vitro* Assays

[0050] One assay is the binding assay. Binding of a molecule to a target may be inhibitory, due to steric, allosteric or charge-charge interactions. Binding assays can be performed in solution or on a solid phase support. Binding assays may be used as a first round screen to rapidly eliminate certain compounds before moving into more sophisticated screening assays. The target may be either free in solution, fixed to a support, or expressed in or on the

surface of a cell. Examples of supports include, but are not limited to nitrocellulose, column, gel, or surface of a plasmon resonance (SPR) device (Szabo *et al.*, 1995). Either the target or the compound can be labeled, thereby permitting determination of binding.

[0051] In another embodiment, the assay may measure the enhanced binding of a target to a natural or artificial substrate or binding partner. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with the binding moiety's function. One may measure the amount of free label versus bound label to determine binding or inhibition of binding. In other embodiments, binding is determined by gel electrophoresis, gel filtration chromatography, fluorescence quenching, flow cytometry, ELISA, solid phase immunoassay, confocal microscopy, or surface plasmon resonance (SPR). Downstream signal transduction is indicative of binding to a receptor, for example transactivation of ErbB2.

[0052] A technique for high throughput screening of compounds is described in PCT Application WO 84/03564. In high throughput screening, large numbers of candidate inhibitory test compounds, which may be small molecules, natural substrates and ligands, or may be fragments or structural or functional mimetics thereof, are synthesized on a solid substrate, such as plastic pins or some other surface. Alternatively, purified target molecules can be coated directly onto plates or supports for use in drug screening techniques. Also, fusion proteins containing a reactive region (preferably a terminal region) may be used to link an active region of an enzyme to a solid phase, or support. The test compounds are reacted with the target molecule, and bound test compound is detected by various methods (see, *e.g.*, Coligan *et al.*, 1991).

B. *In Vivo* Assays

[0053] *In vivo* assays involve the use of various animal models, including transgenic animals that have been engineered to have specific defects, or carry markers that can be used to measure the ability of a candidate substance to reach and affect different cells within the organism. Due to their size, ease of handling, and information on their physiology and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses, and monkeys (including, but not limited to chimps, gibbons and baboons). Assays for modulators may be conducted using an animal model derived from any of these species and others.

[0054] In such assays, one or more candidate substances are administered to an animal and the ability of the candidate substance(s) to alter the physiology of the animal or the model condition to be treated, as compared to a similar animal not treated with the candidate substance(s), are assessed. The characteristics may be any of those discussed above with regard to the function of a particular compound (*e.g.*, enzyme, receptor, and hormone levels or activity) or cell (*e.g.*, growth, tumorigenicity, survival), or a broader indication such as behavior, anemia, immune response, etc.

[0055] Treatment of these animals with candidate substances will involve the administration of the compound in an appropriate form. Administration can be by any route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated routes are systemic intravenous injection, regional administration via blood or lymph supply, or directly to an affected site.

C. Arrays

[0056] Hi-throughput assays, for example, arrays comprising a plurality of ligands arranged on a solid support, represent an important diagnostic tool provided by the invention. The use of arrays involves the placement and binding of nucleic acids, or another type of ligand having affinity for a molecule in a test sample, to known locations, termed sectors, on a solid support.

[0057] Devices employing such arrays might be employed as combinatorial chemical or drug screening devices, antibody arrays, peptide arrays, cell arrays, enzymatic activity arrays, or DNA or other polynucleotide arrays that will be selective for binding to related proteins or other biomolecules. In addition, encapsulated cells or biomolecules coated onto the walls of microcapillary tubes will function as flow through devices having single or multiple channels, which might be employed as screening devices or as biosensors on systems, such as in liquid chromatography or in "lab-on-a-chip" devices. Signal readout from such devices might be via binding of 5 fluorescent proteins or of antigens, to be measured by subsequent antibody-based detection methods (possibly employing additional arrays), or via reaction with endogenous biopathways which will result in the formation of a detectable species, *e.g.* enzymatic conversion

of a substrate to a fluorescent dye molecule, or change in the electrical properties, e.g. conductivity, of the cell and/or surrounding matrix resulting from exposure to the specific agent.

[0058] In certain aspects to the invention, binding of a labeled ECD may be used to identify, confirm, or detect a binding affinity of a ligand for the ECD or the ECD for the ligand.

[0059] Arrays can be brought into contact with a test sample to determine the presence or absence of a given molecule in the sample. By including any additional other target nucleic acids or other types of ligands. Potentially thousands of target molecules in a test sample can be screened. Many different methods for preparation of arrays comprising target substances arranged on solid supports are known to those of skill in the art and could be used in accordance with the invention.

[0060] Specific methods for preparation of such arrays are disclosed in, for example, Affinity Techniques, Enzyme Purification: Jakoby and Wilchek, (1974). Examples of other techniques which have been described for the attachment of test materials to arrays include the use of successive application of multiple layers of biotin, avidin, and extenders (U.S. Patent No. 4,282,287); methods employing a photochemically active reagent and a coupling agent which attaches the photoreagent to the substrate (U.S. Patent No. 4,542,102); use of polyacrylamide supports on which are immobilized oligonucleotides (PCT Patent Publication 90/07582); use of solid supports on which oligonucleotides are immobilized *via* a 5'-dithio linkage (PCT Patent Publication 91/00868); and through use of a photoactivateable derivative of biotin as the agent for immobilizing a biological polymer of interest onto a solid support (see U.S. Patent No. 5,252,743; and PCT Patent Publication 91/07087). In the case of a solid support made of nitrocellulose or the like, standard techniques for UV-crosslinking may be of particular utility (Sambrook *et al.*, 2001).

IV. PROTEIN SYNTHESIS

[0061] It is contemplated that the protein or peptide compositions described herein may be synthesized *de novo*. For example, it is contemplated that peptide-based antagonists or selective agonists of a B1 GPCR, *e.g.*, CRFR2, may be synthesized using the techniques described herein. Such peptide-based modulators may be modified or improved analogs of CRF, urocortin, or astressin B, among others. It is contemplated that in certain embodiments of the invention, the native chemical ligation procedure is used. The general procedure follows the

method of Hackeng *et al.* (1999). Peptide fragments are synthesized using the Boc protocol on MBHA or CM resins. Thioester-containing fragments are synthesized on TAMPAL resin (trityl-associated-mercaptopropionic-acid-leucine), which is a MBHA resin modified with a thioamide linker. Leucine is first coupled to MBHA resin followed by S-tritylmercaptopropionic acid (Peptides Int'l.) to give the modified TAMPAL-MBHA resin. The trityl group is removed by two 5 min treatments of TFA/scavenger cocktail (95 TFA: 2.5 EDT: 2.5 H₂O). The remainder of the peptide is synthesized using the Boc method of solid phase peptide synthesis.

[0062] After resin cleavage and purification of the peptide fragments, 1.5 equiv. thioester-peptide and 1 equivalent of Cys-peptide are dissolved in the ligation buffer at a concentration of 1-3 mM. The ligation buffer consists of 6 M guanidine-HCl and 0.1 N sodium phosphate, adjusted to pH 8.5 with NaOH, which normalizes to ~pH 7 upon addition of the peptide-TFA salts. Thiophenol and benzylmercaptan (4% v/v each) are also added to the reaction mixture to promote the thioester exchange reaction. The ligation reaction is then heated at 38 °C and progress is monitored by HPLC. The ligation of [Cys²¹]-oCRF(21-41) or [HCys²¹]-oCRF(21-41) to [His¹³(DNP)]-oCRF(1-20)-MPAL-NH₂ reaches maximal completion at 24 or 48 h, respectively.

[0063] Another method that is contemplated for use in the present invention uses the Staudinger ligation between a C-terminal fragment containing a phosphinothioester and an N-terminal azido-peptide. The coupling and rearrangement occurs in high yield in aqueous THF at room temperature for 12 h and without epimerization. This method has been used in the synthesis of ribonuclease A, consisting of 124 amino acids. Since RNase A contains a cysteine at position 110, fragment (110-124) was coupled to fragment (1-109) *via* NCL. Fragment (110-214) was synthesized from two smaller fragments using the Staudinger ligation method. Alternatively, other investigators have incorporated a removable thiol-containing auxiliary that is attached to the N-terminal amine of the peptide to be ligated.

[0064] An aspect of NCL that limits its practicality is that it is labor intensive at each step. To increase efficiency, solid-phase ligation strategies have been explored. Camarero *et al.* (1998) have used a thio-linked PEGA support to produce the peptide thioester, which eliminates handling of the free thioester in solution. The resin is stable to HF, which cleaves off the protecting groups, but is displaced during the 2-3 h ligation reaction with the Cys-peptide fragment in the presence of aromatic thiol cofactors. Another approach developed by the

Dawson group utilizes a safety catch acid-labile linker on DADPA gel (Pierce), upon which successive NCL reactions may build up the desired protein. The support is stable to all solid phase synthetic reaction conditions until it is concomitantly reduced with SiCl_4 and cleaved with TFA to produce the peptide-amide.

[0065] Faced with the dilemma of synthesizing proteins that contained neither Cys, Met or Val, that certain amino acids would best qualify as a conservative substitute that would not affect structure and biological. The primary method for predicting the utility of amino acid replacement has been the use of mutation matrices as first published by Dayhoff and Eck (1968) using protein evolutionary data. This method has been expanded over the years by many investigations to include physiochemical and structural properties and the hierarchical clustering of these data. This work has matured into the AAIndex (See Kawashima and Kanehisa, 2000), which forms the basis of the current analysis.

[0066] A hypothesis emerging from inspection of the mutation matrices contained within the AAIndex is that amino acid replacement can be highly context specific. Given the four major clusters originally identified by Nakai and co-workers (*i.e.*, α -helix and turn propensities, β -strand propensity, hydrophobicity, and physiochemical properties) an examination of the role of Met, and more specifically of the likelihood of successful replacement, is possible. Regarding hydrophobicity, the work of George *et al.* (1990) on mutational frequency suggests that Met is promiscuous, replacing Ala, Asn, Cys, Gln, Gly, His, Ile, Leu, Phe, Pro, Ser, Thr, Tyr and Val. However, the work of Johnson and Overington toward identifying a structural basis for sequence comparison suggests that Met is much more severely restricted, replacing only Ile and Leu. The sequence alignment approach to quantifying conformational similarity of Kolaskar and Kulkarni-Kale suggests that Met can replace Arg, Leu, Lys, Phe or Trp. A significant body of work conducted by Luthy and co-workers examined the role of secondary structural context in amino acid replacement. This work suggests that Met could replace Ile, Leu or Phe in an outside helix, but Met is a poor replacement for any amino acid in an "inside helix" context, as it is poor in the "inside other" and "inside beta" contexts. However, in an "outside other" context, Met replaces Ile, Leu, Phe and Val, and in an "outside beta" context, Met replaces Ile, Leu, Pro and Val. The structurally-derived correlation method of Niefind and Schomburg suggests that Met replaces Ala, Gln, Glu and Ile. Finally, the work of Riek *et al.*, (1995) on the evolutionary conservation of hydrophilic and hydrophobic residues in transmembrane sequences suggests that Met can replace Glu, His, Lys, and Thr.

V. PEPTIDE CHARACTERIZATION

[0067] HPLC, CZE, CD, $[\alpha]_D$, FPLC, NMR, LSIMS, and other available techniques such as counterion (AcOH and TFA) content using HPLC may be used to characterize peptides in the present invention. In one embodiment, peptides are characterized by well-documented build-up of the peptide sequence on the polymer to insure us that the proper sequence was assembled on the synthesizer. HPLC using the TEAP buffer at different pHs and 0.1% TFA in CH₃CN and several columns (C₁₈, C₄ and diphenyl) are able to provide an appreciation of the amounts and relative properties of the contaminants. The TEAP buffer in the presence of acetonitrile for size exclusion chromatography is appropriate

[0068] In one embodiment, capillary zone electrophoresis (CZE) is used for the quantitative analysis of the peptides and their impurities. CZE is carried out using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 Model 50Z and using a ChromJet integrator. Several buffers have been used and conditions optimized (addition of CH₃CN or TFE in the buffers) for the elution of CRF analogs.

[0069] In another embodiment, optical rotation is used for the characterization of novel amino acids or scaffolds. Optical rotations of peptides are measured (sodium D line) in an adequate solvent ($c = 0.2-1.0$) using a Perkin-Elmer 241 polarimeter and a 100- μ L cell.

[0070] It is contemplated that fast performance liquid chromatography, FPLC, is used for the characterization and purification of the analogs, such as peptide ligands for CRFR2 or mutations of CRFR2. Recent results indicate that ion-exchange chromatography of small peptides and proteins using recently developed supports can be extremely resolute and can be used for both analytical and preparative purposes.

[0071] It is contemplated that high field NMR spectrometry is used for the characterization of some selected constrained and B1 GPCR-selective analogs, specifically CRFR-selective analogs, the structures of which will be useful for our understanding of the structural basis for receptor selectivity and mechanism of action.

[0072] Circular dichroism (CD) spectroscopy is contemplated in certain embodiments of the invention to correlate theoretical and observed CD spectra of selected CRF agonists and antagonists using the CaPPS package of Applequist and co-workers.

[0073] Mass spectrometry can be used for the identification and systematic characterization of peptides and amino acid derivatives synthesized in the laboratory.

VI. PROTEIN EXPRESSION AND PURIFICATION

[0074] Embodiments of the present invention may require the use of certain proteins, polypeptides, peptides, or enzymes, *e.g.*, B1 GPCR or CRFR2. CRFR2 or the extracellular domain of CRFR2, *e.g.*, may be obtained by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques (Sambrook, 2001), the isolation of proteinaceous compounds from natural sources, or the chemical synthesis of proteinaceous materials.

[0075] Various host-expression vector systems may be utilized to express the ECDs of the invention. Such host-expression systems represent vehicles to produce and subsequently purify the polypeptides of interest, but also represent cells that may, when transformed or transfected with the appropriate coding sequences, exhibit the protein of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing protein coding sequences; yeast (*e.g.* *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the protein coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the protein coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing protein coding sequences; or mammalian cell systems (*e.g.* COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5 K promoter).

[0076] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the protein being expressed. For example, when a large quantity of such a protein is produced for use in the generation of antibodies or to screen peptide libraries and the like, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to

the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983), in which the protein coding sequence may be ligated in frame with the lac Z coding region producing a fusion protein (Inouye and Inouye, 1985; Van Heeke *et al.*, 1989). pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned CRFR2 protein can be released from the GST moiety.

[0077] In an insect system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes and encoded proteins. The virus grows in *Spodoptera frugiperda* cells. The coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (see Smith, *et al.*, 1983; U.S. Pat. No. 4,745,051).

[0078] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the gene coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the protein in infected hosts (*e.g.*, see Logan *et al.*, 1984). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire coding sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals including the ATG initiation codon may be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and

initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (See Bitter, *et al.*, 1987).

[0079] In addition, a host cell may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

[0080] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines may be engineered to stably express a polypeptide. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*). Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A selectable marker in the recombinant plasmid confers resistance to the selection and allows cells that stably integrate the plasmid into their chromosomes to grow and form foci, which are cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express a polypeptide. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of a polypeptide.

[0081] In one embodiment, timing or quantity of expression of the recombinant protein can be controlled using an inducible expression construct. Inducible constructs and systems for inducible expression of recombinant proteins are known to those skilled in the art. Examples of such inducible promoters or other gene regulatory elements include, but are not limited to, tetracycline, metallothionine, ecdysone, and other steroid-responsive promoters, rapamycin responsive promoters, and the like (No *et al.*, 1996; Furth *et al.*, 1994). Additional control

elements that can be used include promoters requiring specific transcription factors such as viral, particularly HIV, promoters. In one embodiment, a Tet inducible gene expression system is utilized. (Gossen *et al.*, 1992; Gossen, *et al.*, 1995). Using such a system, expression of the recombinant protein is placed under the control of the tetO operator sequence and transfected or transformed into a host cell. In the presence of TetR, which is co-transfected into the host cell, expression of the recombinant protein is repressed due to binding of the TetR protein to the tetO regulatory element. High-level, regulated gene expression can then be induced in response to varying concentrations of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox), which compete with tetO elements for binding to TetR. Constructs and materials for tet inducible gene expression are available commercially from CLONTECH Laboratories, Inc., Palo Alto, Calif.

[0082] When used as a component in an assay system, a polypeptide may be labeled, either directly or indirectly, to facilitate detection of a complex formed between the polypeptide and a test substance. Any of a variety of suitable labeling systems may be used including, but not limited to radioisotopes; enzyme labeling systems that generate a detectable calorimetric signal or light when exposed to substrate; and fluorescent labels. Where recombinant DNA technology is used to produce a polypeptide for such assay systems, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization, and/or detection.

[0083] Indirect labeling involves the use of a protein, such as a labeled antibody, which specifically binds to the protein. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library.

[0084] In certain embodiments a B1 GPCR, for example CRFR2, or the extracellular domain of a B1- GPCR may be purified. Generally, "purified" will refer to a specific protein, polypeptide, or peptide composition that has been subjected to fractionation to remove various other proteins, polypeptides, or peptides, and which composition substantially retains its activity, as may be assessed, for example, by the protein assays. There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Examples of purification techniques include, but are not limited to, column chromatography, High Performance Liquid Chromatography (HPLC), medium pressure liquid chromatography, Gel chromatography, Affinity Chromatography, ion exchange chromatography, hydrophobic

interaction chromatography, size-exclusion chromatography, preparative gel electrophoresis, or isoelectric focusing chromatography (Sambrook, 2001).

VII. CANDIDATE AGENTS

[0085] The term “candidate agents” refers to any antagonist or agonist that may potentially modulate (negatively or positively) signal transduction of a B1 GPCR, in particular aspects a CRFR2. An agonist refers to a substance that increases the effective level of B1 GPCR activity through interaction with the extracellular domain. An antagonist refers to a substance that decreases B1 GPCR activity through interaction with the extracellular domain.

[0086] Candidate substances can include fragments or parts of naturally-occurring compounds. Candidate substance also includes various synthetic and recombinant molecules or libraries of such compounds or molecules. In one embodiment, the candidate substances are small molecules. In yet other embodiments, candidate substances may be synthetic or natural peptides. Examples of small molecules that may be screened include, but are not limited to, small organic molecules, peptides or fragments thereof, peptide-like molecules, nucleic acids, polypeptides, peptidomimetics, carbohydrates, lipids, or other organic (carbon-containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention to identify compounds that modulate a B1 GPCR.

[0087] Alternatively, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed or modeled as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide, polynucleotide, small molecule inhibitors, or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators. Other suitable candidate substances, compounds, or modulators of the present invention will function to regulate the activity of a B1 GPCR. Such candidate substances may include, but are not limited to, monoclonal and polyclonal antibodies, aptamers, and aptazymes.

A. Peptide Mimetics

[0088] As used herein, the terms "mimetic" or "peptide mimetic" may be used interchangeably, and refer to a compound that biologically mimics determinants on hormones, cytokines, enzyme substrates, viruses, ligands, or other bio-molecules. Mimetics may antagonize, stimulate, or otherwise modulate the physiological activity of the receptors for natural ligands. Certain mimetics that mimic elements of protein secondary and tertiary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of an antibody or an antigen. Thus, a peptide mimetic permits molecular interactions similar to the natural molecule. Molecules are designed to mimic amino acid residues in alpha-helix or beta-turn conformations on the surface of a protein. Such molecules disrupt certain protein-protein interactions involved in disease or aberrant physiology.

[0089] Peptide mimetics can be designed and produced by techniques known to those of skill in the art. (See *e.g.*, U.S. Pat. Nos. 4,612,132; 5,643, 873 and 5,654,276, the teachings of which are herein incorporated by reference). These mimetics can be based, for example, on a specific B1 GPCR ligand and maintain the relative positions in space of the corresponding ligand. These peptide mimetics possess biological activity (*e.g.*, GPCR inhibiting or stimulating activity) similar to the biological activity of the corresponding peptide compound, but possess a "biological advantage" over the corresponding natural ligand with respect to one or more of the following properties that include, but are not limited to affinity, activity (inhibitory or stimulatory), solubility, pharmacokinetics, stability, and susceptibility to hydrolysis and proteolysis.

[0090] Methods for preparing peptide mimetics include modifying the N- terminal amino group, the C-terminal carboxyl group, and/or changing one or more of the amino linkages in the peptide to a non-amino linkage. Two or more such modifications can be coupled in one peptide mimetic. Modifications of peptides to produce peptide mimetics are described in U.S. Patents 5,643,873 and 5,654,276, the teachings of which are incorporated herein by reference.

[0091] Where the peptide mimetics of present invention comprise amino acids, the test substance can also be cyclic protein, peptides, and cyclic peptide mimetics. Such cyclic test

substances can be produced using known laboratory techniques (*e.g.*, as described in U.S. Patent 5,654,276, the teachings of which are herein incorporated in their entirety by reference).

[0092] The mimetics of the present invention can comprise either the 20 naturally occurring amino acids or other synthetic amino acids or at least one modified or unusual amino acid, including but not limited to those shown on Table 1 below.

TABLE 1 Modified and Unusual Amino Acids			
Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
Baad	3- Aminoadipic acid	Hyl	Hydroxylysine
Bala	β -alanine, β -Amino-propionic acid	Ahyl	Allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Aile	Allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
Baib	3-Aminoisobutyric acid	Melle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		N-methyllleucine
Aph	4-minophenylalanine	Agl (acylated)	Acylated aminoglycine (eqv to betidaminoacids)

[0093] Synthetic amino acids encompassed by the present invention include, for example, naphthylalanine, L-hydroxypropylglycine, L-3,4- dihydroxyphenylalanyl, alpha-amino acids such as L-alpha- hydroxylysyl and D-alpha-methylalanyl, L-alpha-methyl-alanyl, beta amino-acids such as beta-alanine, and isoquinolyl. In other aspects C-alpha-methyl amiono acids, in particular C-alpha-methyl-leucine may be included in an engineered mimetic or library of such compounds.

[0094] D-amino acids and other non-naturally occurring synthetic amino acids can be incorporated into the test substances of the present invention. Such non-naturally occurring synthetic amino acids include those where the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) are replaced with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclic.

[0095] As used herein, "lower alkyl" refers to straight and branched chain alkyl groups having from 1 to 6 carbon atoms, such as methyl, ethyl propyl, butyl and so on. "Lower alkoxy" encompasses straight and branched chain alkoxy groups having from 1 to 6 carbon atoms, such as methoxy, ethoxy and so on.

[0096] Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic. Heterocyclic groups typically contain one or more nitrogen, oxygen, and/or sulphur heteroatoms, including, but not limited to furazanyl, furyl, imidazolidinyl, imidazolyl, imidazolinyl, isothiazolyl, isoxazolyl, morpholinyl (*e.g.*, morpholino), oxazolyl, piperazinyl (*e.g.*, 1-piperazinyl), piperidyl (*e.g.*, 1-piperidyl, piperidino), pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolidinyl (*e.g.*, 1-pyrrolidinyl), pyrrolinyl, pyrrolyl, thiadiazolyl, thiazolyl, thienyl, thiomorpholinyl (*e.g.*, thiomorpholino), and triazolyl. The heterocyclic groups can be substituted or unsubstituted. Where a group is substituted, the substituent can be alkyl, alkoxy, halogen, oxygen, or substituted or unsubstituted phenyl. (See U.S. Patents 5,654, 276 and 5,643,873, the teachings of which are herein incorporated by reference).

[0097] The peptide analogs or mimetics of the invention include isosteres. The term "isostere" as used herein refers to a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes, but is not limited to peptide back-bone modifications (*i.e.*, amide bond mimetics). Such modifications include modifications of the amide nitrogen, the alpha-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. Several peptide backbone modifications are known, including $\phi[\text{CH}_2 \text{ S}]$, $\phi[\text{CH}_2 \text{ NH}]$, $\phi[\text{C(S)NH}_2]$, $\phi[\text{NHCO}]$, $\phi[\text{C(O)CH}_2]$, and $\phi[(\text{E}) \text{ or } (\text{Z}) \text{ CH.dbd.CH}]$. In the nomenclature used above, ϕ indicates the absence of an amide bond.

The structure that replaces the amide group is specified within the brackets. Other examples of isosteres include peptides substituted with one or more benzodiazepine molecules (see *e.g.*, James *et al.* 1993).

[0098] Other possible modifications include an N-alkyl (or aryl) substitution (ϕ [CONR]), backbone crosslinking to construct lactams and other cyclic structures, or retro-inverso amino acid incorporation (ϕ [NHCO]). "Inverso" means replacing L-amino acids of a sequence with D-amino acids, and "retro-inverso" or "enantio-retro" means reversing the sequence of the amino acids ("retro") and replacing the L-amino acids with D-amino acids. For example, if the parent peptide is Thr-Ala-Tyr, the retro modified form is Tyr-Ala-Thr, the inverso form is thr-ala-tyr, and the retro-inverso form is tyr-ala-thr (lower case letters refer to D-amino acids). Compared to the parent peptide, a retro-inverso peptide has a reversed backbone while retaining substantially the original spatial conformation of the side chains, resulting in a retro-inverso isomer with a topology that closely resembles the parent peptide and is able to bind the selected domain. See Goodman *et al.*, 1981). See also U.S. Patent 4,522,752 for further description of "retro-inverso" peptides.

EXAMPLES

[0099] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: MATERIAL AND METHODS

[0100] *Mutagenesis.* The myc-mCRFR2 β , in which a c-myc epitope is inserted between residues 29 and 30, as well as all the point mutants were created by overlap extension PCR using mCRFR2 β as the template. The PCR products were subcloned into pCDNA3 and the sequences were confirmed by automated sequencing.

[0101] *Protein expression.* A cDNA encoding amino acids 39-133 of mouse CRFR2 β was inserted into pET-32a(+) (Novagen) with *Kpn*I and *Xho*I, and its integrity

confirmed by automated sequencing. The sequence of the protein is: GSGMKETAAAKFERQHMDSPDLGT (mCRF- R2 β (39-133) (SEQ ID NO:16), in which an S-tag sequence (used for purification) is underlined and the additional amino acids are part of the thrombin cleavage site and the *KpnI* cloning site. The N-terminal residues are highly flexible as determined by NMR. The protein was expressed in minimal media containing 4 g/l ^{13}C -D-glucose and 1 g/l ^{15}N -ammonium sulfate. Protein purification was carried out as described (See Perrin *et al.*, 2003). Twenty liters of expression media yielded one sample of ^{13}C , ^{15}N -labeled ECD $_1$ -CRF- R2 β with a concentration of ~ 0.2 mM.

[0102] *Radioreceptor assays.* Mutant receptors or myc-mCRFR2 β , were transiently transfected into COSM6 cells followed by binding to crude membrane preparations. Binding to myc-mCRFR2 β was performed in triplicate, as described (See Perrin *et al.*, 2003).

[0103] *NMR Experiments.* All the NMR spectra were recorded at 25°C on Bruker 700 MHz spectrometer equipped with four radio-frequency channels and triple resonance cryo-probe with shielded z-gradient coil. The NMR samples contained 0.2 mM of ^{13}C , ^{15}N -labeled ECD $_1$ -CRFR2 β in 10 mM BisTris(HCl), 95% H $_2$ O/5% D $_2$ O at pH 7.4. Sequential assignment and structure determination was performed with the standard protocol for ^{13}C , ^{15}N -labeled samples. ^1H , ^{13}C and ^{15}N backbone resonances were assigned using the triple resonance experiments HNCA and CBCA(CO)NH and 3D ^{15}N -resolved [^1H , ^1H]-NOESY experiments. The side chain signals were assigned from HCCH-COSY and ^{13}C -resolved [^1H , ^1H]-NOESY experiments. Aromatic side chain assignments were obtained with 2D DQF-COSY, 2D [^1H , ^1H]-NOESY in D $_2$ O and 3D ^1H -TOCSY-relayed ct- ^{13}C , ^1H]-HMQC experiments. Distance constraints for the calculation of the 3D structure were derived from 3D ^{13}C -, ^{15}N -resolved [^1H , ^1H]-NOESY and 2D [^1H , ^1H]-NOESY spectra recorded with a mixing time of 80 ms.

EXAMPLE 2: STRUCTURE DETERMINATION

[0104] 3881 NOEs are observed in the NOESY spectra leading to 1089 meaningful distance restraints and 362 angle restraints (Table 2). These structural restraints were used as an input for the structure calculation with the program CYANA, followed by restrained energy minimization using the program INSIGHT. A total of 100 conformers were initially generated by CYANA and the bundle of 20 conformers with the lowest target function is used to represent the three-dimensional NMR structure. The small residual constraint violations in the 20 refined conformers and the good coincidence of experimental NOEs and short interatomic distances

show that the input data represent a self-consistent set, and that the restraints are well satisfied in the calculated conformers (Table 2). The deviations from ideal geometry are minimal, and similar energy values were obtained for all 20 conformers. The quality of the structures determined is reflected by the small backbone RMSD values relative to the mean coordinates of residues 58-83 and 99-113 of ~ 0.8 Å (see Table 2 and FIG. 1B). The bundle of 20 conformers representing the NMR structure is deposited in the PDB database with accession code 1U34, which is incorporated herein by reference in its entirety. The a set atomic coordinates for a representative conformer is provided in Table 3.

[0105] *Chemical shift perturbation experiments.* [^{15}N , ^1H]-HMQC experiments of 0.05 mM ECD₁-CRFR2 β in 10 mM BisTris(HCl), 95% H₂O/5% D₂O at pH 5 were measured in the absence and presence of an equimolar concentration of either astressin or CRF. Backbone assignment at pH 5 has been achieved following pH-dependent chemical shift changes of the cross-peaks in a series of [^{15}N , ^1H]-HMQC experiments measured at pH 7, 6.5, 5.5 and 5. The assignment was then verified by the measurement of an HNCA experiment at pH 5.

Table 2: Parameters characterizing the NMR structure of ECD₁-CRFR2 β

Number of distance constraints	1089
Number of dihedral angle constraints	362
Average upper limit distance constraint violations	1.88 ± 0.94 Å
Average dihedral angle constraint violations	11.8 ± 10.3
Intra-protein energy after minimization ¹ (kcal mol ⁻¹)	-2092.3 ± 49.8
Coordinate precision (Å) residues 58-83, 99-113	
R.m.s.d ² to the mean for N, C α and C β	0.81 ± 0.20 Å
R.m.s.d to the mean for all the heavy atoms	1.30 ± 0.25 Å
Structural quality – Ramachandran plot ³ (%)	
Percentage in most favored region	54.9 ± 2.57
Percentage in the allowed region	31.9 ± 2.29
Percentage in the additionally allowed region	7.4 ± 1.62
Percentage in the disallowed region ⁴	4.8 ± 1.09

The parameters are given for an ensemble of twenty lowest-energy conformers (out of 100 structures calculated); None of these final structures exhibit NOE-derived violations greater than 0.2 Å or dihedral angle restraint violations greater than 5°. ¹The cyana structures were parameterized with the cff91 force field. The minimizations were conducted in vacuum for 500 steps of conjugate gradient minimization using InsightII. ²R.m.s.d – Root mean square deviation; ³Structure quality was analyzed using PROCHECK; ⁴Most of the angles in the disallowed region are in the disordered region.

Table 3: The atomic coordinates for a representative conformer (without Hydrogens).

ATOM N	1	N	GLY A	15	8.551	-42.265	12.281	1.00	0.00
ATOM C	2	CA	GLY A	15	8.834	-40.964	11.698	1.00	0.00
ATOM C	3	C	GLY A	15	8.059	-39.859	12.420	1.00	0.00
ATOM O	4	O	GLY A	15	7.517	-40.082	13.502	1.00	0.00
ATOM N	5	N	SER A	16	8.032	-38.693	11.792	1.00	0.00
ATOM C	6	CA	SER A	16	7.333	-37.554	12.362	1.00	0.00
ATOM C	7	C	SER A	16	6.933	-36.578	11.253	1.00	0.00
ATOM O	8	O	SER A	16	7.357	-36.726	10.108	1.00	0.00
ATOM C	9	CB	SER A	16	8.196	-36.844	13.406	1.00	0.00
ATOM O	10	OG	SER A	16	7.448	-36.492	14.567	1.00	0.00
ATOM N	11	N	GLY A	17	6.120	-35.602	11.632	1.00	0.00
ATOM C	12	CA	GLY A	17	5.657	-34.603	10.684	1.00	0.00
ATOM C	13	C	GLY A	17	5.802	-33.193	11.260	1.00	0.00
ATOM O	14	O	GLY A	17	4.806	-32.527	11.537	1.00	0.00
ATOM N	15	N	MET A	18	7.051	-32.780	11.422	1.00	0.00
ATOM C	16	CA	MET A	18	7.338	-31.461	11.959	1.00	0.00
ATOM C	17	C	MET A	18	8.820	-31.115	11.800	1.00	0.00
ATOM O	18	O	MET A	18	9.681	-31.765	12.391	1.00	0.00
ATOM C	19	CB	MET A	18	6.963	-31.421	13.443	1.00	0.00
ATOM C	20	CG	MET A	18	6.524	-30.015	13.859	1.00	0.00
ATOM S	21	SD	MET A	18	5.434	-30.108	15.268	1.00	0.00
ATOM C	22	CE	MET A	18	5.104	-28.373	15.520	1.00	0.00
ATOM N	23	N	LYS A	19	9.072	-30.093	10.996	1.00	0.00
ATOM C	24	CA	LYS A	19	10.435	-29.652	10.749	1.00	0.00
ATOM C	25	C	LYS A	19	10.416	-28.460	9.789	1.00	0.00
ATOM O	26	O	LYS A	19	9.350	-28.015	9.368	1.00	0.00
ATOM C	27	CB	LYS A	19	11.296	-30.819	10.264	1.00	0.00
ATOM C	28	CG	LYS A	19	12.505	-31.027	11.178	1.00	0.00

ATOM C	59	CA	ALA	A	24	15.294	-13.408	11.584	1.00	0.00
ATOM C	60	C	ALA	A	24	14.857	-12.010	11.144	1.00	0.00
ATOM O	61	O	ALA	A	24	14.647	-11.767	9.956	1.00	0.00
ATOM C	62	CB	ALA	A	24	16.607	-13.843	10.930	1.00	0.00
ATOM N	63	N	LYS	A	25	14.733	-11.127	12.124	1.00	0.00
ATOM C	64	CA	LYS	A	25	14.325	-9.759	11.851	1.00	0.00
ATOM C	65	C	LYS	A	25	15.365	-8.797	12.427	1.00	0.00
ATOM O	66	O	LYS	A	25	16.392	-9.228	12.951	1.00	0.00
ATOM C	67	CB	LYS	A	25	12.906	-9.512	12.367	1.00	0.00
ATOM C	68	CG	LYS	A	25	11.890	-9.566	11.224	1.00	0.00
ATOM C	69	CD	LYS	A	25	10.713	-10.475	11.581	1.00	0.00
ATOM C	70	CE	LYS	A	25	9.585	-10.339	10.556	1.00	0.00
ATOM N	71	NZ	LYS	A	25	8.430	-11.181	10.940	1.00	0.00
ATOM N	72	N	PHE	A	26	15.065	-7.512	12.310	1.00	0.00
ATOM C	73	CA	PHE	A	26	15.962	-6.485	12.813	1.00	0.00
ATOM C	74	C	PHE	A	26	15.191	-5.419	13.595	1.00	0.00
ATOM O	75	O	PHE	A	26	13.969	-5.329	13.489	1.00	0.00
ATOM C	76	CB	PHE	A	26	16.620	-5.832	11.596	1.00	0.00
ATOM C	77	CG	PHE	A	26	17.341	-6.817	10.675	1.00	0.00
ATOM C	78	CD1	PHE	A	26	16.693	-7.343	9.601	1.00	0.00
ATOM C	79	CD2	PHE	A	26	18.630	-7.168	10.930	1.00	0.00
ATOM C	80	CE1	PHE	A	26	17.362	-8.258	8.746	1.00	0.00
ATOM C	81	CE2	PHE	A	26	19.300	-8.083	10.074	1.00	0.00
ATOM C	82	CZ	PHE	A	26	18.652	-8.608	9.000	1.00	0.00
ATOM N	83	N	GLU	A	27	15.938	-4.639	14.362	1.00	0.00
ATOM C	84	CA	GLU	A	27	15.340	-3.582	15.161	1.00	0.00
ATOM C	85	C	GLU	A	27	14.447	-2.697	14.288	1.00	0.00
ATOM O	86	O	GLU	A	27	14.856	-2.268	13.211	1.00	0.00
ATOM C	87	CB	GLU	A	27	16.415	-2.752	15.865	1.00	0.00
ATOM C	88	CG	GLU	A	27	16.599	-3.208	17.313	1.00	0.00

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ATOM C	89	CD	GLU	A	27	17.302	-2.131	18.143	1.00	0.00
ATOM O	90	OE1	GLU	A	27	16.852	-0.969	18.063	1.00	0.00
ATOM O	91	OE2	GLU	A	27	18.275	-2.496	18.838	1.00	0.00
ATOM N	92	N	ARG	A	28	13.246	-2.449	14.788	1.00	0.00
ATOM C	93	CA	ARG	A	28	12.292	-1.622	14.067	1.00	0.00
ATOM C	94	C	ARG	A	28	11.089	-1.302	14.957	1.00	0.00
ATOM O	95	O	ARG	A	28	10.303	-2.190	15.286	1.00	0.00
ATOM C	96	CB	ARG	A	28	11.807	-2.322	12.798	1.00	0.00
ATOM C	97	CG	ARG	A	28	12.279	-1.578	11.547	1.00	0.00
ATOM C	98	CD	ARG	A	28	13.298	-2.411	10.766	1.00	0.00
ATOM N	99	NE	ARG	A	28	12.615	-3.176	9.700	1.00	0.00
ATOM C	100	CZ	ARG	A	28	13.248	-3.940	8.799	1.00	0.00
ATOM N	101	NH1	ARG	A	28	14.584	-4.045	8.831	1.00	0.00
ATOM N	102	NH2	ARG	A	28	12.547	-4.600	7.867	1.00	0.00
ATOM N	103	N	GLN	A	29	10.983	-0.033	15.320	1.00	0.00
ATOM C	104	CA	GLN	A	29	9.889	0.415	16.166	1.00	0.00
ATOM C	105	C	GLN	A	29	9.025	1.434	15.420	1.00	0.00
ATOM O	106	O	GLN	A	29	7.835	1.205	15.203	1.00	0.00
ATOM C	107	CB	GLN	A	29	10.413	0.998	17.479	1.00	0.00
ATOM C	108	CG	GLN	A	29	9.639	0.439	18.674	1.00	0.00
ATOM C	109	CD	GLN	A	29	10.260	0.901	19.994	1.00	0.00
ATOM N	110	NE2	GLN	A	29	9.979	2.161	20.313	1.00	0.00
ATOM O	111	OE1	GLN	A	29	10.950	0.163	20.678	1.00	0.00
ATOM N	112	N	HIS	A	30	9.657	2.538	15.046	1.00	0.00
ATOM C	113	CA	HIS	A	30	8.961	3.592	14.329	1.00	0.00
ATOM C	114	C	HIS	A	30	9.951	4.695	13.950	1.00	0.00
ATOM O	115	O	HIS	A	30	10.992	4.845	14.588	1.00	0.00
ATOM C	116	CB	HIS	A	30	7.777	4.114	15.144	1.00	0.00
ATOM C	117	CG	HIS	A	30	6.641	4.647	14.306	1.00	0.00
ATOM C	118	CD2	HIS	A	30	6.515	5.817	13.615	1.00	0.00

ATOM N	119	ND1	HIS	A	30	5.461	3.949	14.109	1.00	0.00
ATOM C	120	CE1	HIS	A	30	4.670	4.675	13.333	1.00	0.00
ATOM N	121	NE2	HIS	A	30	5.325	5.831	13.028	1.00	0.00
ATOM N	122	N	MET	A	31	9.593	5.438	12.914	1.00	0.00
ATOM C	123	CA	MET	A	31	10.437	6.523	12.442	1.00	0.00
ATOM C	124	C	MET	A	31	10.041	7.849	13.095	1.00	0.00
ATOM O	125	O	MET	A	31	10.107	8.901	12.462	1.00	0.00
ATOM C	126	CB	MET	A	31	10.312	6.645	10.923	1.00	0.00
ATOM C	127	CG	MET	A	31	8.979	7.287	10.532	1.00	0.00
ATOM S	128	SD	MET	A	31	8.180	6.314	9.268	1.00	0.00
ATOM C	129	CE	MET	A	31	6.655	7.226	9.093	1.00	0.00
ATOM N	130	N	ASP	A	32	9.638	7.756	14.354	1.00	0.00
ATOM C	131	CA	ASP	A	32	9.233	8.935	15.100	1.00	0.00
ATOM C	132	C	ASP	A	32	8.026	9.577	14.411	1.00	0.00
ATOM O	133	O	ASP	A	32	7.908	9.533	13.188	1.00	0.00
ATOM C	134	CB	ASP	A	32	10.356	9.970	15.147	1.00	0.00
ATOM C	135	CG	ASP	A	32	10.027	11.247	15.923	1.00	0.00
ATOM O	136	OD1	ASP	A	32	9.600	11.108	17.089	1.00	0.00
ATOM O	137	OD2	ASP	A	32	10.210	12.334	15.333	1.00	0.00
ATOM N	138	N	SER	A	33	7.159	10.158	15.228	1.00	0.00
ATOM C	139	CA	SER	A	33	5.966	10.808	14.714	1.00	0.00
ATOM C	140	C	SER	A	33	5.002	11.116	15.861	1.00	0.00
ATOM O	141	O	SER	A	33	5.007	10.431	16.881	1.00	0.00
ATOM C	142	CB	SER	A	33	5.277	9.940	13.660	1.00	0.00
ATOM O	143	OG	SER	A	33	5.713	10.254	12.341	1.00	0.00
ATOM N	144	N	PRO	A	34	4.177	12.177	15.650	1.00	0.00
ATOM C	145	CA	PRO	A	34	3.209	12.584	16.654	1.00	0.00
ATOM C	146	C	PRO	A	34	2.020	11.622	16.692	1.00	0.00
ATOM O	147	O	PRO	A	34	2.107	10.502	16.190	1.00	0.00
ATOM C	148	CB	PRO	A	34	2.816	14.001	16.269	1.00	0.00

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ATOM C	149	CG	PRO	A	34	3.220	14.165	14.812	1.00	0.00
ATOM C	150	CD	PRO	A	34	4.143	13.012	14.453	1.00	0.00
ATOM N	151	N	ASP	A	35	0.937	12.095	17.291	1.00	0.00
ATOM C	152	CA	ASP	A	35	-0.268	11.290	17.400	1.00	0.00
ATOM C	153	C	ASP	A	35	-1.330	11.837	16.444	1.00	0.00
ATOM O	154	O	ASP	A	35	-1.391	13.041	16.202	1.00	0.00
ATOM C	155	CB	ASP	A	35	-0.838	11.343	18.819	1.00	0.00
ATOM C	156	CG	ASP	A	35	-2.185	10.640	19.002	1.00	0.00
ATOM O	157	OD1	ASP	A	35	-2.203	9.632	19.741	1.00	0.00
ATOM O	158	OD2	ASP	A	35	-3.165	11.127	18.398	1.00	0.00
ATOM N	159	N	LEU	A	36	-2.140	10.926	15.927	1.00	0.00
ATOM C	160	CA	LEU	A	36	-3.196	11.301	15.002	1.00	0.00
ATOM C	161	C	LEU	A	36	-2.615	11.414	13.590	1.00	0.00
ATOM O	162	O	LEU	A	36	-3.138	10.817	12.650	1.00	0.00
ATOM C	163	CB	LEU	A	36	-3.901	12.572	15.481	1.00	0.00
ATOM C	164	CG	LEU	A	36	-5.429	12.548	15.435	1.00	0.00
ATOM C	165	CD1	LEU	A	36	-6.022	13.622	16.351	1.00	0.00
ATOM C	166	CD2	LEU	A	36	-5.937	12.678	13.998	1.00	0.00
ATOM N	167	N	GLY	A	37	-1.543	12.186	13.486	1.00	0.00
ATOM C	168	CA	GLY	A	37	-0.886	12.384	12.206	1.00	0.00
ATOM C	169	C	GLY	A	37	-0.860	13.867	11.828	1.00	0.00
ATOM O	170	O	GLY	A	37	-1.889	14.540	11.866	1.00	0.00
ATOM N	171	N	THR	A	38	0.328	14.333	11.472	1.00	0.00
ATOM C	172	CA	THR	A	38	0.503	15.724	11.088	1.00	0.00
ATOM C	173	C	THR	A	38	0.080	15.932	9.633	1.00	0.00
ATOM O	174	O	THR	A	38	0.192	17.035	9.101	1.00	0.00
ATOM C	175	CB	THR	A	38	1.958	16.110	11.359	1.00	0.00
ATOM C	176	CG2	THR	A	38	2.925	15.498	10.344	1.00	0.00
ATOM O	177	OG1	THR	A	38	2.003	17.507	11.083	1.00	0.00
ATOM N	178	N	THR	A	39	-0.399	14.853	9.029	1.00	0.00

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ATOM C	179	CA	THR	A	39	-0.839	14.905	7.645	1.00	0.00
ATOM C	180	C	THR	A	39	-1.474	13.573	7.239	1.00	0.00
ATOM O	181	O	THR	A	39	-0.852	12.520	7.364	1.00	0.00
ATOM C	182	CB	THR	A	39	0.362	15.293	6.780	1.00	0.00
ATOM C	183	CG2	THR	A	39	1.397	14.171	6.679	1.00	0.00
ATOM O	184	OG1	THR	A	39	-0.175	15.391	5.464	1.00	0.00
ATOM N	185	N	LEU	A	40	-2.707	13.665	6.762	1.00	0.00
ATOM C	186	CA	LEU	A	40	-3.434	12.480	6.336	1.00	0.00
ATOM C	187	C	LEU	A	40	-2.798	11.927	5.059	1.00	0.00
ATOM O	188	O	LEU	A	40	-2.238	12.681	4.265	1.00	0.00
ATOM C	189	CB	LEU	A	40	-4.926	12.789	6.196	1.00	0.00
ATOM C	190	CG	LEU	A	40	-5.318	13.680	5.015	1.00	0.00
ATOM C	191	CD1	LEU	A	40	-6.657	13.242	4.419	1.00	0.00
ATOM C	192	CD2	LEU	A	40	-5.326	15.155	5.420	1.00	0.00
ATOM N	193	N	LEU	A	41	-2.905	10.616	4.903	1.00	0.00
ATOM C	194	CA	LEU	A	41	-2.347	9.954	3.737	1.00	0.00
ATOM C	195	C	LEU	A	41	-3.380	9.960	2.608	1.00	0.00
ATOM O	196	O	LEU	A	41	-3.144	9.395	1.542	1.00	0.00
ATOM C	197	CB	LEU	A	41	-1.845	8.555	4.103	1.00	0.00
ATOM C	198	CG	LEU	A	41	-1.060	8.446	5.412	1.00	0.00
ATOM C	199	CD1	LEU	A	41	-1.145	7.030	5.985	1.00	0.00
ATOM C	200	CD2	LEU	A	41	0.388	8.901	5.222	1.00	0.00
ATOM N	201	N	GLU	A	42	-4.504	10.606	2.882	1.00	0.00
ATOM C	202	CA	GLU	A	42	-5.575	10.693	1.905	1.00	0.00
ATOM C	203	C	GLU	A	42	-5.884	12.158	1.585	1.00	0.00
ATOM O	204	O	GLU	A	42	-7.034	12.512	1.331	1.00	0.00
ATOM C	205	CB	GLU	A	42	-6.826	9.964	2.394	1.00	0.00
ATOM C	206	CG	GLU	A	42	-7.849	9.810	1.266	1.00	0.00
ATOM C	207	CD	GLU	A	42	-7.176	9.335	-0.024	1.00	0.00
ATOM O	208	OE1	GLU	A	42	-6.762	10.217	-0.806	1.00	0.00

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ATOM O	209	OE2	GLU	A	42	-7.092	8.100	-0.197	1.00	0.00
ATOM N	210	N	GLN	A	43	-4.837	12.969	1.607	1.00	0.00
ATOM C	211	CA	GLN	A	43	-4.981	14.386	1.324	1.00	0.00
ATOM C	212	C	GLN	A	43	-5.997	14.601	0.200	1.00	0.00
ATOM O	213	O	GLN	A	43	-6.846	15.486	0.287	1.00	0.00
ATOM C	214	CB	GLN	A	43	-3.632	15.016	0.971	1.00	0.00
ATOM C	215	CG	GLN	A	43	-2.746	14.026	0.213	1.00	0.00
ATOM C	216	CD	GLN	A	43	-1.607	13.518	1.099	1.00	0.00
ATOM N	217	NE2	GLN	A	43	-1.575	12.195	1.233	1.00	0.00
ATOM O	218	OE1	GLN	A	43	-0.810	14.276	1.625	1.00	0.00
ATOM N	219	N	TYR	A	44	-5.874	13.776	-0.831	1.00	0.00
ATOM C	220	CA	TYR	A	44	-6.771	13.865	-1.971	1.00	0.00
ATOM C	221	C	TYR	A	44	-6.482	12.756	-2.983	1.00	0.00
ATOM O	222	O	TYR	A	44	-7.401	12.207	-3.587	1.00	0.00
ATOM C	223	CB	TYR	A	44	-6.491	15.220	-2.623	1.00	0.00
ATOM C	224	CG	TYR	A	44	-5.214	15.253	-3.465	1.00	0.00
ATOM C	225	CD1	TYR	A	44	-5.296	15.388	-4.838	1.00	0.00
ATOM C	226	CD2	TYR	A	44	-3.981	15.149	-2.855	1.00	0.00
ATOM C	227	CE1	TYR	A	44	-4.095	15.418	-5.630	1.00	0.00
ATOM C	228	CE2	TYR	A	44	-2.779	15.179	-3.648	1.00	0.00
ATOM C	229	CZ	TYR	A	44	-2.896	15.313	-4.997	1.00	0.00
ATOM O	230	OH	TYR	A	44	-1.761	15.342	-5.746	1.00	0.00
ATOM N	231	N	CYS	A	45	-5.200	12.459	-3.137	1.00	0.00
ATOM C	232	CA	CYS	A	45	-4.777	11.425	-4.067	1.00	0.00
ATOM C	233	C	CYS	A	45	-5.206	11.841	-5.476	1.00	0.00
ATOM O	234	O	CYS	A	45	-5.213	13.026	-5.801	1.00	0.00
ATOM C	235	CB	CYS	A	45	-5.338	10.054	-3.681	1.00	0.00
ATOM S	236	SG	CYS	A	45	-4.293	8.634	-4.166	1.00	0.00
ATOM N	237	N	HIS	A	46	-5.554	10.842	-6.273	1.00	0.00
ATOM C	238	CA	HIS	A	46	-5.983	11.088	-7.639	1.00	0.00

ATOM C	239	C	HIS	A	46	-7.400	11.667	-7.635	1.00	0.00
ATOM O	240	O	HIS	A	46	-7.897	12.093	-6.594	1.00	0.00
ATOM C	241	CB	HIS	A	46	-5.863	9.818	-8.484	1.00	0.00
ATOM C	242	CG	HIS	A	46	-4.996	9.976	-9.710	1.00	0.00
ATOM C	243	CD2	HIS	A	46	-5.189	9.568	-10.997	1.00	0.00
ATOM N	244	ND1	HIS	A	46	-3.770	10.617	-9.683	1.00	0.00
ATOM C	245	CE1	HIS	A	46	-3.257	10.591	-10.905	1.00	0.00
ATOM N	246	NE2	HIS	A	46	-4.139	9.941	-11.718	1.00	0.00
ATOM N	247	N	ARG	A	47	-8.008	11.664	-8.813	1.00	0.00
ATOM C	248	CA	ARG	A	47	-9.357	12.184	-8.958	1.00	0.00
ATOM C	249	C	ARG	A	47	-10.315	11.444	-8.022	1.00	0.00
ATOM O	250	O	ARG	A	47	-11.240	12.042	-7.475	1.00	0.00
ATOM C	251	CB	ARG	A	47	-9.850	12.040	-10.399	1.00	0.00
ATOM C	252	CG	ARG	A	47	-9.404	10.706	-11.001	1.00	0.00
ATOM C	253	CD	ARG	A	47	-8.233	10.904	-11.966	1.00	0.00
ATOM N	254	NE	ARG	A	47	-8.735	11.349	-13.285	1.00	0.00
ATOM C	255	CZ	ARG	A	47	-7.946	11.652	-14.325	1.00	0.00
ATOM N	256	NH1	ARG	A	47	-6.615	11.561	-14.206	1.00	0.00
ATOM N	257	NH2	ARG	A	47	-8.489	12.047	-15.485	1.00	0.00
ATOM N	258	N	THR	A	48	-10.060	10.153	-7.868	1.00	0.00
ATOM C	259	CA	THR	A	48	-10.889	9.325	-7.008	1.00	0.00
ATOM C	260	C	THR	A	48	-12.337	9.819	-7.025	1.00	0.00
ATOM O	261	O	THR	A	48	-12.720	10.655	-6.209	1.00	0.00
ATOM C	262	CB	THR	A	48	-10.264	9.320	-5.612	1.00	0.00
ATOM C	263	CG2	THR	A	48	-10.379	10.678	-4.914	1.00	0.00
ATOM O	264	OG1	THR	A	48	-11.116	8.467	-4.853	1.00	0.00
ATOM N	265	N	THR	A	49	-13.102	9.281	-7.962	1.00	0.00
ATOM C	266	CA	THR	A	49	-14.498	9.657	-8.097	1.00	0.00
ATOM C	267	C	THR	A	49	-15.356	8.427	-8.399	1.00	0.00
ATOM O	268	O	THR	A	49	-15.596	8.103	-9.562	1.00	0.00

ATOM C	269	CB	THR	A	49	-14.596	10.744	-9.169	1.00	0.00
ATOM C	270	CG2	THR	A	49	-14.440	10.185	-10.585	1.00	0.00
ATOM O	271	OG1	THR	A	49	-15.954	11.169	-9.110	1.00	0.00
ATOM N	272	N	ILE	A	50	-15.795	7.773	-7.334	1.00	0.00
ATOM C	273	CA	ILE	A	50	-16.620	6.585	-7.472	1.00	0.00
ATOM C	274	C	ILE	A	50	-17.143	6.171	-6.095	1.00	0.00
ATOM O	275	O	ILE	A	50	-16.620	6.606	-5.070	1.00	0.00
ATOM C	276	CB	ILE	A	50	-15.849	5.479	-8.195	1.00	0.00
ATOM C	277	CG1	ILE	A	50	-16.798	4.388	-8.696	1.00	0.00
ATOM C	278	CG2	ILE	A	50	-14.741	4.910	-7.306	1.00	0.00
ATOM C	279	CD1	ILE	A	50	-16.086	3.447	-9.670	1.00	0.00
ATOM N	280	N	GLY	A	51	-18.170	5.334	-6.115	1.00	0.00
ATOM C	281	CA	GLY	A	51	-18.770	4.857	-4.881	1.00	0.00
ATOM C	282	C	GLY	A	51	-20.217	5.340	-4.751	1.00	0.00
ATOM O	283	O	GLY	A	51	-20.565	6.013	-3.783	1.00	0.00
ATOM N	284	N	ASN	A	52	-21.019	4.978	-5.742	1.00	0.00
ATOM C	285	CA	ASN	A	52	-22.419	5.367	-5.751	1.00	0.00
ATOM C	286	C	ASN	A	52	-22.523	6.878	-5.962	1.00	0.00
ATOM O	287	O	ASN	A	52	-23.173	7.574	-5.183	1.00	0.00
ATOM C	288	CB	ASN	A	52	-23.093	5.027	-4.420	1.00	0.00
ATOM C	289	CG	ASN	A	52	-24.600	4.838	-4.601	1.00	0.00
ATOM N	290	ND2	ASN	A	52	-25.340	5.750	-3.977	1.00	0.00
ATOM O	291	OD1	ASN	A	52	-25.061	3.922	-5.265	1.00	0.00
ATOM N	292	N	PHE	A	53	-21.872	7.343	-7.018	1.00	0.00
ATOM C	293	CA	PHE	A	53	-21.882	8.760	-7.341	1.00	0.00
ATOM C	294	C	PHE	A	53	-21.315	9.007	-8.740	1.00	0.00
ATOM O	295	O	PHE	A	53	-21.839	9.832	-9.489	1.00	0.00
ATOM C	296	CB	PHE	A	53	-20.992	9.459	-6.312	1.00	0.00
ATOM C	297	CG	PHE	A	53	-21.229	10.967	-6.209	1.00	0.00
ATOM C	298	CD1	PHE	A	53	-20.764	11.797	-7.181	1.00	0.00

ATOM C	299	CD2	PHE	A	53	-21.903	11.479	-5.144	1.00	0.00
ATOM C	300	CE1	PHE	A	53	-20.983	13.196	-7.085	1.00	0.00
ATOM C	301	CE2	PHE	A	53	-22.122	12.879	-5.048	1.00	0.00
ATOM C	302	CZ	PHE	A	53	-21.658	13.708	-6.021	1.00	0.00
ATOM N	303	N	SER	A	54	-20.255	8.277	-9.052	1.00	0.00
ATOM C	304	CA	SER	A	54	-19.611	8.408	-10.348	1.00	0.00
ATOM C	305	C	SER	A	54	-18.854	7.123	-10.688	1.00	0.00
ATOM O	306	O	SER	A	54	-17.629	7.074	-10.585	1.00	0.00
ATOM C	307	CB	SER	A	54	-18.661	9.607	-10.373	1.00	0.00
ATOM O	308	OG	SER	A	54	-19.173	10.677	-11.164	1.00	0.00
ATOM N	309	N	GLY	A	55	-19.614	6.113	-11.085	1.00	0.00
ATOM C	310	CA	GLY	A	55	-19.029	4.830	-11.440	1.00	0.00
ATOM C	311	C	GLY	A	55	-19.156	4.568	-12.942	1.00	0.00
ATOM O	312	O	GLY	A	55	-20.208	4.138	-13.413	1.00	0.00
ATOM N	313	N	PRO	A	56	-18.041	4.843	-13.671	1.00	0.00
ATOM C	314	CA	PRO	A	56	-18.019	4.641	-15.109	1.00	0.00
ATOM C	315	C	PRO	A	56	-17.917	3.154	-15.452	1.00	0.00
ATOM O	316	O	PRO	A	56	-18.851	2.574	-16.002	1.00	0.00
ATOM C	317	CB	PRO	A	56	-16.828	5.447	-15.601	1.00	0.00
ATOM C	318	CG	PRO	A	56	-15.957	5.691	-14.379	1.00	0.00
ATOM C	319	CD	PRO	A	56	-16.778	5.354	-13.146	1.00	0.00
ATOM N	320	N	TYR	A	57	-16.772	2.578	-15.112	1.00	0.00
ATOM C	321	CA	TYR	A	57	-16.536	1.169	-15.377	1.00	0.00
ATOM C	322	C	TYR	A	57	-16.228	0.413	-14.084	1.00	0.00
ATOM O	323	O	TYR	A	57	-16.388	0.952	-12.991	1.00	0.00
ATOM C	324	CB	TYR	A	57	-15.311	1.111	-16.292	1.00	0.00
ATOM C	325	CG	TYR	A	57	-14.486	2.400	-16.308	1.00	0.00
ATOM C	326	CD1	TYR	A	57	-13.798	2.794	-15.178	1.00	0.00
ATOM C	327	CD2	TYR	A	57	-14.429	3.168	-17.453	1.00	0.00
ATOM C	328	CE1	TYR	A	57	-13.022	4.006	-15.193	1.00	0.00

ATOM C	329	CE2	TYR	A	57	-13.652	4.381	-17.469	1.00	0.00
ATOM C	330	CZ	TYR	A	57	-12.987	4.739	-16.337	1.00	0.00
ATOM O	331	OH	TYR	A	57	-12.253	5.886	-16.353	1.00	0.00
ATOM N	332	N	THR	A	58	-15.791	-0.828	-14.251	1.00	0.00
ATOM C	333	CA	THR	A	58	-15.459	-1.664	-13.110	1.00	0.00
ATOM C	334	C	THR	A	58	-13.945	-1.689	-12.890	1.00	0.00
ATOM O	335	O	THR	A	58	-13.392	-2.704	-12.472	1.00	0.00
ATOM C	336	CB	THR	A	58	-16.061	-3.050	-13.350	1.00	0.00
ATOM C	337	CG2	THR	A	58	-16.031	-3.926	-12.095	1.00	0.00
ATOM O	338	OG1	THR	A	58	-17.446	-2.795	-13.563	1.00	0.00
ATOM N	339	N	TYR	A	59	-13.318	-0.558	-13.179	1.00	0.00
ATOM C	340	CA	TYR	A	59	-11.880	-0.437	-13.018	1.00	0.00
ATOM C	341	C	TYR	A	59	-11.511	-0.207	-11.551	1.00	0.00
ATOM O	342	O	TYR	A	59	-12.378	0.075	-10.726	1.00	0.00
ATOM C	343	CB	TYR	A	59	-11.466	0.789	-13.833	1.00	0.00
ATOM C	344	CG	TYR	A	59	-11.632	2.116	-13.091	1.00	0.00
ATOM C	345	CD1	TYR	A	59	-12.688	2.292	-12.219	1.00	0.00
ATOM C	346	CD2	TYR	A	59	-10.726	3.138	-13.291	1.00	0.00
ATOM C	347	CE1	TYR	A	59	-12.844	3.541	-11.520	1.00	0.00
ATOM C	348	CE2	TYR	A	59	-10.882	4.387	-12.591	1.00	0.00
ATOM C	349	CZ	TYR	A	59	-11.934	4.526	-11.740	1.00	0.00
ATOM O	350	OH	TYR	A	59	-12.082	5.706	-11.080	1.00	0.00
ATOM N	351	N	CYS	A	60	-10.222	-0.338	-11.272	1.00	0.00
ATOM C	352	CA	CYS	A	60	-9.727	-0.148	-9.918	1.00	0.00
ATOM C	353	C	CYS	A	60	-9.928	1.320	-9.535	1.00	0.00
ATOM O	354	O	CYS	A	60	-9.875	2.202	-10.390	1.00	0.00
ATOM C	355	CB	CYS	A	60	-8.264	-0.577	-9.786	1.00	0.00
ATOM S	356	SG	CYS	A	60	-7.976	-2.377	-9.947	1.00	0.00
ATOM N	357	N	ASN	A	61	-10.154	1.535	-8.247	1.00	0.00
ATOM C	358	CA	ASN	A	61	-10.362	2.881	-7.740	1.00	0.00

ATOM C	449	CD	PRO	A	72	-1.751	5.817	-8.189	1.00	0.00
ATOM N	450	N	GLN	A	73	-6.198	5.284	-9.327	1.00	0.00
ATOM C	451	CA	GLN	A	73	-7.048	4.309	-9.988	1.00	0.00
ATOM C	452	C	GLN	A	73	-6.296	3.648	-11.146	1.00	0.00
ATOM O	453	O	GLN	A	73	-5.414	4.258	-11.746	1.00	0.00
ATOM C	454	CB	GLN	A	73	-8.347	4.954	-10.475	1.00	0.00
ATOM C	455	CG	GLN	A	73	-8.991	5.792	-9.369	1.00	0.00
ATOM C	456	CD	GLN	A	73	-9.009	7.275	-9.743	1.00	0.00
ATOM N	457	NE2	GLN	A	73	-7.908	7.688	-10.367	1.00	0.00
ATOM O	458	OE1	GLN	A	73	-9.958	7.996	-9.484	1.00	0.00
ATOM N	459	N	SER	A	74	-6.674	2.409	-11.423	1.00	0.00
ATOM C	460	CA	SER	A	74	-6.047	1.658	-12.499	1.00	0.00
ATOM C	461	C	SER	A	74	-7.090	0.795	-13.212	1.00	0.00
ATOM O	462	O	SER	A	74	-8.286	0.921	-12.954	1.00	0.00
ATOM C	463	CB	SER	A	74	-4.908	0.786	-11.969	1.00	0.00
ATOM O	464	OG	SER	A	74	-4.122	1.466	-10.994	1.00	0.00
ATOM N	465	N	ALA	A	75	-6.599	-0.062	-14.094	1.00	0.00
ATOM C	466	CA	ALA	A	75	-7.472	-0.946	-14.846	1.00	0.00
ATOM C	467	C	ALA	A	75	-7.455	-2.337	-14.210	1.00	0.00
ATOM O	468	O	ALA	A	75	-6.477	-2.717	-13.568	1.00	0.00
ATOM C	469	CB	ALA	A	75	-7.036	-0.970	-16.312	1.00	0.00
ATOM N	470	N	PRO	A	76	-8.577	-3.077	-14.415	1.00	0.00
ATOM C	471	CA	PRO	A	76	-8.699	-4.418	-13.868	1.00	0.00
ATOM C	472	C	PRO	A	76	-7.855	-5.414	-14.666	1.00	0.00
ATOM O	473	O	PRO	A	76	-7.684	-5.262	-15.874	1.00	0.00
ATOM C	474	CB	PRO	A	76	-10.186	-4.728	-13.916	1.00	0.00
ATOM C	475	CG	PRO	A	76	-10.785	-3.745	-14.910	1.00	0.00
ATOM C	476	CD	PRO	A	76	-9.754	-2.659	-15.170	1.00	0.00
ATOM N	477	N	GLY	A	77	-7.348	-6.411	-13.955	1.00	0.00
ATOM C	478	CA	GLY	A	77	-6.525	-7.432	-14.581	1.00	0.00

ATOM C	479	C	GLY	A	77	-5.136	-6.886	-14.919	1.00	0.00
ATOM O	480	O	GLY	A	77	-4.334	-7.571	-15.551	1.00	0.00
ATOM N	481	N	ALA	A	78	-4.896	-5.658	-14.484	1.00	0.00
ATOM C	482	CA	ALA	A	78	-3.619	-5.012	-14.733	1.00	0.00
ATOM C	483	C	ALA	A	78	-2.701	-5.226	-13.528	1.00	0.00
ATOM O	484	O	ALA	A	78	-2.894	-6.162	-12.754	1.00	0.00
ATOM C	485	CB	ALA	A	78	-3.846	-3.530	-15.037	1.00	0.00
ATOM N	486	N	LEU	A	79	-1.721	-4.342	-13.406	1.00	0.00
ATOM C	487	CA	LEU	A	79	-0.773	-4.422	-12.308	1.00	0.00
ATOM C	488	C	LEU	A	79	0.157	-3.208	-12.354	1.00	0.00
ATOM O	489	O	LEU	A	79	0.683	-2.864	-13.411	1.00	0.00
ATOM C	490	CB	LEU	A	79	-0.036	-5.763	-12.334	1.00	0.00
ATOM C	491	CG	LEU	A	79	0.413	-6.254	-13.711	1.00	0.00
ATOM C	492	CD1	LEU	A	79	1.884	-5.914	-13.962	1.00	0.00
ATOM C	493	CD2	LEU	A	79	0.134	-7.750	-13.877	1.00	0.00
ATOM N	494	N	VAL	A	80	0.331	-2.592	-11.193	1.00	0.00
ATOM C	495	CA	VAL	A	80	1.189	-1.424	-11.088	1.00	0.00
ATOM C	496	C	VAL	A	80	2.517	-1.828	-10.445	1.00	0.00
ATOM O	497	O	VAL	A	80	2.883	-1.311	-9.390	1.00	0.00
ATOM C	498	CB	VAL	A	80	0.467	-0.313	-10.321	1.00	0.00
ATOM C	499	CG1	VAL	A	80	1.443	0.789	-9.905	1.00	0.00
ATOM C	500	CG2	VAL	A	80	-0.688	0.259	-11.145	1.00	0.00
ATOM N	501	N	GLU	A	81	3.203	-2.749	-11.108	1.00	0.00
ATOM C	502	CA	GLU	A	81	4.482	-3.228	-10.614	1.00	0.00
ATOM C	503	C	GLU	A	81	5.620	-2.366	-11.164	1.00	0.00
ATOM O	504	O	GLU	A	81	5.569	-1.922	-12.310	1.00	0.00
ATOM C	505	CB	GLU	A	81	4.689	-4.702	-10.969	1.00	0.00
ATOM C	506	CG	GLU	A	81	4.966	-4.872	-12.464	1.00	0.00
ATOM C	507	CD	GLU	A	81	6.412	-5.310	-12.708	1.00	0.00
ATOM O	508	OE1	GLU	A	81	7.052	-4.691	-13.586	1.00	0.00

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ATOM O	509	OE2	GLU	A	81	6.844	-6.254	-12.012	1.00	0.00
ATOM N	510	N	ARG	A	82	6.620	-2.154	-10.321	1.00	0.00
ATOM C	511	CA	ARG	A	82	7.769	-1.354	-10.709	1.00	0.00
ATOM C	512	C	ARG	A	82	9.013	-1.800	-9.937	1.00	0.00
ATOM O	513	O	ARG	A	82	9.572	-1.032	-9.156	1.00	0.00
ATOM C	514	CB	ARG	A	82	7.518	0.132	-10.443	1.00	0.00
ATOM C	515	CG	ARG	A	82	6.221	0.597	-11.107	1.00	0.00
ATOM C	516	CD	ARG	A	82	6.147	2.124	-11.157	1.00	0.00
ATOM N	517	NE	ARG	A	82	5.340	2.631	-10.024	1.00	0.00
ATOM C	518	CZ	ARG	A	82	5.374	3.895	-9.584	1.00	0.00
ATOM N	519	NH1	ARG	A	82	6.174	4.791	-10.178	1.00	0.00
ATOM N	520	NH2	ARG	A	82	4.608	4.264	-8.548	1.00	0.00
ATOM N	521	N	PRO	A	83	9.419	-3.072	-10.190	1.00	0.00
ATOM C	522	CA	PRO	A	83	10.587	-3.630	-9.528	1.00	0.00
ATOM C	523	C	PRO	A	83	11.877	-3.062	-10.122	1.00	0.00
ATOM O	524	O	PRO	A	83	12.306	-3.478	-11.198	1.00	0.00
ATOM C	525	CB	PRO	A	83	10.460	-5.134	-9.710	1.00	0.00
ATOM C	526	CG	PRO	A	83	9.483	-5.335	-10.858	1.00	0.00
ATOM C	527	CD	PRO	A	83	8.782	-4.011	-11.109	1.00	0.00
ATOM N	528	N	CYS	A	84	12.461	-2.121	-9.396	1.00	0.00
ATOM C	529	CA	CYS	A	84	13.695	-1.492	-9.837	1.00	0.00
ATOM C	530	C	CYS	A	84	13.401	-0.706	-11.116	1.00	0.00
ATOM O	531	O	CYS	A	84	12.423	-0.982	-11.809	1.00	0.00
ATOM C	532	CB	CYS	A	84	14.811	-2.518	-10.042	1.00	0.00
ATOM S	533	SG	CYS	A	84	14.854	-3.864	-8.802	1.00	0.00
ATOM N	534	N	PRO	A	85	14.289	0.286	-11.398	1.00	0.00
ATOM C	535	CA	PRO	A	85	14.136	1.114	-12.581	1.00	0.00
ATOM C	536	C	PRO	A	85	14.537	0.348	-13.843	1.00	0.00
ATOM O	537	O	PRO	A	85	13.725	0.167	-14.749	1.00	0.00
ATOM C	538	CB	PRO	A	85	15.006	2.333	-12.324	1.00	0.00

ATOM C	569	CD2	PHE	A	88	20.011	-0.771	-12.965	1.00	0.00
ATOM C	570	CE1	PHE	A	88	21.550	-1.514	-10.810	1.00	0.00
ATOM C	571	CE2	PHE	A	88	19.599	-0.484	-11.637	1.00	0.00
ATOM C	572	CZ	PHE	A	88	20.378	-0.862	-10.588	1.00	0.00
ATOM N	573	N	ASN	A	89	21.793	-4.344	-13.092	1.00	0.00
ATOM C	574	CA	ASN	A	89	22.526	-5.294	-12.270	1.00	0.00
ATOM C	575	C	ASN	A	89	23.968	-4.812	-12.104	1.00	0.00
ATOM O	576	O	ASN	A	89	24.867	-5.278	-12.801	1.00	0.00
ATOM C	577	CB	ASN	A	89	22.563	-6.676	-12.928	1.00	0.00
ATOM C	578	CG	ASN	A	89	21.166	-7.104	-13.383	1.00	0.00
ATOM N	579	ND2	ASN	A	89	20.214	-6.891	-12.479	1.00	0.00
ATOM O	580	OD1	ASN	A	89	20.968	-7.593	-14.483	1.00	0.00
ATOM N	581	N	GLY	A	90	24.144	-3.882	-11.176	1.00	0.00
ATOM C	582	CA	GLY	A	90	25.461	-3.330	-10.909	1.00	0.00
ATOM C	583	C	GLY	A	90	25.599	-1.927	-11.502	1.00	0.00
ATOM O	584	O	GLY	A	90	26.486	-1.679	-12.318	1.00	0.00
ATOM N	585	N	ILE	A	91	24.709	-1.047	-11.071	1.00	0.00
ATOM C	586	CA	ILE	A	91	24.719	0.326	-11.549	1.00	0.00
ATOM C	587	C	ILE	A	91	23.989	1.217	-10.544	1.00	0.00
ATOM O	588	O	ILE	A	91	23.343	0.721	-9.622	1.00	0.00
ATOM C	589	CB	ILE	A	91	24.151	0.403	-12.967	1.00	0.00
ATOM C	590	CG1	ILE	A	91	25.253	0.701	-13.985	1.00	0.00
ATOM C	591	CG2	ILE	A	91	23.008	1.418	-13.045	1.00	0.00
ATOM C	592	CD1	ILE	A	91	25.812	2.111	-13.790	1.00	0.00
ATOM N	593	N	LYS	A	92	24.115	2.519	-10.754	1.00	0.00
ATOM C	594	CA	LYS	A	92	23.476	3.485	-9.878	1.00	0.00
ATOM C	595	C	LYS	A	92	22.023	3.681	-10.317	1.00	0.00
ATOM O	596	O	LYS	A	92	21.761	4.276	-11.361	1.00	0.00
ATOM C	597	CB	LYS	A	92	24.284	4.783	-9.830	1.00	0.00
ATOM C	598	CG	LYS	A	92	24.464	5.264	-8.389	1.00	0.00

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ATOM C	599	CD	LYS	A	92	25.098	6.656	-8.350	1.00	0.00
ATOM C	600	CE	LYS	A	92	25.921	6.848	-7.074	1.00	0.00
ATOM N	601	NZ	LYS	A	92	25.140	7.594	-6.062	1.00	0.00
ATOM N	602	N	TYR	A	93	21.116	3.169	-9.497	1.00	0.00
ATOM C	603	CA	TYR	A	93	19.697	3.281	-9.788	1.00	0.00
ATOM C	604	C	TYR	A	93	19.162	4.657	-9.387	1.00	0.00
ATOM O	605	O	TYR	A	93	19.872	5.447	-8.768	1.00	0.00
ATOM C	606	CB	TYR	A	93	19.007	2.212	-8.938	1.00	0.00
ATOM C	607	CG	TYR	A	93	19.359	2.277	-7.450	1.00	0.00
ATOM C	608	CD1	TYR	A	93	18.953	3.357	-6.691	1.00	0.00
ATOM C	609	CD2	TYR	A	93	20.082	1.258	-6.866	1.00	0.00
ATOM C	610	CE1	TYR	A	93	19.283	3.419	-5.292	1.00	0.00
ATOM C	611	CE2	TYR	A	93	20.414	1.320	-5.466	1.00	0.00
ATOM C	612	CZ	TYR	A	93	19.998	2.397	-4.748	1.00	0.00
ATOM O	613	OH	TYR	A	93	20.311	2.456	-3.426	1.00	0.00
ATOM N	614	N	ASN	A	94	17.914	4.902	-9.759	1.00	0.00
ATOM C	615	CA	ASN	A	94	17.275	6.170	-9.447	1.00	0.00
ATOM C	616	C	ASN	A	94	16.209	5.947	-8.373	1.00	0.00
ATOM O	617	O	ASN	A	94	15.914	6.849	-7.589	1.00	0.00
ATOM C	618	CB	ASN	A	94	16.588	6.758	-10.681	1.00	0.00
ATOM C	619	CG	ASN	A	94	17.616	7.184	-11.731	1.00	0.00
ATOM N	620	ND2	ASN	A	94	18.485	6.231	-12.054	1.00	0.00
ATOM O	621	OD1	ASN	A	94	17.619	8.303	-12.215	1.00	0.00
ATOM N	622	N	THR	A	95	15.660	4.741	-8.369	1.00	0.00
ATOM C	623	CA	THR	A	95	14.633	4.390	-7.403	1.00	0.00
ATOM C	624	C	THR	A	95	13.391	5.260	-7.604	1.00	0.00
ATOM O	625	O	THR	A	95	13.411	6.454	-7.309	1.00	0.00
ATOM C	626	CB	THR	A	95	15.239	4.510	-6.003	1.00	0.00
ATOM C	627	CG2	THR	A	95	14.191	4.371	-4.898	1.00	0.00
ATOM O	628	OG1	THR	A	95	16.040	3.339	-5.871	1.00	0.00

ATOM N	629	N	THR	A	96	12.339	4.628	-8.106	1.00	0.00
ATOM C	630	CA	THR	A	96	11.090	5.331	-8.350	1.00	0.00
ATOM C	631	C	THR	A	96	9.948	4.671	-7.577	1.00	0.00
ATOM O	632	O	THR	A	96	8.925	4.312	-8.159	1.00	0.00
ATOM C	633	CB	THR	A	96	10.858	5.371	-9.862	1.00	0.00
ATOM C	634	CG2	THR	A	96	12.082	5.873	-10.630	1.00	0.00
ATOM O	635	OG1	THR	A	96	10.756	3.998	-10.234	1.00	0.00
ATOM N	636	N	ARG	A	97	10.160	4.530	-6.277	1.00	0.00
ATOM C	637	CA	ARG	A	97	9.159	3.919	-5.417	1.00	0.00
ATOM C	638	C	ARG	A	97	8.787	2.530	-5.940	1.00	0.00
ATOM O	639	O	ARG	A	97	7.657	2.307	-6.370	1.00	0.00
ATOM C	640	CB	ARG	A	97	7.899	4.782	-5.340	1.00	0.00
ATOM C	641	CG	ARG	A	97	7.976	5.763	-4.169	1.00	0.00
ATOM C	642	CD	ARG	A	97	8.032	5.020	-2.833	1.00	0.00
ATOM N	643	NE	ARG	A	97	9.302	5.324	-2.138	1.00	0.00
ATOM C	644	CZ	ARG	A	97	9.631	6.535	-1.668	1.00	0.00
ATOM N	645	NH1	ARG	A	97	8.785	7.563	-1.817	1.00	0.00
ATOM N	646	NH2	ARG	A	97	10.806	6.718	-1.050	1.00	0.00
ATOM N	647	N	ASN	A	98	9.760	1.632	-5.885	1.00	0.00
ATOM C	648	CA	ASN	A	98	9.550	0.271	-6.348	1.00	0.00
ATOM C	649	C	ASN	A	98	8.379	-0.347	-5.582	1.00	0.00
ATOM O	650	O	ASN	A	98	8.063	0.080	-4.472	1.00	0.00
ATOM C	651	CB	ASN	A	98	10.787	-0.593	-6.097	1.00	0.00
ATOM C	652	CG	ASN	A	98	11.303	-0.412	-4.668	1.00	0.00
ATOM N	653	ND2	ASN	A	98	12.502	-0.946	-4.454	1.00	0.00
ATOM O	654	OD1	ASN	A	98	10.655	0.174	-3.817	1.00	0.00
ATOM N	655	N	ALA	A	99	7.765	-1.342	-6.205	1.00	0.00
ATOM C	656	CA	ALA	A	99	6.635	-2.024	-5.595	1.00	0.00
ATOM C	657	C	ALA	A	99	6.027	-2.997	-6.607	1.00	0.00
ATOM O	658	O	ALA	A	99	6.405	-2.999	-7.777	1.00	0.00

ATOM C	719	CB	ASN	A	106	-13.186	-8.236	-9.490	1.00	0.00
ATOM C	720	CG	ASN	A	106	-14.634	-8.728	-9.455	1.00	0.00
ATOM N	721	ND2	ASN	A	106	-14.766	-10.038	-9.647	1.00	0.00
ATOM O	722	OD1	ASN	A	106	-15.571	-7.968	-9.267	1.00	0.00
ATOM N	723	N	GLY	A	107	-11.618	-5.415	-10.659	1.00	0.00
ATOM C	724	CA	GLY	A	107	-11.373	-4.015	-10.362	1.00	0.00
ATOM C	725	C	GLY	A	107	-10.995	-3.823	-8.892	1.00	0.00
ATOM O	726	O	GLY	A	107	-11.566	-2.976	-8.207	1.00	0.00
ATOM N	727	N	THR	A	108	-10.036	-4.624	-8.452	1.00	0.00
ATOM C	728	CA	THR	A	108	-9.575	-4.553	-7.075	1.00	0.00
ATOM C	729	C	THR	A	108	-8.055	-4.721	-7.013	1.00	0.00
ATOM O	730	O	THR	A	108	-7.454	-5.302	-7.914	1.00	0.00
ATOM C	731	CB	THR	A	108	-10.337	-5.606	-6.268	1.00	0.00
ATOM C	732	CG2	THR	A	108	-11.854	-5.455	-6.392	1.00	0.00
ATOM O	733	OG1	THR	A	108	-10.053	-6.831	-6.939	1.00	0.00
ATOM N	734	N	TRP	A	109	-7.480	-4.200	-5.939	1.00	0.00
ATOM C	735	CA	TRP	A	109	-6.041	-4.285	-5.747	1.00	0.00
ATOM C	736	C	TRP	A	109	-5.725	-5.656	-5.144	1.00	0.00
ATOM O	737	O	TRP	A	109	-6.600	-6.515	-5.053	1.00	0.00
ATOM C	738	CB	TRP	A	109	-5.533	-3.123	-4.890	1.00	0.00
ATOM C	739	CG	TRP	A	109	-5.450	-1.791	-5.636	1.00	0.00
ATOM C	740	CD1	TRP	A	109	-6.184	-0.687	-5.437	1.00	0.00
ATOM C	741	CD2	TRP	A	109	-4.548	-1.464	-6.713	1.00	0.00
ATOM C	742	CE2	TRP	A	109	-4.795	-0.165	-7.106	1.00	0.00
ATOM C	743	CE3	TRP	A	109	-3.554	-2.241	-7.336	1.00	0.00
ATOM N	744	NE1	TRP	A	109	-5.822	0.323	-6.306	1.00	0.00
ATOM C	745	CZ2	TRP	A	109	-4.092	0.473	-8.135	1.00	0.00
ATOM C	746	CZ3	TRP	A	109	-2.861	-1.590	-8.362	1.00	0.00
ATOM C	747	CH2	TRP	A	109	-3.098	-0.282	-8.770	1.00	0.00
ATOM N	748	N	ALA	A	110	-4.471	-5.816	-4.747	1.00	0.00

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ATOM C	779	C	ASN A 114	6.726	-5.721	-2.814	1.00	0.00
ATOM O	780	O	ASN A 114	6.437	-5.680	-4.009	1.00	0.00
ATOM C	781	CB	ASN A 114	5.306	-7.101	-1.329	1.00	0.00
ATOM C	782	CG	ASN A 114	5.114	-7.999	-2.552	1.00	0.00
ATOM N	783	ND2	ASN A 114	3.929	-7.865	-3.140	1.00	0.00
ATOM O	784	OD1	ASN A 114	5.983	-8.763	-2.937	1.00	0.00
ATOM N	785	N	TYR A 115	7.962	-5.812	-2.344	1.00	0.00
ATOM C	786	CA	TYR A 115	9.102	-5.873	-3.243	1.00	0.00
ATOM C	787	C	TYR A 115	10.406	-6.049	-2.462	1.00	0.00
ATOM O	788	O	TYR A 115	10.963	-5.079	-1.950	1.00	0.00
ATOM C	789	CB	TYR A 115	9.135	-4.529	-3.973	1.00	0.00
ATOM C	790	CG	TYR A 115	9.095	-3.315	-3.043	1.00	0.00
ATOM C	791	CD1	TYR A 115	10.269	-2.692	-2.669	1.00	0.00
ATOM C	792	CD2	TYR A 115	7.885	-2.841	-2.579	1.00	0.00
ATOM C	793	CE1	TYR A 115	10.231	-1.550	-1.794	1.00	0.00
ATOM C	794	CE2	TYR A 115	7.847	-1.699	-1.703	1.00	0.00
ATOM C	795	CZ	TYR A 115	9.021	-1.109	-1.355	1.00	0.00
ATOM O	796	OH	TYR A 115	8.985	-0.029	-0.529	1.00	0.00
ATOM N	797	N	SER A 116	10.855	-7.294	-2.396	1.00	0.00
ATOM C	798	CA	SER A 116	12.084	-7.608	-1.687	1.00	0.00
ATOM C	799	C	SER A 116	13.100	-8.227	-2.649	1.00	0.00
ATOM O	800	O	SER A 116	14.205	-8.585	-2.245	1.00	0.00
ATOM C	801	CB	SER A 116	11.817	-8.557	-0.516	1.00	0.00
ATOM O	802	OG	SER A 116	11.152	-9.746	-0.934	1.00	0.00
ATOM N	803	N	HIS A 117	12.690	-8.333	-3.905	1.00	0.00
ATOM C	804	CA	HIS A 117	13.550	-8.902	-4.928	1.00	0.00
ATOM C	805	C	HIS A 117	14.315	-7.782	-5.635	1.00	0.00
ATOM O	806	O	HIS A 117	14.884	-7.994	-6.706	1.00	0.00
ATOM C	807	CB	HIS A 117	12.744	-9.773	-5.894	1.00	0.00
ATOM C	808	CG	HIS A 117	12.023	-10.922	-5.231	1.00	0.00

ATOM C	809	CD2	HIS	A	117	10.691	-11.198	-5.125	1.00	0.00
ATOM N	810	ND1	HIS	A	117	12.687	-11.946	-4.578	1.00	0.00
ATOM C	811	CE1	HIS	A	117	11.786	-12.795	-4.106	1.00	0.00
ATOM N	812	NE2	HIS	A	117	10.550	-12.329	-4.446	1.00	0.00
ATOM N	813	N	CYS	A	118	14.304	-6.615	-5.010	1.00	0.00
ATOM C	814	CA	CYS	A	118	14.991	-5.461	-5.566	1.00	0.00
ATOM C	815	C	CYS	A	118	16.097	-5.046	-4.596	1.00	0.00
ATOM O	816	O	CYS	A	118	17.236	-4.824	-5.005	1.00	0.00
ATOM C	817	CB	CYS	A	118	14.022	-4.312	-5.854	1.00	0.00
ATOM S	818	SG	CYS	A	118	14.668	-3.030	-6.989	1.00	0.00
ATOM N	819	N	GLU	A	119	15.724	-4.953	-3.328	1.00	0.00
ATOM C	820	CA	GLU	A	119	16.671	-4.567	-2.295	1.00	0.00
ATOM C	821	C	GLU	A	119	17.965	-5.371	-2.434	1.00	0.00
ATOM O	822	O	GLU	A	119	19.053	-4.801	-2.477	1.00	0.00
ATOM C	823	CB	GLU	A	119	16.064	-4.742	-0.901	1.00	0.00
ATOM C	824	CG	GLU	A	119	16.248	-3.477	-0.060	1.00	0.00
ATOM C	825	CD	GLU	A	119	16.663	-3.826	1.370	1.00	0.00
ATOM O	826	OE1	GLU	A	119	17.839	-3.559	1.701	1.00	0.00
ATOM O	827	OE2	GLU	A	119	15.795	-4.351	2.102	1.00	0.00
ATOM N	828	N	PRO	A	120	17.799	-6.719	-2.503	1.00	0.00
ATOM C	829	CA	PRO	A	120	18.941	-7.609	-2.637	1.00	0.00
ATOM C	830	C	PRO	A	120	19.501	-7.571	-4.060	1.00	0.00
ATOM O	831	O	PRO	A	120	18.801	-7.899	-5.016	1.00	0.00
ATOM C	832	CB	PRO	A	120	18.420	-8.980	-2.240	1.00	0.00
ATOM C	833	CG	PRO	A	120	16.906	-8.898	-2.347	1.00	0.00
ATOM C	834	CD	PRO	A	120	16.525	-7.431	-2.455	1.00	0.00
ATOM N	835	N	ILE	A	121	20.759	-7.166	-4.155	1.00	0.00
ATOM C	836	CA	ILE	A	121	21.422	-7.081	-5.445	1.00	0.00
ATOM C	837	C	ILE	A	121	22.865	-7.571	-5.307	1.00	0.00
ATOM O	838	O	ILE	A	121	23.438	-7.525	-4.219	1.00	0.00

ATOM C	839	CB	ILE	A	121	21.305	-5.667	-6.016	1.00	0.00
ATOM C	840	CG1	ILE	A	121	19.869	-5.368	-6.451	1.00	0.00
ATOM C	841	CG2	ILE	A	121	22.306	-5.449	-7.153	1.00	0.00
ATOM C	842	CD1	ILE	A	121	19.536	-6.073	-7.766	1.00	0.00
ATOM N	843	N	LEU	A	122	23.412	-8.028	-6.423	1.00	0.00
ATOM C	844	CA	LEU	A	122	24.777	-8.526	-6.439	1.00	0.00
ATOM C	845	C	LEU	A	122	25.740	-7.354	-6.640	1.00	0.00
ATOM O	846	O	LEU	A	122	26.573	-7.378	-7.544	1.00	0.00
ATOM C	847	CB	LEU	A	122	24.931	-9.634	-7.483	1.00	0.00
ATOM C	848	CG	LEU	A	122	24.599	-9.247	-8.925	1.00	0.00
ATOM C	849	CD1	LEU	A	122	25.832	-9.367	-9.823	1.00	0.00
ATOM C	850	CD2	LEU	A	122	23.422	-10.069	-9.456	1.00	0.00
ATOM N	851	N	ASP	A	123	25.592	-6.355	-5.782	1.00	0.00
ATOM C	852	CA	ASP	A	123	26.438	-5.176	-5.853	1.00	0.00
ATOM C	853	C	ASP	A	123	26.041	-4.200	-4.744	1.00	0.00
ATOM O	854	O	ASP	A	123	25.302	-3.246	-4.987	1.00	0.00
ATOM C	855	CB	ASP	A	123	26.272	-4.460	-7.196	1.00	0.00
ATOM C	856	CG	ASP	A	123	27.490	-3.654	-7.653	1.00	0.00
ATOM O	857	OD1	ASP	A	123	27.711	-2.575	-7.061	1.00	0.00
ATOM O	858	OD2	ASP	A	123	28.172	-4.134	-8.584	1.00	0.00
ATOM N	859	N	ASP	A	124	26.549	-4.472	-3.551	1.00	0.00
ATOM C	860	CA	ASP	A	124	26.256	-3.630	-2.404	1.00	0.00
ATOM C	861	C	ASP	A	124	26.920	-4.222	-1.159	1.00	0.00
ATOM O	862	O	ASP	A	124	26.823	-5.424	-0.912	1.00	0.00
ATOM C	863	CB	ASP	A	124	24.749	-3.554	-2.147	1.00	0.00
ATOM C	864	CG	ASP	A	124	24.204	-2.143	-1.908	1.00	0.00
ATOM O	865	OD1	ASP	A	124	24.573	-1.562	-0.865	1.00	0.00
ATOM O	866	OD2	ASP	A	124	23.431	-1.680	-2.773	1.00	0.00
ATOM N	867	N	LYS	A	125	27.578	-3.352	-0.407	1.00	0.00
ATOM C	868	CA	LYS	A	125	28.257	-3.775	0.806	1.00	0.00

ATOM C	869	C	LYS	A	125	29.054	-2.599	1.375	1.00	0.00
ATOM O	870	O	LYS	A	125	30.234	-2.439	1.068	1.00	0.00
ATOM C	871	CB	LYS	A	125	29.104	-5.021	0.540	1.00	0.00
ATOM C	872	CG	LYS	A	125	28.631	-6.198	1.396	1.00	0.00
ATOM C	873	CD	LYS	A	125	28.934	-5.956	2.876	1.00	0.00
ATOM C	874	CE	LYS	A	125	28.536	-7.167	3.722	1.00	0.00
ATOM N	875	NZ	LYS	A	125	27.065	-7.224	3.884	1.00	0.00
ATOM N	876	N	GLN	A	126	28.377	-1.807	2.193	1.00	0.00
ATOM C	877	CA	GLN	A	126	29.008	-0.651	2.807	1.00	0.00
ATOM C	878	C	GLN	A	126	28.476	-0.448	4.227	1.00	0.00
ATOM O	879	O	GLN	A	126	27.580	-1.167	4.667	1.00	0.00
ATOM C	880	CB	GLN	A	126	28.797	0.604	1.958	1.00	0.00
ATOM C	881	CG	GLN	A	126	29.901	0.748	0.909	1.00	0.00
ATOM C	882	CD	GLN	A	126	29.988	2.187	0.398	1.00	0.00
ATOM N	883	NE2	GLN	A	126	28.925	2.580	-0.298	1.00	0.00
ATOM O	884	OE1	GLN	A	126	30.957	2.895	0.621	1.00	0.00
ATOM N	885	N	ARG	A	127	29.052	0.534	4.906	1.00	0.00
ATOM C	886	CA	ARG	A	127	28.646	0.841	6.267	1.00	0.00
ATOM C	887	C	ARG	A	127	28.990	-0.324	7.198	1.00	0.00
ATOM O	888	O	ARG	A	127	28.762	-1.483	6.858	1.00	0.00
ATOM C	889	CB	ARG	A	127	27.144	1.120	6.345	1.00	0.00
ATOM C	890	CG	ARG	A	127	26.874	2.608	6.579	1.00	0.00
ATOM C	891	CD	ARG	A	127	25.454	2.982	6.151	1.00	0.00
ATOM N	892	NE	ARG	A	127	25.308	2.822	4.687	1.00	0.00
ATOM C	893	CZ	ARG	A	127	25.868	3.637	3.783	1.00	0.00
ATOM N	894	NH1	ARG	A	127	26.613	4.675	4.186	1.00	0.00
ATOM N	895	NH2	ARG	A	127	25.681	3.415	2.474	1.00	0.00
ATOM N	896	N	LYS	A	128	29.534	0.026	8.355	1.00	0.00
ATOM C	897	CA	LYS	A	128	29.913	-0.975	9.337	1.00	0.00
ATOM C	898	C	LYS	A	128	30.364	-0.278	10.621	1.00	0.00

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ATOM O	899	O	LYS A 128	29.742	-0.438	11.671	1.00	0.00
ATOM C	900	CB	LYS A 128	30.956	-1.930	8.755	1.00	0.00
ATOM C	901	CG	LYS A 128	30.876	-3.304	9.422	1.00	0.00
ATOM C	902	CD	LYS A 128	31.862	-3.406	10.588	1.00	0.00
ATOM C	903	CE	LYS A 128	32.473	-4.806	10.669	1.00	0.00
ATOM N	904	NZ	LYS A 128	31.466	-5.784	11.140	1.00	0.00
ATOM N	905	N	TYR A 129	31.443	0.480	10.497	1.00	0.00
ATOM C	906	CA	TYR A 129	31.986	1.203	11.635	1.00	0.00
ATOM C	907	C	TYR A 129	32.234	0.260	12.815	1.00	0.00
ATOM O	908	O	TYR A 129	31.843	-0.906	12.776	1.00	0.00
ATOM C	909	CB	TYR A 129	30.922	2.226	12.031	1.00	0.00
ATOM C	910	CG	TYR A 129	31.314	3.104	13.221	1.00	0.00
ATOM C	911	CD1	TYR A 129	32.457	3.876	13.163	1.00	0.00
ATOM C	912	CD2	TYR A 129	30.526	3.123	14.354	1.00	0.00
ATOM C	913	CE1	TYR A 129	32.826	4.702	14.283	1.00	0.00
ATOM C	914	CE2	TYR A 129	30.895	3.949	15.474	1.00	0.00
ATOM C	915	CZ	TYR A 129	32.027	4.698	15.384	1.00	0.00
ATOM O	916	OH	TYR A 129	32.376	5.478	16.441	1.00	0.00
ATOM N	917	N	ASP A 130	32.882	0.800	13.836	1.00	0.00
ATOM C	918	CA	ASP A 130	33.187	0.022	15.025	1.00	0.00
ATOM C	919	C	ASP A 130	34.069	0.852	15.960	1.00	0.00
ATOM O	920	O	ASP A 130	34.611	1.880	15.558	1.00	0.00
ATOM C	921	CB	ASP A 130	33.947	-1.256	14.668	1.00	0.00
ATOM C	922	CG	ASP A 130	33.168	-2.554	14.883	1.00	0.00
ATOM O	923	OD1	ASP A 130	32.795	-3.169	13.860	1.00	0.00
ATOM O	924	OD2	ASP A 130	32.961	-2.902	16.065	1.00	0.00
ATOM N	925	N	LEU A 131	34.187	0.373	17.190	1.00	0.00
ATOM C	926	CA	LEU A 131	34.995	1.058	18.185	1.00	0.00
ATOM C	927	C	LEU A 131	35.959	0.059	18.828	1.00	0.00
ATOM O	928	O	LEU A 131	35.558	-1.043	19.201	1.00	0.00

[illegible]

EXAMPLE 3: DESCRIPTION OF THE STRUCTURE.

[0107] The NMR structure of ^{13}C , ^{15}N -labeled ECD₁-CRFR2 β has been determined using triple resonance experiments for the backbone assignment and NOESY experiments for the distance restraints (Table 2). The NMR structure of ECD₁-CRFR2 β contains two antiparallel β -sheet regions comprising residues 63-64 (β 1 strand), 70-71 (β 2 strand), 79-82 (β 3 strand) and 99-102 (β 4 strand) (FIG. 1A). The polypeptide fold is stabilized by three disulfide bonds between residues Cys45-Cys70, Cys60-Cys103, and Cys84-Cys118 and by a central core consisting of a salt-bridge involving Asp65-Arg101, sandwiched between the aromatic rings of Trp71 and Trp109 (FIG. 2A). The two β -sheets, interconnected by this core, form the scaffold flanked by two disordered regions (residues 39-58, and 84-98).

[0108] Furthermore, the core is surrounded by a second layer of highly conserved residues, Thr69, Val80, Arg82, and of conservatively conserved residues Thr63, Ser74, Ile67 (boxed residues in FIG. 2C). The other conserved residues include Pro72 and Pro83, which are presumably important for ending the β -strands, as well as Gly77, Asn106 and Gly107 located in the hinge regions of the two β -sheets, probably important for their relative orientation. Another cluster of conserved residues is present in the disordered loop between strands β 3 and β 4 (Gly92, Phe93, Asn94 and Thr96). In contrast, the disordered loop from residues 39-58 is highly variable in amino acid sequence. The structure of ECD₁-CRFR2 β is identified as a short consensus repeat (SCR) commonly found in proteins of the complement system including the first SCR module of the human β 2-glycoprotein (PDB code 1C1Z), the closest structure found by the DALI server. Among GPCRs, the SCR domain has been predicted to occur in the N-terminal domain only of the GABA receptor.

EXAMPLE 4: HORMONE PEPTIDE BINDING SITE.

[0109] To obtain detailed structural insights about the binding interface, the interaction between the potent peptide antagonist, astressin, and the ECD₁-CRFR2 β was studied using NMR chemical shift perturbation experiments. FIG. 3A shows the HMQC spectra of ^{15}N -labeled ECD₁-CRFR2 β in the absence and presence of equimolar astressin. Largest chemical shift perturbations are observed in the segments comprising residues 67-69, 90-93, 102-103 and 112-116, respectively (FIGS. 3B). These residues are clustered in the cleft region between the tip of the first β -sheet and the edge of the "palm" of the second β -sheet (FIG. 3C). The observed

changes in the chemical shifts in the disordered loop region 85-98 are indicative of a folding upon ligand binding. This interpretation is supported by the circular dichroism data that revealed a conformational change towards a more structured ECD₁-CRFR2 β upon ligand binding. A structure-evolution approach that assumes the conservation of the ligand-receptor interface within the CRF-R family and that concomitantly screens the surface of ECD₁ for patches with conserved and similar amino acids highlights the same surface region also (FIG. 2B). Furthermore, studies of mutant CRFR2 β , that show reduced binding affinity, serve to confirm the integrity of the binding site in the full-length receptor. The mutation, R112E in myc-mCRFR2 β results in a ~7-fold decrease in the affinity for astressin: The inhibitory binding constants, K_i 's, are: 7.2 (6.3-8.3) nM for myc-mCRFR2 β (R112E) compared to 1.1 (0.8-1.5) nM for myc-mCRFR2 β . Introducing the mutation, I67E, results in a larger decrease in the affinity for astressin: K_i =128(85-191)nM (FIG. 3D). The I67E mutation also reduces (by ~3-fold) the affinity for agonist sauvagine. Mutations of residues T69 or N114, which show only small chemical shift perturbations upon binding to astressin, do not significantly influence the binding affinities. These data suggest that these chemical shift perturbations are an indirect effect of binding. Mutagenesis studies of CRF receptors reported in literature (See Dautzenberg *et al.*, 2002 and 2004; Wille *et al.*, 1999) are also consistent with the proposed interaction surface (FIGS. 2C). The hormone-binding site also provides a structural basis for explaining the binding specificity of ligands. As mentioned above, CRFR2 β binds with high affinity to Ucn 1, Ucn 2, Ucn 3, and to the antagonist astressin, but with lower affinity to CRF. On the other hand, CRF binds to CRF-R1 with higher affinity than does Ucn 2 or Ucn 3. These different binding specificities of CRF receptors are explained by the presence of different amino acids in the binding pocket (FIGS. 2 and 3). For example, the point mutations, R112W and K92Q, replacing the residues R112 or K92 in mCRFR2 β with the residues found in xCRF-R1, results in a 2-3-fold lower binding affinity for astressin: K_i =1.9 (1.0-3.4) nM for myc-mCRFR2 β (R112W) and K_i =3.1 (2.4-3.9) nM for myc-mCRFR2 β (K92Q). To ensure the conservation of the proposed binding site for different ligands, the chemical shift perturbation experiment was also performed with CRF. In the presence of CRF, the same cross-peaks of ECD₁-CRFR2 β were affected as were influenced by astressin. However, instead of a chemical shift change, the cross-peaks were broadened beyond detection, probably due to slow conformational exchange induced by the low binding affinity of CRF.

EXAMPLE 5: MODEL FOR RECEPTOR ACTIVATION.

[0110] The surface potential of the 3D structure provides an insight into receptor activation mechanism. An accumulated distribution of positive charges on the “back-side” of the structure displayed in FIGS. 1-3 (Arg47, Arg82, Arg97) suggests its orientation towards the negatively charged extracellular domains 2-4 and the transmembrane segment of CRFR2 β (FIG. 4B). This orientation is further supported by the observation that amino acid replacements between CRFR2 and CRFR1 with negative charges on the “back-side” of the ECD₁ have their counterpart in replacements with positive charges in the extracellular domains 2-4. The relative orientation of the ECD₁ and the 3D structure of ECD₁-CRFR2 β and astressin B (FIG. 4), indicates that hormone binding and receptor activation occurs in two steps. First, the ligand binds with its C-terminal segment to the solvent exposed binding site of the ECD₁. Second, for an agonist ligand, the N-terminal segment, known to be important for signaling, penetrates into the transmembrane segment of the receptor producing activation of the receptor (FIG. 4B). In contrast, the peptide antagonist, astressin, lacks the first eleven N-terminal residues and, hence, is unable to penetrate the transmembrane and other ECD's of the receptor and fails to activate it. An important prerequisite for this two-step mechanism is the observed kink in the peptide ligand, astressin (FIG. 4) (Grace *et al.*, 2004). The B1 receptors are encoded by fifteen genes in humans; the ligands for these receptors are polypeptide hormones of 27-141 amino acid residues. A structure-based analysis of the amino acid sequences of this receptor subfamily suggests that the SCR fold of the ECD₁ domain must be conserved in all the B1 family receptors (FIG. 2D). This prediction is based on (i) the conserved disulfide bonds and their identical arrangement in the ECD₁'s of CRF-R1, CRFR2 β , PTHR and GLP-1R and, (ii) the conserved salt bridge (Asp65 and Arg101) surrounded by the two conserved tryptophan residues (Trp71 and Trp109), which have been identified as the key residues in the core of ECD₁. Additionally, two prolines, which have been proposed to be crucial for ending the β -sheet (Pro72, Pro84), and Gly77, are also conserved in the receptor subfamily.

[0111] Initial analysis of the three dimensional structure of the ECD₁ provides a explanation for the profound effect of the Asp60Ala mutation (position 65 in CRFR2 β) in another member of this family, namely, the mouse growth hormone releasing factor (GRF) receptor. This mutant GRF receptor is impaired in its ability to bind and transduce the GRF-induced cAMP response, with the physiological consequences of a hypoplastic pituitary and a dwarf (*little*) phenotype. This mutation in the SCR motif would prevent the formation of the

structurally important core salt-bridge thereby hindering the correct folding of the ECD₁ and concomitantly high affinity ligand binding.

REFERENCES

[0112] All patents and publications mentioned in the specifications are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

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U.S. Patent 4,812,128

U.S. Patent 4,906,122

U.S. Patent 5,030,103

U.S. Patent 5,252,743

U.S. Patent 5,583,973

U.S. Patent 5,612,894

U.S. Patent 5,786,203

U.S. Patent 6,080,576

U.S. Patent 6,093,573

U.S. Patent 6,348,466

U.S. Patent 6,500,839

U.S. Patent 6,514,982

U.S. Patent 6,531,475

U.S. Patent 6,541,469

U.S. Patent 6,583,143

U.S. Patent 6,664,261

U.S. Patent 6,747,034

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[0113] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one will readily appreciate from the disclosure, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

DEMANDES OU BREVETS VOLUMINEUX

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COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS

1. A three dimensional model of an extracellular domain of a B1 G-protein coupled receptor (B1 GPCR) generated by using atomic coordinates of an extracellular domain of a B1 GPCR.
2. The model of claim 1, wherein the coordinates are obtained from PDB ID NO: 1U34.
3. The model of claim 1, wherein the B1 GPCR is corticotropin releasing factor receptor (CRFR).
4. The model of claim 3, wherein the CRFR is CRFR2.
5. The model of claim 4, wherein the extracellular domain comprises amino acids 39-133 of SEQ ID NO:2.
6. The model of claim 4, wherein the extracellular domain comprises amino acids 8-102 of SEQ ID NO:4.
7. The model of claim 1, wherein the coordinates are obtained by using NMR spectroscopy.
8. A method of identifying an antagonist or agonist of a B1 GPCR comprising:
 - a) analyzing a structural representation of an extracellular domain of a B1 GPCR;
 - b) designing an agent that binds to the extracellular domain of a B1 GPCR; and
 - c) assessing the antagonist or agonist activity of the agent.
9. The method of claim 8, wherein the B1 GPCR is CRFR2.
10. The method of claim 8, wherein the CRFR2 comprises the coordinate as set forth in PDB ID NO: 1U34.
11. The method of claim 9, wherein the CRFR2 is an α , β , or γ splice variant.
12. The method of claim 9, wherein the extracellular domain of CRFR2 comprises amino acids 39-133 of SEQ ID NO:2.
13. The method of claim 8, wherein the extracellular domain of CRFR2 comprises amino acids 8-102 of SEQ ID NO:4.

14. The method of claim 8, wherein the structure of the extracellular domain of the B1 GPCR is obtained by using NMR spectroscopy.
15. The method of claim 8, wherein designing the agent comprises performing computer fitting analysis.
16. The method of claim 8, wherein the antagonist or agonist binds to one or more conserved amino acids in the extracellular domain of the B1 GPCR.
17. The method of claim 8, wherein the antagonist or agonist binds to a short consensus repeat domain of the extracellular domain of the B1 GPCR.
18. The method of claim 8, wherein the agent is a peptide.
19. The method of claim 8, wherein the agent is a peptidomimetic.
20. The method of claim 8, wherein the agent is a small molecule.
21. A program storage device readable by a machine, tangibly embodying a program of instructions executable by the machine to perform the method steps of claim 8.
22. A method of improving binding of an antagonist or agonist of a B1 GPCR comprising:
 - a) analyzing the structural representation of an extracellular domain of the B1 GPCR bound to the antagonist or agonist;
 - b) designing a modified antagonist or modified agonist in accordance with the structural analysis; and
 - c) assessing the modified antagonist or modified agonist for improved activity.
23. The method of claim 22, wherein the B1 GPCR is CRFR2.
24. The method of claim 23, wherein the CRFR2 is an α , β , or γ splice variant.
25. The method of claim 23, wherein the antagonist is astressin, astressin B, astressin₂-B, urocortin I, urocortin II, or urocortin III.

26. The method of claim 23, wherein the extracellular domain of CRFR2 comprises amino acids 39-133 of SEQ ID NO:2.
27. The method of claim 23, wherein the extracellular domain of CRFR2 comprises amino acids 8-102 of SEQ ID NO:4.
28. The method of claim 22, wherein the structure of the extracellular domain of the B1 GPCR is a NMR structure.
29. The method of claim 22, wherein structural analysis comprises performing computer fitting analysis.
30. The method of claim 22, wherein the antagonist or agonist binds to one or more conserved amino acids in the extracellular domain of the B1 GPCR.
31. The method of claim 22, wherein the antagonist or agonist binds to a short consensus repeat domain of the extracellular domain of B1 GPCR.
32. The method of claim 22, wherein the agent is a peptide.
33. The method of claim 22, wherein the agent is a peptidomimetic.
34. The method of claim 22, wherein the agent is a small molecule.
35. A program storage device readable by a machine, tangibly embodying a program of instructions executable by the machine to perform the method steps of claim 22.
36. A method for identifying a ligand for the extracellular domain of a B1 GPCR comprising the steps of:
 - a) analyzing a three-dimensional structure of the extracellular domain of a B1 GPCR;
 - b) identifying a binding site on the extracellular domain of the B1 GPCR;
 - c) identifying a molecule having a structure that binds the binding site; and
 - d) assessing the binding of the molecule to the extracellular domain of the B1 GPCR.

37. The method of claim 36, wherein the molecule is a small molecule.
38. The method of claim 36, wherein the molecule is a peptidomimetic.
39. The method of claim 36, wherein the molecule is a peptide.
40. The method of claim 36, wherein the molecule is a second receptor.
41. The method of claim 40, wherein the second receptor is an EGF-like receptor.
42. A computer readable medium comprising computer-executable instructions for
 - a) analyzing a coordinate representation of an extracellular domain of a B1 GPCR;
 - b) designing an agent that binds to the extracellular domain of a B1 GPCR; and
 - c) assessing the antagonist or agonist activity of the agent.
43. The computer readable medium of claim 42, further comprising instructions for obtaining the coordinate representation of the extracellular domain of the B1 GPCR.
44. The computer readable medium of claim 43, wherein obtaining the coordinate representation comprises of the extracellular domain of a CRFR2 as set fourth in PDB ID NO: 1U34.
45. The computer readable medium of claim 42, further comprising providing instructions for identifying a binding site on the extracellular domain of the B1 GPCR.

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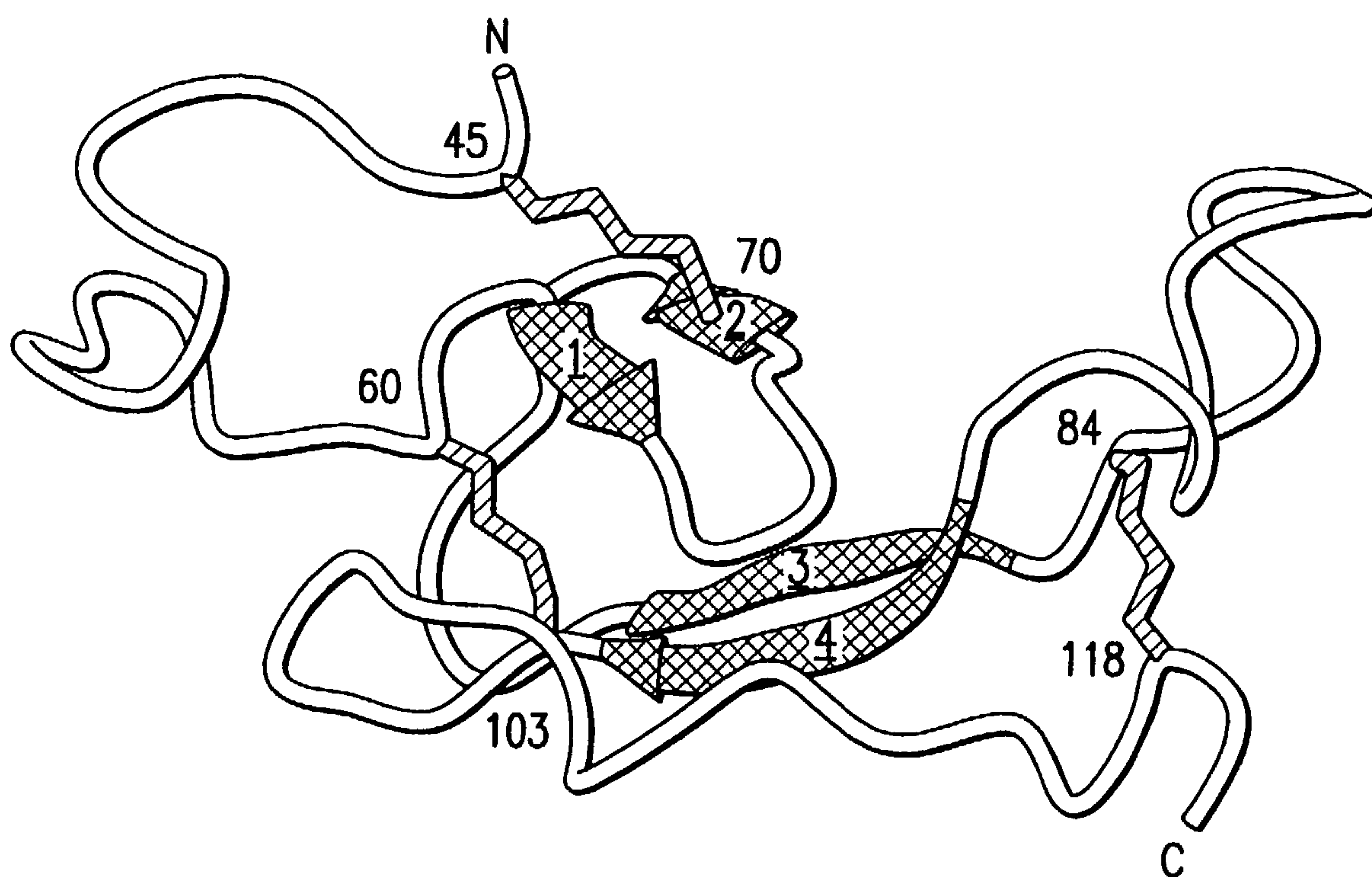


FIG. 1A

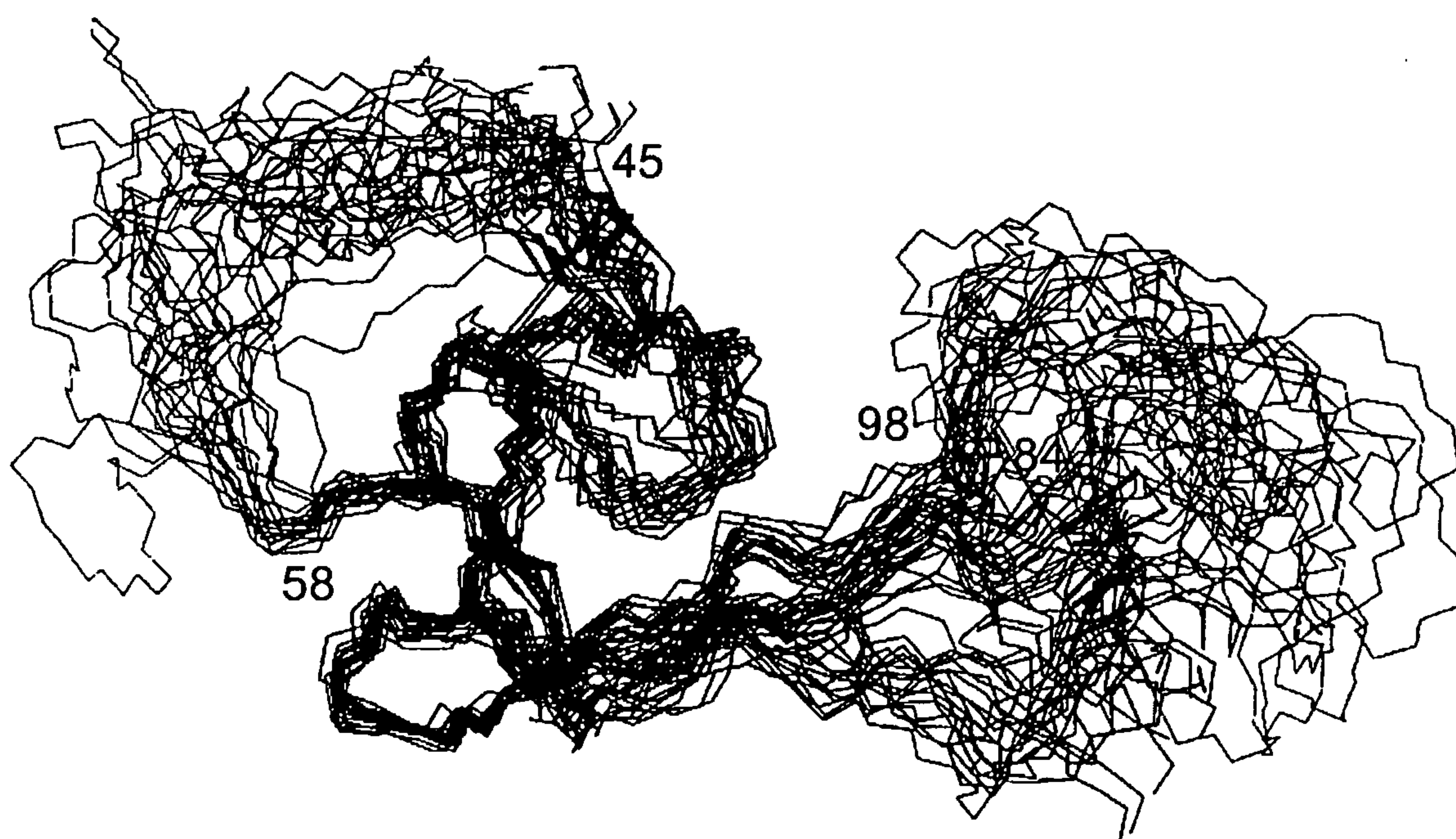


FIG. 1B

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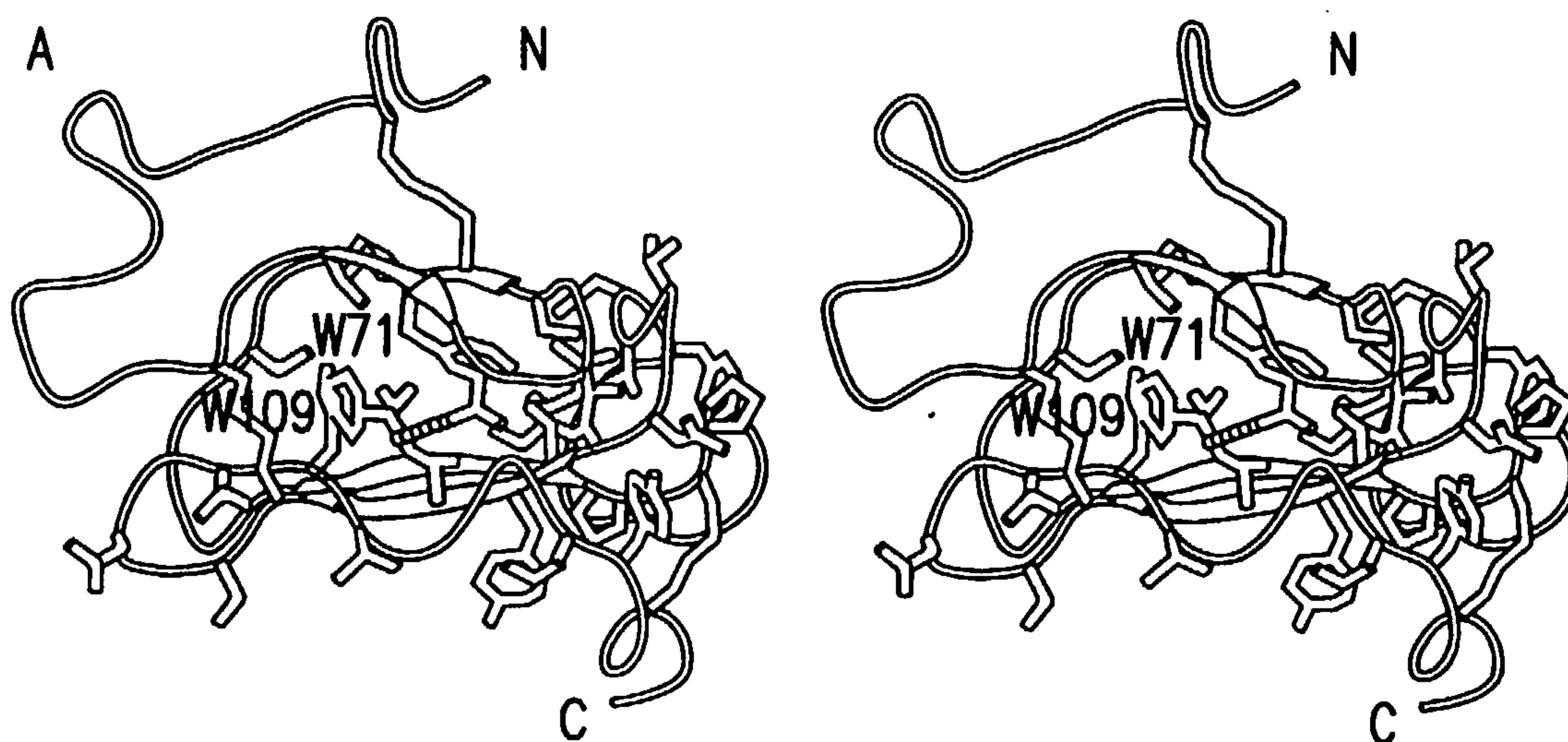


FIG.2A

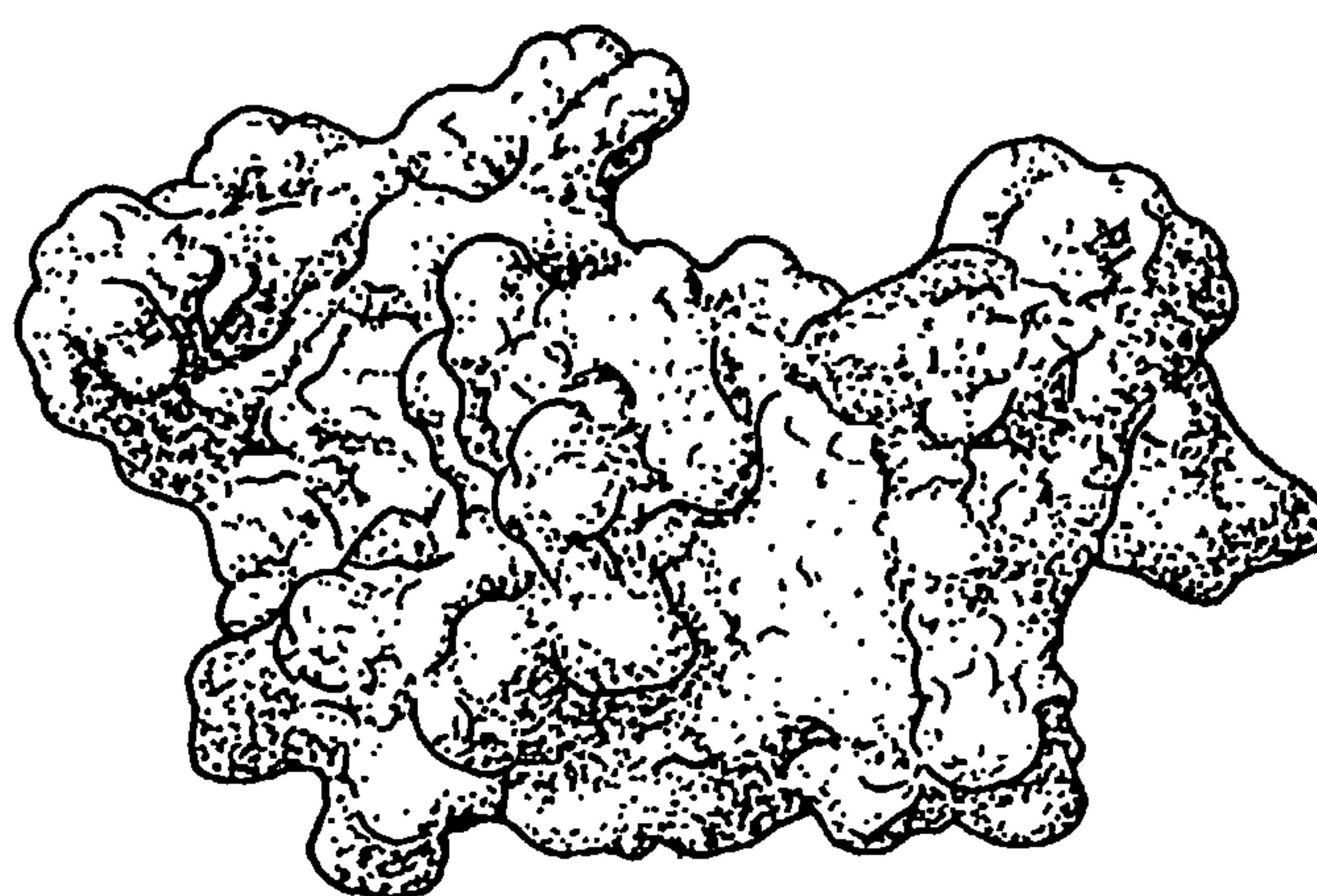


FIG.2B

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CRFR2_MOUSE	TLLEQYCHR	TTIGNFS...	.GPYTYCNIT	LDQIGTQMPQ	SAPGALVERP	CPEYFNGIKY	NTITRNAYREC	LENGTWASRV	NYSHCE
CRFR1_HUMAN	SLQDQHCHES	LSLAS	NISGLQCNAS	VDLIGTQMPR	SPAGQLWRP	CPAFFYGVRY	NTITNGYREC	LANGSWAARV	NYSECC
CRFR1_MOUSE	SLQDQQCES	LSLAS	NVSGLQCNAS	VDLIGTQMPR	SPAGQLWRP	CPAFFYGVRY	NTITNGYREC	LANGSWAARV	NYSECC
CRFR1_TS	SLQDQHCHES	LSPTS	NVSGLQCNAS	VDLIGTQMPR	SPAGQLWRP	CPAFFYGVRY	NTITNGYREC	LANGSWAARV	NYSECC
CRFR1_RAT	SLQDQRCKN	LSLTS	NVSGLQCNAS	VDLIGTQMPR	SPAGQLWRP	CPAFFYGVRY	NTITNGYREC	LANGSWAARV	NYSECC
CRFR1_SHEEP	SLQDQHCHES	LSLAS	NVSGLQCNAS	VDLNGTQMPQ	SPAGQLWRP	CLVFFYGVRY	NTITNGYRVC	LANGTWAARV	NHSECC
CRFR1_CHICK	SIQEQYCES	LLPTT	NHTGPQCNAS	VDLIGTQMPR	SAVGQLVARP	CPEYFYGVRY	NTITNGYREC	LANGSWARRV	NYSQCC
CRFR1_AmNebu	ATAELTCDT	LLNATGTSS	NATGLFCNIS	IDGIGTQMPR	SNAGEIVSRP	CPEYFNGIKY	NTITNMYREC	LANGTWAKKG	NYSQCC
CRFR1_KETA	TTADLTCDT	LLNLTLTPSN	VTAGLFCNMS	IDPIGTQMPK	STAGEWMLRP	CPEYFYGVRY	NTITNMYREC	LSNGSWAVKG	NYTQCC
CRFR_FUGU	SLANLTCEA	LLMSLMNATS	TCSGLYCETS	IDGIGTQMPR	SAAGHMVSRP	CPEYFYGVRY	NTITNMYRKC	LANGTWALKKG	NYSMCK
CRFR1_XENLA	SLQDQ.CET	LQHNS	NFTGLACNAS	IDMIGTQMPK	TAAGQMVSRP	CPEYFNGVQY	NTITNMYREC	HLNGSWACRG	DYAQCC
CRFR2_TuBe1	SLLEANCSL	ELAEELLDDG	EGPYSYCNITI	LDQIGTQMPR	SAAGALVERP	CPEYFNGVQY	NATRNAYREC	LENGTWASRI	NYSQCE
CRFR2_HUMAN	SLLEANCSL	ALAEELLDDG	EYPYSYCNITI	LDQIGTQMPR	SAAGALVERP	CPEYFNGVQY	NTITRNAYREC	LENGTWASKI	NYSQCE
CRFR2_RAT	SLLEANCSL	ALAEELLDDG	EGPYSYCNITI	LDQIGTQMPQ	SAPGALVERP	CEPYFNGIKY	NTITRNAYREC	LENGTWASRI	NYSHCE
CRFR2_KETA	EDGDPNCSV	MDSFQDSFY	NASGLYCNAT	TDEIGTQMPK	SNTRMVERP	CPEYIINGVQY	NTITRSAYREC	IDNGTWALKS	NYSSCE
CRFR2_XENLA	DEFDANCSL	LDAFQDSFLH	SESGYPCSAT	IDQIGTQMPR	SLAGELVERP	CPDSFNGIRY	NTITRNAYREC	PENG TWASIM	NYSQCV
CRFR2_AmNebu	LSVEVNCSL	ADAFGDPAYG	NASALYCNAT	ADEIGTQMPR	SCAGRVVARP	CPDFIINGVQY	NTITRSAYREC	LENGTWAFKI	NYSSCE
Mutagenesis results	*****	*****	*****	*****	*****	*****	*****	*****	*****
Chemical shift perturbation				***			***	***	***

FIG.2C

CRFR2_MOUSE	45	60	70	84	103	118
VIPR1_HUMAN	TILLEQY...	CHRTTIGNFSGPYT..	YCNNTLDQIGTOMFQ	SAPGALVERPCPE	YFNGIKYNTTRNAYR	ECLENGTHASRVN.....YSHCE
GHRHR_HUMAN	QMIEVQHKQC...	LEEAAQ.LENETIGCSKMDNL.	TCMPATPRGQVWVLA	CPILFKLFSSIQGRNVS	RSCIDEGLMTHLEPG..	PYP.IA.CG
GLP1R_HUMAN	TQLREDESAC...	LQAAEEMPNTTLGCPATWDGL.	LQMPITAGSGE	WVTLPCPDFFSHFS	SESG.AVKRDC	TIIG.WSEPFP...PYP.VA.CP
PTHR2_HUMAN	REYRRQ...	CORSLTEDP.PPATDLFCNRTFDEY.	ACMPDGEFGSFVN	SCPWYLPWASSVPQGHVYR	FTAEGLMLQKDNSSL	PWRDLSECE
	LVLKAKVQ.CE..	LNITAQIQEGEGNCFPEWDGL.	ICMPRGTVGKISAV	PCPPYIYDFN..	HKGVAFRHCNPNGT	WDFMHSLNKTKWANYSDCL

FIG.2D

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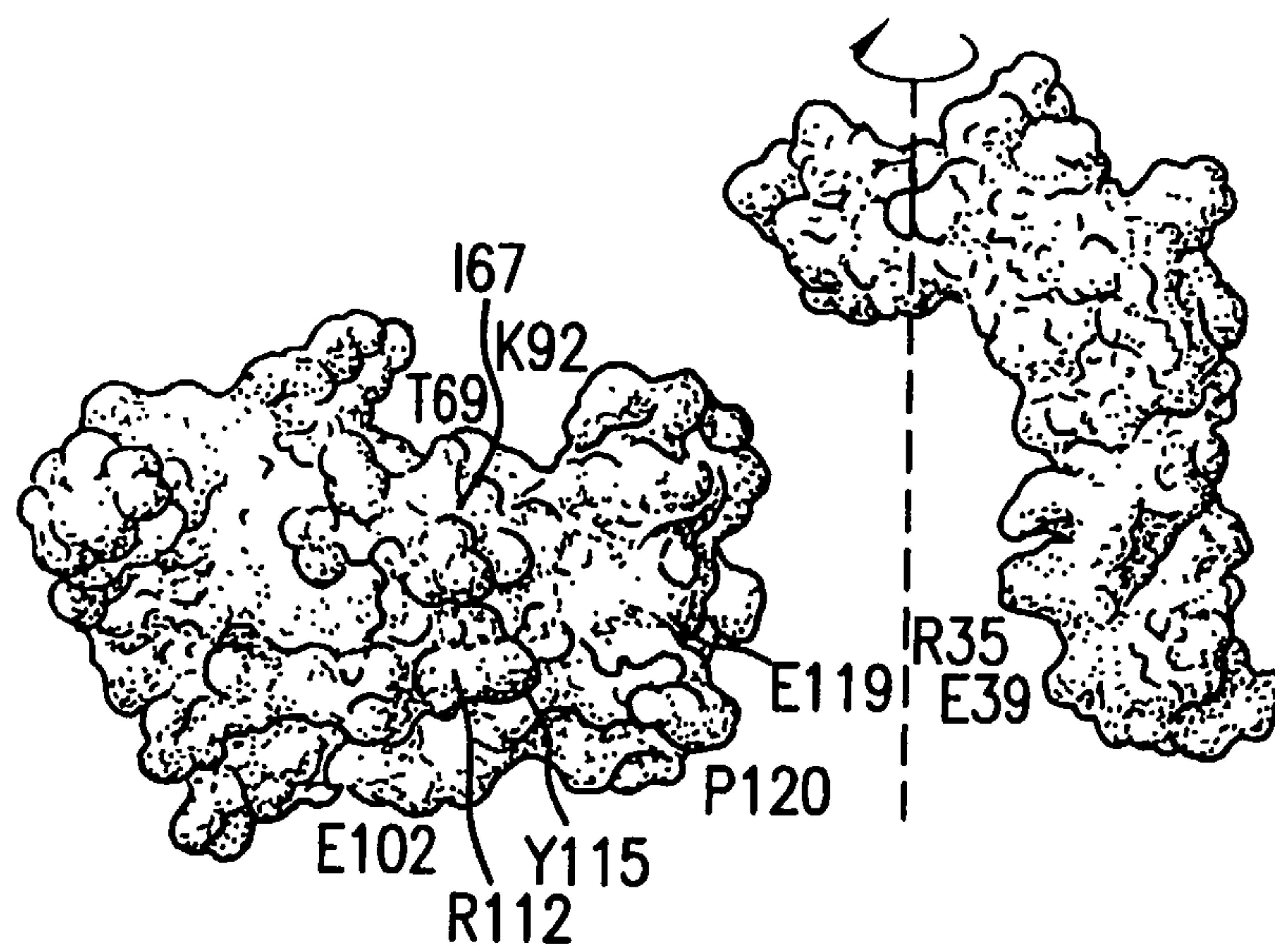


FIG.3A

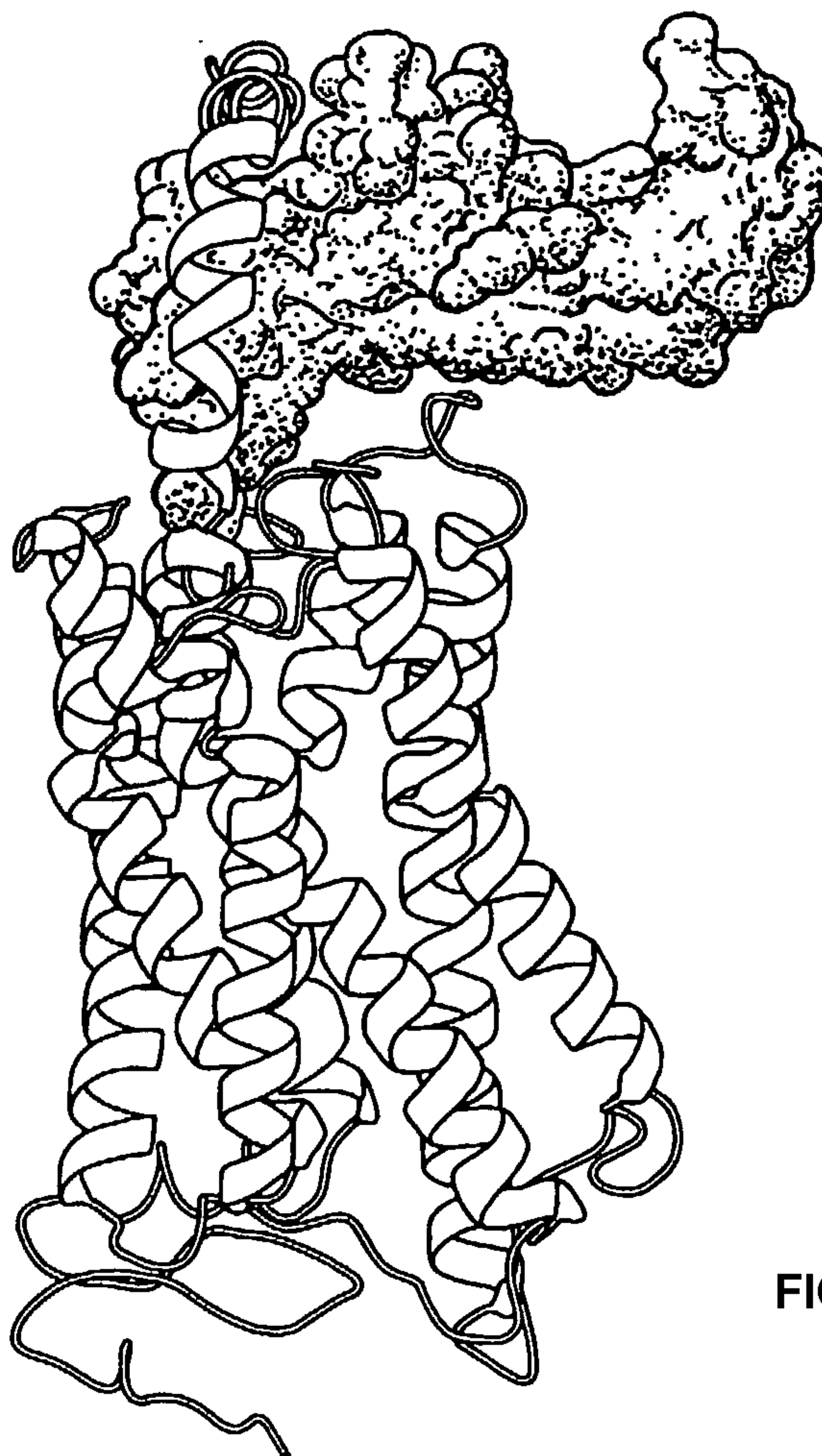


FIG..3B

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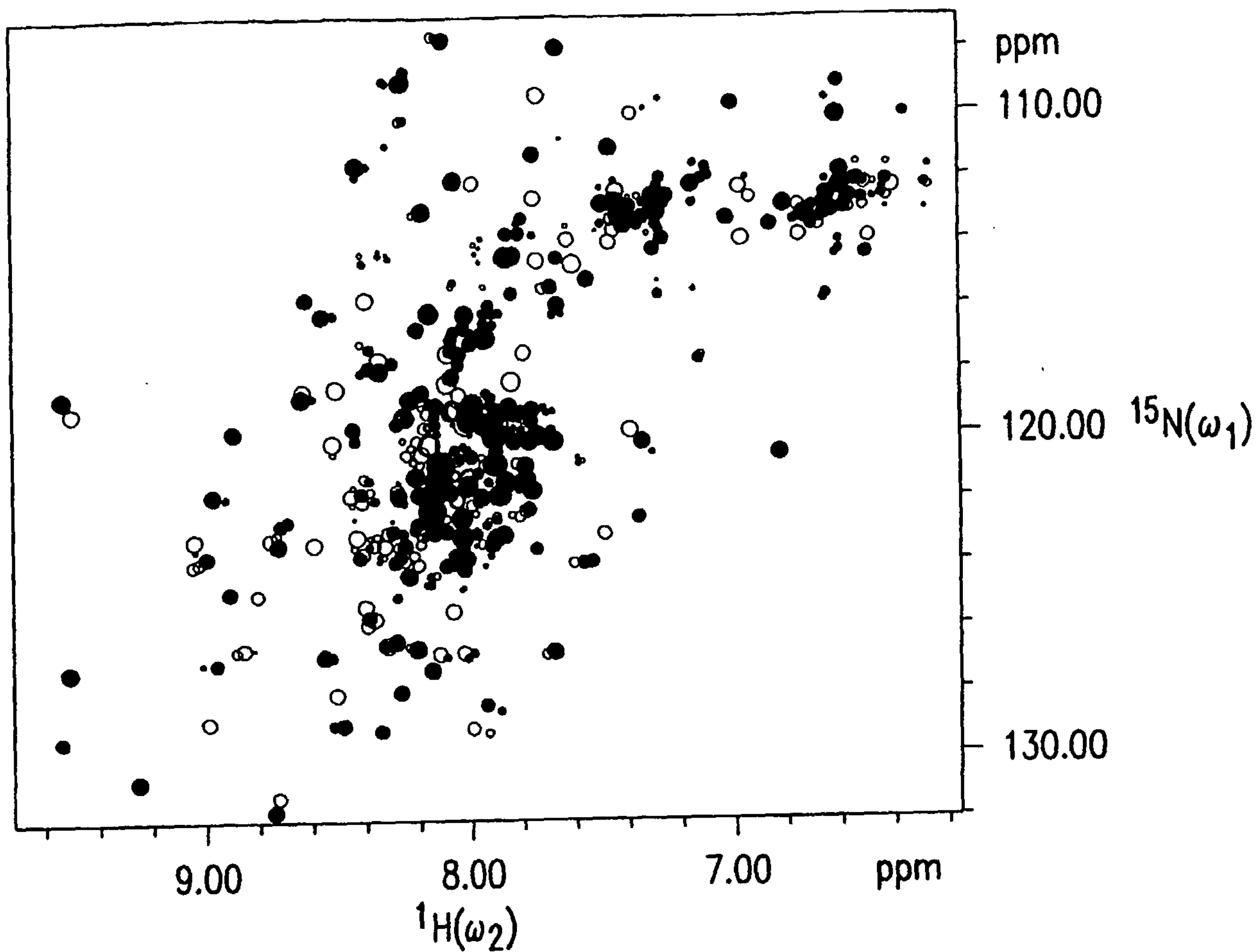


FIG.4A

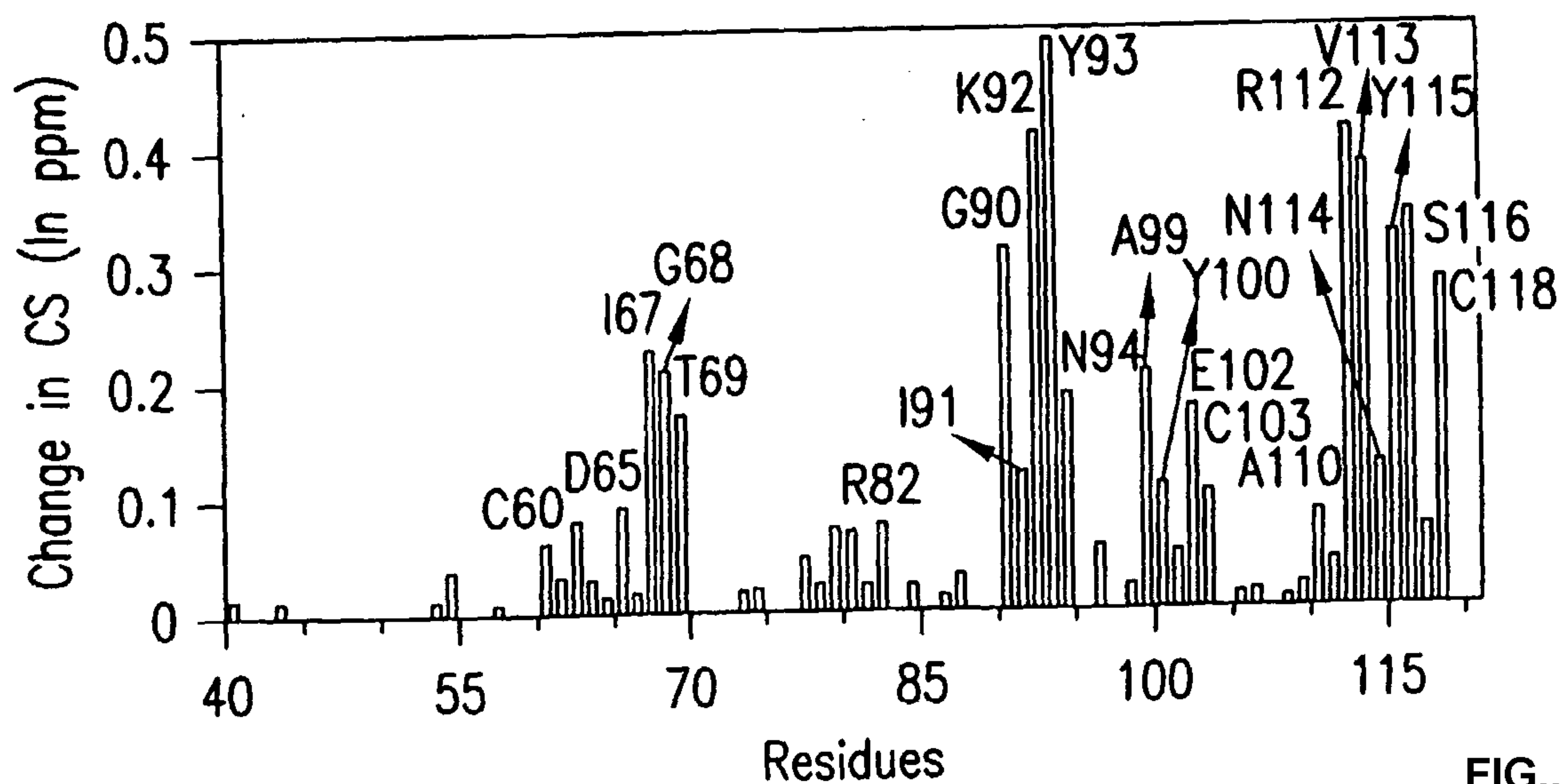


FIG.4B

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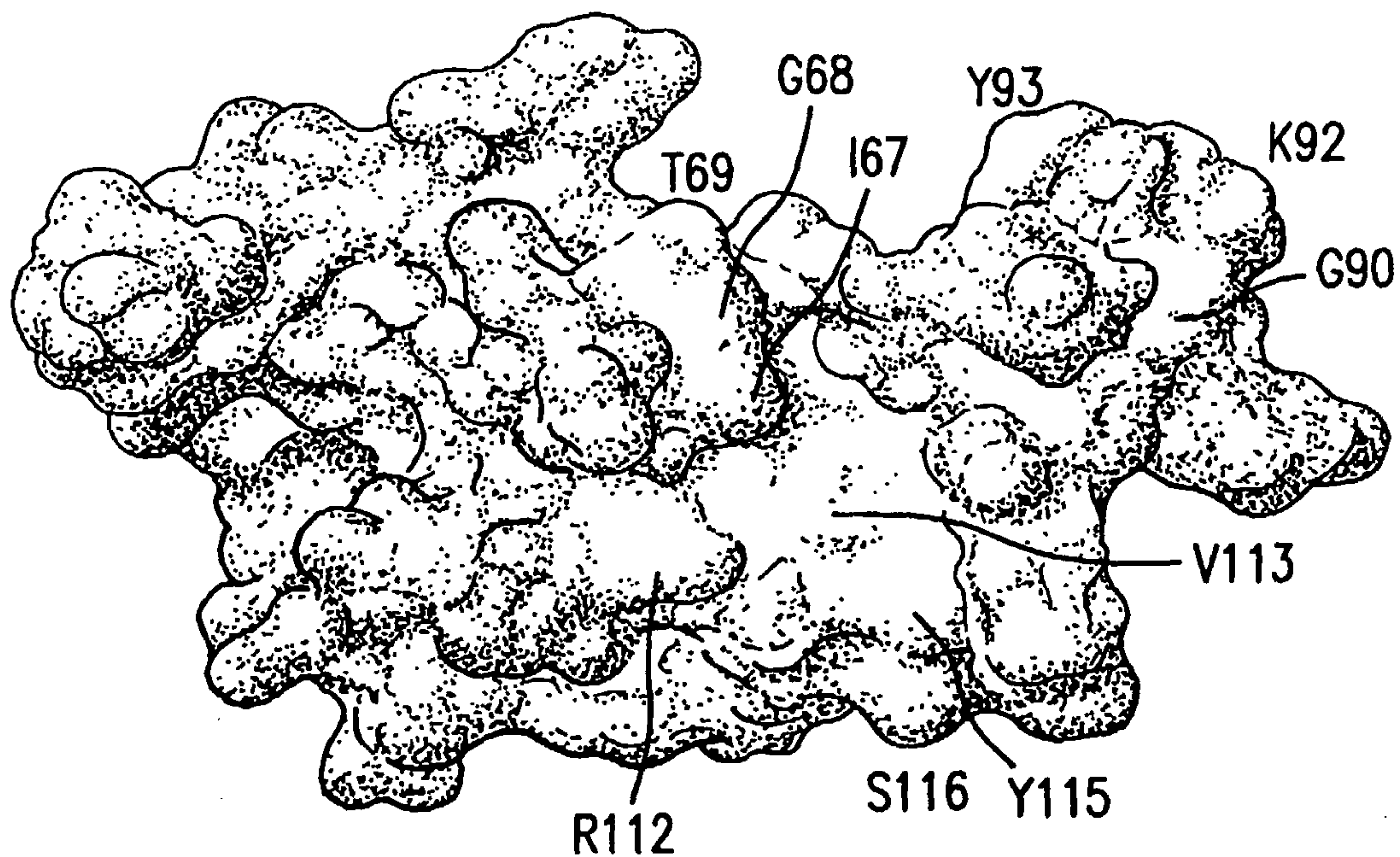


FIG.4C

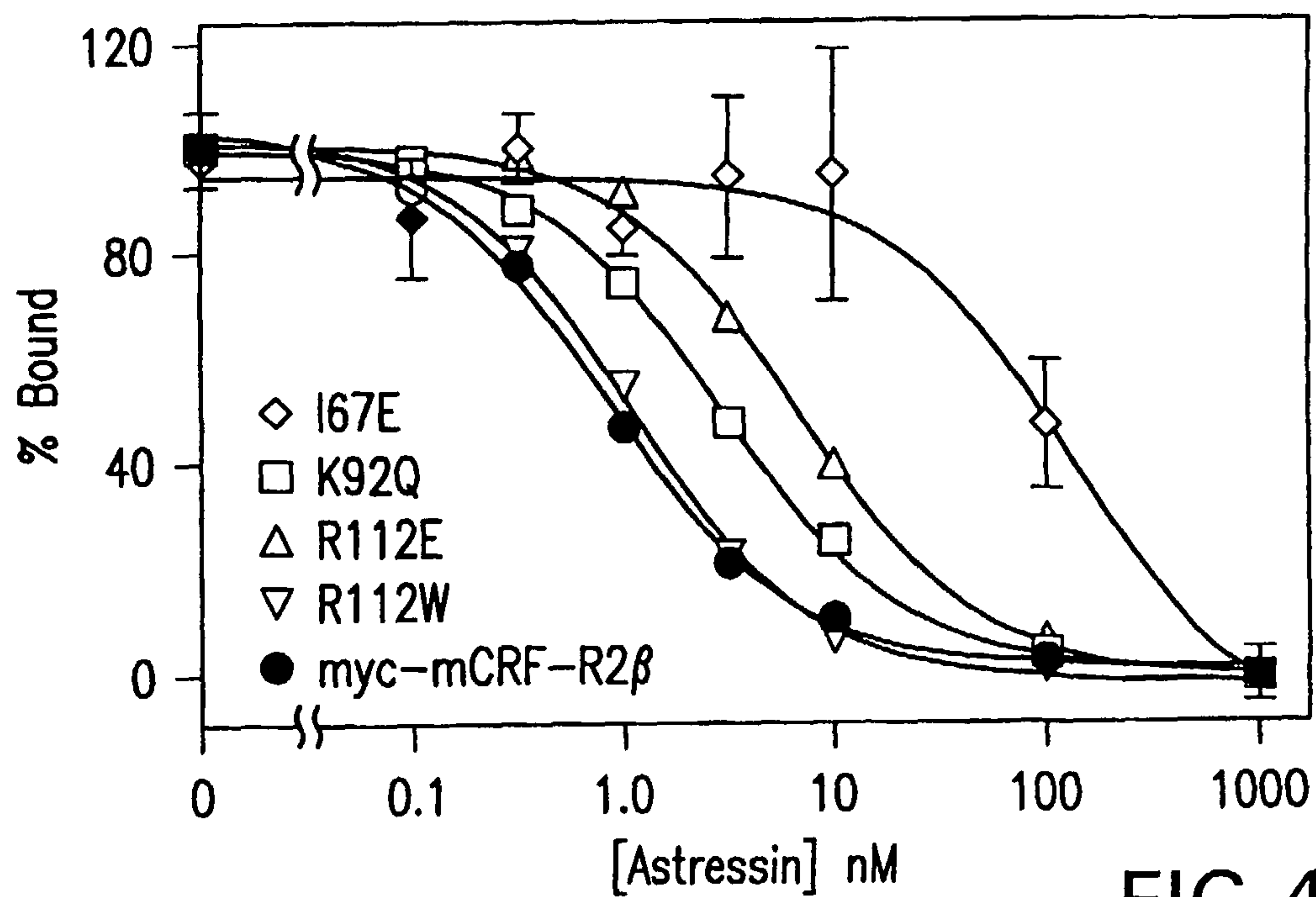


FIG.4D

