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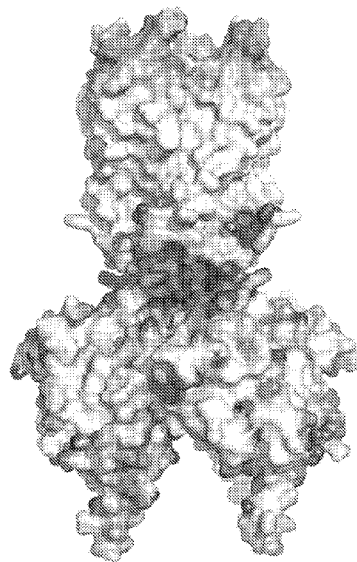
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(54) Title: METHODS FOR IDENTIFYING COMPOUNDS THAT MODULATE ION CHANNEL ACTIVITY OF A KIR CHANNEL



(57) Abstract: Methods for identifying compounds that modulate the ion channel activity of a Kir channel are provided. Methods for identifying compounds that selectively modulate the ion channel activity of specific types of Kir channels based on the turret region of a Kir channel are also provided. Methods for identifying compounds to treat conditions associated with abnormal ion channel activity are also provided. Compounds including purified antibodies and methods of making antibodies which bind to the turret region of a Kir channel are provided. Purified polypeptides including at least a portion of the turret region of a Kir channel and nucleic acid sequences encoding these polypeptides are also provided.

FIG. 7



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Methods for Identifying Compounds That Modulate Ion Channel Activity of a Kir Channel

FIELD OF INVENTION

The present invention relates to Kir channel proteins and methods for identifying
5 compounds that modulate ion channel activity by Kir channels. In particular, the present
invention relates to identifying compounds which are useful for treating diseases related to the
function of Kir channel proteins.

BACKGROUND OF THE INVENTION

10 Inward rectifier K⁺ channels (Kir channel proteins) are involved in the control of many
physiological processes that are important to human health. Kir channel proteins normally
function as K⁺ (potassium) selective pores that span cell membranes. The Kir channels are
referred to as inward rectifier K⁺ (Kir) channels based on a fundamental ion conduction property
of these channels: given an equal but opposite electrochemical driving force K⁺ conductance
15 into the cell far exceeds conductance out of the cell.

Among their many functions Kir channel proteins control the pace of the heart, regulate
secretion of hormones into the blood stream, generate electrical impulses underlying information
transfer in the nervous system and control airway and vascular smooth muscle tone. It is
believed that various disease states are directly related to the function of Kir channel proteins.

20 Members of this channel family include Kir1 – Kir7. (Kubo et al., Pharmacological Rev.,
57:509-526, 2005) Hypertension, atrial fibrillation, and type 2 diabetes are related to Kir
channel protein function and are serious conditions for which new therapies are needed. Specific
links between Kir channel proteins and disease have been found. Kir1.1 channels are present in
the kidney and regulate salt secretion into the urine. Heritable mutations involving Kir1.1 cause
25 Barter's syndrome and hypotension. Compounds which selectively inhibit Kir1.1 have the
potential to serve as a new form of anti-hypertensive agent in which hypokalemia, a major side-
effect of currently used diuretics, should in principle not be a problem. Thus, hypertensive
individuals could benefit from Kir1.1 inhibitor-based therapies. Kir3.1 and Kir3.4 channels,
which assemble to form a heteromultimer, are called G-protein-gated K⁺ channels (GIRK).

30 These channels control heart rate through stimulation by the parasympathetic nervous system.

GIRK channel knock-out mice do not develop atrial fibrillation under any of the usual stimuli that induce this arrhythmia in mice. (Claphan et al., JACC 37, 2136-2143 (June 15, 2001)) Accordingly, inhibition of GIRK channels in humans might provide effective treatment for atrial fibrillation. Kir6 channels are expressed in beta cells of the pancreas and control insulin secretion. With the identification of compounds that selectively inhibit the Kir6 channel new therapies could be realized for the treatment of type 2 diabetes. Accordingly, Kir channel proteins are good targets for the treatment of various diseases.

The Kir channel family of proteins are very similar to each other in both sequence and, by inference, structure; thus, it has been very difficult to identify compounds that can specifically modulate one kind of Kir channel protein without cross-reacting with other types of Kir channel proteins.

For the first time the structure of a eukaryotic Kir channel has been determined, and a structural feature "the turret region" has been identified that is highly ordered in structure and, based on the amino acid sequences will differ among Kir channels. Prior to this structure, only the structure of a prokaryotic Kir channel had been determined. (Nishida et al., EMBO, vol. 26, pp. 4005-4015 (2007)) The turret is an important functional region of the protein and faces the outside of the cell making this region an attractive target for identifying potential therapeutic compounds. Given the identification of the turret region in the various Kir channel proteins and the structure in a prototype, the present invention provides a variety of methods by which the turret region may be used to identify compounds having therapeutic utility for treating the various diseases related to the function of Kir channels.

The present invention provides for the first time the expression and purification of a eukaryotic Kir channel as explained in detail below. Study of the structure of this eukaryotic Kir channel resulted in a realization of the importance of the turret region and the invention of methods which allow identification of therapeutic compounds that can selectively bind to different members of the Kir channel family of proteins.

SUMMARY OF THE INVENTION

The present invention relates to methods for identifying a compound that modulates ion channel activity of a Kir channel including identifying a compound which binds the turret region of a Kir channel; and determining if the compound modulates ion channel activity of the Kir channel.

In particular embodiments, the method may be used to identify an antibody that binds the turret region of a Kir channel and modulates the Kir channel's activity. The antibody may be human, chimeric or humanized. The antibody may also be a polyclonal antibody, monoclonal antibody, an intact immunoglobulin molecule, an antibody fragment, a scFv, a Fab, a F(ab)2, a Fv, or a disulfide linked Fv.

In another embodiment, the method may be used to identify suitable nucleic acid molecules that can modulate a Kir channel's activity by binding to its turret region. In such an embodiment, the nucleic acid may be a DNA or RNA molecule. In certain embodiments the nucleic acid is an aptamer. The method may also include identifying a suitable nucleic acid by using *in vitro* selection techniques.

In another embodiment, the method is used to identify a protein or peptide that can bind a turret region of a Kir channel and modulate the Kir channel. In this embodiment, the protein/peptide may be attached to a protein scaffold or displayed on the surface of a phage.

In another embodiment, the method discussed above is used to screen for small molecules that can modulate Kir channel activity by binding to the Kir channel's turret region.

In any of the methods discussed above, the Kir channel may be a human Kir channel or a chicken/human hybrid Kir channel. Typically, the chicken/human hybrid Kir channel will comprise a human Kir channel turret region.

Various standard biochemical assays may be used to identify whether a compound binds to the turret region of a Kir channel in the method of the present invention. For example, the identifying step may comprise an ELISA and a Western blot to determine if the compound binds to a properly folded Kir channel but not to a denatured Kir channel. Moreover, the identifying step may comprise determining if the compound binds to a Kir channel with a normal turret region but not a mutated turret region.

Regarding the determining whether a compound modulates the activity of a Kir channel, various electrophysiological assays may be used such as two-electrode voltage clamp, patch clamp, and planar lipid bilayer assays. Alternatively or additionally, the determining step may include a fluorescent assay such as one utilizing a thallium specific fluorescent dye.

5 In another aspect, the present invention relates to a method for identifying a compound that selectively modulates ion channel activity of a specific type of Kir channel including identifying a compound which binds the turret region of a specific type of Kir channel but does not bind to other types of Kir channels; and determining if the compound modulates the activity of the Kir channel.

10 In another embodiment, the present invention relates to a method of identifying a compound to treat a condition associated with abnormal ion channel activity by a Kir channel including identifying a compound which binds the turret region of a Kir channel; determining if the compound modulates ion channel activity of the Kir channel; and administering the compound which modulates ion channel activity to a subject to determine if the compound is
15 able to treat the condition. In such a method, the condition may be diabetes mellitus, hypertension, cardiac arrhythmia, or epilepsy.

In another aspect, the present invention relates to a purified antibody that specifically binds to an epitope in the turret region of a Kir channel. In this embodiment, the purified antibody may be a polyclonal antibody, a monoclonal antibody, an intact immunoglobulin
20 molecule, an antibody fragment, a scFv, a Fab, a F(ab)2, a Fv, or a disulfide linked Fv. The antibody may specifically bind to a human Kir channel such as a Kir1, Kir2, Kir3, Kir4, Kir5, Kir6, or Kir7 channel. The antibody preferably binds to an epitope within the turret region of a human Kir channel such as Kir1.1, Kir1.2, Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1, or Kir6.2 channel. Even more preferably, the antibody binds to the
25 variable portion of the turret region of a human Kir channel.

In another embodiment, the present invention relates to a method of making an antibody that specifically binds to an epitope in the turret region of a human Kir channel, including providing a chicken/human hybrid Kir channel, wherein the chicken/human hybrid comprises a human Kir channel turret region; immunizing a non-human animal with the chicken/human
30 hybrid Kir channel; and determining whether the antibody is binding to the human Kir channel

turret region. In this embodiment, the chicken portion of the chicken/human hybrid Kir channel may be derived from a chicken Kir2.2 channel. Moreover, in this embodiment, the human Kir channel turret region may be derived from Kir1, Kir2, Kir3, Kir4, Kir5, Kir6, or Kir7.

Preferably, the human Kir channel turret region is derived from a human Kir1.1, Kir1.2, Kir2.1,

5 Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1, or Kir6.2 channel.

In another embodiment, the present invention relates to a method of making an antibody that specifically binds to an epitope in the turret region of a human Kir channel, including providing a human Kir channel; immunizing a non-human animal with the Kir channel; and determining whether the antibody is binding to the human Kir channel turret region.

10 In another embodiment, the present invention relates to a purified polypeptide that consists of the turret region of human Kir channels such as Kir1.1, Kir1.2, Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1, or Kir6.2. In another aspect, the present invention relates to an isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide that consists of the turret region of human Kir channels such as Kir1.1, Kir1.2,

15 Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1, or Kir6.2.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A-1C shows key residues in eukaryotic Kir channels. A sequence alignment of chicken Kir2.2 (GI:118097849), human Kir2.2 (GI:23110982), human Kir2.1 (GI:8132301),

20 human Kir1.1 (GI:1352479), human Kir3.1 (GI:1352482), human Kir3.4 (GI:1352484), human Kir6.1 (GI:2493600), human Kir7.1 (GI:3150184), KirBac1.1 (GI:33357898), KcsA (GI:39654804), and rat Kv1.2 (GI:73536156) is shown. For all the Kir sequences only the core region corresponding to the expressed protein and atomic structure of Kir2.2 is included in the alignment. For Kv1.2 only the transmembrane pore region is shown. Secondary structure

25 elements are indicated above the sequences and the turret is shown in small dotted lines above the sequence. Residues discussed in the text are boxed in a series of alternating dashes and dots (acidic residues), a series of large dots (two disulfide-bonded cysteines), alternating dashes and pairs of dots (the inner helix bundle activation gate), series of small dashes (conserved residues

among the turrets of eukaryotic Kir channels), a series of small dots (the selectivity filter and E139), and a series of large dashes (critical residues for channel-PIP₂ interactions).

FIG 2A-2E illustrates a structure of Kir2.2. (FIG 2A) Stereoview of a ribbon representation of the Kir2.2 tetramer from the side with the extracellular solution above. Four subunits of the channel are shown. Approximate boundaries of the lipid bilayer are shown as bars. (FIG 2B) A close-up view of the pore-region of a single subunit (in ribbon representation) with the turret, pore helix and selectivity filter labeled. Side chains of residues E139, R149 and a pair of disulfide-bonded cysteines (C123 and C155) are shown as sticks. Ionized hydrogen-bonds are indicated by dashed black lines. The region flanked by the two disulfide-bonded cysteines is stippled. (FIG 2C) Electron density (wire mesh, $2F_o - F_c$, calculated from 50-3.1 Å using phases from the final model and contoured at 1.0 σ) is shown for the side chains of E139 and R149 forming a salt-bridge. (FIG 2D) (FIG 2E) K⁺ selectivity filter of the Kir2.2 channel (FIG 2 D) compared with that of the Kv1.2-Kv2.1 paddle chimera channel (FIG 2E, PDB ID 2R9R). For clarity, only two of the four subunits are shown. K⁺ (cross hatched circles), water molecules (solid spheres), and hydrogen bonds between R149 and E139 (Kir, dashed black lines), or between D379, M380 and waters (Kv, dashed black lines) are shown.

FIG 3A-3G illustrates the cavity and gates region of a Kir channel. (FIG 3A) (FIG 3B) Electron density in the cavity of the Kir2.2 channel ($A, F_o - F_c$ omit map, calculated from 50-3.1 Å using phases from the final model and contoured at 2.0 σ) and of the KcsA channel (FIG 3B, PDB ID 1K4C, $F_o - F_c$ omit map, calculated from 50-3.1 Å using phases from the final model and contoured at 2.8 σ). The channels are shown as ribbon representations with the subunit closest to the viewer removed. Only the side chains facing the cavity are shown (sticks). (FIG 3C) (FIG 3D) Comparison of the transmembrane inner helix bundle activation gate of Kir2.2 (FIG 3C) with the KcsA structure (FIG 3D, PDB ID 1K4C). For clarity, only two of the four subunits (ribbon) are shown. Side chains of the residues in the bundle crossing are shown as sticks and van der Waals surfaces. K⁺ ions are shown as cross hatched spheres. Inner and Outer helices are indicated. (FIG 3E) Superposition of the chicken Kir2.2 cytoplasmic domain (α -carbon trace) and the mouse Kir2.1 cytoplasmic domain (α -carbon trace, PDB ID 1U4F) in stereo viewed from the extracellular side. (FIG 3F) (FIG 3G) Comparison of the apex (G-loop) of the cytoplasmic pores of Kir2.2 (FIG 3F) and mouse Kir2.1 (FIG 3G), with the same view as FIG

3E. The cytoplasmic domains are shown as α -carbon traces, with residues 303-309 (Kir2.2) and 302-308 (Kir2.1) shown as CPK models.

FIG 4A-4F illustration of ion binding sites. (FIG 4A) (FIG 4B) (FIG 4C) Electron density (wire mesh) of Rb^+ (FIG 4A, F_o-F_c map calculated to 4.0 Å, contoured at 3.5 σ for density in the filter and 2.0 σ for density elsewhere), Sr^{2+} (FIG 4B, 10 mM, F_o-F_c map calculated to 3.3 Å, contoured at 1.5 σ for density in the cavity and 3.0 σ for density elsewhere) and Eu^{3+} (FIG 4C, 10 mM, anomalous difference map calculated to 6.0 Å, contoured at 2.8 σ) inside the Kir2.2 channel ion conduction pathway. Kir2.2 is represented as a α -carbon trace with the transmembrane domain and cytoplasmic domain closest to viewer removed for clarity. The ions are shown as spheres. (FIG 4D) Electron density (200 mM Sr^{2+} , F_o-F_c map calculated from 50-3.8 Å, contoured at 2.5 σ , wire mesh) of Sr^{2+} (spheres) in the cavity of Kir2.2. The channel is shown as a ribbon with the subunit closest to the viewer removed. Only the side chains facing the cavity are shown (sticks). (FIG 4E) Stereoview of the ion binding site near the upper ring of charges in the cytoplasmic domain of Kir2.2, viewed from the extracellular side. Residues E225, H227, E300, and Q311 are shown as sticks, and hydrogen bonds between them are indicated as dashed black lines. Electron density (200 mM Sr^{2+} , F_o-F_c map calculated from 50-3.8 Å, contoured at 4.5 σ) of Sr^{2+} (spheres) is shown as wire mesh. (FIG 4F) Stereoview of the ion binding site at the lower ring of charges in the cytoplasmic domain of Kir2.2, viewed from the intracellular side. Residues F255, D256, and K257 are shown as sticks, and hydrogen bonds between D256 from different subunits are indicated as dashed black lines. Electron density (200 mM Sr^{2+} , F_o-F_c map calculated from 50-3.8 Å, contoured at 4.5 σ) of Sr^{2+} (spheres) is shown as wire mesh.

FIG 5A-5D illustrates the unique structure of the extracellular entryway. (FIG 5A) (FIG 5B) Surface representation of chicken Kir2.2 (FIG 5A) and Kv1.2-Kv2.1 paddle chimera (FIG 5B, PDB ID 2R9R) in stereo, viewed from the extracellular side. The four protrusions formed by the top of the turrets are highlighted with a black perimeter and F148 in Kir2.2 is labeled. (FIG 5C) Stereo representation of electron density (wire mesh) for the turret region ($2F_o-F_c$, calculated from 50-3.1 Å using phases from the final model and contoured at 1.0 σ). The turret is shown as sticks (colored according to atom types), and residues corresponding to the highlighted protrusions in panel A are labeled. (FIG 5D) A close-up view of the turret region in a single

subunit in stereo. Side chains of those conserved residues among the turrets of eukaryotic Kir channels, as well as C155 are shown as sticks. Hydrogen bonds between H108, D110 and C123 are indicated as dashed black lines.

FIG 6A-6D results showing the chicken Kir2.2 channel is a strong inward rectifier. (FIG 6A) (FIG 6B) Macroscopic currents are shown from an uninjected oocyte (FIG 6A) and a chicken Kir2.2 channel injected oocyte (FIG 6B) without subtracting leak and capacitive currents. The currents were recorded from oocytes using two-electrode voltage-clamp. Voltage pulses: holding potential (h.p.) 0 mV, depolarizing steps: -80 mV to +80 mV, $\Delta V = 10$ mV, stepping back to 0mV. (FIG 6C) Macroscopic currents recorded from oocytes using patch-clamp. The three current traces show a current trace recorded on-cell (labeled B), a trace recorded immediately after excision of the inside-out patch (labeled C), and a trace recorded approximately 10 minutes after the excision (labeled A) Voltage pulses: ramp from -80 mV to +80 mV over 10 seconds duration. (FIG 6D) I-V curve from a patch containing only a few channels. The single channel current is graphed as a function of voltage (inset).

FIG 7 provides a surface representation of Kir2.2, viewed from the side with the extracellular side above. The surface is shaded for qualitative assessment of the negative and positive electrostatic potential at the surface.

FIG 8 illustrates ion binding sites of Kir2.2 in the selectivity filter, central cavity, upper and lower rings of charges are shown as sticks (oxygens and stippled). The channel is represented as a α -carbon trace with the transmembrane domain and cytoplasmic domain closest to viewer removed for clarity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based in part on the discovery of an important structural feature present in Kir channel proteins. In particular, the present invention relates to the discovery of a "turret region" which is highly ordered in structure and which differs in sequence among different Kir channel proteins. In addition, this turret region faces the outside of the cell making the protein accessible to compounds that bind or otherwise interact with this turret region thereby affecting the ability of the Kir channel to function. The discovery of the fact that this turret

region, which differs in sequence among members of the Kir channel family, is structured provides a basis to identify compounds which can treat disease states related to Kir channel functions.

Example 1 provided below presents a determination of the crystal structure of a eukaryotic Kir channel protein. In particular, the crystal structure of the chicken Kir channel protein, Kir2.2 is presented. Excluding unstructured amino and carboxy termini, the chicken Kir2.2 protein is 90% identical to human Kir2.2. More importantly, for the purposes of the present invention, these structural studies demonstrate that Kir channels have a large structured turret region which provide the basis for the development of compounds that may be used to bind and interact with these turrets and treat disease states related to the functioning of Kir channels. In particular, these turret regions suggest approaches to the development of inhibitory compounds which will bind to a specific member of the Kir channel family of proteins and inhibit Kir channel function.

The turret region of a variety of Kir channel proteins are identified in Figures 1A-1C and in the sequence listings of the present application. In particular, Figures 1A-1C illustrates that a sequence alignment of various human Kir channels indicates that the turret region begins with a consensus sequence HGDL (or minor sequence variations thereof) and extends six amino acid residues after a highly conserved cysteine residue labeled as C123 in Figures 1A-1C. This turret region is highly conserved and most of the variation that occurs in the sequence is located in a variable portion located after the sequence HGDL up to the cysteine labeled as C123. This variable portion within the turret region constitutes a basis for differentiating Kir channels from one another and provides a target for mutagenesis assays to identify compounds capable of turret specific binding.

Given the identification of the structured turret region in the crystal structure, the turret region of other Kir channels may be identified using sequence alignment programs and the teachings of the present invention relating to the consensus sequence and structural features of the Kir channels.

Based on this structural information, methods are presented below in which the identification of the turret region and knowledge of the amino acid sequence of the turret may be used to develop assays to identify therapeutic compounds which include, but are not limited to,

antibodies, nucleic acids, peptides and small molecules that are capable of selective binding to Kir channel proteins.

In general the methods of the present invention for identifying a compound that modulates ion channel activity of a Kir channel comprises a two step process: a first step of identifying a compound which binds the turret region of a Kir channel; and a second step of determining if the compound modulates the ion channel activity of the Kir channel.

Production of Antibodies

In a first method for identifying compounds that modulate the ion channel activity of a Kir Channel, antibodies are prepared against a Kir channel. A variety of Kir channels are known and the methods described below may be used to prepare antibodies against any Kir channel. Given the present discovery of the importance of the turret region in distinguishing one Kir channel from another, it is particularly useful to obtain antibodies which bind the turret region.

The antigens and assay reagents

Two types of Kir channel proteins may be of particular utility in preparing antibodies. The first type are human Kir channel proteins. The second type are chimeric constructs which utilize a non-human sequence, preferably a eukaryotic sequence, such as a chicken sequence, in particular a chicken Kir 2.2 sequence into which a human Kir channel turret region has been inserted, thereby replacing the native turret region. As an example, chimeric proteins which utilize a chicken Kir2.2 "scaffold" into which the turret region from a given human Kir channel is inserted may be used to prepare antibodies which are specific for different human Kir channel proteins. Both human and chimeric Kir channels may be full length proteins or may contain deletions at the amino and/or carboxy termini of the protein if desired.

Conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art may be used in order to prepare human and chimeric Kir channel proteins.

Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D. N. Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed. 1984); Nucleic Acid Hybridization [B. D. Hames & S. J. Higgins eds.

(1985)]; Transcription And Translation [B. D. Hames & S. J. Higgins, eds. (1984)]; Animal Cell Culture [R. I. Freshney, ed. (1986)]; Immobilized Cells And Enzymes [IRL Press, (1986)]; B. Perbal, A Practical Guide To Molecular Cloning (1984); F. M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994); Molecular Cloning: A Laboratory Manual Third Edition [Joseph Sambrook and David W. Russell Cold Spring Harbor Laboratory Press (2001)]; The Condensed Protocols from Molecular Cloning: A Laboratory Manual [Joseph Sambrook and David W. Russell, Cold Spring Harbor Laboratory Press (2006)]; Gene Cloning and Manipulation Second Edition [Christopher Howe, Cambridge University Press (2007)].

10 The cDNA sequences for exemplary human Kir channels are presented in SEQ ID NOS 30-43. The cDNA sequence of the chicken Kir2.2 channel is presented in SEQ ID NO: 45. DNA and cDNA sequences for other types of Kir channels are available in public databases. The turret regions of exemplary human Kir proteins are identified in SEQ ID NOS: 46-56. ...

Expression of chicken Kir 2.2

15 As an example of expression and purification of a eukaryotic Kir channel a protocol for preparing a chicken Kir 2.2 channel protein is provided below. Using standard techniques this procedure may be modified to prepare any of the human Kir channel proteins or a desired chimeric Kir channel protein.

20 To prepare the chicken Kir 2.2 channel, a synthetic gene fragment (Bio Basic, Inc.) encoding residues 38 to 369 of chicken Kir2.2 channel (GI:118097849) was ligated into the XhoI/EcoRI cloning sites of a modified pPICZ-B vector (Invitrogen). The resulting protein has green fluorescent protein (GFP) and a 1D4 antibody recognition sequence (TETSQVAPA) on the C terminus (1), separated by a PreScission protease cleavage site (SNSLEVLFG/GP).

25 The construct was linearized using PmeI and transformed into a HIS⁺ strain of SMD1163 of *Pichia pastoris* (Invitrogen) by electroporation (BioRad Micropulser). Transformants were selected on YPDS plates containing 400-1200 μ /ml Zeocin (Invitrogen). Resistant colonies were tested for expression by anti-1D4 tag Western Blot. For large-scale expression, small cultures grown from the best expressing colony were diluted into BMGY media (Invitrogen) and inoculated at 29 °C overnight, until OD600 reached between 20-30. Cells were then pelleted,

resuspended in BMM media (Invitrogen) and expressed overnight at 24 °C. Cells were harvested, flash-frozen in liquid N₂, and stored at -80 °C until needed.

Cells were lysed in a Retsch, Inc. Model MM301 mixer mill (5 x 3.0 minutes at 25 cps). The lysis buffer contained 150 mM KCl, 50 mM TRIS-HCl pH 8.0, 0.1 mg/ml

5 deoxyribonuclease I, 0.1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0.1 mg/ml soy trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml AEBSF, with 1 mM phenylmethanesulfonyl fluoride added just before lysis (3.0 ml lysis buffer/g cells). pH of the lysate was adjusted to 8.0 with KOH. The lysate was extracted with 100 mM DM (n-dodecyl-β-D-maltopyranoside, Anatrace, solgrade) at room temperature for 1 hour with stirring, and then centrifuged for 40
10 minutes at 30,000g, 10 °C. Supernatant was added to 1D4-affinity resin pre-equilibrated with 150 mM KCl, 50 mM TRIS-HCl pH 8.0, and 4 mM DM. Suspension was layered with Argon and mixed by inversion for 2 hours at room temperature. Beads were collected on a column by gravity, washed with 2 column volumes of buffer (150 mM KCl, 50 mM TRIS-HCl pH 8.0, 1 mM EDTA pH 8.0, and 4 mM DM), and eluted with buffer plus 1 mg/ml 1D4 peptide (AnaSpec,
15 Inc.) over 1 hour at room temperature. 20 mM DTT (Dithiothreitol) and 3 mM TECP were added to eluted protein. The protein was then digested with PreScission protease (20:1 w/w ratio) overnight at 4 °C. Concentrated protein was further purified on a Superdex-200 gel filtration column in 150 mM KCl, 20 mM TRIS-HCl pH 8.0, 4 mM DM (anagrade), 3 mM TCEP, 20 mM DTT and 1 mM EDTA at 4 °C. In a preferred embodiment, the protein extract is maintained in a
20 mild detergent, such as DM, which will maintain the three-dimensional structure of the Kir channel.

Preparation of Human/Chicken Hybrid Kir Channels

Using standard techniques in molecular biology, chimeric Kir channel protein may be prepared by inserting the turret region of a human Kir channel protein into the Kir2.2 chicken
25 sequence described above. The location of the turret regions are identified in Figures 1A-1C.

By way of example, site-directed mutagenesis procedures may be used to insert the coding sequence for a human turret region into a eukaryotic “scaffold” Kir channel coding region. In a preferred embodiment, Stratagene’s QuickChange® is used. QuickChange® utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two
30 synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers,

each complementary to opposite strands of the vector, are extended during temperature cycling by PfuTurbo DNA polymerase. The desired mutation (in this case - the insertion of the human turret region) should be in the middle of the primer with about 10-15 bases of correct sequence on both sides. Incorporation of the oligonucleotide primers generates a mutated plasmid

5 containing staggered nicks. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5'-Gm⁶ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all E. coli strains is Dam methylated and therefore susceptible to Dpn I digestion. The nicked vector DNA containing the
10 desired mutations is then transformed into XL1-Blue supercompetent cells. See, e.g. U.S. Patent Nos. 5,789,166, 5,932,419, and 6,391,548.

As an example, the chicken Kir2.2 protein may be used as a scaffold and the human Kir2.2 channel turret region synthesized for insertion. This methodology can be repeated with any combination of scaffold protein and human turret region.

15 **Preparation of Mutated turret regions**

Given the identification of the turret regions in the human Kir channels site directed mutagenesis or other techniques known in the art may be used to prepare proteins having mutations in the DNA sequence of the turret. In a preferred embodiment, Strategene's QuickChange® is used. Such mutations should be non-silent mutations – that is the mutations
20 should result in amino acid changes at positions within the turret region.

Generation of Antibodies

A human Kir protein or a chimeric Kir channel protein is prepared using standard techniques such as those outlined herein, and used in standard techniques to obtain antibodies.

A variety of antibodies may be used in the present invention and such antibodies include
25 but are not limited to polyclonal, monoclonal, human, humanized chimeric, an intact immunoglobulin molecule, an antibody fragment, single chain, ScFv, Fab fragments, F(ab)₂ Fab, Fv and a disulfide linked Fv.

Various procedures known in the art may be used for the production of antibodies. Host animals can be immunized by injection with a human Kir channel protein, or chimeric Kir

protein or fragments of these proteins. Animals which may be used to generate antibodies include, but are not limited to, rabbits, mice, rats, sheep, goats, and others known in the art. The human and chimeric proteins of the present invention may also be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various
5 adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

10 For preparation of monoclonal antibodies directed toward a Kir channel protein of the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [Nature 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., Immunology Today
15 4:72 1983]; Cote et al., Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 (1985)]. In addition, techniques developed for the production of "chimeric antibodies" [Morrison et al., J. Bacteriol. 159:870 (1984); Neuberger et al., Nature 312:604-608 (1984); Takeda et al., Nature 314:452-454 (1985)]
20 by splicing the genes from a mouse antibody molecule specific for an isolated Kir channel protein of the present invention, or conserved variants thereof, together with a fragment of a human antibody molecule of appropriate biological activity can be used.

Human antibodies can be prepared using any technique. Examples of techniques for human monoclonal antibody production include those described by Cole et al. (Monoclonal
25 Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985) and by Boerner et al. (J. Immunol., 147(1):86-95, 1991). Human antibodies (and fragments thereof) can also be produced using phage display libraries (Hoogenboom et al., J. Mol. Biol., 227:381, 1991; Marks et al., J. Mol. Biol., 222:581, 1991). Information on monoclonal and other types of therapeutic antibodies can also be found in Cellular and Molecular Immunology, 6th Edition, [A.K. Abbas, A.H. Lichtman,
30 S. Pillai (Saunders Elsevier Press, 2007)], and US patents 7,390,887 and 7,629,171. For a

discussion of various types of therapeutic antibodies, see Strategies and Challenges for the Next Generation of Therapeutic Antibodies, A. Beck, T. Wurch, C. Bailly and N. Corvaia, *Nature Rev. Immuno.* 10, 345-352 (2010).

Human antibodies can also be obtained from transgenic animals. For example,
5 transgenic, mutant mice that are capable of producing a full repertoire of human antibodies, in response to immunization, have been described (see, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551-255 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993)).

Humanized antibodies may also be used in the present invention. Antibody humanization
10 techniques generally involve the use of recombinant DNA technology to manipulate the DNA sequence encoding one or more polypeptide chains of an antibody molecule. Accordingly, a humanized form of a non-human antibody (or a fragment thereof) is a chimeric antibody or antibody chain (or a fragment thereof, such as an Fv, Fab, Fab', or other antigen-binding portion of an antibody) which contains a portion of an antigen binding site from a non-human (donor)
15 antibody integrated into the framework of a human (recipient) antibody. Methods for humanizing non-human antibodies are well known in the art. For example, humanized antibodies can be generated according to the methods of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986), Riechmann et al., *Nature*, 332:323-327 (1988), Verhoeven et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the
20 corresponding sequences of a human antibody. Methods that can be used to produce humanized antibodies are also described in U.S. Patent No. 4,816,567 (Cabilly et al.), U.S. Patent No. 5,565,332 (Hoogenboom et al.), U.S. Patent No. 5,721,367 (Kay et al.), U.S. Patent No. 5,837,243 (Deo et al.), U.S. Patent No. 5,939,598 (Kucherlapati et al.), U.S. Patent No. 6,130,364 (Jakobovits et al.), and U.S. Patent No. 6,180,377 (Morgan et al.).

25 Techniques described for the production of single chain antibodies [U.S. Pat. Nos. 5,476,786 and 5,132,405 to Huston; U.S. Pat. No. 4,946,778] can be adapted to produce single chain antibodies specific for a Kir channel protein. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab
30 fragments with the desired specificity for the Kir channel proteins.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Antibodies or fragments thereof, whether attached to other sequences or not, can also include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the antibody or antibody fragment is not significantly altered or impaired compared to the non-modified antibody or antibody fragment. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic acid encoding the antibody or antibody fragment.

Nucleic Acids

The compounds of the present invention include nucleic acids. In particular, nucleic acid sequences capable of binding to a Kir channel may be used in the practice of the present invention. These nucleic acids may be identified using in vitro selection of sequences which bind Kir channel proteins, in particular the turret region of the Kir channel proteins. One type of nucleic acid that is of particular interest is an aptamer. Typically aptamers are small nucleic acid sequences ranging from 15-50 bases in length that fold into defined secondary and tertiary structures that bind to another molecule. This binding is not the typical nucleic acid to nucleic acid hydrogen bond formation but the binding of aptamers can include all other types of covalent and noncovalent binding. In a preferred embodiment, the nucleic acid is DNA, however, other nucleic acids such as RNA may be used. The nucleic acids may be modified or prepared using techniques known in the art to increase the stability of nucleic acids. Representative examples of how to make and use aptamers to bind a variety of different target molecules can be found in the following United States patents: 5,582,981; 5,595,877; 5,637,459; 6,020,130; 6,028,186; 6,030,776; and 6,051,698. See also Published U.S. patent application 11/917,884 (publication No. US2009/0155779A1); Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ (Feb 1992). "Selection of single-stranded DNA molecules that bind and inhibit human thrombin" *Nature*

355(6360): 564-6; Bunka DH, Stockley PG (Aug 2006) "Aptamers come of age – at last" *Nat Rev Microbio.* 4(8): 588-96.

Small Protein/Peptide Compounds

Small Protein/Peptide Compounds may also be used in the practice of the present invention. In particular, small proteins may be prepared and screened for the ability to bind to a Kir channel protein based on binding assays disclosed herein and known in the art. Small molecules such as toxins may also be used in the practice of the invention. In particular, small proteins/peptides modeled on toxins which bind to Kir channel proteins may be prepared and tested for the ability to bind and modulate the activity of Kir channel proteins. (Ramu, et al. (2008) Engineered specific and high affinity inhibition for a subtype of inward rectifier Kir channels Proc. Nat'l Acid Sci USA 105:10774-10778)

A variety of toxins may provide information useful in designing a compound useful in the practice of the present invention. In particular, scorpion toxins (Lu and Mackinnon, 1997 Biochemistry, vol. 36, no. 23, pp 6936 to 6940) snake toxins (for example, the 57 amino acid δ -dendrotoxin from the green mamba snake which inhibits Kir 1.1 channels, (J.P. Imredy, C. Chen, R. Mackinnon,, BioChemistry 37, 14867 (Oct. 20, 1998)) and bee venom toxins (Ramu, et al. (2008) Engineered specific and high affinity inhibition for a subtype of inward rectifier Kir channels Proc. Nat'l Acid Sci USA 105:10774-10778) may be helpful in synthesizing libraries of protein/peptide compounds that can bind and effect a Kir channel. Known toxins are often small proteins typically between 20 and 50 amino acids in size containing disulfide bridges. For some of these toxins, the surface important for binding to a Kir channel protein is known and stretches of amino acids of less than 10 amino acids are believed to be important for binding specificity.

A library of these toxin-based compounds may be prepared while maintaining the key amino acids such as cysteine residues that are important for the folding and structure of the proteins. The amino acid residues important for binding to a Kir channel may be randomized to generate proteins/peptides with enhanced binding strength and turret based specificity for the different members of the Kir channel family of proteins.

Phage Display

One method known in the art to rapidly screen a large variety of potential binding proteins/peptides is a phage display assay.

Phage display libraries may be prepared using known protocols. "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface". *Science* **288** (4705): 1315-1317. Smith GP, Petrenko VA (1997). "Phage display". *Chem. Rev.* **97** (2): 391-410. Kehoe JW, Kay BK (2005). "Filamentous phage display in the new millennium". *Chem. Rev.* **105** (11): 4056-4072. Hufton SE, Moerkerk PT, Meulemans EV, de Br  ne A, Arends JW, Hoogenboom HR (1999). "Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands." *J. Immunol. Methods* **231** (1-2): 39-51. Lunder M, Bratkovic T, Doljak B, Kreft S, Urleb U, Strukelj B, Plazar N. (2005). "Comparison of bacterial and phage display peptide libraries in search of target-binding motif". *Appl. Biochem. Biotechnol.* **127** (2): 125-31. Lunder M, Bratkovic T, Kreft S, Strukelj B (2005). "Peptide inhibitor of pancreatic lipase selected by phage display using different elution strategies". *J. Lipid Res.* 2005 **46** (7): 1512-6.)

Using phage display the protein/peptide constructs may be expressed on the outer coat of the phage. To identify useful sequences a Kir channel protein may be immobilized on the surface of a well of a standard assay plate, and a phage that displays a protein that binds to Kir channels will bind the target Kir channel protein and remain bound while non-binding phage are removed by washing the plates. The bound phage can be eluted and used to produce more phage for further binding assays. These binding assays may be performed with Kir channels having mutated turrets and wild type turrets to select for proteins/peptides that bind in the turret region. Repeated cycles of these binding assays ('panning') results in the identification of phage containing potentially strong binding sequences.

Phage that contain these strong binding sequences can be used to infect a suitable bacterial host, and phagemids are collected and the DNA sequence of interest encoding the binding region excised and sequenced to identify the protein/peptide compound which be further tested using the assays described below.

Small Molecules

Small molecules may also be used in the practice of the present invention. In particular, small molecules may be prepared and screened for the ability to bind to a Kir channel protein based on binding assays disclosed herein and known in the art. See for example U.S. Patent No. 6,641,997. Additionally, small molecule libraries may also be screened.

Immunoassays

Once an antibody has been generated by the methods described above, a variety of different immunoassays may be performed to identify antibodies that bind properly folded Kir channels, are specific for the turret region of the Kir channel and can differentiate between different members of the Kir family based on the turret region.

It is believed that ELISA and Western blot assays are straightforward and efficient assays to identify such antibodies before performing functional assays such as electrophysiological assays.

In general, immunoassays involve contacting a Kir channel protein with an anti-Kir channel antibody under conditions effective, and for a period of time sufficient, to allow the formation of immune complexes (primary immune complexes). Forming such complexes is generally a matter of simply bringing into contact the antibody and the Kir channel protein sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, i.e., to bind to, any molecule (e.g., antigens) present to which the antibodies can bind.

In many forms of immunoassay, the sample-antibody composition, such as an ELISA plate or Western blot, can then be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected. Immunoassays can include methods for detecting or quantifying the amount of a molecule of interest (such as the disclosed biomarkers or their antibodies) in a sample. In general, the detection of an immunocomplex formation is well known in the art and can be achieved by numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, fluorescent, biological or enzymatic tags or any other known label. Such assays include but are not limited to ELISA, western blots,

radioimmunoassay, (enzyme-linked immunosorbant assay), “sandwich” immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays),
5 complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, and other assays known in the art.

Antibody binding can be detected by detecting a label on the primary antibody or the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. For some assays, the secondary antibody is labeled. Many means are known
10 in the art for detecting binding in an immunoassay and are within the scope of the present invention.

The use of immunoassays to detect a specific protein can involve the separation of the proteins by electrophoresis. Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the
15 net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique. Electrophoresis is used in the Western blots described below.

20 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA), or more generically termed EIA (Enzyme ImmunoAssay), is an immunoassay that can detect an antibody specific for a protein. In such an assay, a detectable label bound to either an antibody-binding or antigen-binding reagent is an enzyme. When exposed to its substrate, this enzyme reacts in such a manner as to produce a
25 chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Enzymes which can be used to detectably label reagents useful for detection include, but are not limited to, horseradish peroxidase, alkaline phosphatase, glucose oxidase, β -galactosidase, ribonuclease, urease, catalase, malate dehydrogenase, staphylococcal nuclease, asparaginase, yeast alcohol dehydrogenase, alpha.-glycerophosphate dehydrogenase, triose
30 phosphate isomerase, glucose-6-phosphate dehydrogenase, glucoamylase and

acetylcholinesterase. For descriptions of ELISA procedures, see Voller, A. et al., J. Clin. Pathol. 31:507-520 (1978); Butler, J. E., Meth. Enzymol. 73:482-523 (1981); Maggio, E. (ed.), Enzyme Immunoassay, CRC Press, Boca Raton, 1980; Butler, J. E., In: Structure of Antigens, Vol. 1 (Van Regenmortel, M., CRC Press, Boca Raton, 1992, pp. 209-259; Butler, J. E., In: van Oss, C. J. et al., (eds), Immunochemistry, Marcel Dekker, Inc., New York, 1994, pp. 759-803; Butler, J. E. (ed.), Immunochemistry of Solid-Phase Immunoassay, CRC Press, Boca Raton, 1991); Crowther, "ELISA: Theory and Practice," In: Methods in Molecule Biology, Vol. 42, Humana Press; New Jersey, 1995; U.S. Patent 4,376,110, each of which is incorporated herein by reference in its entirety and specifically for teachings regarding ELISA methods.

10 In preferred embodiments of the present invention, ELISA assays are used to identify antibodies that bind to the Kir channel proteins and are specific to the turret region.

As illustrated in FLOWCHART 1, a first ELISA assay is performed to identify antibodies that bind to the Kir channel protein. As an example, if a human Kir 2.2 channel protein is used as an antigen, an ELISA Assay is performed to identify antibodies that bind to human Kir 2.2

15 channel protein.

By way of an example ELISA assay, solutions are prepared as follows:

Buffer A: Protein buffer containing detergent slightly above CMC

Coating Solution - Buffer A + 20ug/ml Protein (50ul/well, 5.0 ml./plate)

20 Wash Solution - Buffer A (200ul/well x 14 washes; 2.8ml/well, 280ml total /plate)

Blocking Solution - Buffer A + 5% BSA (0.45u filtered) (400ul/well, 40 ml./plate)

Primary Ab solution - Cell culture supernatant (diluted 1:1 with 2X Buffer A/2% BSA) or control sera (1:100 in Buffer A + 2% BSA)

25 Secondary Ab Solution - 1:10,000 goat α mouse-Horseradish peroxidase conjugate in Buffer A + 2% BSA (100ul/well, 10 ml/plate)

Substrate Solution - 1:1 TMB:H₂O₂ (100ul/well, 10ml/plate)

Stop Solution - 2M H₂SO₄ (100ul/well, 10 ml./plate)

The following steps are then performed:

- 5 a) Add 50ul of coating solution to each well. Prepare coated plates the day before the assay and store at 4⁰C overnight, or prepare on day of assay and allow to shake for 1 hour at room temperature. Add solution directly to the bottom of the well, avoiding the sides as much as possible. Coat at least 2 more wells than you have samples for (+) and (-) controls. Leave at least 2 wells uncoated (just wash solution) as negative controls.
- b) Remove coating solution by pouring out and smacking plate face down on a paper towel. Wash wells 3X by adding 200ul of wash solution to each well, shaking for 1 minute, pouring out wash solution and smacking plates face down on paper towels.
- 10 c) Add 300ul of blocking solution to each well and let plates sit at room temperature for 2 hour.
- d) Remove blocking solution and wash wells 3X with 200ul of washing solution.
- e) Add 50 ul 2X Buffer to each well (except controls)
- 15 f) Add 50ul primary antibody solution to the 50 ul 2X buffer in each well and mix. Add diluted (+) control serum to a coated and uncoated well and diluted (-) control serum to coated and uncoated well. Let plates sit at room temperature for 1 hour on orbital shaker.
- g) Remove Primary Ab solution and wash wells 3X with 200ul washing solution.
- h) Add 100ul secondary Ab solution to each well and let plates sit at room temperature
20 for 1 hour.

The plates are then examined to determine if antibodies for a Kir channel protein are present using standard techniques as described above.

WESTERN BLOT

- 25 Western blotting or immunoblotting allows the determination of the molecular mass of a protein and the measurement of relative amounts of the protein present in different samples. Detection methods include chemiluminescence and chromagenic detection. Standard methods for Western blot analysis can be found in, for example, D.M. Bollag et al., Protein Methods (2d edition 1996) and E. Harlow & D. Lane, Antibodies, a Laboratory Manual (1988), U.S. Patent

4,452,901, each of which is herein incorporated by reference in their entirety for teachings regarding Western blot methods. Generally, proteins are separated by gel electrophoresis, usually SDS-PAGE. For the assays used in the present invention for initial antibody screening, it is preferred to use an SDS-PAGE so as to denature the Kir channel proteins used in the blot. The proteins are transferred to a sheet of special blotting paper, e.g., nitrocellulose, though other types of paper, or membranes, can be used. The proteins retain the same pattern of separation they had on the gel. The blot is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the nitrocellulose. An antibody is then added to the solution which is able to bind to its specific protein.

The attachment of specific antibodies to specific immobilized antigens can be readily visualized by indirect enzyme immunoassay techniques, usually using a chromogenic substrate (e.g. alkaline phosphatase or horseradish peroxidase) or chemiluminescent substrates. Other possibilities for probing include the use of fluorescent or radioisotope labels (e.g., fluorescein, ¹²⁵I). Probes for the detection of antibody binding can be conjugated anti-immunoglobulins, conjugated staphylococcal Protein A (binds IgG), or probes to biotinylated primary antibodies (e.g., conjugated avidin/ streptavidin).

As illustrated in FLOWCHART 1, a western blot is performed to determine if an antibody binds to the denatured form of a Kir channel protein. The combination of the ELISA and the Western blot Kir channel assays as illustrated in FLOWCHART 1 facilitates the identification of antibodies that recognize the properly folded native Kir channel (ELISA Positive) but not the denatured (Western Negative) form of the protein.

In particular, if a given antibody binds to a Kir channel protein in an ELISA assay ("ELISA Positive"), but fails to bind to the same Kir channel protein in a Western blot (Western Negative), then the antibody is binding to the native conformation of the protein but not the denatured form.

Identification of Antibodies with Turret Specificity

Given the discovery in the present invention of the importance of the turret regions and the identification of the region of the Kir proteins which constitute the turret region it is possible to prepare Kir channel proteins which contain mutations located in the turret region. This in turn

provides the basis for identification of antibodies that are specific for the turret region of the Kir channel proteins. In particular, the determination of the atomic structure of what constitutes the turret region of the Kir channels identifies where to introduce such mutations so as to selectively identify anti-Kir Channel antibodies that are directed against the turret. Such mutations may be made in a variety of places, such as following the L residue in the sequence HGDL (or slight variations of that sequence) and up to but not including the conserved cysteine labeled C123 in the structure of the proteins (see Figures 1A-1C). Examples of these variable portions of certain turret regions is provided in SEQ ID NOs 1-13. Kir channel proteins which contain mutations in the turret region, or preferably in the variable portion, may be used in assays described below.

To identify antibodies that are specific for the turret region of the Kir channel further ELISA assays may be performed as illustrated in FLOWCHART 1. These ELISA assays utilize Kir channels with mutated turret regions. Antibodies that bind a normal Kir channel in an ELISA assay but do not bind a channel with a mutated turret will be isolated since these antibodies may be considered turret specific – that is the epitope for the antibody is located in the turret region of the protein. The source of these antibodies will be used to prepare monoclonal antibodies using standard techniques as described above. An additional assay described below and presented in FLOWCHART 2 identifies antibodies or other compounds with the ability to bind the turret region using a fluorescent assay.

Assays for Kir Channel Activity

Even if an antibody or other type of compound binds the turret region its utility as a therapeutic compound is based on its functional effect on a Kir channel. Accordingly, the next step is to determine if an antibody which binds the turret region of a Kir channel is capable of modulating electrolyte processing. Monoclonal antibodies prepared from turret specific antibodies identified above can be used in electrophysiological assays as can other compounds found to have binding specificity for the turret region of Kir channels.

There are a variety of electrophysiological assays known to those with skill in the art which may be used to determine whether the compounds of the present invention have an effect on the electrophysiological state of a Kir Channel.

Useful electrophysiological assays include a variety of in vitro and in vivo assays, e.g., measuring voltage, current, measuring membrane potential, measuring ion flux, e.g., potassium or rubidium, measuring potassium concentration, measuring second messengers and transcription levels, and using e.g., voltage-sensitive dyes, radioactive tracers, electrode voltage clamps and patch-clamp electrophysiology. Such assays can be used to test for both inhibitors and activators of Kir channels.

Modulators of the Kir channels may be tested using biologically active, functional Kir channels, either recombinant or naturally occurring. In recombinantly based assays, the subunits are typically expressed and modulation is tested using one of the in vitro or in vivo assays described herein.

In brief, samples or assays that are treated with a potential Kir channel turret binding compounds inhibitors or activators are compared to control samples without the test compound, to examine the extent of modulation. Control samples e.g, those untreated with the compounds are assigned a relative Kir channel activity value of 100. Inhibition is present when Kir channel activity value relative to the control is about 90%, preferably 50%, more preferably 25%.

It should be noted that the compounds may also result in activation of Kir channels. Activation of channels is achieved when the select Kir channel activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher. It is possible that for treating some diseases states such activating compounds may be useful alone or in combination with inhibitors.

Changes in ion flux may be assessed by determining changes in polarization (i.e., electrical potential) of the cell or membrane expressing the Kir channels of this invention. A preferred means to determine changes in cellular polarization is by measuring changes in current (thereby measuring changes in polarization) with voltage-clamp and patch-clamp techniques, e.g., the "outside-out" mode, and the "whole cell" mode (see, e.g., Ackerman et al., New Engl. J. Med. 336:1575-1595 (1997) and *Single Channel Recording*, Plenum Press, B. Sakmann and E. Neher eds). Whole cell currents are conveniently determined using the standard methodology (see, e.g., Hamil et al., Pflugers. Archiv. 391:85 (1981). Other known assays include: radiolabeled rubidium flux assays and fluorescence assays using voltage-sensitive dyes (see, e.g., Vestergaard-Bogind et al., J. Membrane Biol. 88:67-75 (1988); Daniel et al., J. Pharmacol. Meth.

25:185-193 (1991); Holevinsky et al., J. Membrane Biology 137:59-70 (1994)). Assays for compounds capable of inhibiting or increasing potassium flux through the channel proteins can be performed by application of the compounds to a bath solution in contact with and comprising cells having an channel of the present invention (see e.g., Blatz et al., Nature 323:718-720
5 (1986); Park, J. Physiol. 481:555-570 (1994)). Generally, the compounds to be tested are present in the range from 1 pM to 100 μ M.

The effects of the test compounds upon the function of the Kir channels can be measured by changes in the electrical currents or ionic flux or by the consequences of changes in currents and flux. Changes in electrical current or ionic flux are measured by either increases or decreases
10 in flux of cations such as potassium or rubidium ions. The cations can be measured in a variety of standard ways. They can be measured directly by concentration changes of the ions or indirectly by membrane potential or by radiolabeling of the ions. Consequences of the test compound on ion flux can be quite varied. Accordingly, any suitable physiological change can be used to assess the influence of a test compound on the Kir channels of this invention. The
15 effects of a test compound can be measured by a toxin binding assay. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as transmitter release (e.g., dopamine), hormone release (e.g., insulin), transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), cell volume changes (e.g., in red blood cells), immunoresponses (e.g., T cell activation), changes
20 in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as $[Ca^{2+}]$.

Two Electrode Voltage Clamp Assay

One assay that may be of particular use in the present invention is a two electrode voltage clamp assay. This assay may be performed using any of these Kir channel proteins and
25 compounds of the present invention using modifications readily known to those in the art. In the example below, this assay was conducted using the chicken Kir 2.2 channel. To perform this assay, *Xenopus* oocytes will be harvested from mature female *Xenopus laevis* and defolliculated by collagenase treatment for 1-2 hours. Oocytes will then rinsed thoroughly and stored in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, 50 μ g/ml
30 gentamycin, pH 7.6 with NaOH). Defolliculated oocytes will be selected 2-4 hours after

collagenase treatment and injected with cRNA the next day. The injected oocytes will be incubated in ND96 solution for 1-5 days before recording. All oocytes will be stored in an incubator at 18 °C.

5 The desired human or chimeric Kir channel protein DNA will be sub-cloned into the pGEM vector (Promega). cRNA will be prepared using T7 RNA polymerase (Promega) from NdeI-linearized plasmid DNA.

10 All recordings will be performed at room temperature. For two-electrode voltage-clamp experiments, oocytes will be held at 0 mV and pulsed from -80 mV to +80 mV with 10 mV increment steps. Recording solution will contain 98 mM KCl, 0.3 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES pH 7.6. The ionic currents will be recorded with an oocyte clamp amplifier (OC-725C, Warner Instrument Corp.). The recorded signal will be filtered at 1kHz and sampled at 10kHz using an analogue-to-digital converter (Digidata 1440A, Axon Instruments, Inc) interfaced with a computer. pClamp10.1 software (Axon Instruments, Inc) will be used for controlling the amplifier and data acquisition.

15 **Patch clamp assays**

Patch clamp assays use a micropipette attached to a cell membrane to allow recording from a single ion channel in the cell membrane.

20 To perform this type of assay a micropipette which serves as a microelectrode is positioned next to a cell, and a piece of the cell membrane (the 'patch') is drawn into the microelectrode tip; the glass tip of the micropipette forms a high resistance 'seal' with the cell membrane, then whole cell mode is entered by applying suction. Next, the pipette is moved away from the cell to form an outside-out patch. Examples of useful protocols may be found in *Single Channel Recording*, Plenum Press, B. Sakmann and E. Neher eds. This configuration can be used to study Kir channels present in the isolated patch of membrane. Variations of this technique include the "perforated patch" technique, or the patch of membrane can be pulled away from the rest of the cell.

25 As an example, for patch-clamp experiments in the outside-out mode, each oocyte will be incubated in a hypertonic solution containing 200 mM NaCl, 130 mM KCl, 5 mM K₂EDTA, 5 mM K₂HPO₄, 5 mM KH₂PO₄ pH 7.2 for 5-10 minutes and the vitelline membrane will be

removed before seal formation. Currents will be recorded in either cell-attached or outside-out configuration with an Axopatch 200B amplifier, Digidata 1440A analogue-to-digital converter and pClamp10.1 software to control membrane voltage and record. During the current recordings, the membrane will be first held at 0 mV followed by a 10-second voltage ramp from
5 +80 mV to -80 mV. The pipette solution will contain 140 mM KCl, 5 mM K₂HPO₄, 5 mM KH₂PO₄, 0.3 mM CaCl₂, 1 mM MgCl₂, pH 7.2 with KOH. The bath solution will contain 130 mM KCl, 5 mM K₂EDTA, 5 mM K₂HPO₄, 5 mM KH₂PO₄, pH 7.2 with KOH.

To measure a compound for activity, first a control current is measured while perfusing with the recording solution without the compound present. Then, a second current is recorded
10 while perfusing with the solution and the compound of interest. Any difference in current levels indicates that the compound acts to modulate the activity of the Kir channel. An example of this type of assay may be found in Lu and MacKinnon 1997 Biochemistry, vol. 36, no. 23, pp. 6936-6940 or Namba et al., 1996 FEBS Letters vol. 386, pp. 211-214.

Planar lipid bilayer assay

15 Another electrophysiological assay which may be used in the present invention is a planar lipid bilayer assay. In this type of assay a lipid bilayer is created and the Kir channel protein is introduced into the lipid bilayer. A hydrophobic material such as Teflon is used to prepare the lipid bilayer by making a small hole (an aperture) in a sheet of Teflon. A syringe containing a solution of lipids dissolved in an organic solvent is introduced to the hole and a
20 bilayer is formed in the center of the aperture, with solvent forming the perimeter of the newly formed bilayer.

The Teflon sheet provides a partition between two chambers allowing the placement of electrodes on both sides of the sheet. Preferably, the purified Kir channel is reconstituted into lipid vesicles and then fused with the bilayer after it is formed. The detergent coating facilitates
25 insertion into the bilayer. (See US patent 6,191,254 and Guillermo et al J. Membrane Biol (2008) 223: 13-26).

The amount of lipid desired (preferably PE:PG 3:1) is pipetted into a glass vial (about 5-10 mg). The lipid is dried under argon and then further under a room temperature vacuum for about 3 hours.

The lipid is rehydrated with hydration buffer (10 mM HEPES 7.4 (KOH), 450 mM KCl, 4 mM N-methylglucamine, 2 mM DTT) to a final lipid concentration of 10 mg/ml and vortexed briefly. The glass vial is flushed with argon and the lipid mixture is sonicated mildly, i.e., with short pulses of no longer than 30 seconds each. In between sonication pulses, the lipid mixture is cooled in a room temperature water bath to ensure that the lipid mixture does not get too hot. This procedure is repeated until the lipid mixture becomes translucent with a distinct pink shade.

A solution containing 50 mM DM in the hydration buffer is prepared. The DM solution is added to the lipid mixture to give a final concentration of 10 mM of DM and rotated at room temperature for 2 hours. To the detergent/lipid mixture, the Kir channel is added to the desired ratios (e.g., about 0.05 – 0.1). The concentration of DM is then raised to 17.5 mM and the mixture is rotated at room temperature for 1 hour. The detergent/lipid mixture is then put into dialysis tubing and dialysed against the hydration buffer.

Fluorescent Dye Assay

Another assay which may be used to identify compounds which specifically bind the Kir channel turret region is an assay utilizing a fluorescent dye. An example of a suitable dye is FluxOR™ available from Invitrogen (catalog nos. F10016, F10017). An example of this method is shown in FLOWCHART 2.

The FluxOR™ reagent is a fluorogenic indicator dye, which is loaded into cells as a membrane-permeable Acetoxymethanol (AM) ester. According to the protocol, the FluxOR™ reagent is dissolved in DMSO and further diluted with the FluxOR™ assay buffer, a physiological Hank's balanced salt solution, for loading into cells. Pluronic® surfactants, which disperse and stabilize the dye are used to facilitate loading in aqueous solution.

Mammalian cells such as HEK, COS or CHO cells are grown in culture and incubated with the dye. Inside the cell, the non-fluorescent AM ester form of the FluxOR™ dye is cleaved by endogenous esterases into a fluorescent thallium-sensitive indicator. The thallium-sensitive form is retained in the cytosol and its extrusion is inhibited by water-soluble Probenecid, which blocks organic anion pumps. The dye-loading buffer is replaced with fresh, dye-free assay buffer, composed of physiological HBSS containing Probenecid, before the assay. During the assay, a small amount of thallium is added to the cells with a stimulus solution that opens

potassium-permeant ion channels with a mild depolarization or agonist addition. Thallium then passes into cells through open potassium channels according to a strong inward driving force. Upon binding cytosolic thallium, the de-esterified FluxOR™ dye exhibits a strong increase in fluorescence intensity at its peak emission of 525 nm. Baseline and stimulated fluorescence is monitored in real time to give a dynamic, functional readout of thallium redistribution across the membrane with no interference from quencher dyes.

Inhibitors such as, for example, the compounds of the present invention may slow the rate of entry of thallium and thus reduce the onset of a fluorescent signal. This assay may be used for the selection of compounds that specifically bind to the turret regions of the Kir proteins. To identify such compounds a first group of cells would be transfected with wild type Kir channels and a second group of cells transfected with Kir channel having mutated turrets as illustrated in FLOWCHART 2. Test compounds such as the antibodies identified in the assays above would be added to the cells to screen for those compounds which inhibit or reduce the onset of fluorescence upon addition of the thallium dye due to inhibition of the channel. Compounds which reduced the rate of thallium intake in cells with normal turrets but had no effect on cells with mutant turrets would be classified as turret specific inhibitor compounds.

This assay may also be used to determine the specificity of the compounds for given turrets, in other words, the compounds may be introduced into cells which have been transfected with different versions of the Kir Channel to determine if the compound is specific for a given type of Kir Channel protein.

Assay for Selective Binding to Specific Types of Kir Channels

In order to determine whether a given antibody is specific for a given type of Kir Channel, assays such as an ELISA assay may be performed in which an antibody is tested against a variety of different Kir Channels to determine if the antibody is specific for a single type of Kir Channel. Ideally, antibodies that would be used as therapeutic compounds will bind to only one type of Kir channel in the turret region.

Methods to Identify Compounds to Treat Conditions

Compounds that bind to the turret region of a Kir channel and which modulate the ion channel activity of a Kir channel may be administered to a subject to determine if such

compounds are able to treat a given condition. As an example, a compound may be administered to a subject such as a mammal with a given disease state using known methods of administration and the subject is then monitored clinically and tested using biochemical assays to determine if the compound is able to treat the condition using known assays for the disease state. It is

5 believed that a variety of conditions may be treated with the compounds of the present invention, including, but not limited to, diabetes mellitus, hypertension, cardiac arrhythmia and epilepsy.

The present invention may be better understood by reference to the following non-limiting example, which is provided as exemplary of the invention. This example should in no
10 way be construed, however, as limiting the broad scope of the invention. Example 1, which follows below, provides the first determination of the crystal structure of a eukaryotic Kir channel protein and identification of the structured turret region present in Kir channel proteins. (See *Crystal Structure of the Eukaryotic Strong Inward-Rectifier K⁺ Channel Kir2.2 at 3.1 Å Resolution*, X. Tao, J.L. Avalos, J. Chen and R. MacKinnon, Science 2009 December 18; 326
15 (5960); 1668.)

Example 1 - Crystal Structure of the Eukaryotic Strong Inward-Rectifier K⁺ Channel Kir2.2 at 3.1 Å Resolution.

Inward-rectifier K⁺ channels conduct K⁺ ions most efficiently in one direction, into the cell. Kir2 channels control the resting membrane voltage in many electrically excitable cells and
20 heritable mutations cause periodic paralysis and cardiac arrhythmia. We present the crystal structure of Kir2.2 from chicken, which, excluding the unstructured N- and C-termini, is 90% identical to human Kir2.2. Crystals containing Rb⁺, Sr²⁺ and Eu³⁺ reveal binding sites along the ion conduction pathway that are both conductive and inhibitory. The sites correlate with extensive electrophysiological data and provide a structural basis for understanding rectification.

25 The channel's extracellular surface, with large structured turrets and an unusual selectivity filter entryway, might explain the relative insensitivity of eukaryotic inward rectifiers to toxins. These same surface features also suggest a possible approach to the development of inhibitory agents specific to each member of the inward-rectifier K⁺ channel family.

A crystal structure reveals the structural basis of diode-like conduction properties and relative toxin insensitivity in inward rectifier K⁺ channels.

Introduction

5 In 1949 Bernard Katz introduced the term ‘anomalous rectification’ to distinguish the K⁺ currents he observed in frog skeletal muscle from the ‘delayed rectification’ K⁺ currents of the squid axon action potential (1, 2). Today we know that ‘delayed rectifiers’ are a subset of the large family of voltage-dependent K⁺ (Kv) channels, while ‘anomalous rectifiers’ are members of a different family of channels more commonly known as inward rectifier K⁺ (Kir) channels
10 (3). The name inward rectifier refers to a fundamental ion conduction property exhibited to a greater or lesser degree by all members of the family: given an equal but opposite electrochemical driving force, K⁺ conductance into the cell far exceeds conductance out of the cell. Thus, Kir channels are analogous to one-way conductors, or diodes, in solid-state electronic devices.

15 Electrophysiological experiments have shown that inward rectification is a consequence of voltage-dependent pore blockage by intracellular multivalent cations, especially Mg²⁺ and polyamines (4-8). At internal negative (hyperpolarizing) membrane voltages the blocking ions are cleared from the pore so that K⁺ conducts, whereas at internal positive (depolarizing) membrane voltages the blocking ions are driven into the pore from the cytoplasm so that K⁺
20 conduction is blocked. As a result, Kir channels are conductive when an excitable cell is at rest and non-conductive during excitation. This property is thought to foster energy efficiency because it enables Kir channels to regulate the resting membrane potential, but not dissipate the K⁺ gradient during an action potential (3).

 A central mechanistic question is why are Kir channels blocked by intracellular
25 multivalent cations? Mutational studies have identified several amino acids that confer sensitivity to blocking ions (9-19), but a structural description of these sites has remained elusive. Structures of prokaryotic Kir channels, due to their low sequence similarity to eukaryotic Kir channels, do not contain the specific amino acids that are known to underlie blockage and rectification (20, 21).

Another longstanding puzzle in eukaryotic Kir channel studies is their relative insensitivity to natural toxins that typically inhibit other K⁺ channels (22-24). Snake, spider and scorpion venoms, for example, contain numerous toxins against various Kv channels and Ca²⁺-activated K⁺ channels (25-27). By contrast, Kir channel toxins are rare, and no specific toxins against Kir2 channels have been discovered.

Results and Discussion

Eukaryotic Kir channels as a molecular family

The eukaryotic Kir channels contain several amino acid sequence motifs and conserved amino acids that are essential to their functional properties (Figures 1A-1C). For example, in most other K⁺ channels the selectivity filter comprises the 'canonical' filter sequence TXGYGDX, where X represents an aliphatic amino acid (Figures 1A-1C). The corresponding sequence in eukaryotic Kir channels is TXGYGFR, with F sometimes replaced by another amino acid. In light of the structural importance of DX in the canonical sequence, the amino acids FR signify a marked variation on the filter sequence. Eukaryotic Kir channels also contain an absolutely conserved pair of cysteine residues flanking the pore-region, which is the re-entrant peptide segment that forms the pore-helix and selectivity filter of K⁺ channels. Between the outer helix (the first transmembrane segment) and pore-region the 'turret', though varied amongst inward rectifiers, contains the sequence HGDL that could be considered a signature of eukaryotic Kir channels. Finally, through extensive studies combining electrophysiology and mutagenesis several acidic amino acids (D and E) are known to be critical to inward rectification (9-19), and motifs containing basic amino acids (e.g. PKKR) are critical to PIP₂ activation of Kir channels (28-35). These positions are enclosed in boxes on the sequences in Figures 1A-1C.

The Kir2.2 channel from chicken is 90% identical to the human ortholog (excluding the N- and C-termini) and contains all of the sequence characteristics of a strong inward rectifier (36). Figures 6A-6D shows that the chicken Kir2.2 channel expressed in *Xenopus* oocytes indeed functions as a strong rectifier. In oocyte two-electrode voltage clamp recordings with 98 mM KCl in the bath solution inward currents are much larger than outward currents (Figure 6B). In on-cell and excised gigaseal patch recordings channel activity is observed at hyperpolarizing (negative internal) membrane voltages but not at depolarizing (positive internal) voltages (Figure 6C). The single channel conductance measured near -80 mV is approximately 40 pS, which is

very similar to the values reported for the guinea pig and mouse Kir2.2 channels (37, 38) (Figure 6D, inset). The sharp transition between channel conductance and non-conductance as a function of membrane voltage is characteristic of a strong rectifier (36). Note that upon patch excision from the oocyte surface some outward current is observed at voltages slightly positive to the reversal potential because the concentration of intracellular blockers is decreased (Figure 6C, trace labeled (C)). However, the current still decreases with further depolarization (negative conductance) as channels become blocked in a voltage-dependent manner: this behavior reflects the inherent difficulty in washing away trace yet still active concentrations of polyamine molecules due to their very high affinity for the pore in strong rectifiers (39, 40). Several minutes following patch excision the currents decrease (Figure 6C, trace labeled (A)). This 'run-down' reflects altered channel regulation mediated by kinases, phosphatases and lipid signaling (34, 36, 41, 42).

In order to obtain diffracting crystals the intrinsically disordered N- and C-terminal regions were removed. The electrophysiological recordings shown in Figures 6A-6D were made using a similar construct with N- and C-terminal truncations, confirming that the crystal structure corresponds to a functional channel unit with strong rectifying properties. The Kir2.2 model, consisting of the cytoplasmic domain and transmembrane channel, was refined at 3.1 Å to a free R-factor of 0.27. A ribbon diagram in stereo shows the transmembrane pore (above) and the cytoplasmic pore (below) (Figure 2A). Lateral openings between the transmembrane and cytoplasmic pores, at the level of the lipid membrane headgroup layer, contain many arginine and lysine residues. The high density of positive charges makes it unlikely that K⁺ ions would pass through these openings (Figure 7). In Figure 7 the shading at the top of the Figure illustrates a negative electrostatic potential at the surface and the darker shaded region in the center of the Figure illustrates regions of positive electrostatic potential. The structure is therefore consistent with mutagenesis studies, which support the conclusion that the ion pathway extends across the full length of the transmembrane and cytoplasmic pores (9-19). The overall architecture is similar to prokaryotic Kir channels but with a notable difference: the Kir2.2 channel contains prominent, highly structured turrets on the extracellular face of the channel. These surround as if to protect the pore entryway.

The selectivity filter

At a detailed structural level Kir2.2 is quite different from prokaryotic Kir channels owing to minimal (<20%) sequence conservation. The cysteine pair that is absolutely conserved among eukaryotic Kir channels creates a circularized pore region through covalent linkage of the segment preceding the pore helix (C123) to the segment following the selectivity filter (C155) (Figure 2B). The existence of a disulfide bond was correctly predicted on the basis of mutagenesis studies: mutation of the corresponding cysteines in Kir2.1 led to the absence of currents even though expressed protein was detectable by Western Blot analysis (43, 44). Application of 10 mM DTT or reduced glutathione to the outside of cells expressing the wild-type channels did not affect currents. From these two observations it was concluded that a disulfide bridge must be essential for proper folding, but apparently not for function (43, 44). The structure provides an alternative interpretation. The disulfide bridge is buried beneath the protein surface at the level of the membrane interface. Furthermore, the Kir2.2 channel was purified and crystallized in the presence of 20 mM DTT and 3 mM TCEP, and yet the disulfide bridge remained intact. It is therefore possible that the disulfide bridge remains intact upon exposure to moderate concentrations of DTT, and that the bridge may be important for channel function.

The pore region is further stapled together by an ionized hydrogen bond between R149 in the filter sequence TXGYGFR and E139 (Figures 2B and 2C). The Glu O- ϵ to Arg N- η distance is 2.4 Å, compatible with an energetically strong interaction. Mutations altering this interaction are known to alter channel function (45, 46). On the basis of studies with concatenated subunits the salt bridge was thought to be inter-subunit, but the crystal structure shows that this interaction ties together two segments of the pore-region within a single subunit (46).

Despite the presence of substantially different protein contacts surrounding the selectivity filter, the main-chain structure of the filter in Kir2.2 is the same as in other K⁺ channels (47). For example, the main chain RMSD between Kv1.2 and Kir2.2 is 0.4 Å, which is within the margin of certainty to discriminate atomic positions with 3.1 Å diffraction data (Figures 2D and 2E) (20, 48, 49). One structural difference near the filter could possibly account for important pharmacological differences between Kir and other K⁺ channels. In the canonical filter sequence the Asp (D) residue in the filter sequence is buried, creating a flat surface surrounding the filter

opening. By contrast, in Kir channels the Phe (F) residue at the corresponding position projects directly into aqueous solution, creating four protrusions on the perimeter where the filter opens to the extracellular solution.

The cavity and gates

The pore lining on the intracellular side of the selectivity filter is mainly hydrophobic in nearly all K⁺ channels. Eukaryotic Kir channels are an exception in which the central region of the pore – known as the central cavity – contains four polar amino acids (one from each subunit) projecting toward the ion pathway (Figure 3A). In Kir2.2 and other strong rectifiers these polar amino acids are Asp (D173), whereas in weak rectifiers such as Kir1.1 and Kir6.1 they are Asn (Figure 1A-1C). On the basis of electrophysiological studies, Asp residues in the central cavity of strong rectifiers are hypothesized to influence the affinity of Mg²⁺ and polyamines by an electrostatic mechanism (12, 18).

Beneath the central cavity, residues I177 and M181 on the inner helices form two hydrophobic seals that close off the pore leading to the cytoplasm (Figure 3C). Kir2.2 is therefore physically shut at the ‘activation gate’ (50). Amino acids corresponding to positions 177 and 181 are also large and hydrophobic in most other eukaryotic Kir channels, but not in many other K⁺ channels (Figures 1A-1C). For example, in KcsA, Kv channels and prokaryotic Kir channels, the position corresponding to 177 usually contains a small and sometimes polar amino acid, typically Ala or Thr. In KcsA both seal positions contain small amino acids (Figure 3D). Because of the large hydrophobic residues at positions 177 and 181, the inner helices of Kir2.2 do not come as close together in the closed conformation as in KcsA (Figures 3C and 3D).

Figure 3E shows the cytoplasmic domain tetramer from the Kir2.2 channel superimposed onto the domain from Kir2.1, which was solved by crystallography in the absence of a transmembrane channel (11). Over most of the domain these structures are nearly identical. This observation supports the expectation (based on 80% sequence identity) that Kir2.2 should represent an excellent model for the complete Kir2.1 channel. In addition to the activation gate formed by the transmembrane inner helices, Kir channels have been proposed to have a second gate (G-loop) at the apex of the cytoplasmic domain tetramer (11, 51). The G-loop is physically open in Kir2.2 and closed in the Kir2.1 domain (Figures 3F and 3G). The differences in

conformation are due to local movements of the G-loop rather than rigid body motions of the cytoplasmic domains. Local G-loop movements contrast observations on the cytoplasmic domain of Kir3.1, in which G-loop opening appears associated with rigid body movements of domains in the tetramer (20).

5 Ion binding sites for conduction and inward rectification

Figure 4A-F shows the locations of ions in difference Fourier maps from crystals containing Rb^+ , Sr^{2+} , and Eu^{3+} . Rb^+ is a K^+ analog that conducts. Density for this ion is observed at multiple sites in the selectivity filter and at three positions within the pore on the intracellular side of the selectivity filter, but is absent in the central cavity (Figure 4A). The three occupied
10 intracellular positions are: immediately internal to the activation gate in the transmembrane pore, in the cytoplasmic pore internal to the G-loop, and at the entryway to the cytoplasmic pore. We refer to the two sites in the cytoplasmic pore as the upper and lower rings of charges, respectively (Figure 8). The presence of multiple sites along the pore occupied by conducting ions are a prerequisite for strong voltage-dependent block by intracellular cations that cannot
15 pass through the selectivity filter (12, 52-57).

Crystals of Kir2.2 were grown in the presence of 650 mM Rb^+ and yet electron density for Rb^+ is not observed in the cavity (Figure 4A). This finding is noteworthy because under similar conditions a strong monovalent cation peak is observed in the cavity of KcsA (47, 58). Native crystals of Kir2.2, grown in the presence of 150mM K^+ and 500mM Na^+ , show a weak
20 electron density peak at the cavity center with additional peaks on the perimeter, apparently bridging toward the D173 side-chain (Figure 3A). We can not discern whether these peaks represent a disordered ion, multiple ions, or a low occupancy K^+ (or Na^+) in the center, perhaps surrounded by water molecules hydrogen bonded to the Asp carboxylate. We can conclude, however, that the central cavity in Kir2.2, at least in the closed conformation, has cation
25 attractive properties that are different from KcsA.

The divalent cation Sr^{2+} should behave as an electron dense mimic of Mg^{2+} , a biologically important metal ion inhibitor of eukaryotic Kir channels (7, 8). In F_o-F_c Fourier maps from crystals with 10 mM Sr^{2+} , 500 mM Na^+ and 150 mM K^+ , density peaks due to Sr^{2+} are observed at three sites inside the pore intracellular to the selectivity filter: in the cavity, at the
30 upper ring and at the lower ring of charges (Figures 8 and 4B). The magnitude of the Sr^{2+} peak is

small in the cavity (3.4 σ) compared to the peaks at the upper (9.6 σ) and lower (7.2 σ) rings of charges. Separate experiments with crystals containing 200 mM Sr^{2+} support that the weak cavity peak is indeed due to Sr^{2+} , which is present apparently at relatively low occupancy. Detailed views of these sites are shown (Figures 4D-F). They each consist of planar rings of acidic amino acids arranged on the pore's perimeter. All three sites exhibit a preference for Sr^{2+} : 10 mM Sr^{2+} out competes 150 mM K^+ . This selectivity is likely to be electrostatic in origin. The sites are too wide (10.5 Å, 8.9 Å and 9.3 Å diameter for the cavity, upper and lower ring of charges) to mediate direct coordination of an ion at the center. Presumably ions at the center of these sites interact through bridging water molecules. Since each site has the potential to contain multiple negatively charged carboxyl groups, the resulting strong electric field is expected to create a good match for a multivalent cation. Crystals containing the lanthanide Eu^{3+} , which we assume to be trivalent (59), provide support for this hypothesis. An anomalous difference Fourier map shows that Eu^{3+} binds at only one site, the upper ring of charges. This site appears to be more electronegative than the others because it contains two concentric rings of acidic amino acids, E225 and E300.

Mutagenesis studies have identified several amino acids that, when mutated, affect the affinity of Mg^{2+} and polyamines in strong rectifiers. D173 in the cavity, E225 and E300 forming the upper ring of charges, and D256 forming the lower ring of charges are among those known to be important (9-19). The weak Sr^{2+} peak in the cavity might seem incompatible with the large influence that mutations of the cavity Asp (D173) have on Mg^{2+} affinity. However, the channel in the crystal is not in an applied electric field: in an electric field imposed by a depolarized (positive inside) membrane we expect that the distribution of blocker occupancies among the multiple sites will change. Specifically, we expect the blocking cations to be driven deeper into the pore toward the cavity. In correlating the crystallographic with electrophysiological data, it is most significant that the amino acids forming the Sr^{2+} sites in the crystal are the same amino acids that are known to affect blockage and rectification in electrophysiology experiments (36). Beyond providing a structural basis with which to explain past electrophysiological studies, the Kir2.2 structure also suggests many new experiments. For example, most studies on the mechanism of rectification have focused on electrostatic interactions between the positively charged blocker and negatively charged groups on the protein. But hydrophobic interactions between methylene groups of polyamine molecules and hydrophobic residues in the channel may

be important. In particular, we might anticipate that when the pore opens polyamines could interact strongly with the large hydrophobic amino acids at positions 177 and 181 when the leading amino group of the polyamine reaches into the central cavity (Figure 3C) (54).

Since the earliest investigations of strong inward rectifiers two important properties have been noted: a sharp transition from a conductive state to a non-conductive (blocked) state over a very narrow voltage range, and a dependence of the transition on the extracellular K^+ concentration (60-63). Specifically, the voltage at which the transition occurs shifts to more depolarizing values as extracellular K^+ concentration is increased. Both properties, the sharp transition (i.e. strong voltage dependence) and its dependence on extracellular K^+ , have been attributed to the simple notion that conducting ions and blocking ions compete for sites in the pore (12, 52-57, 64-66). The crystallographic data presented here support this conclusion. We observe in the crystal Rb^+ binding at the same sites that can bind multivalent blocking ions. Therefore a high extracellular K^+ (or Rb^+) concentration should favor occupation of the sites by conducting ions, and a more depolarizing voltage should be required to drive blocking ions into the pore from the cytoplasm to replace the conducting ions. Moreover, as blocking ions enter the pore from the intracellular side, the displaced conducting ions must move through the selectivity filter to the extracellular side. This is to say that movements of blocking and conducting ions must be coupled. Such coupling would have energetic consequences because movement of an ion across the membrane voltage difference constitutes work. In other words a blocking ion entering the pore will exhibit a voltage dependence that results from a combination of its own charge and the charge of the displaced ions. This can be the origin of strong voltage dependent block, which can be the origin of a biologically important property of strong rectifiers – their diode property of a sharp transition from a conductive to a non-conductive state as a function of membrane voltage (12, 52-55, 64).

The extracellular pore entryway and pharmacology of Kir channels

Two aspects of the structure may account for the fact that eukaryotic Kir channels, especially members of the Kir2 subfamily, are relatively insensitive to K^+ channel toxins (22-24). The turrets in Kir2.2 are larger and come closer together, constricting the pore entryway compared to Kv1.2; and F148 in the sequence TXGYGFR creates four protrusions on the surface at the pore opening (Figures 5A and 5B). Thus, in Kv channels the entryway is wider and the

pore opens onto intersecting grooves with a flat base, which form the docking surface for pore-blocking scorpion toxins (Figure 5B). In Kir2.2 the entryway is constricted and the grooves are absent (Figure 5A).

5 Though the shape of the eukaryotic Kir channel pore entryway might offer fewer opportunities for inhibitory protein-protein interactions, inhibition might occur by a somewhat different strategy. Inhibitors of Kir1.1 and Kir3.4 channels have been identified. A bee venom toxin, tertiapin, inhibits both of these channels (22). At 21 amino acids tertiapin is smaller than most other venom toxins so it might fit between the turrets more effectively. Alternatively, the turrets themselves might form the binding site for tertiapin (67-69). At 57 amino acids δ -
10 dendrotoxin from the green mamba snake is rather large and yet it inhibits Kir1.1 channels (23). Compared to tertiapin less is known about the binding site on the channel for δ -dendrotoxin, but one aspect of its inhibition is intriguing: the blocked state reduces single channel conductance to about 10% rather than inhibiting all the way. δ -dendrotoxin most likely binds to the turrets but is too large to fit tightly over the pore, which would imply that binding to the turret may be
15 sufficient to alter the channel's function.

 The idea that binding to the turrets could alter function is not surprising when one considers that the turret in Kir2.2 is not a loop, but forms a highly ordered structure (Figure 5C). The base of the turret is formed and pinned together by the HGDL sequence, which with only minor variation is found in all eukaryotic Kir channels (Figures 1A-1C and Figure 5D.) H108
20 stabilizes D110 through a hydrogen bond. The Asp (D) itself is hydrogen bonded to the amide nitrogen of C123, which effectively holds the two ends of the turret together. L111 projects from the surface of a short 3_{10} helix into the protein interior to make stabilizing hydrophobic interactions. Thus, the turrets are structurally important elements of the channel. Between the sequence HGDL and the first Cys of the disulfide bridge the turret sequence is highly variable
25 among Kir channel subtypes. The Kir2.1 channel becomes sensitive to tertiapin if the variable sequence is mutated to be Kir3.4-like (68). Therefore, the turrets appear to be structures through which specific inhibition of Kir channel subtypes might be achievable through directed evolution of specific protein binding partners.

Summary

This presents the atomic structure of a eukaryotic Kir channel, Kir2.2, a strong inward rectifier. The sequence TXGYGFR gives rise to a K⁺ selectivity filter stabilized by disulfide bridges and salt bridges that distinguish eukaryotic Kir channels. Multiple ion binding sites on the intracellular side of the selectivity filter can be occupied by conducting ions but exhibit higher affinity for multivalent blocking ions. Thus, blocking ions entering from the cytoplasm must displace conducting ions through the pore. This situation is expected to give rise to strong voltage-dependent block and diode-like conduction properties. Structural features of the extracellular pore entryway offer an explanation for the relative insensitivity of Kir channels to venomous toxins and a possible approach to the development of selective Kir channel inhibitors.

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70. The X-ray crystallographic coordinates and structure factor files have been deposited in

the Protein Data Bank with accession ID 3JYC.

Materials and Methods

Cloning, expression and purification

A synthetic gene fragment (Bio Basic, Inc.) encoding residues 38 to 369 of chicken
5 Kir2.2 channel (GI:118097849) was ligated into the XhoI/EcoRI cloning sites of a modified
pPICZ-B vector (Invitrogen). The resulting protein has green fluorescent protein (GFP) and a
1D4 antibody recognition sequence (TETSQVAPA) on the C-terminus (I), separated by a
PreScission protease cleavage site (SNSLEVLFO/GP).

The construct was linearized using PmeI and transformed into a HIS⁺ strain of SMD1163
10 of *Pichia pastoris* (Invitrogen) by electroporation (BioRad Micropulser). Transformants were
selected on YPDS plates containing 400-1200 µg/ml Zeocin (Invitrogen). Resistant colonies
were tested for expression by anti-1D4 tag Western Blot. For large-scale expression, small
cultures grown from the best expressing colony were diluted into BMGY media (Invitrogen) and
inoculated at 29 °C overnight, until OD₆₀₀ reached between 20-30. Cells were then pelleted,
15 resuspended in BMM media (Invitrogen) and expressed overnight at 24 °C. Cells were
harvested, flash-frozen in liquid N₂, and stored at -80 °C until needed.

Cells were lysed in a Retsch, Inc. Model MM301 mixer mill (5 x 3.0 minutes at 25 cps).
The lysis buffer contained 150 mM KCl, 50 mM TRIS-HCl pH 8.0, 0.1 mg/ml
deoxyribonuclease I, 0.1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.1 mg/ml soy
20 trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml AEBSF, with 1 mM phenylmethanesulfonyl
fluoride added just before lysis (3.0 ml lysis buffer/g cells). pH of the lysate was adjusted to 8.0
with KOH. The lysate was extracted with 100 mM DM (n-decyl-β-D-maltopyranoside, Anatrace,
solgrade) at room temperature for 1 hour with stirring, and then centrifuged for 40 minutes at
30,000g, 10 °C. Supernatant was added to 1D4-affinity resin pre-equilibrated with 150 mM KCl,
25 50 mM TRIS-HCl pH 8.0, and 4 mM DM. Suspension was layered with Argon and mixed by
inversion for 2 hours at room temperature. Beads were collected on a column by gravity, washed
with 2 column volumes of buffer (150 mM KCl, 50 mM TRIS-HCl pH 8.0, 1 mM EDTA pH 8.0,
and 4 mM DM), and eluted with buffer plus 1 mg/ml 1D4 peptide (AnaSpec, Inc.) over 1 hour at
room temperature. 20 mM DTT (Dithiothreitol) and 3 mM TECP were added to eluted protein.

The protein was then digested with PreScission protease (20:1 w/w ratio) overnight at 4 °C. Concentrated protein was further purified on a Superdex-200 gel filtration column in 150 mM KCl, 20 mM TRIS-HCl pH 8.0, 4 mM DM (anagrade), 3 mM TCEP, 20 mM DTT and 1 mM EDTA at 4 °C.

5 The fraction corresponding to the tetramer peak was concentrated to about 8 mg/ml, mixed 1:1 with crystallization solution and set up as hanging drops over reservoirs containing 0.1ml crystallization solution. Crystals appeared in 7-20% PEG400 or 2-10% PEG4000, with 500 mM KCl or NaCl, and 50 mM buffer pH 6.0-9.5 at 4 °C overnight and grew to full size within 2-3 days.

10 For studies with RbCl, the protein was purified in a similar fashion except that KCl was replaced with RbCl in all buffer solutions and crystals were grown in 10-20% PEG400, 500 mM RbCl, and 50 mM MES pH 6.5. For studies with 10 mM EuCl₃, crystals were grown in 7-20% PEG400, 1 M ammonium formate, 50 mM TRIS-HCl pH 8.5, and 10 mM EuCl₃. For studies with 10 mM SrCl₂, crystals were grown in 10-20% PEG400, 500 mM NaCl, 50 mM HEPES pH
15 7.5, and 10 mM SrCl₂. For studies with 200 mM SrCl₂, crystals were grown in 3-7% PEG4000, 200 mM SrCl₂, and 50 mM Na Citrate pH 5.6.

Structure determination

Crystals were cryo-protected in reservoir plus 25% glycerol (v/v), 4 mM DM, 20 mM DTT, 3 mM TCEP, and 1 mM EDTA in a step-wise manner (5% glycerol increase each step)
20 and flash-frozen in liquid nitrogen. Diffraction data from native crystals were collected to 3.1 Å at beamline 24ID-C (APS) and for crystals in various metal ions (Rb⁺, Sr²⁺, and Eu³⁺) at beamline X29 (Brookhaven NSLS). Images were processed with DENZO and intensities merged with SCALEPACK (2). Data were further processed using the CCP4 suite (3). The crystals belong to the I4 space group. The structure was solved by molecular replacement using
25 the program MOLREP (4), with the 2.4 Å resolution structure of the cytoplasmic domain of mouse Kir2.1 (PDB 1U4F) as a search model. There is one copy of the subunit in the asymmetric unit. The model was built using O (5) and refined with CNS (50-3.1 Å) to $R_{\text{free}}=27.2\%$ (6). The final model contains residues 43-60 and 70-369 (residues 70 to 78 are modeled as alanines) of chicken Kir2.2, three additional residues SNS on the C-terminus
30 corresponding to the PreScission cleavage site, and five K⁺ ions. During the final minimization

refinement step in CNS, occupancies of the K^+ ions were set to 0.5 (which gave rise to a lower R_{free} compared to occupancy of 1.0) and B-factors of the K^+ ions were set to 85 (roughly the average B-factor of surrounding protein atoms). Crystallographic data and refinement statistics are shown in Table S1. Figures were made using PYMOL (www.pymol.org) (7).

5 Ion binding was assessed by calculating anomalous difference Fourier maps for data with Eu^{3+} and F_o-F_c maps for data with Rb^+ and Sr^{2+} using fft in the CCP4 suite (3). Sr^{2+} was analyzed at two different concentrations to discern whether the weak cavity peak was due to Sr^{2+} . This peak became stronger when Sr^{2+} was increased from 10 mM to 200 mM while the monovalent cation concentration was decreased, consistent with Sr^{2+} being present in the cavity but probably
10 at low occupancy. Phases used to calculate F_o-F_c omit maps were derived from a channel model devoid of ions in the cavity or cytoplasmic domain throughout refinement.

Electrophysiology

Xenopus oocytes were harvested from mature female *Xenopus laevis* and defolliculated by collagenase treatment for 1-2 hours. Oocytes were then rinsed thoroughly and stored in
15 ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, 50 μ g/ml gentamycin, pH 7.6 with NaOH). Defolliculated oocytes were selected 2-4 hours after collagenase treatment and injected with cRNA the next day. The injected oocytes were incubated in ND96 solution for 1-5 days before recording. All oocytes were stored in an incubator at 18 °C.

20 The chicken Kir2.2 (residues 38 to 369) gene was sub-cloned into the pGEM vector (Promega). cRNA was prepared using T7 RNA polymerase (Promega) from NdeI-linearized plasmid DNA.

All recordings were performed at room temperature. For two-electrode voltage-clamp experiments, oocytes were held at 0 mV and pulsed from -80 mV to +80 mV with 10 mV
25 increment steps. Recording solution contained 98 mM KCl, 0.3 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES pH 7.6. The ionic currents were recorded with an oocyte clamp amplifier (OC-725C, Warner Instrument Corp.). The recorded signal was filtered at 1kHz and sampled at 10kHz using an analogue-to-digital converter (Digidata 1440A, Axon Instruments, Inc) interfaced with a computer. pClamp10.1 software (Axon Instruments, Inc) was used for

controlling the amplifier and data acquisition. For patch-clamp experiments, each oocyte was incubated in a hypertonic solution containing 200 mM NaCl, 130 mM KCl, 5 mM K₂EDTA, 5 mM K₂HPO₄, 5 mM KH₂PO₄ pH 7.2 for 5-10 minutes and the vitelline membrane was removed before seal formation. Currents were recorded in either cell-attached or inside-out configuration with an Axopatch 200B amplifier, Digidata 1440A analogue-to-digital converter and pClamp10.1 software to control membrane voltage and record. During the current recordings, the membrane was first held at 0 mV followed by a 10-second voltage ramp from +80 mV to -80 mV. The pipette solution contained 140 mM KCl, 5 mM K₂HPO₄, 5 mM KH₂PO₄, 0.3 mM CaCl₂, 1 mM MgCl₂, pH 7.2 with KOH. The bath solution contained 130 mM KCl, 5 mM K₂EDTA, 5 mM K₂HPO₄, 5 mM KH₂PO₄, pH 7.2 with KOH.

For the single channel I-V curve shown in Figure S1D inset, each data point represents the current difference at a given voltage associated with the opening of a single channel.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

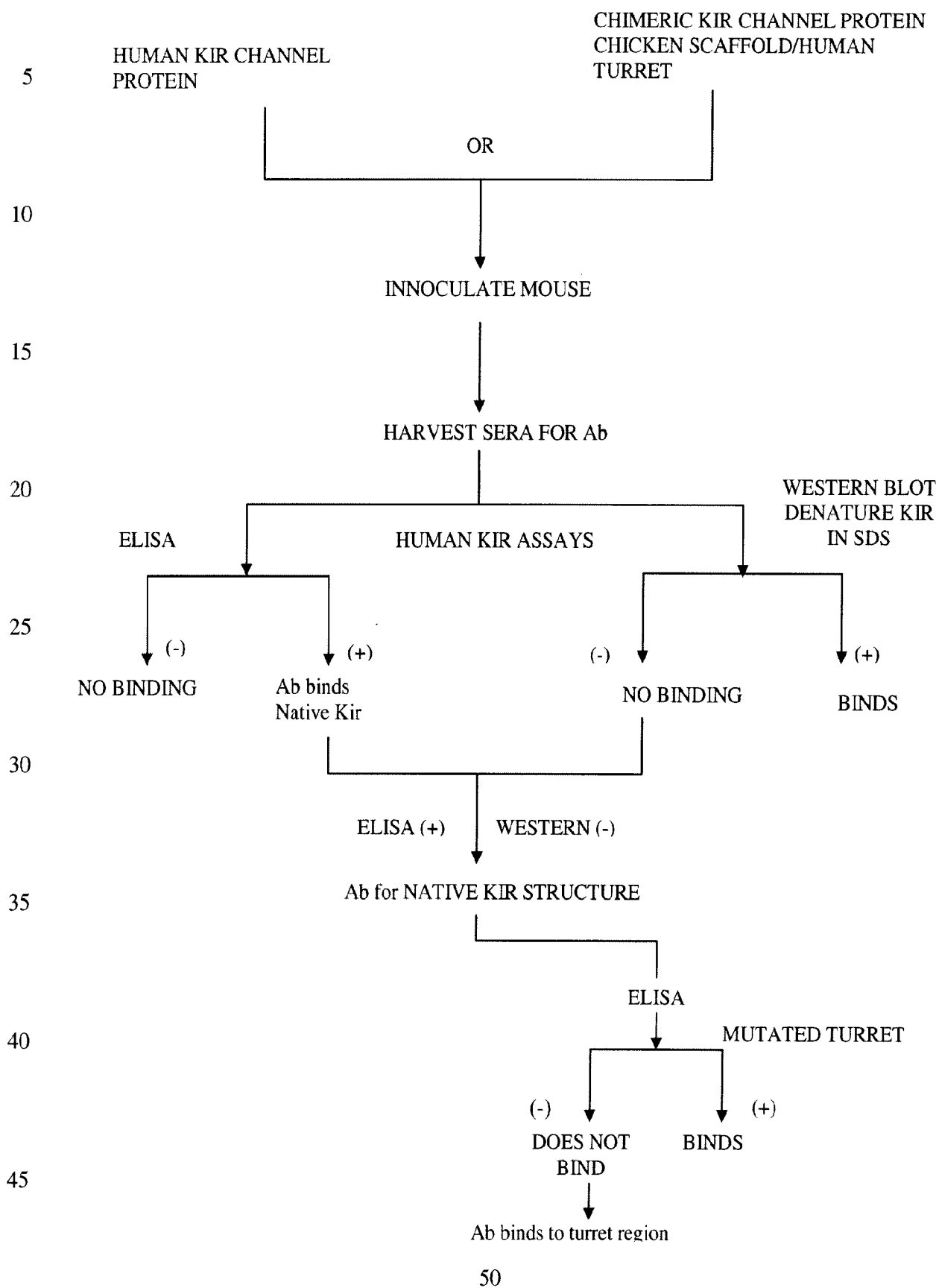
It is further to be understood that all values are approximate, and are provided for description.

All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference.

Table S1 Crystallographic data and refinement statistics

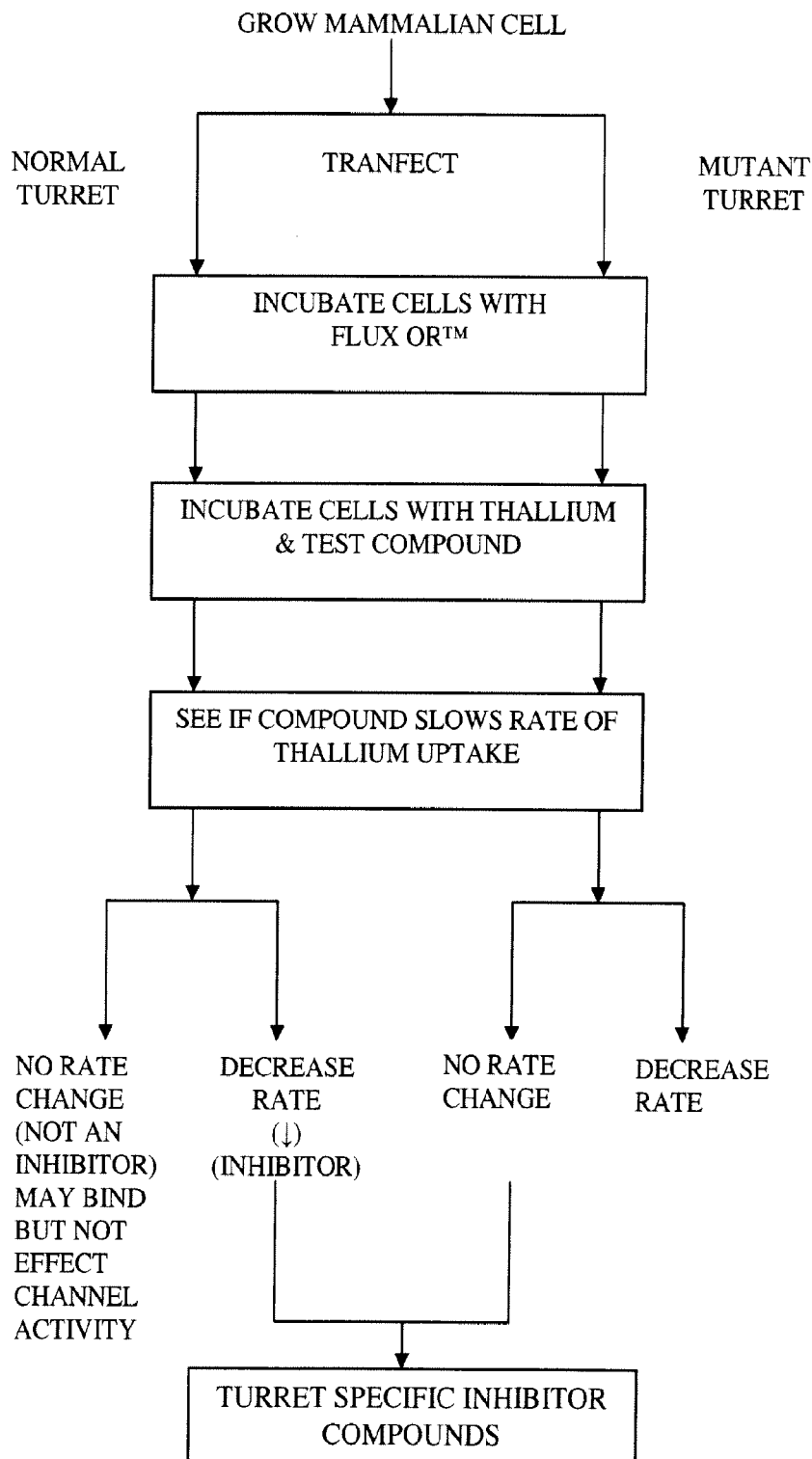
Data Collection		Native	Rb ⁺ (650 mM)	Sr ²⁺ (10 mM)	Sr ²⁺ (200 mM)	Eu ³⁺ (10 mM)
Data set		I4	I4	I4	I4	I4
Space group		a = b = 84.018, c = 196.121	a = b = 82.714, c = 196.142	a = b = 82.806, c = 195.605	a = b = 83.268, c = 197.143	a = b = 84.221, c = 195.915
Lattice constants (Å)		$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$
Source		APS 24ID-C	BNL X29	BNL X29	BNL X29	BNL X29
Wavelength (Å)		0.97949	1.0809	1.0809	1.0809	1.5222
Resolution (Å)		50-3.1	50-4.0	50-3.3	50-3.8	50-6.0
Total / unique observations		65,452 / 12,153	25,333 / 5,231	60,844 / 9,910	31,462 / 6,571	5,649 / 1,517
I / sigma (I) ^a		33.1 (2.3)	20.0 (1.8)	24.3 (2.4)	20.8 (2.7)	24.7 (1.6)
Redundancy ^a		5.4 (4.6)	4.8 (4.3)	6.1 (5.9)	4.8 (4.7)	3.7 (1.4)
Completeness (%) ^a		99.7 (99.9)	93.3 (83.8)	99.8 (100.0)	99.7 (100.0)	87.9 (49.4)
R _{sym} (%) ^{a,b}		6.8 (73.6)	10.1 (78.3)	11.5 (89.4)	9.6 (69.3)	7.0 (35.7)
Model refinement						
Resolution (Å)		50-3.1	50-4.0	50-3.3	50-3.8	50-6.0
Number of reflections		12,122 (609)	5,221 (272)	9,895 (504)	6,569 (346)	1,517 (76)
R _{work} / R _{free}		24.4 / 27.2	29.6 / 35.0	24.9 / 28.6	25.8 / 30.4	36.5 / 39.9
R.m.s. deviation of bond length (Å)		0.010				
R.m.s. deviation of bond angles (°)		1.59				
Protein atoms / K ⁺ ions		2,530 / 5				
Mean B value		112.1				
Ramachandran plot ^c		76.0 / 21.9 / 2.1				
R.m.s., root mean-squared.						

^aNumber in the parentheses represents statistics for data in the highest resolution shell.^bR_{sym} = $\Sigma |I_i - \langle I_i \rangle| / \Sigma I_i$, where $\langle I_i \rangle$ is the average intensity of symmetry equivalent reflections.^cThe three numbers represent the percentage of residues in most favored / additionally allowed / generously allowed regions.

FLOWCHART 1

FLOWCHART 2**FLUORESCENT ASSAY FOR TURRET BINDING SPECIFICITY**

5



SEQUENCES:

1. human Kir 1.1 – variable portion of turret region
PEFHPSANHTP
- 5 2. human Kir 1.2 – variable portion of turret region
LELDPPANHTP
3. human Kir 2.1 – variable portion of turret region
DASKEGKA
- 10 4. human Kir 2.2 – variable portion of turret region
EPAEGRGRTP
5. human Kir 2.3 – variable portion of turret region
EASPGVPAAGGPAAGGGGAAPVAPKP
6. human Kir 2.4 – variable portion of turret region
15 AAPPPPAP
7. human Kir 3.1 – variable portion of turret region
NKAHVGNYTP
8. human Kir 3.4 – variable portion of turret region
DHVGDQEWIP
- 20 9. human Kir 6.1 – variable portion of turret region
YAYMEKSGMEKSGLESTV
10. human Kir 6.2 – variable portion of turret region
APSEGTAEP
11. human Kir 7.1 – variable portion of turret region
25 ELDHDAPPENHTI
12. chicken Kir 2.1 – variable portion of turret region

ENQENNKP

13. chicken Kir 2.2 – variable portion of turret region

ENPGGDDTFKP

5 14. human Kir 1.1 –amino acid sequence

1 MNASSRNVFD TLIRVLTESM FKHLRKWVVT RFFGHSRQRA RLVS KDGR CN IEF GNVEAQS
 61 RFIFFVDIWT TVLDLKWRYK MTIFITAF LG SWFFFGLLWY AVAYIHKDLP EFHPSANHTP
 121 CVENINGLTS AFLFSLETQV TIGYGFR CVT EQCATAIFLL IFQSILGVII NSFMC GAILA
 181 KISRPKKRAK TITFSKNAVI SKRGKKLCLL IRVANLRKSL LIGSHIYGKL LKTTVTPEGE
 10 241 TIILDQININ FVVDAGNENL FFISPLTIYH VIDHNSPFFH MAAETLLQQD FELVVFLDGT
 301 VESTSATCQV RTSYVPEEVL WGYRFAPIVS KTKEGKYRVD FHNFSKTVEV ETPHCAMCLY
 361 NEKDVRARMK RGYDNP NFIL SEVNETDDTK M

15. human Kir 1.2 –amino acid sequence

1 MTSVAKVYYS QTTQTESRPL MGPGIRRRRV LTKDGRSNVR MEHIADKRFL YLKD LWTTFI
 15 61 DMQWRYKLLL FSATFAGTWF LFGVVWYLVA VAHGD LLELD PPANHTPCVV QVHTLTGAFL
 121 FSLESQTTIG YGFRYISEEC PLAIVLLIAQ LVLTTILEIF ITGTFLAKIA RPKKRAETIR
 181 FSQHAVVASH NGKPCLMIRV ANMRKSL LIG CQVTGKLLQT HQTKEGENIR LNQVNVTFQV
 241 DTASDSPFLI LPLTFYHVVD ETSPLKDLPL RSGEGDFELV LILSGTVEST SATCQVRTSY
 301 LP EEILWGYE FTPAISLSAS GKYIADFSLF DQVVKVASPS GLRDSTVRYG DPEK LKLEES
 20 361 LREQAEKEGS ALSVRISNV

16. human Kir 2.1 –amino acid sequence

1 MGSVRTNRY S IVSSEEDGMK LATMAVANGF GNGKSKVHTR QQCRSRFVKK DGH CNVQFIN
 61 VGEKGQRYLA DFTTCVDIR WRWMLVIFCL AFVLSWLFPG CVFWLIAL LH GDLDASKEGK
 121 ACVSEVNSFT AAFLFSIETQ TTIGYGFR CV TDECPIAVFM VVFQSI VGC I IDAFIIGAVM
 25 181 AKMAKPKKR N ETLVFSHNAV IAMRDGKLCL MWRVGNLRKS HLVEAHVRAQ LLKSRTITSEG
 241 EYIPLDQIDI NVGFDSGIDR IFLVSPITIV HEIDEDSPLY DLSKQDIDNA DFEIVVILEG
 301 MVEATAMTTQ CRSSYLANEI LWGHRYPVL FEEKHYKVD YSRFHKTYEV PNTPLCSARD
 361 LAEKKYILSN ANSFCYENEV ALTSKEEDDS ENGVPESTST DTPPDIDLHN QASVPLEPRP
 421 LRRESEI
 30

17. human Kir 2.2 –amino acid sequence

1 MTAASRANPY SIVSSEEDGL HLVTMSGANG FGNGKVHTR RCRNRFVKK GQCNI EFANM
 61 DEKSQRYLAD MFTTCVDIRW RYMLLIFSLA FLASWLLFGI IFWVIAVAHG DLEPAEGRGR
 121 TPCVMQVHGF MAAFLFSIET QTTIGYGLRC VTEEC PAVF MVVAQSIVGC IIDSFMIGAI
 35 181 MAKMARPKKR AQTL LFSHNA VVALRDGKLC LMWRVGNLRK SHIVEAHVRA QLIKPRVTEE
 241 GEYIPLDQID IDVGFDKGLD RIFLVSPITI LHEIDEASPL FGISRQDLET DDFEIVVILE
 301 GMVEATAMTT QARSSYLANE ILWGH RFEPV LFEEKNQYKI DYSHFHKTYE VPSTPRCSAK
 361 DLVENKFLLP SANSFCYENE LAFLSRDEED EADGDQDGRS RDGLSPQARH DFDRLQAGGG
 421 VLEQRPYRRE SEI
 40

18. human Kir 2.3 –amino acid sequence

1 MHGHSRNGQA HVPRRKRNR FVKNGQC NV YFANLSNKSQ RYMADIFTTC VDTRWRYMLM
 61 IFSA AFLVSW LFFGLLFWCI AFFHGDLEAS PGVPAAGGPA AGGGGAAPVA PKPCIMHVNG
 121 FLGAFLSV E TQTTIGYGR CVTEECPLAV IAVVVSIVG CVIDSFMIGT IMAKMARPKK
 45 181 RAQTLLFSHH AVISVRDGKL CLMWRVGNLR KSHIVEAHVR AQLIKPYMTQ EGEYLPDQR

241 DLNVGYDIGL DRIFLVSPII IVHEIDEDSP LYGMGKEELE SEDFEIVVII EGMVEATAMT
 301 TQARSSYLAS EILWGHRFEP VVFEEKSHYK VDYSRFHKTY EVAGTPCCSA RELQESKITV
 361 LPAPPPPPSA FCYENELALM SQEEEEEMEEE AAAAAVAAG LGLEAGSKEE AGIIRMLEFG
 421 SHLDLERMQA SLPLDNISYR RESAI

5

19. human Kir 2.4 –sequence

1 MGLARALRRL SGALDSGDSR AGDEEEAGPG LCRNGWAPAP VQSPVGRRRG RFVKKDGHCHN
 61 VRFVNLGGQG ARYLSDLFTT CVDVRRWRMC LLFSCSFLAS WLLFGLAFWL IASLHGDLAA
 121 PPPAPCFSH VASFLAAFLF ALETQTSIGY GVRSVTEEC P AAVAAVVLQC IAGCVLDAFV
 181 VGAVMAKMAK PKKRNETLVF SENAVVALRD HRLCLMWRVG NLRRSHLVEA HVRAQLLQPR
 241 VTPEGEYIPL DHQDQDVGF D GGTDRIFLVS PITIVHEIDS ASPLYELGRA ELARADFELV
 301 VILEGMVEAT AMTTQCRSSY LPGELLWGHF FEPVLFQGRS QYEVDYRHFH RTYEVPGTPV
 361 CSAKELDERA EQASHSLKSS FPGSLTAFY ENELALSCCQ EEDEDEDETEE GNGVETEDGA
 421 ASPRVLTPTL ALTLPP

10

15

20. human Kir 3.1 –amino acid sequence

1 MSALRRKFGD DYQVVTTSSS GSGLQPQGPQ QDPQQQLVPK KKRQRFVDKN GRCNVQHGNL
 61 GSETSRYLSD LFTTLVDLKW RWNLFIFILT YTVAWLFMAS MWWVIAYTRG DLNKAHVGNV
 121 TPCVANVYNF PSAFLFFIET EATIGYGYRY ITDKCPEGII LFLFQSILGS IVDAFLIGCM
 181 FIKMSQPKKR AETLMFSEHA VISM RDGKLT LMFRVGNLRN SHMVSAQIRC KLLKSRQTPE
 241 GEFLPLDQLE LDVGFSTGAD QLFLVSPLTI CHVIDAKSPF YDLSQRSMQT EQFEIVVILE
 301 GIVETTGMT C QARTSYTEDE VLWGHRRFPV ISLEEGFFKV DYSQFHATFE VPTPPYSVKE
 361 QEEMLLMSSP LIAPAITNSK ERHNSVECLD GLDDITTKLP SKLQKITGRE DFPKKLLRMS
 421 STTSEKAYSL GDLPMKLQRI SSVPGNSEEK LVSKTTKMLS DPMSQSVADL PPKLQKMAGG
 481 AARMENGLPA KLKMNNSDRF T

20

25

21. human Kir 3.4 –amino acid sequence

1 MAGDSRNAME QDMEIGVTPW DPKKIPKQAR DYVPIATDRT RLLAEGKKPR QRYMEKSGKC
 61 NVHHGNVQET YRYLSDLFTT LVDLKWRENL LVFTMVYTVT WLFPGFIWWL IAYIRGDLDH
 121 VGDQEWIPCV ENLSGFVSAF LFSIETETI GYGFRVITEK CPEGIILLV QAILGSIVNA
 181 FMVGC MFVKI SQPKKRAETL MFSNNAVISM RDEKLCMLFR VGDLRNSHIV EASIRAKLIK
 241 SRQTKGEFEI PLNQTDINVG FDTGDDRLFL VSPLIISHEI NQKSPFWEMS QAQLHQEEFE
 301 VVILEGMVE ATGMTCQARS SYMDTEVLWG HRFTPVLTLE KGFYEVDYNT FHDYETNTPT
 361 SCCAKELAEM KREGRLQYL PSPPLGGCA EAGLDAEAEQ NEEDEPKGLG GSREARGSV

30

35

22. human Kir 4.1 – amino acid sequence

1 MTSVAKVYYS QTTQTESRPL MGP GIRRRRV LTKDGRSNVR MEHIADKRFL YLKDLWTTFI
 61 DMQWRYKLLL FSATFAGTW F LFGVVWYLVA VAHGDLEELD PPANHTPCVV QVHTLTGAFL
 121 FSLESQTTIG YGFRYISEEC PLAIVLLIAQ LVLTTILEIF ITGTFLAKIA RPKKRAETIR
 181 FSQHAVVASH NGKPCLMIRV ANMRKSLIG CQVTGKLLQT HQTKEGENIR LNQVNVTFQV
 241 DTASDSPFLI LPLTFYHVVD ETSPLKDLPL RSGEGDFELV LILSGTVEST SATCQVRTSY
 301 LPEEILWGYE FTPAISLSAS GKYIADFSLF DQVVKVASPS GLRDSTVRYG DPEKCLKLEES
 361 LREQAEKEGS ALSVRISNV

40

23. human Kir 4.2 – amino acid sequence

1 MDAIHIGMSS TPLVKHTAGA GLKANRPRVM SKSGHSNVRI DKVDGIYLLY LQDLWTTVID
 61 MKWRYKLTFL AATFVMTWFL FGVIYYAIAF IHGDLEPGEP ISNHTPCIMK VDSLTTGAFL
 121 SLESQTTIGY GVRSITEEC P HAIFLLVAQL VITTLIEIFI TGTFLAKIA RPKKRAETIKF
 181 SHCAVITKQN GKLC LVIQVA NMRKSLIQ CQLSGKLLQTH VTKEGERILL NQATVKFHVD
 241 SSESPEFLIL PMTFYHVLDE TSPLRDLTPQ NLKEKEFELV VLLNATVEST SAVCQSRTSY

50

301 IP EEIYW GFE FVPVVSLSKN GKYVADFSQF EQIRKSPDCT FYCADSEKQQ LEEKYRQEDQ
 361 RERELRTL LL QQSNV

24. human Kir 5.1 – amino acid sequence

5 1 MSYYGSSYHI INADAKYPGY PPEHIIAEKR RARRRLLHKD GSCNVYFKHI FGEWGSYVVD
 61 IFTTLVDTKW RHMVFVIFSL YILSWLIFGS VFWLIAFHG DLLNDPDITP CVDNVHSFTQ
 121 AFLFSLETQT TIGYGYRCVT EECSVAVLMV ILQ SILSCII NTFIIGAALA KMATARKRAQ
 181 TIRFSYFALI GMRDGLCLM WRIGDFRPNH VVEGTVRAQL LRYTEDSEGR MTMAFKDLKL
 241 VNDQIILVTP VTIVHEIDHE SPLYALDRKA VAKDNFEILV TFIYTG DSTG TSHQSRSSYV
 10 301 PREILWGHFR NDVLEVKKRY YKVNCLQFEG SVEVYAPFCS AKQLDWKDQQ LHIEKAPPVR
 361 ESCTSDTKAR RRSFSAVAIV SSCENPEETT TSATHEYRET PYQKALLTLN RISVESQM

25. human Kir 6.1 – amino acid sequence

15 1 MLARKSIPE EYVLARIAAE NLRKPRIRDR LPKARFIAKS GACNLAHKNI REQGRFLQDI
 61 FTTLVDLKW RHTLVIFTSF LCSWLLFAIM WWLVAFAGD IYAYMEKSGM EKSGLESTVC
 121 VTNVRSFTSA FLFSIEVQVT IGFGGRMMTE ECPLAITVLI LQNIIVGLIIN AVMLGCIFMK
 181 TAQAHRRAET LIFSRHAVIA VRNGKLCFME RVGDLRKSMI ISASVRIQVV KKTTTPEGEV
 241 VPIHQDLIPV DNPIESNNIF LVAPLIICHV IDKRSPLYDI SATDLANQDL EVIIVILEGVV
 301 ETTGITTQAR TSYIAEEIQW GHRFVSIVTE EEGVYSVDYS KFGNTVKVAA PRCSARELDE
 20 361 KPSILIQTLQ KSELHQNSL RKRNSMRRNN SMRRNNSIRR NNSSLMPKV QFMTPEGNQN
 421 TSES

26. human Kir 6.2 – amino acid sequence

25 1 MLSRKGIIPE EYVTRLAED PAKPRYRARQ RRARFVSKKG NCNVAHKNI REQGRFLQDV
 61 TTLVDLKWPH TLLIFTMSFL CSWLLFAMAW WLIAFAHGD APSEGTAEP VTSIHSFSSA
 121 FLFSIEVQVT IGFGGRMVTE ECPLAILILI VQNIIVGLMIN AIMLGCIFMK TAQAHRRAET
 181 LIFS KHAVIA LRHGRLCFML RVGDLRKSMI ISATIHMQVV RKTTSPEGEV VPLHQVDIPM
 241 ENGVGGNSIF LVAPLIYHV IDANSPLYDL APSDLHHHQD LEIIVILEGV VETTGITTQA
 301 RTSYLADEIL WQGRFVPIVA EEDGRYSVDY SKFGNTVKVP TPLCTARQLD EDHSLLEALT
 30 361 LASARGPLRK RSVPMAKAKP KFSISPDLS

27. human Kir 7.1 – amino acid sequence

35 1 mdssnckvia pllsqryrm vtkdghstlq mdgaqrgray lrdawgilmd mrwrwmmllvf
 61 sasfvvhwlv favlwyvlae mngdleldhd appenhticv kyitsftaaf sfsletqlti
 121 gygtmfpsgd cpsaiallai qmlglmllea fitgafvaki arpknrafsi rftdtavvah
 181 mdgkpnlifq vantrpsplt svrvsavlyq erengklyqt svdfhldgis sdecpf f f f p
 241 ltyyhsitps splatllqhe npshfelvvf lsamqegtge icqrtsylp seimlh h c f a
 301 slltrgskge yqikmenfdk tpefptplv skspnrtdld ihingqsidn fqisetglte

28. chicken Kir 2.1 – amino acid sequence

40 1 MGSVRTNRY S IVSSEEDGMK LATMAVANGF GNGKSKVHTR QQCRSRFVKK DGHCVNQFIN
 61 VGEKGQRYLA DIFTTCVDIR WRWMLVIFCL TFILSWLFFG CVFWLIAL LH GDLENQENNK
 121 PCVSQVSSFT AAFLFSIETQ TTIGYGFR CV TDECPIAVFM VVFQSI V GCI IDAFIIGAVM
 181 AKMAKPKKR N ETLVFSHNAV VAMRDGKLCL MWRVGNLRKS HLVEAHVRAQ LLKSRTSEG
 241 EYIPLDEIDI NVGFDSGIDR IFLVSPITIV HEIDEDSPLY DLSKQDMDNA DFEIVVILEG
 45 301 MVEATAMTTQ CRSSYLANEI LWGHRYEPVL FEEKNYKVD YSRFHKTYEV PNTPIC SARD
 361 LAEKYILSN ANSFCYENEV ALTSKEEDEI DTGVPESTST DTHPMDHNN QAGVPLEPRP
 421 LRRESEI

29. chicken Kir 2.2 – amino acid sequence

1 mtagrvnpys ivsseedglr lttmpgingf gngkihtrrk crnrfvkkng qcnveftnmd
 61 dkpqryiadm fttcvdirwr ymlllfslaf lvswllfgli fwlialihgd lenpggddtf
 121 kpcvlqvngf vaafllfsiet qttigygfrc vteecplavf mvvvqsivgc iidsfmigai
 181 makmarpkkr aqtlflfshna vvamrdgklc lmwrvgnlrk shiveahvra qlikpritee
 5 241 geyipldqid idvgfdkgld riflvspiti lheinedspl fgisrqdlet ddfeivvile
 301 gmveatamtt qarssylase ilwghrfepv lfeeknqykv dyshfhktye vpstprcsak
 361 dlvenkfillp stnsfcyene lafmsrdede edddsrldd lspdnrhefd rlqatialdq
 421 rsyrresei

10 30. human Kir 1.1 – cDNA

1 atgaatgctt ccagtcggaa tgtgtttgac acgttgatca ggggtgtgac agaaagtatg
 61 ttcaaacatc ttccgaaatg ggctcgctact cgcttttttg ggcattctcg gcaaagagca
 121 aggctagtct ccaaagatgg aaggtgcaac atagaatttg gcaatgtgga ggcacagtca
 181 aggtttatat tctttgtgga catctggaca acggtacttg acctcaagtg gagatacaaa
 15 241 atgaccattt tcatcacagc cttcttgggg agttgggttt tctttggtct cctgtggtat
 301 gcagtagcgt acattcacaa agacctcccg gaattccatc cttctgccaa tcacactccc
 361 tgtgtggaga atattaatgg ottgacctca gcttttctgt tttctctgga gactcaagtg
 421 accattggat atggattcag gtgtgtgaca gaacagtgtg ccactgccat ttttctgctt
 481 atctttcagt ctatacttgg agttataatc aattctttca tgtgtggggc catcttagcc
 20 541 aagatctcca ggcccaaaaa acgtgccaa accattacgt tcagcaagaa cgcagtgatc
 601 agcaaacggg gagggaaagt ttgcctccta atccagtggtg ctaatctcag gaagagcctt
 661 cttattggca gtcacattta tggaaagctt ctgaagacca cagtcactcc tgaaggagag
 721 accattatct tggaccagat caatatcaac tttgtagtgt acgctgggaa tgaatattta
 781 ttcttcatct cccatttgac aatttaccat gtcattgatc acaacagccc tttcttcac
 25 841 atggcagcgg agaccttct ccagcaggac tttgaattag tgggtgtttt agatggcaca
 901 gtggagtcca ccagtgtctac ctgccaaagc cggacatcct atgtcccaga ggaggtgctt
 961 tggggctacc gttttgctcc catagtatcc aagacaaaag aagggaaata ccgagtggtat
 1021 ttccataact ttagcaagac agtggaagtg gagaccctc actgtgccat gtgcctttat
 1081 aatgagaaaag atgttagagc caggatgaag agaggctatg acaaccccaa cttcatcttg
 30 1141 tcagaagtca atgaaacaga tgacaccaa atgtaa

31. human Kir 1.2 – cDNA

1 cttttctgat cccagctccg ggtttaagag tcttggcaag gcccgtcgca cagctctgct
 35 61 cctaactcct gcccgccccg tccgtccatc tgtcccgtg ccccgcgcc catccaaggg
 121 gccactccac ctccgaccca agatgacgtc agttgccaa gtgtattaca gtcagaccac
 181 tcagacagaa agccggcccc taatggggcc agggatacga cggcggagag tctgtacaaa
 241 agatgggtgc agcaacgtga gaatggagca cattgccgac aagcgttcc tctacctcaa
 301 ggacctgtgg acaaccttca ttgacatgca gtggcgctac aagcttctgc tcttctctgc
 40 361 gacctttgca ggcacatggt tctctttgt cgtgggtgtg tatctggtag ctgtggcaca
 421 tggggacctg ctggagctgg acccccgggc caaccacacc cctgtgtgg tacaggtgca
 481 cacactcact ggagccttcc tcttctccct tgaatcccaa accaccattg gctatggctt
 541 ccgtacatc agtgaggaat gtccactagc cattgtgctt cttattgccc agctggtgct
 601 caccaccatc ctggaaatct tcatcacagg taccttctct gcgaagattg cccggcccaa
 45 661 gaagcgggct gagaccatc gtttcagcca gcatgcagtt gtggcctccc acaatggcaa
 721 gccctgcctc atgatccgag ttgccaatat gcgcaaaagc ctctcattg gctgccaggt
 781 gacaggaaaa ctgcttcaga cccaccaaac caaggaaagg gagaacatcc ggctcaacca
 841 ggtcaatgtg actttccaag tagacacagc ctctgacagc cccttcctta ttctaccctt
 901 taccttctat catgtggtag atgagaccag tcccttgaaa gatctccctc ttgcagtggtg
 50 961 tgagggtgac tttgagctgg tgctgatoct aagtgggaca gtggagtcca ccagtgccac
 1021 ctgtcaggtg cgcacttccct acctgccaga ggagatcctt tggggctacg agttcacacc
 1081 tgccatctca ctgtcagcca gtggtaaata catagctgac tttagccttt ttgaccaagt
 1141 tgtgaaagtg gcctctccta gtggcctccg tgacagcact gtacgtacg gagaccctga
 1201 aaagctcaag ttggaggagt cattaagggg gcaagctgag aaggagggca gtgcctttag

1261 tgtgcgcacac agcaatgtct ga

32. human Kir 2.1 – cDNA

```

5      1 atgggcagtg tgcgaaccaa ccgtacacgc atcgtctctt cagaagaaga cggatgaag
      61 ttggccacca tggcagttgc aaatggcttt gggaacggga agagttaaagt ccacaccgga
      121 caacagtgca ggagccgctt tgtgaagaaa gatggccact gtaatgttca gttcatcaat
      181 gtgggtgaga aggggcaacg gtacctcgca gacatcttca ccacgtgtgt ggacattcgc
      241 tggcgggtgga tgcctggttat cttctgcctg gctttcgtcc tgtcatgggt gttttttggc
      301 tgtgtgtttt ggttgatagc tctgtcccat ggggacctgg atgcatccaa agagggcaaa
10     361 gcttgtgtgt ccgaggtcaa cagcttcacg gctgccttcc tcttctccat tgagaccag
      421 acaaccatag gctatggttt cagatgtgtc acggatgaat gcccaattgc tgttttcatt
      481 gtgggtgttcc agtcaatcgt gggctgcacg atcgatgctt tcatcattgg cgcagtcatt
      541 gccaatgttg caaagccaaa gaagagaaac gagactcttg tcttcagtca caatggcgtg
      601 attgccatga gagacggcaa gctgtgtttg atgtggcgag tgggcaatct tcggaaaagc
15     661 cacttggttg aagctcatgt tcgagcacag ctctcctaat ccagaattac ttctgaaggg
      721 gagtatatcc ctctggatca aatagacatc aatgttggtt ttgacagtgg aatcgatcgt
      781 atattttctg tgcctccaat cactatagtc catgaaatag atgaagacag tcttttatat
      841 gatttgagta aacaggacat tgacaacgca gactttgaaa tcgtgggtcat actggaaggc
      901 atggtggaag ccactgccat gacgacacag tgccgtagct cttatctagc aaatgaaatc
20     961 ctgtggggcc accgctatga gcctgtgtc tttgaagaga agcactacta caaagtggac
      1021 tattccaggt tccacaaaac ttacgaagtc cccaacactc ccctttgtag tgccagagac
      1081 ttagcagaaa agaaatatat cctctcaaat gcaaattcat ttgctatga aaatgaagtt
      1141 gccctcacia gcaaagagga agacgacagt gaaaatggag ttccagaaag cactagtagc
      1201 gacacgcccc ctgacataga ccttcacaa caggcaagtg tacctctaga gcccaggccc
25     1261 ttacggcgag agtcggagat atga

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33. human Kir 2.2 – cDNA

```

30     1 atgaccgcgg ccagccgggc caacccctac agcatcgtgt catcggagga ggacgggctg
      61 caccctggta ccatgtcggg cgccaacggc ttgggcaacg gcaaggtgca caccggcgcc
      121 aggtgcccga accgcttcgt caagaagaat ggccagtga acattgagtt cgccaacatg
      181 gacgagaagt cacagcgcta cctggctgac atgttcacca cctgtgtgga catccgctgg
      241 cggtagcatg tgcctcatct ctcgctggcc ttccttgctt cctggctgct gttcggcatc
      301 atcttctggg tcatcgcggt ggcaacgggt gacctggagc cggctgaggg ccggggccgc
      361 acaccctgtg tgatgcaggt gcacggcttc atggcgccct tctcttctc catcgagacg
35     421 cagaccacca tcggctacgg gctgcgctgt gtgacggagg agtgcccggt ggccgtcttc
      481 atggtggttg cccagtcctt cgtgggctgc atcatcgact ccttcattat tgggtccatc
      541 atggccaaga tggcaaggcc caagaagcgg gcacagacgc tgctgttcag ccacaacgcc
      601 gtggtggccc tgcgtgacgg caagctctgc ctcatgtggc gtgtgggtaa cctgcgcaag
      661 agccacattg tggaggccca tgtgcgcgcg cagctcatca agccgcggtt caccgaggag
40     721 ggcgagtaca tcccgttggg ccagatcgac atcgatgtgg gcttcgacaa gggcctggac
      781 cgcactcttc tgggtgtcgc catcaccatc ttgcatgaga ttgacgaggc cagcccgctc
      841 ttccgcatca gccggcagga cctggagacg gacgactttg agatcgtggt catcctggaa
      901 ggcattggtg agggccacag catgaccacc caggcccgca gctcctacct ggccaatgag
      961 atcctgtggg gtcaccgctt tgagcccggt ctcttcgagg agaagaacca gtacaagatt
45     1021 gactactcgc acttcacaaa gacctatgag gtgcctctta cgcctcgctg cagtgcgaag
      1081 gatctggtag agaacaagtt cctgctgccc agcgccaact ccttctgcta cgagaacgag
      1141 ctggccttcc tgagccgtga cgaggaggat gaggcggacg gagaccagga cggccgaagc
      1201 cgggacggcc tcagcccccga ggccaggcat gactttgaca gactccaggc tggcggcggg
50     1261 gtccctggagc agcgcccta cagacgggag tcagagatct ga

```

34. human Kir 2.3 – cDNA

1 atgcacggac acagccgcaa cggccaggcc cactgcccc ggcggaagcg ccgcaaccgc

5 61 ttcgtcaaga agaacggcca atgcaacgtg tacttcgcca acctgagcaa caagtcgcag
 121 cgctacatgg cggacatctt caccacctgc gtggacacgc gctggcgcta catgctcatg
 181 atcttctccg cggccttcc tgtctctcgg ctctttttcg gcctcctctt ctgggtgtatc
 241 gccttcttcc acggtgacct ggaggccagc ccagggggtgc ctgcggcggg gggcccggcg
 301 gcggtgggtg gcggagcagc cccgggtggc cccaagccct gcacatgca cgtgaacggc
 361 ttcttgggtg ccttctgtt ctgggtggag acgcagacga ccatcggtta tgggttccgg
 421 tgcgtgacag aggagtggc gctggcagtc atcgctgtgg tggtcagtc catcgtgggc
 481 tgcgtcatcg actccttcat gattggcacc atcatggcca agatggcgcg gcccaagaag
 541 cgggcgcaga cgttgctgtt cagccaccac gcggtcattt cggtgcgga cggcaagctc
 10 601 tgcctcatgt ggcgctggg caacctggc aagagccaca ttgtggaggc ccacgtgcgg
 661 gcccagctca tcaagcccta catgaccagc gagggcgagt acctgcccct ggaccagcgg
 721 gacctcaacg tgggctatga catcggtcgt gaccgcattt tcttgggtgc gcccatcatc
 781 attgtccacg agatcgacga ggacagcccg ctttatggca tgggcaagga ggagctggag
 841 tcggaggact ttgagatcgt ggtcatcctg gagggcatgg tggaggccac ggccatgacc
 15 901 acccaggccc gcagctccta cctggccagc gagatcctgt ggggccaccg ctttgagcct
 961 gtgggtcttcg aggagaagag ccactacaag gtggactact cacgttttca caagacctac
 1021 gaggtggccg gcacgccctg ctgctcggcc cgggagctgc aggagagtaa gatcacctg
 1081 ctgcccgcgc caccgcccc tccagtgcc ttctgctacg agaacgagct ggccttatg
 1141 agccaggagg aagaggagat ggaggaggag gcagctgcgg cggccgcggg ggccgcaggc
 20 1201 ctgggccttg agcggtgtc caaggaggag gcgggcatca tccggatgct ggagtccggc
 1261 agccacctgg acctggagcg catgcaggct tccctccgc tggacaacat ctctaccgc
 1321 agggagtctg ccactctga

35. human Kir 2.4 – cDNA

25 1 atgggcctgg ccagggccct acgcgcctc agcggcgccc tggattcggg agacagccgg
 61 gcgggcgatg aagaggaggc cgggccggg ttgtgcgcga acgggtgggc gccggcacccg
 121 gtgcagtcac cgtggggcgg gcgcgcgggt cgcttcgtca agaaagacgg gcaactgcaac
 181 gtgcgtttcg taaacctggg tggccagggc gcgcgtacc tgagcgacct gttcaccaca
 241 tgcgtggacg tgcgtggcg ctggatgtgc ctgctcttct cctgctcctt cctgcctcc
 30 301 tggctgctct tggcctggc cttctggctc attgcctcgc tgcacggcga cctggccgcc
 361 ccgccaccgc ccgcgcctg cttctcacac gtggccagct tccctggcgc cttctcttcc
 421 gcgctggaga cgcagacgtc catcggtac ggcgtgcga gcgtaccga ggagtgcgcg
 481 gccgtgtgg ccgcgtggg gctgcagtgc attgcgggt gcgtgctga cgccttcgtc
 541 gtgggtgctg tcatggccaa gatggccaaa cccaagaagc gcaacgagac gctggcttcc
 35 601 agcgagaacg ccgtcgtggc gctgcgcgac caccgcctct gcctcatgtg gcgcgtcggc
 661 aacctgcgcc gcagccacct ggtcgaggcc cacgtgcgtg ccagctgct gcagccccgt
 721 gtgacccagc aggggtgagta catcccgctg gaccaccagg atgtggatgt gggctttgat
 781 ggaggcaccg atcgatctt cctcgtgtcc cccatcacca tcgtccatga gatcgactct
 841 gccagtcctc tgtatgagct aggacgtgcc gagctggcca gggctgactt tgagctgggtg
 40 901 gtcattctcg aggggatggg tgaggccaca gccatgacca cacagtgtcg ctgctcctac
 961 ctccctgggt aactgctctg gggccatcgt tttagccag ttctcttcca gcgtggctcc
 1021 cagtatgagg tcgactatcg ccacttccat cgcacttatg aggtcccagg gacaccggtc
 1081 tgcagtgcga aggagctgga tgaacgggca gagcaggctt cccacagcct caagtctagt
 1141 tccccgggt ctctgactgc attttgttat gagaatgaac ttgctctgag ctgctgccag
 45 1201 gaggaagatg aggacgatga gactgaggaa gggaatgggg tggaaacaga agatggggct
 1261 gctagccccc gagttctcac accaaccctg gcgtgaccc tgcctccatg a

36. human Kir 3.1 – cDNA

50 1 atgtctgcac tccgaaggaa atttggggac gattatcagg tagtgaccac atcgtccagc
 61 ggctcgggct tgcagcccca ggggccaggc caggaccctc agcagcagct tgtgcccagg
 121 aagaagcggc agcggttcgt ggacaagaac ggccggtgca atgtacagca cggcaacctg
 181 ggcagcgaga caagccgcta cctctcggac ctcttcacca cgctgggtgga cctcaagtgg
 241 cgctggaacc tcttcatctt cattctcacc tacaccgtgg cctggctttt catggcgctc
 301 atgtggtggg tgatcgcta cactcggggc gacctgaaca aagccacgt cggtaactac

361 acgccttgcg tggccaatgt ctataacttc ccttctgcct tcctcttctt catcgagacg
 421 gaggccacca tcggctatgg ctaccgatac atcacagaca agtgcctcga gggcatcatc
 481 ctcttctctt tccagtcctt cctgggctcc atcgtggacg ccttctctat cggtgcatg
 541 ttcatacaaga tgtcccagcc caagaagcgc gccgagaccc tcattgttcag cgagcacgcg
 5 601 gtgatctcca tgagggacgg aaaactcacg cttatgttcc ggggtgggcaa cctgcgcaac
 661 agccacatgg tctccgcgca gattcgtctc aagctgctca aatctcgga gacacctgag
 721 ggtgagttcc ttcccttga ccaacttgaa ctggatgtag gttttagtag aggggcagat
 781 caacttttcc ttgtgtcccc cctcacaatt tgccacgtga tcgatgcca aagccctttt
 841 tatgacctat cccagcgaag catgcaaact gaacagttcg agattgtctg catcctagaa
 10 901 ggcattgtgg aaacaactgg gatgacttgt caagctcgaa catcatatac tgaagatgaa
 961 gttcttttgg gtcacgtttt ttttctgtta atttctttag aagagggatt ctttaaagtt
 1021 gattactccc agttccatgc aacatttgaa gtccccacc cactttacag tgtgaaagag
 1081 caggaggaaa tgcttctcat gtcttccct ttaatagcac cagccataac taacagcaaa
 1141 gaaagacata attctgtgga atgcttagat ggactagatg atattactac aaaactacca
 15 1201 tctaagctgc agaaaattac tggaagagaa gactttccca aaaaactctt gaggatgagt
 1261 tctacaactt cagaaaaagc ctacagcttg ggagacttgc ccatgaaact tcaacgaata
 1321 agttcagttc cgggcaactc agaagaaaaa ctggtatcta aaaccacca gatgttatct
 1381 gatcccatga gccagctctg ggctgatttg ccaccaaagc ttcaaaagat ggctggagga
 1441 gcagctagga tggaagggaa ccttcagacc aaattaagaa aatgaactc tgatcgcttc
 20 1501 acataa

37. human Kir 3.4 – cDNA

1 atggtctggcg attctaggaa tgccatgaac caggacatgg agattggagt cactccctgg
 61 gaccccaaga agattccaaa acaggccgcg gattatgtcc ccattgccac agaccgtacg
 25 121 cgctctgtgg ccgagggcaa gaagccacgc cagcgtctaca tggagaagag tggcaagtgc
 181 aacgtgcacc acggcaacgt ccaggagacc taccggtacc tgagtgaact cttcaccacc
 241 ctggtggacc tcaagtggcg cttcaacttg ctctcttcca ccatggttta cactgtcacc
 301 tggtcttctt tcggttctat ttggtggctc attgcttata tccgggggtga cctggaccat
 361 gttggcgacc aagagtggat tccttgtgtt gaaaacctca gtggcttctg gtccgcttcc
 421 ctgttctcca ttgagaccga aacaaccatt ggggtatggc tccgagtcac cacagagaag
 481 tgtccagagg ggattatact cctcttggc caggccatcc tgggtctccat cgtcaatgcc
 541 ttcattgggt gggtcatggt tgtcaagatc agccagccca agaagagagc ggagaccctc
 601 atgttttcca acaacgcagt catctccatg cgggacgaga agctgtgctt catgttccgg
 661 gtggcgacc tccgcaactc ccacatcgtg gaggcctcca tccgggcca gtcacatcaag
 35 721 tccggcgaga ccaaagaggg ggagttcatc cccctgaacc agacagacat caacgtgggc
 781 tttgacacgg gcgacgaccg cctcttctct gtgtctctc tgatcatctc ccatgagatc
 841 aaccagaaga gccctttctg ggagatgtct caggctcagc tgcatcagga agagtttgaa
 901 gttgtggtca ttctagaagg gatggtggaa gccacaggca tgacctgcca agccgggagc
 961 tctacatagg atacagaggt gctctggggc caccgattca caccagtcct cactttggaa
 40 1021 aagggtctct atgaggtgga ctacaacacc ttccatgata cctatgagac caacacaccc
 1081 agctgctgtg ccaaggagct ggcagaaatg aagagggaag gccggtcctt ccagtacctc
 1141 cccagccccc cactgctggg gggctgtgct gaggcagggc tggatgcaga ggctgagcag
 1201 aatgaagaag atgagcccaa ggggtgggt ggggtccagg aggccagggt ctcggtgtga

45 38. human Kir 4.1 – cDNA

1 atgacgtcag ttgccaaggt gtattacagt cagaccactc agacagaaag ccggccccta
 61 atgggcccag ggatacgacg gcggagagtc ctgacaaaag atggctgcag caacgtgaga
 121 atggagcaca ttgcccacaa gcgttctctc tacctcaagg acctgtggac aaccttcatt
 181 gacatgcagt ggcgtctaaa gcttctgtct ttctctgcga cctttgcagg cacatggttc
 50 241 ctcttttggc tgggtgtggt tctggtagct gtggcacatg gggacctgct ggagctggac
 301 ccccgggcca accacacccc ctgtgtggta cagggtcaca cactcactgg agccttctct
 361 ttctcccttg aatcccaaac caccattggc tatggcttcc gctacatcag tgaggaatgt
 421 ccactggcca ttgtgcttct tattgcccag ctggtgtctc ccaccatcct ggaaatcttc
 481 atcacaggta ccttcttggc gaagattgcc cggcccaaga agcgggctga gaccattcgt

541 ttcagccagc atgcagttgt ggccctcccac aatggcaagc cctgcctcat gatccgagtt
 601 gccaatatgc gcaaaagcct cctcattggc tgccaggtga caggaaaact gcttcagacc
 661 caccaaaacca aggaagggga gaacatccgg ctcaaccagg tcaatgtgac tttccaagta
 721 gacacagcct ctgacagccc cttecttatt ctaccctta ccttctatca tgtggtagat
 5 781 gagaccagtc ccttgaaaga tctccctctt cgcagtgggt aggggtgactt tgagctgggtg
 841 ctgatacctaa gtgggacagt ggagtcacac agtgccacct gtcaggtgag cacttctac
 901 ctgccagagg agatcctttg gggctacgag ttcacacctg ccatctcact gtcagccagt
 961 ggtaaataca tagctgactt tagccttttt gaccaagtgt tgaaagtggc ctctcctagt
 1021 ggccctccgtg acagcactgt acgctacgga gaccctgaaa agctcaagtt ggaggagtca
 10 1081 ttaagggagc aagctgagaa ggagggcagt gcccttagtg tgcgcacag caatgtctga

39. human Kir 4.2 – cDNA

1 atggatgcca ttcacatcgg catgtccagc acccccctgg tgaagcacac tgctggggct
 61 gggctcaagg ccaacagacc ccgcgtcatg tccaagagtg ggcacagcaa cgtgagaatt
 15 121 gacaaagtgg atggcatata cctactctac ctgcaagacc tgtggaccac agttatcgac
 181 atgaagtggg gatacaaaact caccctgttc gctgccactt ttgtgatgac ctgggttctt
 241 tttggagtca tctactatgc catcgctgtt attcatgggg acttagaacc cgtgagccc
 301 atttcaaatc ataccccctg catcatgaaa gtggactctc tcaactggggc gtttctcttt
 361 tccctggaat cccagacaac cattggctat ggagtcggtt ccatcacaga ggaatgtcct
 20 421 catgccatct tctgtttggt tgcctcagtg gtcacacga ccttgattga gatcttcate
 481 accggaacct tcttgcccaa aatcgccaga cccaaaaagc gggctgagac catcaagttc
 541 agccactgtg cagtcacac caagcagaat gggaagctgt gcttggtgat tcaggtagcc
 601 aatatgagga agagcctctt gattcagtg cagctctctg gcaagctcct gcagaccac
 661 gtcaccaagg agggggagcg gattctctct aaccaagcca ctgtcaaat ccacgtggac
 25 721 tccctctctg agagccctt cctcattctg cccatgacat tctaccatgt gctggatgag
 781 acgagccccc tgagagacct cacaccccaa aacctaaagg agaaggagtt tgagcttgtg
 841 gtctctctca atgccactgt ggaatccacc agcgtctgtt gccagagccg aacatcttat
 901 atcccagagg aaatctactg gggttttgag tttgtgctg ttggtatctt ctccaaaaat
 961 ggaaaatatg tggctgattt cagtcagttt gaacagattc ggaaaagccc agattgcaca
 30 1021 ttttactgtg cagattctga gaaacagcaa ctcgaggaga agtacaggca ggaggatcag
 1081 agggaaagag aactgaggac acttttatta caacagagca atgtctga

40. human Kir 5.1 – cDNA

1 atgagctatt acggcagcag ctatcatatt atcaatgcgg acgcaaaata cccaggctac
 35 61 ccgccagagc acattatagc tgagaagaga agagcaagaa gacgattact tcacaaagat
 121 ggcagctgta atgtctactt caagcacatt tttggagaat ggggaagcta tgtggttgac
 181 atcttcacca ctcttgtgga caccagtggt cgcctatgtt ttgtgatatt ttctttatct
 241 tatattctct cgtgggtgat atttggtctt gtcttttggc tcatagcctt tcatcatggc
 301 gatctattaa atgatccaga catcacacct tgtgttgaca acgtccattc tttcacaggg
 40 361 gcctttttgt tctccctaga gacccaaacc accataggat atggttatcg ctgtgttact
 421 gaagaatgtt ctgtggcctg gctcatgggt atcctccagt ccatcttaag ttgcatcata
 481 aataccttta tcattggagc tgccttggcc aaaatggcaa ctgctcgaaa gagagcccaa
 541 accattcggt tcagctactt tgcacttata ggtatgagag atgggaagct ttgcctcatg
 601 tggcgcatgt gtgattttcg gccaaaccac gtggtagaag gaacagttag agcccaactt
 45 661 ctccgctata cagaagacag tgaaggagg atgacgatgg catttaaaga cctcaaatat
 721 gtcaacgacc aaatcatcct ggtcaccocg gtaactattg tccatgaaat tgaccatgag
 781 agccctctgt atgccottga ccgcaaagca gtagccaaag ataactttga gattttgggtg
 841 acatttatct atactgggtg ttccactgga acatctcacc aatctagaag ctccatgtgt
 901 ccccgagaaa ttctctgggg ccataggttt aatgatgtct tggaagttaa gaggaagtat
 50 961 taaaaagtga actgcttaca gtttgaagga agtgtggaag tatatgcccc cttttgcagt
 1021 gccaaagcaat tggactggaa agaccagcag ctccacatag aaaaagcacc accagttcga
 1081 gaatcctgca cgtcggacac caaggcgaga cgaaggctcat ttagtgcagt tgccattgtc
 1141 agcagctgtg aaaacctga ggagaccacc acttcgcga cacatgaata tagggaaaca
 1201 ccttatcaga aagctctcct gactttaaac agaattctctg tagaatccca aatgtatg

41. human Kir 6.1 – cDNA

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1 atgtttggcca gaaagagtat catccccgag gagtatgtgc tggcgcgcacat cgccgcagag
5 61 aacctgcgca agccgcgcacat ccgagaccgc ctccccaaag cccgcttcat cgccaagagc
121 ggggcctgca acctggcgca taagaacatc cgtgagcaag gacgctttct acaggacatc
181 ttcaccacct tgggtggacct gaaatggcgc cacacgctgg tcattctttac catgtccttc
241 ctctgcagct ggctgctctt cgctatcatg tgggtggctgg tggccttttg ccattggggac
301 atctatgctt acatggagaa aagtggaaatg gagaaaagtg gtttggagtc cactgtgtgt
361 gtgactaatg tcaggctctt cacttctgct tttctcttct ccattgaagt tcaagttacc
10 421 attgggtttg gagggaggat gatgacagag gaatgccctt tggccatcac ggttttgatt
481 ctocagaata ttgtgggttt gatcatcaat gcagtcattg taggtgcat tttcatgaaa
541 acagctcagg ctacacagaag ggcagaaact ttgattttca gccgcattgc tgtgattgcc
601 gtccgaaatg gcaagctgtg cttcatgttc cgagtgggtg acctgaggaa aagcatgac
661 attagtgcct ctgtgcgcac ccaggtggtc aagaaaacaa ctacacctga aggggagggtg
15 721 gttcctattc accaactgga cattcctgtt gataacccaa tcgagagcaa taacattttt
781 ctggtggccc ctttgatcat ctgccacgtg attgacaagc gcagtccctt gtatgacatc
841 tcagcaactg acctggccaa ccaagacttg gaggtcatag ttattctgga aggagtgggt
901 gaaactactg gcatcaccac acaagcagca acctctaca ttgctgagga gatccaatgg
961 ggccaccgct ttgtgtccat tgtgactgag gaagaaggag tgtattctgt ggattactcc
20 1021 aaatttggca aactgttaa agtagctgct ccacggtgca gtgcccgaga gctggatgag
1081 aaaccttcca tcctattcca gacctccaa aagagtgaac tgtctcatca aaattctctg
1141 aggaagcgca actccatgag aagaaacaat tccatgagga ggaacaattc tatccgaagg
1201 aacaattctt ccctcatggt accaaagggt caatttatga ctccagaagg aaatcaaaac
1261 acatcggaat catga
25

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42. human Kir 6.2 – cDNA

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1 atgtgtgccc gcaagggcat catccccgag gaatacgtgc tgacacgcct ggcagaggac
61 cctgccaaagc ccagggtaccg tgcccgcag cggaggggccc gcttttgtgc caagaaaggc
30 121 aactgcaacg tggcccacaa gaacatccgg gagcaggggc gcttctctga ggacgtgttc
181 accacgctgg tggacctcaa gtggccacac acattgctca tcttcaccat gtccttctctg
241 tgcagctggc tgcctcttgc catggcctgg tggctcatcg ccttcgccca cggtgacctg
301 gccccacgcg agggcactgc tgagccctgt gtcaccagca tccactcctt ctgctctgcc
361 ttccttttct ccattgaggt ccaagtgaat attggctttg gggggcgcat ggtgactgag
421 gagtgcccac tggccatcct gatcctcate gtgcagaaca tegtggggct catgatcaac
35 481 gccatcatgc ttggctgcat cttcatgaag actgccaaag ccaccgcag ggctgagacc
541 ctcatcttca gcaagcatgc ggtgatcgcc ctgcgccacg gccgctctg cttcatgcta
601 cgtgtgggtg acctccgcaa gagcatgac atcagcgcca ccattccacat gcagggtgta
661 cgcaagacca ccagccccga gggcgagggt gtgcccctcc accagggtga catcccatg
721 gagaacggcg tgggtggcaa cagcatcttc ctggtggccc cgtgatcat ctaccatgtc
40 781 attgatgcca acagccact ctacgacctg gcaccacagc acctgcacca ccaccaggac
841 ctcgagatca tcgtcatcct ggaaggcgtg gtggaaacca cgggcatcac caccaggcc
901 cgcacctcct acctggccga tgagatctg tggggccagc gcttttgtgc cattgtagct
961 gaggaggacg gacgttactc tgtggactac tccaagttt gcaacaccgt caaagtgcc
1021 acaccactct gcacggcccc ccagcttgat gaggaccaca gcctactgga agctctgacc
45 1081 ctgcctcag ccgcggggc cctgcgcaag cgcagcgtgc ccattggcaa ggccaagccc
1141 aagttcagca tctctccaga ttccctgtcc tga

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43. human Kir 7.1 – cDNA

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1 atggacagca gtaattgcaa agttattgct cctctcctaa gtcaaagata ccggaggatg
50 61 gtcaccaagg atggccacag cacacttcaa atggatggcg ctcaaagggt tcttgcatat
121 cttcgagatg cttgggggaat cctaattggac atgcgctggc gttggatgat gttggtcttt
181 tctgcttctt ttgttgteca ctggcttgct tttgcagtgc tctggtatgt tctggctgag

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241 atgaatgggtg atctggaact agatcatgat gccccacctg aaaaccacac tatctgtgtc
 301 aagtatatca ccagtttcac agctgcattc tcccttctcc tggagacaca actcacaatt
 361 ggttatggta ccatgttccc cagtgggtgac tgtccaagtg caatcgccctt acttgccata
 421 caaatgtctc taggcctcat gctagaggct tttatcacag gtgcttttgt ggcgaagatt
 5 481 gcccggccaa aaaatcgagc tttttcaatt cgctttactg acacagcagt agtagctcac
 541 atggatggca aacctaatct tatcttccaa gtggccaaca cccgacctag cctctaaacc
 601 agtgtccggg tctcagctgt actctatcag gaaagagaaa atggcaaact ctaccagacc
 661 agtgtggatt tccaccttga tggcatcagt tctgacgaat gtccattctt catctttcca
 721 ctaacgtact atcactccat tacaccatca agtcctctgg ctactctgct ccagcatgaa
 10 781 aatccttctc actttgaatt agttgtattc ctttcagcaa tgcaggaggg cactggagaa
 841 atatgccaaa ggaggacatc ctacctaccg tctgaaatca tgttacatca ctgttttgca
 901 tctctgttga cccgagggtc caaagggtgaa tatcaaatca agatggagaa ttttgacaag
 961 actgtccctg aatttccaac tctctgtgtt tctaaaagcc caaacaggac tgacctggat
 15 1021 atccacatca atggacaaag cattgacaat tttcagatct ctgaaacagg actgacagaa
 1081 taa

44. chicken Kir 2.1 – cDNA

1 atgggcagcg tgcgaaccaa ccgctacagc atcgtgtctt cggaagagga cggcatgaag
 61 ctggcaacca tggccgttgc caatggcttt gggaatggaa aaagtaaggt acacaccagg
 20 121 cagcagtgca ggagccgctt tgtcaaaaa gatggccact gcaacgtcca gtttattaat
 181 gtgggtgaga agggacagcg atacctcgca gacatcttca ccaattgctg ggacatccgc
 241 tggaggtgga tgctggttat cttctgcctg acattcatcc tctcctggct tttctttggc
 301 tgtgtgtttt ggttgattgc gctgttgca ggggatctgg agaaccaaga aaataacaaa
 361 ccgtgtgtct cgcaagtgag cagcttcaact gcagccttcc tgttctccat tgagaccag
 25 421 accacgatcg gctatggctt caggtgcgtc acagacgagt gcccatttgc tgttttcatg
 481 gtggttttcc agtctatagt aggtcgatc attgacgcct tcatcattgg tgcgtcatg
 541 gcaaagatgg ctaagccaaa aaagagaaac gaaactcttg tcttcagcca caatgcctg
 601 gtggccatga gagatgggaa actgtgcctg atgtggcgtg tcggaaacct gaggaaaagc
 661 cacttggttg aggcacacgt gcgagcacag ctcttcaagt ccaggatcac gtcagaaggg
 30 721 gagtacatcc ctttggtatga aatagacatc aatgtagggt ttgacagcgg gatagaccgc
 781 atattcctgg tctccccaat tacaatagta caggaatag atgaagatag tcttttgtat
 841 gacttgagca aacaagacat ggacaatgct gactttgaaa ttgtatgcat tttagagggc
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 961 ctctggggcc accgctatga gccgtactc tttgaagaaa aaaactacta caaagtggac
 35 1021 tattcaaggt tccacaaaac atacgaagtg cccaacacac ccatctgcag tgccagagac
 1081 ttagcagaaa agaaatacat tctctcgaa gcaaaactct tttgctacga gaacgaagtg
 1141 gccctacca gcaaggagga ggacgagatc gacacggggg tgcccagagag cacaagcaca
 1201 gacaccacc cccagatgga ccaccacaac caggcaggag tgcccctaga gccacggcgc
 1261 ctgcccgcgtg agtcggaaat atga

45. chicken Kir 2.2 – cDNA

1 atgactgcag gcagggtcaa cccttacagc atagtgtcct ccgaggaaga cggactgagg
 61 ttgaccacca tgccagggat taacggcttt ggcaatggga aaatccacac caggaggaaa
 121 tgcaggaaca ggtttgtaaa gaagaacggc cagtgcattg tggagtccac caacatggat
 45 181 gacaagccac agagggtacat tgcagacatg ttcaccacgt gcgttgacat ccgttgaggg
 241 tatatgtctt tgcctttctc cctggcattt ctggtatcct ggttattgtt tgggctgatt
 301 ttctggctaa ttgcactcat tcatggagat ctagaaaacc cagggtggaga tgataccttc
 361 aagccttgcg ttctgcaggt caatggcttt gtggtgctt ttctgttctc catcgagacc
 421 caaacgacta ttggttatgg ctccgctgt gtgacagagg agtgcccgtc cgcagtcttc
 50 481 atggtggtgg ttcatgcat cgtggggtgt ataactgact ctttcatgat tgggtgcaata
 541 atggcaaaga tggccaggcc caaaaaagg gccagacat tgcttttcag ccataatgca
 601 gtagtggcaa tgagagatgg aaaactctgc ctgatgtgga gaggttggaa tctccggaaa

5 661 agccacatag tagaagccca cgtacgagct caattaatta agcccagaat cacagaagaa
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 841 ttcgggatca gccgccagga cttggagacg gatgactttg agattgtggt catcctcgaa
 901 ggcatggtag aagccaccgc gatgacgaca caagctcgga gctcctacct ggccagcgag
 961 atcctgtggg gccaccgctt cgagcccgtc ttgttcgagg agaaaaacca gtacaaagta
 1021 gactattccc acttccacaa aacatacgag gtcccgtcca cccccgtctg cagcgccaag
 1081 gacttggtgg agaacaaatt cctgctgccc agcaccact ccttctgcta cgagaatgag
 1141 ctggccttca tgagccgcga tgaggatgag gaggatgatg acagcagggg tttggacgac
 10 1201 ctgagcccag acaacaggca cgagttcgac cggcttcagg caacgatagc gttggatcag
 1261 aggtcatacc ggaggagtc agaaatatga

46. human Kir 1.1 – turret region

HKDLPEFHPSANHTPCVENING

15

47. human Kir 1.2 – turret region

HGDLLELDPPANHTPCVVQVHT

48. human Kir 2.1 – turret region

HGDLIDASKEGKACVSEVNS

20 49. human Kir 2.2 – turret region

HGDLEPAEGRGRTPCVMQVHG

50. human Kir 2.3 – turret region

HGDLEASPGVPAAGGPAAGGGGAAPVAPKPCIMHVNG

51. human Kir 2.4 – turret region

25 HGDLAAPPPPAPCFSHVAS

52. human Kir 3.1 – turret region

RGDLNKAHVGNYPVCVANVYN

53. human Kir 3.4 – turret region

RGDLDHVGDQEWIPCVENLSG

30 54. human Kir 6.1 – turret region

HGDIYAYMEKSGMEKSGLESTVCVTNVR

55. human Kir 6.2 – turret region

HGDLAPSEGTAEPVTSIHS

56. human Kir 7.1 – turret region

NGDLELDHDAPPENHTICVKYITS

57. chicken Kir 2.1 – turret region

HGDLENQENNKPCVSQVSS

5

58. chicken Kir 2.2 – turret region

HGDLENPGGDDTFKPCVLQVNG

59. Kir Bac 1.1 – turret region

10

SPARKPPRGRRIWSGTREVIAYGMPASVWRDLYYWALKVSWPVFFASLAALF
VVNNTLFAILLYQLGDAPIANQSPPGFVGAFFFSVETLATVGYGDMHPQTVYAHA
IATLEIFVGMSGIALSTGLVFARFARPRAKIMFARHAIVRPFNGRMTLMVRAANA
RQNVIAEARAKMRLMRREHSSEGYSLMKIHDLKLVNEHPIFLLGWNMMHVID
ESSPLFGETPESLAEGRAMLLVMIEGSDETTAQVMQARHAWEHDDIRWHHRV
DLMSDVDGMTHIDYTRFNDTEPVEPPGAAPDAQAFAAKPGE

15

60. KcsA – turret region

MAPMLSGLLARLVKLLLGRHGSALHWRAAGAATVLLVIVLLAGSYLAVLAERG
APGAQLITYPRALWWSVETATTVGYGDLYPVTLWGRCVAVVVMVAGITSFGLV
TAALATWFGREQERRGH

61. rKv1.2 – turret region

20

MRELGLLIFFLFIGVILFSSAVYFAEADERDSQFPSIPDAFWWAVVSMTTVGYGD
MVPTTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNIFYHRETEGE

Claims

We claim:

- 5 1. A method for identifying a compound that modulates ion channel activity of a Kir channel comprising:

identifying a compound which binds the turret region of a Kir channel; and

determining if the compound modulates ion channel activity of the Kir channel.
2. The method of claim 1, wherein the compound is an antibody.
- 10 3. The method of claim 2, wherein the antibody is human, chimeric, or humanized.
4. The method of claim 2, wherein the antibody is selected from the group consisting of polyclonal antibodies, monoclonal antibodies, an intact immunoglobulin molecule, an antibody fragment, a scFv, a Fab, a F(ab)₂, a Fv, and a disulfide linked Fv.
5. The method of claim 1, wherein the compound is a nucleic acid.
- 15 6. The method of claim 5, wherein the nucleic acid is selected from the group consisting of DNA and RNA.
7. The method of claim 5, wherein the identifying includes *in vitro* selection of the nucleic acid.
8. The method of claim 1, wherein the compound is a protein/peptide.
- 20 9. The method of claim 8, wherein the protein/peptide is attached to a protein scaffold.
10. The method of claim 8, wherein the protein/peptide is displayed on the surface of a phage.
11. The method of claim 1, wherein the compound is a small molecule.
12. The method of claim 1, wherein the Kir channel is human.

13. The method of claim 1, wherein the Kir channel is a chicken/human hybrid.
14. The method of claim 13, wherein the chicken/human hybrid Kir channel comprises a human Kir channel turret region.
15. The method of claim 1, wherein the identifying step comprises an ELISA and a Western blot to determine if the compound binds to a properly folded Kir channel but not to a denatured Kir channel.
16. The method of claim 1, wherein the identifying step comprises determining if the compound binds to a Kir channel with a normal turret region but not a mutated turret region.
17. The method of claim 1 wherein the determining step comprises an electrophysiological assay.
18. The method of claim 17, wherein the electrophysiological assay is selected from the group consisting of two-electrode voltage clamp, patch clamp, and planar lipid bilayer assays.
19. The method of claim 1, wherein the determining step comprises a fluorescent assay.
20. The method of claim 19, wherein the fluorescent assay utilizes a thallium specific fluorescent dye.
21. A method for identifying a compound that selectively modulates ion channel activity of a specific type of Kir channel comprising:
- identifying a compound which binds the turret region of a specific type of Kir channel but does not bind to other types of Kir channels; and
- determining if the compound modulates the activity of the Kir channel.
22. The method of claim 21, wherein the identifying comprises determining if the compound binds the turret region of the specific type of Kir channel but does not bind the turret region of other type of Kir channels.

23. A method of identifying a compound to treat a condition associated with abnormal ion channel activity by a Kir channel comprising:

identifying a compound which binds the turret region of a Kir channel;

determining if said compound modulates ion channel activity of the Kir channel;

and

administering said compound which modulates ion channel activity to a subject to determine if the compound is able to treat said condition.

24. The method of claim 23, wherein the condition is selected from the group consisting of diabetes mellitus, hypertension, cardiac arrhythmia, and epilepsy.

25. A purified antibody that specifically binds to an epitope in the turret region of a Kir channel.

26. The purified antibody of claim 25 wherein said antibody is human, chimeric or humanized.

27. The purified antibody of claim 25, wherein the antibody is selected from the group consisting of a polyclonal antibody, a monoclonal antibody, an intact immunoglobulin molecule, an antibody fragment, a scFv, a Fab, a F(ab)₂, a Fv, and a disulfide linked Fv.

28. The purified antibody of claim 25, wherein the Kir channel is human.

29. The purified antibody of claim 28, wherein the Kir channel is selected from the group consisting of Kir1, Kir2, Kir3, Kir4, Kir5, Kir6, and Kir7.

30. The purified antibody of claim 25, wherein the epitope is within the variable portion of the turret region.

31. The purified antibody of claim 30, wherein the epitope is within the sequence of SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

32. A method of making an antibody that specifically binds to an epitope in the turret region of a human Kir channel, comprising

providing a chicken/human hybrid Kir channel, wherein the chicken/human hybrid comprises a human Kir channel turret region;

5 immunizing a non-human animal with the chicken/human hybrid Kir channel; and
determining whether the antibody is binding to the human Kir channel turret region.

33. The method of claim 32, wherein the chicken portion of the chicken/human hybrid Kir channel is derived from a chicken Kir2.2 channel.

10 34. The method of claim 33, wherein the human Kir channel turret region is derived from the group consisting of Kir1, Kir2, Kir3, Kir4, Kir5, Kir6, and Kir7.

35. The method of claim 34, wherein the human Kir channel turret region is the amino acid sequence comprising SEQ ID NOS: 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, or 56.

36. A purified polypeptide consisting of at least a portion of a turret region of a Kir channel.

15 37. The purified polypeptide of claim 36, wherein the turret region has the amino acid sequence of SEQ ID NOS: 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, or 56.

38. The purified polypeptide of claim 37, wherein the portion of the turret region has the amino acid sequence of SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.

20 39. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide with the amino acid sequence consisting of at least a portion of a turret region of a Kir channel.

40. The isolated nucleic acid of claim 39, wherein the turret region has the amino acid sequence of SEQ ID NOS: 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, or 56.

41. The isolated nucleic acid of claim 40, wherein the portion of the turret region has the amino acid sequence of SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.
42. The method of claim 5 wherein the nucleic acid is an aptamer.
43. A method of making an antibody that specifically binds to an epitope in the turret region
5 of a human Kir channel, comprising

providing a human Kir channel

immunizing a non-human animal with the Kir channel; and

determining whether the antibody is binding to the human Kir channel turret region.

FIG.1A

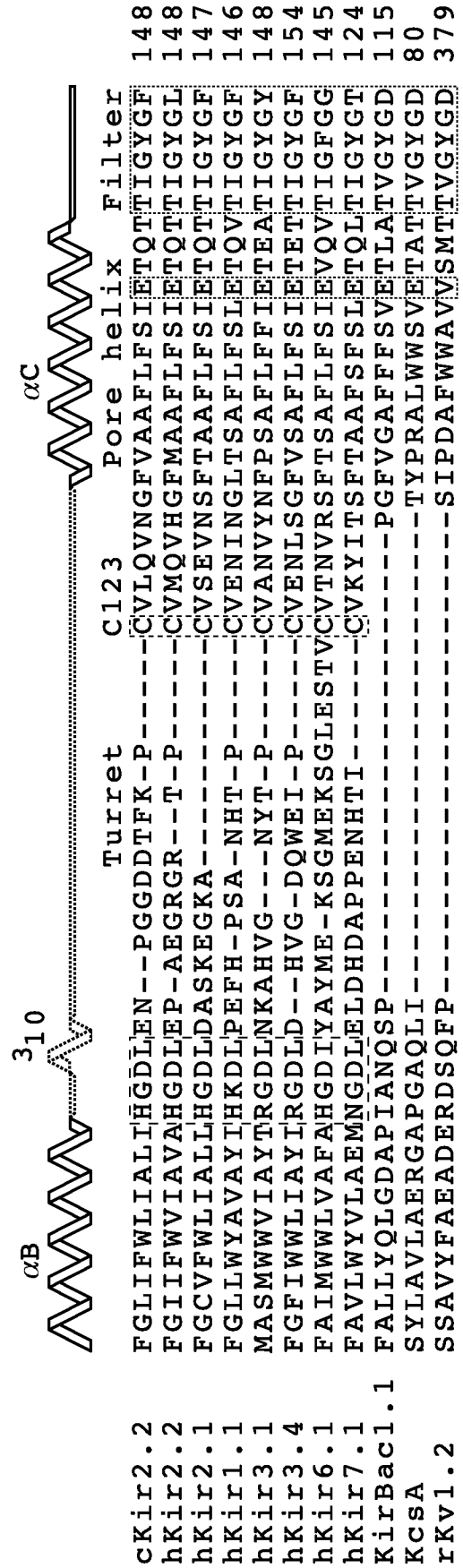
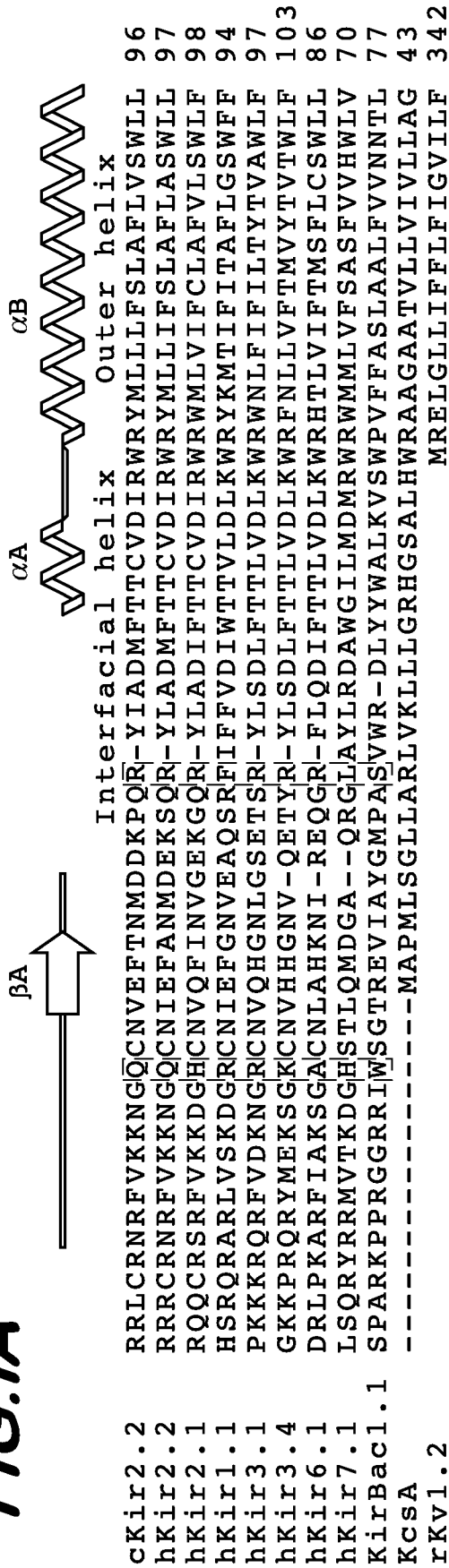


FIG.1B

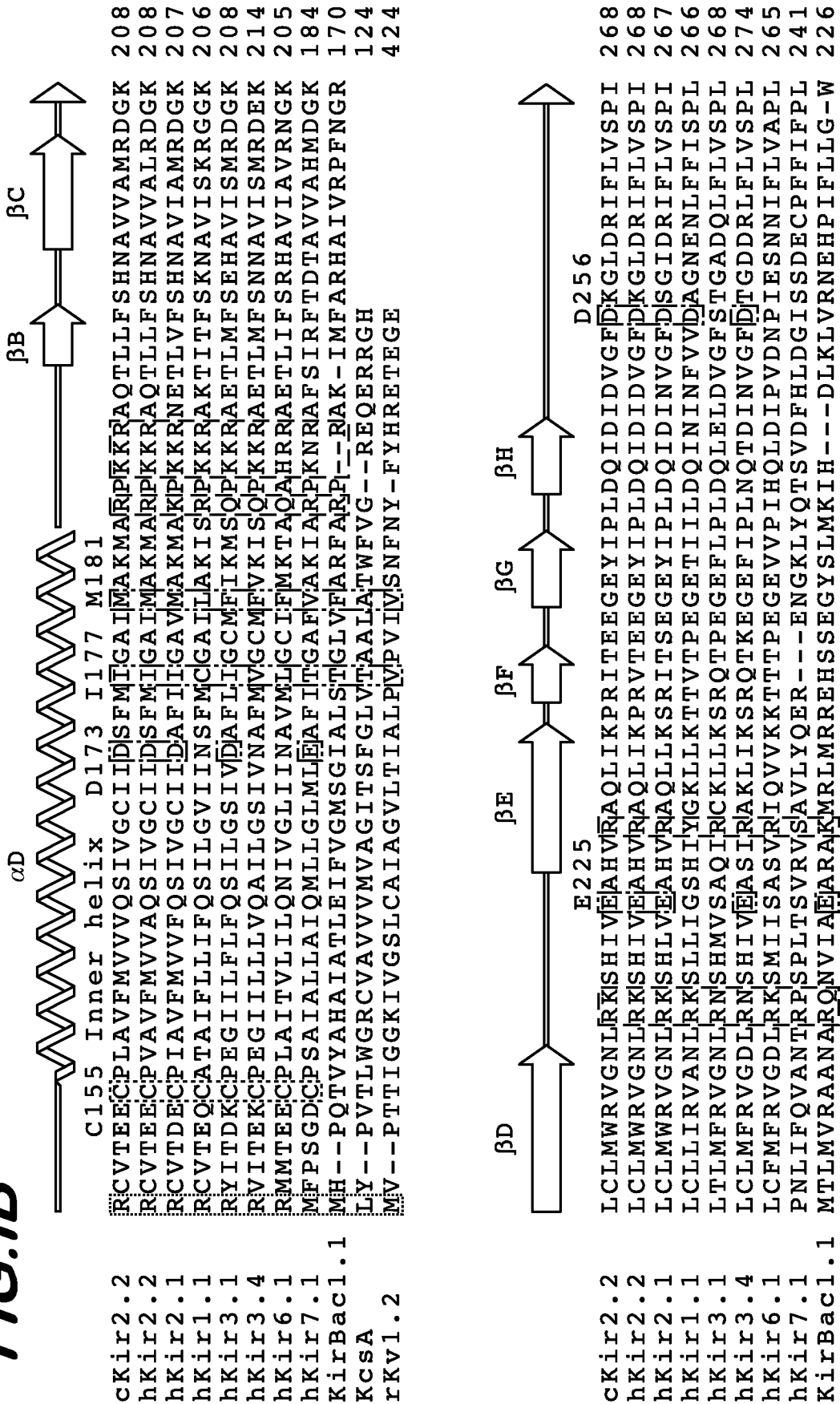
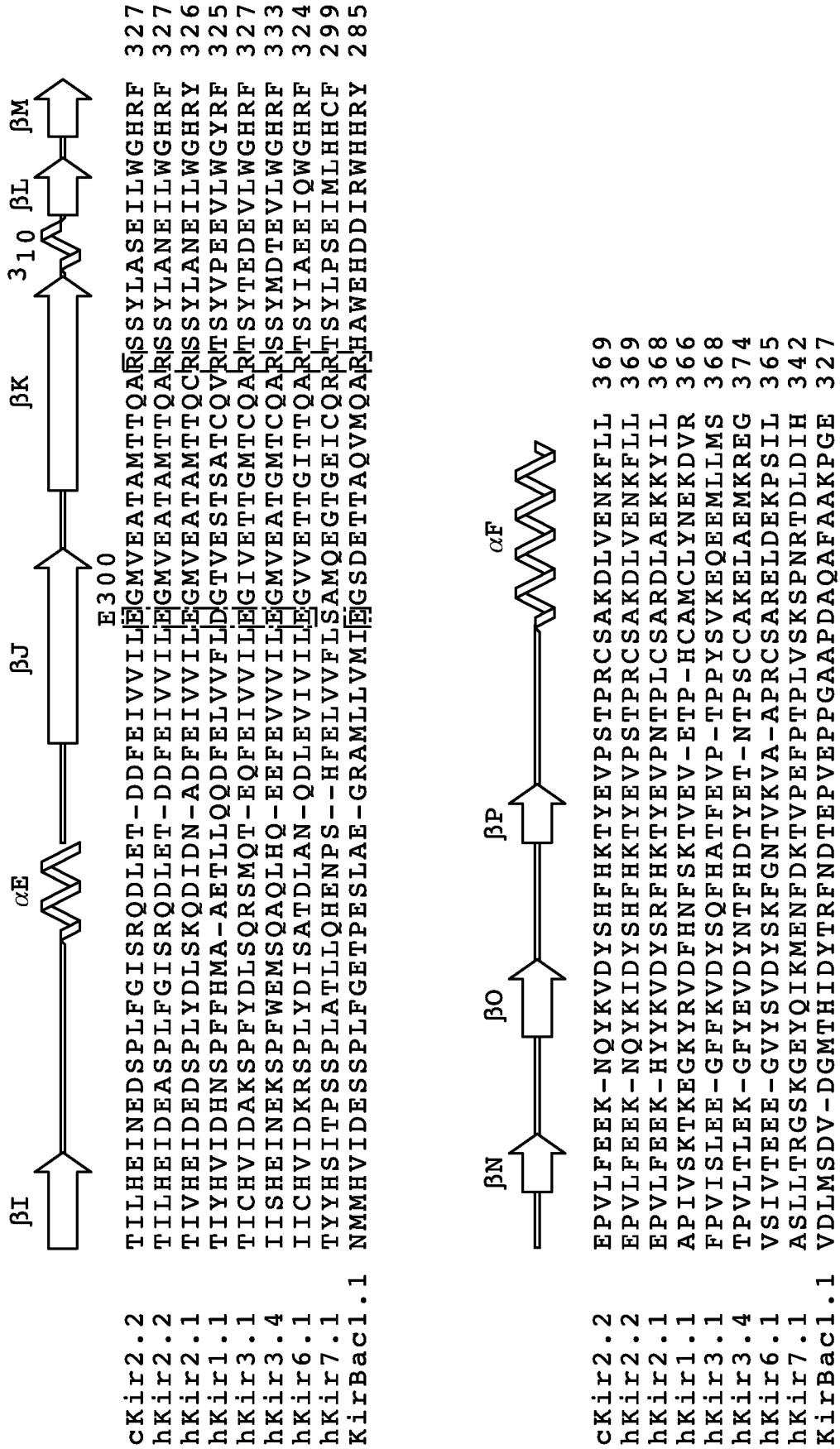


FIG.1C



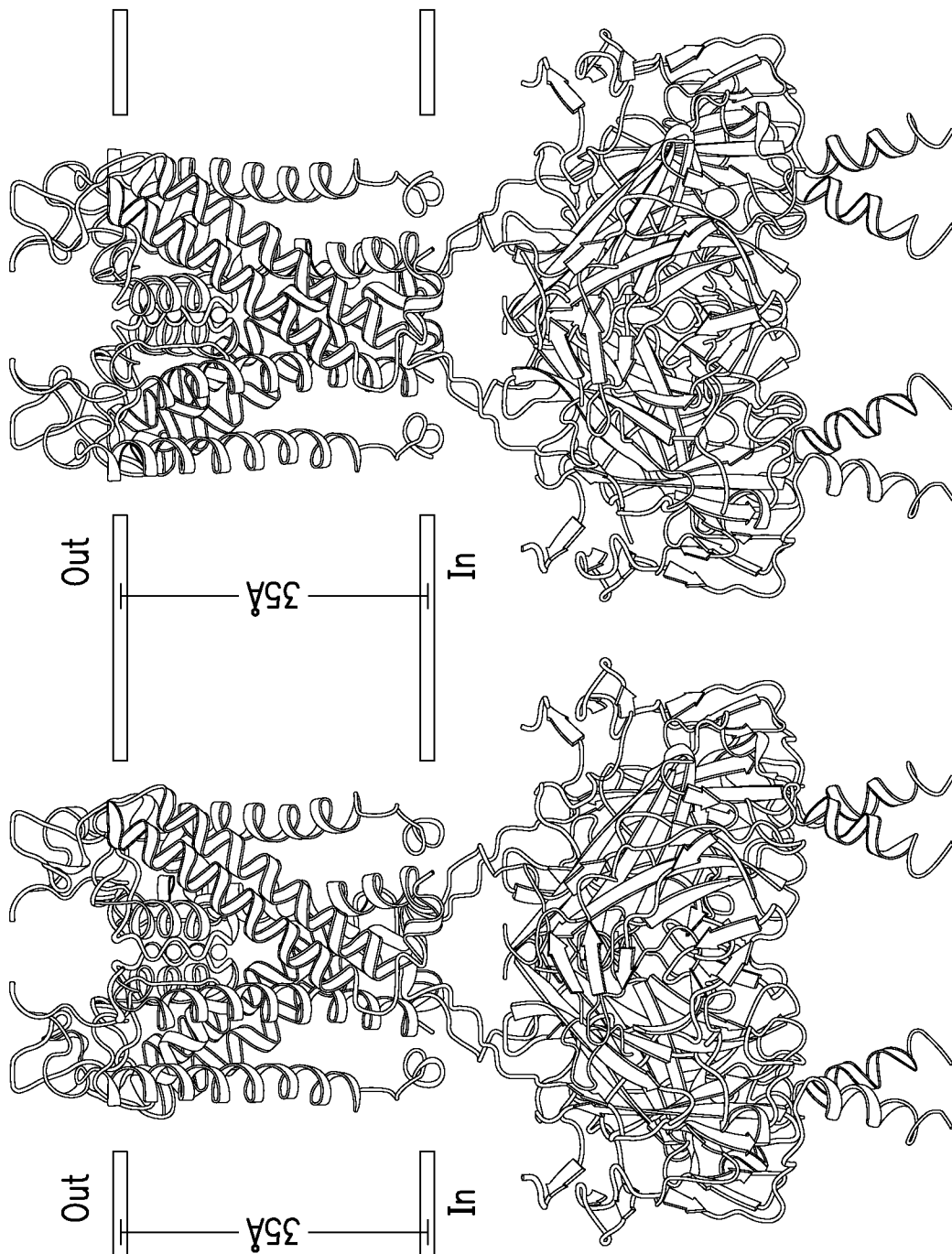


FIG. 2A

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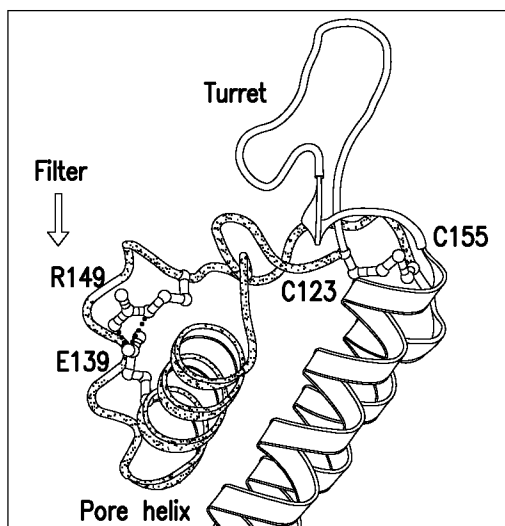


FIG. 2B

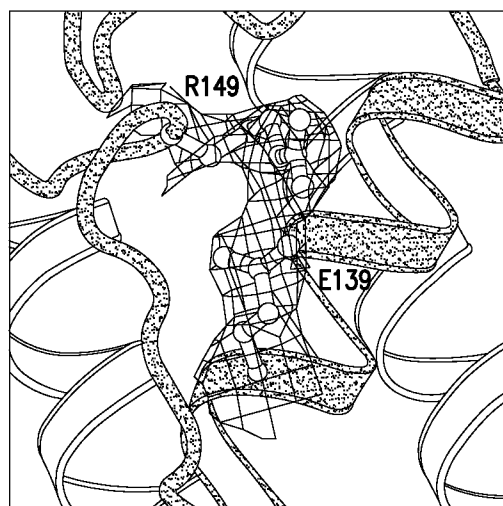


FIG. 2C

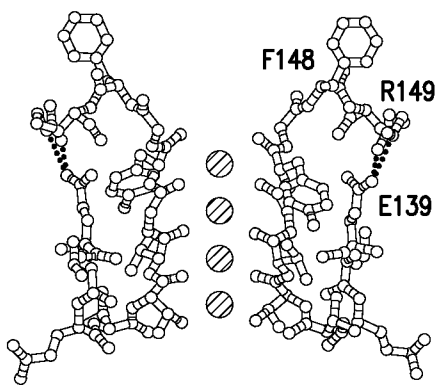


FIG. 2D

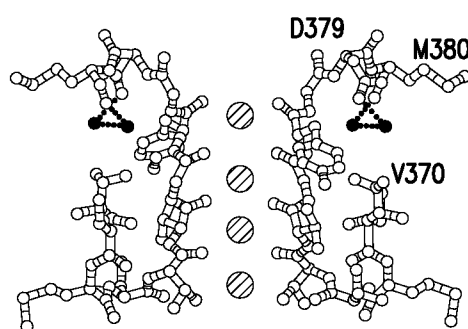


FIG. 2E

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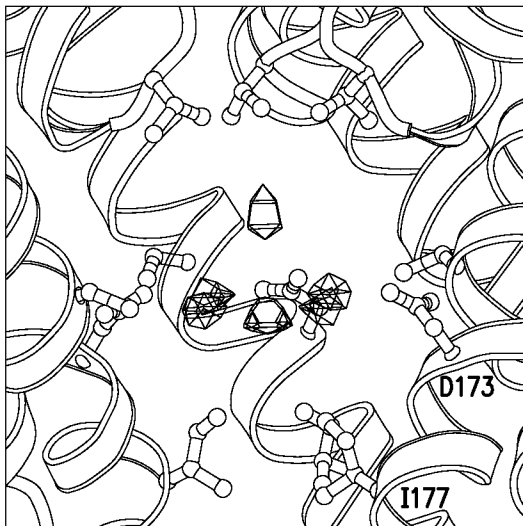


FIG. 3A

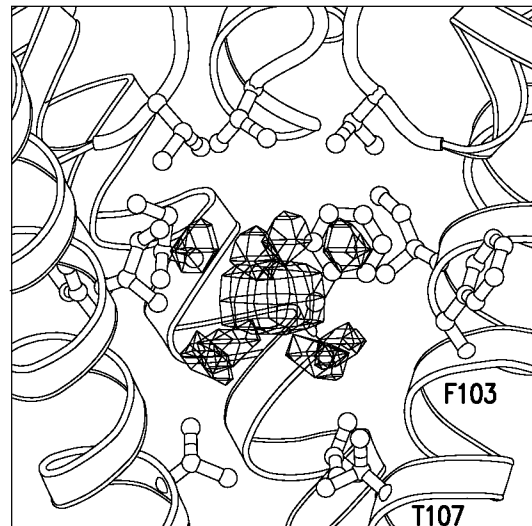


FIG. 3B

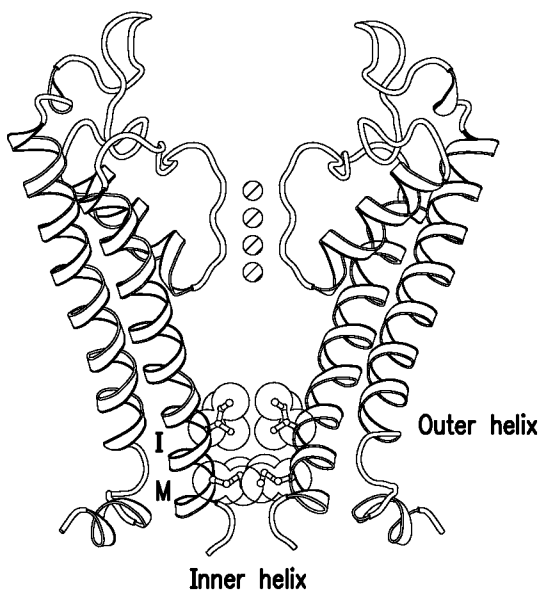


FIG. 3C

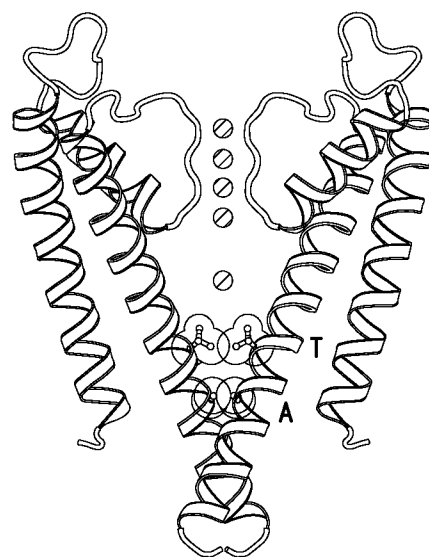


FIG. 3D

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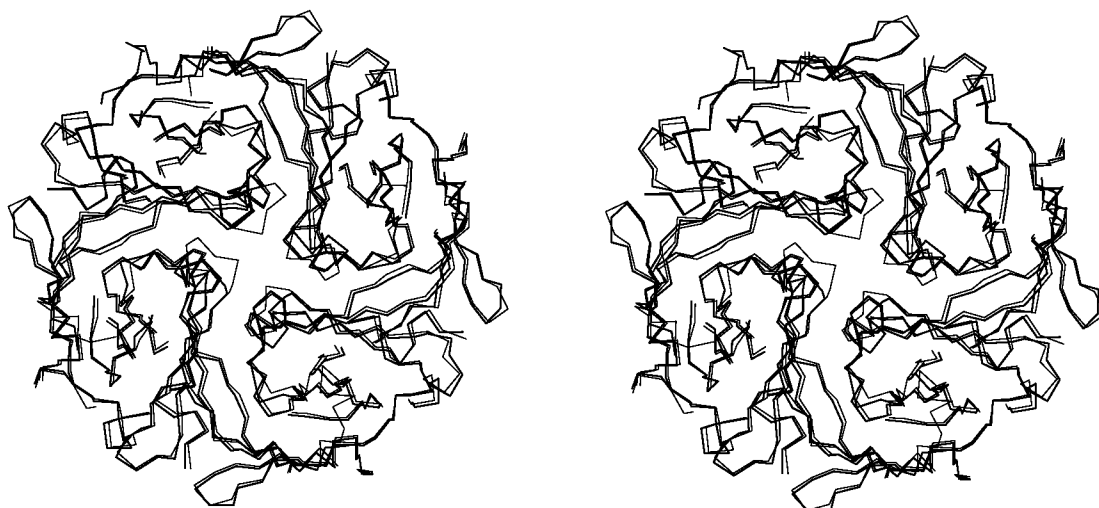


FIG. 3E

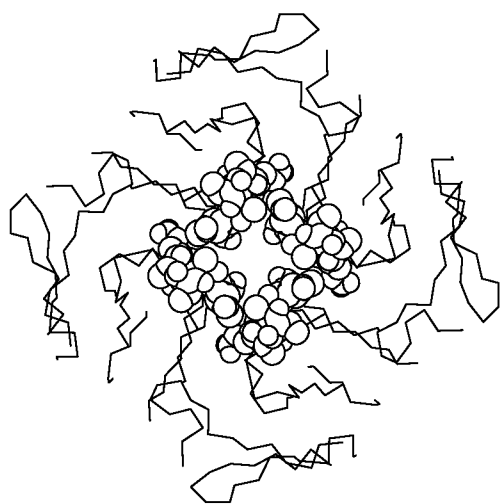


FIG. 3F

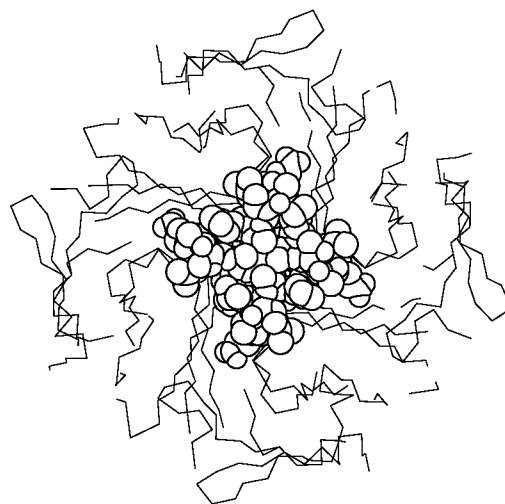


FIG. 3G

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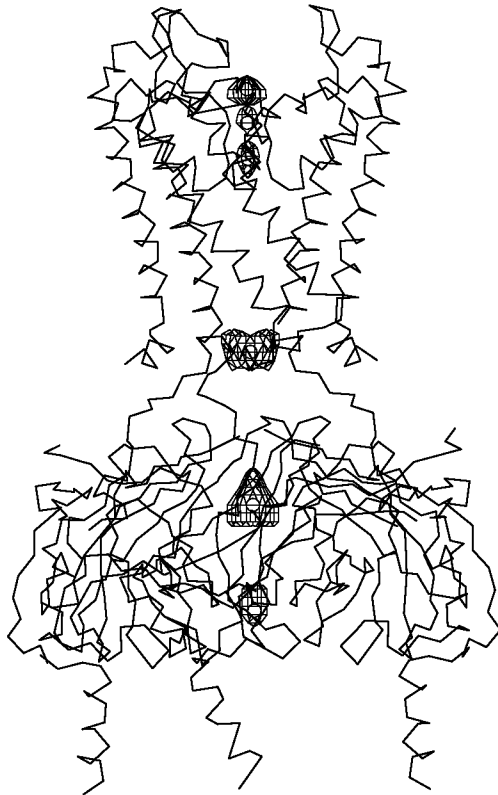


FIG. 4A

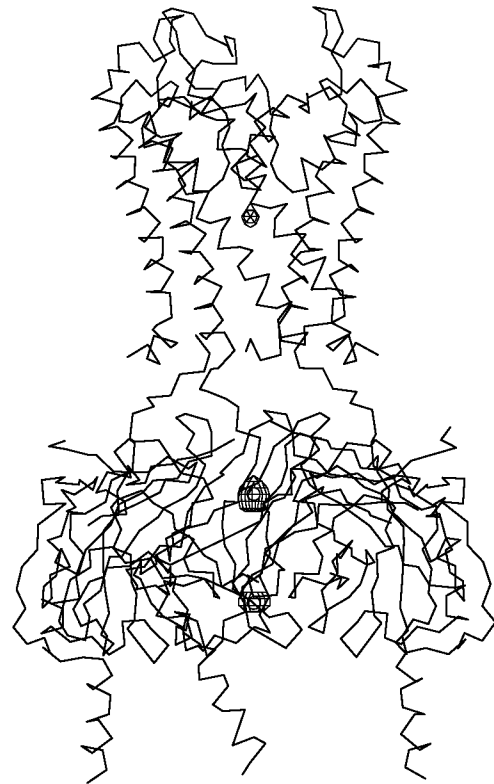


FIG. 4B

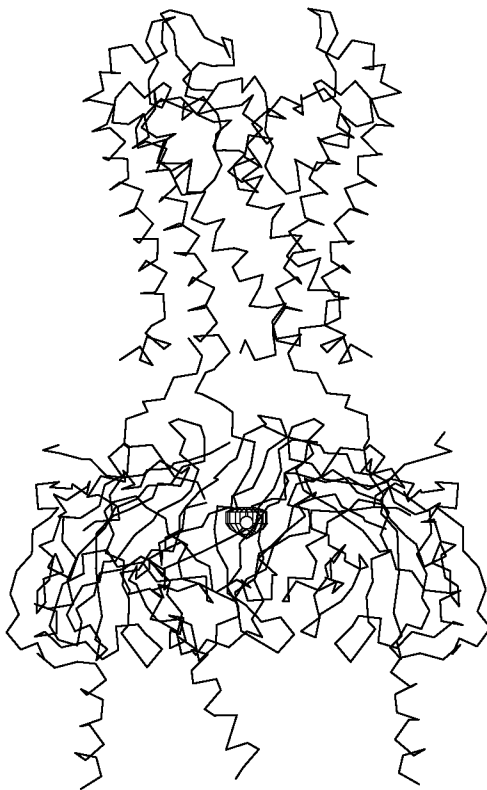


FIG. 4C

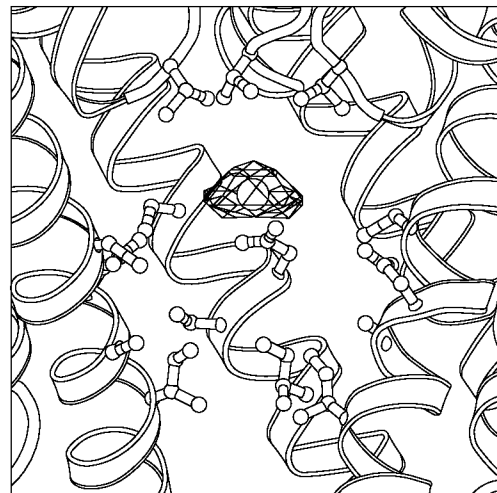


FIG. 4D

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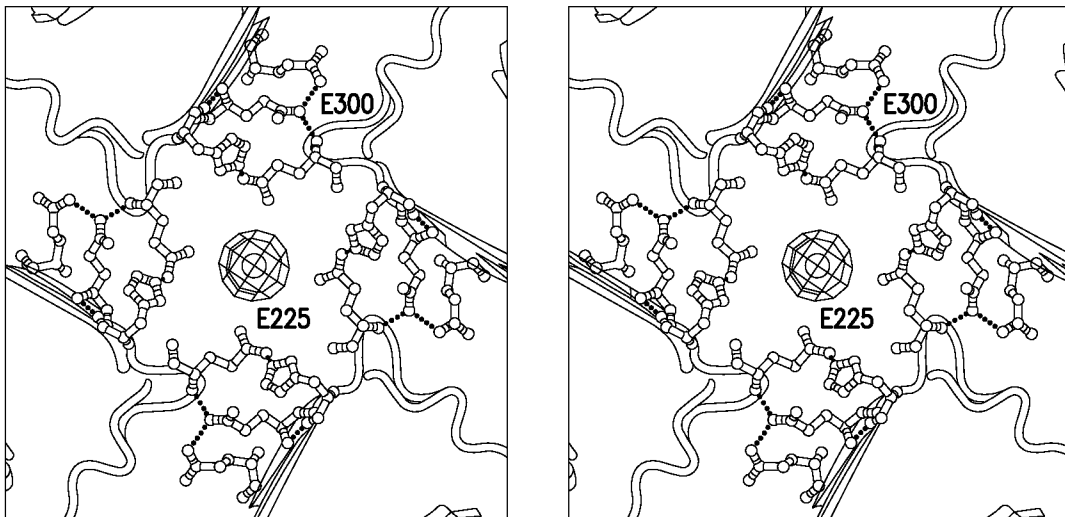


FIG. 4E

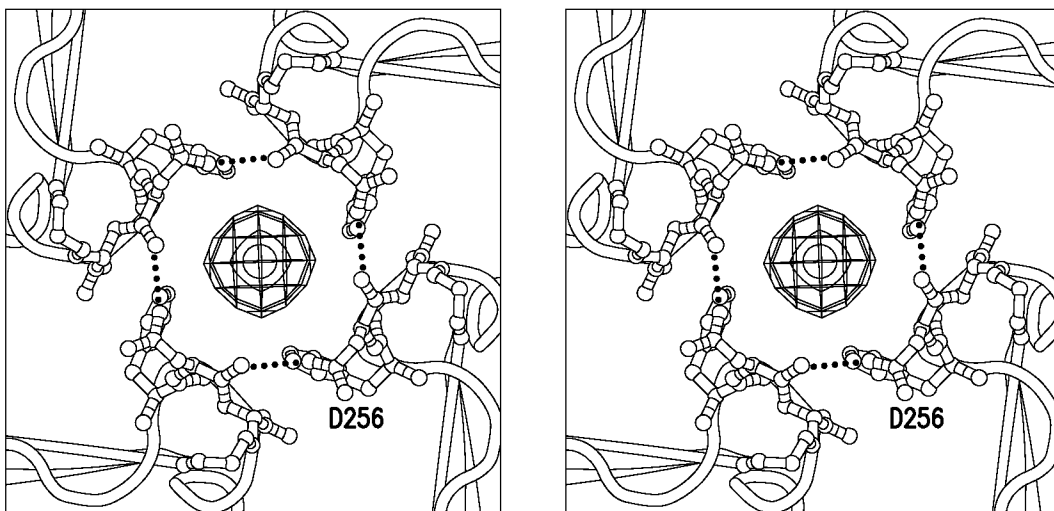
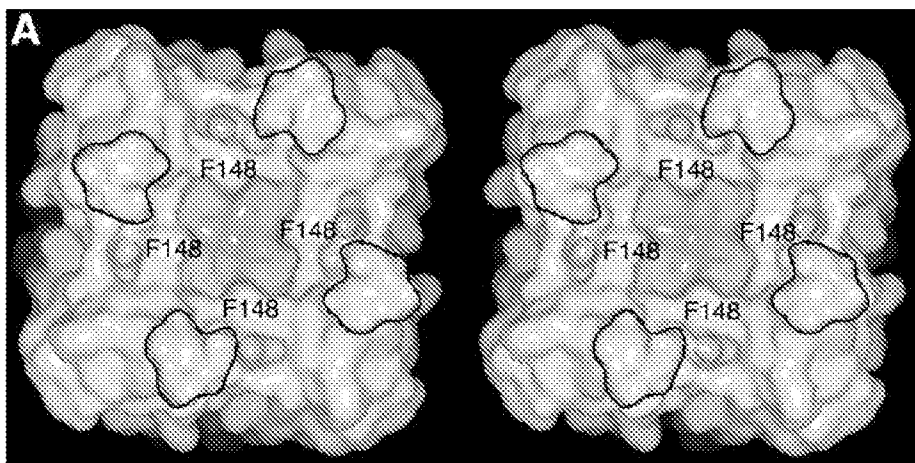
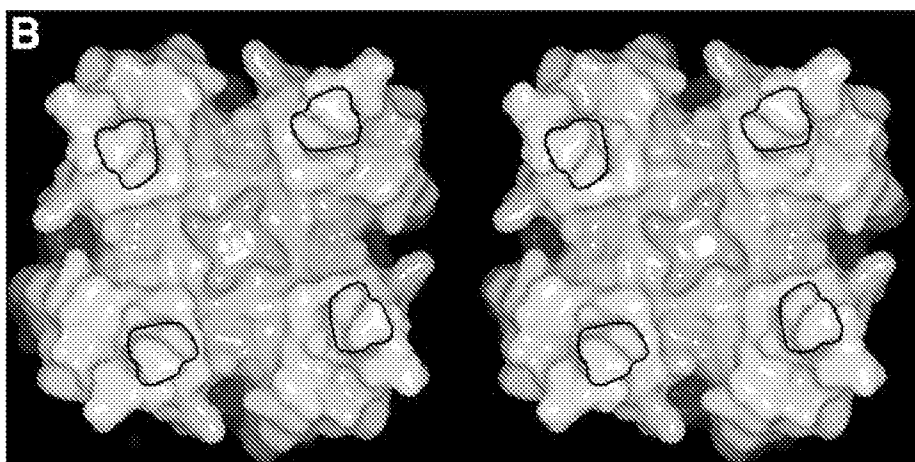


FIG. 4F

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**FIG. 5A****FIG. 5B**

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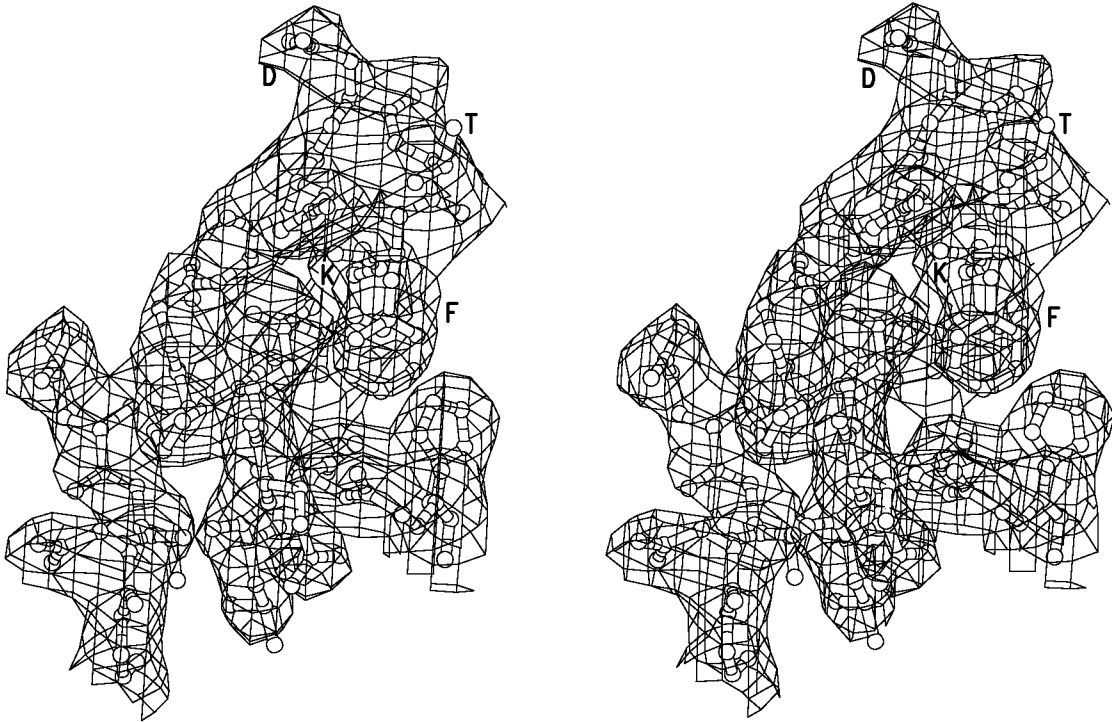


FIG. 5C

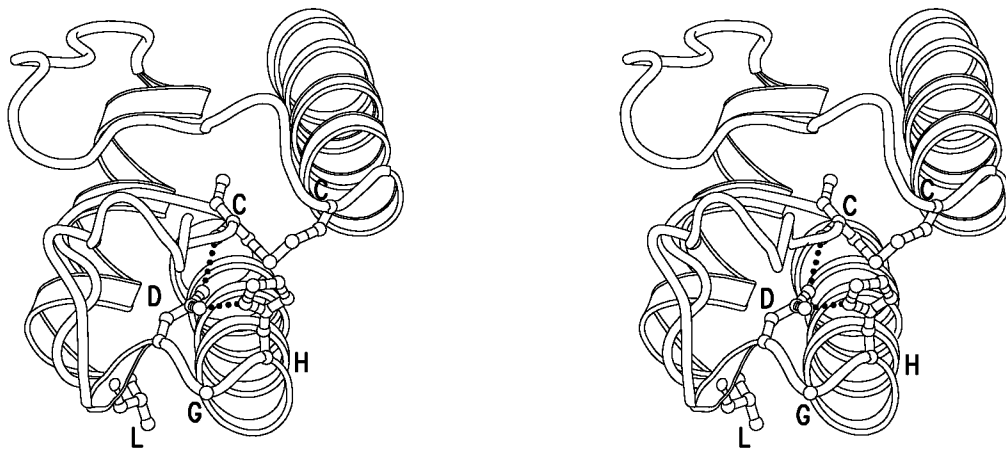


FIG. 5D

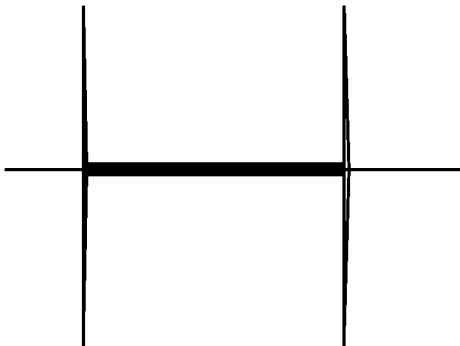


FIG. 6A

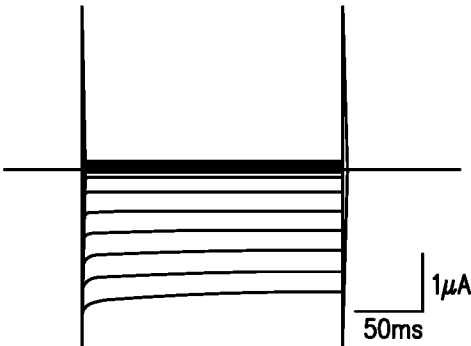


FIG. 6B

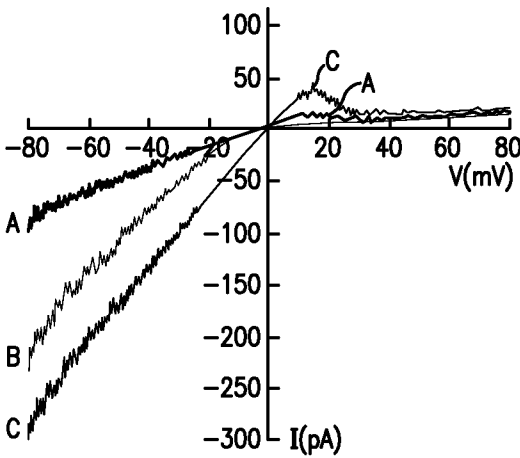


FIG. 6C

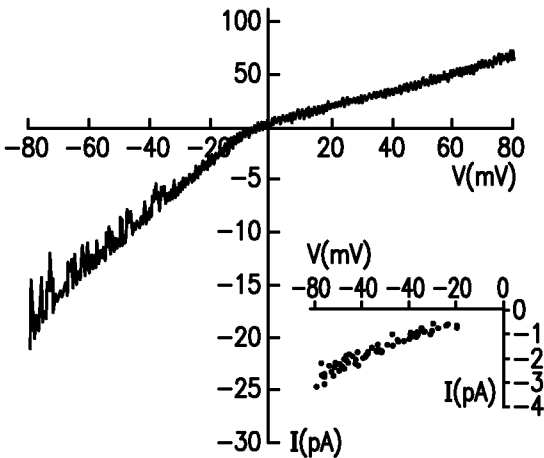


FIG. 6D

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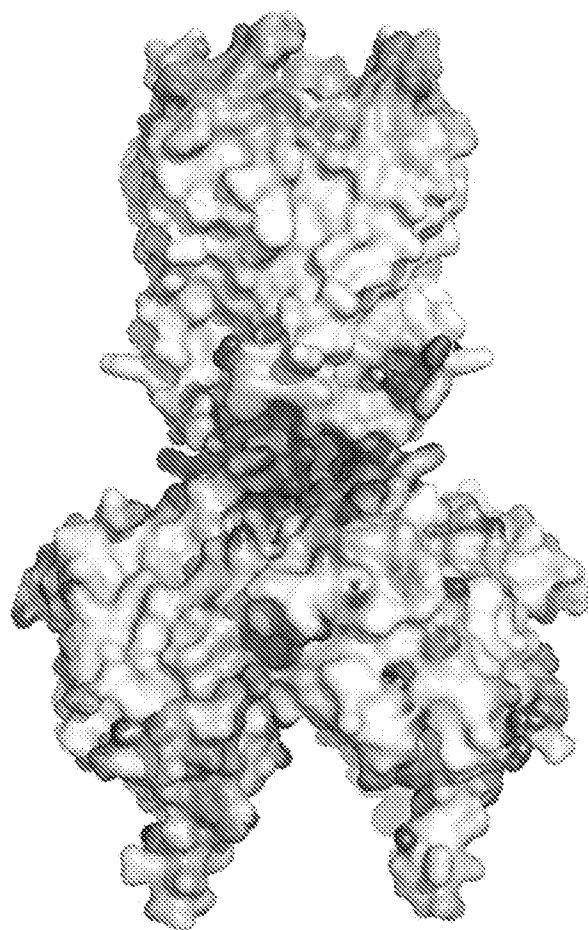


FIG. 7

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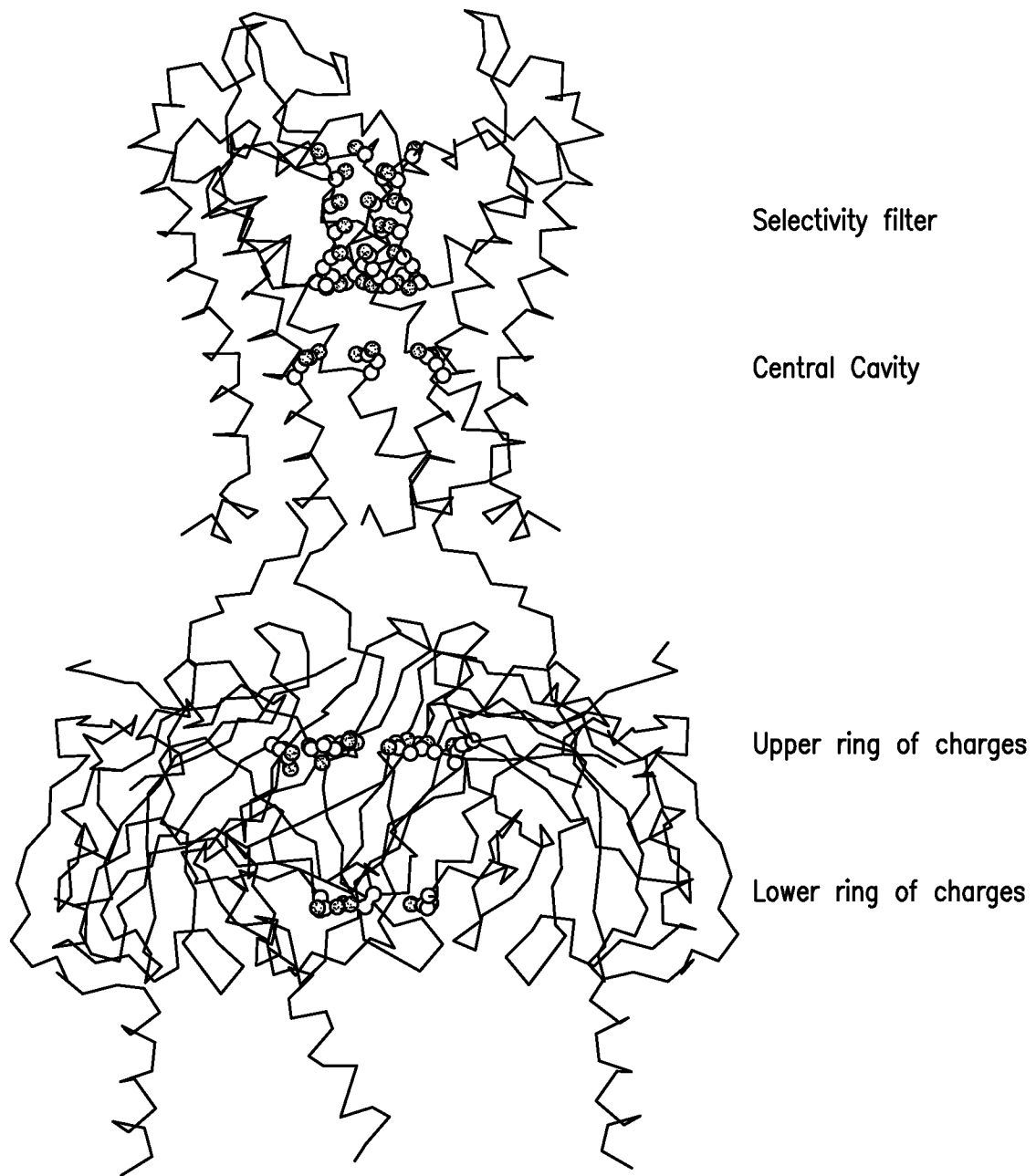


FIG. 8