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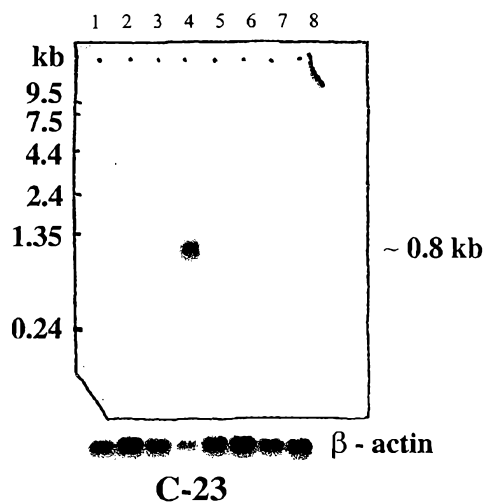
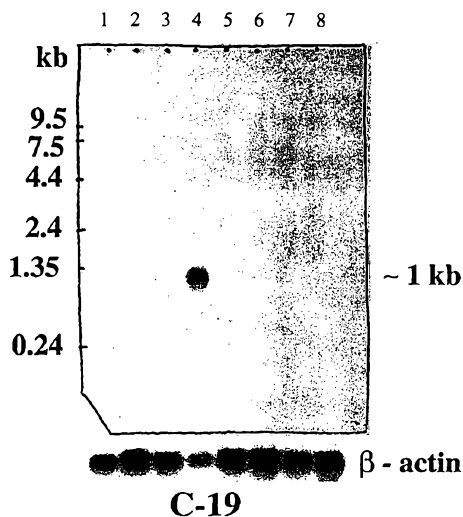
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(54) Title: SPERM SPECIFIC LYSOZYME-LIKE PROTEINS



(57) Abstract: The present invention relates to two novel, testis-specific proteins (C19 and C23) that are lysozyme paralogues. The proteins are believed to play a role in capacitation of sperm and the fertilization of the ovum. Therefore these compounds make ideal targets for the design of contraceptive agents. The C19 and C23 proteins can also be modified to establish lysozyme activity and the modified proteins can then be used in all applications that currently exist for lysozymes.

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Sperm Specific Lysozyme-Like Proteins

This application claims priority under 35 U.S.C. §119(e) to provisional patent application no. 60/176,884, filed January 19, 2000 and provisional patent
5 application no. 60/251,759, filed December 7, 2000.

US Government Rights

This invention was made with United States Government support under Grant No. HD U54 29099, awarded by the National Institutes of Health. The
10 United States Government has certain rights in the invention.

Field of the Invention

The present invention is directed to two novel, testis-specific proteins, designated C19 and C23. These proteins have been designated
15 lysozyme paralogues due to their high degree of conservation of critical amino acids found in other lysozyme-C's.

Background of the Invention

Lysozymes are hydrolases capable of lysing many bacteria. They
20 cleave a beta-glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine of the bacterial cell wall peptidoglycans (murein). Besides this muramidase activity they also display some chitinase (fungal cell wall component) activity. Lysozymes also are credited with antibacterial and antiviral capacities different from the bacteriolytic activity. For example, lysozymes have been
25 demonstrated to have HIV 1 antiviral activity.

Lysozymes have been found in many biological tissues and secretions. Stomach lysozymes (cow, leaf-eating monkey) are even specialized to function at lower pH. There are two types of lysozymes found in the animal kingdom: C-type or chicken-type lysozymes represented by chicken egg white lysozyme, and G-type or
30 goose type lysozymes represented by goose-egg white lysozyme. The C-type lysozymes are actually considered a superfamily including conventional lysozymes,

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calcium-binding lysozymes, and alpha-lactalbumins. All lysozymes have very similar tertiary structures, but vary in amino-acid composition.

Only one lysozyme has been identified and cloned from human tissues and body fluids. The gene coding for the human lysozyme is located on chromosome 12. A second lysozyme C gene was found on chromosome 17, but the corresponding protein has not been described (H. Nomiya, J of Interferon and Cytokine Research 19: 227, 1999). Lysozyme C is a gene of 5856 bp and comprises four exons. The encoded protein is a secretory protein and comprises an 18 amino acid signal sequence and a mature protein of 130 residues. The mature protein contains four disulfide bonds between Cys 6 -- Cys 128, Cys 30 -- Cys 116, Cys 65 -- Cys 81, and Cys 77 -- Cys 95. This protein has been isolated from placenta, amniotic fluid, milk, tears, intestinal cells and leucocytes.

The present invention is directed to two human sperm proteins that have recently been isolated (C19 and C23) and appear to be lysozyme-C paralogues. These proteins are expressed specifically in sperm cell and are believed to function in the events relating to sperm/egg fusion and fertilization.

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

The term "peptide" encompasses a sequence of 3 or more amino acids wherein the amino acids are naturally occurring or synthetic (non-naturally occurring) amino acids. Peptide mimetics include peptides having one or more of the following modifications:

1. peptides wherein one or more of the peptidyl --C(O)NR-- linkages (bonds) have been replaced by a non-peptidyl linkage such as a --CH₂--carbamate linkage

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(--CH₂OC(O)NR--), a phosphonate linkage, a -CH₂-sulfonamide (-CH₂--S(O)₂NR--) linkage, a urea (--NHC(O)NH--) linkage, a --CH₂-secondary amine linkage, or with an alkylated peptidyl linkage (--C(O)NR--) wherein R is C₁-C₄ alkyl;

2. peptides wherein the N-terminus is derivatized to a --NRR₁ group, to a
5 -- NRC(O)R group, to a --NRC(O)OR group, to a --NRS(O)₂R group, to a --NHC(O)NHR group where R and R₁ are hydrogen or C₁-C₄ alkyl with the proviso that R and R₁ are not both hydrogen;

3. peptides wherein the C terminus is derivatized to --C(O)R₂ where R₂ is
10 selected from the group consisting of C₁-C₄ alkoxy, and --NR₃R₄ where R₃ and R₄ are independently selected from the group consisting of hydrogen and C₁-C₄ alkyl.

Naturally occurring amino acid residues in peptides are abbreviated as recommended by the IUPAC-IUB Biochemical Nomenclature Commission as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Norleucine is Nle; Valine is Val or V; Serine is Ser or S; Proline is Pro or P;
15 Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; Glycine is Gly or G, and X is any amino acid. Other naturally occurring amino acids include, by way of example, 4-hydroxyproline, 5-hydroxylysine, and the
20 like.

Synthetic or non-naturally occurring amino acids refer to amino acids which do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein. The resulting "synthetic peptide" contain amino acids other than the 20 naturally occurring, genetically encoded amino acids at one, two,
25 or more positions of the peptides. For instance, naphthylalanine can be substituted for tryptophan to facilitate synthesis. Other synthetic amino acids that can be substituted into peptides include L-hydroxypropyl, L-3,4-dihydroxyphenylalanyl, alpha-amino acids such as L-alpha-hydroxylysyl and D-alpha-methylalanyl, L-alpha.-methylalanyl, beta.-amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids
30 can also be incorporated into the peptides. Other derivatives include replacement of the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) with other side chains.

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As used herein, the term "conservative amino acid substitution" are defined herein as exchanges within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:

Ala, Ser, Thr, Pro, Gly;

5 II. Polar, negatively charged residues and their amides:

Asp, Asn, Glu, Gln;

III. Polar, positively charged residues:

His, Arg, Lys;

IV. Large, aliphatic, nonpolar residues:

10 Met Leu, Ile, Val, Cys

V. Large, aromatic residues:

Phe, Tyr, Trp

As used herein, the term "purified" and like terms relate to the isolation
15 of a molecule or compound in a form that is substantially free of contaminants normally associated with the molecule or compound in a native or natural environment.

As used herein, the term "C19 polypeptide" and like terms refers to polypeptides comprising SEQ ID NO: 2 and biologically active fragments thereof
20 (such as the mature form represented by SEQ ID NO: 8, for example) and the term "C23 polypeptide" and like terms refers to polypeptides comprising SEQ ID NO: 4 and biologically active fragments thereof (such as the mature form represented by SEQ ID NO: 9, for example).

As used herein, the term "biologically active fragment" or "bioactive
25 fragment" of a C19 or C23 polypeptide encompasses natural or synthetic portions of SEQ ID NO: 2 or SEQ ID NO: 4, respectively, that are capable of specific binding to at least one of the natural ligands of the respective native polypeptide.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences or promoters
30 operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

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As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

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Summary of the Invention

The present invention is directed to two lysozyme-like proteins (C19 and C23), nucleic acid sequences encoding those proteins, and antibodies generated against said proteins. Compositions comprising the native C19 or C23 peptides can be used in contraceptive vaccine formulations. Furthermore, antibodies generated against C19 and C23 can be used as diagnostic agents or can be formulated in compositions that are used to interfere with the binding of sperm cells to oocytes. In one embodiment, the present invention is directed to derivatives of the C19 and C23 proteins that have been modified to have lysozyme activity. These modified proteins can be used in any of the applications that currently use human lysozyme C, including antibacterial and antiviral formulations.

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Brief Description of the Drawings

Fig. 1A and 1B is a copy of a multiple tissue Northern Blot, wherein either C19 cDNA (Fig 1A) or C23 cDNA (Fig 1B) was radiolabeled with P³² and hybridized to 2 ug poly-(A)+ mRNAs, revealing a 1 kb (Fig 1A) or 0.8 kb (Fig 1B) message only in testicular RNA. Size of molecular weight markers is indicated at left, lanes 1-8 contain poly-(A)+ mRNA isolated from spleen, thymus, prostate, testis, ovary, small intestine, colon and leucocyte, respectively. The lower panel of Fig. 1A and 1B shows the identical blot probed with β -actin cDNA as a positive control.

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Fig. 2 is a comparison of the mature C19 polypeptide with the mature lysozyme peptides of other species.

Fig. 3 is a comparison of the mature C23 polypeptide with the mature lysozyme peptides of other species.

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Detailed Description of the Invention

Two human sperm proteins have recently been isolated, C19 and C23, that appear to be lysozyme-C paralogues. These proteins are classified as lysozyme paralogues because of their high degree of conservation of critical amino acids found in other lysozyme-C's. However, they differ significantly from the known human lysozyme-C in nucleic acid and amino acid sequence, and their genes are located on different chromosomes. The new proteins C19 and C23 are approximately 15 kDa with pI's of 5.2 and 5.9, respectively. They possess sequence homology to the known human lysozyme-C; however, C19 and C23 are located on chromosome 17 and the X-chromosome, respectively, and thus these two genes represent new human lysozyme-like genes. The nucleic acid sequence and the deduced amino acid sequence of C19 are represented by SEQ ID NO: 1 and SEQ ID NO: 3, respectively, and nucleic acid sequence and the deduced amino acid sequence of C23 are represented by SEQ ID NO: 2 and SEQ ID NO: 4, respectively.

C19 and C23 each contain a signal peptide. The initial C19 polypeptide is synthesized as a 215 amino acid polypeptide (SEQ ID NO: 2) having a MW of 23.4 kDa and a pI of 8.0. The mature C19 peptide is 128 amino acids (SEQ ID NO: 8) and has a MW of about 14.6 kDa and pI of 5.0. The initial C23 polypeptide is synthesized as a 159 amino acid polypeptide (SEQ ID NO: 4) having a MW of 17.9 kDa and a pI of 5.9. The mature C23 peptide is 138 amino acids (SEQ ID NO: 9) and has a MW of about 15.7 kDa and pI of 5.9.

C19 and C23 have 48.8% sequence identity between one another and have 52% and 44% amino-acid sequence identity with the one known mature human lysozyme C, respectively, and 44% and 43% amino-acid sequence identity with the predicted lysozyme homologue on chromosome 17q11.2. C19 is most closely related to human lysozyme (52% sequence identity), whereas C23 is most closely related to chicken lysozyme (51% sequence identity).

The gene encoding C19 is located on Chromosome 17 and is 6012 bp in length. The C19 gene contains 5 exons (109, 309, 159, 79 and 164 bp, respectively) and 4 introns (3436, 1125, 443 and 188 bp, respectively). The gene encoding C23 is located on Chromosome Xp11.1 and is 1950 bp in length. The C23 gene contains 4 exons (169, 159, 79 and 181 bp, respectively) and 3 introns (428, 830,

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and 104 bp, respectively). Interestingly, exons 3 and 4 of C19 have a sequence identity with exons 2 and 3 of C23 greater than the overall sequence identity between the two complete proteins (i.e. greater than 48.8%) and exons 3 and 4 of C19 are identical in size to exons 2 and 3 of C23, respectively.

5 The expression of C19 and C23 is limited to the testes (see Fig 1). To further characterize the expression of C19 and C23, antibodies were generated against C19 and C23. Those antibodies are specific for the target peptide and do not cross react with each other's respective lysozyme-like protein. C19 immunofluorescence and C19 and C23 EM localization experiments demonstrate that expression of the C19
10 and C23 proteins is localized in the sperm acrosome.

 Recombinant C19 and C23 have been expressed in *E. coli* and in yeast. The proteins expressed in yeast were produced in a form that is secreted into the medium, and C19 was purified from the media and used in an assay to test for lysozyme activity. Secretion of the putatively processed forms of C19 and C23 (C23
15 was in crude form) as soluble proteins from *Pichia pastoris* revealed no lysozyme activity for C19 and C23 using *Micrococcus lysodeikticus* as the lysozyme substrate. In particular, *Micrococcus lysodeikticus* was grown to confluence on a petri plate and the cells were contacted with 330 U of human lysozyme C (as a positive control), a reagent blank (as a negative control) and 1650 U of the purified soluble C19 protein
20 (yrC19). Lysozyme activity was observed in the human lysozyme C portion (the positive control) as indicated by a zone of clearance about the introduce sample, but no activity was detected for yrC19. Although these compounds fail to exhibit lysozyme activity in the present assay, these compounds may still exhibit antibacterial/antiviral activity through an unknown mechanism.

25 Of all known lysozyme-C sequences (>75), 20 amino acid residues are invariant (see Figs 2 and 3). C19 contains all but two of those invariable amino acids (E35T, Y54N). The amino acid 35-E is considered a critical amino acid for catalytic function (i.e. cleaving the polysaccharide bond between N-acetylglucosamine and N-acetylmuramic acid). C23 contains all but one (D53E) of the 20 conserved amino
30 acids. The amino acid 53-D is considered a critical amino acid for catalytic function; however, g-type lysozymes do not have a D in the corresponding position. Homologous genes of C19 and C23 have also been isolated by applicants from other

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mammalian species (for example, mice), that contain similar mutations in the catalytic residues of these genes.

In accordance with one embodiment of the present invention, modified versions of the C19 and C23 proteins are provided wherein the 35-T of C19 is
5 converted to 35-E (SEQ ID NO: 5) and the 53-E of C23 is converted to 53-D (SEQ ID NO: 6). It is anticipated that when these single amino acid substitutions are made in each lysozyme-like protein, the modified proteins will exhibit lysozyme activity and thus can be used as alternative compounds in all applications currently utilizing known human lysozyme-C. Furthermore, in one embodiment a modified version of
10 C19 is prepared wherein the 35-T is converted to 35-E and 54-N is converted to 54-Y (SEQ ID NO: 7). This modified version of C19 is also expected to have lysozyme activity.

The C19 and C23 native polypeptides when modified to have lysozyme activity can be used in any of the applications described in US patent 4,945,051, US
15 patent 5,585,257, US patent 5,618,712 and WO 9924589 (DE19749973), the disclosures of which are expressly incorporated herein. The novel lysozymes of the present invention can also be used as the active agent in antibacterial wound dressings, dental plaque preventing formulations, anti-inflammatory throat lozenges, anti-acne compositions, sprays for controlling dry mouth condition and as food
20 additives to prevent spoilage. It has also been reported that lysozyme may be effective against HIV (Lee-Huang, S., PNAS 96:2678, 1999).

In one embodiment, a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 is used as the active agent in an antibacterial and antiviral
25 composition. In one preferred embodiment, a polypeptide comprising an amino acid sequence of SEQ ID NO: 10 or SEQ ID NO: 11 is used as an antibacterial and antiviral agent. The lysozyme proteins of the present invention can also be combined with standard antibacterial and antiviral agents to enhance the efficacy of those agents. In accordance with one embodiment, a composition comprising an amino acid
30 sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 is used as an antibacterial/antiviral additives to intravaginal gels or foams to reduce the risk of sexually transmitted diseases.

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In another embodiment, compositions comprising the native C19 or C23 polypeptides or fragments thereof are used as contraceptive agents. In particular, the unmodified C19 and C23 proteins are anticipated to have sperm specific functions that can be the basis of a contraceptive vaccine, designed to prevent
5 capacitation/fertilization. For example in accordance with one embodiment the C19 or C23 polypeptides or fragments thereof, are used as components of a contraceptive vaccine.

In one aspect of the invention, C19 and C23 polypeptides (either separately or in combination) are delivered to a subject to elicit an active immune
10 response. The vaccine acts as a temporary and reversible antagonist of the function of the egg surface proteins of the invention. For example, such vaccines could be used for active immunization of a subject, to raise an antibody response to temporarily block the sperm's access to the egg plasma antigen. In one aspect of the invention, an antigen could be administered at a certain period of the month, for example during
15 ovulation of a female subject to block fertilization.

In another aspect of the invention, C19 and C23 polypeptides (either separately or in combination) are used as vaccines for permanent sterilization of a subject. Such vaccines can be used to elicit a T-cell mediated attack on the eggs, having an othoritic effect, useful as a method for irreversible sterilization. Methods
20 for generating T-cell specific responses, such as adoptive immunotherapy, are well known in the art (see, for example, Vaccine Design, Michael F. Powell and Mark J. Newman Eds., Plenum Press, New York, 1995, pp 847-867). Such techniques may be particular useful for vetinary contraceptive or sterilization purposes, where a single dose vaccination may be desirable.

25 In one embodiment, the present invention is directed to a purified polypeptide comprising the amino acid sequence of SEQ ID NO: 2, or an amino acid sequence that differs from SEQ ID NO: 2 by one or more conservative amino acid substitutions. More preferably, the purified polypeptide comprises an amino acid sequence that differs from SEQ ID NO: 2 by 10 or less conservative amino acid
30 substitutions. Alternatively, the polypeptide may comprise an amino acid sequence that differs from SEQ ID NO: 2 by 1 to 3 alterations, wherein the alterations are independently selected from a single amino acid deletion, insertion or substitution.

Alternatively, one embodiment of the present invention is directed to a purified polypeptide comprising the amino acid sequence of SEQ ID NO: 4, or an amino acid sequence that differs from SEQ ID NO: 4 by one or more conservative amino acid substitutions. More preferably, the purified polypeptide comprises an amino acid sequence that differs from SEQ ID NO: 4 by 10 or less conservative amino acid substitutions. Alternatively, the polypeptide may comprise an amino acid sequence that differs from SEQ ID NO: 4 by 1 to 3 alterations, wherein the alterations are independently selected from a single amino acid deletion, insertion or substitution.

Another embodiment of the present invention encompasses polypeptides comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 and amino acid sequences that differs from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 or SEQ ID NO: 9 by 10 or less conservative amino acid substitutions. The present invention also encompasses fragments of SEQ ID NO: 2 and SEQ ID NO: 4, wherein the peptide fragment is at least ten amino acids in length and comprises ten contiguous amino acids that are identical in sequence to an ten contiguous amino portion of SEQ ID NO: 2 or SEQ ID NO: 4.

In one embodiment, the present invention provides methods of screening for agents, small molecules, or proteins that interact with polypeptides of SEQ ID NO: 2 or SEQ ID NO: 4. The invention encompasses both *in vivo* and *in vitro* assays to screen small molecules, compounds, recombinant proteins, peptides, nucleic acids, antibodies *etc.* which bind to or modulate the activity of C19 or C23 and are thus useful as therapeutics or diagnostic markers for fertility.

For example, the C19 or C23 polypeptide, or a bioactive fragment thereof, can be used to isolate ligands that bind to the respective native polypeptide under physiological conditions. The method comprises the steps of contacting the C19 or C23 polypeptide with a mixture of compounds under physiological conditions, removing unbound and non-specifically bound material, and isolating the compounds that remain bound to the C19 or C23 polypeptide. Typically, the C19 or C23 polypeptide will be bound to a solid support using standard techniques to allow rapid screening compounds. The solid support can be selected from any surface that has been used to immobilize biological compounds and includes but is not limited to

polystyrene, agarose, silica or nitrocellulose. In one embodiment the solid surface comprises functionalized silica or agarose beads. Screening for such compounds can be accomplished using libraries of pharmaceutical agents and standard techniques known to the skilled practitioner.

5 In accordance with one embodiment the C19 and C28 polypeptides and peptide fragments are used to isolate oocyte proteins that bind to C19 and C28. The procedures for recovering oocyte proteins and screening for ligands that bind to C19 and C23 are well known to those skilled in the art. In one embodiment the C19 or C23 polypeptide is immobilized to a solid support and the proteins are contacted with
10 a solution/suspension of oocyte proteins under conditions that allow binding. Unbound and non-specific bound materials are then washed from the solid support and the remaining bound materials are recovered and analyzed (by microsequencing, for example). Microsequencing of the recovered proteins will allow for the design of nucleic acid probes and primers for the identification and cloning of the corresponding
15 genes that encode the recovered proteins.

 The present invention also encompasses nucleic acid sequences that encode the C19 and C23 polypeptides, and bioactive fragments and derivatives thereof. In particular the present invention is directed to nucleic acid sequences comprising the sequence of SEQ ID NO: 1, or SEQ ID NO: 3, or fragments thereof.
20 In one embodiment, purified nucleic acids comprising at least 20 contiguous nucleotides (*i.e.*, a hybridizable portion) that are identical to any 20 contiguous nucleotides of SEQ ID NO: 1 or SEQ ID NO: 3 are provided. In other embodiments, the nucleic acids comprises at least 25 (contiguous) nucleotides, 50 nucleotides, 100 nucleotides, or 200 nucleotides of SEQ ID NO: 1 or SEQ ID NO: 3.

25 One embodiment of the present invention includes nucleic acids that hybridize (under conditions defined herein) to all or a portion of the nucleotide sequence represented by SEQ ID NO: 1 or its complement. Alternatively, the present invention also includes nucleic acids that hybridize (under conditions defined herein) to all or a portion of the nucleotide sequence represented by SEQ ID NO: 3 or its
30 complement. The hybridizing portion of the hybridizing nucleic acids is typically at least 15 (e.g., 20, 25, 30, or 50) nucleotides in length. Hybridizing nucleic acids of the type described herein can be used, for example, as a cloning probe, a primer (e.g., a

PCR primer), or a diagnostic probe. The DNA sequence of SEQ ID NO: 1, SEQ ID NO: 3, or fragments thereof, can be used as probes to detect homologous genes from other vertebrate species.

Nucleic acid duplex or hybrid stability is expressed as the melting temperature or T_m , which is the temperature at which a nucleic acid duplex dissociates into its component single stranded DNAs. This melting temperature is used to define the required stringency conditions. Typically a 1% mismatch results in a 1°C decrease in the T_m , and the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if two sequences having > 95% identity, the final wash temperature is decreased from the T_m by 5°C). In practice, the change in T_m can be between 0.5°C and 1.5°C per 1% mismatch.

The present invention is directed to the nucleic acid sequence of SEQ ID NO: 1 and SEQ ID NO: 3, and nucleic acid sequences that hybridize to those sequences (or fragments thereof) under stringent or highly stringent conditions. In accordance with the present invention highly stringent conditions are defined as conducting the hybridization and wash conditions at no lower than -5°C T_m . Stringent conditions are defined as involve hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at 68°C. Moderately stringent conditions include hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS and washing in 3x SSC/0.1% SDS at 42°C. Additional guidance regarding such conditions is readily available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

In another embodiment of the present invention, nucleic acid sequences encoding the C19 or C23 polypeptides can be inserted into expression vectors and used to transfect cells to enhance the expression of those proteins on the target cells. In accordance with one embodiment, nucleic acid sequences encoding C19 or C23, or a fragment or a derivative thereof, are inserted into a eukaryotic expression vector in a manner that operably links the gene sequences to the appropriate regulatory sequences, and recombinant C19 or recombinant C23 is expressed in a eukaryotic host cell. Suitable eukaryotic host cells and vectors are known to those skilled in the art.

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In particular, nucleic acid sequences encoding C19 or C23 may be added to a cell or cells *in vitro* or *in vivo* using delivery mechanisms such as liposomes, viral based vectors, or microinjection. Accordingly, one aspect of the present invention is directed to transgenic cell lines that contain recombinant genes that express C19 or C23.

The present invention also encompasses antibodies, including anti-idiotypic antibodies, antagonists and agonists, as well as compounds or nucleotide constructs that inhibit expression of the C19 and C23 genes (transcription factor inhibitors, antisense and ribozyme molecules, or gene or regulatory sequence replacement constructs), or promote expression of C19 and C23 (*e.g.*, expression constructs in which C19 or C23 coding sequences are operatively associated with expression control elements such as promoters, promoter/enhancers, *etc.*). Antagonists of C19 and/or C23 function can be used to interfere with the capacitation of vertebrate sperm and fertilization of an ovum, and thus used as contraceptive agents. Furthermore, antibodies against the C19 or C23 protein can be used for the diagnosis of conditions or diseases characterized by expression or overexpression of C19 or C23, or in assays to monitor patients being treated with C19 or C23 agonists, antagonists or inhibitors.

In accordance with one embodiment, antibodies are provided that specifically bind to C19 or C23. In particular, a C19 or C23 polypeptide, fragments thereof, or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. In accordance with one embodiment of the present invention an antigenic compound is provided for generating antibodies, wherein the compound comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. The antibodies generated can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions. Antibodies to C19 or C23 may be generated using methods that are well known in the art.

In one embodiment, rabbit polyclonal antibodies to an epitope of C19 or C23, is obtained. For the production of antibody, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with a C19 or

C23 peptide. Various adjuvants may be used to increase the immunological response, depending on the host species, and 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
5 limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward an egg surface protein sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For
10 example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional
15 embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer
20 Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for epitopes of C19 or C23 together with genes from a human
25 antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce egg surface protein-specific single chain antibodies. An additional embodiment of the invention
30 utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of

-15-

monoclonal Fab fragments with the desired specificity for egg surface proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the 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, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the C19 or C23 proteins of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

Antibodies generated in accordance with the present invention may include, but are not limited to, polyclonal, monoclonal, chimeric (i.e. "humanized" antibodies), single chain (recombinant), Fab fragments, and fragments produced by a Fab expression library. These antibodies can be used as diagnostic agents for the diagnosis of conditions or diseases characterized by expression or overexpression of C19 or C23, or in assays to monitor patients being treated with C19 or C23 receptor agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule.

In accordance with one embodiment an antibody is provided that specifically binds to a polypeptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. In one preferred embodiment the antibody is a monoclonal antibody.

In one embodiment antibodies against the C19 and/or C23 proteins are used as contraceptive agents that prevent the binding of sperm cells to eggs. An experiment was conducted to determine if the antibodies against C19 and C23 could interfere human sperm's ability to bind to eggs (See Example 2). The assay was

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conducted *in vitro* using human sperm and hamster eggs. C19 and C23 are on the acrosome membrane and are only exposed upon permeabilization of the acrosome. Only approximately 1/3 of sperm undergo acrosome reaction *in vitro*. As seen in Example 2, antibodies against C19 significantly interfered with sperm cells ability to
5 bind to hamster eggs while no effect was observed for the antibody generated against C23. These results suggest that a unique receptor for the C19 protein may exist on mammalian eggs, and this receptor itself could serve as a target for contraceptive agents.

The present invention also encompasses compositions that can be
10 placed in contact with sperm cells to inhibit the function of the C19 and C23 protein (i.e. either by inhibiting the expression of the C19 and C23 proteins or by interfering with the protein's function). In particular the compositions may comprise peptide fragments of C19 or C23, or analogs thereof that are taken up by the sperm cells and compete for binding with C19 and C23's natural ligands. Such inhibitory peptides can
15 be modified to include fatty acid side chains to assist the peptides in penetrating the sperm cell membrane. Compositions comprising a C19 or C23 inhibitory agent can be used to modulate fertility of an individual, and in one embodiment, the inhibitory agents function as a male contraceptive pharmaceutical. In accordance with one embodiment a composition is provided that comprises an eight to fifteen amino acid
20 sequence that is identical to an eight to fifteen contiguous amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 and a pharmaceutically acceptable carrier.

Example 1

Isolation of the C19 and C23 Proteins

25 Materials and Methods

Solubilization and electrophoresis of human spermatozoal proteins

Preparation of semen specimens and solubilization of sperm proteins were performed as previously described (Naaby-Hansen et al, 1997a.) For analytical two-dimensional electrophoresis the detergent/urea extracted proteins were separated
30 by isoelectric focusing (IEF) in acrylamide tube gels prior to second dimensional gel electrophoresis (SDS-PAGE), which was performed in a Protean II xi Multi-Cell apparatus (Bio-Rad, Richmond, CA) or on large format (23 x 23 cm) gels

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(Investigator 2-D Electrophoresis System, ESA) which were also employed for preparative 2D gel electrophoresis. Electrotransfer to nitrocellulose membranes and subsequent visualizing of the proteins by gold staining was accomplished as previously described (Naaby-Hansen et al, 1997) while electrotransfer to PVDF membranes (0.2 mm pore size, Pierce) was carried out as described by Henzel et al. (1993) using the transfer buffer composition of Matsudaira (1987) (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11). The immobilized proteins were visualized by staining in a solution containing 0.1% Coomassie R250, 40% methanol and 0.1% acetic acid for one minute, followed by destaining in a solution of 10% acetic acid and 50% methanol for 3 x 3 minutes.

Generation of antiserum against gel purified C19 and C23

The 86 kDa Coomassie-stained protein spot was cored from three 1.5 mm thick 2-D SDS-PAGE gels of human sperm extracts. The gel cylinders were minced into a slurry in 1 ml of PBS and emulsified with an equal volume of complete Freund's adjuvant. Six hundred μ l of this emulsion was intradermally injected into a New Zealand white rabbit, followed by two monthly subcutaneous booster injections of similarly-prepared antigen with incomplete Freund's adjuvant. Serum was collected 10 days after each booster injection.

Microsequencing of the C19 and C23 proteins

The C19 and C23 stained protein spots were cored from a 1.5 mm thick 2D SDS-polyacrylamide gel and fragmented into smaller pieces. The proteins were destained in methanol, reduced in 10 mM dithiothreitol and alkylated in 50 mM iodoacetamide in 0.1 M ammonium bicarbonate. After removing the reagents, the gel pieces were incubated with 12.5 ng/ml trypsin in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides were extracted from the gel pieces in 50 % acetonitrile in 5% formic acid and microsequenced by tandem mass spectrometry and by Edman degradation at the Biomolecular Research Facility of the University of Virginia. Differentiation of leucine and isoleucine in the sequences were determined by Edman sequencing of HPLC isolated peptides. A degenerate deoxyinosine containing primers were used to isolate the C19 and C23 cDNA clones based on the microsequencing data and using PCR technology.

Northern and dot blot analyses

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A Northern blot containing 2 mg of poly(A)⁺ RNA from eight selected human tissues was obtained from Clontech. The Northern blot was probed with a ³²P-labeled C19 cDNA (Fig. 1A) or ³²P-labeled C23 cDNA (Fig. 1B). Probes were prepared by random oligonucleotide prime labeling (Feinberg and Vogelstein, 1983).
5 Hybridization was performed in ExpressHyb solution (Clontech) at 68 °C for 1 h followed by three washes in 2x SSC, 0.05% SDS at room temperature and two washes in 0.1x SSC, 0.1% SDS for 20 min at 50 °C.

A normalized RNA dot blot containing 89 to 514 ng of mRNA from 50 different human tissues was obtained from Clontech and probed with ³²P-labeled C19
10 cDNA or ³²P-labeled C23 cDNA. The normalized (100-500 ng) poly-(A)⁺ mRNAs present on the grid were isolated from various tissue sources including: whole brain, amygdala, caudate nucleus, cerebellum, cerebral cortex, frontal lobe, hippocampus, medulla oblongata, occipital lobe, putamen, substantia nigra, temporal lobe, thalamus, subthalamic nucleus, spinal chord, heart, aorta, skeletal muscle, colon, bladder, uterus,
15 prostate, stomach, testis, ovary, pancreas, pituitary gland, adrenal gland, thyroid gland, salivary gland, mammary gland, kidney, liver, small intestine, spleen, thymus, peripheral leukocyte, lymph node, bone marrow, appendix, lung, trachea, placenta, fetal brain, fetal heart, fetal kidney, fetal liver, fetal spleen, fetal thymus, fetal lung, and 100 ng total yeast RNA, 100 ng yeast tRNA, 100 ng E. coli rRNA, 100 ng E. coli
20 DNA, 100 ng poly r(A), 100 ng Cot 1 human DNA, 100 ng human DNA, 500 ng human DNA. The blot was hybridized in ExpressHyb solution (Clontech) containing salmon sperm DNA and human placental Cot-1 DNA overnight at 65 °C. The blot was then washed three times in 2x SSC, 1% SDS at 65 °C followed by two additional washes in 0.1x SSC, 0.5% SDS at 55 °C before exposing the filter to X-Ray film.
25 Hybridization was only detected in the testis RNA dot.

Example 2

Human Sperm Binding and Fusion Assay Using Zona-Free Hamster Eggs

Sperm Preparation:

30 Motile sperm were harvested by the swim up method of Bronson and Fusi (1990). Briefly, a 500 µl sperm sample underlaid in 2 ml of BWW media containing 5 mg/ml HSA. Sperm were allowed to swim up for 1.5 – 2 h. Swimup

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sperm were collected and 8 ml of BWW+5 mg/ml HSA was added. The composition was spin at 600xg for 8 min at RT, the supernatant was removed and 8 ml of media was added to the pellet. The resuspended pellet was spun at 600xg for 8 min at RT. The supernatant was removed and 50 ml of BWW containing 30 mg/ml HSA was added to the pellet. Total sperm cells were counted and then incubated overnight in BWW+30 mg/ml HSA at a concentration of 20×10^6 sperm/ml.

Egg Collection:

Female hamsters received i.p. injections of 30 IU PMSG followed by 30 IU of hCG 72 h later. 14-16 h following hCG injection, hamsters were sacrificed and oviducts are collected in BWW media containing 5 mg/ml HSA. Cumulus cells were removed with 1 mg/ml hyaluronidase, the eggs were washed and zona pellucidae removed with 1 mg/ml trypsin. The eggs were then thoroughly washed and allowed to rest in the incubator.

Sperm/Antibody Incubation:

Sperm was diluted to 20×10^6 sperm/ml and incubated with appropriate dilutions of pre-immune or immune sera (initially a 1:10 and 1:50 dilution of sera is tested) in paraffin oil covered microdrops for 1 h.

Hamster eggs were added to the drops containing the sperm+antibody. The gametes were then co-incubated for 3 h.

Assessment of Binding and Fusion:

Eggs were washed free of unbound and loosely bound sperm by serial passage through 5 (50 ml) wash drops. The same pipet is used for all eggs washed in an individual experiment. Eggs are then stained by short-term (5-15 s) exposure to 1 mM acridine orange-3% DMSO in BSA/BWW (30 mg/ml), washed through 4 (50 ml) wash drops and mounted under 22 X 22 mm coverslips. Under UV illumination, unexpanded heads of oolemma-adherent sperm were counted and sperm that had penetrated the ooplasm exhibited expanded green heads. All experiments were repeated 3 times

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Results

1:10 dilution of C19 Antibody

5	<u>Number of sperm bound per egg</u>			
	Pre Immune	38.2	Immune	21.8

P value = 7.78×10^{-6}

10	<u>Number of sperm fused per egg</u>			
	Pre Immune	3.2	Immune	2.9

P value = 0.6

15

1:10 dilution of C23 Antibody

20	<u>Number of sperm bound per egg</u>			
	Pre Immune	28.7	Immune	27.4

P value = 0.79

25

25	<u>Number of sperm fused per egg</u>			
	Pre Immune	1.8	Immune	1.6

30

P value = 0.71

Claims:

1. A purified polypeptide comprising
the amino acid sequence of SEQ ID NO: 2;
an amino acid sequence that differs from SEQ ID NO: 2 by one to ten
5 conservative amino acid substitutions; or
an amino acid sequence that differs from SEQ ID NO: 2 by a single mutation,
wherein the single mutation represents a single amino acid deletion, insertion or
substitution.
2. A purified polypeptide comprising
10 the amino acid sequence of SEQ ID NO: 4;
an amino acid sequence that differs from SEQ ID NO: 4 by one to ten
conservative amino acid substitutions; or
an amino acid sequence that differs from SEQ ID NO: 4 by a single mutation,
wherein the single mutation represents a single amino acid deletion, insertion or
15 substitution.
3. A purified or recombinant polypeptide wherein said polypeptide
comprises an amino acid sequence selected from the group consisting of SEQ ID NO:
2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and
SEQ ID NO: 9.
- 20 4. A purified or recombinant polypeptide wherein said polypeptide
comprises an amino acid sequence selected from the group consisting of SEQ ID NO:
5, SEQ ID NO: 6, SEQ ID NO: 7 or an amino acid sequence that differs from SEQ ID
NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 by one to five conservative amino acid
substitutions.
- 25 5. A purified or recombinant polypeptide wherein said polypeptide
comprises an amino acid sequence selected from the group consisting of SEQ ID NO:
10, SEQ ID NO: 11, or an amino acid sequence that differs from SEQ ID NO: 10 or
SEQ ID NO: 11 by one to five conservative amino acid substitutions.
6. A nucleic acid sequence comprising the sequence of SEQ ID NO: 1, or
30 SEQ ID NO: 3.
7. A nucleic acid sequence that hybridizes to a 100 nucleotide fragment of
SEQ ID NO: 1 or SEQ ID NO: 3 under stringent conditions.

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8. A transgenic host cell comprising the nucleotide sequence of claim 7.
9. A nucleic acid sequence comprising a 25 bp nucleic acid sequence that is identical to a contiguous 25 bp sequence of SEQ ID NO: 1 or SEQ ID NO: 3.
10. A method of screening for potential human therapeutic agents, said
5 method comprising contacting a C19 or C23 protein with a candidate compound; and determining if the candidate compound selectively binds to the C19 or C23 protein.
11. The method of claim 10 wherein the C19 or C23 protein is expressed on the surface of a cell.
12. An antibody that binds specifically to the protein of SEQ ID NO: 2.
- 10 13. An antibody that binds specifically to the protein of SEQ ID NO: 4.
14. An antigenic compound, said compound comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9.

15

FIG 1 A

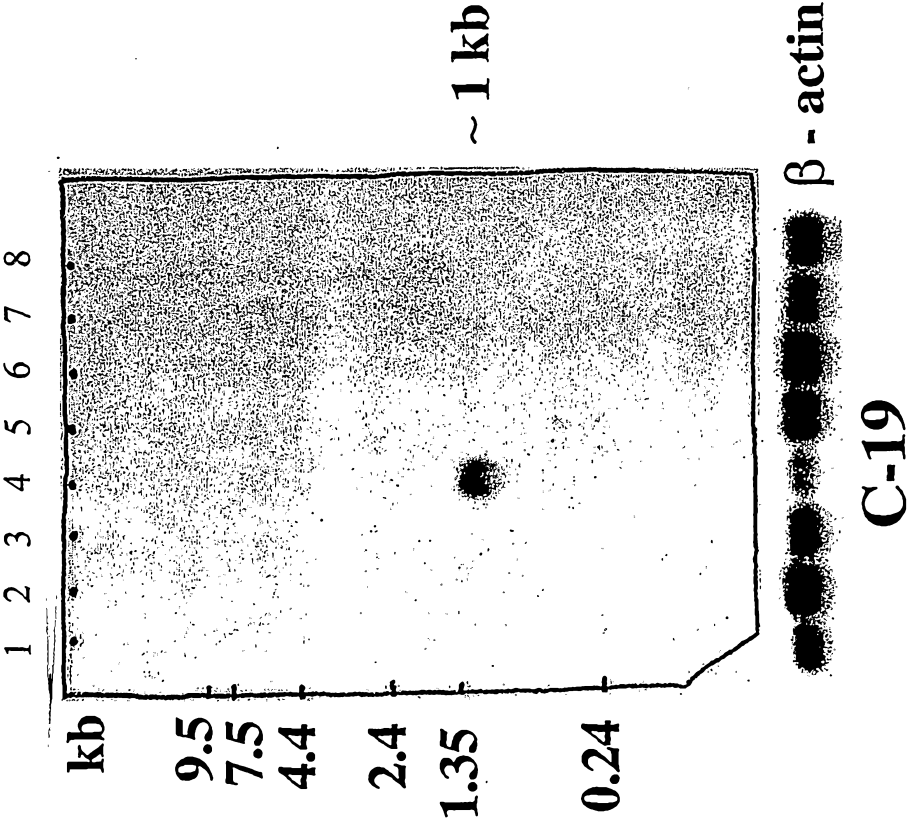
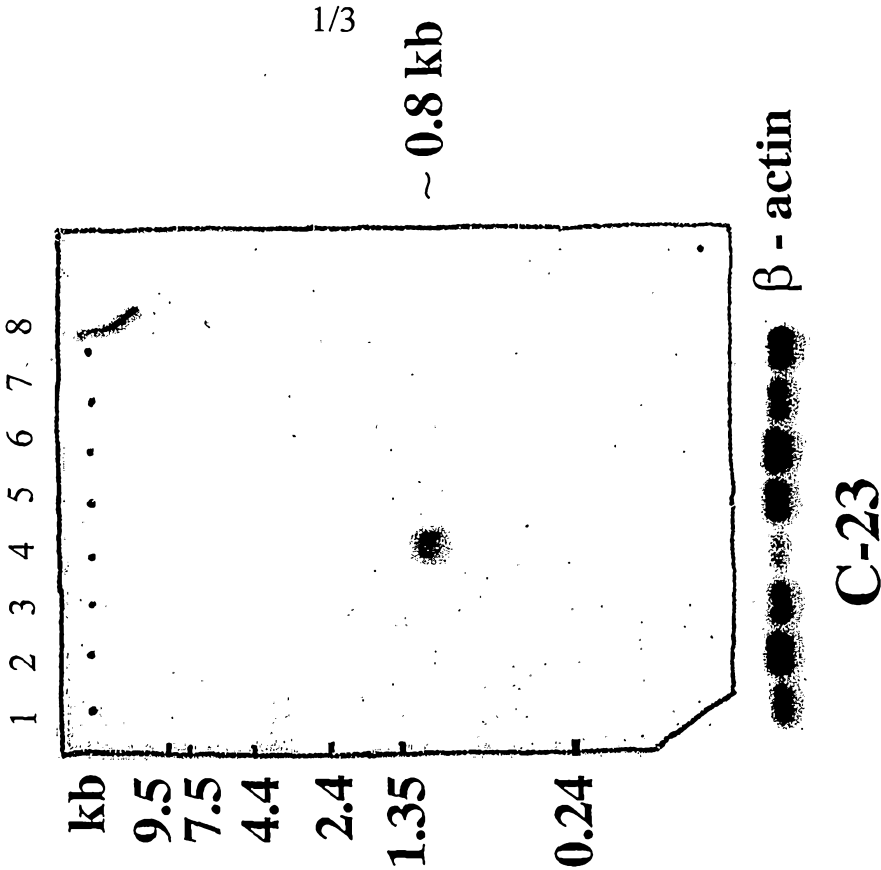


FIG 1 B



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Shetty, Jagathapala
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 Lys Ile Tyr Glu Arg Cys Glu Leu Ala Ala Arg Leu Glu Arg Ala Gly
 1 5 10 15

45

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Leu Asn Gly Tyr Lys Gly Tyr Gly Val Gly Asp Trp Leu Cys Met Ala
 20 25 30

5 His Tyr Glu Ser Gly Phe Asp Thr Ala Phe Val Asp His Asn Pro Asp
 35 40 45

Gly Ser Ser Glu Tyr Gly Ile Phe Gln Leu Asn Ser Ala Trp Trp Cys
 50 55 60

10 Asp Asn Gly Ile Thr Pro Thr Lys Asn Leu Cys His Met Asp Cys His
 65 70 75 80

Asp Leu Leu Asn Arg His Ile Leu Asp Asp Ile Arg Cys Ala Lys Gln
 85 90 95

15 Ile Val Ser Ser Gln Asn Gly Leu Ser Ala Trp Thr Ser Trp Arg Leu
 100 105 110

His Cys Ser Gly His Asp Leu Ser Glu Trp Leu Lys Gly Cys Asp Met
 20 115 120 125

His Val Lys Ile Asp Pro Lys Ile His Pro
 130 135

25

<210> 10
 <211> 128
 <212> PRT
 <213> Homo sapiens

30

<400> 10
 Lys Leu Tyr Gly Arg Cys Glu Leu Ala Arg Val Leu His Asp Phe Gly
 1 5 10 15

35 Leu Asp Gly Tyr Arg Gly Tyr Ser Leu Ala Asp Trp Val Cys Leu Ala
 20 25 30

Tyr Phe Glu Ser Gly Phe Asn Ala Ala Ala Leu Asp Tyr Glu Ala Asp
 35 40 45

40 Gly Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Arg Trp Cys
 50 55 60

Ser Asn Leu Thr Pro Asn Val Pro Asn Val Cys Arg Met Tyr Cys Ser
 45 65 70 75 80

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5  Asp Leu Leu Asn Pro Asn Leu Lys Asp Thr Val Ile Cys Ala Met Lys
    85                                90                                95

10 5  Ile Thr Gln Glu Pro Gln Gly Leu Gly Tyr Trp Glu Ala Trp Arg His
    100                                105                                110

    His Cys Gln Gly Lys Asp Leu Thr Glu Trp Val Asp Gly Cys Asp Phe
    115                                120                                125

15 <210> 11
    <211> 138
    <212> PRT
    <213> Homo sapiens

    <400> 11

20 Lys Ile Tyr Glu Arg Cys Glu Leu Ala Ala Arg Leu Glu Arg Ala Gly
    1                                5                                10                                15

    Leu Asn Gly Tyr Lys Gly Tyr Gly Val Gly Asp Trp Leu Cys Met Ala
    20                                25                                30

25 His Tyr Glu Ser Gly Phe Asp Thr Ala Phe Val Asp His Asn Pro Asp
    35                                40                                45

    Gly Ser Ser Asp Tyr Gly Ile Phe Gln Leu Asn Ser Ala Trp Trp Cys
    50                                55                                60

30 Asp Asn Gly Ile Thr Pro Thr Lys Asn Leu Cys His Met Asp Cys His
    65                                70                                75                                80

    Asp Leu Leu Asn Arg His Ile Leu Asp Asp Ile Arg Cys Ala Lys Gln
    85                                90                                95

35 Ile Val Ser Ser Gln Asn Gly Leu Ser Ala Trp Thr Ser Trp Arg Leu
    100                                105                                110

40 His Cys Ser Gly His Asp Leu Ser Glu Trp Leu Lys Gly Cys Asp Met
    115                                120                                125

    His Val Lys Ile Asp Pro Lys Ile His Pro
    130                                135

45

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<210> 12

<211> 126

<212> PRT

<213> Nasalis concolor

5

<400> 12

Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 1 5 10 15

10 Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

Lys Trp Glu Ser Gly Tyr Asn Thr Glu Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

15

Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

20 Asn Asn Lys Thr Pro Gly Ala Val Asp Ala Cys His Ile Ser Cys Ser
 65 70 75 80

Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 85 90 95

25 Val Val Ser Asp Pro Gln Gly Val Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

His Cys Gln Asn Lys Asp Val Ser Gln Tyr Val Lys Gly Cys
 115 120 125

30

<210> 13

<211> 126

<212> PRT

35 <213> Nasalis concolor

<400> 13

Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 1 5 10 15

40

Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

45 Lys Trp Glu Ser Gly Tyr Asn Thr Glu Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

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Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

5 Asn Asn Lys Thr Pro Gly Ala Val Asp Ala Cys His Ile Ser Cys Ser
 65 70 75 80

Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 85 90 95

10 Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

His Cys Gln Asn Lys Asp Val Ser Gln Tyr Val Lys Gly Cys
 15 115 120 125

<210> 14
 <211> 126
 20 <212> PRT
 <213> Macaca mulatta

<400> 14
 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 25 1 5 10 15

Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

30 Lys Trp Glu Ser Gly Tyr Asn Thr Glu Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 35 50 55 60

Asn Asn Lys Thr Pro Gly Ala Val Asp Ala Cys His Ile Ser Cys Ser
 65 70 75 80

40 Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 85 90 95

Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

45

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His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Lys Gly Cys
 115 120 125

5 <210> 15
 <211> 126
 <212> PRT
 <213> Macaca mulatta

10 <400> 15
 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Arg Leu Gly
 1 5 10 15

15 Leu Asp Gly Tyr Arg Gly Ile Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

Lys Trp Glu Ser Asp Tyr Asn Thr Gln Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

20 Gln Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser His Tyr Trp Cys
 50 55 60

Asn Asn Lys Thr Pro Gly Ala Val Asn Ala Cys Arg Ile Ser Cys Asn
 65 70 75 80

25 Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Val Thr Cys Ala Lys Arg
 85 90 95

30 Val Val Arg Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Gln Gly Cys
 115 120 125

35 <210> 16
 <211> 126
 <212> PRT
 <213> Nasalis concolor

40 <400> 16
 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Arg Leu Gly
 1 5 10 15

45

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Leu Asp Gly Tyr Arg Gly Ile Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

5 Lys Trp Glu Ser Gly Tyr Asn Thr Gln Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

Gln Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser His Tyr Trp Cys
 50 55 60

10 Asn Asn Lys Thr Pro Gly Ala Val Asn Ala Cys His Ile Ser Cys Asn
 65 70 75 80

Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Val Thr Cys Ala Lys Arg
 85 90 95

15 Val Val Arg Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Gln Gly Cys
 20 115 120 125

<210> 17
 <211> 126
 25 <212> PRT
 <213> Gorilla gorilla

<400> 17
 Lys Val Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Arg Leu Gly
 30 1 5 10 15

Met Asp Gly Tyr Arg Gly Ile Ser Leu Ala Asn Trp Met Cys Leu Ala
 20 25 30

35 Lys Trp Glu Ser Gly Tyr Asn Thr Arg Ala Thr Asn Tyr Asn Ala Asp
 35 40 45

Arg Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

40 Asn Asp Lys Thr Pro Gly Ala Val Asn Ala Cys His Leu Ser Cys Ser
 65 70 75 80

Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 45 85 90 95

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Val Val Arg Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

5 Arg Cys Gln Asn Arg Asp Val Arg Gln Tyr Val Gln Gly Cys
 115 120 125

<210> 18
 10 <211> 126
 <212> PRT
 <213> Homo sapiens

<400> 18
 15 Lys Val Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Arg Leu Gly
 1 5 10 15

Met Asp Gly Tyr Arg Gly Ile Ser Leu Ala Asn Trp Met Cys Leu Ala
 20 25 30

Lys Trp Glu Ser Gly Tyr Asn Thr Arg Ala Thr Asn Tyr Asn Ala Asp
 35 40 45

25 Arg Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

Asn Asp Lys Thr Pro Gly Ala Val Asn Ala Cys His Leu Ser Cys Ser
 65 70 75 80

30 Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 85 90 95

Val Val Arg Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 35 100 105 110

Arg Cys Gln Asn Arg Asp Val Arg Gln Tyr Val Gln Gly Cys
 115 120 125

40

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<210> 19
 <211> 126
 <212> PRT
 <213> Leporinus elongatus

5

<400> 19
 Lys Ile Tyr Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 1 5 10 15

10 Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Met Cys Leu Ala
 20 25 30

Lys Trp Glu Ser Ser Tyr Asn Thr Arg Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

15

Lys Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

20 Asn Asp Lys Thr Pro Arg Ala Val Asn Ala Cys His Ile Pro Cys Ser
 65 70 75 80

Ala Leu Leu Lys Asp Asp Ile Thr Gln Ala Val Ala Cys Ala Lys Arg
 85 90 95

25 Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

His Cys Gln Asn Gln Asp Leu Thr Pro Tyr Ile Arg Gly Cys
 115 120 125

30

<210> 20
 <211> 126
 <212> PRT
 35 <213> Colobus guereza

<400> 20
 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 1 5 10 15

40

Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

45 Lys Trp Glu Ser Gly Tyr Asn Thr Asp Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

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Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 50 55 60

5 Asn Asn Lys Thr Pro Gly Ala Val Asn Ala Cys His Ile Ser Cys Asn
 65 70 75 80

Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
 85 90 95

10 Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Lys Lys
 100 105 110

His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Glu Gly Cys
 15 115 120 125

<210> 21
 <211> 126
 20 <212> PRT
 <213> Macaca mulatta

<400> 21
 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Arg Leu Gly
 25 1 5 10 15

Leu Asp Gly Tyr Arg Gly Ile Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

30 Lys Trp Glu Ser Asn Tyr Asn Thr Gln Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

Gln Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser His Tyr Trp Cys
 50 55 60

35 Asn Asn Lys Thr Pro Gly Ala Val Asn Ala Cys His Ile Ser Cys Asn
 65 70 75 80

Ala Leu Leu Gln Asp Asn Ile Ala Asp Ala Val Thr Cys Ala Lys Arg
 40 85 90 95

Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
 100 105 110

45

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His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Gln Gly Cys
 115 120 125

5 <210> 22
 <211> 128
 <212> PRT
 <213> *Aythya americana*

10 <400> 22
 Lys Val Tyr Ser Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Leu Gly
 1 5 10 15

15 Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
 20 25 30

Asn Tyr Glu Ser Gly Phe Asn Thr Gln Ala Thr Asn Arg Asn Thr Asp
 35 40 45

20 Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
 50 55 60

Asp Asn Gly Lys Thr Pro Arg Lys Asn Ala Cys Gly Ile Pro Cys Ser
 65 70 75 80

25 Val Leu Leu Arg Ser Asp Ile Thr Glu Ala Val Arg Cys Ala Lys Arg
 85 90 95

30 Ile Val Ser Asp Gly Asp Gly Met Asn Ala Trp Val Ala Trp Arg Asn
 100 105 110

Arg Cys Arg Gly Thr Asp Val Ser Lys Trp Ile Arg Gly Cys Arg Leu
 115 120 125

35 <210> 23
 <211> 128
 <212> PRT
 <213> *Phasianus colchicus*

40 <400> 23
 Lys Val Tyr Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Leu Gly
 1 5 10 15

45

	Leu	Asp	Asn	Tyr	Arg	Gly	Tyr	Ser	Leu	Gly	Asn	Trp	Val	Cys	Ala	Ala
				20					25					30		
5	Lys	Tyr	Glu	Ser	Asn	Phe	Asn	Thr	His	Ala	Thr	Asn	Arg	Asn	Thr	Asp
			35					40					45			
	Gly	Ser	Thr	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser	Arg	Trp	Trp	Cys
		50					55					60				
10	Asn	Asp	Gly	Lys	Thr	Pro	Gly	Arg	Asn	Leu	Cys	His	Ile	Pro	Cys	Ser
	65					70					75					80
	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser	Val	Asn	Cys	Ala	Lys	Lys
					85					90					95	
15	Ile	Val	Ser	Asp	Gly	Asn	Gly	Met	Asn	Ala	Trp	Val	Ala	Trp	Arg	Asn
				100					105					110		
	Arg	Cys	Lys	Gly	Thr	Asp	Val	Ser	Val	Trp	Thr	Arg	Gly	Cys	Arg	Leu
20			115					120					125			
	<210>	24														
	<211>	128														
25	<212>	PRT														
	<213>	Aythya americana														
	<400>	24														
30	Lys	Val	Tyr	Glu	Arg	Cys	Glu	Leu	Ala	Ala	Ala	Met	Lys	Arg	Leu	Gly
	1				5					10					15	
	Leu	Asp	Asn	Tyr	Arg	Gly	Tyr	Ser	Leu	Gly	Asn	Trp	Val	Cys	Ala	Ala
				20					25					30		
35	Asn	Tyr	Glu	Ser	Ser	Phe	Asn	Thr	Gln	Ala	Thr	Asn	Arg	Asn	Thr	Asp
			35					40					45			
	Gly	Ser	Thr	Asp	Tyr	Gly	Ile	Leu	Glu	Ile	Asn	Ser	Arg	Trp	Trp	Cys
		50					55					60				
40	Asp	Asn	Gly	Lys	Thr	Pro	Arg	Lys	Asn	Ala	Cys	Gly	Ile	Pro	Cys	Ser
	65					70					75					80
	Val	Leu	Leu	Arg	Ser	Asp	Ile	Thr	Glu	Ala	Val	Lys	Cys	Ala	Lys	Arg
45					85					90					95	

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Ile Val Ser Asp Gly Asp Gly Met Asn Ala Trp Val Ala Trp Arg Asn
 100 105 110

Arg Cys Lys Gly Thr Asp Val Ser Arg Trp Ile Arg Gly Cys Arg Leu
 5 115 120 125

<210> 25
 <211> 128
 <212> PRT
 10 <213> Phasianus colchicus

<400> 25
 Lys Val Tyr Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Met Gly
 1 5 10 15

15 Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
 20 25 30

Lys Phe Glu Ser Asn Phe Asn Thr Gly Ala Thr Asn Arg Asn Thr Asp
 20 35 40 45

Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
 50 55 60

25 Asn Asp Gly Arg Thr Pro Gly Lys Asn Leu Cys His Ile Pro Cys Ser
 65 70 75 80

Ala Leu Leu Ser Ser Asp Ile Thr Ala Ser Val Asn Cys Ala Lys Lys
 85 90 95

30 Ile Val Ser Asp Gly Asn Gly Met Asn Ala Trp Val Ala Trp Arg Lys
 100 105 110

His Cys Lys Gly Thr Asp Val Asn Val Trp Ile Arg Gly Cys Arg Leu
 35 115 120 125

<210> 26
 <211> 128
 40 <212> PRT
 <213> Ortalis vetula

<400> 26
 Lys Ile Tyr Lys Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Tyr Gly
 45 1 5 10 15

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Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
 20 25 30

Arg Tyr Glu Ser Asn Tyr Asn Thr Gln Ala Thr Asn Arg Asn Ser Asn
 5 35 40 45

Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
 50 55 60

10 Asn Asp Gly Arg Thr Pro Gly Lys Asn Leu Cys His Ile Ser Cys Ser
 65 70 75 80

Ala Leu Met Gly Ala Asp Ile Ala Pro Ser Val Arg Cys Ala Lys Arg
 85 90 95

15 Ile Val Ser Asp Gly Asp Gly Met Asn Ala Trp Val Ala Trp Arg Lys
 100 105 110

His Cys Lys Gly Thr Asp Val Ser Thr Trp Ile Lys Asp Cys Lys Leu
 20 115 120 125

<210> 27
 <211> 128
 25 <212> PRT
 <213> Phasianus colchicus

<400> 27
 Lys Val Tyr Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Met Gly
 30 1 5 10 15

Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
 20 25 30

35 Lys Phe Glu Ser Asn Phe Asn Thr Gly Ala Thr Asn Arg Asn Thr Asp
 35 40 45

Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
 50 55 60

40 Asn Asp Gly Arg Thr Pro Gly Lys Asn Leu Cys His Ile Pro Cys Ser
 65 70 75 80

Ala Leu Leu Ser Ser Asp Ile Thr Ala Ser Val Asn Cys Ala Lys Lys
 45 85 90 95

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Ile Val Ser Asp Gly Asp Gly Met Asn Ala Trp Val Ala Trp Arg Lys
      100                      105                  110

His Cys Lys Gly Thr Asp Val Asn Val Trp Ile Arg Gly Cys Arg Leu
    5         115                        120                125


<210> 28
<211> 128
10 <212> PRT
   <213> Phasianus colchicus


<400> 28
Lys Val Tyr Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Leu Gly
15     1              5               10             15


Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
          20                   25                 30


20 Lys Phe Glu Ser Asn Phe Asn Thr His Ala Thr Asn Arg Asn Thr Asp
       35                     40           45


Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
        50                    55            60


25 Asn Asp Gly Arg Thr Pro Gly Arg Asn Leu Cys His Ile Pro Cys Ser
     65                       70           75             80


Ala Leu Leu Ser Ser Asp Ile Thr Ala Ser Val Asn Cys Ala Lys Lys
30           85                   90                   95


Ile Val Ser Asp Gly Asn Gly Met Asn Ala Trp Val Ala Trp Arg Asn
      100                      105                  110


35 Arg Cys Lys Gly Thr Asp Val Asn Ala Trp Thr Arg Gly Cys Arg Leu
       115                         120                125


<210> 29
<211> 128
40 <212> PRT
   <213> Phasianus colchicus


<400> 29
Lys Val Tyr Gly Arg Cys Glu Leu Ala Ala Ala Met Lys Arg Leu Gly
45     1              5               10             15
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Leu Asp Asn Tyr Arg Gly Tyr Ser Leu Gly Asn Trp Val Cys Ala Ala
 20 25 30

5 Lys Phe Glu Ser Asn Phe Asn Thr His Ala Thr Asn Arg Asn Thr Asp
 35 40 45

Gly Ser Thr Asp Tyr Gly Ile Leu Gln Ile Asn Ser Arg Trp Trp Cys
 50 55 60

10 Asn Asp Gly Arg Thr Pro Gly Arg Asn Leu Cys His Ile Ser Cys Ser
 65 70 75 80

Ala Leu Leu Ser Ser Asp Ile Thr Ala Ser Val Asn Cys Ala Lys Lys
 15 85 90 95

Ile Val Ser Asp Arg Asn Gly Met Asn Ala Trp Val Ala Trp Arg Asn
 100 105 110

20 Arg Cys Lys Gly Thr Asp Val Asn Ala Trp Ile Arg Gly Cys Arg Leu
 115 120 125

<210> 30
 25 <211> 128
 <212> PRT
 <213> Macaca mulatta

<400> 30

30 Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
 1 5 10 15

Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
 20 25 30

35 Lys Trp Glu Ser Gly Tyr Asn Thr Glu Ala Thr Asn Tyr Asn Pro Asp
 35 40 45

Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
 40 50 55 60

Asn Asn Gly Lys Thr Pro Gly Val Asp Ala Cys His Ile Ser Cys Ser
 65 70 75 80

45

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Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
85 90 95

Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
5 100 105 110

His Cys Gln Asn Arg Asp Val Ser Gln Tyr Val Lys Gly Cys Gly Val
115 120 125

10
<210> 31
<211> 128
<212> PRT
<213> Nasalis concolor

15
<400> 31
Lys Ile Phe Glu Arg Cys Glu Leu Ala Arg Thr Leu Lys Lys Leu Gly
1 5 10 15

20 Leu Asp Gly Tyr Lys Gly Val Ser Leu Ala Asn Trp Val Cys Leu Ala
20 25 30

Lys Trp Glu Ser Gly Tyr Asn Thr Glu Ala Thr Asn Tyr Asn Pro Asp
35 40 45

25
Glu Ser Thr Asp Tyr Gly Ile Phe Gln Ile Asn Ser Arg Tyr Trp Cys
50 55 60

Asn Asn Gly Lys Thr Pro Gly Val Asp Ala Cys His Ile Ser Cys Ser
30 65 70 75 80

Ala Leu Leu Gln Asn Asn Ile Ala Asp Ala Val Ala Cys Ala Lys Arg
85 90 95

35 Val Val Ser Asp Pro Gln Gly Ile Arg Ala Trp Val Ala Trp Arg Asn
100 105 110

His Cys Gln Asn Lys Asp Val Ser Gln Tyr Val Lys Gly Cys Gly Val
115 120 125

40