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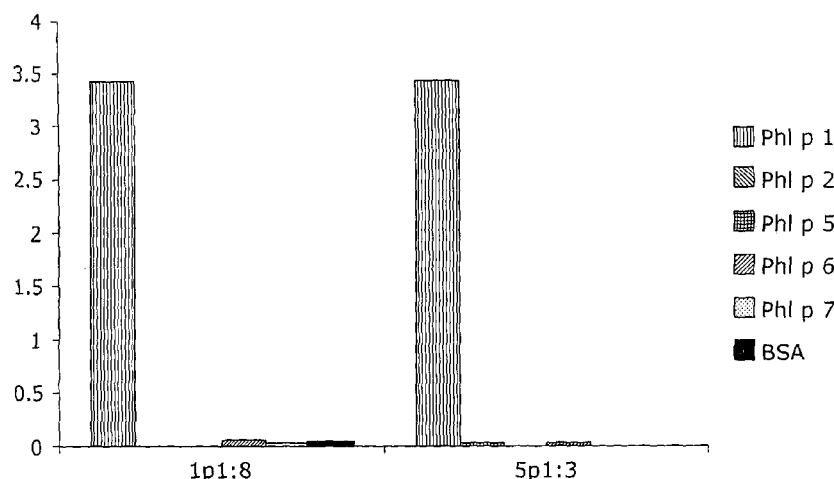


FIGURE 2

(57) Abstract: The present invention provides hypoallergenic polypeptides comprising or consisting of a variant of the amino acid sequence of SEQ ID NO: 1 wherein one or more amino acids of SEQ 1 within an IgE-binding epitope is mutated and wherein the polypeptide exhibits reduced IgE-reactivity compared to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 1. The invention further provides pharmaceutical compositions of such polypeptides, as well as methods for making and using said polypeptides, for example in the prevention of pollen grass allergies. Related aspects of the invention provide antibody polypeptides for passive immunisation against pollen grass allergies.

5

NOVEL THERAPIES

Field of Invention

10 The present invention relates to novel agents for use in the prevention of grass pollen allergies. In particular, the invention provides hypoallergenic polypeptides and antibodies for use in the immunisation of a subject, as well as means for making and using such polypeptides and antibodies.

15

Background

Type I allergy is an increasing, worldwide health issue, today affecting as much as 30% of the population of many countries (Finkelman *et al.*, 2007). At the initiation of an allergic response antigen-specific antibodies of the IgE isotype, bound to the surface of mast cells and basophils via the high affinity IgE receptor (FcεRI), are cross-linked by allergen. Such cross-linkage causes activation of these effector cells triggering them to degranulate, releasing a range of biologically active compounds such as histamines, proteases, cytokines and chemokines, eventually giving rise to symptoms associated with an allergic response (Gould *et al.*, 2003, Gould *et al.*, 2008).

Grass pollen is a major cause of allergic disease that may affect as many as 20 % of the general population (Andersson *et al.*, 2003), severely affecting the quality of life of these patients and causing huge costs to society (Kiotseridis *et al.*, 2011, Hellgren *et al.*, 2010). Specific immunotherapy is today the only disease-modifying treatment method used against grass pollen allergy, with demonstrated long-term clinical efficacy (Durham *et al.*, 1999). For over a century this treatment has been carried out using natural pollen extracts (Noon, 1911). However, such natural extract may be of varying quality, for example variations in amount or even a total lack of certain important allergen components, low stability, presence of contaminations and poor immunogenicity of some allergens are potential pit-falls that all must be considered (Focke *et al.*, 2010). In

addition there is a risk of inducing life-threatening side effects when using these extracts in immunotherapy (Møllerup *et al.*, 2000, Valenta *et al.*, 2011). However, the large progress during the last two decades in characterization of the molecular nature of important allergens offers safer alternatives to the natural extracts, such as the use of recombinant allergens or so called natural or recombinant hypoallergens. Hypoallergens are allergen variants with a reduced IgE-reactivity, while the immunogenicity and T cell reactivity are kept intact. They are thus able to induce production of allergen-specific IgG antibodies and to modify T cell responses, without provoking a potentially lethal systemic IgE-mediated allergic response, with the aim of reducing the sensitivity to the allergen of interest (Valenta *et al.*, 2004, Wachholtz *et al.*, 2003, Mothes *et al.*, 2003, Larche, 2006).

Among the many different allergen groups present in grass pollen, the group 1 allergen is the most common sensitizer, with as many as 90 % of all grass pollen allergic individuals having group 1-reactive IgE (Laffer *et al.*, 1994). Although the molecular nature of many different group 1 allergens as well as their reactivity with human polyclonal IgE has been quite thoroughly investigated (Duffort *et al.*, 2008, Laffer *et al.*, 1994, Laffer *et al.*, 1996, Tamborini *et al.*, 1997, Smith *et al.*, 1994), we still lack detailed knowledge of the interaction of these allergens with human IgE at a clonal level. Only recently have a few studies been published where human monoclonal IgE have been used as tools to gain a detailed understanding of the molecular interaction between IgE and allergen, so crucial for the initiation of an allergic response (Andréasson *et al.*, 2006, Persson *et al.*, 2008a, Flicker *et al.*, 2006, Christenssen *et al.*, 2008, Christenssen *et al.*, 2010, Holm *et al.*, 2011).

Hence, there exists a need for new agents for the treatment and prevention of grass pollen allergies.

Summary of Invention

The first aspect of the invention provides a hypoallergenic polypeptide comprising or consisting of a variant of the amino acid sequence of the C-terminal sequence of the major timothy group 1 pollen allergen, Phl p 1 (SEQ ID NO: 1):

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESWGAIWRID
TPDKL TGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

SEQ ID NO:1

wherein one or more amino acids of SEQ ID NO: 1 within an IgE-binding epitope is mutated and wherein the polypeptide exhibits reduced IgE-reactivity compared to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 1.

By "hypoallergenic polypeptide" we include amino acid variants of the major timothy group 1 pollen allergen, Phl p 1, which exhibit a reduced IgE-reactivity in mammals (such as humans), while the immunogenicity and T cell reactivity are maintained.

By "Phl p 1" we mean the major pollen allergen of timothy grass (*Phleum pratense*), which is known to exist in at least two isoforms, Phl p 1.0102 and Phl p 1.0101. The amino acid sequence of SEQ ID NO:1 corresponds to isoform Phl p 1.0102.

The term 'amino acid' as used herein includes the standard twenty genetically-encoded amino acids and their corresponding stereoisomers in the 'D' form (as compared to the natural 'L' form), omega-amino acids and other naturally-occurring amino acids, unconventional amino acids (e.g., α,α -disubstituted amino acids, N-alkyl amino acids, etc.) and chemically derivatised amino acids (see below).

Preferably, however, the polypeptide comprises or consists of L-amino acids.

When an amino acid is being specifically enumerated, such as 'alanine' or 'Ala' or 'A', the term refers to both L-alanine and D-alanine unless explicitly stated otherwise. Other unconventional amino acids may also be suitable components for polypeptides of the present invention, as long as the desired functional property is retained by the polypeptide. For the peptides shown, each encoded amino acid residue, where

appropriate, is represented by a single letter designation, corresponding to the trivial name of the conventional amino acid.

By 'variant' of the amino acid sequence of SEQ ID NO: 1 we include polypeptides comprising or consisting of an amino acid sequence in which one or more amino acid residues within an IgE-binding epitope of SEQ ID NO: 1 are mutated, for example inserted, deleted and/or substituted, so as to reduce the IgE-reactivity of the polypeptide relative to the wildtype Phl p 1 allergen.

Thus, the polypeptide may comprise one or more insertions and/or one or more deletions and/or one or more substitutions of amino acids relative to the amino acid sequence of SEQ ID NO: 1. For example, one or more amino acids within an IgE-binding epitope of SEQ ID NO:1 may be substituted, either conservatively or non-conservatively.

In one embodiment, the hypoallergenic polypeptide is a non-naturally occurring variant of SEQ ID NO:1.

By "IgE-reactivity" we mean the ability of a polypeptide to interact non-covalently with the antigen-binding site of antibodies of the IgE isotype (or recombinant antibodies carrying an IgE Fc), for example as measured by standard immunochemical assays such as ELISA or test systems commonly used in allergy diagnosis such as ImmunoCAP or ImmunoCAP ISAC (Phadia AB, Uppsala, Sweden).

By "IgE-binding epitope" on Phl p 1 we mean a sequence or conformation of exposed amino acid residues at the surface of the wildtype Phl p 1 allergen which is recognised by IgE antibodies against Phl p 1 produced in response to a challenge with the Phl p 1 allergen or other grass pollen group I allergens (see Examples below and Flicker *et al.*, 2006).

By "amino acids of SEQ ID NO: 1 within an IgE-binding epitope" we include not only the amino acid residues that constitute the IgE-binding epitope, but also those amino acids which are not directly part of the epitope but nevertheless are capable of influencing its structure (and, in particular, the binding of IgE thereto) when mutated.

In a further embodiment of the hypoallergenic polypeptides of the invention, the IgE-binding epitope comprises or consists of one or more of the following amino acids of SEQ ID NO: 1: K8, N11, K36, K38, K45, D55, K59, E77, E79, E84, K87 and E93.

- 5 For example, the hypoallergenic polypeptide may comprise a mutation at amino acid N11 of SEQ ID NO: 1. Alternatively or in addition, the hypoallergenic polypeptide may comprise a mutation at amino acid K8 of SEQ ID NO: 1. Alternatively or in addition, the hypoallergenic polypeptide may comprise a mutation at amino acid D55 of SEQ ID NO: 1. Thus, the hypoallergenic polypeptide comprises a mutation at amino acid K8,
10 N11 and D55 of SEQ ID NO: 1.

It will be appreciated by persons skilled in the art that the mutation may be a deletion, insertion or substitution.

- 15 In one preferred embodiment, the hypoallergenic polypeptide comprises mutations at amino acid residue K8 and/or N11 and/or D55 of SEQ ID NO: 1, which may be defined by reference to the following 3D crystal co-ordinates of the homodimer of Phl p 1 (PDB [Protein Data Bank] accession number: 1N10):

	<i>Residue</i>	<i>Atoms</i>
20	K8	1154
	N11	1157
	D55	1201
25	K8	2154
	N11	2157
	D55	2201

- 30 For example, the hypoallergenic may comprise or consist of the amino acid sequence of SEQ ID NO: 1 having one or more of the following mutations therein: K8A, N11A and/or D55A.

- As stated above, a functional consequence of the introduction of amino acid mutations
35 into an IgE-binding epitope of the C-terminus of Phl p 1 (e.g. SEQ ID NO: 1) is a

reduction in the IgE reactivity of the polypeptide, resulting in the creation of a hypoallergen.

It will be appreciated by persons skilled in the art that the reduction in IgE reactivity must be relevant to the species of mammal in which the hypoallergenic polypeptide is intended to be used (to prevent grass pollen allergy). Thus, for hypoallergenic polypeptides intended to use in humans, the reduction must be in reactivity to human IgE.

The IgE reactivity may be reduced in whole or in part relative to the IgE reactivity of SEQ ID NO: 1. In one embodiment, IgE reactivity is reduced by at least 10%, for example at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and preferably by 100%, relative to the IgE reactivity of SEQ ID NO: 1.

It will be further appreciated by persons skilled in the art that the hypoallergenic polypeptides of the invention may be longer, the same length or shorter than the amino acid sequence of SEQ ID NO: 1.

In one embodiment, the polypeptide is fewer than 500 amino acids in length, for example fewer than 400, 300, 200, 150, 140, 130, 120, 105, 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 70, 60, 50, 40, 30 or fewer amino acids in length.

Thus, the polypeptide may be between 50 and 150 amino acids in length, for example between 70 and 120, between 80 and 110, or between 90 and 100 amino acids in length (e.g. 95 amino acids in length).

The hypoallergenic polypeptides of the invention share partial (but not total) amino acid sequence identity with the amino acid sequence identity with SEQ ID NO:1. In one embodiment, the polypeptide shares at least 50% amino acid sequence identity with SEQ ID NO:1, for example at least 60%, 70%, 80, 90%, 95%, or 99% amino acid sequence identity.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequences have been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (as described in Thompson *et al.*, 1994, *Nuc. Acid Res.* **22**:4673-4680, the relevant disclosures in which document are hereby incorporated by reference).

5 The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

10 Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

15 Alternatively, the BESTFIT program may be used to determine local sequence alignments.

Exemplary hypoallergenic polypeptides of the invention comprise or consist of one of the following amino acid sequences:

20

KVTFHVEAGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 2;

25

KVTFHVEKGSAPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESWG
AIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 3;

30

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEAGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 4;

35

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGADKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 5;

KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELAESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 6;

5 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIATPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 7;

10 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDALTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 8;

15 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTAAEDVIPEGWKADTSYESK
SEQ ID NO: 9;

20 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAADVIPEGWKADTSYESK
SEQ ID NO: 10;

25 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPAGWKADTSYESK
SEQ ID NO: 11;

30 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWAADTSYESK
SEQ ID NO: 12;

35 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYASK
SEQ ID NO: 13; and

KVTFHVEAGSAPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESWG
AIWRIATPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 14.

It will be appreciated by persons skilled in the art that the invention also extends to hypoallergenic polypeptides derived from isoform Phl p 1.0101 of the major timothy group 1 pollen allergen.

5

Thus, in an alternative embodiment of the invention, the polypeptide may comprise or consist of a variant of the amino acid sequence of SEQ ID NO: 15

10

KVTFHVEKGSNPNYLALLVKFVAGDGDVVAVDIKEKGKDKWIALKESWG
AIWRIDTPEVLKGPFTVRYTTEGGTKGEAKDVIPEGWKADTAYESK

SEQ ID NO:15

For example, the polypeptide variant of SEQ ID NO:15 may comprise one or more of the following mutations:

15

K8A, N11A and/or D55A.

The first aspect of the invention extends to hypoallergenic polypeptides wherein one or more amino acids is modified or derivatised.

20

Chemical derivatives of one or more amino acids may be achieved by reaction with a functional side group. Such derivatised molecules include, for example, those molecules in which free amino groups have been derivatised to form amine hydrochlorides, *p*-toluene sulphonyl groups, carboxybenzoxy groups, *t*-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatised to form salts, methyl and ethyl esters or other types of esters and hydrazides. Free hydroxyl groups may be derivatised to form O-acyl or O-alkyl derivatives. Also included as chemical derivatives are those peptides which contain naturally occurring amino acid derivatives of the twenty standard amino acids. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine for lysine. Derivatives also include peptides containing one or more additions or deletions as long as the requisite activity is maintained. Other included modifications are amidation, amino terminal acylation (e.g. acetylation or thioglycolic acid amidation), terminal carboxylamidation (e.g. with ammonia or methylamine), and the like terminal modifications.

35

In order to provide improvement in the pharmacokinetics of the polypeptide-based agents described herein, the present invention provides polypeptides that are linked to polymers which provide increased stability and half-life (as described in detail below).

5 The attachment of polymer molecules (e.g. polyethylene glycol; PEG) to proteins is well established and has been shown to modulate the pharmacokinetic properties of the modified proteins. For example, PEG modification of proteins has been shown to alter the *in vivo* circulating half-life, antigenicity, solubility, and resistance to proteolysis of the protein (Abuchowski *et al.*, *J. Biol. Chem.* 1977, 252:3578; Nucci *et al.*, *Adv. Drug*
10 *Delivery Reviews* 1991, 6:133; Francis *et al.*, *Pharmaceutical Biotechnology* Vol. 3 (Borchardt, R. T. ed.); and *Stability of Protein Pharmaceuticals: in vivo Pathways of Degradation and Strategies for Protein Stabilization*, 1991, pp235-263, Plenum, NY).

Both site-specific and random PEGylation of protein molecules is known in the art (for
15 example, see Zalipsky & Lee, *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*, 1992, pp 347-370, Plenum, NY; Goodson & Katre, 1990, *Bio/Technology*, 8:343; Hershfield *et al.*, 1991, *PNAS* 88:7185). More specifically, random PEGylation of polypeptide molecules has been described at lysine residues and thiolated derivatives (Ling & Mattiasson, 1983, *Immunol. Methods* 59: 327; Wilkinson *et al.*, 1987, *Immunol. Letters*, 15: 17; Kitamura *et al.*, 1991, *Cancer Res.* 51:4310; Delgado
20 *et al.*, 1996 *Br. J. Cancer*, 73: 175; Pedley *et al.*, 1994, *Br. J. Cancer*, 70:1126).

Attachment of a PEG polymer to an amino acid residue of a polypeptide may be achieved using several PEG attachment moieties including, but not limited to N-
25 hydroxysuccinimide (NHS) active ester, succinimidyl propionate (SPA), maleimide (MAL), vinyl sulfone (VS), or thiol. A PEG polymer, or other polymer, can be linked to a polypeptide at either a predetermined position, or may be randomly linked to the polypeptide molecule. It is preferred, however, that the PEG polymer be linked to a polypeptide at a predetermined position. A PEG polymer may be linked to any residue in
30 the a polypeptide, however, it is preferable that the polymer is linked to either a lysine or cysteine, which is either naturally occurring in the polypeptide, or which has been engineered into the polypeptide, for example, by mutagenesis of a naturally occurring residue in the polypeptide to either a cysteine or lysine. PEG-linkage can also be mediated through a peptide linker attached to a polypeptide. That is, the PEG moiety
35 can be attached to a peptide linker fused to a polypeptide, where the linker provides the site, e.g. a free cysteine or lysine, for PEG attachment.

As used herein, "polymer" refers to a macromolecule made up of repeating monomeric units, and can refer to a synthetic or naturally occurring polymer such as an optionally substituted straight or branched chain polyalkylene, polyalkenylene, or polyoxyalkylene polymer or a branched or unbranched polysaccharide. A "polymer" as used herein,
5 specifically refers to an optionally substituted or branched chain poly(ethylene glycol), poly(propylene glycol), or poly(vinyl alcohol) and derivatives thereof.

Thus, "PEG" or "PEG polymer" refers to polyethylene glycol, and more specifically can refer to a derivitized form of PEG, including, but not limited to N-hydroxylsuccinimide (NHS) active esters of PEG such as succinimidyl propionate, benzotriazole active esters,
10 PEG derivitized with maleimide, vinyl sulfones, or thiol groups. Particular PEG formulations can include PEG-O-CH₂CH₂CH₂-CO₂-NHS; PEG-O-CH₂-NHS; PEG-O-CH₂CH₂-CO₂-NHS; PEG-S-CH₂CH₂-CO-NHS; PEG-O₂CNH-CH(R)-CO₂-NHS; PEG-NHCO-CH₂CH₂-CO-NHS; and PEG-O-CH₂-CO₂-NHS; where R is (CH₂)₄NHCO₂(mPEG). PEG polymers useful in the invention may be linear molecules,
15 or may be branched wherein multiple PEG moieties are present in a single polymer.

A "sulfhydryl-selective reagent" is a reagent which is useful for the attachment of a PEG polymer to a thiol-containing amino acid. Thiol groups on the amino acid residue cysteine are particularly useful for interaction with a sulfhydryl-selective reagent. Sulfhydryl-selective reagents which are useful for such attachment include, but are not
20 limited to maleimide, vinyl sulfone, and thiol. The use of sulfhydryl-selective reagents for coupling to cysteine residues is known in the art and may be adapted as needed according to the present invention (for example, see Zalipsky, 1995, *Bioconjug. Chem.* 6:150; Greenwald *et al.*, 2000, *Crit. Rev. Ther. Drug Carrier Syst.* 17:101; Herman *et al.*, 1994, *Macromol. Chem. Phys.* 195:203).

25

The attachment of PEG or another agent, e.g. human serum albumin, to a polypeptide as described herein will preferably not impair the ability of the polypeptide to induce the formation of IgG antibodies to Phl p 1 (whilst still exhibiting reduced IgE reactivity). That is, the PEG-linked polypeptide will retain such activity relative to a non-PEG-linked
30 counterpart. As used herein, "retains activity" refers to a level of activity of a PEG-linked polypeptide which is at least 10% of the level of activity of a non-PEG-linked polypeptide, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80% and up to 90%, preferably up to 95%, 98%, and up to 100% of the activity of a non-PEG-linked polypeptide comprising the same antigen-binding domain or domains. More specifically, the activity of a PEG-
35 linked polypeptide compared to a non-PEG linked polypeptide should be determined on

a polypeptide molar basis; that is equivalent numbers of moles of each of the PEG-linked and non-PEG-linked polypeptides should be used in each trial. In determining whether a particular PEG-linked polypeptide "retains activity", it is preferred that the activity of a PEG-linked polypeptide be compared with the activity of the same polypeptide in the
5 absence of PEG.

As used herein, the term "*in vivo* half-life" refers to the time taken for the serum concentration of a polypeptide, fusion or derivative of the invention to reduce by 50% *in vivo*, for example due to degradation of the polypeptide and/or clearance or
10 sequestration of the polypeptide by natural mechanisms. The polypeptides described herein can be stabilized *in vivo* and their half-life increased by binding to molecules, such as PEG, which resist degradation and/or clearance or sequestration. The half-life of a polypeptide is increased if its functional activity persists, *in vivo*, for a longer period than a similar polypeptide which is not linked to a PEG polymer. Typically, the half life of a
15 PEGylated polypeptide is increased by 10%, 20%, 30%, 40%, 50% or more relative to a non-PEGylated polypeptide. Increases in the range of 2x, 3x, 4x, 5x, 10x, 20x, 30x, 40x, 50x or more of the half life are possible. Alternatively, or in addition, increases in the range of up to 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 150x of the half life are possible.

20 As used herein, "resistant to degradation" or "resists degradation" with respect to a PEG or other polymer-linked polypeptide means that the PEG- or other polymer-linked polypeptide is degraded by no more than 10% when exposed to pepsin at pH 2.0 for 30 minutes and preferably not degraded at all.

25 It will be appreciated by persons skilled in the art that peptidomimetic compounds may also be useful in the present invention. Thus, by 'polypeptide' or 'peptide' we include peptidomimetic compounds which are capable of inducing antibodies to grass pollen group 1 allergens. The term 'peptidomimetic' refers to a compound that mimics the conformation and desirable features of a particular peptide as a therapeutic agent.

30 For example, the polypeptides of the invention include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Meziere *et al.* (1997)
35 *J. Immunol.* **159**, 3230-3237. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse

peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

5 In an alternative embodiment, the polypeptide of the invention is a peptidomimetic compound wherein one or more of the amino acid residues are linked by a $-y(\text{CH}_2\text{NH})-$ bond in place of the conventional amide linkage.

10 Similarly, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the carbon atoms of the amino acid residues is used; it is particularly preferred if the linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

15 It will be appreciated that the peptide may conveniently be blocked at its N- or C-terminus so as to help reduce susceptibility to exoproteolytic digestion.

20 A variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have also been used to modify mammalian peptides. In addition, a presumed bioactive conformation may be stabilised by a covalent modification, such as cyclisation or by incorporation of lactam or other types of bridges, for example see Veber *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* **75**:2636 and Thursell *et al.*, 1983, *Biochem. Biophys. Res. Comm.* **111**:166.

25 A common theme among many of the synthetic strategies has been the introduction of some cyclic moiety into a peptide-based framework. The cyclic moiety restricts the conformational space of the peptide structure and this frequently results in an increased affinity of the peptide for a particular biological receptor. An added advantage of this strategy is that the introduction of a cyclic moiety into a peptide may also result in the peptide having a diminished sensitivity to cellular peptidases.

30

Thus, polypeptides of the invention may comprise terminal cysteine amino acids. Such a polypeptide may exist in a heterodetic cyclic form by disulphide bond formation of the mercaptide groups in the terminal cysteine amino acids or in a homodetic form by amide peptide bond formation between the terminal amino acids. As indicated above, cyclising small peptides through disulphide or amide bonds between the N- and C-terminus
35 cysteines may circumvent problems of affinity and half-life sometime observed with linear

peptides, by decreasing proteolysis and also increasing the rigidity of the structure, which may yield higher affinity compounds. Polypeptides cyclised by disulphide bonds have free amino and carboxy-termini which still may be susceptible to proteolytic degradation, while peptides cyclised by formation of an amide bond between the N-terminal amine and C-terminal carboxyl and hence no longer contain free amino or carboxy termini. Thus, the peptides of the present invention can be linked either by a C-N linkage or a disulphide linkage.

Cyclic peptides may have longer half-lives in serum (see, for example, (Picker and Butcher 1992;Huang et al. 1997). The present invention is not limited in any way by the method of cyclisation of peptides, but encompasses peptides whose cyclic structure may be achieved by any suitable method of synthesis. Thus, heterodetic linkages may include, but are not limited to formation via disulphide, alkylene or sulphide bridges. Methods of synthesis of cyclic homodetic peptides and cyclic heterodetic peptides, including disulphide, sulphide and alkylene bridges, are disclosed in US 5,643,872. Other examples of cyclisation methods are discussed and disclosed in US 6,008,058. Cyclic peptides can also be prepared by incorporation of a type 11' β -turn dipeptide (Doyle *et al.* 1996).

A further approach to the synthesis of cyclic stabilised peptidomimetic compounds is ring-closing metathesis (RCM). This method involves steps of synthesising a peptide precursor and contacting it with an RCM catalyst to yield a conformationally restricted peptide. Suitable peptide precursors may contain two or more unsaturated C-C bonds. The method may be carried out using solid-phase-peptide-synthesis techniques. In this embodiment, the precursor, which is anchored to a solid support, is contacted with a RCM catalyst and the product is then cleaved from the solid support to yield a conformationally restricted peptide.

Another approach, disclosed by D. H. Rich in *Protease Inhibitors*, Barrett and Selveson, eds., Elsevier (1986), has been to design peptide mimics through the application of the transition state analogue concept in enzyme inhibitor design. For example, it is known that the secondary alcohol of statine mimics the tetrahedral transition state of the scissile amide bond of the pepsin substrate.

In summary, terminal modifications are useful, as is well known, to reduce susceptibility by proteinase digestion and therefore to prolong the half-life of the peptides in solutions,

particularly in biological fluids where proteases may be present. Polypeptide cyclisation is also a useful modification and is preferred because of the stable structures formed by cyclisation and in view of the biological activities observed for cyclic peptides.

5 Thus, the polypeptide of the first aspect of the invention may be linear or cyclic.

The present invention also extends to pharmaceutically acceptable acid or base addition salts of the above described polypeptides. The acids which are used to prepare the pharmaceutically acceptable acid addition salts of the aforementioned base compounds
10 useful in this invention are those which form non-toxic acid addition salts, *i.e.* salts containing pharmacologically acceptable anions, such as the hydrochloride, hydrobromide, hydroiodide, nitrate, sulphate, bisulphate, phosphate, acid phosphate, acetate, lactate, citrate, acid citrate, tartrate, bitartrate, succinate, maleate, fumarate, gluconate, saccharate, benzoate, methanesulphonate, ethanesulphonate,
15 benzenesulphonate, *p*-toluenesulphonate and pamoate [*i.e.* 1,1'-methylene-bis-(2-hydroxy-3 naphthoate)] salts, among others.

Pharmaceutically acceptable base addition salts may also be used to produce pharmaceutically acceptable salt forms of the polypeptides. The chemical bases that
20 may be used as reagents to prepare pharmaceutically acceptable base salts of the present compounds that are acidic in nature are those that form non-toxic base salts with such compounds. Such non-toxic base salts include, but are not limited to those derived from such pharmacologically acceptable cations such as alkali metal cations (*e.g.* potassium and sodium) and alkaline earth metal cations (*e.g.* calcium and magnesium),
25 ammonium or water-soluble amine addition salts such as N-methylglucamine- (meglumine), and the lower alkanolammonium and other base salts of pharmaceutically acceptable organic amines, among others.

The polypeptide may be lyophilised for storage and reconstituted in a suitable carrier
30 prior to use. Any suitable lyophilisation method (*e.g.* spray drying, cake drying) and/or reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of activity loss and that use levels may have to be adjusted upward to compensate. Preferably, the lyophilised (freeze dried) polypeptide loses no more than about 20%, or no more than
35 about 25%, or no more than about 30%, or no more than about 35%, or no more than

about 40%, or no more than about 45%, or no more than about 50% of its activity (prior to lyophilisation) when rehydrated.

It will be appreciated by persons skilled in the art that the hypoallergenic polypeptides of the invention may be adapted, using methods well known in the art, in order to prolong their half-life *in vivo*. Any one or more of the following known methods of improving the half-life of proteins may be used for this purpose:

(a) PEGylation

A widely used method for improving the half-life of proteins is the covalent linking of polyethylene glycol (PEG) moieties to the protein (as detailed above).

(b) Fusion proteins

IgG fusion proteins

Human immunoglobulin G (IgG) molecules have circulating half-lives of approximately twenty days. The Fc portion of IgG molecules have been extensively used for the creation of fusion proteins consisting of an Fc part and a protein with a therapeutic use. Such fusion proteins exhibit a prolonged half-life compared to their Fc-lacking counterparts.

Fc-linked proteins are produced by creating fusion proteins between Fc and the protein of interest by standard genetic engineering protocols. The Fc group is fused to the C-terminus of the protein of interest. Due to the presence of cysteine residues in the hinge region of IgG, Fc fusion proteins are expressed as disulfide-linked homodimers. This further increases their effective size and circulating half-lives. In addition, homodimeric constructs may have an increased functional activity due to improved avidity for its receptor / ligand compared to the corresponding monomeric form.

Human serum albumin fusion proteins

Human serum albumin (HSA) is the most abundant naturally occurring blood protein in the circulation and has a half-life of 19 days [Osborn, B.L., et al., *Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in*

cynomolgus monkeys. J Pharmacol Exp Ther, 2002. **303**(2): p. 540-8]. Thus, HSA is a suitable fusion partner for the creation of fusion proteins with improved half-life. HSA fusion proteins exhibit a prolonged half-life due to the capability of HSA to stabilize the protein towards proteolysis and increasing the residence time in the body [Veronese, F.M. and J.M. Harris, *Introduction and overview of peptide and protein pegylation*. Adv Drug Deliv Rev, 2002. **54**(4): p. 453-6]. HSA fusion proteins, including IL-2, IFN- α and - β and growth hormone (GH), have been produced and shown to have improved pharmacokinetic properties. Albuferon (HSA-IFN- α) and albutropin (HSA-GH) exhibit half-lives that are 18 and 6 times longer in cynomolgus monkeys, respectively, than the respective counterparts lacking an HSA group [Osborn, B.L., et al., *Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys*. J Pharmacol Exp Ther, 2002. **303**(2): p. 540-8, Osborn, B.L., et al., *Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys*. Eur J Pharmacol, 2002. **456**(1-3): p. 149-58].

HSA-linked proteins are produced by creating fusion proteins between HSA and the protein of interest by standard genetic engineering protocols. The HSA group may be added at either the N- or the C-terminus. Since the modification is added to the terminus of the protein, the risk of interfering with the structure of the protein and thus with its function is considerably less compared to modifications such as pegylation in the interior of the protein. In addition, the chance of avoiding interference with the active site of the protein is increased by the fact that the HSA group may be added at either the N- or C-terminus of the protein of interest [Osborn, B.L., et al., *Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys*. J Pharmacol Exp Ther, 2002. **303**(2): p. 540-8, Osborn, B.L., et al., *Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys*. Eur J Pharmacol, 2002. **456**(1-3): p. 149-58, Syed, S., K.E. Kelly, and W.P. Sheffield, *Inhibition of thrombin by hirudin genetically fused to wild-type or mutant antithrombin*. Thromb Res, 1996. **84**(6): p. 419-29], depending on which is more likely to result in a fusion protein with maintained biological activity. Thus, in the case of albuferon and albutropin, the C-terminus of the HSA was fused with the N-terminus of IFN- α or GH, respectively, creation of a functionally active hirudin-HSA fusion protein, the HSA group had to be fused to the C-terminus of hirudin. These results indicate that the properties of the target protein determine whether fusion at the N- or C-terminus is optimal.

(c) Glycosylation

The introduction of new sialic acid-containing carbohydrates into a protein (glycoengineering) has been shown to improve *in vivo* half-life. This method may be used
5 for naturally glycosylated proteins or for proteins that normally lack glycosylation [Elliott, S., et al., *Enhancement of therapeutic protein in vivo activities through glycoengineering*. Nat Biotechnol, 2003. **21**(4): p. 414-21].

Glycosylation of proteins may be in the form of N-linked or O-linked carbohydrates. N-
10 linked carbohydrates are typically attached to consensus sequences (Asn-X-Ser/Thr) where X is any amino acid except proline. O-glycosylation occurs at Ser/Thr residues.

For the production of glycosylated proteins, the introduction of novel glycosylation sites may be required. For glycosylation to occur, expression may be performed in yeast,
15 insect or mammalian cell systems. cells may be since the glycosylation pattern is similar to that in mammalian cells whereas cell cycles are shorter and therefore expression process faster. Darbepoetin- α is an example of a modified human erythropoietin expressed in CHO cells. It contains two extra N-glycosylation sites, resulting in a three times improved *in vivo* half-life [Elliott, S., et al., *Enhancement of therapeutic protein in vivo activities through glycoengineering*. Nat Biotechnol, 2003. **21**(4): p. 414-21].
20

An alternative method of glycosylation is the chemical addition of carbohydrate groups to proteins. In this method, the protein is expressed naked, *e.g.* in *E. coli*. Following expression and purification, the protein is glycosylated in a fully synthetic cell-free
25 process. The method offers great flexibility in terms of number, size and type of carbohydrate to be added.

(d) Fatty acid acylation /myristoylation

30 Fatty acids have a high affinity and high capacity of HSA binding. This characteristic can be utilized for improving the half-life of proteins. Thus, fatty acyl can be attached to amino acids of proteins, thus generating fatty acyl acylated proteins. Upon reaching the circulation, the fatty acyl group is capable of binding to circulating HSA, resulting in an improved *in vivo* half-life of the protein.

35

This method was used for the development of Insulin detemir, which was fatty acyl acylated with myristate at Lys^{B29} by treatment of insulin with fatty acid hydroxyl-succinimide esters in dimethyl formamide/DMSO [Kurtzhals, P., et al., *Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo*. Biochem J, 1995. **312** (Pt 3): p. 725-31, Hamilton-Wessler, M., et al., *Mechanism of protracted metabolic effects of fatty acid acylated insulin, NN304, in dogs: retention of NN304 by albumin*. Diabetologia, 1999. **42**(10): p. 1254-63]. This generated an insulin analogue with increased in vivo half-life due to binding of HSA.

(e) Dextran

Dextran results in an immobilization of the protein, resulting in a slow release and thereby improves the half-life of the protein. Dextran-streptokinase, has been marketed in Russia for thrombolytic therapy. In addition, insulin, somatostatin (which is used for therapy and diagnosis of tumours expressing somatostatin receptors) and the ribosome-inactivating drug trichosantin conjugated to dextran, had a significantly improved half-lives [Baudys, M., et al., *Extending insulin action in vivo by conjugation to carboxymethyl dextran*. Bioconjug Chem, 1998. **9**(2): p. 176-83, Chan, W.L., et al., *Lowering of trichosanthin immunogenicity by site-specific coupling to dextran*. Biochem Pharmacol, 1999. **57**(8): p. 927-34, Wulbrand, U., et al., *A novel somatostatin conjugate with a high affinity to all five somatostatin receptor subtypes*. Cancer, 2002. **94**(4 Suppl): p. 1293-7].

In addition to protein-based pharmaceuticals, dextran has been used for improving the half-life of antibiotics and cytotoxic drugs [Yura, H., et al., *Synthesis and pharmacokinetics of a novel macromolecular prodrug of Tacrolimus (FK506), FK506-dextran conjugate*. J Control Release, 1999. **57**(1): p. 87-99, Nakashima, M., et al., *In vitro characteristics and in vivo plasma disposition of cisplatin conjugated with oxidized and dicarboxymethylated dextrans*. Biol Pharm Bull, 1999. **22**(7): p. 756-61, Kim, D.S., Y.J. Jung, and Y.M. Kim, *Synthesis and properties of dextran-linked ampicillin*. Drug Dev Ind Pharm, 2001. **27**(1): p. 97-101].

Dextran conjugation is carried out by reductive amination using periodate-activated dextran or by the use of cyanogens bromide [Wulbrand, U., et al., *A novel somatostatin conjugate with a high affinity to all five somatostatin receptor subtypes*. Cancer, 2002. **94**(4 Suppl): p. 1293-7, Kim, D.S., Y.J. Jung, and Y.M. Kim, *Synthesis and properties of*

dextran-linked ampicillin. Drug Dev Ind Pharm, 2001. **27**(1): p. 97-101]. The dextran used may vary in size, and dextran ranging from 9 to 82 kDa have been used [Kim, D.S., Y.J. Jung, and Y.M. Kim, *Synthesis and properties of dextran-linked ampicillin*. Drug Dev Ind Pharm, 2001. **27**(1): p. 97-101, Behe, M., et al., *Biodistribution, blood half-life, and*
5 *receptor binding of a somatostatin-dextran conjugate*. Med Oncol, 2001. **18**(1): p. 59-64].

In one embodiment of the first aspect of the invention, the polypeptide of the invention is a "fusion" polypeptide comprising a second polypeptide region.

10 In one embodiment, the second polypeptide region enhances *in vivo* half-life and/or immunogenicity.

In an alternative embodiment, the second polypeptide region aids purification.

15 For example, the hypoallergenic polypeptide region may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said polypeptide. Examples of such fusions are well known to those skilled in the art. Similarly, the said polypeptide may be fused to an oligo-histidine tag such as His6 or to
20 an epitope recognised by an antibody, such as the well-known FLAG and Myc tag epitopes.

In a further embodiment of the first aspect of the invention, the polypeptide comprises or consists of tandem repeats (of the same or different polypeptide regions).

25 For example, the polypeptide may comprise or consist of peptide tags that represent antigens to which there is an existing T cell response in most individuals (e.g. tetanus toxin fragments).

However, non-limiting preferred embodiments of the invention include hypoallergenic
30 polypeptides that consist of an amino acid sequence selected from the group consisting of SEQ ID NOS: 2 to 14.

Methods for the production of the hypoallergenic polypeptides of the invention are well known in the art. Conveniently, the polypeptide is a recombinant polypeptide.

35

Thus, a nucleic acid molecule (or polynucleotide) encoding the polypeptide may be expressed in a suitable host and the polypeptide obtained therefrom. Suitable methods for the production of such recombinant polypeptides are well known in the art (for example, see Sambrook & Russell, 2000, *Molecular Cloning, A Laboratory Manual*, Third Edition, Cold Spring Harbor, New York, the relevant disclosures in which document are hereby incorporated by reference).

In brief, expression vectors may be constructed comprising a nucleic acid molecule which is capable, in an appropriate host, of expressing the polypeptide encoded by the nucleic acid molecule.

A variety of methods have been developed to operably link nucleic acid molecules, especially DNA, to vectors, for example, via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, e.g. generated by endonuclease restriction digestion, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerising activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a larger molar excess of linker molecules in the presence of an enzyme that is able to catalyse the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease site are commercially available from a number of sources including International Biotechnologies Inc., New Haven, CN, USA.

5 A desirable way to modify the DNA encoding the polypeptide of the invention is to use PCR. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

10 In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

15 The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide. Thus, the DNA encoding the polypeptide may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention
20 or binding moiety thereof.

In one embodiment, the nucleic acid molecule is codon-optimised for a particular type of host cell in order to promote its expression therein.

25 The DNA (or in the case or retroviral vectors, RNA) encoding the polypeptide may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

30

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally
35 available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector.

Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on
5 another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the expression vector are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can
10 then be recovered.

Many expression systems are known, including bacteria (for example, *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors typically include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of
20 the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing
25 convenient restriction sites for insertion of a DNA segment.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and pTrc99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

- 5 Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmid pRS413-416 is a Yeast Centromere plasmids (Ycps).

10

Other vectors and expression systems are well known in the art for use with a variety of host cells.

15

The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains T7 (available from New England Biolabs, Ipswich, MA, USA), DH5 (available from Bethesda Research Laboratories Inc., Bethesda, MD, USA), and RR1 (available from the American Type Culture Collection (ATCC) of Rockville, MD, USA; No. ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CRL 1658 and 293 cells which are human embryonic kidney cells.

20 Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

25

Transformation of appropriate cell hosts with a DNA construct is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al.* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and *Molecular Cloning: a Laboratory Manual*, 3rd edition, Sambrook & Russell, 2001, Cold Spring Harbor Laboratory Press. Transformation of yeast cells is described in Sherman *et al.* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations,

30

35

are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA. The relevant disclosures in the above documents are hereby incorporated by reference.

- 5 Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cells, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646, the relevant disclosures in which
10 document are hereby incorporated by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5 PEB using 6250V per cm at 25 μ FD.

Methods for transformation of yeast by electroporation are disclosed in Becker &
15 Guarente (1990) *Methods Enzymol.* **194**, 182, the relevant disclosures in which document are hereby incorporated by reference.

Successfully transformed cells, *i.e.* cells that contain a DNA construct encoding a polypeptide, can be identified by well known techniques. For example, cells resulting
20 from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208, the relevant disclosures in which document are hereby incorporated by reference.
25 Alternatively, the presence of the protein in the supernatant or the cell pellet can be detected using antibodies.

In addition to assaying directly for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the
30 recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity.

Samples of cells suspected of being transformed are harvested and assayed for the
35 protein using suitable antibodies.

The host cell may be a host cell within a non-human animal body. Thus, transgenic non-human animals which express a polypeptide by virtue of the presence of the transgene are included. Preferably, the transgenic non-human animal is a rodent such as a mouse. Transgenic non-human animals can be made using methods well known in the art (see
5 below).

Methods of cultivating host cells and isolating recombinant proteins are well known in the art. It will be appreciated that, depending on the host cell, the compounds of the invention (or binding moieties thereof) produced may differ. For example, certain host
10 cells, such as yeast or bacterial cells, either do not have, or have different, post-translational modification systems which may result in the production of forms of compounds of the invention (or binding moieties thereof) which may be post-translationally modified in a different way.

15 In one embodiment, the polypeptides for use in the methods of the invention are produced in a eukaryotic system, such as a mammalian cell.

Polypeptides can also be produced *in vitro* using a commercially available *in vitro* translation system, such as rabbit reticulocyte lysate or wheatgerm lysate (available from
20 Promega). Preferably, the translation system is rabbit reticulocyte lysate. Conveniently, the translation system may be coupled to a transcription system, such as the TNT transcription-translation system (Promega). This system has the advantage of producing suitable mRNA transcript from an encoding DNA polynucleotide in the same reaction as the translation.

25 Thus, a related aspect of the invention provides a nucleic acid molecule, such as an isolated DNA molecule, encoding a hypoallergenic polypeptide according to the first aspect of the invention.

30 For example, the nucleic acid molecule may comprise or consist of one of the following nucleotide sequences:

35 AAAGTGACCTTCCATGTTGAAGCAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG

AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 16;

5 AAAGTGACCTTCCATGTTGAAAAAGGCAGCGCACCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
10 AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 17;

15 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGG
CAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 18;

20 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCGCAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
25 AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 19;

30 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGGCAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
35 AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 20;

40 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGCAACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 21;

45 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATGCACTGACAGGTCCGTTTACCGTTCGTTATACCACCG

AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 22;

5 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
10 AAGGTGGCACCAAAACCGCAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 23;

15 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGCAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 24;

20 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
25 AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGCAGGTTGGAAAGC
AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 25;

30 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGGCAGC
35 AGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 26;

40 AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTGCT
GGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAGA
AAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGCG
TATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACCG
AAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAGC
AGATACCAGCTATGCAAGCAAATAA

SEQ ID NO: 27; and

45 AAAGTGACCTTCCATGTTGAAGCAGGCAGCGCACCGAATTATCTGGCACTGC
TGGTCAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTAAAGAG
AAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAATTTGGC
GTATTGCAACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTATACCACC

GAAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAGGTTGGAAAG
CAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 28.

- 5 A third aspect of the invention provides a vector comprising a nucleic acid molecule according to the second aspect of the invention.

In one embodiment, the vector is an expression vector.

- 10 A fourth aspect of the invention provides a host cell comprising a nucleic acid molecule according to the second aspect of the invention or a vector according to the third aspect of the invention.

- 15 A fifth aspect of the invention provides a method for producing a polypeptide according to the first aspect of the invention comprising culturing a population of host cells comprising a nucleic acid molecule according to the second aspect of the invention or a vector according to the third aspect of the invention under conditions in which the polypeptide is expressed, and isolating the polypeptide therefrom.

- 20 A sixth aspect of the invention provides a pharmacological composition comprising a polypeptide according to the first aspect of the invention and a pharmaceutically acceptable diluent, excipient or carrier.

- 25 As used herein, 'pharmaceutical composition' means a therapeutically effective formulation for use in the methods of the invention.

- A 'therapeutically effective amount', or 'effective amount', or 'therapeutically effective', as used herein, refers to that amount which provides a therapeutic or prophylactic effect for a given condition (grass pollen allergy) and administration regimen. This is a predetermined quantity of active material calculated to produce a desired therapeutic effect in association with the required additive and diluent, *i.e.* a carrier or administration vehicle. Further, it is intended to mean an amount sufficient to reduce and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in a host. As is appreciated by those skilled in the art, the amount of a compound may vary depending on its specific activity. Suitable dosage amounts may contain a predetermined quantity of active composition

calculated to produce the desired therapeutic effect in association with the required diluent. In the methods and use for manufacture of compositions of the invention, a therapeutically effective amount of the active component is provided. A therapeutically effective amount can be determined by the ordinary skilled medical or veterinary worker
5 based on patient characteristics, such as age, weight, sex, condition, complications, other diseases, *etc.*, as is well known in the art.

The polypeptides can be formulated at various concentrations, depending on the efficacy/toxicity/solubility of the particular polypeptide being used. Preferably, the
10 formulation comprises the hypoallergenic polypeptide at a concentration of between 0.1 μ M and 10 mM, more preferably between 1 μ M and 1 mM, between 5 μ M and 500 μ M, between 10 μ M and 300 μ M, and most preferably about 100 μ M.

Thus, the pharmaceutical composition may comprise an amount of a polypeptide
15 sufficient to immunise a subject against a grass pollen allergy.

It will be appreciated by persons skilled in the art that the medicaments containing the hypoallergenic polypeptides of the invention will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the
20 intended route of administration and standard pharmaceutical practice (for example, see *Remington: The Science and Practice of Pharmacy*, 19th edition, 1995, Ed. Alfonso Gennaro, Mack Publishing Company, Pennsylvania, USA, the relevant disclosures in which document are hereby incorporated by reference).

25 For example, the medicaments may be administered orally, buccally or sublingually in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications. The medicaments and agents may also be administered via intracavernosal injection.

30 Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate and glycine, disintegrants such as starch (preferably corn, potato or tapioca starch), sodium starch glycolate, croscarmellose sodium and certain complex silicates, and granulation binders such as
35 polyvinylpyrrolidone, hydroxyl-propylmethylcellulose (HPMC), hydroxy-propylcellulose

(HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules.

5 Preferred excipients in this regard include lactose, starch, cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the polypeptides may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

10

The medicaments can also be administered parenterally, for example, intravenously, intra-articularly, intra-arterially, intraperitoneally, intra-theccally, intraventricularly, intrasternally, intracranially or by injection into secondary lymphoid organs, like lymph nodes, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

20

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

25

30

For oral and parenteral administration to human patients, the dosage level of the medicaments will usually be from 0.1 to 1000 µg per adult, administered in single or divided doses. For example, a dose of 1 to 100 µg protein per administration may be

35

used. Such doses may be administered at set intervals, for example daily, weekly, monthly, every 5 to 6 weeks, every 10 weeks, *etc.*

The medicaments can also be administered intranasally or by inhalation and are
5 conveniently delivered in the form of a dry powder inhaler or an aerosol spray
presentation from a pressurised container, pump, spray or nebuliser with the use of a
suitable propellant, *e.g.* dichlorodifluoromethane, trichlorofluoro-methane,
dichlorotetrafluoro-ethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA
134A3 or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA3), carbon dioxide or other
10 suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined
by providing a valve to deliver a metered amount. The pressurised container, pump,
spray or nebuliser may contain a solution or suspension of the active compound, *e.g.*
using a mixture of ethanol and the propellant as the solvent, which may additionally
contain a lubricant, *e.g.* sorbitan trioleate. Capsules and cartridges (made, for example,
15 from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder
mix of a compound of the invention and a suitable powder base such as lactose or
starch.

Aerosol or dry powder formulations are preferably arranged so that each metered dose
20 or 'puff' contains at least 0.1 µg of a compound of the invention for delivery to the patient.
It will be appreciated that the overall daily dose with an aerosol will vary from patient to
patient, and may be administered in a single dose or, more usually, in divided doses
throughout the day.

25 Alternatively, the medicaments can be administered in the form of a suppository or
pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment
or dusting powder. The compounds of the invention may also be transdermally
administered, for example, by the use of a skin patch. They may also be administered
by the ocular route.

30

For application topically to the skin, the medicaments can be formulated as a suitable
ointment containing the active compound suspended or dissolved in, for example, a
mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum,
propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and
35 water. Alternatively, they can be formulated as a suitable lotion or cream, suspended or
dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan

monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

Formulations suitable for topical administration in the mouth include lozenges comprising
5 the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth;
pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or
sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable
liquid carrier.

10 Proteins and polypeptides can also be delivered by electroincorporation (EI). EI occurs
when small particles of up to 30 microns in diameter on the surface of the skin
experience electrical pulses identical or similar to those used in electroporation. In EI,
these particles are driven through the stratum corneum and into deeper layers of the
skin. The particles can be loaded or coated with drugs or genes or can simply act as
15 "bullets" that generate pores in the skin through which the drugs can enter.

In one proffered embodiment of the sixth aspect of the invention, the pharmaceutical
composition is a vaccine composition.

20 Such vaccines typically contain one or more adjuvants.

Thus, the hypoallergenic polypeptides may be prepared in an immunogenic formulation
containing suitable adjuvants and carriers and administered to the patient in known ways.
Suitable adjuvants include Freund's complete or incomplete adjuvant, muramyl dipeptide,
25 the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminium hydroxide, saponin,
DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil),
liposomes, Pluronic polyols, MF59 or the Ribi adjuvant system (see, for example GB-A-2
189 141). "Pluronic" is a Registered Trade Mark.

30 Adjuvants are included in vaccines to enhance the immune response to the immunogen. It
will be appreciated by persons skilled in the art that such adjuvants may be selected to
mainly boost the immune response or to simultaneously alter the type of immune
response so as to favour a response appropriate for the intended use of the vaccine. It is
expected that a protective response to allergen vaccination involves a shift in the
35 response from a Th2 (T helper type 2) response to a Th1 (T helper type 1) or a Treg (T
regulatory) response (Li and Boussiotis, 2008). For instance, adjuvants that affect the

levels of cytokines like IL-10 or TGF-beta may have such beneficial effects. Indeed, it has been demonstrated that a combination of 1,25-dihydroxyvitamin D3 plus dexamethasone as well as *Lactobacillus plantarum* (Van Overtvelt et al., 2008) enhanced sublingual immunotherapy in mice. Similarly, a Toll-like receptor 2 agonist (Pam3CSK4) was shown to enhance the performance of sublingual immunotherapy (Lombardi et al., 2008). Furthermore, incorporation of heat-killed *Listeria monocytogenes* in the adjuvant in an allergen-specific immunotherapy setting reduced the symptoms caused by subsequent allergen challenge as well as the levels of allergen-specific IgE following specific immunotherapy (Frick et al., 2005). Similarly, targeting of allergen by use of mucoadhesive chitosan particles to dendritic cells, a procedure that has been shown to increase T cell proliferation and interferon-gamma/IL-10 secretion in vitro, enhances tolerance induction and improves vaccine performance (Saint-Lu et al., 2009). It is consequently preferred that formulations that incorporate the hypoallergenic polypeptide also incorporate components that favour responses of the Th1- or Treg-phenotype.

It will be appreciated by persons skilled in the art that the pharmaceutical compositions of the invention may comprise a single hypoallergenic polypeptide of the invention or may comprise one or more additional antigens.

20

In one embodiment, the one or more additional antigens comprise or consist of other grass pollen allergens, for example those belonging to grass pollen allergen groups 1, 2, 3, 4, 5, 6, 7, 10, 11, 12 and 13 (reviewed by Andersson K and Lidholm J (2003) *Int Arch Allergy Immunol* 130, 87–107; DOI: 10.1159/000069013). Similarly the pharmaceutical preparation may contain allergens from other sources like birch pollen allergen Bet v 1 for use in vaccination against combined grass and tree pollen allergy.

A seventh aspect of the invention provides a hypoallergenic polypeptide according to the first aspect of the invention for use in medicine.

30

Persons skilled in the art will further appreciate that the hypoallergenic polypeptides of the present invention have utility in both the medical and veterinary fields. Thus, the medicaments may be used in the treatment of both human and non-human animals (such as horses, dogs and cats).

35

Preferably, however, the patient is human.

By 'treatment' we include both therapeutic and prophylactic treatment of the patient. The term 'prophylactic' is used to encompass the use of a polypeptide or formulation described herein which either prevents or reduces the likelihood of grass pollen allergies in a patient or subject.

As discussed above, the term 'effective amount' is used herein to describe concentrations or amounts of compounds according to the present invention which may be used to produce a favourable change in a disease or condition treated, whether that change is a remission, a favourable physiological result, a reversal or attenuation of a disease state or condition treated, the prevention or the reduction in the likelihood of a condition or disease state occurring, depending upon the disease or condition treated.

In one embodiment, the hypoallergenic polypeptide of the invention is for use as a vaccine, for example for the prevention of grass pollen allergies (including allergic rhinitis).

A related, eighth embodiment of the invention provides a polypeptide according to the first aspect of the invention in the preparation of a medicament for use as a vaccine, for example for the prevention of grass pollen allergies (including allergic rhinitis).

A ninth aspect of the invention provides a method for active immunisation of a subject comprising administering to the subject a polypeptide according to the first aspect of the invention, in particular for the prevention of grass pollen allergies (including allergic rhinitis).

In addition of the active immunisation aspects of the invention detailed above, the present invention also provides agents for use in the passive immunisation against grass pollen.

Thus, a tenth aspect of the invention provides an antibody with specificity for major timothy group 1 pollen allergen, Phl p 1, or an antigen-binding fragment or derivative thereof, wherein the antibody, fragment or derivative competes for binding to Phl p 1 with one or more of the following polypeptides:

EVQLVESGGGLGQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGIS
 WNSGRIGYADSVKGRFTISRDNAKNSLHLQMNSLRAEDTALYYCARERLPGNWN
 YDLWGRGRTLVTVSSGGGGSGGGGSGGGGSSQSALTQPPSVSGAPGQRVTISCT
 GSSSNFGAGYHVHWYQQFPGTAPKLLIQNNIRPSGVPDRFSASKSGTSASLAIT
 5 GLQPDDEADYYCQSYDSSVSGSVFGGGTKLTVL

SEQ ID NO:29

QVQLVQSGAEVKKSGASLKVSCKASGYTFTDYGISWVRQAPGQGLEWMGWINW
 NNGNTHYAQKLQGRVTMTTDTSTKTAYMELKSLRYDDTAVYYCAAGFHYWGQG
 10 TLVTVSSGGGGSGGGGSGGGGSSQSVLTQPPSVSVSPGQTARITCSADALANQY
 GYWYQQKPGQAPVLLIYKDNERPSGIPERFSGSSSGTTVTLTISGVQAEDEADYY
 CQSSDRFGSRYVFGTGTKLTVL

SEQ ID NO:30

- 15 By “antibody” we include substantially intact antibody molecules, as well as chimaeric antibodies, humanised antibodies, human antibodies (wherein at least one amino acid is mutated relative to the naturally occurring human antibodies), single chain antibodies, bispecific antibodies, antibody heavy chains, antibody light chains, homodimers and heterodimers of antibody heavy and/or light chains, and antigen binding fragments and
 20 derivatives of the same.

The term ‘antibody’ also includes all classes of antibodies, including IgG, IgA, IgM, IgD and IgE. Thus, the antibody may be an IgG molecule, such as an IgG1, IgG2, IgG3, or IgG4 molecule.

25

The term “antibody” also encompasses antibody mimetics, such as:

- Affibodies (see Nygren, 2008, *FEBS J.* **275** (11): 2668–76);
- Affilins (see Ebersbach, 2007, *J. Mol. Biol.* **372** (1): 172–85);
- 30 • Affitins (see Krehenbrink, 2008, *J. Mol. Biol.* **383** (5): 1058–68);
- Anticalins (see Skerra, 2008, *FEBS J.* **275** (11): 2677–83);
- Avimers (see Silverman, 2005, *Nat. Biotechnol.* **23** (12): 1556–61);
- DARPs (see Boersma & Plückthun, 2011 Opinion in Biotechnology (in press); Stumpp, 2008, *Drug Discov. Today* **13** (15–16): 695–701);
- 35 • Fynomers (see Grabulovski, 2007, *J Biol Chem* **282** (5): 3196–3204);

- Kunitz domain peptides (see Nixon, 2006, *Curr Opin Drug Discov Devel* **9** (2): 261–8); and/or
- Monobodies (see Koide, 2007, *Methods Mol. Biol.* **352**: 95–109).

5 By “antigen-binding fragment” we mean a functional fragment of an antibody that is capable of binding to an antigen.

Preferably, the antigen-binding fragment is selected from the group consisting of Fv fragments (e.g. single chain Fv and disulphide-bonded Fv), Fab-like fragments (e.g. Fab
10 fragments, Fab' fragments and F(ab)2 fragments), single variable domains (e.g. VH and VL domains) and domain antibodies (dAbs, including single and dual formats [i.e. dAb-linker-dAb]).

By “competes for binding” we include that the antibody, fragment or derivative of he
15 invention binds at or near the same epitope on Phl p 1 as the antibody polypeptide of SEQ Id NO: 29 and/or 30.

Competitive binding may be determined by methods well known to those skilled in the art such as ELISA, immunohistochemistry, immunoprecipitation, Western blots,
20 radiolabelling experiments and other methods known in the art.

In one embodiment, the antibody, fragment or derivative comprises one or more of the following CDRs:

25	<i>Heavy chain:</i>	GFTFDDYA	SEQ ID NO:31
		ISWNSGRI	SEQ ID NO:32
		ARERLPGNWNLYDL	SEQ ID NO:33
	<i>Light chain:</i>	SSNFGAGYH	SEQ ID NO:34
30		NNN	SEQ ID NO:35
		QSYDSSVSGSV	SEQ ID NO:36

For example, the antibody, fragment or derivative may comprise one or two or three (e.g. SEQ ID NOS 31 to 33 or SEQ ID NOS 34 to 36), or four or five or six of the above
35 CDRs.

Persons skilled in the art will appreciate that there are different nomenclatures to define CDR regions, such as Kabat, Cothia, Honnegger and IMGT. The CDRs are defined herein in accordance with the IMGT system (see WHO-IUIS Nomenclature SubCommittee for IG and TR, Report Aug 2007).

5

For example, the antibody, fragment or derivative may comprise or consist of the amino acid sequence of SEQ ID NO: 29.

In an alternative embodiment, the antibody, fragment or derivative comprises one or more of the following CDRs:

10

<i>Heavy chain:</i>	GYTFTDYG	SEQ ID NO:37
	INVNNGNT	SEQ ID NO:38
	AAGFHY	SEQ ID NO:39

15

<i>Light chain:</i>	ALANQY	SEQ ID NO:40
	KDN	SEQ ID NO:41
	QSSDRFGSRYV	SEQ ID NO:42

For example, the antibody, fragment or derivative may comprise one or two or three (e.g. SEQ ID NOS 37 to 39 or SEQ ID NOS 40 to 42), or four or five or six of the above CDRs.

For example, the antibody, fragment or derivative may comprise or consist of the amino acid sequence of SEQ ID NO: 30.

25

In a further embodiment of the tenth aspect of the invention, the antibody, fragment or derivative is fused to an Fc region, or portion thereof.

The Fc portion may be from an IgG or IgA antibody, such as IgG4.

30

An eleventh aspect of the invention provides a nucleic acid molecule (e.g. a DNA molecule) encoding an antibody, fragment or derivative according to the tenth aspect of the invention, or a component polypeptide chain thereof (such as a heavy chain, light chain, or variable region thereof).

35

Preferred aspects and examples of the eleventh aspect of the invention are detailed above in relation to the second aspect of the invention. For example, wherein the nucleic acid molecule may be codon optimised.

- 5 In one embodiment, the nucleic acid molecule comprises or consists of the following nucleotide sequence:

10 GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGGACAGCCTGGCAGGT
CCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTGATGATTATGCCA
TGCAGTGGGTCCGGCAAGCTCCAGGGAAGGGCCTGGAGTGGGTCTCAGG
TATTAGTTGGAATAGTGGTCGCATAGGCTATGCGGACTCTGTGAAGGGCC
GATTCACCATCTCCAGAGACAACGCCAAGAACTCCCTGCATCTGCAAATGA
ACAGTCTACGAGCTGAGGACACGGCCTTATATTACTGCGCAAGAGAGAGG
CTGCCTGGGAACTGGAACACGATCTCTGGGGCCGTGGCACCTGGTCAC
15 CGTCTCCTCAGGTGGAGGCGGTTGAGGCGGAGGTGGATCCGGCGGTGGC
GGATCGCAGTCTGCCCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAG
GGCAGAGGGTCACCATCTCCTGCACTGGGAGCAGCTCCAACTTCGGGGCA
GGTTATCATGTACACTGGTACCAGCAATTTCCAGGAACAGCCCCCAAATC
CTCATCCAGAATAACAACATTCGGCCCTCAGGGGTCCCTGACCGATTCTCT
20 GCCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCACTGGGCTCCAGCC
TGACGATGAGGCTGATTATTACTGCCAGTCGTATGACAGCAGCGTGAGTG
GTTTCGGTTTTTCGGCGGAGGCACCAAGCTGACCGTCCTC

SEQ ID NO: 43

- 25 In an alternative embodiment, the nucleic acid molecule comprises or consists of the following nucleotide sequence:

30 CAGGTACAGCTGGTGCAATCTGGAGCTGAGGTGAAGAAGTCTGGGGCCTC
ACTGAAGGTCTCCTGCAAGGCTTCTGGTTACACCTTTACCGACTATGGTAT
CAGCTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGATGG
ATCAACGTCAACAATGGTAATACACACTATGCACAGAAGCTCCAGGGCAGA
GTCACCATGACTACAGACACATCCACGAAAACAGCCTACATGGAAGTGAAG
AGCCTGAGATATGACGACACGGCCGTGTATTACTGTGCGGCCGGCTTTCA
CTATTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGTGGAGGCGGTT
35 CAGGCGGAGGTGGATCCGGCGGTGGCGGATCGCAGTCTGTGCTGACTCA
GCCACCCTCGGTGTCAGTGTCCCAGGACAGACGGCCAGGATCACCTGCT

CTGCAGATGCATTGGCAAACCAATATGGTTATTGGTACCAGCAGAAGCCAG
GCCAGGCCCTGTGTTACTGATATATAAAGATAATGAGAGGCCCTCAGGGA
TCCCTGAGCGATTCTCTGGCTCCAGCTCAGGGACAACAGTCACGTTGACC
ATCAGTGGAGTCCAGGCAGAAGACGAGGCTGACTACTACTGTCAATCATCA
5 GACAGGTTTGGTAGTCGTTATGTCTTCGGCACAGGGACCAAGCTGACCGT
CCTA

SEQ ID NO: 44

10 A twelfth aspect of the invention provides a vector comprising a nucleic acid molecule according to the eleventh aspect of the invention.

Preferred aspects and examples of the twelfth aspect of the invention are detailed above in relation to the third aspect of the invention.

15 For example, the vector may be an expression vector.

A thirteenth aspect of the invention provides a host cell comprising a nucleic acid molecule according to the eleventh aspect of the invention or a vector according to the twelfth aspect of the invention.

20 Preferred aspects and examples of the thirteenth aspect of the invention are detailed above in relation to the fourth aspect of the invention.

25 A fourteenth aspect of the invention provides a method for producing an antibody, fragment or derivative according to the tenth aspect of the invention comprising culturing a population of host cells comprising a nucleic acid molecule according to the eleventh aspect of the invention or a vector according to the twelfth aspect of the invention under conditions in which the polypeptide is expressed, and isolating the polypeptide therefrom.

30 A fifteenth aspect of the invention provides a pharmacological composition comprising an antibody, fragment or derivative according to the tenth aspect of the invention and a pharmaceutically acceptable diluent, excipient or carrier.

35 Preferred aspects and examples of the fifteenth aspect of the invention are detailed above in relation to the sixth aspect of the invention.

A sixteenth aspect of the invention provides an antibody, fragment or derivative according to the tenth aspect of the invention for use in medicine.

5 In one embodiment, the antibody, fragment or derivative is for use as a passive vaccine, e.g. for the prevention of grass pollen allergies.

A seventeenth aspect of the invention provides of an antibody, fragment or derivative according to the tenth aspect of the invention in the preparation of a medicament for use as a vaccine, for the prevention of grass pollen allergies.

10

An eighteenth aspect of the invention provides a method for passive immunisation of a subject comprising administering to the subject an antibody, fragment or derivative according to the tenth aspect of the invention. In one embodiment, the method is for the prevention of grass pollen allergies.

15

Preferred aspects and examples of the sixteenth, seventeenth and eighteenth aspects of the invention are detailed above in relation to the seventh, eighth and ninth aspects of the invention.

20 A nineteenth aspect of the invention provides a method for standardization of diagnostic procedures comprising use of antibody fragments or derivatives according to the 10th aspect of the invention. In one embodiment the method is for the standardization of tools/chips/reagents/devices used for diagnosis of allergy by measurement of allergen-specific IgE.. Defined monoclonal reagents (antibody fragments or antibody fragments
25 fused Fc of antibodies such as human IgE Fc) as described in the tenth aspect of the invention used alone or in combination can be utilized to determine, using immunological assays, such as ELISA and western blot known to persons skilled in the art, the amount of timothy group 1 allergen content in raw material (recombinant allergens, purified natural allergens, or complex extracts/mixtures of allergens) used for production of such
30 tools/chips/reagents/devices or in the final tool/chip/reagent/device product.

A twentieth aspect of the invention provides a process for the development of hypoallergenic polypeptide comprising the steps of:

- 35 (a) providing a lymphocyte-containing sample from an allergic individual;
(b) isolating RNA from the lymphocyte-containing sample provided in step (a);

- (c) generating cDNA from the RNA isolated in step (b);
- (d) isolating from the cDNA generated in step (c) IgE heavy chain variable domain-encoding genes;
- (e) (optionally) isolating from the cDNA generated in step (c) antibody light chain variable domain-encoding genes;
- (f) constructing one or more polynucleotide library comprising or consisting of the IgE heavy chain variable domain-encoding genes isolated in step (d) and (optionally) antibody light chain variable domain-encoding genes isolated in step (e);
- (g) selecting one or more polynucleotide encoding allergen-specific antibody or antigen-binding fragment thereof from the one or more polynucleotide library;
- (h) (optionally) cloning the one or more polynucleotide selected in step (g) into another vector system;
- (i) expressing the one or more allergen-specific antibody or antigen-binding fragment thereof;
- (j) (optionally) determining the binding specificity (and, optionally, the binding affinity) to the cognate allergen of the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i);
- (k) generating and expressing one or more variant of the cognate allergen;
- (l) analysing the binding affinity of the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i) to the one or more variant of the cognate allergen generated in step (k); and
- (m) selecting one or more variant of the cognate allergen that has a lower affinity for the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i) than the cognate allergen.

Optionally, the process of the twentieth aspect of the invention comprises the further steps of:

- (n) determining the ability of the one or more variant of the cognate allergen selected in step (m) to recognize polyclonal IgE in serum or plasma provided from a subject allergic to the cognate allergen;
- (o) determining the ability of the one or more variant of the cognate allergen selected in step (m) to activate basophils (carrying IgE) provided from a subject allergic to the cognate allergen; and

- (p) selecting one or more variant of the cognate allergen that has lower affinity for polyclonal IgE as found in serum or plasma of subjects allergic to the cognate allergen and/or lower ability to activate basophils (carrying IgE) derived from subjects allergic to the cognate allergen.

5

Optionally, the process of the twentieth aspect of the invention comprises the further steps of:

- (q) determining the ability of the one or more variant of the cognate allergen to induce expression of cognate allergen-specific antibodies in a subject.
- (r) selecting one or more variant of the cognate allergen that is capable of inducing the expression of cognate allergen-specific antibodies in a subject.

10

By "cognate allergen" we mean the (or an) allergen to which the allergic individual from which a lymphocyte-containing sample was obtained from is allergic. The cognate allergen is used to select one or more polynucleotide encoding cognate allergen-specific antibody or antigen-binding fragment thereof from the one or more DNA library in step (g).

15

In step (a) the lymphocyte-containing sample provided is preferably a blood sample (as used by Andréasson et al., 2006), lymph node tissue, spleen tissue, bone marrow tissue, or mucosal tissue (such as sinus mucosa as used by Levin et al., 2011) involved in the allergic response. Preferably the lymphocytes are B cell lineage cells (B cells and differentiated cells thereof such as plasma cells) (typically more than 1 %). The cells may be enriched for IgE-producing B cells by sorting using flow cytometry. Hence, in step (b), the isolated RNA preferably comprises or consists lymphocyte-derived RNA, for example, B cell-derived RNA.

20

Generation of cDNA can be performed using any suitable method known in the art (for example, see Sambrook & Russell, 2000, *Molecular Cloning, A Laboratory Manual*, Third Edition, Cold Spring Harbor, New York, the relevant disclosures in which document are hereby incorporated by reference). For example, the RNA isolated in step (b) could be sequenced and cDNA could be synthesised *in vitro*. However, cDNA will typically be generated using reverse transcriptase (i.e., conventional first strand cDNA synthesis – see, for example, Myers, T.W. and Gelfand, D.H. (1991) Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase.

30

35

Biochemistry 30, 7661-6). To ensure that heavy chain variable domain encoding sequences are derived from mRNA encoding IgE, primers used for cDNA synthesis may be selected so as to specifically hybridize to IgE-encoding mRNA (example of such primer is provided by Andréasson et al. (2006)),

5

In Steps (d-e), genes are preferably isolated and/or amplified using polymerase chain reaction (PCR), however, any other suitable method known in the art may be used. In one embodiment, only polynucleotide encoding antibody variable region (light chain and/or heavy chain) may be isolated. Alternatively, polynucleotide encoding antibody
10 variable and constant region (light chain and/or heavy chain) may be isolated. Polynucleotide encoding entire antibody may be isolated. To ensure that amplification of heavy chain variable domain encoding sequences are derived from cDNA encoding IgE, primers used for PCR may be selected so as to specifically hybridize to and amplify IgE-encoding cDNA (example of such primer is provided by Andréasson et al. (2006)).
15 Typically, the heavy chain variable domain make up for the specificity-determining part of antibodies (Xu and Davis, 2000). Consequently, it is preferred that the library is made from sequences encoding the variable domain of the heavy chain of IgE. Light chain encoding genes may be derived from IgE-producing B lymphocytes (e.g. ensured by sorting of IgE-producing B lymphocytes by flow cytometry) or from other B lymphocytes,
20 including B lymphocytes derived from other individuals or derived synthetically.

An antibody gene library is a collection of vectors that have been transformed with the genes for the variable regions of different antibodies. The variable region genes can either be synthesized *in vitro* based on sequence information of IgE variable domain
25 encoding mRNA or cDNA or amplified from the genetic material in human antibody-producing B cells, or in the case of the light chain encoding genes, be synthetically derived. Variable region genes are typically used instead of genes for whole antibody molecules because fragments of antibodies are more easily assembled in microorganisms than whole antibody molecules and the variable regions of an antibody
30 are the most important fragments in terms of function. Each variable region gene is spliced into a vector. Vectors may then be inserted into a microorganism.

Hence, in step (f), the one or more DNA library is preferably constructed using a vector suitable for functional antibody and/or antibody fragment expression. Where required for
35 functional antibody/antibody fragment expression (for example if these regions are absent from the cloned polynucleotides) the expression vector may include one or more

antibody constant regions (e.g., heavy chain constant regions alpha (α), delta (δ), epsilon (ϵ), gamma (γ) or mu (μ) found in IgA, IgD, IgE, IgG and IgM, respectively; and/or light chain constant region lambda (λ) or kappa (κ)). However, in one embodiment, the one or more DNA library is a variable region expression library, for instance in the scFv format
5 as described by Andréasson et al. (2006).

In step (g) cognate allergen-specific antibody fragments may be selected from the antibody library (enriched by binding to the cognate allergen) using any suitable method known in the art. However, preferred methods include phage display, yeast display,
10 bacterial display, mRNA display and ribosome display (mRNA display utilizes covalent mRNA-polypeptide complexes linked through puromycin, whereas ribosome display utilizes stalled, non-covalent ribosome-mRNA-polypeptide complexes).

The display of proteins and polypeptides on the surface of bacteriophage (phage), fused
15 to one of the phage coat proteins, provides a powerful tool for the selection of specific ligands. This 'phage display' technique was originally used by Smith in 1985 (*Science* **228**, 1315-7) to create large libraries of peptides for the purpose of selecting those with high affinity for a particular antigen. More recently, the method has been employed to present antibody fragments (e.g. as described by McCafferty *et al.*, 1990, *Nature* **348**,
20 552-554), domains of proteins and intact proteins at the surface of phages in order to identify ligands having desired properties.

The principles behind phage display technology are as follows:

- 25 (i) Nucleic acid encoding the protein or polypeptide for display is cloned into a phage;
 - (ii) The cloned nucleic acid is expressed fused to the coat-anchoring part of one of the phage coat proteins (typically the p3 or p8 coat proteins in the case of filamentous phage), such that the foreign protein or polypeptide is displayed on the surface of the phage;
 - 30 (iii) The phage displaying the protein or polypeptide with the desired properties is then selected (e.g. by affinity chromatography) thereby providing a genotype (linked to a phenotype) that can be sequenced, multiplied and transferred to other expression systems.
- 35 Alternatively, the foreign protein or polypeptide may be expressed using a phagemid vector (*i.e.* a vector comprising origins of replication derived from a phage and a plasmid)

that can be packaged as a single stranded nucleic acid in a bacteriophage coat. When phagemid vectors are employed, a "helper phage" is used to supply the functions of replication and packaging of the phagemid nucleic acid. The resulting phage will express both the wild type coat protein (encoded by the helper phage) and the modified coat protein (encoded by the phagemid), whereas only the modified coat protein is expressed when a phage vector is used.

Methods of selecting phage expressing a protein or peptide with a desired specificity are known in the art. For example, a widely used method is "panning", in which phage stocks displaying ligands are exposed to solid phase coupled target molecules, e.g. using affinity chromatography.

Alternative methods of selecting phage of interest include SAP (Selection and Amplification of Phages; as described in WO 95/16027) and SIP (Selectively-Infective Phage; EP 614989A, WO 99/07842), which employ selection based on the amplification of phages in which the displayed ligand specifically binds to a ligand binder. In one embodiment of the SAP method, this is achieved by using non-infectious phage and connecting the ligand binder of interest to the N-terminal part of p3. Thus, if the ligand binder specifically binds to the displayed ligand, the otherwise non-infective ligand-expressing phage is provided with the parts of p3 needed for infection. Since this interaction is reversible, selection can then be based on kinetic parameters (see Duenas *et al.*, 1996, *Mol. Immunol.* **33**, 279-285).

The use of phage display to isolate ligands that bind biologically relevant molecules has been reviewed in Felici *et al.* (1995) *Biotechnol. Annual Rev.* **1**, 149-183, Katz (1997) *Annual Rev. Biophys. Biomol. Struct.* **26**, 27-45 and Hoogenboom *et al.* (1998) *Immunotechnology* **4**(1), 1-20. Several randomised combinatorial peptide libraries have been constructed to select for polypeptides that bind different targets, e.g. cell surface receptors or DNA (reviewed by Kay, 1995, *Perspect. Drug Discovery Des.* **2**, 251-268; Kay and Paul, 1996, *Mol. Divers.* **1**, 139-140). Proteins and multimeric proteins have been successfully phage-displayed as functional molecules (see EP 0349578A, EP 0527839A, EP 0589877A; Chiswell and McCafferty, 1992, *Trends Biotechnol.* **10**, 80-84). In addition, functional antibody fragments (e.g. Fab, single chain Fv [scFv]) have been expressed (McCafferty *et al.*, 1990, *Nature* **348**, 552-554; Barbas *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**, 7978-7982; Clackson *et al.*, 1991, *Nature* **352**, 624-628), and some of the shortcomings of human monoclonal antibody technology have been superseded

since human high affinity antibody fragments have been isolated (Marks *et al.*, 1991, *J. Mol. Biol.* **222**, 581-597; Hoogenboom and Winter, 1992, *J. Mol. Biol.* **227**, 381-388). Further information on the principles and practice of phage display is provided in *Phage display of peptides and proteins: a laboratory manual* Ed Kay, Winter and McCafferty
5 (1996) Academic Press, Inc ISBN 0-12-402380-0, the disclosure of which is incorporated herein by reference.

In yeast display (or yeast surface display; Boder, E.T., Wittrup, K.D.; Nat. Biotech., 1997, 15, 553-57) a protein of interest is displayed as a fusion to the Aga2p protein on the
10 surface of yeast. The Aga2p protein is naturally used by yeast to mediate cell-cell contacts during yeast cell mating. As such, display of a protein via Aga2p projects the protein away from the cell surface, minimizing potential interactions with other molecules on the yeast cell wall. The use of magnetic separation and flow cytometry in conjunction with a yeast display library is a highly effective method to isolate high affinity protein
15 ligands.

In bacterial display (or bacteria display or bacterial surface display) libraries of polypeptides displayed on the surface of bacteria are screened using flow cytometry or iterative selection procedures (biopanning) (see, for example, Francisco, J. A.; Campbell, R.; Iverson, B. L.; Georgiou, G. (1993). "Production and Fluorescence-Activated Cell
20 Sorting of Escherichia coli Expressing a Functional Antibody Fragment on the External Surface". *Proc. Nat. Acad. Sci. U.S.A.* **90**: 10444-48).

Ribosome display and mRNA are *in vitro* peptide screening methods. mRNA display
25 utilizes covalent mRNA-polypeptide complexes linked through puromycin, whereas ribosome display utilizes stalled, non-covalent ribosome-mRNA-polypeptide complexes. For further information on these methods see, for example, Hanes J, Plückthun A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* 94, 4937-4942 and Liu R, Barrick JE, Szostak JW, Roberts RW (2000).
30 "Optimized synthesis of RNA-protein fusions for *in vitro* protein selection". *Meth Enzymol.* **318**: 268-93.

In step (h), any vector suitable for the expression of antibody or antigen-binding protein may be used (as described above). The expression vector may be, for example, for
35 encoding antibody fragments (as described by Persson *et al.*, 2008b), intact antibody (as described by Andréasson *et al.*, 2006), or antibody fragments fused to constant domains

of antibodies (as described by Carlsson et al., 2012, and by Moutel et al., 2009, and the present accompanying Examples).

5 In step (j) it is preferred that the binding specificity to the cognate allergen of more than one allergen-specific antibody or antigen-binding fragment thereof selected in step (g) and expressed in step (i) is determined, for example, the binding specificity to the cognate allergen of at least at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 10 2000, 2500 or at least 3000 allergen-specific antibodies or antigen-binding fragments thereof expressed in step (i) may be determined.

In step (k) cognate allergen variants may be generated using rational design (i.e., site-directed mutagenesis of one or more residues) or random mutagenesis (for 15 example, error-prone PCR or gene shuffling). However, any suitable method mutagenic method known in the art may be used. By 'cognate allergen variant' we include polypeptides comprising or consisting of an amino acid sequence in which one or more amino acid residues of the cognate allergen are mutated, for example inserted, deleted and/or substituted (preferably substituted). Preferably, surface-exposed residues 20 thought to be important to allergenicity and/or surface-exposed residues thought to be important to immunogenicity are mutated. Preferably, the minimum number of mutations are made for example, 15 or less, 14 or less, 13 or less, 12 or less, 11 or less, 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less or 1 mutation may be made relative to the cognate allergen.

25 In one embodiment, one or more IgE epitopes relative to the cognate allergen are not mutated. In one embodiment, one or more IgG epitopes relative to the cognate allergen are not mutated. In one embodiment, one or more IgA epitopes relative to the cognate allergen are not mutated.

30 Step (l) may optionally include determining the ability of the one or more variant of the cognate allergen selected in step (m) to recognize polyclonal IgE as found in serum or plasma of subjects allergic to the cognate allergen and/or the ability of the one or more variant of the cognate allergen selected in step (m) to activate basophils (carrying IgE) 35 derived from subjects allergic to the cognate allergen.

In step (m), the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i) preferably has affinity for the one or more variant of the cognate allergen of at least 80% or less than for the cognate allergen, for example 75% or less, 70% or less, 65% or less, 60% or less, 55% or less, 50% or less, 45% or less, 40% or less, 35% or less, 30% or less, 25% or less, 20% or less, 15% or less, 10% or less, 5% or less, 4% or less, 3% or less, 2% or less or 1% or less.

In step (n), IgE of serum or plasma derived from individuals that are allergic to the cognate allergen may bind to the one or more variant of the cognate allergen identified in step (m) with an affinity of at least 80% or less than for the cognate allergen, for example 75% or less, 70% or less, 65% or less, 60% or less, 55% or less, 50% or less, 45% or less, 40% or less, 35% or less, 30% or less, 25% or less, 20% or less, 15% or less, 10% or less, 5% or less, 4% or less, 3% or less, 2% or less or 1% or less.

In step (o) basophils or other cells carrying receptors for IgE and that also carry IgE specific for cognate allergen, cells typically isolated from individuals allergic against cognate allergen, require more of the variant of the cognate allergen identified in step (m) than of cognate antigen to achieve cell activation, preferably at least 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3 times as much, but at least 2 times as much.

20

In step (q), individuals (i.e., humans) or animals (such as, but not limited to, mice, rats, guinea pigs, rabbits, goats or sheep) immunized with a variant of the cognate allergen identified in step (m) induce antibodies that also recognize cognate allergen in a binding assay. Such antibodies may be the IgG isotype, in man commonly of the IgG4 subclass but also of the IgG1, IgG2 and IgG3 subclasses, but additionally/alternatively of other isotypes such as the IgA isotype. Such antibodies should prevent activity of processes involved in allergic disease by IgE antibodies as outlined by Flicker et al. (2011).

A twenty-first aspect of the invention provides a polypeptide obtained or obtainable by the process of the twentieth aspect of the invention.

30

A twenty-second aspect of the invention provides a nucleic acid molecule encoding the polypeptide according to the twenty-first aspect of the invention.

A twenty-third aspect of the invention provides a vector (preferably an expression vector) comprising the nucleic acid molecule according to the twenty-second aspect of the invention.

- 5 A twenty-fourth aspect of the invention provides a host cell containing the nucleic acid molecule or the vector according to the twenty-second or twenty-third aspects of the invention.

10 A twenty-fifth aspect of the invention provides a pharmaceutical composition comprising a polypeptide, a nucleic acid, a vector or a host cell according to the twenty-first, twenty-second, twenty-third, or twenty-fourth aspects of the invention, a pharmaceutically acceptable diluent, excipient or carrier, and (optionally) an adjuvant. Preferably, the pharmaceutical composition is a vaccine composition.

- 15 The pharmaceutical composition according to the twenty-fifth aspects of the invention may comprise one or more adjuvant. Preferably, the one or more adjuvant is capable of reducing Th2-type immunity against the polypeptide, a nucleic acid, a vector of the pharmaceutical composition in mammals (such as humans). Preferably the one or more adjuvant is capable of increasing Th1-type and/or T regulatory (Treg)-type immunity
20 against the polypeptide, a nucleic acid, a vector of the pharmaceutical composition in mammals (such as humans).

By "capable of reducing Th2-type immunity" we mean that the Th2-type response is capable of being reduced compared to negative control(s) lacking the one or more
25 adjuvant. Preferably the Th2-type response is reduced compared negative control(s) lacking the one or more adjuvant by at least 5%, for example, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%,
30 at least 99.9% or at least 100%.

Th2-type immunity levels may be determined by any suitable means known in the art. In one embodiment, Th2-type immunity levels are determined through the production of IL-4, IL-5 and IL-13 by Th2-type T cells (Akdis and Akdis, 2007, 2011). In an additional or
35 alternative embodiment Th2-type immunity levels are determined using the methodology described by Akdis et al., (1996).

By "capable of increasing Th1-type and/or T regulatory (Treg)-type immunity" we mean that the Th1-type and/or T regulatory (Treg)-type immunity is capable of being increased compared to negative control(s) lacking the one or more adjuvant by at least 5%, for example, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% at least 100%, at least 125%, at least 150%, at least 175%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900% or at least 1000%.

Th1-type immunity levels may be determined using any suitable means known in the art. In one embodiment, Th1-type immunity levels are determined through the production of interferon-gamma by Th1-type T cells (Akdis and Akdis, 2007, 2011). In an additional or alternative embodiment, Th1-type immunity levels are determined using the methodology described by Akdis et al. (1996).

Treg-type cellular immunity levels may be determined using any suitable means known in the art. In one embodiment Treg-type cellular immunity levels are determined through the production of IL-10 and TGF-beta by Treg-type T cells (Akdis and Akdis, 2007, 2011). In an additional or alternative embodiment Treg-type cellular immunity levels are determined using the methodology described by Akdis et al., (1998) and Jutel et al., (2003).

25

The one or more adjuvant may be selected from the group consisting of:

- (i) 1,25-dihydroxyvitamin D3 plus dexamethasone;
- (ii) *Lactobacillus plantarum*;
- (iii) Pam3CSK4 (a Toll-like receptor 2 agonist);
- (iv) heat-killed *Listeria monocytogenes*; and
- (v) mucoadhesive chitosan particles.

30

A twenty-sixth aspect of the invention provides a hypoallergenic polypeptide comprising or consisting of a variant of the amino acid sequence of SEQ ID NO: 71 carrying a K246 mutation relative to the Phl p 5.0101 sequence (SEQ ID NO: 70).

35

VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYE
 SYKFIPALEAAVAQAYAATVATAPEVKYTVFETALKKAITAMSEAQKAAKPAA
 AATATATAAVGAATGAATAATGGYKV

SEQ ID NO: 71

5

Preferably the mutation is a substitution or a deletion. The amino acid substitution may be with any natural amino acid, but is preferably with alanine (A). Preferably, K246 is mutated.

10

Preferred, non-limiting examples which embody certain aspects of the invention will now be described, with reference to the following figures:

15

Figure 1. Basic outline of the process used to create a hypoallergenic protein variant based on the binding properties of human monoclonal IgE. The process described herein is based on selection of specific binders from phage-displayed antibody fragment libraries but is applicable with minor modifications to human monoclonal IgE derived by other approaches used for antibody development such as, but not limited to, those based on ribosomal display (Hanes and Plückthun, 1997) or sorting of antigen-specific B cells (Meijer *et al.*, 2006).

20

Figure 2. Binding specificity of novel scFv selected on Phl p 1 as determined by ELISA on different recombinant timothy grass allergens and BSA. All clones are highly specific for Phl p 1, with no detectable cross-reactivity.

25

Figure 3. Sequence (SEQ ID NO:46) of Phl p 1.0102 (gray + black) highlighting its C-terminal domain (black). 12 mutant versions of the C-terminal domain were produced, each carrying a single mutation in one of the underlined positions, and tested for IgE binding. All mutated amino acids were replaced with an alanine.

30

Figure 4. Binding of Phl p 1-specific IgE to a C-terminal fragment was confirmed by ELISA. All five human IgE (1p1:8, 5p1:3, p1-15, p1-20, clone 10) do bind the C-terminal domain at levels comparable to Phl p 1-binding. In contrast, only one of the mouse monoclonals (1.8 but not 1.10 and 1.21) reacts with the C-terminal domain.

35

Figure 5. Cross-reactivity of Phl p 1-binding human monoclonal IgE against protein extracts of 10 grass species with group 1 allergens with high sequence identity to Phl p 1 as determined by ImmunoCAP. Values are normalized and compared to Phleum pratense (100%).

5

Figure 6. 3D visualization of the C-terminal part of Phl p 1 (green) with mutations K8A, N11A and D55A, shown to be important for IgE-binding, marked in blue. In red are shown amino acids that failed to affect IgE-binding when mutated.

Figure 7. Inhibition of binding of five human IgE (A, 1p1:8; B, 5p1:3; C, p1-15; D, p1-20; E, clone 10) and one mouse monoclonal (F, 1.8) to immobilized Phl p 1 using soluble proteins as determined by ELISA. Inhibition with the C-terminal domain of Phl p 1, the combined mutant versions of the C-terminal domain (carrying mutations K8A, N11A and D55A) and GST was evaluated. The combined mutant failed to inhibit binding of human IgE (A-E), while it did inhibit the binding of the mouse monoclonal (F) to a level comparable to that of the unmutated C-terminal domain.

Figure 8. Reactivity of serum IgE from Phl p 1-positive donors against the C-terminal domain of Phl p 1, the potentially hypoallergenic variant and GST, as determined using ELISA. The introduction of three mutations (K8A, N11A and D55A) significantly reduces the reactivity against polyclonal IgE from human serum.

Figure 9. Sequence alignment of the VH of set of Phl p 1 specific human IgE with their corresponding germline genes (**SEQ ID NOS: 47 to 54**).

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Figure 10. Cross reactivity of the set of human IgE (A, 1p1:8; B, 5p1:3; C, p1-15; D, p1-20; E, clone 10) and three mouse monoclonals (F, 1.8; G, 1.10; H, 1.21) to different isoforms of Phl p 1, as determined by ELISA.

Figure 11. Sequence alignment of group 1 allergens from the 10 different grass species included in the ImmunoCAP analysis

Phl p 1.0101 (Timothy grass)	SEQ IS NO:55;
Phl p 1.0102 (Timothy grass)	SEQ IS NO:56;
Pha a 1 (Canary grass)	SEQ IS NO:57;
Dac g 1.01 (Cocksfoot)	SEQ IS NO:58;

35

	Poa p 1 (Kentucky blue grass)	SEQ IS NO:59;
	Lol p 1 (Rye grass)	SEQ IS NO:60;
	Hol l 1 (Velvet grass)	SEQ IS NO:61;
	Tri a 1 (Wheat)	SEQ IS NO:62;
5	Cyn d 1 (Bermuda grass)	SEQ IS NO:63;
	Pas n 1 (Bahia grass)	SEQ IS NO:64; and
	Zea m 1 (Maize)	SEQ IS NO:65;

10 **Figure 12.** Sequence relationship of C-terminal domains of group 1 grass pollen allergens. Method. Neighbor joining; midpoint rooting

Figure 13. 3D visualization of the 12 amino acids chosen for mutation (red) spread evenly over the surface of the C-terminal domain of Phl p 1 (green). In **B** figure **A** is rotated 180°.

15

Figure 14. Reactivity of five the human IgE clones 1p1:8 (A), 5p1:3 (B), p1-15 (C), p1-20 (D) and clone (E) against Phl p 1, the C-terminal domain of Phl p 1, GST and the 12 mutant versions of the C-terminal domain as determined by ELISA.

20 **Figure 15.** Inhibition of binding of five human IgE (A, 1p1:8; B, 5p1:3; C, p1-15; D, p1-20; E, clone 10) and one mouse monoclonal (D, 1.8) to immobilized Phl p 1 using soluble proteins as determined by ELISA. Inhibition with the C-terminal domain of Phl p 1, the 12 mutant versions of the C-terminal domain and GST was evaluated. Mutants K8A, N11A and D55A and GST all to some extent failed to inhibit binding of human IgE (A-E), while
25 only mutant E84A and GST failed to inhibit the mouse monoclonal (F).

Figure 16. Reactivity of serum IgE from Phl p 1-positive donors against Phl p 1, the C-terminal domain of Phl p 1, 12 mutants of the C-terminal domain, each carrying one mutation (as denoted in legend), and GST as determined by ELISA.

30

Figure 17. Inhibition of binding of five human IgE (A, 1p1:8; B, 5p1:3; C, p1-15; D, p1-20; E, clone 10) and one mouse monoclonal (F, 1.8) to immobilized Phl p 1.0102 using soluble proteins as determined by ELISA. Inhibition with the C-terminal domain of Phl p 1.0102, a mutant version of the C-terminal domain of Phl p 1.0101 (carrying mutations K8A, N11A and D55A) and GST was evaluated. The combined mutant version of the C-terminal domain of Phl p 1.0101 failed to inhibit binding of human IgE (A-E), while it did
35

inhibit the binding of the mouse monoclonal (F), although at a slightly lower level compared to that of the unmutated C-terminal domain of Phl p 1.0102.

Figure 18. Gating strategy for the basophil activation test. Basophils were identified as CD123+/HLADR- cells in the upper left corner of the plot (A) and their expression levels of the activation markers CD203c and CD63 were further assessed for e.g. unstimulated basophils (B), and basophils stimulated with Phl p 1 (C) or the N11A mutant of the C-terminal domain of Phl p 1 fused to GST (D). Note the formation of a new CD63+ population for the stimulated samples (C, D) (in particular in samples stimulated with intact Phl p 1 (C)) compared to the unstimulated sample (B). These types of changes in marker levels were observed in all investigated donors.

Figure 19. Patterns of changes in basophil activation in three individual grass pollen allergic donors (D1-3). Basophils were stimulated with the wild type protein Phl p 1 (filled diamonds) and the C-terminal domain of Phl p 1 fused to GST (filled squares), as well as with N11A (open triangles) or K8A, N11A, D55A (open circles) mutated variants of the C-terminal domain of Phl p 1 fused to GST. Changes in CD63 expression upon degranulation were assessed. Stimulation index is calculated from the mean fluorescence intensity (MFI) of CD63 expression levels and % CD63+ cells from the stimulated samples as compared to the unstimulated control samples.

Figure 20. Characterization of protein produced in larger scale. A. SDS-PAGE separation of GST-hypoallergen (with K8A, N11A, D55A mutations) obtained following chromatographic purification on a GSTrap FF column. The expected product is indicated by an arrow. B. SDS-PAGE separation of two different batches of hypoallergen (with K8A, N11A, D55A mutations) without GST (SEQ ID NO: 67) obtained following treatment of GST-hypoallergen with PreScission protease and subsequent chromatographic removal of GST, uncleaved product, proteolytic enzyme and other impurities by size exclusion chromatography. A product with a purity of >95 % was obtained. C. Peptide mass fingerprint of one batch of GST-free hypoallergen (with K8A, N11A, D55A mutations) (SEQ ID NO:67).

Figure 21. SDS-PAGE of proteins produced in E.coli. The proteins correspond to the GST tag alone (lanes 2,3) and the GST tag fused to the C-terminal domain of Phl p 5.0101 (lanes 4,5), and to mutated versions K246A (lanes 6,7), K270A (lanes 8,9) and K280A (lanes 10,11) thereof. Duplicate lanes of each sample represent different fractions

obtained during the elution procedure used to purify the proteins. Lanes 1 and 12 are a molecular weight markers.

Figure 22. Structure (PDB: 1L3P) of C-terminal domain of Phl p 5. The positions of lysine side chains corresponding to K246, K270 and K280 of Phl p 5.0101 are highlighted in dark grey colour.

Figure 23. Binding of Phl p 5-specific scFv to GST, Phl p 5, and fusion proteins of GST with the C-terminal domain of Phl p 5.0101, and K246A, K270A and K280A mutants of that domain.

EXAMPLES

Materials & Methods

5 Recombinant allergens, mouse monoclonal antibodies and patient sera

Recombinant allergens Phl p 1.0102, Phl p 2, Phl p 5, Phl p 6 and Phl p 7 were purchased from Biomay AG (Vienna, Austria). Phl p 1.0101 was purchased from INDOOR Biotechnologies Ltd (Wiltshire, United Kingdom). Three mouse monoclonal
 10 antibodies (1.8, 1.10, 1.21) (Duffort *et al.*, 2008) specific for Phl p 1 were kindly provided by Dr. D. Barber (ALK-Abelló, Madrid, Spain). Sera were obtained from grass pollen positive allergic patients. These studies were approved by the local ethical committee.

Production of the C-terminal domain of Phl p 1 and mutant version thereof

15 Codon optimized genes encoding the C-terminal domain of Phl p 1.0102, mutant versions thereof and a mutant version of the C-terminal domain of Phl p 1.0101 were purchased from GeneArt and cloned into the pGEX-6P-1 expression vector (GE Healthcare) to allow for production of GST-fusion proteins. For example, the nucleotide
 20 sequence of the pGEX-6P-1 containing an insert encoding the N11A variant of SEQ ID NO:1 is shown below as SEQ ID NO: 66 (wherein the sequence encoding the hypoallergenic polypeptide is shown in bold type, the Bam HI and Not I restriction sites are underlined and the codon encoding the N11A mutation is shown boxed in bold italics):

25
 30
 35
 40
 ACGTTATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAG
 CTGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCG
 CACTCCCGTTCTGGATAATGTTTTTTCGCCGACATCATAACGGTTCTGGCAAATAT
 TCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAG
 CGGATAACAATTCACACAGGAAACAGTATTCATGTCCCCTATACTAGGTTATTGGA
 AAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGGAATATCTTGAAGAAAAA
 TATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGT
 TTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTAAAT
 TAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAAACATGTTGGGT
 GGTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATA
 TTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTT
 GATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTTGAAGATCGTTTATGTCA
 TAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATGACG
 CTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCAAAATTA
 GTTTGTTTTAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATC
 CAGCAAGTATATAGCATGGCCTTTCAGGGCTGGCAAGCCACGTTTGGTGGTGGC

58

CTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATT
 ACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTT
 CCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCC
 CATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGC
 5 AAATCGCGCTGTTAGCGGGGCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCT
 GGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGG
 CGACTGGAGTGCCATGTCCGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCA
 TCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCG
 CGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATAC
 10 GACGATACCGAAGACAGCTCATGTTATATCCCGCCGTCAACCACCATCAAACAGGA
 TTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGC
 CAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCAC
 CCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGC
 AGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTA
 15 ATGTGAGTTAGCTCACTCATTAGGCACCCAGGCTTTACACTTTATGCTTCCGGCTC
 GTATGTTGTGTGGAATTGTGAGCGGATAACAATTTACACAGGAAACAGCTATGACC
 ATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGG
 CGTTACCCAACTTAATCGCCTTGCAAGCACATCCCCCTTTCGCCAGCTGGCGTAATA
 GCGAAGAGGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGA
 20 ATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAGCTGGCTGGAG
 TGCGATCTTCCTGAGGCCGATACTGTCTGTCGTCCTCCCTCAAACCTGGCAGATGCACGG
 TTACGATGCGCCCATCTACACCAACGTAACCTATCCCATACGGTCAATCCGCCGTT
 TGTTCACGAGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAG
 CTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTGGAATT

SEQ ID NO:66

Vectors carrying the inserted genes were transformed into T7 Express competent
Escherichia coli (New England Biolabs, Ipswich, MA, USA) and grown in 2xYT-medium
 (supplemented with 100 µg/ml carbencillin) until OD₆₀₀=0.4, at which time induction of
 30 protein production was achieved by addition of isopropyl β-D-1-thiogalactopyranoside
 (IPTG) to a final concentration of 1 mM. The production was allowed to continue for 3
 hours at 37°C before cells were harvested and treated with lysozyme (Sigma-Aldrich, St.
 Louis, MO, USA). Soluble GST-fusion proteins were purified using affinity
 chromatography with GSTrap FF columns (GE Healthcare, Piscataway, NJ, USA).

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Phl p 1-specific human antibody fragments

Phl p 1-specific single chain antibody fragments (scFv) p1-15 and p1-20 have previously
 been isolated (Persson *et al.*, 2007) by selection on Phl p 1.0102 from a phage display
 40 library established using heavy chain variable (VH) domain-encoding sequences of the
 IgE-encoding transcriptome of an allergic patient (Andreasson *et al.*, 2006). The
 sequence encoding Phl p 1-specific antibody fragment clone10 (Flicker *et al.*, 2006) was
 collected from GenBank (accession numbers AJ512649 and AJ512646). A codon-
 optimized gene encoding clone10 in scFv format was obtained from GeneArt. Four N-

terminal codons, not found in the published sequence, were added to the construct (encoding EVQL as defined by the IGHV3-9*01 gene).

The construction of the two combinatorial scFv libraries used in this study for selection of additional Phl p 1-specific IgE has previously been described (Levin *et al.*, 2011). To isolate Phl p 1-specific binders from the library, phage display selections were performed on recombinant Phl p 1.0102. Three selection rounds were performed in Immuntubes (Nunc A/S, Roskilde, Denmark) coated with allergen diluted to 5 µg/ml in phosphate buffered saline (PBS). After a pre-selection performed in tubes incubated with selection buffer (1% w/v BSA, 0.05% Tween 20 v/v in PBS) the phage library was applied to the allergen-coated tubes and incubated for 2 hours. Unbound phages were washed away and bound phages were eluted by addition of trypsin, which cleaves at a trypsin sensitive site between the scFv and phage protein III. After the third selection round clones were picked at random and phage stocks produced for specificity analysis.

Construction of vectors for production of scFv and scFv-CH₂-4 fusion proteins

To enable production of soluble scFv, genes encoding scFv were transferred in a single cloning step into a production vector (Persson *et al.*, 2008b). Ligated DNA was transformed into chemically competent One Shot Top10 *E. coli* (Invitrogen, Carlsbad, CA, USA).

To allow for production of scFv-CH₂-4 fusion proteins the pFUSE-hlgG-Fc2 vector (Moutel *et al.*, 2009) was modified to produce scFv fused to the IgE CH₂-4 domains. A codon-optimized gene encoding the IgE CH₂-4 domains, with codon 2 in CH₂ mutated to alanine, was purchased from GeneArt and cloned into the pFUSE-hlgG-Fc2 vector between the *NotI* and *NheI* restriction sites. In a second cloning step genes encoding scFv were cloned into the vector using *NcoI* and *NotI*. The ligated DNA was transformed into chemically competent XL1-Blue *E. coli* (Agilent Technologies, Santa Clara, CA, USA).

Production of scFv in *E. coli*

Cells carrying the vector encoding scFv were grown in 2xYT-medium (supplemented with 100 µg/ml carbencillin). At OD₆₀₀=0.9 protein production was induced by the addition of IPTG to a final concentration of 1 mM. Production was allowed to proceed for 16 hours at

30°C. Cells were harvested and treated with lysozyme (Sigma-Aldrich). Soluble scFv were finally purified using affinity chromatography with Ni-NTA agarose columns (Qiagen GmbH, Hilden, Germany).

5 Production of scFv-CH ϵ 2-4 fusion proteins in HEK293 cells

For production of scFv-CH ϵ 2-4 fusion proteins HEK293 cells were grown in Minimum Essential Medium (Invitrogen) containing 2mM L-glutamine and 10% HyClone Fetal Bovine Serum (Hyclone Laboratories, Utah, USA) in 5% CO₂ at 37°C until 90%
10 confluency was reached. Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were grown for 72 hours at which time the supernatant containing scFv-CH ϵ 2-4 was collected and sterilized by filtration (0.45 μ m) prior to analysis.

15 ELISA binding assays

The ability of the investigated recombinant proteins either displayed on phage, as soluble scFv or as scFv-CH ϵ 2-4 fusion proteins to bind intact allergens, the C-terminal domain of Phl p 1.0102 or mutant versions thereof was determined using ELISA. Antigens were
20 coated in microtiterplates at a concentration of 5 μ g/ml. Either BSA or GST was used as negative control. Blocking was performed with 1% w/v milk, 0.05% v/v Tween 20 in PBS. Bound phages were detected with an horseradish peroxidase (HRP)-labeled anti-M13 monoclonal antibody (mAb) (GE Healthcare), scFv with an HRP-labeled anti-FLAG M2 mAb (Sigma Aldrich), scFv-CH ϵ 2-4 fusion proteins with an HRP-labeled anti-IgE
25 antiserum (KPL, Guilford, UK) and mouse monoclonal antibodies with a HRP-labeled polyclonal rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) using 1-Step Ultra TMB - ELISA Substrate (Pierce, Rockford, IL, USA) as chromogen. Absorbance was measured at 450 nm.

30 The IgE-reactivity of serum from 11 Phl p 1-positive donors to Phl p 1.0102, the C-terminal domain of Phl p 1.0102, mutant versions of this domain and GST was analyzed using ELISA. Antigens were coated and wells were blocked as described above. Bound IgE was detected using HRP-labeled anti-IgE antiserum. Detection was performed as described above.

35

ELISA blocking assay

A blocking assay was performed to identify the presence, or not, of multiple epitopes on the C-terminal domain of Phl p 1.0102. Briefly, microtiterplates were coated with the Phl p 1 C-terminal domain or BSA at a concentration of 0.2 µg/ml. Blocking was performed as described above. Wells were pre-incubated with Phl p 1-specific soluble scFv or Phl p 1-specific mouse antibodies for 1 hour before addition of scFv-CH ϵ 2-4 fusion proteins. Bound scFv-CH ϵ 2-4 fusion proteins were detected using the HRP-labeled anti-IgE antibody and a chemiluminescent substrate (SuperSignal ELISA Femto Maximum Sensitivity Substrate, Pierce).

Genetic analysis

Inserts encoding Phl p 1-specific binders made in this study were sequenced by GATC Biotech AG (Konstanz, Germany). The genetic origin of sequences encoding VH and variable light (VL) domains and mutational status of the sequences included in this study were determined using the IMGT/V-QUEST web tool (program version 3.2.20; reference directory release: 201135-3) (Brochet *et al.*, 2008). Genes were annotated according to the IMGT nomenclature (Lefranc, 1999) Sequence alignments were performed using MacVector 12.0.3 (MacVector Inc., Cary, NC, USA).

ImmunoCAP analysis

Crude, diluted supernatants containing scFv-CH ϵ 2-4 were assessed for binding to allergens found in pollen extracts using the standard ImmunoCAP system (Phadia AB, Uppsala, Sweden) All samples were assessed in technical duplicates.

Basophil degranulation assay

Basophils are cells of the immune system with major roles during sensitization to allergens and subsequent allergic reactions as reviewed by Karasuyama *et al.* (2011). The degree of basophil activation upon allergen challenge can be measured through basophil activation tests, which together with recombinant allergens, can be used to diagnose allergy as discussed by Valent *et al.* (2004) and reviewed by Ebo *et al.* (2006). In this study, we have investigated the ability of two Phl p 1 mutants, mut2 and mut13, to

induce degranulation of basophils from peripheral blood from three grass pollen allergic donors, compared to the wild-type proteins.

5 Heparinized peripheral blood was collected from three grass pollen allergic donors (D1, D2 and D3) by the Department of Otorhinolaryngology at Lund University hospital. All donors were positive in a skin prick test and had circulating allergen-specific IgE levels, as determined by ImmunoCap (Phadia, Uppsala, Sweden). The study was approved by the local Ethics Committee.

10 Peripheral blood mononuclear cells (PBMC) were isolated from whole blood through density gradient centrifugation (Lymphoprep, Axis-Shield PoC, Oslo, Norway) immediately after sampling. Cells were resuspended in RPMI 1640 (without L-glutamin, Thermo Fisher Scientific, MA, USA) with 0.5% (w/v) BSA (Cohn fraction V, Saveen & Werner, Limhamn, Sweden). Cell count and viability were determined using an
15 automated cell counter (Countess, Invitrogen, CA, USA).

PBMC were challenged with the C-terminal domain fused to GST at 1, 0.1, 0.01 or 0.001 µg/ml or equimolar concentrations of full-length Phl p 1 (Biomay, Vienna, Austria), the N11A mutant or the K8A, N11A, D55A mutant of the C-terminal domain of Phl p 1 fused
20 to GST. A control with an equimolar concentration of GST relative the highest concentration of the C-terminal domain fused to GST and a positive control with 20 µM fMLF (Sigma-Aldrich, MO, USA) were included. 2 ng/ml human IL-3 (Miltenyi Biotec, Bergisch Gladbach, Germany) was added and after 60 min incubation in a water bath (37°C, 5% CO₂) the degranulation was stopped by addition of 20 mM EDTA. One
25 unstimulated control sample, incubated in 37°C and treated with EDTA and IL-3, and one untreated control, kept on ice and not treated with EDTA or IL-3, were also included. Cells were thereafter washed and resuspended in PBS (DPBS/modified, without Ca²⁺ and Mg²⁺, Thermo Fisher Scientific) with 0.5% (w/v) BSA and 2.5 mM EDTA. Non-specific IgG binding was blocked using 6 µg/ml mouse IgG (Jackson Immunoresearch,
30 PA, USA) and the cells were stained for 20 min at 4°C with HLA-DR-PerCPCy5.5 (BioLegend, CA, USA), CD123-PE (BD Pharmingen, NJ, USA), and CD63-FITC (BD Pharmingen). CD203c conjugated with allophycocyanin (APC) (Miltenyi Biotec) was included for some donors. An unstimulated control, only stained with HLA-DR-PerCPCy5.5 and CD123-PE, was included. After washing, the cells were resuspended in
35 PBS with 0.5% (w/v) paraformaldehyde (Thermo Fisher Scientific) and analyzed with a flow cytometer (FACSCanto II, BD Biosciences, NJ, USA) and FCS Express 4 (De Novo

Software, CA, USA). 500-2000 basophils, gated as CD123+/HLA-DR- cells, were acquired for each sample. The degree of degranulation is visualized as stimulation index, calculated as (MFI (CD63) x percent CD63+ cells of stimulated cells) / (MFI (CD63) x percent CD63+ cells of unstimulated cells).

5

Production and characterization of GST-free hypoallergen

E. coli TUNER(DE3) (Novagen) cells carrying the gene encoding the K8A, N11A, D55A mutant of the C-terminal domain of Phl p 1.0102 cloned into the pGEX-6P-1 vector was grown in of Luria Broth (Difco) supplemented with 100 µg/ml ampicillin in 5 L Erlenmeyer
10 flasks with indentations (1 L / flask) at 18°C, 200 rpm. At OD600 ~ 0.5, IPTG was added to a final concentration of 1 mM. 18 hours after induction, cells were harvested in a JLA 8.1000 rotor, 8000xg, 4°C, 15 min, and the pellets were stored at -80°C or used directly for preparation of soluble extract.

15

The pellets from 1 L culture described above were resuspended in 20 ml PBS, pH 7.3, supplemented with one tablet Complete Protease Inhibitor, EDTA-free (Roche). The cell suspension was passed twice through a French Pressure cell at 18 000 psi. The lysate was ultracentrifuged in a Ti 50.2 rotor, 45 000 rpm, 60 min, 4°C, and the supernatant
20 (soluble fraction) was passed through a 0.45 µm filter.

The soluble fraction was used for affinity chromatography. A 5 ml GSTrap FF column (GE Healthcare) was connected to an ÄKTA Avant system. The column was run at room temperature, while fractions were collected at 4°C. The column was washed with 5
25 column volumes (CV) H₂O and was then equilibrated with 5 CV PBS, pH 7.3, and the sample was applied using the air sensor. The column was washed with PBS, pH 7.3 until a stable UV signal was obtained, and bound protein was eluted with 10 CV 50 mM Tris-HCl, 150 mM NaCl, 40 mM reduced glutathione, pH 8.0. The flow rate was set to 5 ml/min, except during sample application and elution, when the flow rate was 0.5 ml/min.
30 The flow through and wash fractions were saved, and during elution, 2 ml fractions were collected. Peak fractions were analyzed with SDS-PAGE, and fractions containing GST-tagged hypoallergen were pooled. The pooled fractions were concentrated and the buffer was exchanged to 50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0 using Vivaspin Turbo 15 ultrafiltration spin columns (MWCO 10,000).

35

The GST-tag was removed from the purified product with PreScission protease (13 u / mg protein) to produce a protein product with an expected sequence as illustrated in SEQ ID NO: 67 (a product that incorporates a glycine-proline-leucine-glycine-serine sequence attached to the N-terminal part of hypoallergen sequence). DTT was added to the reaction mixture to a final concentration of 1 mM. The cleavage reaction was left at 4°C for 1-3 days.

GPLGSKVTFHVEAGSAPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESWGAIWRI
ATPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

SEQ ID NO:67

To separate hypoallergen from PreScission protease and GST and other contaminating proteins, size exclusion chromatography was performed. The hypoallergen/PreScission protease reaction mixture was passed through a 0.22 µm filter before loading the sample using a superloop onto a 329 ml HiLoad 26/600 Superdex 200 pg gel filtration column (GE Healthcare) connected to an ÄKTA Purifier system. The column was run at 4°C, with a flow rate of 2.6 ml/min using a buffer consisting of 50 mM Tris, 150 mM NaCl, pH 8.0. 4 ml fractions were collected. Peak fractions were analyzed with SDS-PAGE, and fractions containing hypoallergen were pooled. The pooled fractions were concentrated and the buffer was exchanged to PBS using Vivaspin Turbo 15 ultrafiltration spin columns (MWCO 10,000). The concentrated protein solution was passed through a 0.22 µm filter and was stored on ice in the cold room until delivery.

Mass spectrometry analysis was performed to verify that the correct protein had been purified. Samples of hypoallergen were diluted 10x in 50 mM ammonium bicarbonate/acetonitrile (1:1) in a total volume of 20 µl. 3 µl 100 ng/µl trypsin was added, and the mixture was incubated at 37°C for two hours. A 10x dilution of the tryptsinated sample was spotted on a Maldi plate. The plate was analyzed using a 4700 Proteomics Analyzer (Applied Biosystems/MDS SCIEX, USA). A peptide mass fingerprint was obtained in positive reflector mode. The obtained peptide list was compared with the expected protein sequence using the GPMW (General Protein/Mass Analysis for Windows) software.

Studies of Phl p 5 and its recognition by human IgE

To further test the methodology to develop hypoallergenic variants of allergens we also
 5 used a set of antibody fragments specific for the major grass pollen allergen Phl p 5. The
 antibody fragments (scFv) 4.3, 4.4, 4.12 and 4.13 (Andréasson et al., 2006) and p5-MA5
 and p5-AB5 (Persson et al., 2008c), all originally derived by phage display selection for
 binding to Phl p 5, were produced as scFv-CH₂-3 of human IgG2 in HEK293 cells as
 described by Carlsson et al. (2012). A codon-optimized gene sequence (SEQ ID NO: 68)
 10 encoding clone 5 (Steinberger et al., 1996) in scFv format was synthesised by Life
 Technologies and used to produce an scFv-CH₂-3 fusion protein in HEK293 cells.
 These seven antibody fragments are clonally unrelated in terms of immunoglobulin V
 gene usage (Table 1) and/or type of V, D J gene rearrangement (Andréasson et al.,
 2006; Persson et al., 2007; Persson et al., 2008c; Steinberger et al. 1996) as manifested
 15 by differences in CDRH3 length (Table 1) and thus represent different solutions for the
 creation of Phl p 5-specific antibodies.

GAAGTTCAGCTGCTGGAAAGCGGTGGTGGTGTTCAGCCTGGTCGTAGCCTGC
 20 GTCTGAGCTGTGCAGCAAGCGAATTTGCATTTGATAGCTATGAGATTTATTGGGTTC
 GTCAGGCACCGGGTAAAGGTCTGGAATGGGTTGCAATGATTAGCATTGATGAAACC
 AACAAACACTATGCCGATAGCGTTAAAGGTCGCTTTACCATTTTTTCGCGATAACAGC
 AATAAAACCGTGTCATCTGCAAATGAATAATCTGCGTAGCGAAGATAGCGGTGTGTAT
 TATTGTGCACGTTGGAATAATTGGAATCATCGTGGTATGGTGTTCCTTTGATCTG
 25 TGGGGTCAGGGTACAATGGTTACCGTTAGCAGCGGTGGCGGTGGTAGTGGTGGTG
 GCGGTAGCGGAGGTGGTGGTTCAGATATTCAGATGACCCAGAGCCCGAGCAGCGT
 TAGCGCAAGCGTTGGTGATCGTGTTACCATTACCTGTCGTGCAAGCCAGGGTATTA
 GCAGCTGGCTGGCATGGTATCAGCAGAAACCTGGTAAAGCACCGAAACTGCTGATT
 TATAGCGCCAGCAGCCTGCAGAGCGGTGTTCCGAGCCGTTTTAGCGGTAGTGGTA
 30 GCGGCACCGATTTTAGCCTGACCATTAGCAGTCTGCAGCCGGAAGATAGTGCAACC
 TATTATTGTCAGCAGGCAAATAGCTTTCCGTATACCTTTGGTCAGGGCACCAAAGTT
 GAAATCAAACGC

SEQ ID NO:68

35

Table 1

Genetic origin of the heavy chain V and J genes used by Phl p 5-specific scFv.

ScFv clone	GenBank accession numbers	Heavy chain germline gene origin		CDRH3 length
		IGHV	IGHJ	
4.3	DQ065731	IGHV3-11	IGHJ3	15
4.4	DQ065732	IGHV3-23	IGHJ6	21
4.12	DQ065734	IGHV1-69	IGHJ4	20
4.13	DQ065735	IGHJ1-18	IGHJ6	23
p5-AB5	EF601887	IGHV3-23	IGHJ4	16
p5-MA5	EF601894	IGHV1-18	IGHJ4	15
clone 5	X95746	IGHV3-30 or IGHV3-30-3	IGHJ3	17

5

A codon-optimized gene (synthesized by Life Technologies) (SEQ ID NO: 69) encoding the C-terminal fragment (residues 181-312 of UNIPROT: Q40960) of Phl p 5.0101 (SEQ ID NO: 70) was cloned into the pGEX-6P-1 vector (as described above). Furthermore, codon-optimized genes encoding mutant variants (K246A (SEQ ID NO: 71), K270A (SEQ ID NO: 72) or K280A (SEQ ID NO: 73)) of the C-terminal fragment of Phl p 5.0101 were similarly cloned into the pGEX6P-1 vector. These three mutations were selected as the corresponding residues in another isoform of Phl p 5 were known to be exposed on the surface of the protein as judged from a structure of the C-terminal domain of this allergen (PDB. 1L3P) and as they were among residues proposed to contribute to recognition of another isoform of Phl p 5 by IgE (Gehlhar et al., 2006). These fusion proteins were produced in T7 Express *Escherichia coli* (New England Biolabs) and purified using affinity chromatography with GSTrap FF columns, as described above.

20 GTTATTCCGGCAGGCGAACTGCAGGTTATTGAAAAAGTTGATGCAGCCTTTAAAGTT
GCAGCAACCGCAGCAAATGCAGCACCGGCAAATGATAAATTCACCGTTTTTTGAAGC
CGCATTCAACGATGCAATTAAAGCAAGCACCGGTGGTGCATATGAAAGCTATAAATT
CATTCCGGCACTGGAAGCAGCAGTTAAACAGGCATATGCAGCGACCGTTGCAACCG
CACCGGAAGTGAAATATACCGTGTTTGAACAGCACTGAAAAAAGCAATTACCGCAA
25 TGAGCGAAGCACAGAAAGCAGCAAACCGGCAGCAGCAGCCACCGCAACCGCCAC
CGCAGCAGTTGGTGCAGCAACAGGTGCAGCCACAGCCGCAACCGGTGGTTATAAA
GTT

SEQ ID NO:69

VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYESYKFIPAL
 EAAVKQAYAATVATAPEVKYTVFETALKKAITAMSEAQKAAKPAAAATATATAAVGAAT
 5 GAATAATGGYKV

SEQ ID NO: 70

VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYESYKFIPAL
 10 EAAVAQAYAATVATAPEVKYTVFETALKKAITAMSEAQKAAKPAAAATATATAAVGAAT
 GAATAATGGYKV

SEQ ID NO: 71

VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYESYKFIPAL
 15 EAAVKQAYAATVATAPEVKYTVFETALKAAITAMSEAQKAAKPAAAATATATAAVGAAT
 GAATAATGGYKV

SEQ ID NO: 72

VIPAGELQVIEKVDAAFKVAATAANAAPANDKFTVFEEAFNDAIKASTGGAYESYKFIPAL
 20 EAAVKQAYAATVATAPEVKYTVFETALKKAITAMSEAQAAPAAAATATATAAVGAAT
 GAATAATGGYKV

SEQ ID NO: 73

25

For immunological characterization, recombinant allergens, fragments thereof or
 fragments thereof carrying mutations (or the similarly produced GST tag alone) were
 coated onto microtitreplates at 0.2-1 µg/ml. Plates were washed and scFv-CH₂-3 fusion
 30 proteins were added. After incubation, plates were washed again and HRP-labelled anti-
 human IgG was used to detect binding of the recombinant scFv-CH₂-3 fusion protein to
 the immobilized protein using 1-Step Ultra TMB - ELISA Substrate (Pierce, Rockford, IL,
 USA) as chromogen. Absorbance was measured at 450 nm.

35

Results

Isolation of additional Phl p 1-specific human monoclonal IgE

To allow for analysis of the IgE immune response against grass pollen group 1 allergens, the available set of specific binders (Flicker et al., 2006; Persson et al., 2007) was expanded by phage display selections of antibody fragment libraries on recombinant Phl p 1. These new libraries had been established using VH-encoding sequences of the IgE-encoding transcriptome of patients suffering from chronic rhinosinusitis (Levin et al., 2011). Three rounds of selections were performed and randomly chosen clones were screened for Phl p 1-specificity using phage-ELISA. The process and the subsequent analysis are summarized in Fig. 1. Two novel specific binders (1p1:8 and 5p3:1) were isolated to complement the three previously available clones (p1-15, p1-20 and clone 10), creating a set of clones derived from 4 different IgE libraries. The two new clones were highly Phl p 1-specific, not showing any detectable signs of cross-reactivity with other timothy grass allergens (Fig. 2).

Genetic composition of Phl p 1-specific human monoclonal IgE

Analysis of the sequences of five Phl p 1-specific antibody fragments revealed a diversity amongst the set of binders in terms of both heavy and light chain germline gene origin and length of the third complementarity determining region of the heavy chain (CDRH3) (Table 2).

Table 2
Genetic origin of Phl p 1-specific scFv.

ScFv clone	GenBank accession numbers	Heavy chain germline gene origin			CDRH3 length	Light chain germline gene origin		CDRL3 length
		IGHV	IGHD	IGHJ		IGLV	IGLJ	
1p1:8	JX169823	IGHV3-9	IGHD3-9	IGHJ2	13	IGLV1-40	IGLJ2 or IGLJ3	11
5p1:3	JX169824	IGHV1-18	IGHD1-14	IGHJ4	6	IGLV3-25	IGLJ1	11
p1-15	EF607094	IGHV3-53	IGHD3-16	IGHJ4	11	IGKV2-28 or IGKV2D-28	IGKJ1	9
p1-20	EF607096	IGHV1-18	IGHD1-26	IGHJ4 or IGHJ5	7	IGLV3-25	IGLJ3	10
clone10	AJ512649 and AJ512646	IGHV3-9	IGHD2-15	IGHJ-4	14	IGKV1-39 or IGKV1D-39	IGKJ4	9

5

Both frequently used (IGHV1-18) and less common (IGHV3-9 and IGHV3-53) variable heavy chain genes (Boyd *et al.*, 2010) were utilized. The length of the product of the gene rearrangement, encoding the CDRH3, believed to be of substantial importance for antibody specificity (Xu *et al.*, 2000), also shows variability ranging from 6-14 codons (mean = 10.2), a range shorter than that described for polyclonal IgE repertoires, such as those reported by Kerzel *et al.* (mean = 15.9; $p=0.006$) and Andreasson *et al.* (mean = 14.8; $p=0.027$). The five clones, i.e. including those that originate from the same heavy chain germline gene, uses different VDJ rearrangements and have substantially different mutational patterns (Fig. 9). One particularly notable exception is a shared double mutation in codon 32 in CDRH1 of clones originating from germline gene IGHV1-18, a set of mutations that results in an S→D substitution (Fig. 9).

The set of human IgE are all specific for a C-terminal domain of Phl p 1

In an effort to map important binding epitopes of human Phl p 1-specific IgE a 95 amino acids long C-terminal fragment of Phl p 1 (Fig. 3), which has previously been shown to be immunodominant and to be recognized by a human monoclonal antibody of IgE isotype (Flicker *et al.* 2006), was produced. The ability of the five available Phl p 1-specific human antibodies and three mouse antibodies detecting distinct epitopes on Phl p 1 to bind the fragment was determined using ELISA (Fig. 4). All five tested human antibodies did bind the C-terminal fragment, confirming its immunodominance in human IgE responses. In contrast only one (1.8) of the three mouse monoclonal antibodies bound this allergen fragment.

Cross reactivity to different isoforms of Phl p 1

Grasses often produce different isoforms of their allergens, a fact that may complicate
 5 diagnosis and immunotherapy if the epitopes are not shared between the isoforms. The
 two isoforms, defined by the Allergome database (Mari *et al.*, 2009), of the group 1
 allergen of timothy, Phl p 1.0101 and Phl p 1.0102, differ in 15 out of 240 residues (94%
 identity) found in the mature protein and in 9 out of 95 residues (91% identity) of the C-
 terminal domain. To assess the ability of various antibodies to recognize different
 10 isoforms, we tested their binding activity in ELISA (Fig. 10). The mouse antibodies, which
 had been induced by immunization of natural Phl p 1 (Duffort *et al.*, 2008), recognized
 Phl p 1.0101 equally well or slightly better than Phl p 1.0102. Human antibodies (in the
 form of scFv-CH ϵ 2-4), which all had originally been isolated for binding to Phl p 1.0102,
 also recognized both isoforms of Phl p 1, although several of them, in particular clone p1-
 15 15 were less reactive towards Phl p 1.0101 as compared to Phl p 1.0102. Epitopes are
 thus shared, although not completely, between the two isoforms of Phl p 1.

Cross reactivity to group 1 allergens

20 To investigate the cross-reactivity amongst Phl p 1-binding human IgE towards pollen
 extracts from a range of other species, the five binders in the format of scFv-CH ϵ 2-4
 fusion proteins were analyzed for reactivity using ImmunoCAP (Fig. 5) with allergens
 derived from 9 grass species with group 1-allergens carrying high sequence similarity to
 Phl p 1 (Fig. 11). All five clones did to some extent cross-react with several of the
 25 analyzed extracts and one of the clones (clone 10) showed reactivity against all tested
 species except *Zea mays* (maize). This is largely in agreement with previous studies
 demonstrating a broadly cross-reactive behavior of this clone although ImmunoCAP
 analysis did not detect binding to maize extracts defined as weakly positive by
 immunoblot (Flicker *et al.*, 2006). Nevertheless, several grass extracts, in particular that
 30 derived from *Cynodon dactylon* but also those derived from *Triticum sativum* and
Paspalum notatum, were recognized by only one or a few members in this collection of
 clones. This is in agreement with the fact that the sequences of the C-terminal domain of
 the group 1 allergen of these grasses differ extensively from that of Phl p 1 (Fig. 12).
 Also extracts likely containing more closely related group 1 allergens were not
 35 recognized by one of the human antibodies (1p1:8). Altogether, these findings indicate
 that a polyclonal IgE preparation like a serum sample that test positive for several

extracts still may be very restricted in terms of repertoire clonality when targeting some of these allergen sources.

Epitope mapping of Phl p1-specific human IgE

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Blocking assays using the available human scFv and the mouse monoclonal antibody (1.8) reactive with the C-terminal domain of Phl p 1 revealed that this domain holds adjacent, but not overlapping, IgE-binding epitopes (Table 3).

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Table 3
Blocking of binding of scFv-Fcε2-4 to the C-terminal fragment of Phl p 1 by mouse IgG antibody or soluble human scFv.

Inhibitor [†]	Binding of scFv-Fcε2-4 detected for				
	1p1:8	5p1:3	p1-15	p1-20	clone10
1.8 IgG	**	**	-	**	**
1p1:8 scFv	-	-	-	-	-
5p1:3 scFv	-	**	-	**	-
p1-15 scFv	*	**	*	**	-
clone10 scFv	**	-	*	-	**
** = >80% inhibition; * = >50% inhibition; - = <50% inhibition					
[†] p1-20 did not produce soluble scFv in quantities sufficient for analysis					

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Although all antibody fragments were not efficient inhibitors of allergen binding of other antibody fragments and one scFv was not available in soluble form as it could not be produced, it is evident that 5p1:3 and p1-20, two binders that are encoded by similar, yet distinct, germline rearrangements (Table 2, Fig. 9), recognize overlapping sequences. Similarly, clone 10 blocks not only itself but also allergen binding of 1p1:8. These two scFv also originate from an identical IGHV gene. The mouse monoclonal antibody 1.8 seems to bind a third epitope, overlapping the two epitopes defined above for human scFv originating from IgE repertoires. ScFv p1-15 shows epitope reactivity in-between that of the two other sets of human scFv in this assay setup.

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To further pinpoint areas important for IgE-binding on Phl p 1, 12 mutant versions of the C-terminal fragment (Fig. 3), each carrying a single mutation of a surface-exposed amino acid, were produced and the effect of the mutations on antibody binding was evaluated in a direct binding assay. The amino acids chosen for investigation are spread across the surface of the C-terminal part of Phl p 1 (Fig. 13). Several mutations affected the binding of human antibodies to protein immobilized on microtiter plates. However, 3 different

mutations (K8A, N11A and D55A) that in particular reduced the reactivity with one or several of the tested Phl p 1-binding IgE were identified (Fig. 14). To further confirm binding specificity the ability of the mutants in soluble form to prevent binding of antibody specificities to immobilized Phl p 1 was investigated. These studies confirmed the relevance of one or several of the 3 identified amino acids one by one for the binding of 1p1:8, 5p1:3, p1-20 and clone 10 (Fig. 15). Phl p 1-C-N11A failed to inhibit the binding of 5p1:3 and p1:20, binders that had been identified as recognizing overlapping epitopes. This mutant of the C-terminal fragment of Phl p 1 were also partly defective in its recognition of clone 10, a binder that was even less reactive to Phl p 1-C-D55A and Phl p 1-C-K8A. Allergen binding of 1p1:8, a binder with specificity overlapping that of clone 10, was also prevented by the K8A mutation. Specific binder p1-15 was poorly inhibited by several soluble proteins, but in particular by Phl p 1-C-K87A. In contrast, the only mouse antibody, 1.8, that recognized the C-terminal domain of Phl p 1, was not affected by mutations in any of these residues. Instead its binding to soluble antigen was reduced by the E84A mutation, indicating a different specificity of this mouse antibody.

Identified epitopes constitutes a significant part of the IgE-binding epitopes on Phl p 1

To further assess the importance of these amino acids for the IgE reactivity, as found in serum of allergic donors, samples derived from 11 Phl p 1-positive donors were assayed against the 12 mutants by ELISA (Fig. 16). This study confirmed the particular influence of mutation N11A also on reactivity of polyclonal human serum IgE to the C-terminal immunodominant domain of Phl p1.

The three mutations (K8A, N11A and D55A) identified above as important for the binding of human monoclonal IgE to Phl p 1 are closely located on the surface of the C-terminal fragment (Fig. 6), constituting a potential human IgE-binding hot spot. Based on this novel knowledge of important residues for IgE-binding on Phl p 1 yet another potential hypoallergenic variant was created, based on the C-terminal domain carrying the three mutations K8A, N11A and D55A. The new combined mutant failed to fully inhibit the allergen-binding potential of any of the five Phl p 1-specific human antibodies included in this study (Fig. 7). Importantly, just as Phl p 1-C-N11A, it showed a significantly ($p < 0.0001$) reduced reactivity with human serum IgE (Fig. 8), an indication that a majority of IgE present in these sera targets epitopes affected by the introduced mutations. However, the combined mutant did bind the mouse monoclonal, 1.8, further emphasizing the difference in epitopes targeted by this murine antibody and the five human IgE.

As antibodies cross-react between different isoforms of Phl p 1, we also investigated the effect of introducing the mutations shown to be important for binding to Phl p 1.0102, into the sequence of Phl p 1.0101. This recombinant C-terminal protein domain in fusion to GST, also demonstrated a substantially reduced level of binding by human recombinant scFv-CHe2-4 as compared to the wildtype protein sequence (Fig. 17). These mutations can thus be used also to render the C-terminal domain of Phl p 1.0101 hypoallergenic.

Mutated variants of Phl p 1 C-terminal domain do not efficiently induce degranulation of basophils

The basophil activation test was performed on cells from grass pollen allergic donors in order to investigate the capacity of two mutated recombinant versions (N11A and K8A, N11A, D55A) of the the C-terminal domain of the grass pollen allergen Phl p 1, to induce degranulation, as compared to wild-type allergens. The expression levels of the degranulation marker CD63, as well as the percentage of CD63-positive cells, were assessed after allergen stimulation of basophils, defined as CD123+/HLA-DR- cells in the PBMC population (Fig. 18). Basophils were stimulated with four different concentrations of wild-type proteins Phl p 1 and GST fusion proteins of the C-terminal fragment of Phl p 1 and mutated (N11A and K8A, N11A, D55A, respectively) variants thereof. The stimulation index, based on changes in MFI of CD63 expression and percent CD63+ cells in comparison to the relevant controls, was evaluated. This analysis demonstrated that a higher concentration of the mutated variants of the C-terminal domain of Phl p 1 was required to achieve degranulation of basophils, highlighting their hypoallergenic nature (Fig. 19).

Production and characterization of GST-free hypoallergen

For certain applications it is expected that a product lacking a large protein tag, such as GST, is preferred. To accomplish production of a largely tag-free product (exemplified by SEQ ID NO:67) the fusion protein was initially produced in E. coli TUNER(DE3). The cells were homogenized and the fusion protein was purified on a GSTrap FF column. The product (>85% pure) displayed a molecular weight close to the theoretically expected (37.3 kDa) (Fig. 20A). After removal of the GST-tag with PreScission Protease and size exclusion chromatography a product with an apparent molecular weight (as determined by SDS-PAGE) only slightly higher than the expected (10.9 kDa) was obtained (Fig. 20B). A mass spectrometry-based peptide mass fingerprint assay

comparing observed peptide masses of two batches of produced protein to a theoretical peptide list of the GST-free hypoallergen indicated sequence coverage of 94% (Fig. 20C; Table 4) confirming isolation of the intended protein (SEQ ID NO:67).

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Table 4

Mass spectrometry-based identification of peptides from two batches of GST-free hypoallergen (carrying mutations K8A, N11A, D55A)¹ after trypsin cleavage.

Batch	Mass input / found	dev.	mc ²	Residues ³
1	890.688 / 889.502	-0.0201	0	65-72
	1004.676 / 1003.488	-0.0181	0	51-58
	1516.141 / 1514.846	-0.0190	1	59-72
	1674.207 / 1672.873	-0.0195	1	46-58
	1721.210 / 1719.868	-0.0194	1	26-41
	1917.365 / 1915.994	-0.0190	2	44-58
	2029.538 / 2028.104	-0.0210	0	7-25
	2102.604 / 2101.111	-0.0231	3	42-58
	2359.785 / 2358.248	-0.0224	4	40-58
	3093.204 / 3091.415	-0.0253	1	73-100
	3804.853 / 3802.968	-0.0231	5	26-58
	890.515 / 889.502	-0.0006	0	65-72
	1004.500 / 1003.488	-0.0005	0	51-58
	1515.882 / 1514.846	-0.0019	1	59-72
2	1673.918 / 1672.873	-0.0023	1	46-58
	1720.918 / 1719.868	-0.0025	1	26-41
	1917.041 / 1915.994	-0.0020	2	44-58
	2029.188 / 2028.104	-0.0037	0	7-25
	2102.228 / 2101.111	-0.0052	3	42-58
	2359.388 / 2358.248	-0.0056	4	40-58
	3092.636 / 3091.415	-0.0069	1	73-100

¹ The final product is 100 residues long as it incorporates 5 N-terminal residues between the site cut by PreScission protease and the hypoallergen sequence itself (SEQ ID NO:67).

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² Missed cleavage

³ Numbers refer to SEQ ID NO:67.

Human monoclonal scFv specific for Phl p 5 and recognition of the C-terminal domain of Phl p 5.

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Recombinant proteins representing GST fused to the C-terminal part of Phl p 5.0101, and mutated versions thereof, were efficiently produced in E. coli (Fig. 21). The mutated versions of the protein all introduce amino acid modifications of residues exposed on the surface of the C-terminal domain of Phl p 5 (Fig. 22). Human monoclonal scFv 4.4, 4.12, 4.13, p5-MA5 and p5-AB5 fused to CH₂-3 of IgG2 all bound recombinant C-terminal

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fragment of Phl p 5.0101 (Fig. 23). Similar CH₂-3 fusion proteins carrying scFv clone 5 or 4.3 did not bind the C-terminal allergen fragment (Fig. 23) in agreement with their proven recognition of a more N-terminal part of Phl p 5 (Andréasson et al., 2006). ScFv 4.4 and 4.13 fused to CH₂-3 of IgG2 bound all three mutated variants of the C-terminal
5 fragment of Phl p 5.0101. In contrast, scFv 4.12, p5-MA5 and p5-AB5 fused to CH₂-3 of IgG2 recognized two mutated variants (K270A and K280A) of the C-terminal fragment of Phl p 5.0101 but not the K246A variant. These results demonstrate that we, by use of human scFv derived from the IgE repertoire, have defined a residue, K246A, that is important for recognition of Phl p 5 by at least a subset of human IgE. The methodology
10 outlined in Fig.1 is thus applicable to several different allergenic proteins.

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CLAIMS

1. A hypoallergenic polypeptide comprising or consisting of a variant of the amino acid sequence of SEQ ID NO: 1

5

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESWGAIWR
IDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

SEQ ID NO:1

- 10 wherein one or more amino acids of SEQ ID NO: 1 within an IgE-binding epitope is mutated

and wherein the polypeptide exhibits reduced IgE-reactivity compared to a polypeptide consisting of the amino acid sequence of SEQ ID NO: 1.

15

2. A polypeptide according to Claim 1 wherein the IgE-binding epitope comprises or consists of one or more of the following amino acids of SEQ ID NO: 1:

N11, K8, K36, K38, K45, D55, K59, E77, E79, E84, K87 and E93.

20

3. A polypeptide according to Claim 1 or 2 comprising a mutation at amino acid N11 of SEQ ID NO: 1.

4. A polypeptide according to any one of the preceding claims comprising a mutation at amino acid K8 of SEQ ID NO: 1.

25

5. A polypeptide according to any one of the preceding claims comprising a mutation at amino acid D55 of SEQ ID NO: 1.

- 30 6. A polypeptide according to any one of the preceding claims comprising a mutation at amino acid K8, N11 and D55 of SEQ ID NO: 1.

7. A polypeptide according to any one of Claims 3 to 6 wherein the mutation is a substitution.

35

8. A polypeptide according to any one of the preceding claims comprising mutations at amino acid positions K8 and/or N11 and/or D55.

5 9. A polypeptide according to any one of the preceding claims comprising one or more of the following mutations:

K8A, N11A and/or D55A.

10 10. A polypeptide according to any one of the preceding claims wherein IgE reactivity is reduced by at least 10%, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and preferably by 100%.

11 11. A polypeptide according to any one of the preceding claims wherein the IgE is human IgE.

12 12. A polypeptide according to any one of the preceding claims wherein the polypeptide is fewer than 500 amino acids in length, for example fewer than 400, 300, 200, 150, 140, 130, 120, 105, 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 85, 80, 70, 60, 50, 40, 30 or fewer amino acids in length.

20 13. A polypeptide according to any one of the preceding claims wherein the polypeptide is between 50 and 150 amino acids in length, for example between 70 and 120, between 80 and 110, or between 90 and 100 amino acids in length.

25 14. A polypeptide according to any one of the preceding claims wherein the polypeptide is 95 amino acids in length.

30 15. A polypeptide according to any one of the preceding claims wherein the polypeptide shares at least 50% amino acid sequence identity with SEQ ID NO:1, for example at least 60%, 70%, 80, 90%, 95%, or 99% amino acid sequence identity.

16. A polypeptide according to any one of the preceding claims wherein the polypeptide comprises or consists of one of the following amino acid sequences:

KVTFHVEAGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

5

SEQ ID NO: 2;

KVTFHVEKGSAPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESWG
AIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

10

SEQ ID NO: 3;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEAGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

15

SEQ ID NO: 4;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGADKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

20

SEQ ID NO: 5;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELAESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

SEQ ID NO: 6;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW
GAIWRIATPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

25

SEQ ID NO: 7;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW
GAIWRIDTPDALTPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

30

SEQ ID NO: 8;

KVTFHVEKGSNPNYLALLVKYVNGDGDVVAVDIKEKGKDKWIELKESW
GAIWRIDTPDKLTGPFTVRYTTEGGTKTAAEDVIPEGWKADTSYESK

35

SEQ ID NO: 9;

KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
 GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAADVIPEGWKADTSYESK
SEQ ID NO: 10;

5 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
 GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPAGWKADTSYESK
SEQ ID NO: 11;

10 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
 GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWAADTSYESK
SEQ ID NO: 12;

15 KVTFHVEKGSNPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESW
 GAIWRIDTPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYASK
SEQ ID NO: 13; and

20 KVTFHVEAGSAPNYLALLVKYVNGDGDVAVDIKEKGKDKWIELKESWG
 AIWRIATPDKLTGPFTVRYTTEGGTKTEAEDVIPEGWKADTSYESK
SEQ ID NO: 14.

17. A polypeptide according to any one of the preceding claims comprising or consisting of a variant of the amino acid sequence of SEQ ID NO: 15

25 KVTFHVEKGSNPNYLALLVKFVAGDGDVAVDIKEKGKDKWIALKESWG
 AIWRIDTPEVLKGPFTVRYTTEGGTKGEAKDVIPEGWKADTAYESK
SEQ ID NO: 15

18. A polypeptide according to Claim 17 comprising one or more of the following mutations:

30 K8A, N11A and/or D55A.

19. A polypeptide according to any one of the preceding claims wherein one or more amino acids is modified or derivatised.

35 20. A polypeptide according to Claim 19 wherein the polypeptide is PEGylated.

21. A polypeptide according to Claim 19 or 20 wherein the polypeptide is glycosylated.

5 22. A polypeptide according to any one of the preceding claims wherein the polypeptide is a fusion polypeptide comprising a second polypeptide region.

23. A polypeptide according to Claim 22 wherein the second polypeptide region enhances immunogenicity and/or aids purification.

10

24. A polypeptide according to any one of the preceding claims wherein the polypeptide comprises or consists of tandem repeats.

15

25. A polypeptide according to any one of the preceding claims wherein the polypeptide consists of an amino acid sequence selected from the group consisting of SEQ ID NOS: 2 to 14.

20

26. A nucleic acid molecule encoding a polypeptide according to any one of Claims 1 to 25.

27. A nucleic acid molecule according to Claim 26 wherein the nucleic acid molecule is a DNA molecule.

25

28. A nucleic acid molecule according to Claim 26 or 27 wherein the nucleic acid molecule is codon optimised.

29. A nucleic acid molecule according to any one of Claims 26 to 28 comprising or consisting of one of the following nucleotide sequences:

30

AAAGTGACCTTCCATGTTGAAGCAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAAACCGAAGCAGAAGATGTTATTCCGGAAG
35 GTTGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 16;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCGCACCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 17;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGGCAGGCCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 18;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCCGCGACACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 19;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCCAAAGACAAATGGATTGAACTGGCAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 20;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGCAACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 21;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATGCACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 22;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGCAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 23;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGCAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 24;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGCAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 25;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGGCAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 26;

AAAGTGACCTTCCATGTTGAAAAAGGCAGCAATCCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGATACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGCAAGCAAATAA

SEQ ID NO: 27; and

AAAGTGACCTTCCATGTTGAAGCAGGCAGCGCACCGAATTATCTGGCACTG
CTGGTGAAATATGTGAATGGTGATGGTGATGTTGTGGCCGTTGATATTTAA
GAGAAAGGCAAAGACAAATGGATTGAACTGAAAGAAAGCTGGGGTGCAAT
TTGGCGTATTGCAACACCGGATAAACTGACAGGTCCGTTTACCGTTCGTTA
TACCACCGAAGGTGGCACCAAACCGAAGCAGAAGATGTTATTCCGGAAG
GTTGGAAAGCAGATACCAGCTATGAAAGCAAATAA

SEQ ID NO: 28.

30. A vector comprising a nucleic acid molecule according to any one of Claims 26 to 29.
31. A vector according to Claim 30 wherein the vector is an expression vector.
- 5 32. A host cell comprising a nucleic acid molecule according to any one of Claims 26 to 29 or a vector according to Claim 30 or 31.
- 10 33. A method for producing a polypeptide according to any one of Claims 1 to 25 comprising culturing a population of host cells comprising a nucleic acid molecule according to any one of Claims 26 to 29 or a vector according to Claim 31 under conditions in which the polypeptide is expressed, and isolating the polypeptide therefrom.
- 15 34. A pharmacological composition comprising a polypeptide according to any one of Claims 1 to 25 and a pharmaceutically acceptable diluent, excipient or carrier.
35. A pharmacological composition according to Claim 34 wherein the composition comprises an adjuvant.
- 20 36. A pharmacological composition according to Claim 34 or 35 wherein the composition is a vaccine composition.
- 25 37. A pharmacological composition according to any one of Claims 34 to 36 further comprising one or more additional antigens.
38. A pharmacological composition according to Claim 37 wherein the one or more additional antigens are grass pollen allergens.
- 30 39. A polypeptide according to any one of Claims 1 to 25 for use in medicine.
40. A polypeptide according to Claim 39 for use as a vaccine.
- 35 41. A polypeptide according to Claim 39 or 40 for the prevention of grass pollen allergies.

42. Use of a polypeptide according to any one of Claims 1 to 25 in the preparation of a medicament for use as a vaccine.

5 43. The use according to Claim 42 wherein the medicament is for the prevention of grass pollen allergies.

44. A method for active immunisation of a subject comprising administering to the subject a polypeptide according to any one of Claims 1 to 25.

10 45. A method according to Claims 44 for the prevention of grass pollen allergies.

46. An antibody with specificity for major timothy group 1 pollen allergen, Phl p 1, or an antigen-binding fragment or derivative thereof, wherein the antibody, fragment or derivative competes for binding to Phl p 1 with one or more of the
15 following polypeptides:

EVQLVESGGGLGQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGI
SWNSGRIGYADSVKGRFTISRDNKNSLHLMNSLRAEDTALYYCARERLPG
NWNNDLWGRGTLTVSSGGGGSGGGGSGGGGSQSALTQPPSVSGAPGQR
20 VTISCTGSSSNFGAGYHVHWYQQFPGTAPKLLIQNNIRPSGVPDRFSASKSG
TSASLAITGLQPDDEADYYCQSYDSSVSGSVFGGGTKLTVL

SEQ ID NO:29

25 QVQLVQSGAEVKKSGASLKVSCKASGYTFTDYGISWVRQAPGQGLEWMGWI
NVNNGNTHYAQKLQGRVTMTTDTSTKTAYMELKSLRYDDTAVYYCAAGFHY
WGQGTLLTVSSGGGGSGGGGSGGGGSQSVLTQPPSVSVSPGQTARITCSA
DALANQYGYWYQQKPGQAPVLLIYKDNERPSGIPERFSGSSSGTTVTLTISGV
QAEDEADYYCQSSDRFGSRYVFGTGKLTVL

SEQ ID NO:30

30

47. An antibody, fragment or derivative according to Claim 46 comprising the following CDRs:

5	<i>Heavy chain:</i>	GFTFDDYA	SEQ ID NO:31
		ISWNSGRI	SEQ ID NO:32
		ARERLPGNWNLYDL	SEQ ID NO:33
10	<i>Light chain:</i>	SSNFGAGYH	SEQ ID NO:34
		NNN	SEQ ID NO:35
		QSYDSSVSGSV	SEQ ID NO:36

48. An antibody, fragment or derivative according to Claim 46 or 47 comprising or consisting of the amino acid sequence of SEQ ID NO: 29.

15

49. An antibody, fragment or derivative according to Claim 46 comprising the following CDRs:

20	<i>Heavy chain:</i>	GYTFTDYG	SEQ ID NO:37
		INVNNGNT	SEQ ID NO:38
		AAGFHY	SEQ ID NO:39
25	<i>Light chain:</i>	ALANQY	SEQ ID NO:40
		KDN	SEQ ID NO:41
		QSSDRFGSRYV	SEQ ID NO:42

50. An antibody, fragment or derivative according to Claim 46 or 49 comprising or consisting of the amino acid sequence of SEQ ID NO: 30.

30

51. An antibody, fragment or derivative according to any one of Claims 46 to 50 fused to an Fc region, or portion thereof.

52. An antibody, fragment or derivative according to Claim 51 wherein the Fc portion is from an IgG or IgA antibody.

35

53. A nucleic acid molecule encoding an antibody, fragment or derivative according to any one of Claims 46 to 52, or a component polypeptide chain thereof.

54. A nucleic acid molecule according to Claim 53 wherein the nucleic acid molecule is a DNA molecule.

55. A nucleic acid molecule according to Claim 53 or 54 wherein the nucleic acid molecule is codon optimised.

56. A nucleic acid molecule according to any one of Claims 53 to 55 comprising or consisting of the following nucleotide sequence:

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGGACAGCCTGGCAGGT
 CCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTGATGATTATGCCA
 TGCAGTGGGTCCGGCAAGCTCCAGGGAAGGGCCTGGAGTGGGTCTCAGG
 TATTAGTTGGAATAGTGGTCGCATAGGCTATGCGGACTCTGTGAAGGGCC
 GATTCACCATCTCCAGAGACAACGCCAAGAACTCCCTGCATCTGCAAATGA
 ACAGTCTACGAGCTGAGGACACGGCCTTATATTACTGCGCAAGAGAGAGG
 CTGCCTGGGAACTGGAACCTACGATCTCTGGGGCCGTGGCACCCCTGGTCAC
 CGTCTCCTCAGGTGGAGGCGGTTGAGGCGGAGGTGGATCCGGCGGTGGC
 GGATCGCAGTCTGCCCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAG
 GGCAGAGGGTCACCATCTCCTGCACTGGGAGCAGCTCCAACCTTCGGGGCA
 GGTTATCATGTACACTGGTACCAGCAATTTCCAGGAACAGCCCCCAAACCTC
 CTCATCCAGAATAACAACATTCGGCCCTCAGGGGTCCCTGACCGATTCTCT
 GCCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCACTGGGCTCCAGCC
 TGACGATGAGGCTGATTATTACTGCCAGTCGTATGACAGCAGCGTGAGTG
 GTTCGGTTTTTCGGCGGAGGCACCAAGCTGACCGTCCTC

SEQ ID NO: 43

57. A nucleic acid molecule according to any one of Claims 53 to 55 comprising or consisting of the following nucleotide sequence:

CAGGTACAGCTGGTGAATCTGGAGCTGAGGTGAAGAAGTCTGGGGCCTC
 ACTGAAGGTCTCCTGCAAGGCTTCTGGTTACACCTTTACCGACTATGGTAT
 CAGCTGGGTGCGACAGGCCCCCTGGACAAGGGCTTGAGTGGATGGGATGG
 ATCAACGTCAACAATGGTAATACACACTATGCACAGAAGCTCCAGGGCAGA

GTCACCATGACTACAGACACATCCACGAAAACAGCCTACATGGAAGTGAAG
 AGCCTGAGATATGACGACACGGCCGTGTATTACTGTGCGGCCGGCTTTCA
 CTATTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGTGGAGGCGGTT
 CAGGCGGAGGTGGATCCGGCGGTGGCGGATCGCAGTCTGTGCTGACTCA
 5 GCCACCCTCGGTGTCAGTGTCCCCAGGACAGACGGCCAGGATCACCTGCT
 CTGCAGATGCATTGGCAAACCAATATGGTTATTGGTACCAGCAGAAGCCAG
 GCCAGGCCCTGTGTTACTGATATATAAAGATAATGAGAGGCCCTCAGGGA
 TCCCTGAGCGATTCTCTGGCTCCAGCTCAGGGACAACAGTCACGTTGACC
 ATCAGTGGAGTCCAGGCAGAAGACGAGGCTGACTACTACTGTCAATCATCA
 10 GACAGGTTTGGTAGTCGTTATGTCTTCGGCACAGGGACCAAGCTGACCGT
 CCTA

SEQ ID NO: 44

- 15 58. A vector comprising a nucleic acid molecule according to any of Claims 53 to 57.
59. A vector according to Claim 58 wherein the vector is an expression vector.
- 20 60. A host cell comprising a nucleic acid molecule according to any of Claims 53 to 57 or a vector according to Claim 58 or 59.
- 25 61. A method for producing an antibody, fragment or derivative according to any one of Claims 1 to 25 comprising culturing a population of host cells comprising a nucleic acid molecule according to any of Claims 53 to 57 or a vector according to Claim 59 under conditions in which the polypeptide is expressed, and isolating the polypeptide therefrom.
- 30 62. A pharmacological composition comprising an antibody, fragment or derivative according to any one of Claims 46 to 52 and a pharmaceutically acceptable diluent, excipient or carrier.
63. A pharmacological composition according to Claim 62 wherein the composition comprises an adjuvant.
- 35 64. A pharmacological composition according to Claim 62 or 63 wherein the composition is a vaccine composition.

65. An antibody, fragment or derivative according to any one of Claims 46 to 52 for use in medicine.
- 5 66. An antibody, fragment or derivative according to Claim 65 for use as a passive vaccine.
67. An antibody, fragment or derivative according to Claim 65 or 66 for the prevention of grass pollen allergies.
- 10 68. Use of an antibody, fragment or derivative according to any one of Claims 46 to 52 in the preparation of a medicament for use as a vaccine.
69. The use according to Claim 68 wherein the medicament is for the prevention of grass pollen allergies.
- 15 70. A method for passive immunisation of a subject comprising administering to the subject an antibody, fragment or derivative according to any one of Claims 46 to 52.
- 20 71. A method according to Claims 70 for the prevention of grass pollen allergies.
72. An antibody, fragment or derivative as described in claims 46 to 61 for use in standardization of timothy group 1 allergen content in a material (such as recombinant allergens, purified natural allergens, or complex extracts/mixtures of such allergens).
- 25 73. A process for the development of hypoallergenic polypeptide comprising the steps of:
- 30 (a) providing a lymphocyte-containing sample from an allergic individual;
- (b) isolating RNA from the lymphocyte-containing sample provided in step (a);
- 35 (c) generating cDNA from the RNA isolated in step (b);

- 5 (d) isolating from the cDNA generated in step (c) IgE heavy chain variable domain-encoding genes;
- (e) (optionally) isolating from the cDNA generated in step (c) antibody light chain variable domain-encoding genes;
- 10 (f) constructing one or more polynucleotide library comprising or consisting of the IgE heavy chain variable domain-encoding genes isolated in step (d) and (optionally) antibody light chain variable domain-encoding genes isolated in step (e);
- (g) selecting one or more polynucleotide encoding allergen-specific antibody or antigen-binding fragment thereof from the one or more polynucleotide library;
- 15 (h) (optionally) cloning the one or more polynucleotide selected in step (g) into another vector system;
- (i) expressing the one or more allergen-specific antibody or antigen-binding fragment thereof;
- 20 (j) (optionally) determining the binding specificity (and, optionally, the binding affinity) to the cognate allergen of the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i);
- (k) generating and expressing one or more variant of the cognate allergen;
- 25 (l) analysing the binding affinity of the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i) to the one or more variant of the cognate allergen generated in step (k); and
- 30 (m) selecting one or more variant of the cognate allergen that has a lower affinity for the one or more allergen-specific antibody or antigen-binding fragment thereof expressed in step (i) than the cognate allergen.

74. The process according to Claim 73 optionally comprises the further steps of:

- 35 (n) determining the ability of the one or more variant of the cognate allergen selected in step (m) to recognize polyclonal IgE in serum or plasma provided from a subject allergic to the cognate allergen;

- (o) determining the ability of the one or more variant of the cognate allergen selected in step (m) to activate basophils (carrying IgE) provided from a subject allergic to the cognate allergen; and
- (p) selecting one or more variant of the cognate allergen that has lower affinity for polyclonal IgE in serum or plasma provided from a subject allergic to the cognate allergen and/or lower ability to activate basophils (carrying IgE) provided from a subject allergic to the cognate allergen.

and/or the steps of:

- (q) determining the ability of the one or more variant of the cognate allergen to induce expression of cognate allergen-specific antibodies in a subject.
- (r) selecting one or more variant of the cognate allergen that is capable of inducing the expression of cognate allergen-specific antibodies in a subject.

75. The process according to Claim 73 or 74 wherein the lymphocyte-containing sample provided in step (s) is a blood sample, lymph node tissue, spleen tissue, bone marrow tissue, or mucosal tissue.

76. The process according to Claim 75 wherein the lymphocytes are B cells.

77. The process according to any one of Claims 73 to 76 wherein steps (d-e) are performed using polymerase chain reaction (PCR).

78. The process according to any one of Claims 73 to 77 wherein step (g) is performed using a method selected from the group consisting of phage display, yeast display, bacterial display, mRNA display and ribosome display.

79. The process according to any one of Claims 73 to 78 wherein in step (j) the binding specificity to the cognate allergen of more than one cognate allergen-specific antibody or antigen-binding fragment thereof selected in step (f) and expressed in step (i) is determined.

80. The process according to any one of Claims 73 to 78 wherein in step (k) cognate allergen variants are generated using rational design or random mutagenesis.
- 5 81. A polypeptide obtained or obtainable by the process defined in any one of Claims 73 to 80.
82. A nucleic acid molecule encoding a polypeptide defined in Claim 81.
- 10 83. A vector comprising a nucleic acid molecule defined in Claim 82.
84. A host cell containing a nucleic acid molecule defined in Claim 82 or the vector defined in Claim 83.
- 15 85. A pharmaceutical composition comprising a polypeptide defined in Claim 81, a nucleic acid defined in Claim 82, a vector defined in Claim 83 or a host cell defined in Claim 84 and a pharmaceutically acceptable diluent, excipient or carrier.
- 20 86. The pharmaceutical composition according to Claim 85 comprising an adjuvant.
87. A hypoallergenic polypeptide substantially as herein described with reference to the description and figures.
- 25 88. A nucleic acid molecule substantially as herein described with reference to the description and figures.
89. A pharmaceutical composition substantially as herein described with reference to the description and figures.
- 30 90. A method for active immunisation of a subject comprising administering to the subject a polypeptide according to Claim 81, a nucleic acid defined in Claim 82, a vector defined in Claim 83 or a pharmaceutical composition defined in Claim 86.
- 35

91. A method for immunisation of a subject substantially as herein described with reference to the description and figures.
- 5 92. An antibody, fragment or derivative substantially as herein described with reference to the description and figures.
93. A method for identifying a candidate antibody, or an antigen-binding fragment or derivative thereof, substantially as herein described with reference to the description and figures.
- 10 94. A hypoallergenic polypeptide comprising or consisting of a variant of the amino acid sequence of SEQ ID NO: 70 comprising a mutation of K246.
- 15 95. The hypoallergenic polypeptide according to Claim 93 wherein the mutation is a substitution or a deletion
96. The hypoallergenic polypeptide according to Claim 94 wherein the mutation is a substitution with Alanine (A).
- 20 97. The hypoallergenic polypeptide according to any one of Claims 93 to 95 wherein K246 is mutated.

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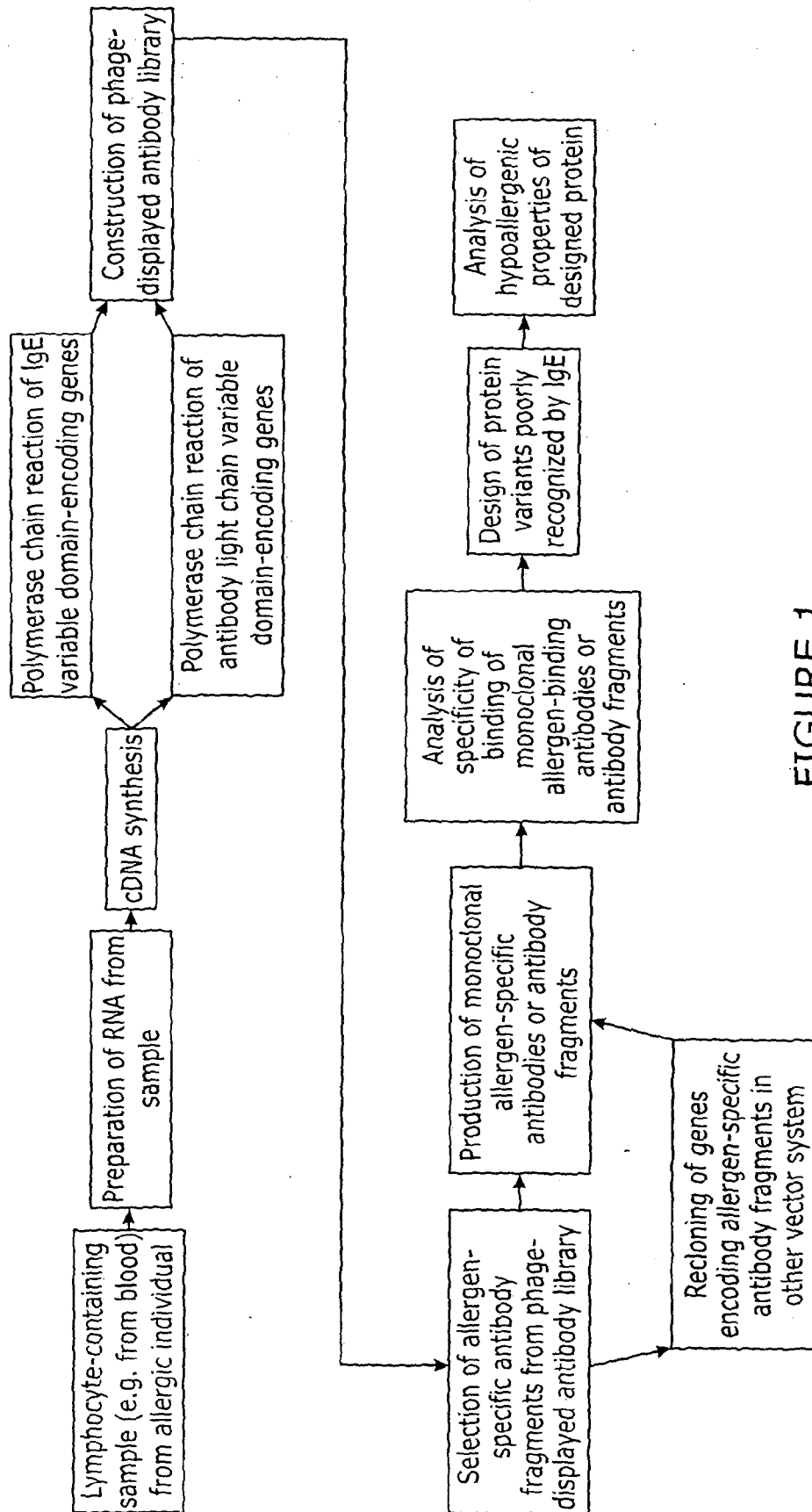


FIGURE 1

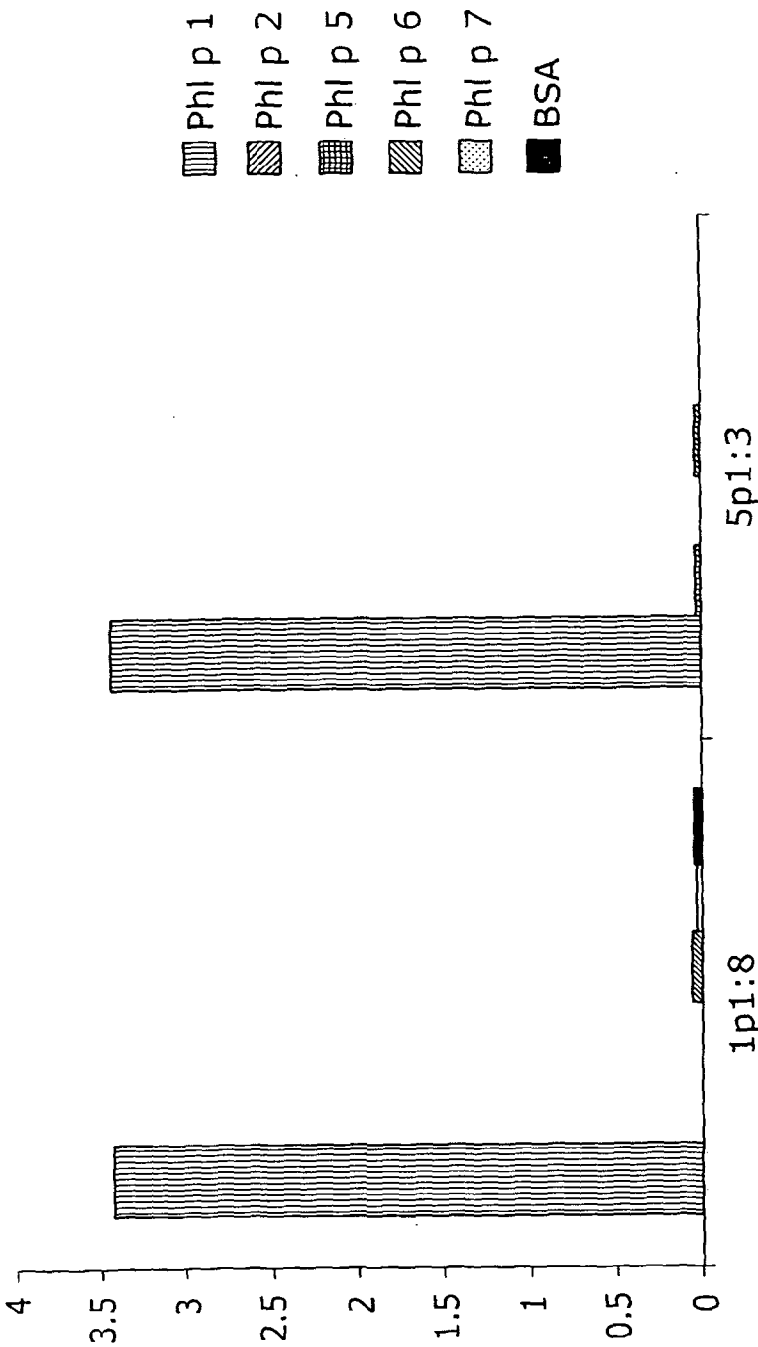


FIGURE 2

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MASSSSVLLV VVLFAVFLGS AYGIPKVPPG PNITATYGDK WLDKSTWYG 50
KPTGAGPKDN GGACGYKDVD KPPFSGMTGC GNTPIFKSGR GCGSCFEIKC 100
TKPEACSGEP VVVHITDDNE EPIAPYHFDL SGHAFGAMAK KGDEQKL RSA 150
GELELQFRRV KCKYPEGT 167
KVTFHVEKGS NPNYLALLVK YVNGDGDVVA VDIKEKGKDK WIELKESWGA 217
IWRIDTPDKL TGPFTVRYTT EGGTKTEAED VIPEGWKADT SYESK 263

SEQ ID NO:46

FIGURE 3

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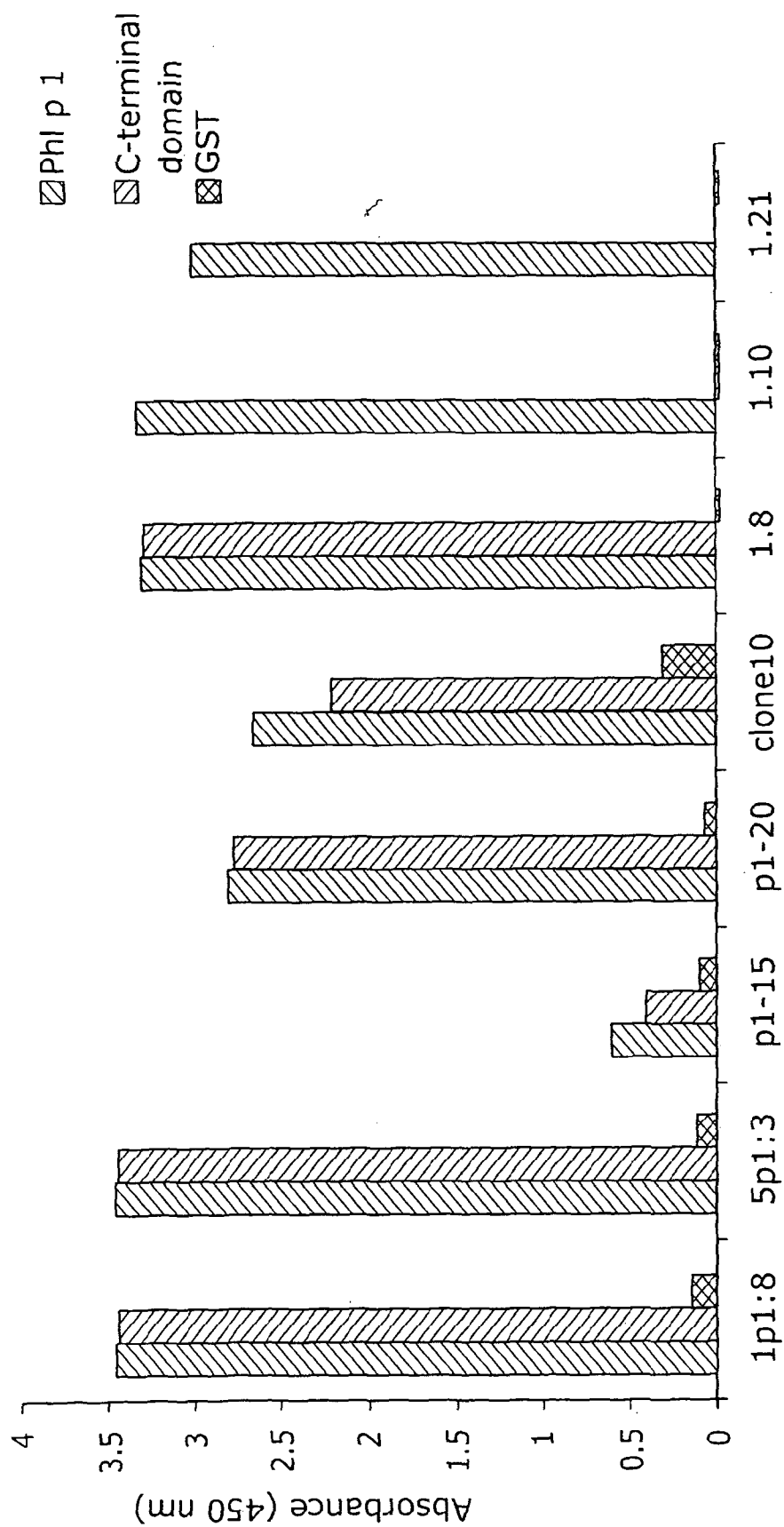
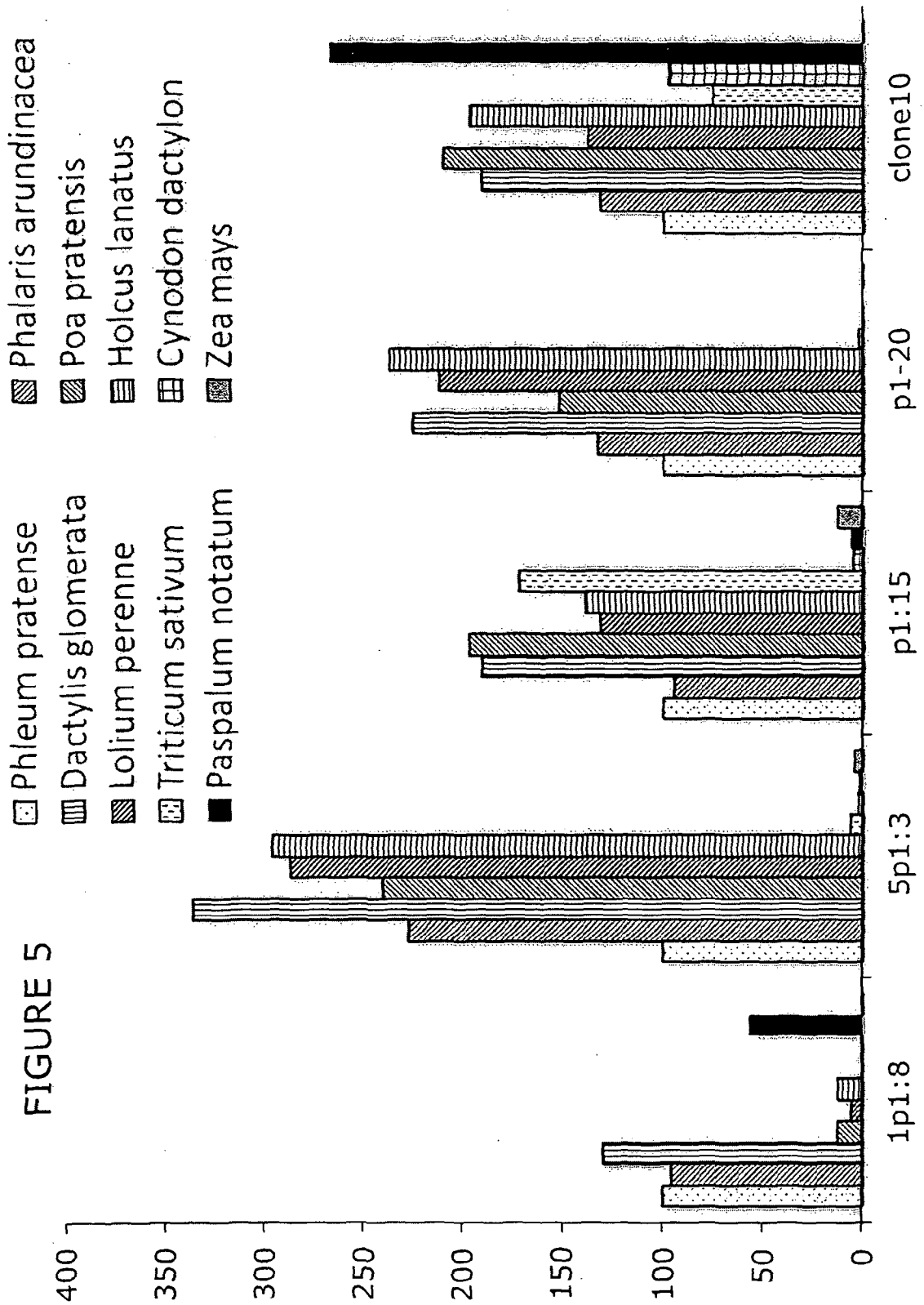


FIGURE 4

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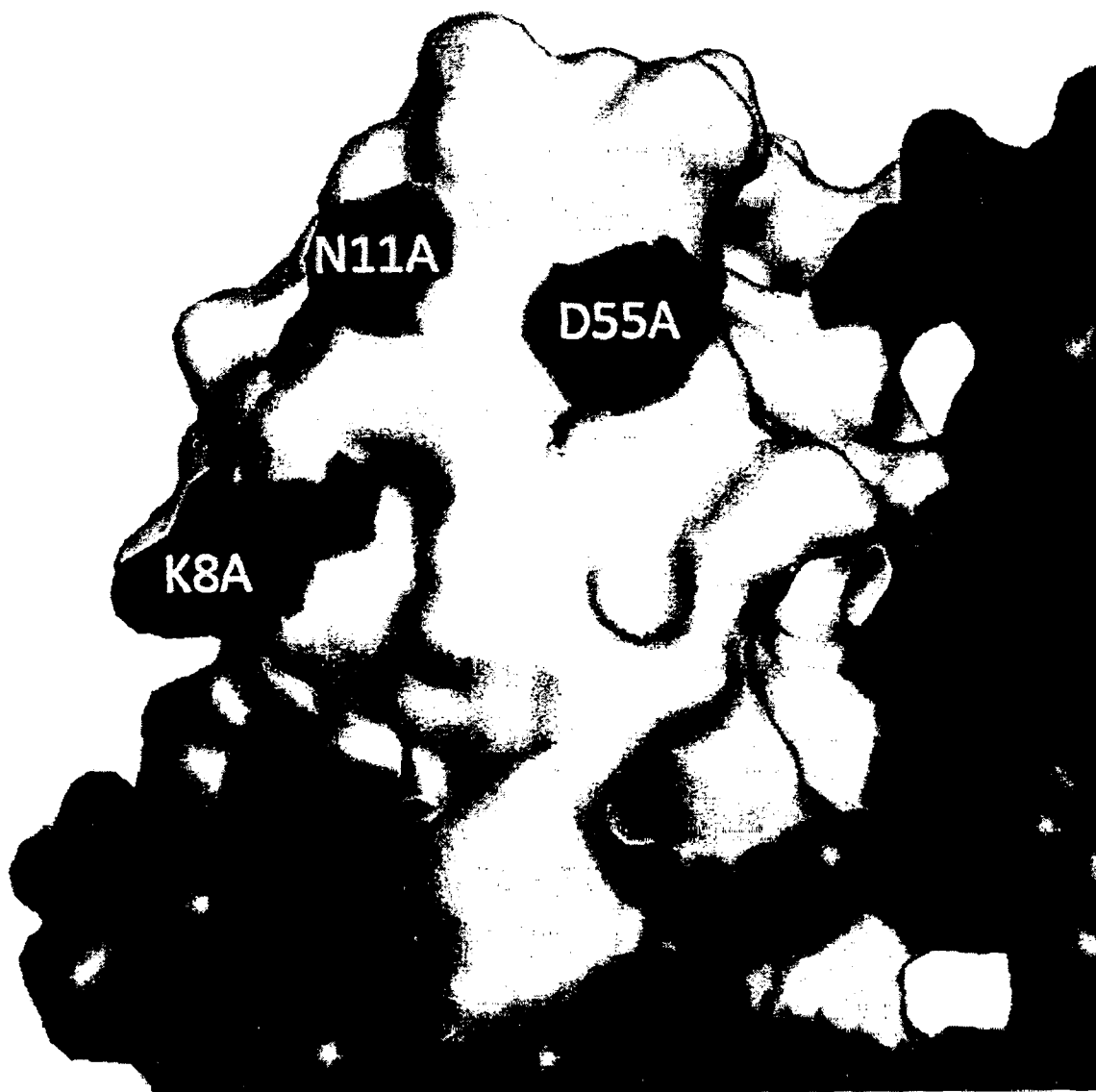


FIGURE 6

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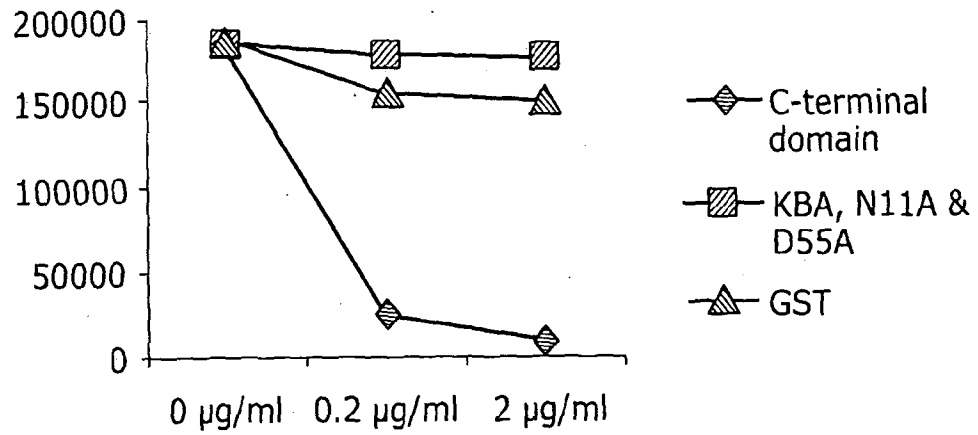


FIGURE 7A

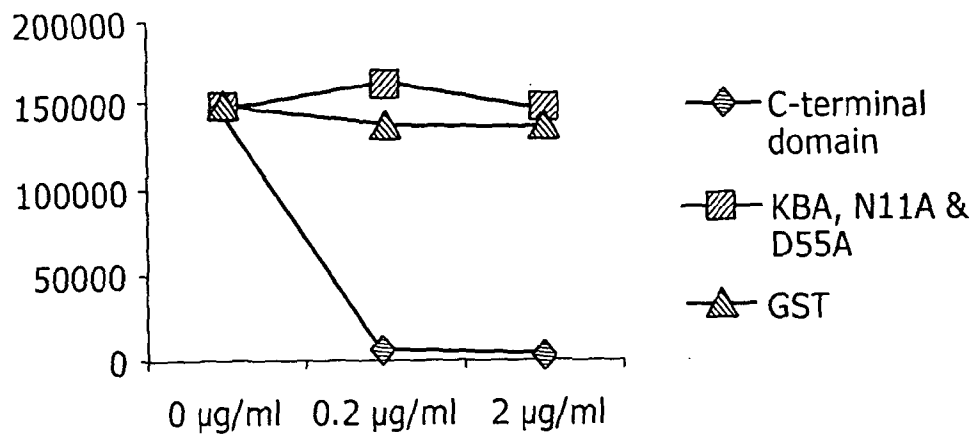


FIGURE 7B

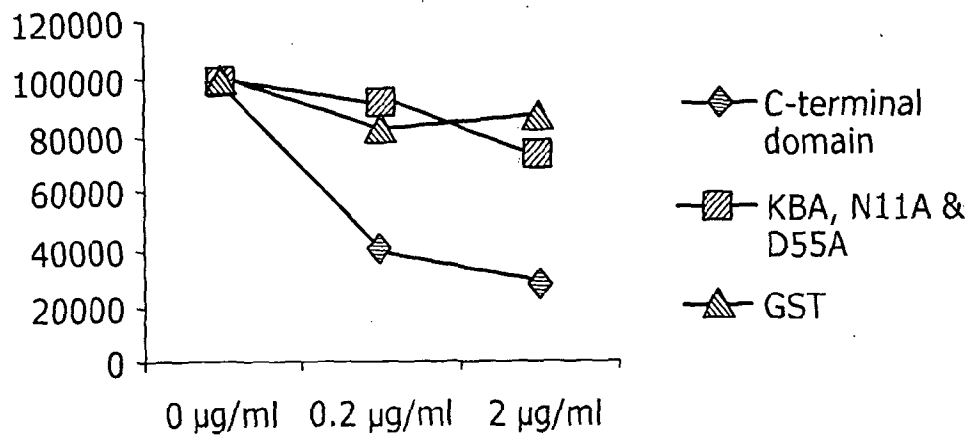


FIGURE 7C

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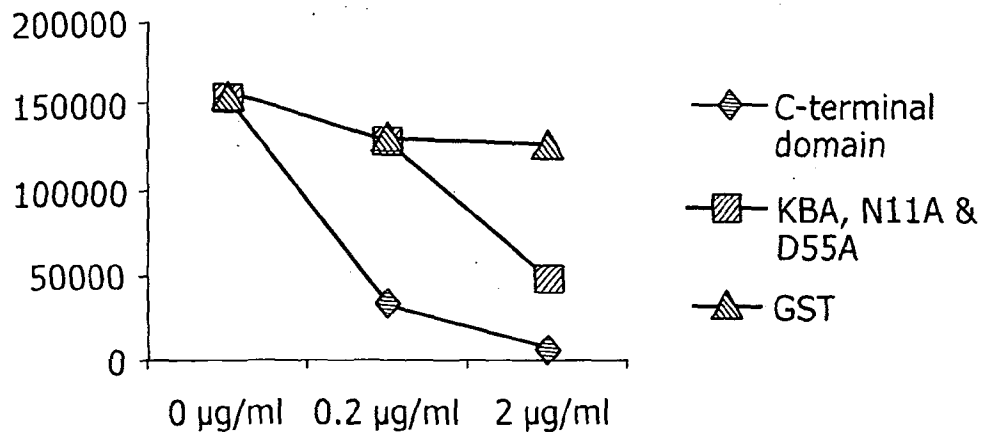


FIGURE 7D

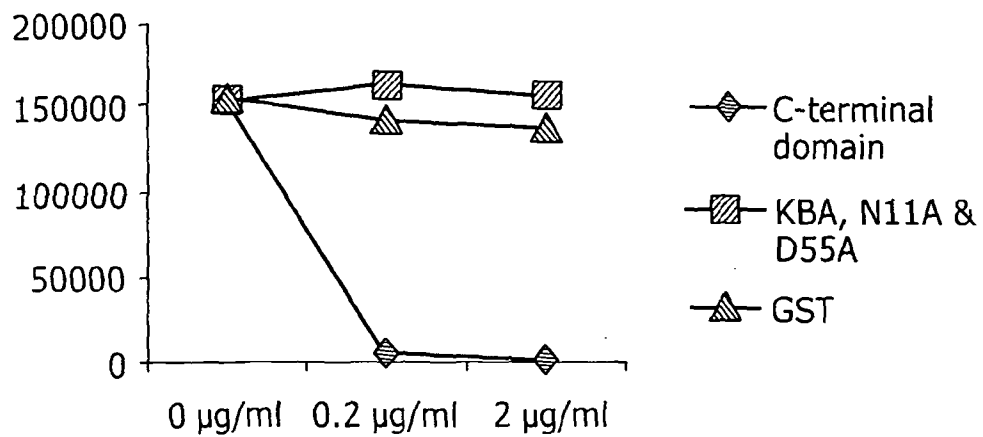


FIGURE 7E

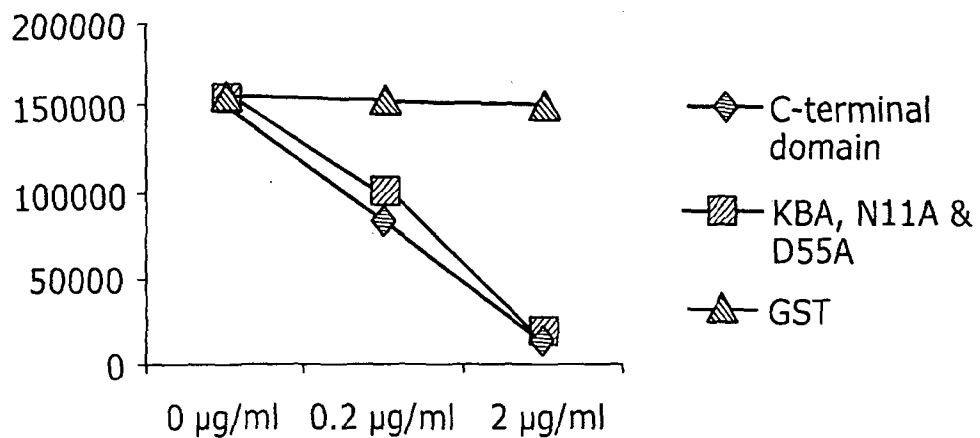


FIGURE 1F

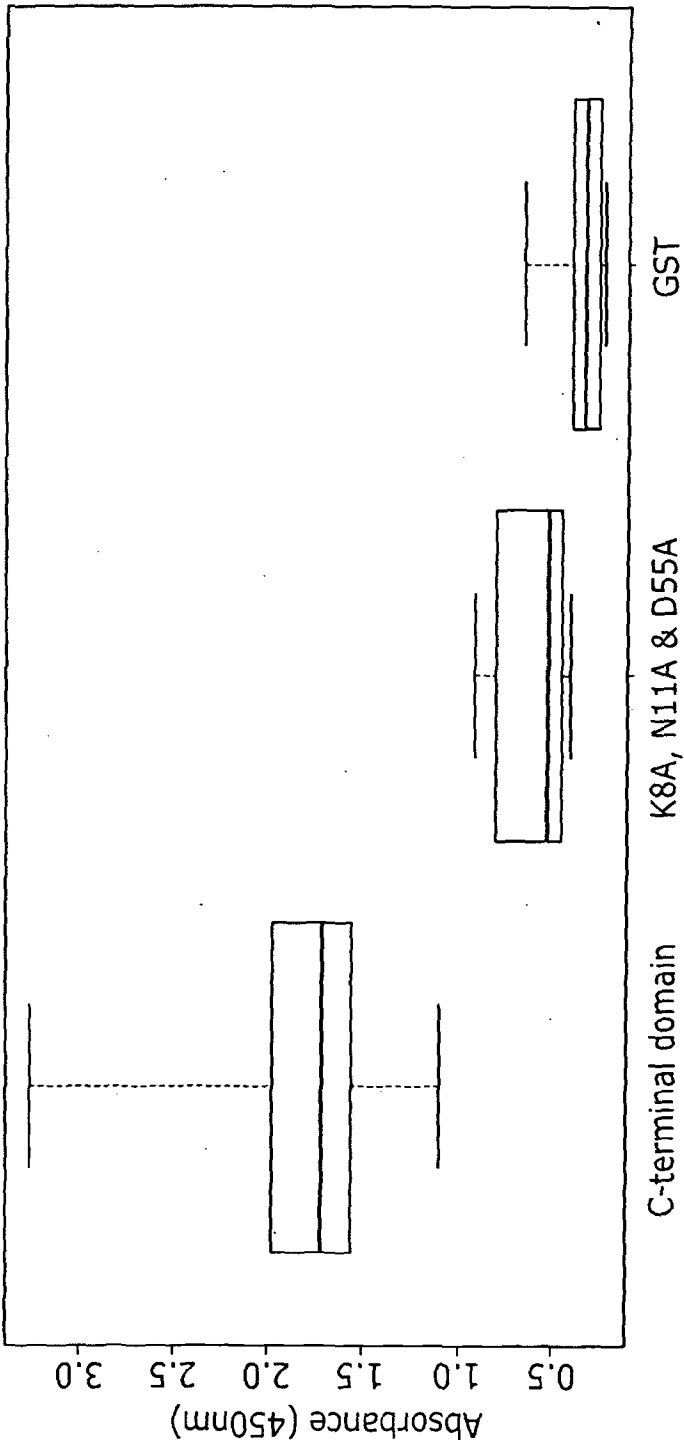


FIGURE 8

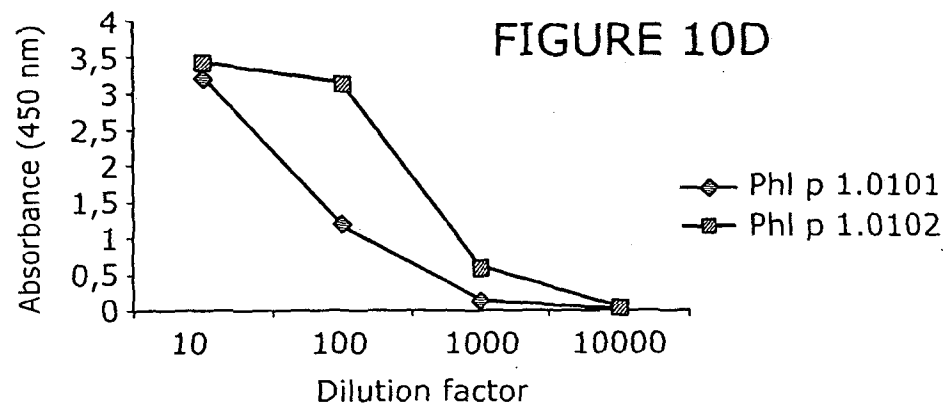
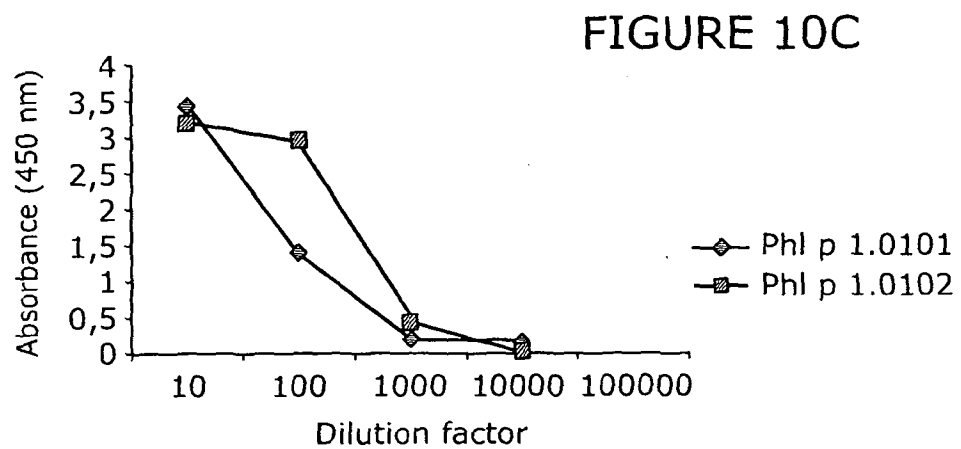
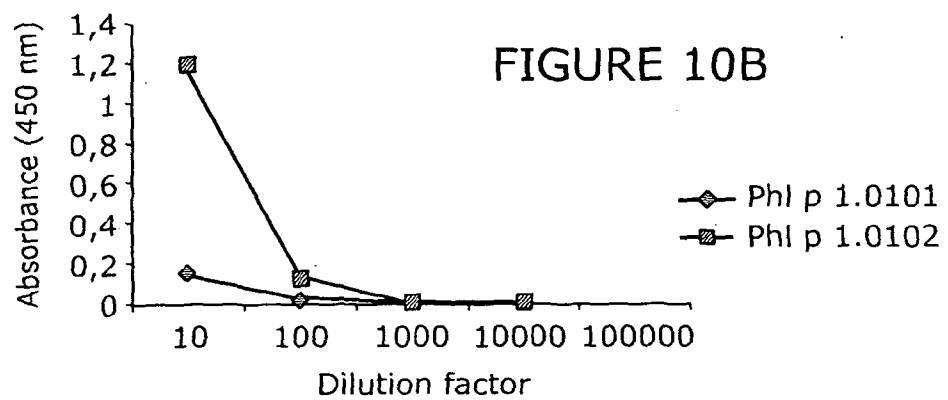
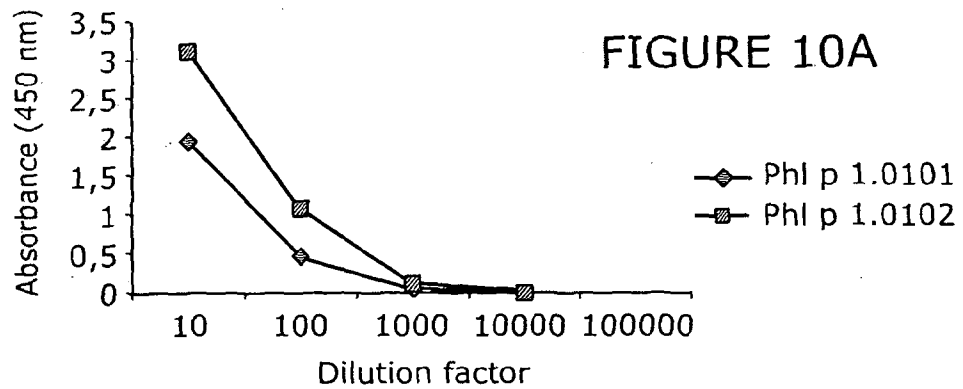
IGHV1-18*01	Q V Q L V Q S G A	E V K K P G A S V K V S C K A S G Y T F T S Y G	I S W V R Q A P G Q G L	50
p1-20 VH	E V Q L V Q S G T	E V K K P G A S V K V S C R A S G Y M F T D Y G	I S W V R Q A P G Q G L	40
5p1-3 VH	Q V Q L V Q S G A	E V K K S G A S L K V S C K A S G Y T F T D Y G	I S W V R Q A P G Q G L	30
				20
IGHV1-18*01	E W M G W I S A Y N G N T	N Y A Q K L Q	G R V T M T T D T S T	90
p1-20 VH	E W M G W I S A Y S G K T	H Y A Q K V Q	G R L T M T T N T S T	80
5p1-3 VH	E W M G W I N V N G N T	H Y A Q K L Q	G R V T M T T D T S T	70
				60
IGHV1-18*01	V Y Y C A R			
p1-20 VH	V Y Y C A R G S			
5p1-3 VH	V Y Y C A A G			
				100

FIGURE 9A

IGHV3-9*01 1p1-8 VH clone 10	10 E V Q L V E S G G E V Q L V E S G G - - - L E S G G	20 G L V Q P G R S L R L S C A A S G F T F D D Y A G L G Q P G R S L R L S C A A S G F T F D D Y A G W V Q P G R S L R L S C A A S G F T F D D H A	30 - - - - - - - - -	40 M H W V R Q A P G K G L M H W V R Q A P G K G L M H W V R Q A P G K G L	50 - - - - - - - - -
IGHV3-9*01 1p1-8 VH clone 10	60 E W V S G I S W N S G S I E W V S G I S W N S G R I E W V A G V S W N S G T R	70 G Y A D S V K G Y A D S V K A Y A D S V K	80 G R F T I S R D N A K N S L Y L Q M N S L R A E D T A G R F T I S R D N A K N S L H L Q M N S L R A E D T A G R F T I S R D N A K N S L Y L E M K S L R A E D T A	90 - - - - - - - - -	100 - - - - - - - - -
IGHV3-9*01 1p1-8 VH clone 10	L Y Y C A K D L Y Y C A R E I Y Y C A R D	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -

FIGURE 9B

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FIGURE 10E

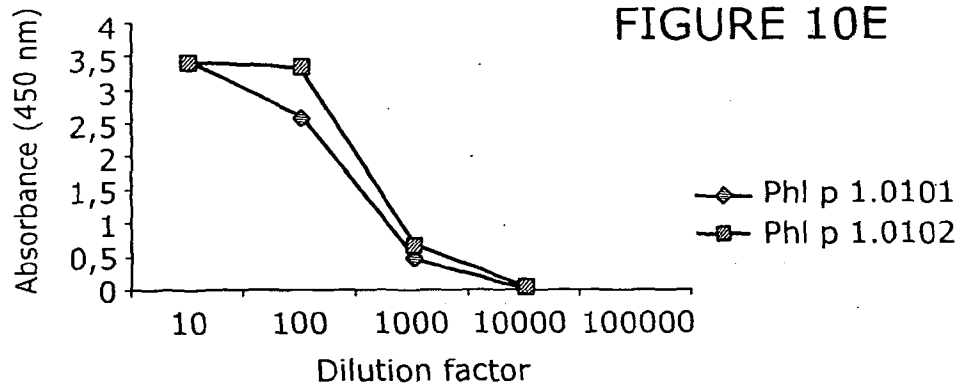


FIGURE 10F

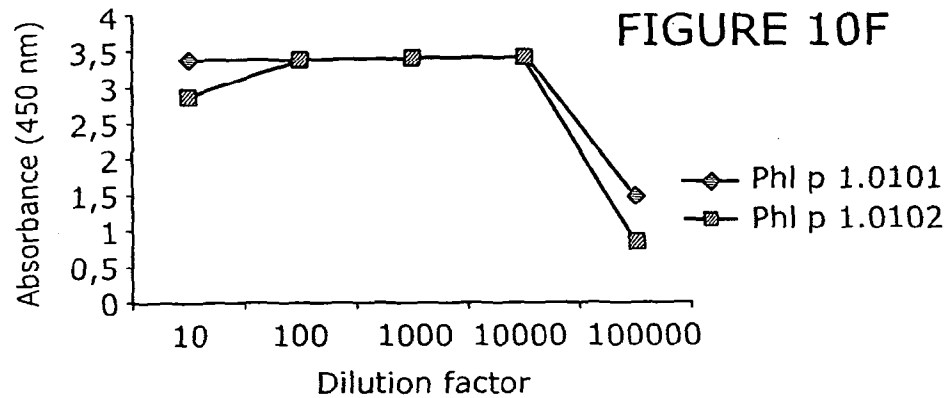


FIGURE 10G

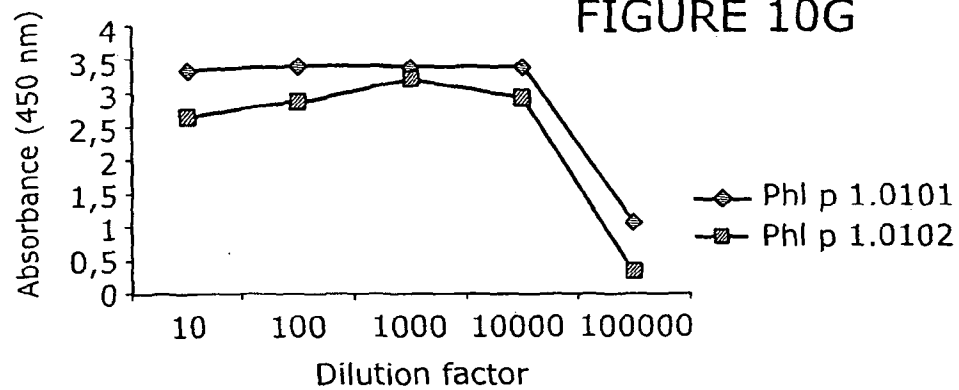
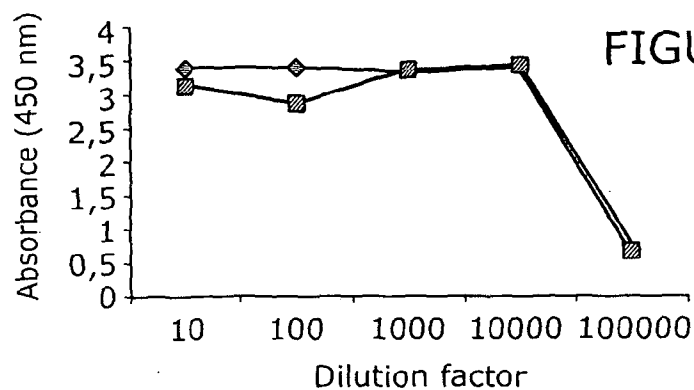


FIGURE 10H



20

40

60

SUBSTITUTE SHEET (RULE 26)

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	80	100
Phl p 1.0101 {Timothy grass}	V D K P P F S	G M T G C G N T P I F K S G R G C G
Phl p 1.0102 {Timothy grass}	V D K P P F S	G M T G C G N T P I F K S G R G C G
Pha a 1 {Canary grass}	V D K A P F N	G M T G C G N T P I F K D G R G C G
Dac g 1.01 {Cocksfoot}	V D K A P F N	G M T G C G N T P I F K D G R G C G
Poa p 1 {Kentucky Blue grass}	V D K A P F S	G M T G C G N T P I F K S G R G C G
Lol p 1 {Rye grass}	V D K A P F N	G M T G C G N T P I F K D G R G C G
Hol l 1 {Velvet grass}	V D K P P F S	G M T G C G N T P I F K D G R G C G
Tri a 1 {Wheat}	V N Q Y P I S	S M G A C G N E P I F A G G E G C G
Cyn d 1 {Bermuda grass}	V D K P P F D	G M T A C G N E P I F K D G L G C R
Pas n 1 {Bahia Grass}	V N L P P Y N	G F T A C G N P P I F K D G K G C G
Zea m 1 {Maize}	V N K P P F N	S M G A C G N I P I F K D G L G C G

	120
Phl p 1.0101 {Timothy grass}	S C F E I K C T - - K P E A C S G E P V V V H I T
Phl p 1.0102 {Timothy grass}	S C F E I K C T - - K P E A C S G E P V V V H I T
Pha a 1 {Canary grass}	S C F E L K C S - - K P E S C S G E P I T V H I T
Dac g 1.01 {Cocksfoot}	S C F E I K C T - - K P E S C S G E A V T V H I T
Poa p 1 {Kentucky Blue grass}	S C F E I K C T - - K P E S C S G E P V L V H I T
Lol p 1 {Rye grass}	S C F E I K C T - - K P E S C S G E A V T V T I T
Hol l 1 {Velvet grass}	S C F E I K C T - - K P E S C S G E P V T V H I T
Tri a 1 {Wheat}	M C Y E I K C E Y S N N P S C S G Q P R R I V I T
Cyn d 1 {Bermuda grass}	A C Y E I K C K - - E P V E C S G E P V L V K I T
Pas n 1 {Bahia Grass}	S C Y E I R C N - - K P E - C S G Q P V T V F I T
Zea m 1 {Maize}	S C F E I K C D - - K P V E C S G K P V V V H I T

	140
Phl p 1.0101 {Timothy grass}	D D N E E P I A A Y H F D L S G I A F G S M A K K
Phl p 1.0102 {Timothy grass}	D D N E E P I A P Y H F D L S G H A F G A M A K K
Pha a 1 {Canary grass}	D D N E E P I A P Y H F D L S G H A F G S M A K K
Dac g 1.01 {Cocksfoot}	D D N E E P I A P Y H F D L S G H A F G S M A K K
Poa p 1 {Kentucky Blue grass}	D D N E E P I A A Y H F D L S G K A F G A M A K K
Lol p 1 {Rye grass}	D D N E E P I A P Y H F D L S G H A F G S M A K K
Hol l 1 {Velvet grass}	D D N E E P I A P Y H F D L S G H A F G S M A K K
Tri a 1 {Wheat}	D M N Y Y P V A R Y H L D L S G T A F G S M A K Y
Cyn d 1 {Bermuda grass}	D K N Y E H I A A Y H F D L S G K A F G A M A K K
Pas n 1 {Bahia Grass}	D M N Y E P I A P Y H F D L S G K A F G A M A K P
Zea m 1 {Maize}	D M N Y E P I A A Y H F D L A G T A F G A M A K K

FIGURE 11
(Continued)

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	160
Phl p 1.0101 {Timothy grass}	G D E Q K L R S A G E V E I Q F R R V K C K Y P E
Phl p 1.0102 {Timothy grass}	G D E Q K L R S A G E L E L Q F R R V K C K Y P E
Pha a 1 {Canary grass}	G E E E N V R G A G E L E L Q F R R V K C K Y P D
Dac g 1.01 {Cocksfoot}	G E E Q K L R S A G E L E L Q F R R V K C K Y P E
Poa p 1 {Kentucky Blue grass}	G E E Q K L R S A G E L E L K F R R V K C E Y P E
Lol p 1 {Rye grass}	G E E Q N V R S A G E L E L Q F R R V K C K Y P D
Hol l 1 {Velvet grass}	G E E Q K L R S A G E L E L K F R R V K C K Y P D
Tri a 1 {Wheat}	G L N D R L R H A G I I D M Q F R R V P C N P P .
Cyn d 1 {Bermuda grass}	G Q E D K L R K A G E L T L Q F R R V K C K Y P S
Pas n 1 {Bahia Grass}	G L N D K L R H Y G I F D L E F R R V R C K Y Q G
Zea m 1 {Maize}	G E E E K L R K A G I I D M Q F R R V K C K F . .

	180	200
Phl p 1.0101 {Timothy grass}	G T K V T F H V E K G S N P N Y L A L L V K F V A	
Phl p 1.0102 {Timothy grass}	G T K V T F H V E K G S N P N Y L A L L V K Y V N	
Pha a 1 {Canary grass}	G T K P T F H V E K G S N P N Y L A L L V K Y V D	
Dac g 1.01 {Cocksfoot}	G T K L T F H V E K G S N P N Y L A L L V K Y V D	
Poa p 1 {Kentucky Blue grass}	G T K V T F H V E K G S N P N Y L A L L V K Y V T	
Lol p 1 {Rye grass}	D T K P T F H V E K A S N P N Y L A I L V K Y V D	
Hol l 1 {Velvet grass}	G T K P T F H V E K G S N P N Y L A L L V K Y I D	
Tri a 1 {Wheat}	G M K V T F H V Q R G S N P N Y L A V L V E Y V N	
Cyn d 1 {Bermuda grass}	G T K I T F H I E K G S N D H Y L A L L V K Y A A	
Pas n 1 {Bahia Grass}	G Q K I V F H V E K G S N P N Y L A M L V K F V A	
Zea m 1 {Maize}	G S K V S F H L E K G C G P N Y L A L L V K Y V D	

	220
Phl p 1.0101 {Timothy grass}	G D G D V V A V D I K E K G K D K W I A L
Phl p 1.0102 {Timothy grass}	G D G D V V A V D I K E K G K D K W I E L
Pha a 1 {Canary grass}	G D G D V V A V D I K E K G K D K W I E L
Dac g 1.01 {Cocksfoot}	G D G D V V A V D I K E K G K D K W I A L
Poa p 1 {Kentucky Blue grass}	G D G D V V A V D I K E K G K D K W I E L
Lol p 1 {Rye grass}	G D G D V V A V D I K E K G K D K W I E L
Hol l 1 {Velvet grass}	G D G D V V A V D I K E K G K D K W I E L
Tri a 1 {Wheat}	V D G T V V R M E L M Q T M N G R P T G Y W Q D M
Cyn d 1 {Bermuda grass}	G D G N I V A V D I K P R D S D E F I P M
Pas n 1 {Bahia Grass}	D D G D I V L M E L K E K S S D . W K P M
Zea m 1 {Maize}	G D G D I V P V D I K E K G S D T Y E P L

FIGURE 11
(Continued)

240

260

280

FIGURE 11
(Continued)

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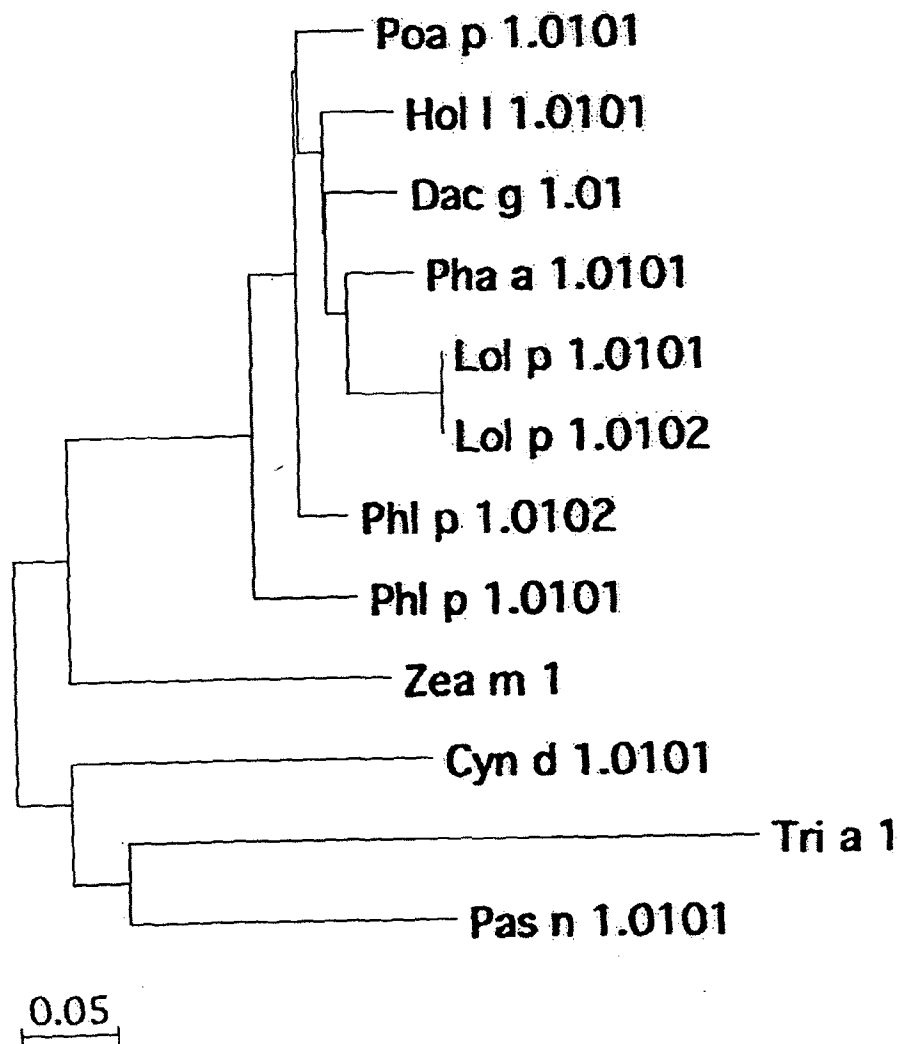


FIGURE 12

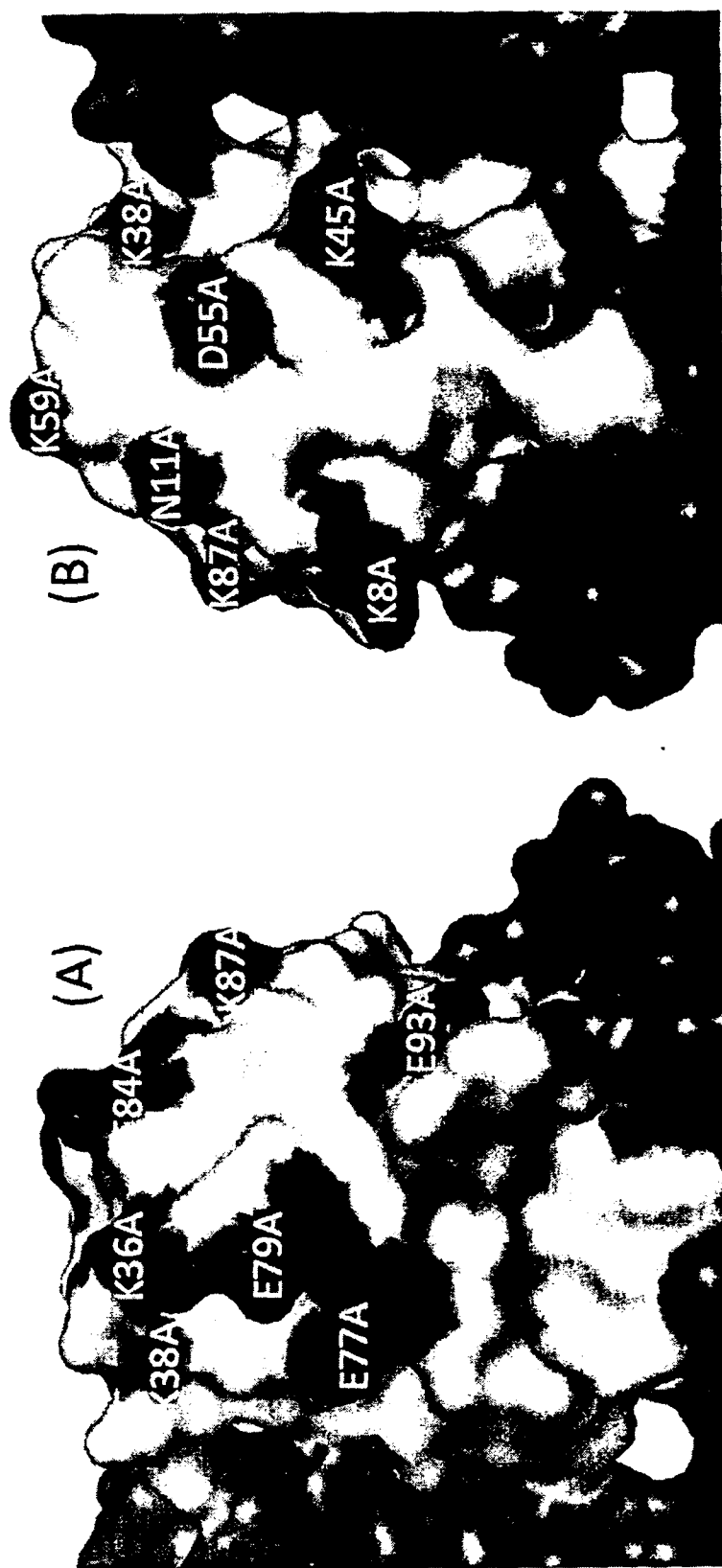


FIGURE 13

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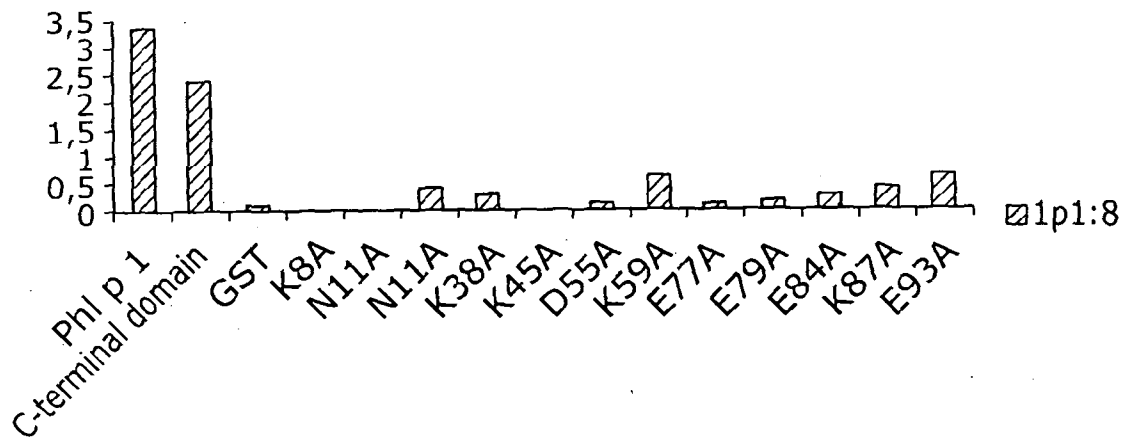


FIGURE 14 A

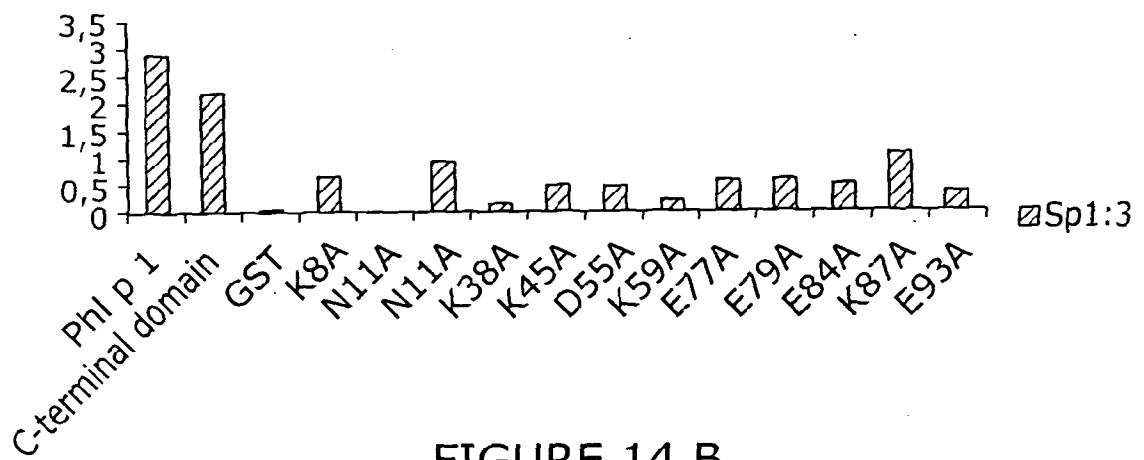


FIGURE 14 B

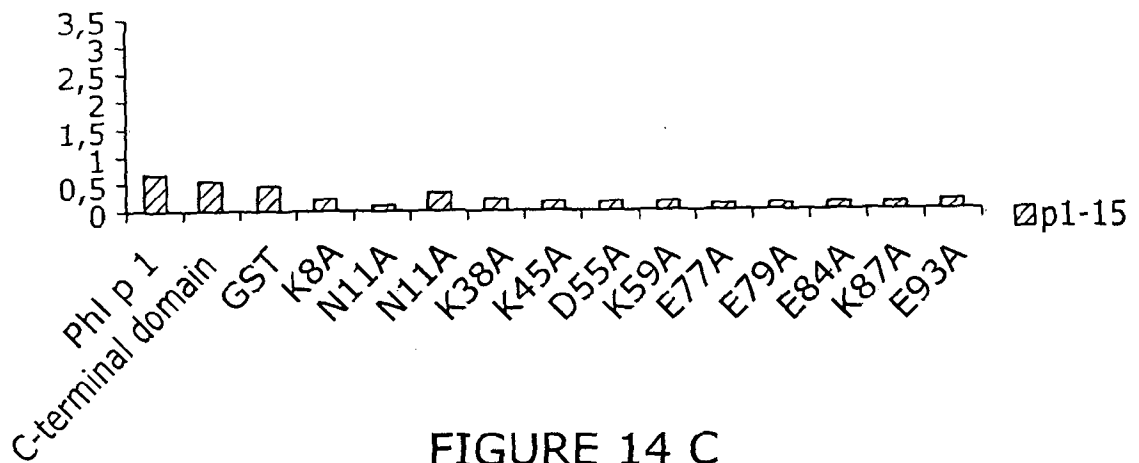


FIGURE 14 C

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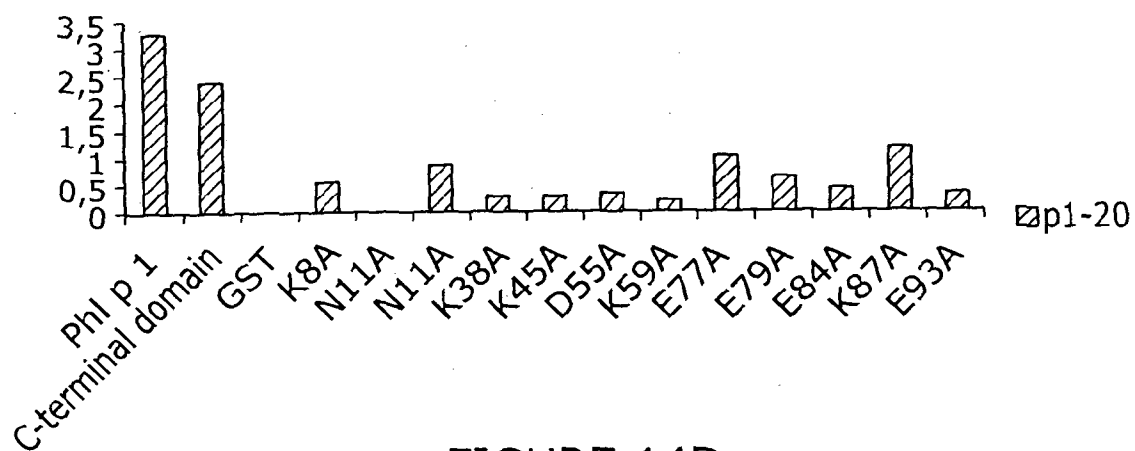


FIGURE 14D

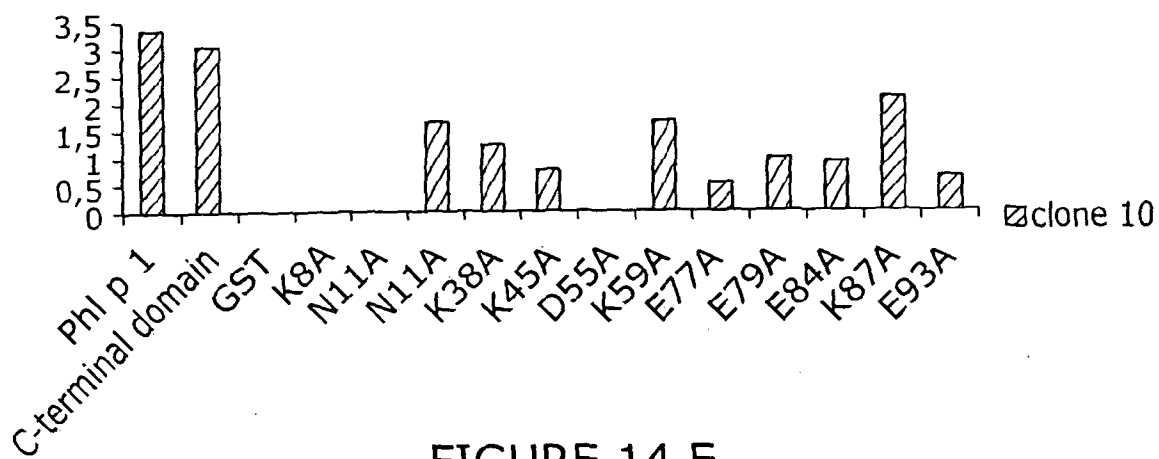


FIGURE 14 E

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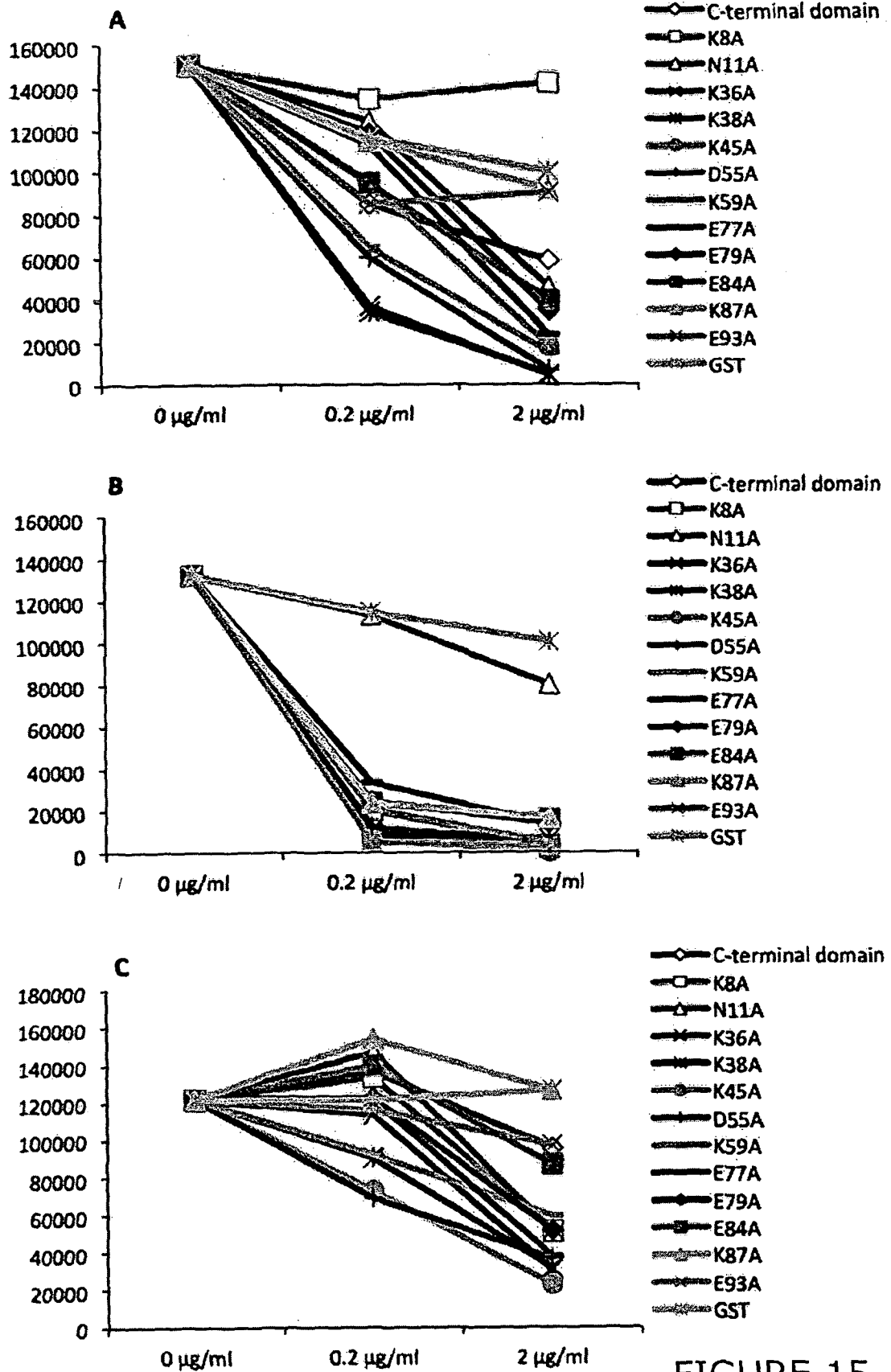


FIGURE 15

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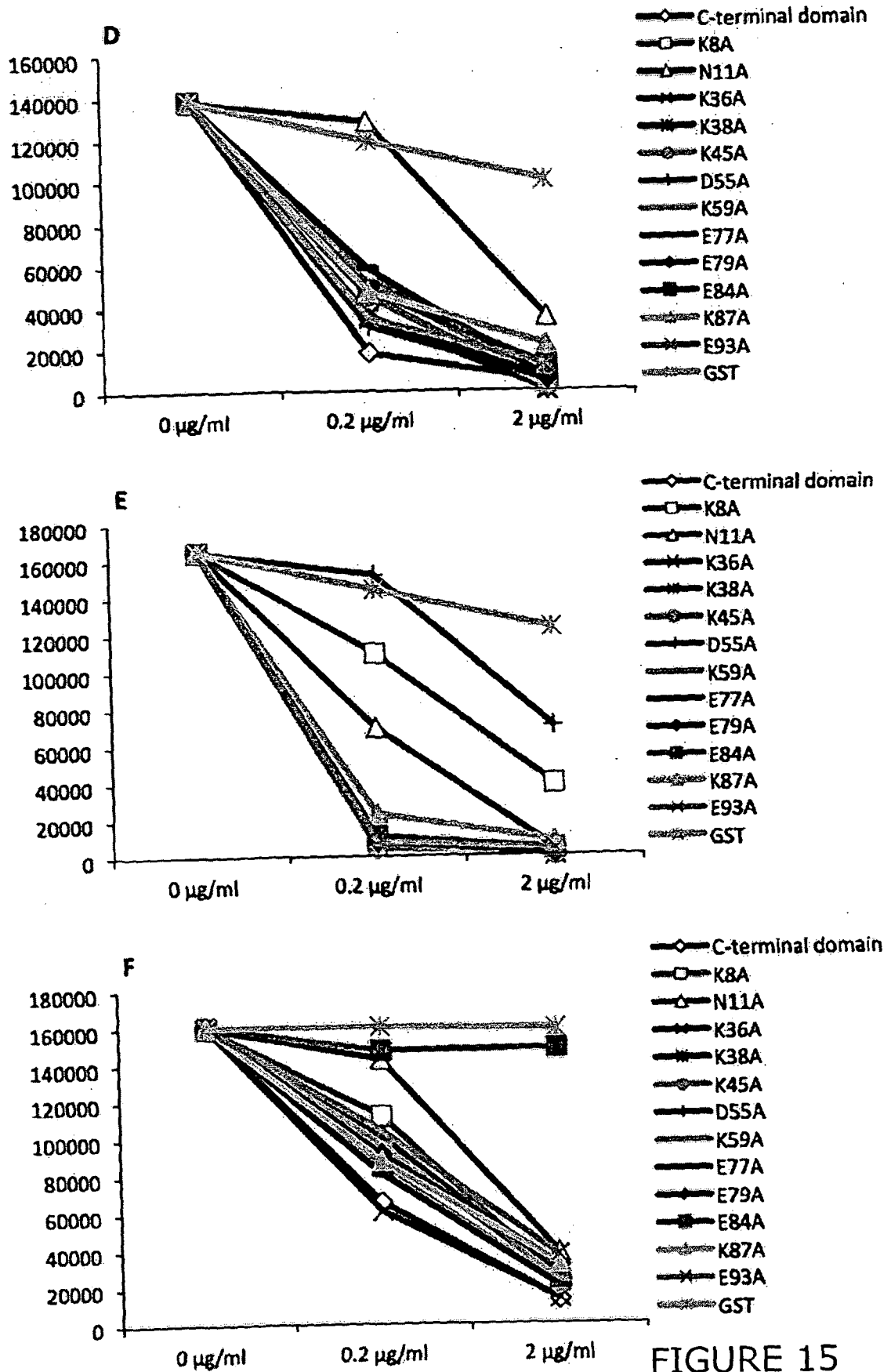


FIGURE 15

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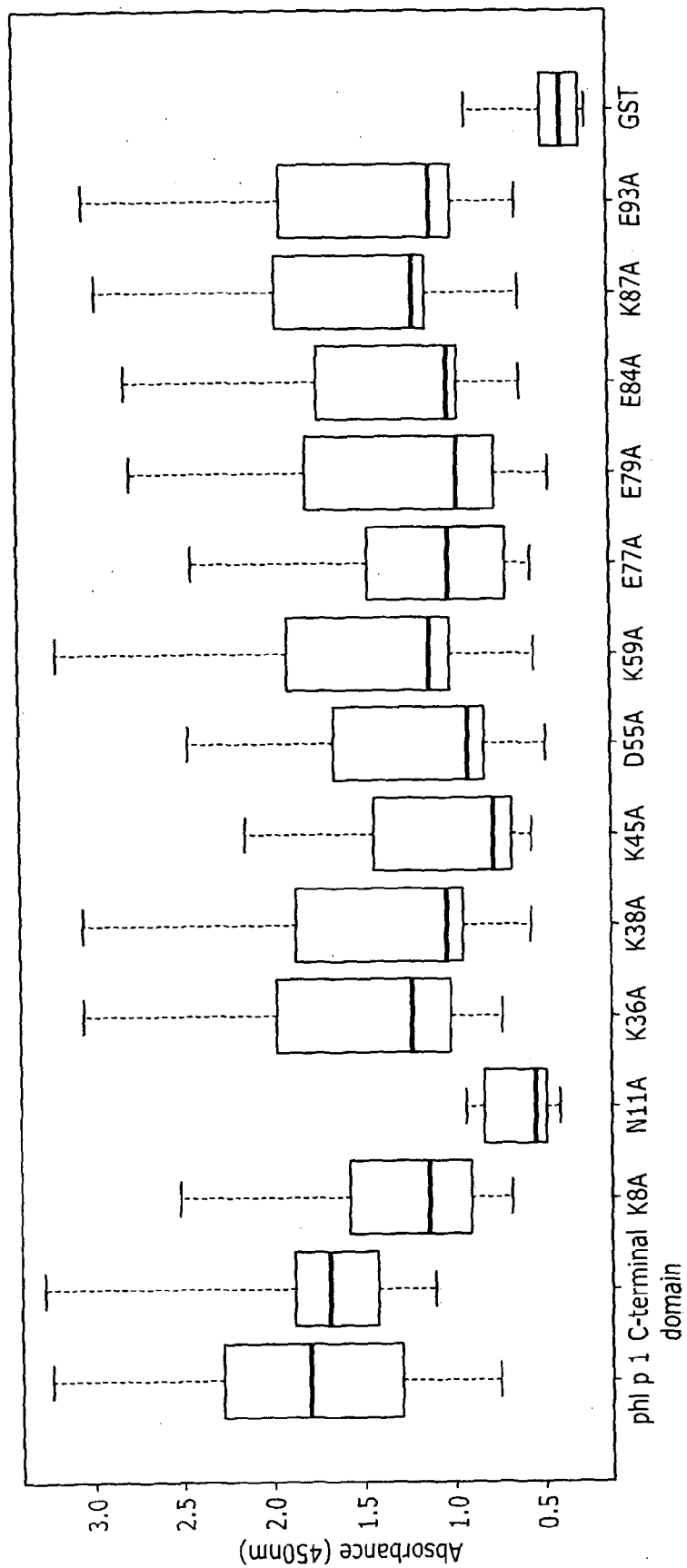


FIGURE 16

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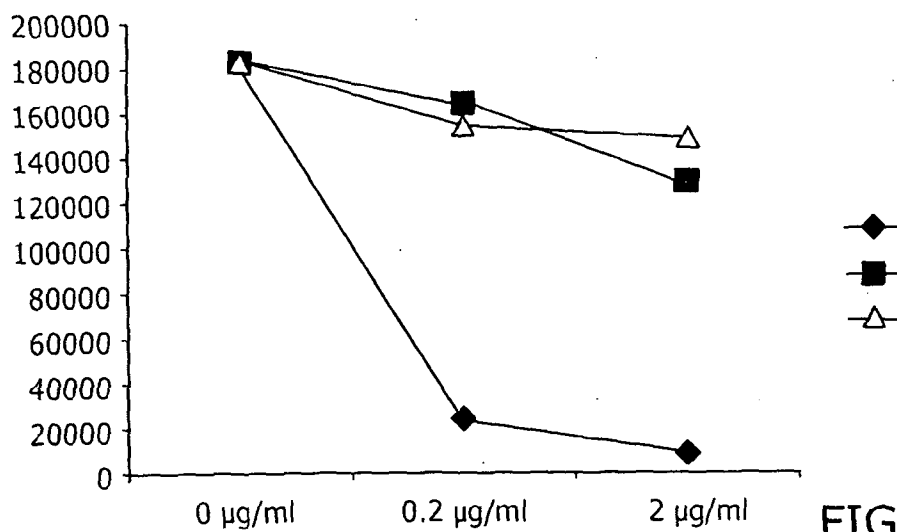


FIGURE 17A

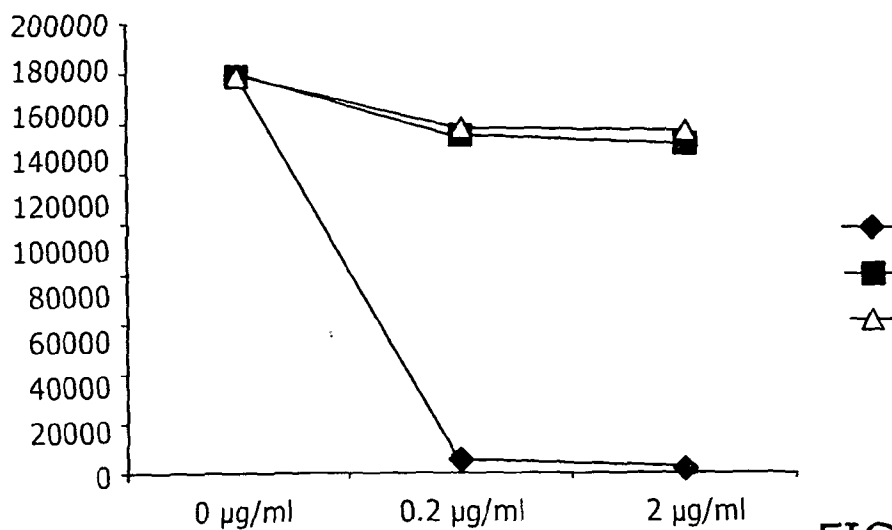


FIGURE 17B

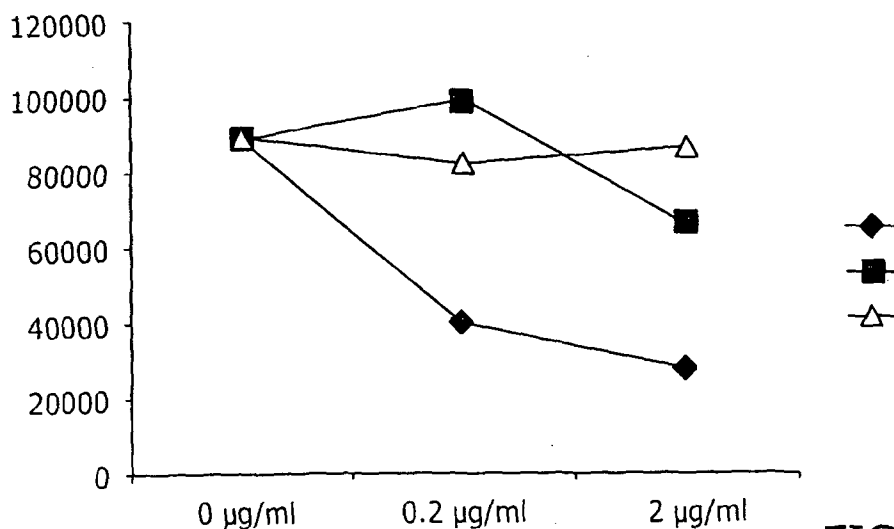


FIGURE 17C

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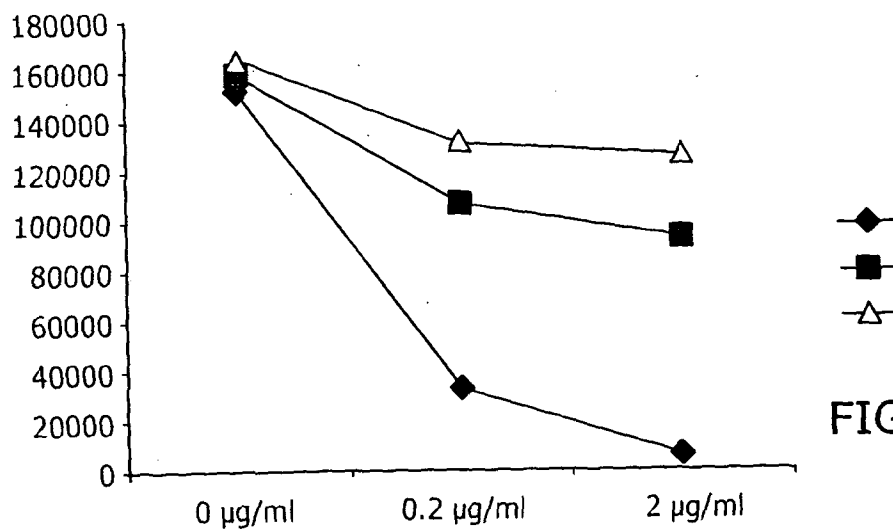


FIGURE 17D

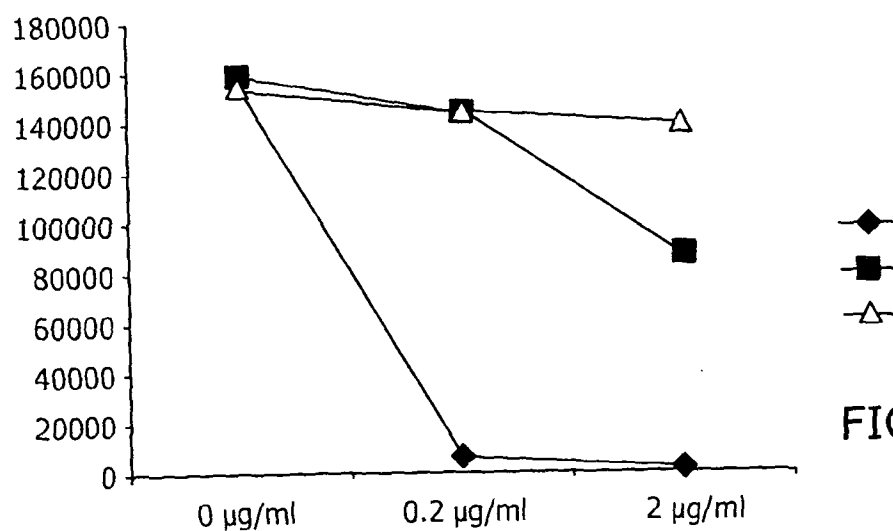


FIGURE 17E

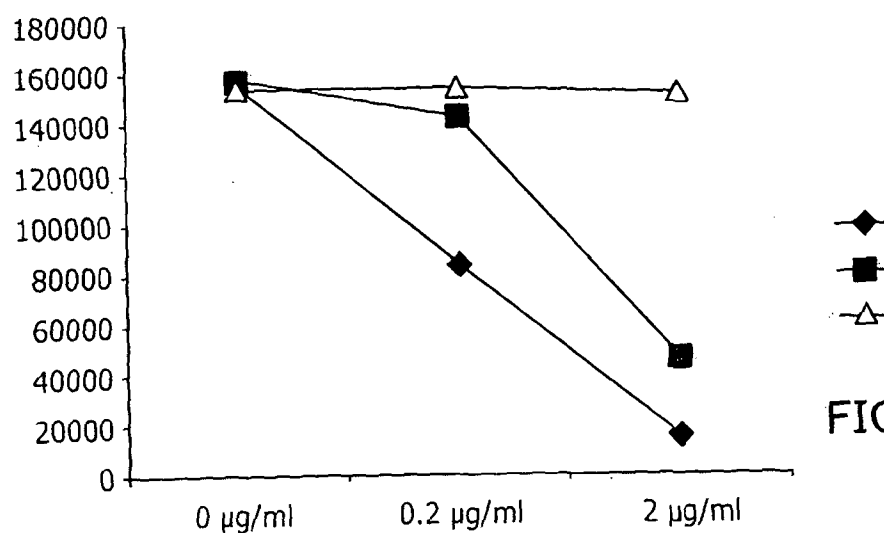


FIGURE 17F

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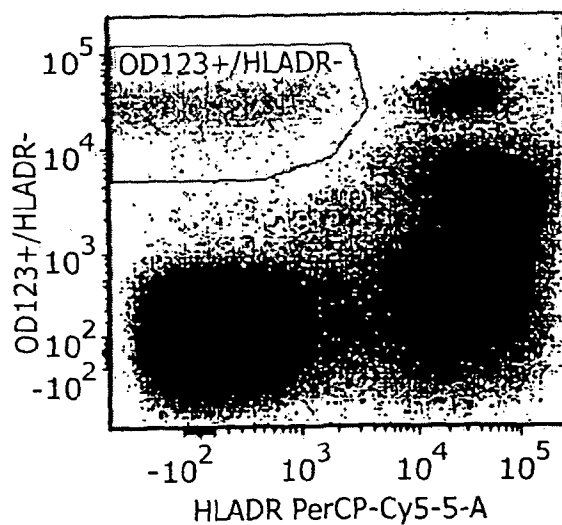


FIGURE 18A

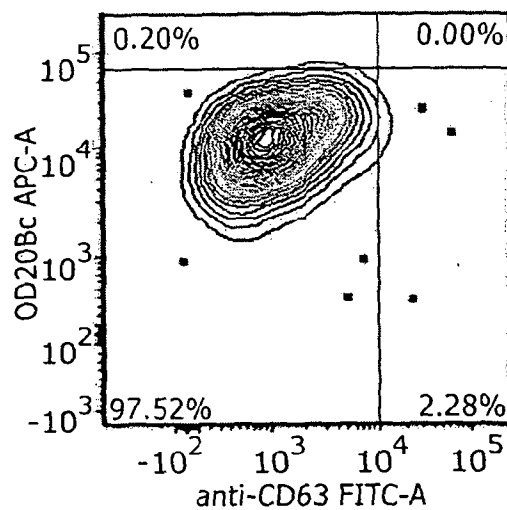


FIGURE 18B

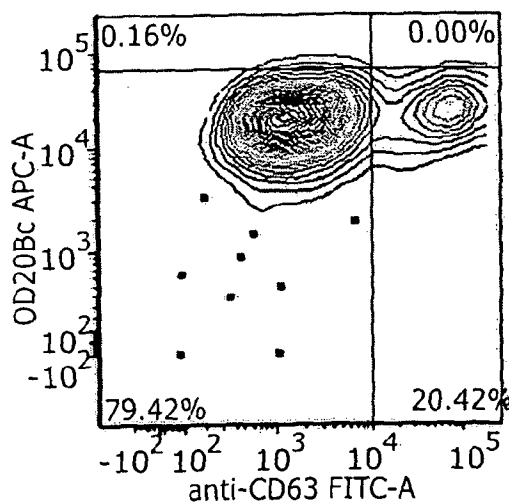


FIGURE 18C

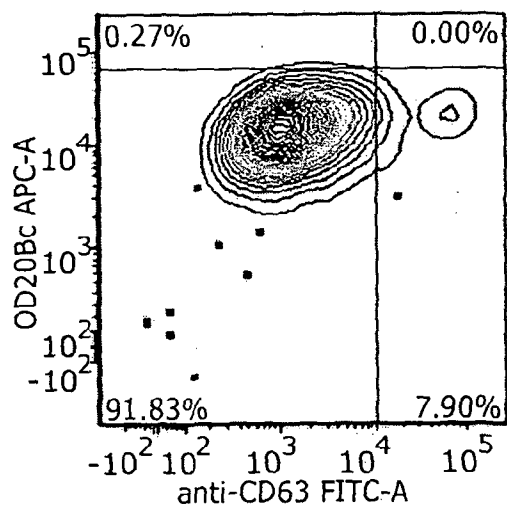
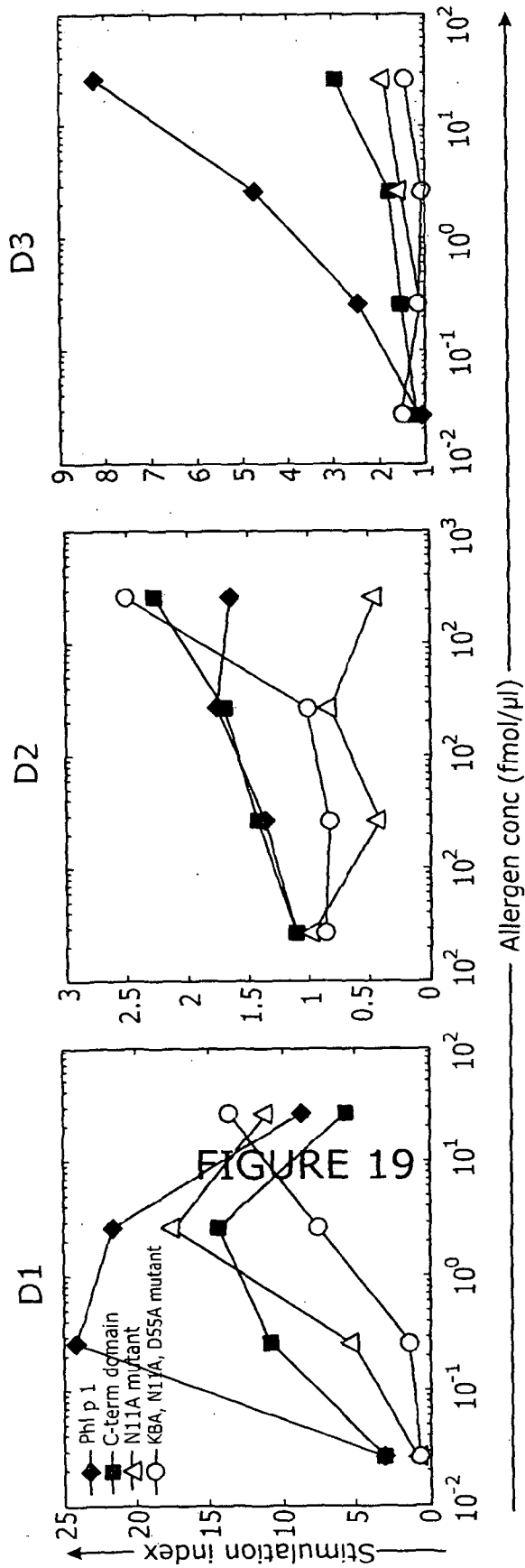


FIGURE 18D

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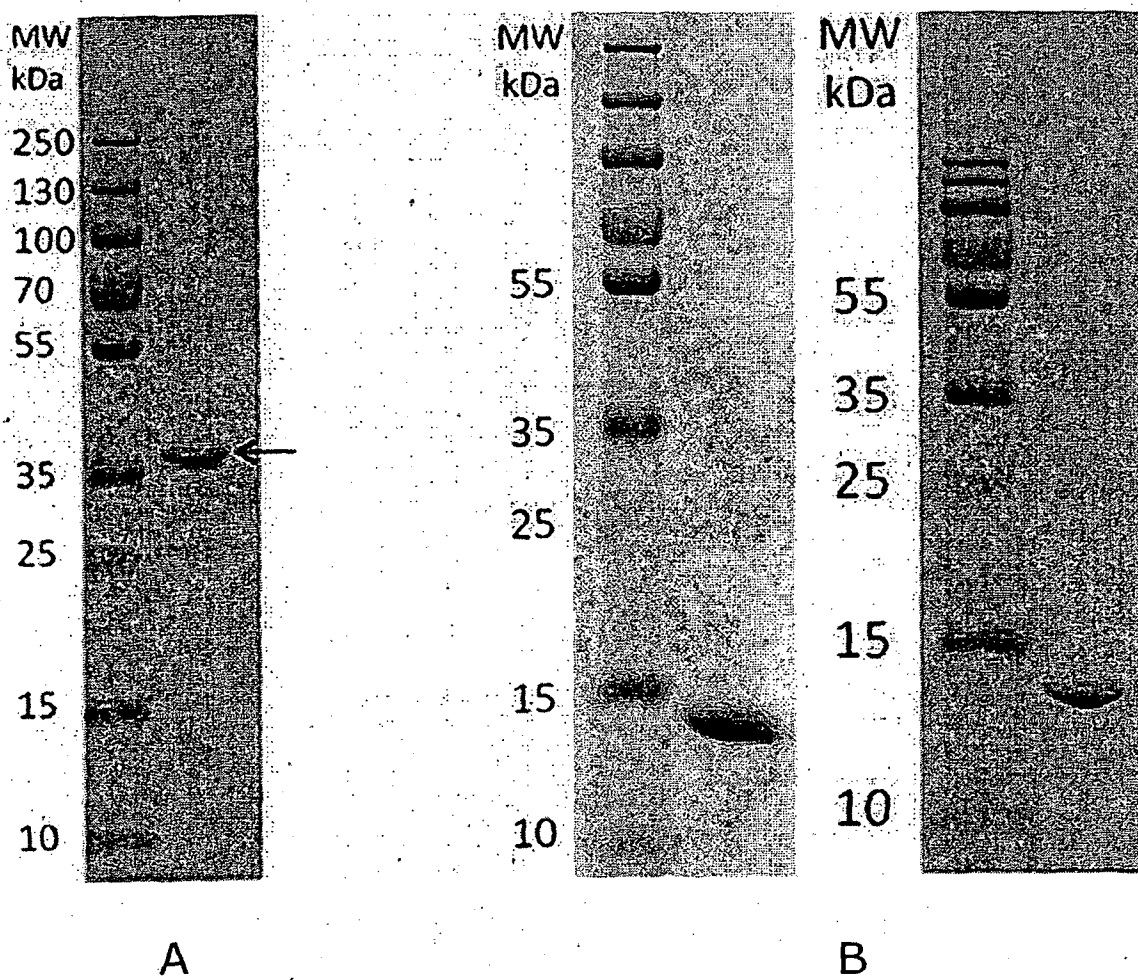


FIGURE 20

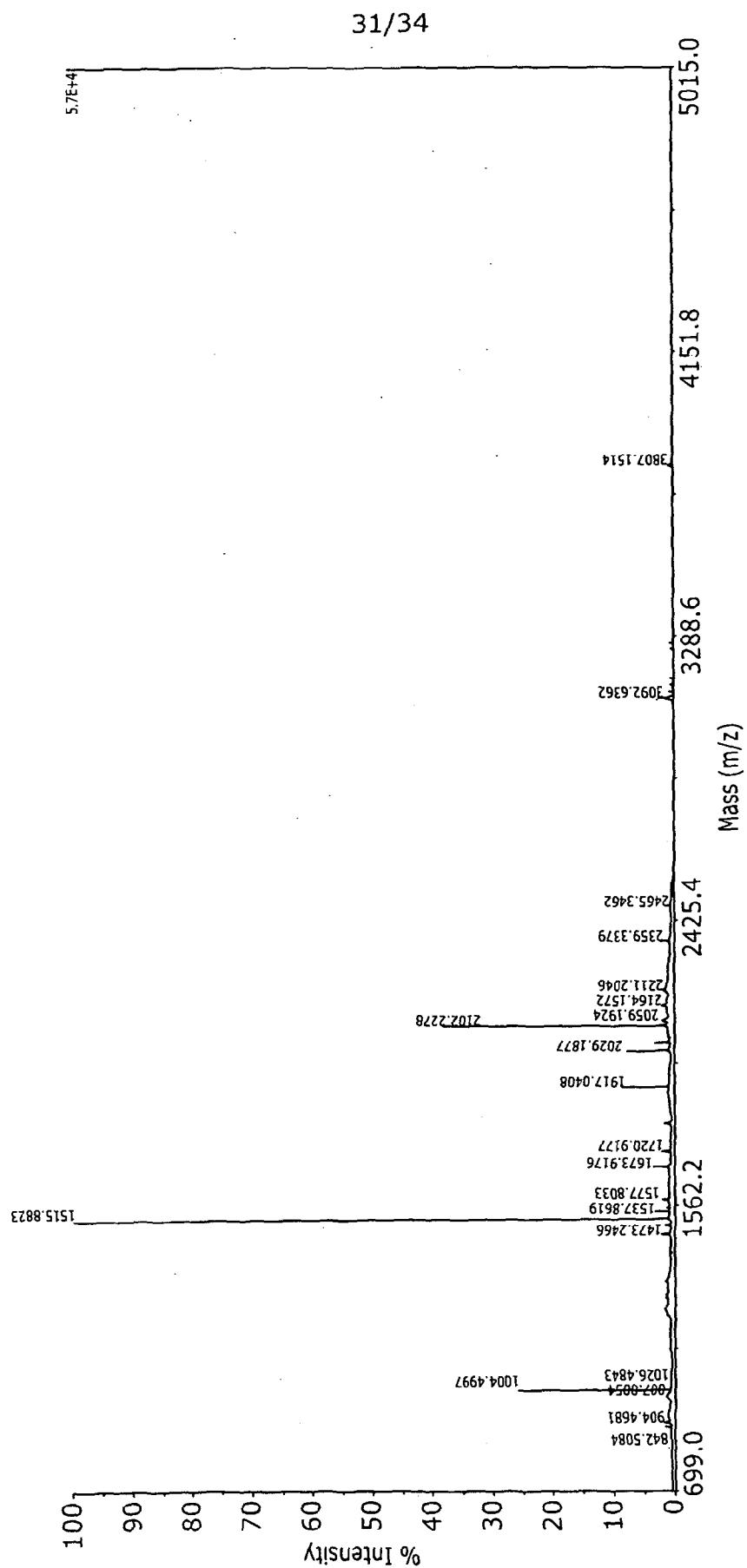


FIGURE 20C

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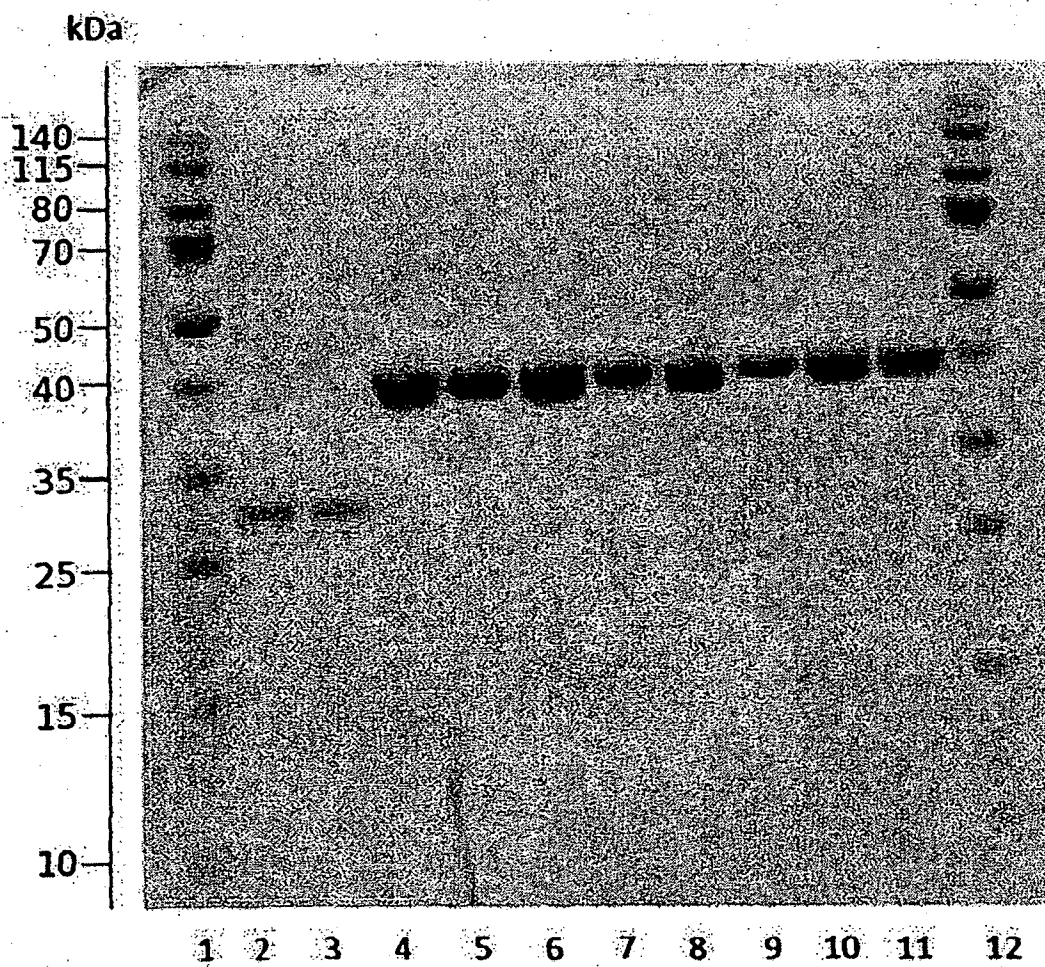


FIGURE 21

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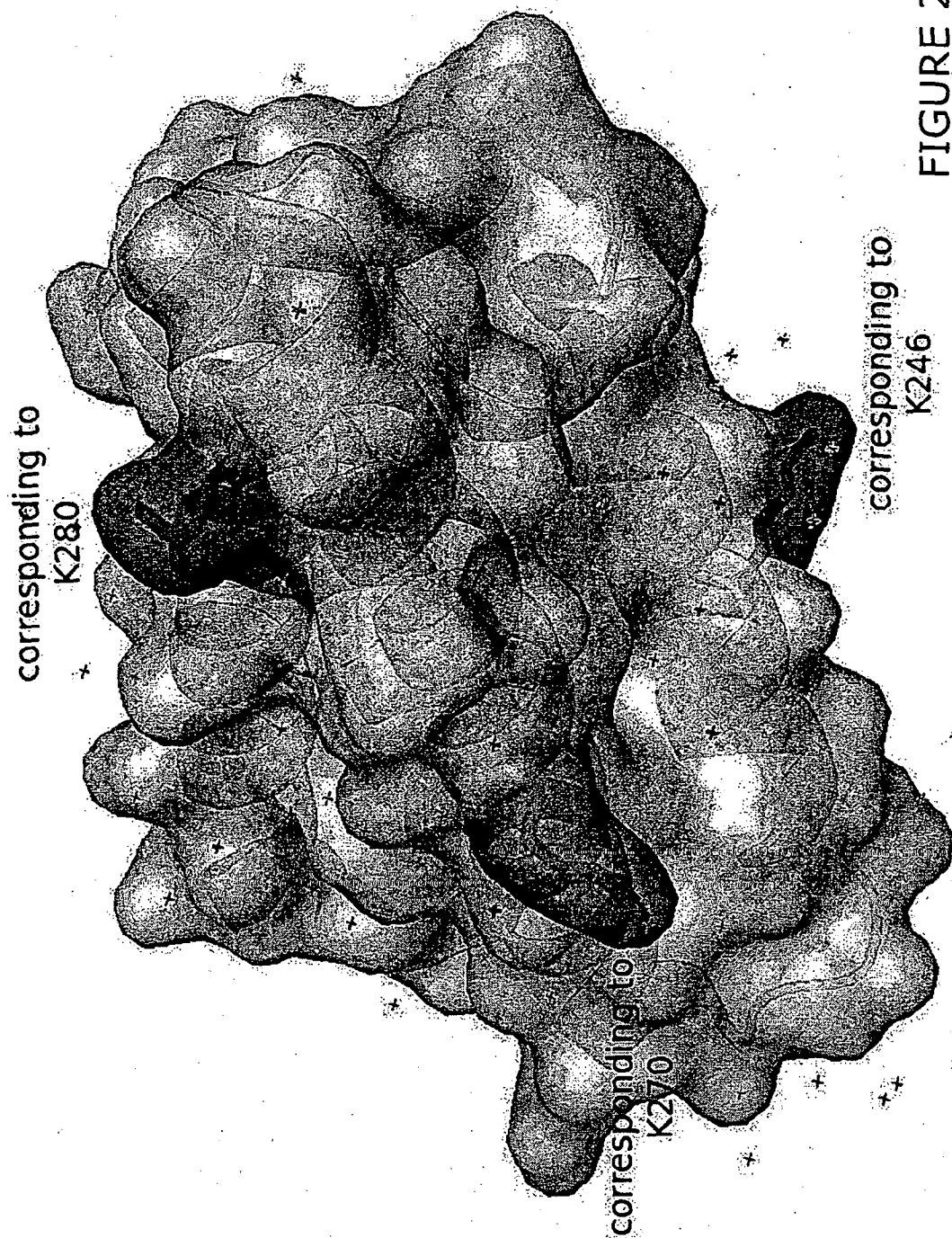


FIGURE 22

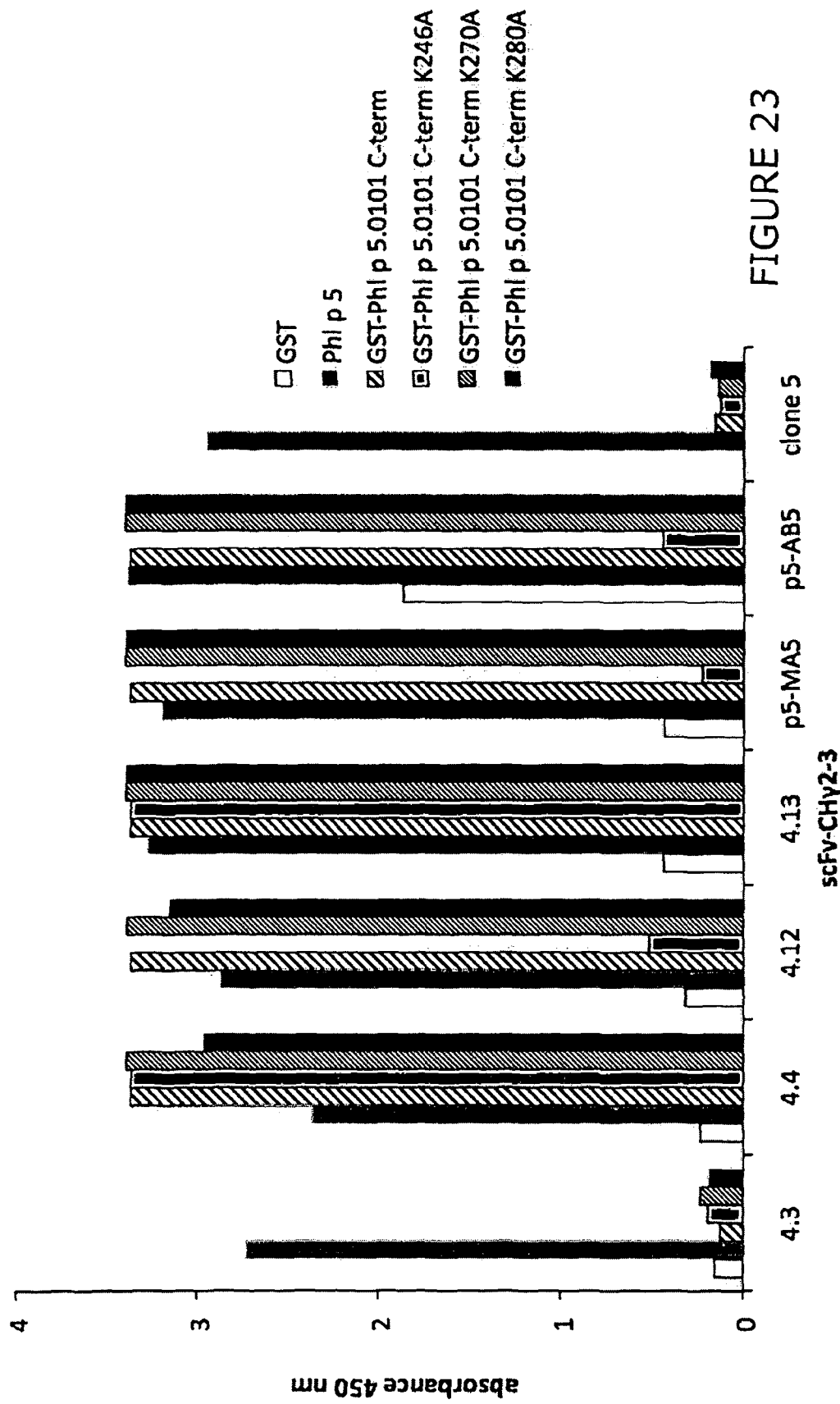


FIGURE 23