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(54) Title: MUTEINS OF TEAR LIPOCALIN

PCT. No. 1

SEQ ID NO: 3

SEQ ID NO: 6

SEQ ID NO: 4

SEQ ID NO: 5

PCR A

PCR B

202

348

500

190

190

378

500

PCT No. 2

SEQ ID NO: 7

SEQ ID NO: 8

SEQ ID NO: 9

SEQ ID NO: 9

PCR

BadX1

BadX1

179

513

(57) Abstract: The present invention relates to novel muteins derived from tear lipocalin or a functional homologue thereof. In particular, the invention relates to a mutein of human tear lipocalin. The invention also refers to a corresponding nucleic acid molecule encoding such a mutein and to a method for its generation. The invention further refers to a method for producing such a mutein. Finally, the invention is directed to pharmaceutical composition comprising such a lipocalin mutein as well as to various use of the mutein.
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MUTEINS OF TEAR LIPOCALIN

The present invention relates to novel muteins derived from tear lipocalin or a functional homologue thereof. In particular, the invention relates to a mutein of human tear lipocalin. The invention also refers to a corresponding nucleic acid molecule encoding such a mutein and to a method for its generation. The invention further refers to a method for producing such a mutein. Finally, the invention is directed to a pharmaceutical composition comprising such a lipocalin mutein as well as to various use of the mutein.

The members of the lipocalin protein family (Pervaiz, S., and Brew, K. (1987) FASEB J. 1, 209-214) are typically small, secreted proteins which are characterized by a range of different molecular-recognition properties: their ability to bind various, principally hydrophobic molecules (such as retinoids, fatty acids, cholesterol, prostaglandins, biliverdins, pheromones, tastants, and odorants), their binding to specific cell-surface receptors and their formation of macromolecular complexes. Although they have, in the past, been classified primarily as transport proteins, it is now clear that the lipocalins fulfill a variety of physiological functions. These include roles in retinol transport, olfaction, pheromone signaling, and the synthesis of prostaglandins. The lipocalins have also been implicated in the regulation of the immune response and the mediation of cell homoeostasis (reviewed, for example, in Flower, D.R. (1996) Biochem. J. 318, 1-143 and Flower, D.R. et al. (2000) Biochim. Biophys. Acta 1482, 9-24).

The lipocalins share unusually low levels of overall sequence conservation, often with sequence identities of less than 20%. In strong contrast, their overall folding pattern is highly conserved. The central part of the lipocalin structure consists of a single eight-stranded anti-parallel β-sheet closed back on itself to form a continuously hydrogen-bonded β-barrel. One end of the barrel is sterically blocked by the N-terminal peptide segment that runs across its bottom as well as three peptide loops connecting the β-strands. The other end of the β-barrel is open to the solvent and encompasses a target-binding site which is formed by four peptide loops. It is this diversity of the loops in the otherwise rigid lipocalin scaffold that gives rise to a variety of different binding modes each capable of accommodating targets of different size, shape, and chemical character (reviewed, e.g., in Flower, D.R. (1996), supra; Flower, D.R. et al. (2000), supra, or Skerra, A. (2000) Biochim. Biophys. Acta 1482, 337-350).

Human tear pre-albumin, now called tear lipocalin (TLPC), was originally described as a major protein of human tear fluid (approximately one third of the total protein content) but has recently
also identified in several other secretory tissues including prostate, nasal mucosa and tracheal mucosa. Homologous proteins have been found in rat, pig, dog and horse. Tear lipocalin is an unusual lipocalin member because of its high promiscuity for relative insoluble lipids and binding characteristics that differ from other members of this protein family (reviewed in Redl, B. (2000) *Biochim. Biophys. Acta* **1482**, 241-248). A remarkable number of lipophilic compounds of different chemical classes such as fatty acids, fatty alcohols, phospholipids, glycolipids and cholesterol are endogenous ligands of this protein. Interestingly, in contrast to other lipocalins the strength of ligand (target) binding correlates with the length of the hydrocarbon tail both for alkyl amides and fatty acids. Thus, tear lipocalin binds most strongly the least soluble lipids (Glasgow, B.J. et al. (1995) *Curr. Eye Res.* **14**, 363-372; Gasymov, O.K. et al. (1999) *Biochim. Biophys. Acta* **1433**, 307-320).

The precise biological function of human tear lipocalin has not been fully elucidated so far and is still a matter of controversy. In tear fluid, it appears to be most important for the integrity of the tear film by removing lipids from the mucous surface of the eye to the liquid phase (reviewed in Gasymov, O.K. et al. (1999), *supra*). However, it displays additional activities *in vitro* that are very unusual among lipocalins, namely inhibition of cystein proteinases as well as non-specific endonuclease activity (van’t Hof, W. et al. (1997) *J. Biol. Chem.* **272**, 1837-1841; Yusifov, T.N. et al. (2000) *Biochem. J.* **347**, 815-819). Recently, it has been demonstrated that tear lipocalin is able to bind several lipid peroxidation products in vitro resulting in the hypothesis that it might function as a physiological oxidative-stress-induced scavenger of potentially harmful lipophilic molecules (Lechner, M. et al. (2001) *Biochem. J.* **356**, 129-135).

Proteins, which selectively bind to their corresponding targets by way of non-covalent interaction, play a crucial role as reagents in biotechnology, medicine, bioanalytics as well as in the biological and life sciences in general. Antibodies, i.e. immunoglobulins, are a prominent example of this class of proteins. Despite the manifold needs for such proteins in conjunction with recognition, binding and/or separation of ligands/targets, almost exclusively immunoglobulins are currently used. The application of other proteins with defined ligand-binding characteristics, for example the lectins, has remained restricted to special cases.

Rather recently, members of the lipocalin family have become subject of research concerning proteins having defined ligand-binding properties. The PCT publication WO 99/16873 discloses the class of anticalins®; polypeptides of the lipocalin family, in which amino acid positions in the region of all four peptide loops are mutated, which are arranged at the end of the cylindrical β-
barrel structure encompassing the binding pocket, and which correspond to those segments in the linear polypeptide sequence comprising the amino acid positions 28 to 45, 58 to 69, 86 to 99 and 114 to 129 of the bilin-binding protein of *Pieris brassicae*. The PCT publication WO 00/75308 discloses muteins of the bilin-binding protein, which specifically bind digoxigenin, whereas the International Patent Applications WO 03/029463 and WO 03/029471 relate to muteins of the human neutrophil gelatinase-associated lipocalin and apolipoprotein D, respectively. In order to further improve and fine tune ligand affinity, specificity as well as folding stability of a lipocalin variant various approaches using different members of the lipocalin family have been proposed (Skerra, A. (2001) *Rev. Mol. Biotechnol.* **74**, 257-275; Schlehuber, S., and Skerra, A. (2002) *Biophys. Chem.* **96**, 213-228) such as the "shaping" of the ligand-binding pocket or the replacement of additional amino acid residues.

However, for various applications it could also be advantageous to have more than one binding site per molecule available – either the natural binding pocket plus an engineered additional (protein)-binding site or two different engineered binding sites. For example, it could be considered to use lipocalin muteins as adapter or linker molecules which may be attached to a given binding partner via binding site I, whereas binding site II is used for screening/selection purposes or the like. One possibility to achieve this goal, is the use of fusion proteins comprising two lipocalin muteins of same or different binding specificity which are coupled to each other by a peptide linker. Such fusion proteins, also called "duocalins", are described in WO 99/16873 and also by Schlehuber, S. & Skerra, A. (2001), *Biol. Chem.* **382**, 1335-1342, for example.

Recently high-affinity histamine-binding proteins have been identified in the saliva of *Rhipicephalus appendiculatus* ticks (Paesen, G.C. et al. (1999) *Mol. Cell* **3**, 661-671). These proteins sequester histamine at the wound site, outcompeting histamine receptors for the ligand in order to suppress inflammation during blood feeding. The crystal structure of these histamine-binding proteins reveals a lipocalin fold novel in containing two binding sites for histamine having different binding affinities. The sites, one of which is a typical lipocalin binding site, are orthogonally arranged and highly rigid, forming an unusually polar internal surface that specifically complements the properties of histamine. A related protein termed SHBP, which is secreted by a rodent- and cattle-feeding tick, binds both histamine and serotonin at the two different binding sites (Sangamnatdej, S. et al. (2002) *Insect Mol. Biol.* **11**, 79-86). The high-affinity binding site lies perpendicular to the long axis of the β-barrel leading to distortions in the protein structure compared to other lipocalins. Thus, it appears as if such a binding site cannot be
engineered in any given lipocalin. On the other hand, since the binding sites are rather buried in the core of the β-barrel there appear to be sterical limitations with regard to ligand size.

Thus, there remains a need for the generation of binding proteins that uses different binding sites and/or alternative lipocalin scaffolds, simply for the reason to have more options for practical realisation.

Accordingly, it is an object of the invention to provide alternative lipocalin muteins having binding affinity to a given target.

This object is accomplished by a lipocalin mutein having the features of the independent claims as well as the method for its generation.

In one embodiment such a lipocalin mutein is a mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof, wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the N-terminal peptide stretch as well as the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and wherein the mutein binds a given target with detectable affinity.

In more illustrative terms, this embodiment is based on the finding of the inventors that amino acids in the three loops at the closed end of the internal ligand binding site of a tear lipocalin and/or the N-terminal peptide stretch of the tear lipocalin (cf. Fig. 4) can be mutated in order to obtain lipocalin muteins that bind a given target with determinable affinity. Thus, the invention provides a structurally new class of lipocalin muteins with antibody-like binding properties. This means that these muteins can be used in the same way for the generation of new binding proteins with a predetermined specificity as the class of the above mentioned anticalins® (lipocalin muteins which are derived from the proteins of the lipocalin family such as the bilin-binding protein of Pieris brassicae, in which amino acid positions in the four peptide loops positioned on the open end of the ligand binding site are mutated). For this reason, these new lipocalin muteins of the present invention are also called anticalins®.

In another embodiment, a mutein of the invention is also a mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof. In this case, the mutein comprises at least two mutated amino acid residues at any sequence position in the four peptide loops AB, CD, EF, and
GH encompassing the natural lipocalin binding pocket, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and wherein the mutein binds a given target with detectable affinity. Accordingly, this embodiment provides for a new class of scaffold in which amino acids in the four loops at the open end of the ligand binding site of the lipocalins can be mutated for the generation of binding molecules against a desired target.

In yet another embodiment the invention relates to a mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof,

wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the N-terminal region as well as the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket,

wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the four peptide loops AB, CD, EF, and GH encompassing the natural lipocalin binding pocket,

wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and

wherein the mutein binds at least one given target with detectable affinity.

Thus, the invention also provides for the first time a monomeric lipocalin mutein or anticalin® that due to the presence of two binding sites can have binding specificity for two given ligands. Such a bispecific molecule can be considered to be functionally equivalent to a bispecific antibody molecule such as a bispecific diabody. However, compared to a bispecific diabody (or antibody fragment in general), this new class of bispecific anticalins® has the advantage that it is composed only of one polypeptide chain whereas a diabody consists of two polypeptide chains which are non covalently associated with each other.

A bispecific lipocalin mutein of this new class of binding proteins may be used as an adapter molecule. For example, when having binding affinity to two different receptors, such a bispecific lipocalin molecule can cross-link these receptors. An example of such an anticalin® would be a mutein, wherein the first binding site binds to a apoptosis receptor such as the CD95 (also known as Fas or Apo 1 receptor) and the second binding site can bind to a cell surface receptor which is expressed on the same cell. Binding of such a bispecific mutein in a bicellular manner may result in mutual cross-linking of the CD95 apoptosis receptor and the second cell surface receptor target antigen, which can effectively induce apoptosis of the cells (cf. Jung, G. et al. (2001) Cancer Res.
61, 1846-1848). However such a bispecific mutein may also have only binding affinity for one given target. Such a mutein may be useful as a molecular storage for drugs that are to be slowly released into the blood stream.

The term "mutagenesis" as used herein means that the experimental conditions are chosen such that the amino acid naturally occurring at a given sequence position of the lipocalin used can be substituted by at least one amino acid that is not present at this specific position in the respective natural polypeptide sequence. The term "mutagenesis" also includes the (additional) modification of the length of sequence segments by deletion or insertion of one or more amino acids. Thus, it is within the scope of the invention that, for example, one amino acid at a chosen sequence position is replaced by a stretch of three random mutations, leading to an insertion of two amino acid residues compared to the length of (the respective segment of) the wild-type protein. The term "random mutagenesis" refers to that no predetermined single amino acid (mutation) is present at a certain sequence position but that at least two amino acids can be incorporated into a selected sequence position during mutagenesis with a certain probability.

Such experimental conditions can, for example, be achieved by incorporating codons with a degenerate base composition into a nucleotide acid encoding the respective lipocalin employed. For example, use of the codon NNK or NNS (wherein N = adenine, guanine or cytosine or thymine; K = guanine or thymine; S = adenine or cytosine) allows incorporation of all 20 amino acids plus the amber stop codon during mutagenesis, whereas the codon VVS limits the number of possibly incorporated amino acids to 12, since it excludes the amino acids Cys, Ile, Leu, Met, Phe, Trp, Tyr, Val from being incorporated into the selected position of the polypeptide sequence; use of the codon NMS (wherein M = adenine or cytosine), for example, restricts the number of possible amino acids to 11 at a selected sequence position since it excludes the amino acids Arg, Cys, Gly, Ile, Leu, Met, Phe, Trp, Val from being incorporated at a selected sequence position. In this respect it is noted that codons for other amino acids (than the regular 20 naturally occurring amino acids) such as selenocysteine or pyrrolysine can also be incorporated into a nucleic acid of a mutein. It is also possible to use as described by Wang, L., et al., Science, 292:498-500, 2001 or Wang, L., Schultz, P.G., Chem. Comm., 1:1-11, 2002 "artificial" codons such as UAG which are usually recognized as stop codons in order to insert other unusual amino acids, for example O-methyl-L-tyrosine or p-aminophenylalanine.

The term "tear lipocalin" as used herein is not limited to the human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424) but is meant to include all polypeptides having the
structurally conversed lipocalin fold and a sequence homology with respect to the amino acid sequence of the human tear lipocalin of at least 60%. The term lipocalin fold is used in its regular meaning as used, e.g., in Flower, D.R. (1996), *supra*, to describe the typical three-dimensional lipocalin structure with a conformationally conserved β-barrel as a central motif made of a cylindrically closed β-sheet of eight antiparallel strands, wherein the open end of the barrel the β-strands are connected by four loops in a pairwise manner so that the binding pocket is formed (see also Fig. 4).

The definition of the peptide loops as used in the present invention is also in accordance with the regular meaning of the term lipocalin fold and is as follows and also illustrated in Fig. 4: The peptide loop (segment) AB connects the β-strands A and B of the cylindrically closed β-sheet, the peptide loop CD connects the β-strands C and D, the peptide loop EF connects the β-strands E and F, the peptide loop GH connects the β-strands G and H, the peptide loop BC connects the β-strands B and C, the loop DE connects the β-strands D and E, and the loop FG connects the β-strands F and G. As can be seen from Fig. 4 the loops AB, CD, EF and GH form the known binding site of the lipocalins (which was therefore called the open end), whereas, as found in the present invention, the loops BC, DE and FG can be used together with the N-terminal peptide stretch to form a second binding site which is located at the closed end of the β-barrel.

In accordance with the above, the term "tear lipocalin" includes structural homologues, already identified or yet to be isolated, from other species which have an amino acid sequence homology of more than about 60%. The term "homology" as used herein in its usual meaning and includes identical amino acids as well as amino acids which are regarded to be conservative substitutions (for example, exchange of a glutamate residue by a aspartate residue) at equivalent positions in the linear amino acid sequence of two proteins that are compared with each other.

The percentage of homology is determined herein using the program BLASTP, version blastp 2.2.5 (November 16, 2002; cf. Altschul, S. F. et al. (1997) *Nucleic Acids Res.* 25, 3389-3402). The percentage of homology is based on the alignment of the entire polypeptide sequences (cutoff value set to 10^{−3}) including the propeptide sequences, using the human tear lipocalin as reference in a pairwise comparison. It is calculated as the percentage of numbers of "positives" (homologues amino acids) indicated as result in the BLASTP program output divided by the total number of amino acids selected by the program for the alignment. It is noted in this connection that this total number of selected amino acids can differ from the length of the tear lipocalin (176 amino acids including the propeptide) as it is seen in the following.
Examples of homologues proteins are Von Ebners gland protein 1 of *Rattus norvegicus* (VEGP protein; SWISS-PROT Data Bank Accession Numbers P20289) with a sequence homology of ca. 70 % (125 positives/178 positions including the propeptide; when the 18 residues long propeptides containing 13 "positives" are not taken into account: 112 positives/160, resulting also in an homology of ca. 70 %), Von Ebners gland protein 2 of *Rattus norvegicus* (VEG protein 2; SWISS-PROT Data Bank Accession Numbers P41244) with a sequence homology of ca. 71 % (127 positives/178 including the propeptide; when the 18 residues long propeptides are not taken into account: 114 positives/160, the homology is determined to be also ca. 71 %), Von Ebners gland protein 2 of *Sus scrofa* (pig) (LCN1; SWISS-PROT Data Bank Accession Numbers P53715) with a sequence homology of about 74 % (131 positives/176 positions including the propeptide; when the 18 residues long propeptides containing 16 "positives" are not taken into account: 115 positives/158, resulting in an homology of ca. 73.0 %), or the Major allergen Can f1 precursor of dog (ALL 1, SWISS-PROT Data Bank Accession Numbers O18873) with a sequence homology of ca. 70.0 %, (122 positives/174 positions, or 110 positives/156 = ca. 70 % homology, when the propeptides with 12 positives are excluded) as determined with the program BLASTP as explained above. Such a structural homologue of the tear lipocalin can be derived from any species, i.e. from prokaryotic as well as from eukaryotic organisms. In case of eukaryotic organisms, the structural homologue can be derived from invertebrates as well as vertebrates such as mammals (e.g., human, monkey, dog, rat or mouse) or birds or reptiles.

In case a protein other than tear lipocalin is used in the present invention, the definition of the mutated sequence positions given for tear lipocalin can be assigned to the other lipocalin with the help of published sequence alignments or alignments methods which are available to the skilled artisan. A sequence alignment can, for example, be carried out as explained in WO 99/16873 (cf. Fig. 3 therein), using an published alignment such as the one in Fig. 1 of Redl, B. (2000) *Biochim. Biophys. Acta*, **1482**, 241-248. If the three-dimensional structure of the lipocalins are available structural superpositions can also be used for the determination of those sequence positions that are to be subjected to mutagenesis in the present invention. Other methods of structural analysis such as multidimensional nuclear magnetic resonance spectroscopy can also be employed for this purpose.

The homologue of tear lipocalin can also be a mutein protein of tear lipocalin itself, in which amino acid substitutions are introduced at positions other than the positions selected in the present invention. For example, such a mutein can be a protein in which positions at the solvent exposed
surface of the β-barrel are mutated compared to the wild type sequence of the tear lipocalin in order to increase the solubility or the stability of the protein.

In general, the term "tear lipocalin" includes all proteins that have a sequence homology of more than 60%, 70% 80%, 85%, 90% or 95% in relation to the human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424).

In one preferred embodiment of the invention the mutein as disclosed herein is derived from human tear lipocalin. In other preferred embodiments the mutein is derived from the VEGP protein, VEG protein 2, LCN 1 or ALL 1 protein.

If the binding site at the closed end of the β-barrel is used, the mutein according to the invention typically comprises mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 41-49, 69-77, and 87-98 of the linear polypeptide sequence of human tear lipocalin. The positions 7-14 are part of the N-terminal peptide stretch, the positions 41-49 are comprised in the BC loop, the positions 60-77 are comprised in the DE loop and the positions 87-98 are comprised in the FG loop.

In more specific embodiments of those muteins the mutations are introduced at those sequence positions which correspond to the positions 8, 9, 10, 11, 12, 13, 43, 45, 47, 70, 72, 74, 75, 90, 92, 94, and 97 of human tear lipocalin. Usually, such a mutein comprises mutations at 5-10 or 12-16 or all 17 of the sequence positions.

In case the binding site at the open end of the β-barrel is subjected to mutagenesis a lipocalin mutein according to the invention comprises mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 24-36, 53-66, 79-84, and 103-110 of the linear polypeptide sequence of human tear lipocalin. The positions 24-36 are comprised in the AB loop, the positions 53-66 are comprised in the CD loop, the positions 69-77 are comprised in the EF loop and the positions 103-110 are comprised in the GH loop.

Accordingly, a mutein having two binding sites comprises mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 41-49, 69-77, and 87-97 of the linear polypeptide sequence of human tear lipocalin and additional mutations at any two or more of the sequence positions in the peptide segments corresponding to
the sequence positions 24-36, 53-66, 79-84, and 103-110 of the linear polypeptide sequence of human tear lipocalin.

In this respect it is noted that the number of the segments (loops) defined above which are used for mutagenesis can vary (the N-terminal peptide stretch is included in the meaning of the term segment or loop). It is not necessary to mutate all four of these segments altogether of each of two binding sites, for example in a concerted mutagenesis. But it is also possible to introduce mutations only in one, two or three segments of each binding site in order to generate a mutein having detectable affinity to a given target. Therefore, it is possible to subject, for example, only two or three segments at the closed end of the β-barrel to mutagenesis if a binding molecule with only one engineered binding site is wanted. If this molecule is then wanted to have binding affinity towards a second target, sequence positions in any of the four loops of the second binding site can then be mutated.

The lipocalin muteins of the invention may comprise the wild-type (natural) amino acid sequence outside the mutated segments. On the other hand, the lipocalin muteins disclosed herein may also contain amino acid mutations outside the sequence positions subjected to mutagenesis as long as those mutations do not interfere with the binding activity and the folding of the mutein. Such mutations can be accomplished very easily on DNA level using established standard methods (Sambrook, J. et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Possible alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with a chemically similar amino acid residue. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan. One the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence.

Such modifications of the amino acid sequence include directed mutagenesis of single amino acid positions in order to simplify sub-cloning of the mutated lipocalin gene or its parts by incorporating cleavage sites for certain restriction enzymes. Furthermore, mutations can be introduced in order to improve certain characteristics of the mutein.
The lipocalin muteins of the invention are able to bind the desired target with detectable affinity, i.e. with an affinity constant of preferably at least 10^5 M^-1. Lower affinities are generally no longer measurable with common methods such as ELISA and therefore of secondary importance. Especially preferred are lipocalin muteins, which bind the desired target with an affinity of at least 10^6 M^-1, corresponding to a dissociation constant of the complex of 1 μM. The binding affinity of a mutein to the desired target can be measured by a multitude of methods such as fluorescence titration, competition ELISA or surface plasmon resonance.

It is clear to the skilled person that complex formation is dependent on many factors such as concentration of the binding partners, the presence of competitors, ionic strength of the buffer system etc. Selection and enrichment is generally performed under conditions allowing the isolation of lipocalin muteins having an affinity constant of at least 10^5 M^-1 to the target. However, the washing and elution steps can be carried out under varying stringency. A selection with respect to the kinetic characteristics is possible as well. For example, the selection can be performed under conditions, which favor complex formation of the target with muteins that show a slow dissociation from the target, or in other words a low k_{off} rate.

A tear lipocalin mutein of the invention may be used for complex formation with a given target. The target (ligand) may be any chemical compound in free or conjugated form which exhibits features of an immunological hapten, a hormone such as steroid hormones or any biopolymer or fragment thereof, for example, a protein or protein domain, a peptide, an oligodeoxynucleotide, a nucleic acid, an oligo- or polysaccharide or conjugates thereof. In a preferred embodiment of the invention the target is a protein. The protein can be any globular soluble protein or a receptor protein, for example, a trans-membrane protein involved in cell signaling, a component of the immune systems such as an MHC molecule or cell surface receptor that is indicative of a specific disease. The mutein may also be able to bind only fragments of a protein. For example, a mutein can bind to a domain of a cell surface receptor, when it is part of the receptor anchored in the cell membrane as well as to the same domain in solution, if this domain can be produced as a soluble protein as well. However the invention is by no means limited to muteins that only bind such macromolecular targets. But it is also possible to obtain muteins of tear lipocalin by means of mutagenesis which show specific binding affinity to ligands of low(er) molecular weight such as biotin, fluorescein or digoxigenin.

For some applications, it is useful to employ the muteins of the invention in a labeled form. Accordingly, the invention is also directed to lipocalin muteins which are conjugated to a label
selected from the group consisting of enzyme labels, radioactive labels, colored labels, fluorescent labels, chromogenic labels, luminescent labels, haptens, digoxigenin, biotin, metal complexes, metals, and colloidal gold. The mutein may also be conjugated to an organic molecule. The term "organic molecule" as used herein preferably denotes an organic molecule comprising at least two carbon atoms, but preferably not more than seven rotatable carbon bonds, having a molecular weight in the range between 100 and 2000 Dalton, preferably 1000 Dalton, and optionally including one or two metal atoms.

In general, it is possible to label the lipocalin mutein with any appropriate chemical substance or enzyme, which directly or indirectly generates a detectable compound or signal in a chemical, physical or enzymatic reaction. An example for a physical reaction is the emission of fluorescence upon irradiation or the emission of X-rays when using a radioactive label. Alkaline phosphatase, horseradish peroxidase or β-galactosidase are examples of enzyme labels which catalyze the formation of chromogenic reaction products. In general, all labels commonly used for antibodies (except those exclusively used with the sugar moiety in the Fe part of immunoglobulins) can also be used for conjugation to the muteins of the present invention. Such conjugates can be produced by methods well known in the art.

For several applications of the muteins disclosed herein it may be advantageous to use them in the form of fusion proteins. In preferred embodiments the inventive lipocalin mutein is fused at its N-terminus or its C-terminus to a protein, a protein domain or a peptide such as a signal sequence and/or an affinity tag.

The fusion partner may confer new characteristics to the inventive lipocalin mutein such as enzymatic activity or binding affinity for other molecules. Examples of suitable fusion proteins are alkaline phosphatase, horseradish peroxidase, glutathion-S-transferase, the albumin-binding domain of protein G, protein A, antibody fragments, oligomerization domains, lipocalin muteins of same or different binding specificity (which results in the formation of "duocalins", cf. Schlehuber, S. & Skerra, A. (2001), *Biol. Chem.* 282, 1335-1342), or toxins. If two bispecific lipocalin mutein of the inventions (i.e. each of them has two binding sites) are combined into a "duocalin", a tetravalent molecule is formed. If for example a duocalin is generated from only one mutein having two binding sites that specifically bind biotin, a tetravalent molecule (homodimer) comparable to streptavidin (which is a homotetramer, in which each monomer binds one biotin molecule) can be obtained. Due to expected avidity effects such a mutein might be a useful analytical tools in methods that make use of the detection of biotin groups.
Affinity tags such as the Strep-tag® or Strep-tag® II (Schmidt, T.G.M. et al. (1996) *J. Mol. Biol.* **255**, 753-766), the myc-tag, the FLAG-tag, the His₆-tag or the HA-tag or proteins such as glutathione-S-transferase also allow easy detection and/or purification of recombinant proteins are further examples of preferred fusion partners. Finally, proteins with chromogenic or fluorescent properties such as the green fluorescent protein (GFP) or the yellow fluorescent protein (YFP) are suitable fusion partners for a lipocalin mutein of the invention as well.

The term "fusion protein" as used herein also comprises lipocalin muteins according to the invention containing a signal sequence. Signal sequences at the N-terminus of a polypeptide direct this polypeptide to a specific cellular compartment, for example the periplasm of *E. coli* or the endoplasmatic reticulum of eukaryotic cells. A large number of signal sequences is known in the art. A preferred signal sequence for secretion a polypeptide into the periplasm of *E. coli* is the OmpA-signal sequence.

The present invention also relates to nucleic acid molecules (DNA and RNA) comprising nucleotide sequences coding for muteins as described herein. Since the degeneracy of the genetic code permits substitutions of certain codons by other codons specifying the same amino acid, the invention is not limited to a specific nucleic acid molecule encoding a fusion protein of the invention but includes all nucleic acid molecules comprising nucleotide sequences encoding a functional fusion protein.

In one preferred embodiment of the nucleic acid molecule of invention its sequence is derived from the coding sequence of human tear lipocalin. In other preferred embodiments the nucleic acid is derived from the VEGP protein, VEG protein 2, LCN 1 or ALL 1 protein.

In another preferred embodiment the nucleic acid sequence encoding a mutein according to the invention comprises mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 43-49, 70-77, and 87-97 of the linear polypeptide sequence of human tear lipocalin, with the sequence positions corresponding to the positions 8, 9, 10, 11, 12, 13, 43, 45, 47, 70, 72, 74, 75, 90, 92, 94, and 97 of human tear lipocalin being particularly preferred.

In a further preferred embodiment the nucleic acid sequence encoding a mutein according to the invention comprises mutations at any two or more of the sequence positions in the peptide...
segments corresponding to the sequence positions 24-36, 53-66, 79-84, and 103-110 of the linear polypeptide sequence of human tear lipocalin.

Also preferred are nucleic acid molecules encoding a mutein of the invention comprising mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 43-49, 70-77, and 87-97 of the linear polypeptide sequence of human tear lipocalin mutations and additional mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 24-36, 53-66, 79-84, and 103-110 of the linear polypeptide sequence of human tear lipocalin.

The invention as disclosed herein also includes nucleic acid molecules encoding TLPc muteins, which comprise additional mutations outside the segments of experimental mutagenesis. Such mutations are often tolerated or can even prove to be advantageous, for example if they contribute to an improved folding efficiency, protein stability or ligand binding affinity of the mutein.

A nucleic acid molecule disclosed in this application may be "operably linked" to a regulatory sequence (or regulatory sequences) to allow expression of this nucleic acid molecule.

A nucleic acid molecule, such as DNA, is referred to as "capable of expressing a nucleic acid molecule" or capable "to allow expression of a nucleotide sequence" if it comprises sequence elements which contain information regarding to transcriptional and/or translational regulation, and such sequences are "operably linked" to the nucleotide sequence encoding the polypeptide. An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed are connected in a way that enables gene expression. The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions comprise a promoter which, in prokaryotes, contains both the promoter per se, i.e. DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5' non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5'-capping elements in eukaryotes. These regions can also include enhancer or repressor elements as well as translated signal and leader sequences for targeting the native polypeptide to a specific compartment of a host cell.
In addition, the 3’ non-coding sequences may contain regulatory elements involved in transcriptional termination, polyadenylation or the like. If, however, these termination sequences are not satisfactory functional in a particular host cell, then they may be substituted with signals functional in that cell.

Therefore, a nucleic acid molecule of the invention can include a regulatory sequence, preferably a promoter sequence. In another preferred embodiment, a nucleic acid molecule of the invention comprises a promoter sequence and a transcriptional termination sequence. Suitable prokaryotic promoters are, for example, the tet promoter, the lacUV5 promoter or the T7 promoter. Examples of promoters useful for expression in eukaryotic cells are the SV40 promoter or the CMV promoter.

The nucleic acid molecules of the invention can also be comprised in a vector or any other cloning vehicles, such as plasmids, phagemids, phage, baculovirus, cosmids or artificial chromosomes. In a preferred embodiment, the nucleic acid molecule is comprised in a phasmid. A phasmid vector denotes a vector encoding the intergenic region of a temperent phage, such as M13 or f1, or a functional part thereof fused to the cDNA of interest. After superinfection of the bacterial host cells with such an phagemid vector and an appropriate helper phage (e.g. M13K07, VCS-M13 or R408) intact phage particles are produced, thereby enabling physical coupling of the encoded heterologous cDNA to its corresponding polypeptide displayed on the phage surface (reviewed, e.g., in Kay, B.K. et al. (1996) Phage Display of Peptides and Proteins - A Laboratory Manual, 1st Ed., Academic Press, New York NY; Lowman, H. B. (1997) Annu. Rev. Biophys. Biomol. Struct. 26, 401–424 or Rodi, D. J. & Makowski, L. (1999) Curr. Opin. Biotechnol. 10, 87–93).

Such cloning vehicles can include, aside from the regulatory sequences described above and a nucleic acid sequence encoding a lipocalin mutein of the invention, replication and control sequences derived from a species compatible with the host cell that is used for expression as well as selection markers conferring a selectable phenotype on transformed or transfected cells. Large numbers of suitable cloning vectors are known in the art, and are commercially available.

The DNA molecule encoding lipocalin muteins of the invention, and in particular a cloning vector containing the coding sequence of such a lipocalin mutein can be transformed into a host cell capable of expressing the gene. Transformation can be performed using standard techniques
(Sambrook, J. et al. (1989), *supra*). Thus, the invention is also directed to a host cell containing a nucleic acid molecule as disclosed herein.

The transformed host cells are cultured under conditions suitable for expression of the nucleotide sequence encoding a fusion protein of the invention. Suitable host cells can be prokaryotic, such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*, or eukaryotic, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, SF9 or High5 insect cells, immortalized mammalian cell lines (e.g. HeLa cells or CHO cells) or primary mammalian cells.

The invention also relates to a method for the generation of a mutein according to the invention or a fusion protein thereof, comprising:

(a) subjecting a nucleic acid molecule encoding a tear lipocalin of a functional homologue thereof, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, to mutagenesis at two or more different nucleotide triplets, resulting in one or more mutein nucleic acid molecules(s);

(b) expressing the one or more mutein nucleic acid molecule(s) obtained in (a) in a suitable expression system, and

(c) enriching at least one mutein having a detectable binding affinity for a given target by means of selection and/or isolation.

In further embodiments of this method, the nucleic acid molecule can be individually subjected to mutagenesis at two or more different nucleotide triplets in any one, two, three or all four above-mentioned peptide segments arranged at either end of the β-barrel structure.

In the method of generation a mutein or a fusion protein thereof is obtained starting from the nucleic acid encoding tear lipocalin or a functional homologue thereof, which is subjected to mutagenesis and introduced into a suitable bacterial or eukaryotic host organism by means of recombinant DNA technology (as already outlined above).

The coding sequence of, for example, human tear lipocalin (Redl, B. et al. (1992) *J. Biol. Chem.* 267, 20282-20287) can serve as a starting point for mutagenesis of the peptide segments selected in the present invention. For the mutagenesis of the amino acids in the N-terminal peptide stretch and the three peptide loops BC, DE, and FG at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket as well as the four peptide loops AB, CD, EF, and
GH encompassing said binding pocket, the person skilled in the art has at his disposal the various established standard methods for site-directed mutagenesis (Sambrook, J. et al. (1989), supra). A commonly used technique is the introduction of mutations by means of PCR (polymerase chain reaction) using mixtures of synthetic oligonucleotides, which bear a degenerate base composition at the desired sequence positions. The use of nucleotide building blocks with reduced base pair specificity, as for example inosine, is another option for the introduction of mutations into a chosen sequence segment. A further possibility is the so-called triplet-mutagenesis. This method uses mixtures of different nucleotide triplets each of which codes for one amino acid for the incorporation into the coding sequence.

One possible strategy for introducing mutations in the selected regions of the respective polypeptides is based on the use of four oligonucleotides, each of which is partially derived from one of the corresponding sequence segments to be mutated (cf. Fig. 3). When synthesizing these oligonucleotides, a person skilled in the art can employ mixtures of nucleic acid building blocks for the synthesis of those nucleotide triplets which correspond to the amino acid positions to be mutated so that codons encoding all natural amino acids randomly arise, which at last results in the generation of a lipocalin peptide library. For example, the first oligonucleotide corresponds in its sequence - apart from the mutated positions - to the coding strand for the peptide segment to be mutated at the most N-terminal position of the lipocalin polypeptide. Accordingly, the second oligonucleotide corresponds to the non-coding strand for the second sequence segment following in the polypeptide sequence. The third oligonucleotide corresponds in turn to the coding strand for the corresponding third sequence segment. Finally, the fourth oligonucleotide corresponds to the non-coding strand for the fourth sequence segment. A polymerase chain reaction can be performed with the respective first and second oligonucleotide and separately, if necessary, with the respective third and fourth oligonucleotide.

The amplification products of both of these reactions can be combined by various known methods into a single nucleic acid comprising the sequence from the first to the fourth sequence segments, in which mutations have been introduced at the selected positions. To this end, both of the products can for example be subjected to a new polymerase chain reaction using flanking oligonucleotides as well as one or more mediator nucleic acid molecules, which contribute the sequence between the second and the third sequence segment. This procedure is schematically reproduced in Fig. 3. In the choice of the number and arrangement within the sequence of the oligonucleotides used for the mutagenesis, the person skilled in the art has numerous alternatives at his disposal.
The nucleic acid molecules defined above can be connected by ligation with the missing 5'- and 3'-sequences of a nucleic acid encoding a lipocalin polypeptide and/or the vector, and can be cloned in a known host organism. A multitude of established procedures are available for ligation and cloning (Sambrook, J. et al. (1989), supra). For example, recognition sequences for restriction endonucleases also present in the sequence of the cloning vector can be engineered into the sequence of the synthetic oligonucleotides. Thus, after amplification of the respective PCR product and enzymatic cleavage the resulting fragment can be easily cloned using the corresponding recognition sequences.

Longer sequence segments within the gene coding for the protein selected for mutagenesis can also be subjected to random mutagenesis via known methods, for example by use of the polymerase chain reaction under conditions of increased error rate, by chemical mutagenesis or by using bacterial mutator strains. Such methods can also be used for further optimization of the target affinity or specificity of a lipocalin mutein. Mutations possibly occurring outside the segments of experimental mutagenesis are often tolerated or can even prove to be advantageous, for example if they contribute to an improved folding efficiency or folding stability of the lipocalin mutein.

After expression of the nucleic acid sequences that were subjected to mutagenesis in an appropriate host, the clones carrying the genetic information for the plurality of respective lipocalin muteins, which bind a given target can be selected from the library obtained. Well known techniques can be employed for the selection of these clones, such as phage display (reviewed in Kay, B.K. et al. (1996) supra; Lowman, H. B. (1997) supra or Rodi, D. J. & Makowski, L. (1999) supra), colony screening (reviewed in Pini, A. et al. (2002) Comb. Chem. High Throughput Screen. 5, 503-510), ribosome display (reviewed in Amstutz, P. et al. (2001) Curr. Opin. Biotechnol. 12, 400-405) or mRNA display as reviewed in Wilson, D.S. et al. (2001) Proc. Natl. Acad. Sci. USA 98, 3750-3755.

An embodiment of the phage display technique (reviewed in Kay, B.K. et al. (1996) supra; Lowman, H. B. (1997) supra or Rodi, D. J. & Makowski, L. (1999) supra) using temperent M13 phage is given as an example of a selection method according to the invention. However, it is noted that other temperent phage such as f1 or lytic phage such as T7 may be employed as well. For the exemplary selection method, M13 phagemids (cf. also above) are produced which allow the expression of the mutated lipocalin nucleic acid sequence as a fusion protein with a signal
sequence at the N-terminus, preferably the OmpA-signal sequence, and with the capsid protein pIII of the phage M13 or fragments thereof capable of being incorporated into the phage capsid at the C-terminus. The C-terminal fragment ΔpIII of the phage capsid protein comprising amino acids 217 to 406 of the wild-type sequence is preferably used to produce the fusion proteins. Especially preferred is a C-terminal fragment of pIII, in which the cysteine residue at position 201 is missing or is replaced by another amino acid.

The fusion protein may comprise additional components such as an affinity tag, which allows the immobilization and/or purification of the fusion protein or its parts. Furthermore, a stop codon can be located between the sequence regions encoding the lipocalin or its muteins and the phage capsid gene or fragments thereof, wherein the stop codon, preferably an amber stop codon, is at least partially translated into an amino acid during translation in a suitable suppressor strain.

For example, the phagemid vector pTLPC7 (Fig. 1) can be used for the construction of a phage library encoding human tear lipocalin muteins. The inventive nucleic acid molecules coding for the mutated peptide segments are inserted into the vector using the BsrXI restriction sites. Recombinant vectors are then transformed into a suitable host strain such as E. coli XL1-Blue. The resulting library is subsequently superinfected in liquid culture with an appropriate M13-helper phage in order to produce functional phage. The recombinant phagemid displays the lipocalin mutein on its surface as a fusion with the coat protein pIII or a fragment thereof, while the N-terminal signal sequence of the fusion protein is normally cleaved off. On the other hand, it also bears one or more copies of the native capsid protein pIII supplied by the helper phage and is thus capable of infecting a recipient, in general a bacterial strain carrying a F- or F'-plasmid. During or after infection gene expression of the fusion protein comprised of the lipocalin mutein and the capsid protein pIII can be induced, for example by addition of anhydrotetracycline. The induction conditions are chosen such that a substantial fraction of the phage obtained displays at least one lipocalin mutein on their surface. Various methods are known for isolating the phage, such as precipitation with polyethylene glycol. Isolation typically occurs after an incubation period of 6-8 hours.

The isolated phage are then subjected to a selection process by incubating them with a given target, wherein the target is present in a form allowing at least a temporary immobilization of those phage displaying muteins with the desired binding activity. Several immobilization methods are known in the art. For example, the target can be conjugated with a carrier protein such as serum albumin and be bound via this carrier to a protein-binding surface such as
polystyrene. Microtiter plates suitable for ELISA techniques or so-called "immunosticks" are preferred. Alternatively, conjugates of the target can also be implemented with other binding groups such as biotin. The target can then be immobilized on surfaces, which will selectively bind this group, such as microtiter plates or paramagnetic particles coated with avidin or streptavidin.

For example, the phage particles are captured by binding to the respective target immobilized on the surface. Unbound phage particles are subsequently removed by iterative washing. For the elution of bound phage, free target (ligand) molecules can be added to the samples as a competitor. Alternatively, elution can also be achieved by adding proteases or under moderately denaturing conditions, e.g. in the presence of acids, bases, detergents or chaotropic salts. A preferred method is the elution using buffers having pH 2.2, followed by neutralization of the solution. The eluted phage may then be subjected to another selection cycle. Preferably, selection is continued until at least 0.1 % of the clones comprise lipocalin muteins with detectable affinity for the respective target. Depending on the complexity of the library employed 2-8 cycles are required to this end.

For the functional analysis of the selected lipocalin muteins, an E. coli host strain is infected with the phagemids obtained and phagemid DNA is isolated using standard techniques (Sambrook, J. et al. (1989), supra). The mutated sequence fragment or the entire lipocalin mutein nucleic acid sequence can be sub-cloned in any suitable expression vector. The recombinant lipocalin muteins obtained can be purified from their host organism or from a cell lysate by various methods known in the art such as gel filtration or affinity chromatography.

However, the selection of lipocalin muteins can also be performed using other methods well known in the art. Furthermore, it is possible to combine different procedures. For example, clones selected or at least enriched by phage display can subsequently be subjected to a colony-screening assay in order to directly isolate a particular lipocalin mutein with detectable binding affinity for a given target. Additionally, instead of generating a single phage library comparable methods can be applied in order to optimize a mutein with respect to its affinity or specificity for the desired target by repeated, optionally limited mutagenesis of its coding nucleic acid sequence.

The invention also relates to a method for the production of a mutein of the invention, wherein the mutein, a fragment of the mutein or a fusion protein of the mutein and another polypeptide is produced starting from the nucleic acid coding for the mutein by means of genetic engineering methods. The method can be carried out in vivo, the mutein can for example be produced in a
bacterial or eucaryotic host organism and then isolated from this host organism or its culture. It is also possible to produce a protein in vitro, for example by use of an *in vitro* translation system.

When producing the mutein *in vivo* a nucleic acid encoding a mutein of the invention is introduced into a suitable bacterial or eucaryotic host organism by means of recombinant DNA technology (as already outlined above). For this purpose, the host cell is first transformed with a cloning vector comprising a nucleic acid molecule encoding a mutein of the invention using established standard methods (Sambrook, J. et al. (1989), *supra*). The host cell is then cultured under conditions, which allow expression of the heterologous DNA and thus the synthesis of the corresponding polypeptide. Subsequently, the polypeptide is recovered either from the cell or from the cultivation medium. Since many lipocalins comprise intramolecular disulfide bonds, it can be preferred to direct the polypeptide to a cell compartment having an oxidizing redox-milieu using an appropriate signal sequence. Such an oxidizing environment is provided in the periplasm of Gram-negative bacteria such as *E. coli* or in the lumen of the endoplasmatic reticulum of eukaryotic cells and usually favors the correct formation of the disulfide bonds. It is, however, also possible to generate a mutein of the invention in the cytosol of a host cell, preferably *E. coli*. In this case, the polypeptide can, for instance, be produced in form of inclusion bodies, followed by renaturation *in vitro*. A further option is the use of specific host strains having an oxidizing intracellular milieu, which thus allow the production of the native protein in the cytosol.

However, a mutein of the invention may not necessarily be generated or produced only by use of genetic engineering. Rather, a lipocalin mutein can also be obtained by chemical synthesis such as Merrifield solid phase polypeptide synthesis. It is for example possible that promising mutations are identified using molecular modeling and then to synthesize the wanted (designed) polypeptide *in vitro* and investigate the binding activity for a given target.

The invention also relates to a pharmaceutical composition comprising at least one inventive mutein or a fusion protein thereof and a pharmaceutically acceptable excipient.

The lipocalin muteins according to the invention can be administered via any parenteral or non-parenteral (enteral) route that is therapeutically effective for proteinaceous drugs. Parenteral application methods comprise, for example, intracutaneous, subcutaneous, intramuscular or intravenous injection and infusion techniques, e.g. in the form of injection solutions, infusion solutions or tinctures, as well as aerosol installation and inhalation, e.g. in the form of aerosol mixtures, sprays or powders. Non-parenteral delivery modes are, for instance, orally, e.g. in the
form of pills, tablets, capsules, solutions or suspensions, or rectally, e.g. in the form of suppositories. The muteins of the invention can be administered systemically or topically in formulations containing conventional non-toxic pharmaceutically acceptable excipients or carriers, additives and vehicles as desired.

In a preferred embodiment of the present invention the pharmaceutical is administered parenterally to a mammal, and in particular to humans, with aerosol installation being the most preferable application method due to the low molecular weight of the muteins.

Accordingly, the muteins of the present invention can be formulated into compositions using pharmaceutically acceptable ingredients as well as established methods of preparation (Gennaro, A.L. and Gennaro, A.R. (2000) Remington: The Science and Practice of Pharmacy, 20th Ed., Lippincott Williams & Wilkins, Philadelphia, PA). To prepare the pharmaceutical compositions, pharmaceutically inert inorganic or organic excipients can be used. To prepare e.g. pills, powders, gelatin capsules or suppositories, for example, lactose, talc, stearic acid and its salts, fats, waxes, solid or liquid polyols, natural and hardened oils. Suitable excipients for the production of solutions, suspensions, emulsions, aerosol mixtures or powders for reconstitution into solutions or aerosol mixtures prior to use include water, alcohols, glycerol, polyols, and suitable mixtures thereof as well as vegetable oils.

The pharmaceutical composition may also contain additives, such as, for example, fillers, binders, wetting agents, glidants, stabilizers, preservatives, emulsifiers, and furthermore solvents or solubilizers or agents for achieving a depot effect. The latter is that fusion proteins may be incorporated into slow or sustained release or targeted delivery systems, such as liposomes and microcapsules.

The formulations can be sterilized by numerous means, including filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile medium just prior to use.

As is evident from the above disclosure, a mutein of the present invention or a fusion protein or a conjugate thereof can be employed in many applications. In general, such a mutein can be used in all applications antibodies are used, except those with specifically rely on the glycosylation of the Fc part.
A mutein of the invention can also be used for the targeting of a compound to a preselected site. For such a purpose the mutein is contacted with the compound of interest in order to allow complex formation. Then the complex comprising the mutein and the compound of interest are delivered the preselected site. This use is in particular suitable, but not restricted to, for delivering a drug (selectively) to the site such an infected body part or organ which is supposed to be treated with the drug.

Another use of the inventive muteins is the binding/detection of a given target or target molecule, comprising contacting the mutein with a test sample supposed to contain said target, and detecting of the mutein/target complex by a suitable signal. A mutein can also be used for the separation of a given target, comprising contacting the mutein with a sample supposed to contain said target in order to allow complex formation, and separating the mutein/target complex from the sample. In such uses the complex comprising the mutein and the target may be immobilized on any suitable solid phase.

The detectable signal can be caused by a label, as explained above, or by a change of physical properties due to the binding, i.e. the complex formation, itself. One example is plasmon surface resonance, the value of which is changed during binding of binding partners from which one is immobilized on a surface such as a gold foil.

The muteins disclosed herein and its derivatives can thus be used in many fields similar to antibodies or fragments thereof. In addition to their use for binding to a support, allowing the target of a given mutein or a conjugate or a fusion protein of this target to be immobilized or separated, the muteins can be used for labeling with an enzyme, an antibody, a radioactive substance or any other group having biochemical activity or defined binding characteristics. By so doing, their respective targets or conjugates or fusion proteins thereof can be detected or brought in contact with them. For example, muteins of the invention can serve to detect chemical structures by means of established analytical methods (e.g. ELISA or Western Blot) or by microscopy or immunosensorsics. Here, the detection signal can either be generated directly by use of a suitable mutein conjugate or fusion protein or indirectly by immunochemical detection of the bound mutein via an antibody.

Numerous possible applications for the inventive muteins also exist in medicine. In addition to their use in diagnostics and drug delivery, a mutant polypeptide of the invention, which binds, for example, tissue- or tumor-specific cellular surface molecules can be generated. Such a mutein
may, for example, be employed in conjugated form or as a fusion protein for "tumor imaging" or directly for cancer therapy.

Another related and preferred use of a mutein described herein is target validation, i.e. the analysis whether a polypeptide assumed to be involved in the development or progress of a disease or disorder is indeed somehow causative of that disease or disorder. This use for validating a protein as a pharmacological drug target takes advantage of the ability of a mutein of the present invention to specifically recognize a surface area of a protein in its native conformation, i.e. to bind to a native epitope. In this respect, it is to be noted that this ability has been reported only for a limited number of recombinant antibodies. However, the use of an inventive mutein for validation of a drug target is not limited to the detection of proteins as targets, but also includes the detection of protein domains, peptides, nucleic acid molecules, organic molecules or metal complexes.

The invention is further illustrated by the following Figures and Examples in which.

**Figure 1** schematically depicts the phagemid vector pTLPC7;

**Figure 2** schematically depicts the phasmid vector pTLPC6;

**Figure 3** schematically illustrates the generation of the library of tear lipocalin muteins at the nucleic acid level;

**Figure 4** schematically depicts the structure of the lipocalin fold;

**Figure 5** schematically depicts the expression vector pASK75-strepII-CD47;

**Figure 6** shows the polypeptide sequence of mature human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424); and

**Figure 7** shows a schematic drawing of the expression vector pBBP46.

**Fig. 1** shows a schematic drawing of the vector pTLPC7 encoding a fusion protein comprised of the OmpA signal sequence (OmpA), a modified TLPC with the amino acid substitutions Ala5Asp, Ser6Gly, Asp7Gly, Cys101Ser, and Glu104Gln (for the TLPC cDNA, see Redl et al.,
supra) and a truncated form of the M13 coat protein pIII, comprising amino acids 217 to 406 (pIII). Gene expression is under the control of the tetracycline promoter/operator (tetO) system. Transcription is terminated at the lipoprotein transcription terminator (tLPP). The vector further comprises an origin of replication (ori), the intergenic region of the filamentous phage f1 (f1-IG), the ampicillin resistance gene (bla) coding for β-lactamase and the tetracycline repressor gene (tetR). An amber stop codon, which is partially translated into Gln in SupE amber suppressor host strain, is located between the TLPC coding region and the coding region for the truncated phage coat protein pIII. Both the BstXI-restriction sites used for the cloning of the mutated gene cassette and the restriction sites flanking the structural gene are labeled. The nucleic acid sequence of a XbaI-HindIII segment of pTLPC7 is shown together with the encoded amino acid sequence in the sequence listing as SEQ ID NO: 1. The vector sequence outside this region is identical with that of pASK75, the complete nucleotide sequence of which is given in the German patent publication DE 44 17 598 A1.

Fig. 2 shows a schematic drawing of the vector pTLPC6. pTLPC6 encodes a fusion protein comprised of the OmpA signal sequence, a modified TLPC according to Fig. 1, and the Streptag® II affinity tag. Otherwise, the vector is identical to pTLPC7. The nucleic acid sequence of a XbaI-HindIII segment of pTLPC6 is shown together with the encoded amino acid sequence in the sequence listing as SEQ ID NO: 2. The vector sequence outside this region is identical with that of pASK75, the complete nucleotide sequence of which is given in the German patent publication DE 44 17 598 A1.

Fig. 3 schematically shows a strategy for the concerted mutagenesis of 17 selected amino acid positions in the modified TLPC by repeated polymerase chain reaction (PCR). For the sequence near the N-terminus as well as for each of the three peptide loops BC, DE, and FG, respectively, in which the amino acids are to be mutated, an oligodeoxynucleotide was synthesized, (SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6), bearing random nucleotides as indicated in the sequence listing. Due to the composition chosen, from the altogether three possible stop codons only the amber stop codon, TAG, was allowed at the mutated codons, which is translated as glutamine in the E. coli supE strains XL1-blue (Bullock et al. (1987) BioTechniques 5, 376-378) or TG1 (Sambrook et al., supra). For certain applications, for example gene expression in other bacterial strains or organisms, such a nonsense codon can be substituted by a glutamine-encoding codon, e.g., by site-directed mutagenesis. A nucleic acid fragment with 159 base pairs was amplified (PCR No. 1, A) with the respective primers SEQ ID NO: 3 and SEQ ID NO: 4 using the pTLPC6 plasmid-DNA (SEQ ID NO: 2) as a template. In another PCR, a
nucleic acid fragment with 123 base pairs was amplified (PCR no. 1, B) with the primers SEQ ID NO: 5 and SEQ ID NO: 6, respectively, also using pTLPC6 as template. The mixture of both PCR products served as a template in another amplification (PCR No. 2) with the two 5'-biotinylated flanking PCR primers, namely SEQ ID NO: 7 and SEQ ID NO: 8, and a mediating primer SEQ ID NO: 9, resulting in the amplification of a DNA fragment of 341 base pairs. This fragment comprising a mixture of all 17 mutated codons was subsequently cloned into the vector pTPLC7 using the two BstXI restriction sites, the special arrangement of which led to two non-compatible overhanging DNA ends enabling a particularly efficient ligation. The ligation efficiency could be improved by purification of the digested PCR-fragment by paramagnetic streptavidin coated beads. The amino acid substitution Glu104Gln as well as the silent mutations in the codon for Ala-3 of the ompA signal sequence, in the codon for Ala21 and His106 were previously accomplished during the construction of pTLPC6 in order to introduce both of the BstX1 restriction sites into the TLPC coding sequence.

Fig. 4 schematically illustrates the characteristic features of the lipocalin fold (according to Flower, D.R. (1996), supra). The eight β-strands of the antiparallel β-sheet which form the β-barrel) are shown as arrows and labeled A to H (a ninth β-strand, designated I which is additionally present in some lipocalins, is also schematically shown). The hydrogen-bonded connection of two strands is indicated by a pair of dotted lines between them. The connecting loops are shown as solid curved lines. The two ends of the β-barrel are topologically distinct. One end has four β-hairpins (loops AB, CD, EF and GH), the opening of the known ligand binding site of the lipocalins is here and called the open end. The other end of the β-barrel has three loops (BC, DE and FG) which together with the N-terminal polypeptide region build the closed end and are used in the present invention to introduce an alternative binding site. The parts which form the three main structurally conserved regions (SCRs) of the fold, SCR1, SCR2 and SCR3, are marked as boxes.

Fig. 5 shows a schematic drawing of the expression vector pASK75-strepII-CD47. pASK75-strepII-CD47 codes for a fusion protein made of the OmpA signal sequence, a modified extracellular domain of human CD47 comprising amino acids 1 to 119 of the mature protein with the amino acid substitution Cys15 to Ala and the Strep-tag® II affinity tag. All further genetic elements are identical with the generic vector pASK75. A relevant segment from the nucleic acid sequence of pASK75-strepII-CD47 is reproduced together with the encoded amino acid sequence in the sequence listing as SEQ ID NO: 10. The segment begins with the XbaI restriction site and ends with the HindIII restriction site. The vector elements outside this region are identical with
the vector pASK75, the complete nucleotide sequence of which is exhibited in the German patent publication DE 44 17 598 A1.

**Fig. 6** shows the polypeptide sequence of mature human tear lipocalin (SWISS-PROT Data Bank Accession Number M90424, 158 amino acids, cf. also Redl B. (2000) *Biochim. Biophys. Acta, supra*). In this respect it is noted that a human protein that was modified as follows was used in the following examples for the generation of lipocalin muteins. First, the first four N-terminal amino acid residues of the deposited sequence of human tear lipocalin (HHELL) were deleted. Second, the last two C-terminal amino acid residues (SD) were also deleted. Third, the wild type sequence at sequence positions 5 to 7 (ASD) was changed to GGD. These changes are reflected in the attached sequence listings, in which the amino acids GGD are indicated as first three residues of the used tear lipocalin. The four segments (AB, CD, EF and GH) at the open end of the β-barrel in which amino acids are exchanged are marked below the sequence of TLPC by double underlining. The segments BC, DE and FG as well as the N-terminal peptide stretch in which mutations are introduced to create a binding site at the closed end of the β-barrel are marked in bold and single underlining. The sequences position of TLPC which are mutated in the examples are additionally labelled with asterisks.

**Fig. 7** shows a schematic drawing of the expression vector pBBP46. pBBP46 codes for a fusion protein of the OmpA signal sequence and the T7 detection tag (T7) with a modified bilin binding protein of *Pieris brassicae* (cf. SEQ ID NO: 11) including the C-terminal Strep-tag® II. This structural gene is followed by the dsbC structural gene (including its ribosomal binding site) from *E. coli* (Zapun et al., Biochemistry 34 (1995), 5075-5089) as a second cistron (dsbC). A relevant segment of the nucleic acid sequence of pBBP46 is reproduced together with the encoded amino acid sequence in the sequence listing as SEQ ID NO: 11. The segment begins with the *Xba*I restriction site and ends with the *Hind*III restriction site. The vector elements outside this region are identical with the vector pASK75, the complete nucleotide sequence of which is exhibited in the German patent publication DE 44 17 598 A1.

**Examples**

**Example 1: Generation of a library with about 10 billion independent TLPC muteins**

Unless otherwise indicated, established recombinant genetic methods were used, for example as described in Sambrook et al. (*supra*).
A random library of TLPC with high complexity was prepared by concerted mutagenesis of in total 17 selected amino acid positions near the N-terminus and in the peptide loops BC, DE as well as FG using PCR in multiple steps according to Fig. 3. The PCR reactions were performed in a volume of 100 µl in both of the first amplification steps (PCR No. 1, A and B), wherein 10 ng pTLPC6 plasmid-DNA (Fig. 1, SEQ ID NO: 2) was employed as template together with 50 pmol of each pair of primers (SEQ ID NO: 3 and SEQ ID NO: 4 or SEQ ID NO: 5 and SEQ ID NO: 6, respectively), which were synthesized according to the conventional phosphoramidite method. In addition, the reaction mixture contained 10 µl 10 x Taq buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂, 1% v/v Triton X-100) and 2 µl dNTP-Mix (10 mM dATP, dCTP, dGTP, dTTP). After bringing to volume with water, 5 U Taq DNA-polymerase (5 U/µl, Promega) were added and 20 cycles of 1 minute at 94°C, 1 minute at 62°C and 1.5 minutes at 72°C were carried out in a thermocycler with a heated lid (Eppendorf), followed by an incubation for 5 minutes at 60°C for final extension. The desired amplification products were isolated by preparative agarose gel electrophoresis from GTQ Agarose (Roth) using the Jetsorb DNA extraction kit (Genomed).

For the subsequent amplification step a 2000 µl mixture was prepared, wherein approximately 1000 fmol of both of these respective fragments were used as templates, in the presence of 1000 pmol of each of the assembly primers SEQ ID NO: 7, SEQ ID NO: 8 and 20 pmol of the mediating primer SEQ ID NO: 9. Both assembly primers had a biotin group at their 5'-ends allowing the purification of the PCR-product after BstXI cleavage via streptavidin-coated paramagnetic beads. Additionally, 200 µl 10 x Taq buffer, 40 µl dNTP-Mix (10 mM dATP, dCTP, dGTP, dTTP), 100 u Taq DNA-polymerase (5 U/µl, Promega) and water were added to bring the mixture to the final volume of 2000 µl. The mixture was divided into 100 µl aliquots and PCR was performed with 20 cycles of 1 minute at 94°C, 1 minute at 60°C, 1.5 minutes at 72°C, followed by a subsequent incubation for 5 minutes at 60°C. The PCR product was purified using the E.Z.N.A. Cycle-Pure Kit (PeqLab).

For cloning purposes, this fragment representing the library of TPLC muteins in nucleic acid form was first cut with the restriction enzyme BstXI (Promega) according to the instructions of the manufacturer and then purified by preparative agarose gel electrophoresis as described above, resulting in a double stranded DNA-fragment of 303 nucleotides in size. DNA-fragments not or incompletely digested were removed via their 5'-biotin tags using streptavidin-coated paramagnetic beads (Merck).
Therefore, 200 µl of the commercially available suspension of the paramagnetic particles in a concentration of 10 mg/ml were washed three times with 100 µl TE-buffer. The particles were then drained and mixed with 100 pmol of the DNA-fragment in 100 µl TE-buffer for 15 minutes at room temperature. The paramagnetic particles were collected at the wall of the Eppendorf vessel with the aid of a magnet and the supernatant containing the purified DNA fragment was recovered for further use in the following ligation reaction.

The DNA of the vector pTLPC7 (Fig. 2) was cut with BstXI as described above and the larger of the two resulting fragments (3907 bp) was purified by preparative agarose gel electrophoresis. For the ligation reaction, 5.99 µg (30 pmol) of the PCR fragment and 77.3 µg (30 pmol) of the vector fragment were incubated in the presence of 833 Weiss Units of T4 DNA ligase (Promega) in a total volume of 8330 µl (50 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) for 24 h at 16°C. The DNA in the ligation mixture was then precipitated by adding 208 µl yeast tRNA (10 mg/ml solution in H₂O (Roche)), 8330 µl 5 M ammonium acetate, and 33.3 ml ethanol. Incubation at RT for 1 h was followed by centrifugation (30 minutes, 16000 g, 4°C). The precipitate was washed with 5 ml ethanol (70% v/v, RT), centrifuged (10 minutes, 16000 g, 4°C), and air dried until the DNA pellet appeared glossy and uncolored. Finally, the DNA was dissolved to a final concentration of 200 µg/ml in a total volume of 416.5 µl water.

The preparation of electrocompetent E. coli XL1-Blue (Bullock et al., supra) was carried out according to the methods described by Tung and Chow (Trends Genet. 11 (1995), 128-129) and by Hengen (Trends Biochem. Sci. 21 (1996), 75-76). 1 L LB-medium was adjusted to an optical density at 600 nm of OD₆₀₀ = 0.08 by addition of a stationary XL1-Blue overnight culture and was incubated at 140 rpm and 26°C in a 2 L Erlenmeyer flask. After reaching an OD₆₀₀ = 0.6, the culture was cooled for 30 minutes on ice and subsequently centrifuged for 15 minutes at 4000 g and 4°C. The cells were washed twice with 500 ml ice-cold 10% w/v glycerol and finally re-suspended in 2 ml of ice-cold GYT-medium (10% w/v glycerol, 0.125% w/v yeast extract, 0.25% w/v tryptone). The cells were then aliquoted (200 µl), shock-frozen in liquid nitrogen and stored at -80°C.

The Micro Pulser system (BioRad) was used in conjunction with cuvettes from the same vendor (electrode separation 2 mm) for electroporation. All steps were carried out at room temperature employing pre-chilled cuvettes at a temperature of -20°C. Each 10 µl of the DNA solution (2 µg) was mixed with 100 µl of the cell suspension, incubated for 1 minute on ice, and transferred to
the pre-chilled cuvette. Electroporation was performed (5 ms, 12.5 kV/cm) and the suspension was immediately diluted in 2 ml SOC-medium, followed by incubation for 60 minutes at 37°C and 140 rpm. Afterwards, the culture was diluted in 4 l 2 x YT-medium containing 100 µg/ml ampicillin (2 YT/Amp) resulting in an OD<sub>550</sub> of 0.26. By employing a total of 78.61 µg ligated DNA about 1.0 x 10<sup>10</sup> transformants were obtained in 42 electroporation runs.

**Example 2: Selection and identification of TLPC muteins with determinable binding affinity towards a given target**

The selection and identification of muteins of the invention with determinable binding affinity towards a given target can be carried in accordance with any protocol known to the skilled persons, for example, in accordance the respective protocols described in any of the PCT publications WO 99/16873, WO 00/75308, WO 03/029463 and WO 03/029471 as well the protocols described above. Principally, in a first step TLPC muteins can be presented and selected by means of phagemids to a given target that is immobilized on a solid phase. Then, selected muteins can be identified.

A more detailed suitable protocol for the generation of muteins that can bind to proteins is described in the following. As exemplary protein CD47 can be used.

**Example 2.1: Production and purification of the extracellular domain of CD47**

For the production of CD47 as a target protein for the isolation of CD47-specific TLPC muteins, *E. coli* JM83 (Yanisch-Perron et al. (1985) *Gene* 33, 103-119) was transformed with the expression plasmid pASK75-strepII-CD47 (Fig. 5) harboring the cDNA encoding the extracellular domain of CD47 (AS 1-119 of the mature protein) with a single mutation resulting in a Cys→Ala exchange at amino acid position 15 (for the CD47 cDNA, see Lindberg et al. (1993) *J. Cell Biol.* 123, 485-496). 100 ml LB-medium containing 100 µg/ml ampicillin (LB/Amp) was inoculated with a single colony of the JM83 transformant, and incubated overnight at 37°C, 160 rpm. Then, 2 l LB/Amp-medium in a 5 l-Erlenmeyer flask were inoculated with 40 ml of this pre-culture and were shaken at 26°C, 160 rpm to an OD<sub>550</sub> = 0.4. Subsequently, the temperature was lowered to 22°C and upon reaching an OD<sub>550</sub> = 0.5 the production of the recombinant protein was induced by adding 200 µg/l anhydro-tetracycline (200 µl of a 2 mg/ml stock solution in DMF) followed by shaking for 3-4 further hours at 22°C and 160 rpm.
The cells from one flask were centrifuged (10 minutes, 5500 g, 4°C) and re-suspended in 20 ml periplasmic release buffer (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA), followed by cooling on ice for 30 minutes. Subsequently, the spheroplasts were removed in two successive centrifugation steps (25 minutes, 5300 g, 4°C and 10 minutes, 27000 g, 4°C, respectively). The supernatant containing the periplasmic protein extract was dialyzed against SA-buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), sterile-filtered, and subjected to chromatographic purification.

The purification was performed by using the Strep-Tag® II-affinity tag (Skerra and Schmidt (2000) Methods Enzymol. 326, 271-304) located at the C-terminus of the CD47 protein and by employing Streptactin Superflow material (IBA). A chromatographic column with a bed volume of 8 ml was filled with this affinity matrix and equilibrated with 20 ml SA-buffer at 4°C at a flow rate of 60 ml/h. Chromatography was monitored by measuring the absorption at 280 nm of the eluate in a flow-through photometer. After application of the periplasmic protein extract, the column was washed with SA-buffer until reaching the base line and the bound CD47 was subsequently eluted with ca. 15 ml of a solution of 2.5 mM D-Desthiobiotin (IBA) in SA-buffer collecting fractions of the eluate. The fractions containing purified CD47 were analyzed via SDS-polyacrylamide gel electrophoresis (Fling und Gregerson (1986) Anal. Biochem. 155, 83-88), combined and dialyzed against an appropriate buffer, if needed. The protein yield was approximately 370 μg per 1 l culture.

**Example 2.2: Phagemid presentation and selection of TPLC muteins against the extracellular domain of human CD47 employing polystyrol sticks**

A 2 ml aliquot of the stored phagemids from Example 1 containing a suitable number of phagemids (for example 10^13 phagemids) can be centrifuged (30 minutes, 21460 g, 4°C), the supernatant can then be removed, and the sedimanted phagemid particles then dissolved in a buffer such as 1 ml PBS (4 mM KH2PO4, 16 mM Na2HPO4, 115 mM NaCl, pH 7.4). After incubation for 30 minutes on ice the solution is centrifuged (5 minutes, 18500 g, 4°C) to remove residual aggregates.

Immuno-sticks (NUNC) can be used for the affinity enrichment of the recombinant phagemids carrying the BBP mutein fusion proteins. These sticks are first coated for a suitable period of time (for instance) overnight at 4°C with 800 μl of the extracellular domain of human CD47 (CD47)
(100 µg/ml) in SA-buffer as a target protein. Unoccupied binding sites on the surface of the Immuno-Stick are then saturated by incubation with 1.2 ml 2 % w/v BSA in PBST (PBS with 0.1 % v/v Tween 20) for 2 hours at room temperature. Afterwards, the Immuno-stick can be briefly washed for example three or four times with 1.2 ml PBST and subsequently incubated with a mixture of 500 µl of the phagemid solution (ca. 5×10^{12} cfu) and of 250 µl of PBS containing 6 % w/v BSA and 0.3 % v/v Tween 20 for 1 hour at room temperature.

For the removal of unbound phagemids, repeated washing can be performed, for examples six to eight times, each time with 950 µl PBST for 2 minutes. Adsorbed phagemids are then finally eluted by treating the Immuno-stick for 10 minutes with 950 µl 0.1 M glycine/HCl pH 2.2, followed by immediate neutralisation of the pH of the elution fraction by mixing it with 150 µl 0.5 M Tris.

For the amplification, this phagemid solution (1.1 ml, containing between 10^6 and 10^8 cfu, depending on the selection cycle) can be shortly warmed to 37 °C, mixed with 3 ml of an exponentially growing culture of E. coli XL1-blue (OD_{550} = 0.5), and incubated for 30 minutes at 37 °C, 140 rpm. The cells infected with the phagemids are subsequently sedimented (2 minutes, 4420 g, 4 °C), resuspended in 600 µl of the culture medium, and plated out onto three agar plates with LB/Amp-medium (LB/Amp agar; 145 mm diameter).

After incubation for 14 hours at 32 °C, the cells are scraped from the agar plates, each with addition of 10 ml 2xYT/Amp, are transferred to a sterile Erlenmeyer-flask, and are shaken for 30 minutes at 37 °C, 140 rpm for complete suspension. For the repeated production and affinity enrichment of phagemid particles 50 ml of 2xYT/Amp can be inoculated to an OD_{550} of ca. 0.08 with an appropriate volume of this suspension and incubated at 37 °C, 160 rpm until OD_{550} reached 0.5.

After infection with VCS-M13 helper phage (Stratagene) at a moi of approximately 10 the culture can be shaken for additional 45 minutes at 37 °C, 140 rpm. Kanamycin (70 µg/ml) is subsequently added, the incubator temperature lowered to 26 °C and, after 10 minutes, anhydrotetracycline is added at 25 µg/l (6.2 µl of a 0.2 mg/ml stock solution in DMF) in order to induce gene expression. Incubation can continue for another 12-15 hours at 26 °C, 140 rpm.

The cells are sedimented by centrifugation (15 minutes, 12100 g, 4 °C) and the supernatant containing the phagemid particles sterile-filtered (0.45 µm), mixed with 1/4 volume (12.5 ml) 20
% w/v PEG 8000, 15 % w/v NaCl, and incubated on ice for 1 hour. After centrifugation (20 minutes, 18000 g, 4 °C) the precipitated phagemid particles are dissolved in 2 ml of cold BBS/E. The solution can be distributed to two 1.5 ml reaction vessels and incubated on ice for 30 minutes. After centrifugation of undissolved components (5 minutes, 21460 g, 4 °C) each supernatant is transferred to a new reaction vessel.

Mixture with 1/4 volume 20 % w/v PEG 8000, 15 % w/v NaCl and incubation for 30 to 60 minutes on ice can serve to reprecipitate the phagemid particles. After centrifugation (20 minutes, 21460 g, 4 °C) the supernatant is removed and the precipitated phagemid particles are dissolved and combined in a total of 1 ml PBS. After incubation for 30 minutes on ice the solution is centrifuged (5 minutes, 21460 g, 4 °C) in order to remove residual aggregates and the complete supernatant containing between 10^{11} and 5 \times 10^{12} phagemids is directly used for the next round of affinity enrichment. An appropriate number of further selection cycles (usually 4 or 5) with the CD47 can be carried out in this way.

**Example 2.3: Identification of CD47-binding BBP muteins by use of a high-throughput ELISA screening method**

For the analytical production of the TLPC muteins equipped with an N-terminal T7 detection tag (Novagen) as well as a C-terminal Strep-tag® II affinity tag and their characterization by high-throughput ELISA screening, a vector analogues to the vector the pBBP46 (Fig. 7) can be used. The gene cassette containing the TLPC between the two BstXI cleavage sites can be subcloned from the vector pTLPC7 into the vector analogues to pBBP46 (Fig. 7).

For this purpose the plasmid DNA is isolated from the mixture of the *E. coli* clones obtained by infection with the phagemids from Example 2.2 eluted as a result of the last selection cycle, using the Plasmid Miniprep Spin kit (Genomed). The DNA is cut with the restriction enzyme BstXI and the smaller of the two fragments is purified by preparative agarose-gel electrophoresis as described in Example 1. The DNA of the vector that is analogues to pBBP46 is likewise cut with BstXI and the larger of the two fragments (4165 bp) was isolated in the same way.

For the ligation, each 50 fmol of the two DNA-fragments are mixed with 3 Weiss Units T4 DNA ligase (Promega) in a total volume of 20 μl (30 mM Tris/HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP), followed by incubation for 2 h at 22 °C. *E. coli* TG1-F* (E. coli K12 TG1,
which had lost its episome) is transformed with 5 μl of this ligation mixture according to the CaCl$_2$-method (Sambrook et al., supra) and plated on LB/Amp agar plates (22 cm x 22 cm).

Single *E. coli* colonies obtained after the transformation harbouring the respective TLPC plasmids coding for the TLPC muteins are picked from these agar plates into 70 μl per well 2xYT/Amp in flat bottom 384 well plates (Greiner) by means of an automated colony picker (Genetix) and grown overnight at 37 °C at 700 rpm on a benchtop shaker (Bühler) in a humidified incubator (MMM Medcenter) at 60 % relative humidity (rH). The cultures can be diluted 1:100 into 100 μl 2xYT/Amp in round bottom 96 well plates (Nunc) by means of a 96 pin replicating head (Genetix) and grown for ca. 1 h at 37 °C and 60 % rH, followed by an incubation for 3 h at 22 °C and 60 % rH, both at 700 rpm, until the OD$_{550}$ reached approximately 0.6. The 384 well plates are kept as "master" plates at -80 °C after adding 25 μl 60 % v/v glycerol to each well.

Recombinant TLPC muteins are produced in the 96 well plates by adding 20 μl per well of 1.2 μg/ml anhydrated tetracyclin in 2xYT (obtained by diluting a 2 mg/ml stock solution 1:1667 in 2xYT; final concentration 0.2 μg/ml) to the bacterial cultures and incubation overnight at 22 °C and 700 rpm at 60 % rH. Afterwards, 40 μl of lysis buffer (400 mM Na-borate pH 8.0, 320 mM NaCl, 4 mM EDTA, 0.3 % w/v lysozyme) is added to each well and the plate was incubated for 1 h at 22 °C and 700 rpm at 60 % rH. To minimize non-specific binding interactions in the subsequent ELISA experiment, obtained crude cell extracts can be supplemented with 40 μl/well PBS containing 10 % w/v BSA and 0.05 % v/v Tween 20 (final concentration 2 % BSA) for 1 h at 22 °C and 700 rpm at 60 % rH.

For the detection of binding, the crude cell extracts containing the TLPC muteins are tested for their reactivity with the prescribed target protein CD47 and the unrelated control protein aldolase (Roche), respectively, in ELISA experiments. Therefore, wells of black Fluotrac 600 ELISA plates (Greiner; 384 well) are coated overnight with 20 μl of a solution of recombinant CD47 (produced as described in Example 2.1) or the control protein at 4 °C, each at a concentration of 20 μg/ml in PBS. Plates are washed five times with 100 μl PBS containing 0.05 % v/v Tween 20 (PBST/0.05) per well with an automated ELISA plate washer (Molecular Devices) leaving a residual volume of 10 μl of the washing buffer in each well after the last washing step. Residual binding sites are blocked by incubation with 100 μl PBST/0.05 containing 2 % w/v BSA for 2 h at room temperature. Afterwards, plates are again washed five times as described above.

For complex formation between the TLPC muteins and the immobilized proteins, the wells can be incubated with 10 μl of the cell extract from above for 1 hour at room temperature.
Subsequently, plates are washed again five times and 10 μl of an anti-T7 monoclonal antibody-HRP-conjugate (Amersham), diluted 1:5000 in PBST/0.05 containing 0.5 % w/v non-fat dry milk powder (Vitalia), is added to each well and incubated for 1 hour at room temperature. Plates are again washed five times and 10 μl of the fluorogenic HRP-substrate QuantaBli™ (Pierce, diluted as described by the manufacturer) is added to detect bound TPLC muteins by means of the attached anti-T7 monoclonal antibody-HRP-conjugate. After 60 minutes at room temperature fluorescence is excited at a wavelength of 320 nm (± 12.5 nm) and measured at 430 nm (± 17.5 nm) in a GENiosPlus plate reader (Tecan).

Those analyzed TPLC muteins that showed a binding signal on the prescribed target protein (CD47) which exceeded the binding signal on the unrelated control protein (aldolase) at least by a factor of three (signal-to-control ratio of at least 3) can be subsequently subjected to a secondary high-throughput ELISA screening experiment in order to study the binding specificity of these muteins in greater detail. Therefore, these clones are transferred from the 384 well master plates described above onto LB/Amp agar, and grown overnight at 37 °C. 100 μl 2xYT/Amp in round bottom 96 well plates (Nunc) was inoculated with single colonies from these agar plates and grown overnight at 37 °C at 700 rpm and 60 % rH. The cultures are diluted 1:100 into 100 μl 2xYT/Amp in round bottom 96 well plates (Nunc) and production of recombinant TPLC muteins as well as preparation of the bacterial lysates was performed as described above.

For the detection of target-specificity of the TPLC muteins, wells of black Fluotrac 600 ELISA plates (Greiner; 384 well) are coated overnight at 4 °C with 20 μl of a solution of recombinant CD47 or, as a control, with BSA (Roth), transferrin (Roche), aldolase (Roche), ovalbumin (Sigma) as well as a conjugate of RNaseA (Fluka) and digoxigenin, each at a concentration of 20 μg/ml in PBS.

This conjugate is prepared by reacting RNaseA at a twofold molar ratio of digoxigenin-3-O-methylcarbonyl-ε-amidocaproic acid-N-hydroxy-succinimide ester (DIG-NHS; Roche) according to the instructions of the manufacturer. Excess reactant is removed from the RNaseA-conjugate by means of size exclusion chromatography using a HiTrap column (Amersham) according to the instructions of the manufacturer employing PBS as running buffer.

After overnight incubation, the plates are washed as described above and blocked by the addition of 100 μl/well PBST/0.05 containing 2 % w/v BSA at the conditions described above, followed again by washing of the plates. 10 μl of the blocked bacterial lysates of the selected TPLC muteins mentioned above are transferred to each of the wells coated with either CD47 or the
unrelated control proteins and incubated for 1 h at ambient temperature. Bound TPLC muteins are detected with anti-T7 monoclonal antibody-HRP-conjugate and the fluorogenic HRP-substrate QuantaBlu™ as described above.

5 The nucleotide sequence of chosen TLPC clones can then be determined. Furthermore, these clones can then be chosen for the determination of their binding affinities for CD47.
What is claimed is:

1. A mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof, wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the N-terminal peptide stretch as well as in the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and wherein the mutein binds a given target with detectable affinity.

2. The mutein of claim 1, wherein the mutein is derived from human tear lipocalin.

3. The mutein of claim 1 or 2, wherein the mutein comprises amino acid mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 43-49, 70-77, and 87-97 of the linear polypeptide sequence of human tear lipocalin.

4. The mutein of any of claims 1 to 3, wherein the mutein comprises amino acid mutations at any two or more of the sequence positions corresponding to the sequence positions 8, 9, 10, 11, 12, 13, 43, 45, 47, 70, 72, 74, 75, 90, 92, 94, and 97 of the linear polypeptide sequence of human tear lipocalin.

5. A mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof, wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the four peptide loops AB, CD, EF, and GH encompassing the natural lipocalin binding pocket, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and wherein the mutein binds a given target with detectable affinity.

6. The mutein of claim 5, wherein the mutein is derived from human tear lipocalin.

7. The mutein of claim 5 or 6, wherein the mutein comprises amino acid mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 24-36, 53-66, 79-84, and 103-110 of the linear polypeptide sequence of human tear lipocalin.
8. The mutein of any of claims 5 to 7, further comprising at least two mutated amino acid residues at any sequence position in the N-terminal peptide stretch as well as the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket.

9. The mutein of claim 8, comprising amino acid mutations at any two or more of the sequence positions in the peptide segments corresponding to the sequence positions 7-14, 43-49, 70-77, and 87-97 of the linear polypeptide sequence of human tear lipocalin.

10. The mutein of claim 8 or 9, comprising amino acid mutations at any two or more of the sequence positions corresponding to the sequence positions 8, 9, 10, 11, 12, 13, 43, 45, 47, 70, 72, 74, 75, 90, 92, 94, and 97 of the linear polypeptide sequence of human tear lipocalin.

11. A mutein derived from a polypeptide of tear lipocalin or a functional homologue thereof, wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the N-terminal region as well as the three peptide loops BC, DE, and FG arranged at the end of the β-barrel structure that is located opposite of the natural lipocalin binding pocket,

   wherein the mutein comprises at least two mutated amino acid residues at any sequence position in the four peptide loops AB, CD, EF, and GH encompassing the natural lipocalin binding pocket,

   wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, and

   wherein the mutein binds at least one given target with detectable affinity.

12. The mutein of any of claims 1 to 10, wherein the mutein is conjugated to a label selected from the group consisting of organic molecules, enzyme labels, radioactive labels, colored labels, fluorescent labels, chromogenic labels, luminescent labels, haptens, digoxigenin, biotin, metal complexes, metals, and colloidal gold.

13. The mutein of any of claims 1 to 13, wherein the mutein is fused at its N-terminus or its C-terminus to a protein, a protein domain or a peptide.
14. A nucleic acid molecule comprising a nucleotide sequence encoding a mutein of any of claims 1 to 13.

15. The nucleic acid molecule of claim 15 comprised in a vector.

16. The nucleic acid molecule of claim 15 comprised in a phagemid vector.

17. A host cell containing a nucleic acid molecule of any of claims 14 to 16.

18. A method for the generation of a mutein of any of claims 1 to 13, comprising:
(a) subjecting a nucleic acid molecule encoding a tear lipocalin of a functional homologue thereof, wherein said tear lipocalin or functional homologue thereof has at least 60% sequence homology with human tear lipocalin, to mutagenesis at two or more different nucleotide triplets,
(b) expressing the at least one mutein nucleic acid molecules obtained in (a) in a suitable expression system, and
(c) enriching at least one mutein having a detectable binding affinity for a given target by means of selection and/or isolation.

19. A method for the production of a mutein according to any of claims 1 to 13, wherein the mutein, a fragment of the mutein or a fusion protein of the mutein and another polypeptide is produced starting from the nucleic acid coding for the mutein by means of genetic engineering methods.

20. The method of claim 19, wherein the mutein is produced in a bacterial or eukaryotic host organism and is isolated from this host organism or its culture.

21. A pharmaceutical composition comprising at least one mutein of any of claims 1 to 13.

22. Use of a mutein of any of claims 1 to 13 for the detection of a given target, comprising the steps of:
(a) contacting the mutein with a test sample supposed to contain said target, and
(b) detecting of the mutein/target complex by a suitable signal.
23. Use of claim 22, wherein the given target is a protein or protein domain, a peptide, a nucleic acid molecule, an organic molecule or a metal complex and the detection is carried out for validation of the protein as pharmacological drug target.

24. Use of a mutein of any of claims 1 to 13 for the separation of a given target, comprising:
   (a) contacting the mutein with a sample supposed to contain said target, and
   (b) separating the mutein/target complex from the sample.

25. The use of any of claim 22 to 24, wherein the mutein/target complex is bound onto a solid phase.

26. Use of a mutein of any of claims 1 to 13 for the targeting of a compound to a preselected site comprising
   (a) contacting the mutein with said compound, and
   (b) delivering the mutein/compound complex to the preselected site

27. Use of a mutein of any of claims 1 to 13 for complex formation with a given target.
pTLPC7
4210 bp

Fig. 1
pTLPC6
3667 bp

Fig. 2
PCR No. 1

SEQ ID NO: 3

SEQ ID NO: 4

PCR A

SEQ ID NO: 5

SEQ ID NO: 6

PCR B

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PCR No. 2

SEQ ID NO: 7

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BstXI

SEQ ID NO: 9

BstXI

PCR

179 519

Fig. 3
**Fig. 6**

1. HHLAS**DEET** QDVS**TAG**TWYLK AMTVDFEPF E MNLESVTPMT

41. LT**TLEGGNLE** AKVTMLISGR CQEVKAVLEK TDEPGKYTAD

81. GGKHVAY**TYR** SHV**KHD**YTFY CEGELHGBKV RGVKLVGRDP

121. KNNLEALEDF EKAAGARGLS TESILIPRQS ETCSPGSD 158

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**Fig. 4**

Diagram showing the structure with labeled parts and annotations. The diagram includes the following labels:

- AB
- CD
- DE
- EF
- GH
- FG
- SCR1
- SCR2
- SCR3
- N-terminal peptide stretch
- Al
- N
- Open end
- Closed end

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Figure 5

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**INTERNATIONAL SEARCH REPORT**

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, MEDLINE, SEQUENCE SEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>GASYMOROKTAY K ET AL: &quot;Site-directed tryptophan fluorescence reveals the solution structure of tear lipocalin: Evidence for features that confer promiscuity in ligand binding&quot; BIOCHEMISTRY, vol. 40, no. 49, 11 December 2001 (2001-12-11), pages 14754-14762, XP002269552 ISSN: 0006-2960 figure 4</td>
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Relevant to claim No.

1-17, 22-27

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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search: 22 March 2004

Date of mailing of the international search report: 08/04/2004

Name and mailing address of the SA:

European Patent Office, P.B. 3518 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2000, Tr. 31 551 epo nl
Fax (+31-70) 340-3016

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