Amino acid sequence presenting fusion polypeptide and its use

Herein is reported a fusion polypeptide comprising one or more fragments of one or more peptidyl-prolyl cis/trans isomerase or FKBP family members and its use in methods for antibody screening/selection, for epitope mapping as well as its use as immunogen for the production of antibodies specifically binding an immunogenic peptide or secondary structure presented by the fusion polypeptide.

Background of the Invention

In recent years the production of therapeutic antibodies has steadily increased and it is likely that therapeutic antibodies will become the biggest group of therapeutics available for the treatment of various diseases in the near future. The impact of therapeutic antibodies emerges from their specificity, such as the specific target recognition and binding function.

Antibodies can be obtained from an experimental animal that has been immunized with an immunogen. The immunogen is in most cases a polypeptide or a fragment of a polypeptide. To provide the immunogen in sufficient quantity and purity a recombinantly produced immunogen can be employed.

Generally prokaryotic and eukaryotic cells can be used for the recombinant production of polypeptides. The recombinant polypeptides can be obtained either in soluble form or as precipitate (inclusion body). Prior to chromatographic purification the insoluble polypeptide contained in the inclusion bodies has to be solubilized.

Generally the immunogen is a synthetic or a peptidic or a recombinantly produced or a fusion or a chimeric or a support conjugated polypeptide. For immunization the immunogen can be administered either alone or in combination with an adjuvant, such as Freud's adjuvant.

Knappe, T.A., et al. (J. Mol. Biol. 368 (2007) 1458-1468) reported that the Flap-region of FKBP12 can be replaced by the IF domain of the structurally related E.coli chaperone SlyD. The chimeric FKBP12-SlyD fusion polypeptide has a 200-times increase peptidyl-prolyl-cis/trans isomerase activity compared to the isolated polypeptide.

The E.coli SlyD and FKBP12 (wild type and mutants C23A and C23S) can be recombinantly produced in E.coli in high yield in soluble form (Standaert, R.F., et al., Nature 346 (1990) 671-674).

FKBP derived from thermophilic organisms and E.coli SlyD can be used as chaperons in the recombinant expression of fusion polypeptides in E.coli (Ideno, A., et al., Appl. Microbiol. Biotechnol. 64 (2004) 99-105). The E.coli SlyD and FKBP12 polypeptides are reversibly folding polypeptides (Scholz, C., et al., J. Biol. Chem. 271 (1996) 12703-12707).

The amino acid sequence of the FKBP12 polypeptide comprises a single tryptophan residue at position 60. Thus, FKBP12 mutants can be analyzed for structural integrity simply by analyzing the tryptophan fluorescence (DeCenzo, M.T., et al., Protein Eng. 9 (1996) 173-180). A test for remaining catalytic activity of the FKBP12 mutant can be performed by determining the remaining rotamase activity (Brecht, S., et al., Neuroscience 120 (2003) 1037-1048; Schories, B., et al., J. Pept. Sci. 13 (2007) 475-480; Timerman, A.P., et al., J. Biol. Chem. 270 (1995) 2451-2459). It is also possible to determine the structural integrity of FKBP12 mutants by determining the FK506- or Rapamycin binding (DeCenzo, M.T., et al., Protein Eng. 9 (1996) 173-180).

McNamara, A., et al. (J. Org. Chem. 66 (2001) 4585-4594) report peptides constrained by an aliphatic linkage between two C(alpha) sites: design, synthesis, and unexpected conformational properties of an i,(i+4)-linked peptide.

Suzuki, et al. (Suzuki, R., et al., J. Mol. Biol. 328 (2003) 1149-1160) report the three-dimensional solution structure of an archaic FKBP with a dual function of peptidyl-prolyl-cis-trans isomerase and chaperone-like activities. Expression vector, host, fused polypeptide, process for producing fused polypeptide and process for producing protein are reported in EP 1 516 928. Knappe, T. A., et al., reports that the insertion of a chaperone domain converts FKBP12 into a powerful catalyst of protein folding (J. Mol. Biol. 368 (2007) 1458-1468). A chimeric fusion polypeptide with superior chaperone and folding activities is reported in WO 2007/077008. In WO 03/000878 the use of FKBP chaperones as expression tool is reported. In EP 1 621 555 an immunogen, composition for immunological use, and method of producing antibody using the same are reported. Rebuzzini, G. (PhD work at the University of Milano-Bicocca (Italy) (2009)) reports a study of the

hepatitis C virus NS3 helicase domain for application in a chemiluminescent immunoassay.

In WO 2007/077008 chimeric fusion proteins with superior chaperone and folding activities are reported. The conversion of FKBP12 into a powerful catalyst of protein folding by insertion of a chaperone domain is reported by Knappe et al. (Knappe, T.A., et al., J. Mol. Biol. 368 (2007) 1458-1468).

Summary of the Invention

The fusion polypeptides as reported herein are fusion polypeptides comprising i) one or more parts derived from one, i.e. the same, or different polypeptides with PPIase activity or belonging to the FKBP family, and ii) an immunogenic polypeptide inserted therein in between.

The fusion polypeptides as reported herein can be used for the immunization of an animal for the generation of antibodies specifically binding to the immunogenic polypeptide inserted into the one or more parts derived from the one or more polypeptides with PPIase activity or belonging to the FKBP family.

One aspect as reported herein is a fusion polypeptide according to formula I

$$NH_2\text{-}S_2\text{-}X_1\text{-}S_1\text{-}COOH \qquad \text{(formula I)}$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are non-overlapping amino acid sequences derived from a second polypeptide, and

- denotes a peptide bond,

wherein the second polypeptide is a polypeptide with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or is derived from the FKBP domain family.

It has been found that with the fusion polypeptides as reported herein antibodies, which specifically bind to internal (so called hidden or buried) epitopes of a (naturally occurring) amino acid sequence, can be obtained. Internal epitopes are not accessible in classical immunization protocols as these are e.g. only accessible

upon activation and concomitant conformational changes of the antigenic polypeptide (such as a receptor). Furthermore, antibodies can be obtained that specifically bind to immunogenic polypeptides, which are derived from structures otherwise difficult to be provided in sufficient amount or quality.

The fusion polypeptides as reported herein are chimeric, recombinant polypeptides that can be used for peptide, secondary and tertiary structure display e.g. in methods for antibody screening/selection or for epitope mapping as well as immunogen for the production of antibodies specifically binding the presented antigenic amino acid sequence or secondary structure. The polypeptides as reported herein can be recombinantly produced, are thermodynamically stable, monomeric and soluble in aqueous solutions.

One aspect as reported herein is a fusion polypeptide according to formula II

$$NH_2\text{-}S_4\text{-}X_2\text{-}S_3\text{-}S_2\text{-}X_1\text{-}S_1\text{-}S_0\text{-}COOH \qquad \text{(formula II)}$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are non-overlapping amino acid sequences derived from a second polypeptide,

S_3 and S_0 are either absent or non-overlapping amino acid sequences derived from a third polypeptide,

S_4 is either absent or an amino acid sequence derived from a fourth polypeptide,

X_2 is either absent or a peptidic linker sequence, and

- denotes a peptide bond,

wherein the second polypeptide and the third polypeptide and the fourth polypeptide are different from each other and are polypeptides with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or which are derived from the FKBP domain family.

In one embodiment of all aspects as reported herein the second polypeptide with peptidyl-prolyl cis/trans-isomerase activity or derived from the FKBP domain family is SlyD.

In one embodiment of all aspects as reported herein the second polypeptide is a polypeptide from a thermophile.

In one embodiment the thermophile is a thermophile bacterium. In one embodiment the thermophile bacterium is from the family of Thermaceae. In one embodiment the thermophile is Thermus thermophilus.

In one embodiment the thermophile is a thermophile Archaea. In one embodiment the thermophile Archaea is a hyperthermophilic Archaea. In one embodiment the thermophile is from the class of Thermococci. In one embodiment the thermophile is Thermococcus gammatolerans

In one embodiment the thermophile has an optimal growth temperature of at least 60 °C.

In one embodiment of all aspects as reported herein the immunogenic sequence is comprised in the X_1 amino acid sequence. In one embodiment the X_1 amino acid sequences comprises the immunogenic sequence and one or more parts derived from a further polypeptide with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or derived from a further polypeptide from the FKBP-fold domain family, whereby the further polypeptide is different from the second polypeptide.

The amino acid sequence of X_1 is inserted in place of the insert-in-flap-domain (IF-domain) of the second polypeptide. Thus, if X_1 is identical to the IF-domain, i.e. has the amino acid sequence of the IF-domain, the fusion polypeptide S_2-X_1-S_1 is identical to the corresponding part of the naturally occurring second polypeptide.

In one embodiment of all aspects as reported herein the S_2 and S_1 amino acid sequences derived from a second polypeptide are connected (directly) to each other by the IF-domain in the wild-type (naturally occurring) second polypeptide.

In one embodiment of all aspects as reported herein X_1 is inserted in place of the insert-in-flap-domain (IF-domain) of the second polypeptide.

One aspect as reported herein is a polypeptide that has at least 70 % amino acid sequence identity to the polypeptide of formula I when determined excluding X_1, or

formula II when determined excluding X_1, X_2 and the absent sequences. In one embodiment the polypeptide has at least 80 % amino acid sequence identity. In one embodiment the polypeptide has at least 90 % amino acid sequence identity. In one embodiment the polypeptide has at least 95 % amino acid sequence identity. In one embodiment the polypeptide has at least 98 % amino acid sequence identity.

In one embodiment of all aspects as reported herein X_1 comprises an amino acid sequence that corresponds to a hidden epitope.

In one embodiment of all aspects as reported herein X_1 has an amino acid sequence length from 4 to about 500 amino acid residues. In one embodiment X_1 has an amino acid sequence length from 5 to about 100 amino acid residues. In one embodiment X_1 has an amino acid sequence length of about 7 to about 60 amino acid residues.

In one embodiment of all aspects as reported herein at least one amino acid residue of X_1 comprises a post-translational modification. In one embodiment one, or two, or three, or four, or five, or six, or seven, or eight, or nine, or ten amino acid residues of X_1 comprise a post-translational modification.

In one embodiment the fusion polypeptide is according to the following formula

$$NH_2\text{-}S_3\text{-}S_2\text{-}X_1\text{-}S_1\text{-}S_0\text{-}COOH$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are non-overlapping amino acid sequences derived from a second polypeptide,

S_3 and S_0 are either absent or non-overlapping amino acid sequences derived from a third polypeptide, and

- denotes a peptide bond,

wherein the second polypeptide and the third polypeptide are different from each other and are polypeptides with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or which are derived from the FKBP domain family.

In one embodiment of all aspects as reported herein the second polypeptide and the third polypeptide and the fourth polypeptide are from different species.

In one embodiment of all aspects as reported herein the second polypeptide is a human polypeptide, or a plant polypeptide, or a bacterial polypeptide, or an archaic polypeptide.

In one embodiment of all aspects as reported herein the third polypeptide is a human polypeptide, or a bacterial polypeptide, or an archaic polypeptide.

In one embodiment of all aspects as reported herein the fourth polypeptide is a bacterial polypeptide, or an archaic polypeptide.

In one embodiment of all aspects as reported herein the fourth polypeptide is a bacterial polypeptide. In one embodiment the bacterial polypeptide is a polypeptide from a thermophilic bacterium. In one embodiment the thermophile is from the family of Thermaceae. In one embodiment the thermophile is Thermus thermophilus.

In one embodiment of all aspects as reported herein the fourth polypeptide is an archaic polypeptide. In one embodiment the archaic polypeptide is a polypeptide from a hyperthermophilic Archaea. In one embodiment the thermophile is from the class of Thermococci. In one embodiment the archaic organism is Thermococcus gammatolerans.

In one embodiment of all aspects as reported herein the thermophile has an optimal growth temperature of at least 60 °C.

In one embodiment of all aspects as reported herein the first polypeptide is a human polypeptide.

In one embodiment of all aspects as reported herein X_1 is either a random amino acid sequence or an amino acid sequence derived from a first polypeptide wherein the dipeptide GS is added at the N-terminus and the tripeptide GSS is added at the C-terminus.

In one embodiment of all aspects as reported herein X_1 is a polypeptide of formula III

$$X_aX_bX_cX_d\text{-}X_0\text{-}X_eX_fX_gX_h \qquad \text{(formula III)}$$

wherein X_0 is either a random amino acid sequence or an amino acid sequence of a first polypeptide, and

wherein each of X_a to X_h denotes an (naturally occurring) amino acid residue and any of X_{a-h} may be individually present or absent.

In one embodiment of all aspects as reported herein X_1 is a polypeptide selected from the group of formula IV to formula XIII, with

GS-X_0-GSS	(formula IV),
AGS-X_0-GSS	(formula V),
CG-X_0-GC	(formula VI),
C-X_0-GC	(formula VII),
G-X_0-G	(formula VIII),
S-X_0-GSS	(formula IX),
GG-X_0-GG	(formula X),
G-X_0-TGG	(formula XI),
GGGS-X_0-GGGS	(formula XII),
GGNP-X_0-GPT	(formula XIII),

wherein X_0 is either a random amino acid sequence or an amino acid sequence derived from a first polypeptide.

In one embodiment X_0 is flanked at its N- and C-terminus by individual (single) cysteine residues.

In one embodiment X_1 comprises a cysteine residue within the N-terminal amino acid residues and a cysteine residue within the C-terminal amino acid residues. In one embodiment the N- or C-terminal amino acid residues are the eight terminal residues. In one embodiment X_1 comprises one cysteine residue at its N-terminus and one cysteine residue at its C-terminus.

In one embodiment X_1 is a circularly constrained polypeptide.

In one embodiment X_1 is a circular polypeptide.

In one embodiment the cysteine residues of X_1 have an alpha carbon atom distance of 4.3 Angstroms to 6.5 Angstroms. In one embodiment the cysteine residues of X_1 have an alpha carbon distance of 4.5 Angstroms. In one embodiment the cysteine residues of X_1 have a mean alpha carbon atoms distance of 5.6 Angstroms.

In one embodiment of all aspects as reported herein X_1 or X_0 has a length from 4 to about 500 amino acid residues.

In one embodiment of all aspects as reported herein X_2 is a linker amino acid sequence of from about 10 to about 30 amino acid residues.

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4, X_2, S_3, and S_0 are absent. In one embodiment S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), and S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), and S_4, X_2, S_3, and S_0 are absent. In one embodiment S_2 has the amino acid sequence SEQ ID NO: 04, and S_1 has the amino acid sequence SEQ ID NO: 05, S_4, X_2, S_3, and S_0 are absent, and X_1 is a polypeptide of formula IV (GS-X_0-GSS).

In one embodiment of all aspects as reported herein the second polypeptide is human FKBP12 (SEQ ID NO: 06), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4, X_2, S_3, and S_0 are absent. In one embodiment S_2 has the amino acid sequence SEQ ID NO: 07, and S_1 has the amino acid sequence SEQ ID NO: 08 (LVFDVELLKLE), and S_4, X_2, S_3, and S_0 are absent, and X_1 is a polypeptide of formula III (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Thermus thermophilus SlyD (SEQ ID NO: 09), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4, X_2, S_3, and S_0 are absent. In one embodiment S_2 has the amino acid sequence SEQ ID NO: 10, and S_1 has the amino acid sequence SEQ ID NO: 11, and S_4, X_2, S_3, and S_0 are absent, and X_1 is a polypeptide of formula V (AGS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the third polypeptide is human FKBP12 (SEQ ID NO: 06), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4 and X_2 are absent. In one embodiment S_3 has the amino acid sequence of SEQ ID NO: 07, S_2 has the

amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 08 (LVFDVELLKLE), S_4 and X_2 are absent, and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the third polypeptide is Thermus thermophilus SlyD (SEQ ID NO: 09), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4 and X_2 are absent. In one embodiment S_3 has the amino acid sequence of SEQ ID NO: 10, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 11 (LVFDVELLKLE), S_4 and X_2 are absent, and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the third polypeptide is human FKBP12 (SEQ ID NO: 06), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4 and X_2 are absent. In one embodiment S_3 has the amino acid sequence of SEQ ID NO: 07, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 08 (LVFDVELLKLE), S_4 and X_2 are absent, and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, the third polypeptide is human FKBP12 (SEQ ID NO: 06), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity. In one embodiment S_4 has the amino acid sequence SEQ ID NO:12, X_2 has the amino

acid sequence SEQ ID NO: 13, S_3 has the amino acid sequence of SEQ ID NO: 07, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 08 (LVFDVELLKLE), and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, the third polypeptide is Thermus thermophilus SlyD (SEQ ID NO: 09), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity. In one embodiment S_4 has the amino acid sequence SEQ ID NO:12, X_2 has the amino acid sequence SEQ ID NO: 13, S_3 has the amino acid sequence of SEQ ID NO: 10, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 11 (LVFDVELLKLE), and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Thermus thermophilus SlyD (SEQ ID NO: 09), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_3 and S_0 are absent. In one embodiment S_4 has the amino acid sequence SEQ ID NO: 12, X_2 has the amino acid sequence SEQ ID NO: 13, S_2 has the amino acid sequence SEQ ID NO: 10, S_1 has the amino acid sequence SEQ ID NO: 11, S_3 and S_0 are absent, and X_1 is a polypeptide of formula V (AGS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_3 and S_0 are absent. In one

embodiment S_4 has the amino acid sequence SEQ ID NO: 12, X_2 has the amino acid sequence SEQ ID NO: 13, S_2 has the amino acid sequence SEQ ID NO: 04, S_1 has the amino acid sequence SEQ ID NO: 05, S_3 and S_0 are absent, and X_1 is a polypeptide of formula IV (GS-X_0-GSS) or formula VI ((P)CG-X_0-GC).

In one embodiment of all aspects as reported herein the second polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4, X_2, S_3, and S_0 are absent. In one embodiment S_2 has the amino acid sequence SEQ ID NO: 107, S_1 has the amino acid sequence SEQ ID NO: 108, S_4, X_2, S_3, and S_0 are absent, and X_1 is a polypeptide selected from formula IV to formula XIII.

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the third polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_4 and X_2 are absent. In one embodiment S_3 has the amino acid sequence of SEQ ID NO: 107, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 108, S_4 and X_2 are absent, and X_1 is a polypeptide selected from formula IV to formula XIII.

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, the third polypeptide is human FKBP12 (SEQ ID NO: 06), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity. In one embodiment S_4 has the amino acid sequence SEQ ID NO: 106, X_2 has the amino acid sequence SEQ ID NO: 13, S_3 has the amino acid sequence of SEQ ID NO: 07, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03

(CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 08 (LVFDVELLKLE), and X_1 is a polypeptide selected from formula IV to formula XIII.

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, the third polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity. In a specific embodiment S_4 has the amino acid sequence SEQ ID NO: 12, X_2 has the amino acid sequence SEQ ID NO: 13, S_3 has the amino acid sequence of SEQ ID NO: 107, S_2 has the amino acid sequence SEQ ID NO: 02 (DRGAGC), S_1 has the amino acid sequence SEQ ID NO: 03 (CLIPPASV), S_0 has the amino acid sequence SEQ ID NO: 108, and X_1 is a polypeptide selected from formula IV to formula XIII.

In one embodiment of all aspects as reported herein the second polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is E.coli SlyD (SEQ ID NO: 12), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_3 and S_0 are absent. In one embodiment S_4 has the amino acid sequence SEQ ID NO: 12, X_2 has the amino acid sequence SEQ ID NO: 13, S_2 has the amino acid sequence SEQ ID NO: 107, S_1 has the amino acid sequence SEQ ID NO: 108, S_3 and S_0 are absent, and X_1 is a polypeptide selected from formula IV to formula XIII.

In one embodiment of all aspects as reported herein the second polypeptide is Arabidopsis thaliana FKBP13 (SEQ ID NO: 01), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and the fourth polypeptide is Thermococcus gammatolerans SlyD (SEQ ID NO: 106), or a polypeptide with at least 70 %, or at least 80 %, or at least 90 %, or at least 95 %, or at least 98 % amino acid sequence identity, and S_3 and S_0 are absent. In one embodiment S_4 has the amino acid sequence SEQ ID NO: 107, X_2 has the amino acid sequence SEQ ID NO: 13, S_2 has the amino acid

sequence SEQ ID NO: 04, S_1 has the amino acid sequence SEQ ID NO: 05, S_3 and S_0 are absent, and X_1 is a polypeptide selected from formula IV to formula XIII.

The fusion polypeptides of the aspects as reported herein have many applications because they can be produced recombinantly in good yields e.g. in E.coli. For example the fusion polypeptides can be used for presenting amino acid sequence for immunization, antibody generation, antibody screening, antibody epitope mapping, or immunohistochemistry screening.

In one embodiment of all aspects as reported herein X_2 has the amino acid sequence GGGSGGGSGGGSGGGSGGGSGGG (SEQ ID NO: 14).

In one embodiment of all aspects as reported herein X_1 has the amino acid sequence GGGSGGNPX$_0$GPTGGGS (SEQ ID NO: 32), wherein X_0 is an amino acid sequence from 4 to 85 amino acid residues.

In one embodiment a fusion polypeptide comprising E.coli SlyD and human FKBP12 amino acid sequences is used for the presentation of non-stable conformational polypeptides.

In one embodiment a fusion polypeptide comprising human FKBP12 and Arabidopsis thaliana FKBP13, or solely Thermus thermophilus SlyD, or Thermus thermophilus SlyD and Arabidopsis thaliana FKBP13, or solely Thermococcus gammatolerans SlyD, or Thermococcus gammatolerans SlyD and Arabidopsis thaliana FKBP13 amino acid sequences is used for the presentation of stable secondary structures.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for eliciting an immune response against X_1 or X_0 in an animal.

One aspect as reported herein is a method for eliciting an immune response in an animal against a polypeptide comprising the step of administering to the animal a fusion polypeptide as reported herein for at least one time, whereby X_0 is the immunogenic amino acid sequence.

One aspect as reported herein is a method for obtaining a nucleic acid encoding an antibody specifically binding to a target antigen comprising the following steps:

a) administering to an animal a fusion polypeptide as reported herein for at least one time, whereby the amino acid sequence of X_1 comprises the amino acid sequence of the target antigen,

b) recovering from the animal three to ten days after the last administration of the polypeptide B-cells that produce the antibody specifically binding to the target antigen, and

c) obtaining from the B-cells the nucleic acid encoding an antibody specifically binding to a target antigen.

One aspect as reported herein is a method for the production of an antibody specifically binding to a target antigen comprising the following steps:

a) administering to an animal a fusion polypeptide as reported herein for at least one time, whereby the amino acid sequence of X_1 comprises the amino acid sequence of the target antigen,

b) recovering from the animal three to ten days after the last administration of the polypeptide B-cells that produce the antibody specifically binding to the target antigen,

c) optionally obtaining from the B-cells the nucleic acid encoding the antibody specifically binding to the target antigen, and

d) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the target antigen and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

One aspect as reported herein is a method for the production of an antibody specifically binding to a target antigen comprising the following steps:

a) recovering from an experimental animal after the administration of a fusion polypeptide as reported herein B-cells that produce the antibody specifically binding to the target antigen that has the amino acid sequence of X_0, and

b) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the amino acid sequence of X_0 and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for epitope mapping, whereby the amino acid sequence of X_1 comprises the epitope.

One aspect as reported herein is a method for selecting an antibody specifically binding to a target antigen comprising the following steps:

a) determining the binding affinity of a plurality of antibodies to a target antigen, whereby the amino acid sequence of X_1 of a fusion polypeptide as reported herein comprises the target antigen amino acid sequence,
b) selecting the antibody having an apparent complex stability above a pre-defined threshold level.

One aspect as reported herein is a method for selecting an antibody suitable for immunohistochemical analysis of a target polypeptide comprising the following steps:

a) determining the binding kinetics of a plurality of antibodies,
c) selecting the antibody having an apparent complex stability above a pre-defined threshold level.

One aspect as reported herein is a method for mapping a binding site of an antibody to a target amino acid sequence comprising the following steps:

a) contacting a solid support to which a fusion polypeptide as reported herein is immobilized, whereby the amino acid sequence of X_1 comprises the target amino acid sequence, with an antibody,
b) determining the kinetic properties of the antibody with the fusion polypeptide as reported herein,
c) selecting the antibody having an apparent complex stability above a pre-defined threshold level.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for determining structure-function-relationships, whereby the amino acid sequence of X_1 comprises the polypeptide for which the structure-function-relationship shall be determined.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for presenting a polypeptide with its correct secondary and/or tertiary structure, whereby the amino acid sequence of X_1 comprises the polypeptide.

One aspect as reported herein is the use of a fusion polypeptide as reported herein in a screening method.

In one embodiment the screening method is a screening method for identifying or selecting molecules that specifically bind to X_1. In one embodiment the molecule is a small molecule or a polypeptide. In one embodiment the polypeptide is an antibody, or antibody-fragment, or antibody-fusion polypeptide.

One aspect as reported herein is the use of a fusion polypeptide as reported herein in ribosome display.

One aspect as reported herein is the use of a fusion polypeptide as reported herein in phage display.

One aspect as reported herein is the use of a polypeptide as reported herein in cell surface display. In one embodiment the cell is a prokaryotic cell. In one embodiment the prokaryotic cell is a bacterial cell. In one embodiment the cell is a eukaryotic cell. In one embodiment the eukaryotic cell is a CHO cell, or a HEK cell, or a BHK cell, or a Sp2/0 cell, or a NS0 cell, or a yeast cell.

One aspect as reported herein is the antibody produced by a method as reported herein.

One aspect as reported herein is a pharmaceutical formulation comprising the fusion polypeptide as reported herein and a pharmaceutically acceptable carrier.

One aspect as reported herein is a diagnostic formulation comprising the fusion polypeptide as reported herein conjugated to a detectable label.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for the manufacture of a medicament.

One aspect as reported herein is the use of a fusion polypeptide as reported herein for the treatment of a disease.

One aspect as reported herein is a method of treating an individual comprising administering to the individual an effective amount of the fusion polypeptide as reported herein.

Detailed Description of the Invention

In general, the antigen or antigenic or immunogenic amino acid sequence, e.g. comprised in the amino acid sequence of X_1 is inserted in place of the insert-in-flap-domain (IF-domain) of the SlyD part of the second polypeptide in the fusion polypeptide as reported herein.

In one embodiment of all aspects as reported herein X_0 is selected from a fragment of a naturally occurring polypeptide or is a randomized amino acid sequence. In one embodiment the naturally occurring polypeptide is a human polypeptide.

The fusion polypeptide comprising the X_1 amino acid sequence can be used on the one hand for the immunization of animals to generate antibodies and on the other hand for the screening of antibody libraries obtained by randomization or after immunization. Specific binders can also be identified using any screening and display methodology, such as ribosome display, phage display, cell surface display, viral display and a fusion polypeptide based display as reported herein.

Thermus thermophilus SlyD as well as Thermococcus gammadurans SlyD are highly stable proteins with the ability to reversibly fold even when their Flap domain is being replaced by a foreign amino acid insertion X1. These molecules can be used in ribosome display, basically according to the method of Mattheakis, L. C., et al. Proc. Natl. Acad. Sci USA 91 (1994) 9022-9026) to display a polypeptide sequence X1 in the frames of Thermus thermophilus SlyD or Thermococcus gammadurans SlyD. The so called ternary complex consists of (1) the ribosomal subunits attached to the (2) mRNA encoding the genetic information of the (3) ribosomally presented fusion polypeptide.

The ternary complexes can be used in panning procedures versus antibodies or antibody fragments, which specifically recognize the X1 amino acid sequence.

The fusion polypeptide as reported herein can be used for the screening/selection of antibodies obtained by the immunization of animals using the fusion polypeptide as reported herein, wherein the fusion polypeptide used for the immunization of the animal and the fusion polypeptide used for the screening of the obtained antibodies have the identical X_1 amino acid sequence and the remaining amino acid sequence is different. This allows deselecting antibodies that specifically bind to the scaffold and not to the immunogenic peptide X_1.

In one embodiment has the fusion polypeptide used for the immunization and the fusion polypeptide used for the screening a sequence identity of less than 20 %. In one embodiment the sequence identity is less than 10 %.

Herein is reported as one aspect a fusion polypeptide comprising at its N-terminus an N-terminal fragment of the Thermus thermophilus SlyD polypeptide (SEQ ID NO: 10), i.e. residues 2 to 64 of the Thermus thermophilus SlyD polypeptide (numbering starts with M as residue 1 of SEQ ID NO: 09). Thereafter the immunogenic sequence comprised in X_1 is inserted. The C-terminus of the fusion polypeptide is formed by amino acid residues 123 to 149 of the Thermus

thermophilus SlyD polypeptide (SEQ ID NO: 11) and an optional purification tag with the amino acid sequence GSRKHHHHHHHH (SEQ ID NO: 16).

Herein is reported as one aspect a fusion polypeptide comprising at its N-terminus an N-terminal fragment of the Thermococcus gammatolerans SlyD polypeptide (SEQ ID NO: 106), i.e. residues 2 to 85 of the Thermococcus gammatolerans SlyD polypeptide (numbering starts with M as residue 1 of SEQ ID NO: 107). Thereafter the immunogenic sequence comprised in X_1 is inserted. The C-terminus of the fusion polypeptide is formed by amino acid residues 137 to 156 of the Thermococcus gammatolerans SlyD polypeptide (SEQ ID NO: 108) and an optional purification tag with the amino acid sequence GSRKHHHHHHHH (SEQ ID NO: 16).

Herein is reported as one aspect a polypeptide comprising at its N-terminus an N-terminal fragment of the E.coli SlyD polypeptide, i.e. residues 1 to 165 of the E.coli SlyD polypeptide (numbering starts with M as residue 1). Thereafter a linker is inserted which connects the C-terminus of the E.coli SlyD fragment with a fragment of the N-terminus of the human FKBP12 polypeptide, i.e. residues 2 to 84 of the human FKBP12 polypeptide (numbering starts with M as residue 1). Thereafter an amino acid sequence from 5 to 500 amino acid residues can be inserted. The C-terminus of the polypeptide is formed by amino acid residues 97 to 108 of the human FKBP12 polypeptide and a purification tag with the amino acid sequence GSRKHHHHHHHH (SEQ ID NO: 16).

One aspect as reported herein is a variant of the fusion polypeptide as reported herein that has at least 70 % amino acid sequence identity with respect to the parent polypeptide, and that has a melting point that is increased compared to the parent polypeptide. In one embodiment the melting point is at least 55 °C. In one embodiment the melting point is at least 60 °C. In one embodiment the melting point is at least 65 °C.

The term “derived from a polypeptide” denotes that a fragment of the full length amino acid sequence of the respective polypeptide is present, whereby the fragment has at least 70 % amino acid sequence identity to the respective sequence in the full length polypeptide.

The X_1 and X_0 amino acid sequence, respectively, can freely be chosen as long it is at least 5 amino acid residues in length. For example the inserted sequence can be derived from, i.e. can comprise a fragment of, the leukocyte markers, CD2, CD3,

CD4, CD5, CD6, CD7, CD8, CD11a,b,c, CD13, CD14, CD18, CD19, CD20, CD22, CD23, CD27 and its ligand, CD28 and its ligands B7.1, B7.2, B7.3, CD29 and its ligand, CD30 and its ligand, CD40 and its ligand gp39, CD44, CD45 and isoforms, CDw52 (Campath antigen), CD56, CD58, CD69, CD72, CTLA-4, LFA-1 and TCR; the histocompatibility antigens, MHC class I or II, the Lewis Y antigens, SLex, SLey, SLea, and SLeb; the integrins, VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6, αVβ3, and LFA-1, Mac-1, and pl50,95, αVβ1, gpIIbIIIa, αR β3, α6β4, αVβ 5, αVβ6, and αV 62 7; the selectins, L-selectin, P-selectin, and E-selectin and their counter receptors VCAM-1, ICAM-1, ICAM-2, and LFA-3; the interleukins, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15; the interleukin receptor is selected from the group consisting of IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-8R, IL-9R, IL-10R, IL-11R, IL-12R, IL-13R, IL-14R, and IL-15R; the chemokine is selected from the group consisting of PF4, RANTES, MIP1α, MCP1, NAP-2, Groα, Groβ, and IL-8; the growth factor is selected from the group consisting of TNFalpha, TGFbeta, TSH, VEGF/VPF, VEGFA, VEGFB, VEGF111, VEGF121, VEGF165, VEGF189, VEGF206, PTHrP, EGF family, FGF, PDGF family, endothelin, Fibrosin (FSF-1), human Laminin, and gastrin releasing peptide (GRP), PLGF, HGH, HGHR; the growth factor receptor is selected from the group consisting of TNFalphaR, RGFbetaR, TSHR, VEGFR/VPFR, FGFR, EGFR, PTHrPR, PDGFR family, EPO-R, GCSF-R and other hematopoietic receptors; the interferon receptor is selected from the group consisting of IFNCαR, IFNβR, and IFNλR; the Ig and its receptor is selected from the group consisting of IgE, FcγRI, and FcγRII; the tumor antigen is selected from the group consisting of her2-neu, mucin, CEA and endosialin; the allergen is selected from the group consisting of house dust mite antigen, lol p1 (grass) antigens, and urushiol; the viral polypeptide is selected from the group consisting of CMV glycoproteins B, H, and gCIII, HIV-1 envelope glycoproteins, RSV envelope glycoproteins, HSV envelope glycoproteins, HPV envelope glycoproteins, Hepatitis family surface antigens; the toxin is selected from the group consisting of pseudomonas endotoxin and osteopontin/uropontin, snake venom, spider venom, and bee venom conotoxin; the blood factor is selected from the group consisting of complement C3b, complement C4a, complement C4b-9, Rh factor, fibrinogen, fibrin, and myelin associated growth inhibitor; and the enzyme is selected from the group consisting of cholesterol ester transfer polypeptide, membrane bound matrix metalloproteases, and glutamic acid decarboxylase (GAD).

The term "peptidic linker sequence" denotes peptide linkers of natural and/or synthetic origin. They consist of a linear amino acid chain wherein the 20 naturally occurring amino acids are the monomeric building blocks. The chain has a length of from 10 to 50 amino acids, particularly of from 10 to 30 amino acids. The linker may contain repetitive amino acid sequences or sequences of naturally occurring polypeptides, such as polypeptides with a hinge-function. In one embodiment the peptidic linker amino acid sequence is a "synthetic linker amino acid sequence" that is designated to be rich in glycine, glutamine, and/or serine residues. These residues are arranged e.g. in small repetitive units of up to five amino acids, such as GGGGS, QQQQG, or SSSSG (SEQ ID NO: 17, 18, or 19). This small repetitive unit may be repeated for two to six times to form a multimeric unit. At the amino- and/or carboxy-terminal ends of the multimeric unit up to six additional arbitrary, naturally occurring amino acids may be added. Other synthetic peptidic linkers are composed of a single amino acid, that is repeated between 10 to 20 times and may comprise at the amino- and/or carboxy-terminal end up to six additional arbitrary, naturally occurring amino acids, such as e.g. serine in the linker GSSSSSSSSSSSSSSG (SEQ ID NO: 20). Particular linker amino acid sequences are shown in the following Table. In one embodiment the linker amino acid sequence is selected from $[GQ_4]_3GNN$ (SEQ ID NO: 21), $G_3[SG_4]_2SG$ (SEQ ID NO: 22), $G_3[SG_4]_2SG_2$ (SEQ ID NO: 23), $(G_3S)_5GGG$ (SEQ ID NO: 24). All peptidic linkers can be encoded by a nucleic acid molecule and therefore can be recombinantly expressed.

Table.

linker amino acid sequence	**SEQ ID NO:**
$(GQ_4)_3$	25
$(GQ_4)_3G$	26
$(GQ_4)_3GNN$	27
$(G_2S)_3$	28
$(G_2S)_4$	29
$(G_2S)_5$	30
$(G_3S)_3$	31
$(G_3S)_4$	32
$(G_3S)_5$	33
$(G_3S)_5GGG$	34
$(G_4S)_2$	35
$(G_4S)_2G$	36

linker amino acid sequence	SEQ ID NO:
$(G_4S)_2GG$	37
$(G_4S)_2GGG$	38
$(G_4S)_2GN$	39
$(G_4S)_3$	40
$(G_4S)_3G$	41
$(G_4S)_3T$	42
$(G_4S)_3GG$	43
$(G_4S)_3GGT$	44
$(G_4S)_3GGN$	45
$(G_4S)_3GAS$	46
$(G_4S)_4$	47
$(G_4S)_5$	48
$(G_4S)_5G$	49
$(G_4S)_5GG$	50
$(G_4S)_5GAS$	51
$G(S)_{15}G$	52
$G(S)_{15}GAS$	53

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech,

Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "animal" denotes an animal selected from the group of mouse, rat, rabbit, sheep, cat, dog, hamster, cynomolgus, and chimpanzee. Particularly, the animal is a mouse or a rabbit or a hamster or a rat. In one embodiment the animal is a non-human animal.

In one embodiment the term "recovering" comprises a) (i) immortalizing B-cells from the animal immunized with the target antigen, and (ii) screening the resulting immortalized cells for the secretion of the antibody specifically binding to the target antigen, or b) (i) co-cultivating single deposited B-cells in the presence of feeder cells, and (ii) screening the cultivation supernatant for the presence of antibodies specifically binding to the target antigen.

The term "specifically binding to a target antigen" denotes that an antibody is binding to the target antigen with a dissociation constant ($=K_{Diss.}$) of at least 10^{-8} mol/l, particularly with a $K_{Diss.}$ of at least 10^{-10} mol/l. At the same time the property

of not specifically binding to a target antigen is insured by a $K_{Diss.}$ of 10^{-7} mol/l or worse, e.g. of 10^{-5} mol/l.

The term "pharmaceutical formulation" denotes a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

The term “E.coli SlyD” denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL AHGHVHGAHDHHHDHDHD (SEQ ID NO: 12).

The term “denotes a polypeptide that has the following amino acid sequence” denotes a polypeptide of the given amino acid sequence and also includes variants thereof that have the same properties as the polypeptide with respect to X_1. In one embodiment the term denotes a polypeptide with at least 70 % amino acid sequence identity. In one embodiment the term denotes a polypeptide with at least 80 % amino acid sequence identity. In one embodiment the term denotes a polypeptide with at least 90 % amino acid sequence identity. In one embodiment the term denotes a polypeptide with at least 95 % amino acid sequence identity. In one embodiment the term denotes a polypeptide with at least 98 % amino acid sequence identity.

If a polypeptide is produced in or derived from E.coli the amino-terminal methionine residue is usually not efficiently cleaved off by proteases, thus the amino-terminal methionine residue is partially present in the produced polypeptide. In order to account for this all sequence are given with the starting methionine residue.

The term "Thermus thermophilus SlyD" denotes a polypeptide that has the following amino acid sequence:

MKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEG
EAFQAHVPAEKAYGPHDPEGVQVVPLSAFPEDAEVVPGAQFYAQDMEGN
PMPLTVVAVEGEEVTVDFNHPLAGKDLDFQVEVVKVREATPEELLHGHA
H (SEQ ID NO: 09).

The term "Thermococcus gammatolerans SlyD" denotes a polypeptide that has the following amino acid sequence:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPREDLIVPVPIEQFTSAGLE
PVEGMYVMTDAGIAKILKVEEKTVRLDFNHPLAGKTAIFEIEVVEIKKAGE
A (SEQ ID NO: 106).

The term "human FKBP12" denotes a polypeptide that has the following amino acid sequence:

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFM
LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFD
VELLKLE (SEQ ID NO: 06).

The term "Arabidopsis thaliana FKBP13" denotes a polypeptide that has the following amino acid sequence:

ETTSCEFSVSPSGLAFCDKVVGYGPEAVKGQLIKAHYVGKLENGKVFDSSY
NRGKPLTFRIGVGEVIKGWDQGILGSDGIPPMLTGGKRTLRIPPELAYGDRG
AGCKGGSCLIPPASVLLFDIEYIGKA (SEQ ID NO: 01).

The term "FKBP12 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MRSGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFK
FMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYG-X_1-
TLVFDVELLKLE (SEQ ID NO: 109),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the FKBP12 fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-FKBP12 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL AHGHVHGAHDHHHDHDHD-X_2- RSGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKF MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYG-X_1-TLVFDVELLKLE (SEQ ID NO: 110),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-FKBP12 fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "FKBP12/13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MRSGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGDRGAGCGS-X_1- GSSCLIPPASVLVFDVELLKLE (SEQ ID NO: 54),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the FKBP12/13 fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-FKBP12/13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
GVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFML
GKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGDRGAGCGS-X_1-
GSSCLIPPASVLVFDVELLKLE (SEQ ID NO: 55),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-FKBP12/13 fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "Thermus thermophilus SlyD fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MRSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGRE
EGEAFQAHVPAEKAY-X_1-GKDLDFQVEVVKVREATPEELLHGHAH (SEQ
ID NO: 56),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermus thermophilus SlyD fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-Thermus thermophilus SlyD fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
KVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGE

AFQAHVPAEKAY-X_1-GKDLDFQVEVVKVREATPEELLHGHAH (SEQ ID NO: 57),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-Thermus thermophilus SlyD fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "Thermococcus gammatolerans SlyD fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMP-X_1-
AGKTAIFEIEVVEIKKAGEA (SEQ ID NO: 111)

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermococcus gammatolerans SlyD fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-Thermococcus gammatolerans SlyD fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
KVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGA
GEIIPGIEEALLGMELGEKKEVVVPPEKGYGMP-X_1-
AGKTAIFEIEVVEIKKAGEA (SEQ ID NO: 112)

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermococcus gammatolerans SlyD fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein. The term "Thermus thermophilus SlyD-FKBP13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

GDRGAGCGS-X_1-GSSCLIPPASVLDFQVEVVKVREATPEELLHGHAH (SEQ ID NO: 58),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermus thermophilus SlyD-FKBP13 fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-Thermus thermophilus SlyD-FKBP13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
KVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGE
AFQAHVPAEKAYGDRGAGCGS-X_1-
GSSCLIPPASVLDFQVEVVKVREATPEELLHGHAH (SEQ ID NO: 59),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-Thermus thermophilus SlyD-FKBP13 fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "Arabidopsis thaliana FKBP13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

ETTSCEFSVSPSGLAFCDKVVGYGPEAVKGQLIKAHYVGKLENGKVFDSSY
NRGKPLTFRIGVGEVIKGWDQGILGSDGIPPMLTGGKRTLRIPPELAYGDRG
AGCGS-X_1-GSSCLIPPASVLLFDIEYIGKA (SEQ ID NO: 60),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Arabidopsis thaliana FKBP13 fusion polypeptide.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-Arabidopsis thaliana FKBP13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
ETTSCEFSVSPSGLAFCDKVVGYGPEAVKGQLIKAHYVGKLENGKVFDSSY
NRGKPLTFRIGVGEVIKGWDQGILGSDGIPPMLTGGKRTLRIPPELAYGDRG
AGCGS-X_1-GSSCLIPPASVLLFDIEYIGKA (SEQ ID NO: 61),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-Arabidopsis thaliana FKBP13 fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

This amino acid sequence and variants thereof are individual aspects as reported herein.

The term "SlyD-FKBP12/13 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHD-X_2-
GVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFML

GKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGDRGAGC-X_1-CLIPPASVLVFDVELLKLEGGGSRPLLPPLPGG (SEQ ID NO: 113),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD-FKBP12/13 fusion polypeptide, and

wherein X_2 is the amino acid sequence of a linker.

In one embodiment of the above aspects X_2 has the amino acid sequence GGGSGGGSGGGS (SEQ ID NO: 13).

For direct detection the detectable label can be selected from any known detectable marker groups, such as dyes, luminescent labeling groups such as chemiluminescent groups, e.g. acridinium esters or dioxetanes, or fluorescent dyes, e.g. fluorescein, coumarin, rhodamine, oxazine, resorufin, cyanine and derivatives thereof. Other examples of detectable labels are luminescent metal complexes, such as ruthenium or europium complexes, enzymes, e.g. as used for ELISA or for CEDIA (Cloned Enzyme Donor Immunoassay), and radioisotopes. Metal chelates which can be detected by electrochemiluminescense are also in one embodiment signal-emitting groups used as detectable labels, with particular preference being given to ruthenium chelates. In one embodiment the labeling group is a ruthenium $(bispyridyl)_3^{2+}$ chelate.

Indirect detection systems comprise, for example, that the detection reagent, e.g. the detection antibody, is labeled with a first partner of a binding pair. Examples of suitable binding pairs are hapten or antigen/antibody, biotin or biotin analogues such as aminobiotin, iminobiotin or desthiobiotin/avidin or streptavidin, sugar/lectin, nucleic acid or nucleic acid analogue/complementary nucleic acid, and receptor/ligand, e.g. steroid hormone receptor/steroid hormone. In one embodiment the first binding pair member is selected from hapten, antigen and hormone. In one embodiment the hapten is selected from digoxin and biotin and analogues thereof. The second partner of such binding pair, e.g. an antibody, streptavidin, etc., usually is labeled to allow for direct detection, e.g. by the labels as mentioned above.

The fusion polypeptides as reported herein are based on polypeptides from the FKBP domain protein family (i.e. proteins with PPIase activity), such as human FKBP12 (Handschumacher, R.E., et al., Science 226 (1984) 544-547), or Arabidopsis thaliana FKBP13, or E.coli SlyD, or Thermos thermophilus SlyD, or

Thermococcus gammatolerans SlyD. The fusion polypeptides as reported herein are a scaffold for the presentation of polypeptides comprised in the amino acid sequence of X_1.

The amino acid sequence of X_1 can replace the Flap domain (amino acid residues A85 to A96) and the beta-bulge (amino acid residues S39 to P46) in the FKBP12 part and/or the IF domain (amino acid residues G69 to D120) in SlyD part. Therewith it is possible to omit the time consuming recombinant preparation and purification of full length proteinaceous immunogens.

The defined presentation of the inserted amino acid sequences and/or structural motifs related thereto of polypeptides X_1 or X_0, respectively, in the fusion polypeptide as reported herein allows an efficient and cost effective production of the immunogenic amino acid sequence contained in X_1 and X_0, respectively, in sufficient amount, quality and with correct three dimensional structure.

Any amino acid sequence can be inserted, such as helices, helix-turn-helix motifs, coiled coil structures, helix bundles, turn-loop motifs, beta-hairpin structures, beta sheets, sheet-helix motifs, sheet-turn-sheet motifs etc. It is also possible to present defined native tertiary structures, individual domains of a multi-domain polypeptide or subdomains, binding domains, antibody fragments, enzymes etc.

The immunogenic polypeptide can be improved compared to the full length polypeptide from which the amino acid sequence of X_1 or X_0, respectively, is derived, e.g. with respect to solubility and/or reversible folding (naturation/denaturation). The fusion polypeptide as reported herein provides the scaffold into which the amino acid sequence of X_1 that is derived from a polypeptide, to which an antibody shall be obtained, is inserted and it stabilizes the structure of the amino acid sequence of X_1 and X_0, respectively, as the conformational entropy is reduced.

If present, an N-terminal SlyD mediates chaperone functionality and keeps the complete fusion polypeptide as a monomeric, soluble and stable polypeptide. Furthermore, it increases the molecular weight of the fusion polypeptide, which is beneficial for its usage in mass sensitive analyses, like SPR measurements.

The SlyD derived polypeptide portion(s) of the fusion polypeptide, independently of the presence of FKBP12 with its flap-region, folds into the correct (native) three-dimensional conformation. The chimeric FKBP12 domain of the fusion

polypeptide seems to be not correctly folded. In contrast to the wild-type FKBP12 polypeptide, which shows an intrinsic Trp fluorescence emission peak at 320 nm, fluorescence spectroscopic analyses of SlyD-FKBP12 fusion polypeptide showed no peak at 320 nm but a typical extrinsic Trp emission shift at 350 nm. The 350 nm peak is broadened. The single Trp moiety in the SlyD-FKBP12 fusion polypeptide is solvent exposed. This indicates that the FKBP domain within in the SlyD-FKBP12 fusion polypeptide is partly or completely unfolded.

Also a BIAcore binding assay of a SlyD-FKBP12 fusion polypeptide derivative vs. immobilized bi-FK506 showed no binding activity, indicating structure-functional loss of the FKBP12 derived part in the polypeptide as reported herein.

Upon these findings without being bound by theory the current SlyD-FKBP12-insertion structural model is that the polypeptide consists of a well folded SlyD moiety and a structurally handicapped FKBP12 fold, which is at least offering its single core Trp residue to the solvent. The polypeptide is monomeric, soluble, folds reversible and shows sufficient thermal stability for its application.

Therefore, the fusion polypeptides as reported herein are suitable scaffolds for mimicking a structural plurality of peptidic secondary structure motives, as long as the inserted peptidic secondary structure motive does not fold as a separated, autonomously folding structure.

A structural plurality of peptidic secondary structure motives is assumed e.g. to be presented in a paraffin-embedded, formalin-fixed tissue in immune histochemical experiments (Abe, et al., Anal. Biochem. 318 (2003) 118-123).

The amino acid sequence of X_1 or X_0, respectively, can be flanked by a G_3S-linker sequence to ensure sufficient distance and flexibility to the amino acid sequence of the second polypeptide in order to avoid influence on the structural integrity of the amino acid sequence of X_1 or X_0, respectively.

The fusion polypeptides as reported herein have a molecular weight of at least 15 kDa. This eliminates the need to conjugate the polypeptide to carrier proteins (such as KLH or particles) or substances for immunization and reduces the occurrence of neo-epitopes by KLH coupling

Nevertheless, should a conjugation be required this is possible by selectively activating the lysine residue in the sequence motif GSRKHHHHHHHH (SEQ ID

NO: 16), which can be activated by the neighboring histidine and arginine residue with LC-SPDP (succinimidyl 6-(3-[2-pyridyldithio]-propionamido) hexanoate).

A structural or partial/total deformation or the generation of neo-epitopes under conditions of immunohistochemical analysis is normally not recognized during the preparation of proteinaceous immunogens. Especially during the formalin treatment the side chains of the amino acid residues Lys, Tyr, His, and Cys are cross-linked. Additionally the tertiary and quaternary structure of the polypeptides are distorted during the tissue preparation procedure using tissue fixation reagent, (heat-)incubation, paraffin embedding, and the tissue dehydration by alcoholic treatment (see e.g. Fowler, C.B., et al., Lab. Invest. 88 (2008) 785-791). It remains unclear whether antigen retrieval processes are able to completely remove formalin induced crosslinks in order to restore native conformational protein structures. Therefore, new secondary structures can be formed and neo-epitopes can be generated or remain even after a retrieval process.

These novel and non-native structures are not present during the immunization campaign. By using conventional antibody development techniques, no or if at all only a limited number of IHC-suited antibodies can be obtained.

Additionally, free polypeptides in solution have a large conformational entropy leading to a transient structure that makes an immune response against a defined enthalpic secondary structure epitope difficult (see e.g. Scott, K.A., et al., Proc. Natl. Acad. Sci. USA 104 (2007) 2661-2666; Gamacho, C.J., et al., PLoS Comput. Biol. 4 (2008) e1000231 1-8). Free peptides used as immunogens can result in developing only antibodies versus the termini of the respective peptide.

Besides the use in immunizing animals and the generation of an immune response the fusion polypeptides as reported herein can be used for the mapping of antibody epitopes, such as linear or conformational epitopes. Different structural motifs can be presented with the amino acid sequence of X_1 or X_0, respectively (= epitope, antigenic (immunogenic) amino acid sequence). These different structural motifs can also be used for the generation of immunohistochemistry (IHC) suited antibody with specificity for the amino acid sequence of X_1 or X_0, respectively. For this purpose the amino acid sequence of X_1 is selected in a way that only the formation of a limited number of neo-epitopes during formalin treatment can be expected. Especially the number of lysine, tyrosine, histidine, and cysteine residues can be minimized by selecting the appropriate sequence. It is also possible to substitute

single residues, such as cysteine residues, by serine residues. As X_1 sequence e.g. small secondary structure motifs can be used, which show a high probability to refold into their conformational originating structure. By grafting X_1 into the FKBP domain the termini of the inserted polypeptide are no longer free and accessible and the structural enthalpy is increased.

One aspect as reported herein is a fusion polypeptide according to formula I

$$NH_2\text{-}S_2\text{-}X_1\text{-}S_1\text{-}COOH \qquad \text{(formula I)}$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are non-overlapping amino acid sequences derived from a second polypeptide, and

- denotes a peptide bond,

wherein the second polypeptide is a polypeptide with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or a member of the FKBP domain family.

In one embodiment the polypeptide with peptidyl-prolyl cis/trans-isomerase activity is SlyD.

In one embodiment the second polypeptide is a polypeptide from a thermophile.

In one embodiment the thermophile is a thermophile bacterium. In one embodiment the thermophile bacterium is from the family of Thermaceae. In one embodiment the thermophile is Thermus thermophilus.

In one embodiment the thermophile is a thermophile Archaea. In one embodiment the thermophile Archaea a hyperthermophilic Archaea. In one embodiment the thermophile is from the class of Thermococci. In one embodiment the thermophile is Thermococcus gammatolerans.

In one embodiment the thermophile has an optimal growth temperature of at least 60 °C.

One aspect as reported herein is a fusion polypeptide according to formula II

S_4-X_2-S_3-S_2-X_1-S_1-S_0 (formula II)

wherein

X_1 is either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are either non-overlapping amino acid sequences derived from a second polypeptide,

S_3 and S_0 are either absent or non-overlapping amino acid sequences derived from a third polypeptide,

S_4 is either absent or an amino acid sequence derived from a fourth polypeptide,

X_2 is either absent or a peptidic linker sequence, and

- denotes a peptide bond,

wherein the second polypeptide and the third polypeptide and the fourth polypeptide are different from each other and are polypeptides with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or members of the FKBP domain family.

In one embodiment the fusion polypeptide as reported herein comprises an amino acid sequence tag.

In one embodiment the amino acid sequence tag is selected from the group of Poly-his-tag, Avitag, Poly-glu-tag, Poly-arg-tag, Strep-tag, Streptavidin Binding Peptide, Epitope-tags as well as combinations thereof.

In one embodiment the amino acid sequence tag is an octa-histidine-tag.

The term “amino acid sequence tag” denotes a sequence of amino acid residues connected to each other via peptide bonds that has specific binding properties. In one embodiment the amino acid sequence tag is an affinity or purification tag. In an embodiment the amino acid sequence tag is selected from Arg-tag, His-tag, Flag-tag, 3xFlag-tag, Strep-tag, Nano-tag, SBP-tag, c-myc-tag, S-tag, calmodulin-binding-peptide, cellulose-binding-domain, chitin-binding-domain, GST-tag, or MBP-tag. In a further embodiment the amino acid sequence tag is selected from SEQ ID NO: 16 (GSRKHHHHHHHH), or SEQ ID NO: 62

(RRRRR), or SEQ ID NO: 63 (RRRRRR), or SEQ ID NO: 64 (HHHHHH), or SEQ ID NO: 65 (KDHLIHNVHKEFHAHAHNK), or SEQ ID NO: 66 (DYKDDDDK), or SEQ ID NO: 67 (DYKDHDGDYKDHDIDYKDDDDK), or SEQ ID NO: 68 (AWRHPQFGG), or SEQ ID NO: 69 (WSHPQFEK), or SEQ ID NO: 70 (MDVEAWLGAR), or SEQ ID NO: 71 (MDVEAWLGARVPLVET), or SEQ ID NO: 72 (MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP), or SEQ ID NO: 73 (EQKLISEEDL), or SEQ ID NO: 74 (KETAAAKFERQHMDS), or SEQ ID NO: 75 (KRRWKKNFIAVSAANRFKKISSSGAL), or SEQ ID NO: 76 (cellulose binding domain), or SEQ ID NO: 77 (cellulose binding domain), or SEQ ID NO: 78 (TNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPA LWQLQ), or SEQ ID NO: 79 (GST-tag), or SEQ ID NO: 80 (MBP-tag). In one embodiment of all aspect as reported before the amino acid sequence tag has the amino acid sequence selected from SEQ ID NO: 62 to SEQ ID NO: 80. In one embodiment the amino acid sequence tag has the amino acid sequence of SEQ ID NO: 16, or SEQ ID NO: 64, or SEQ ID NO: 68.

If a polypeptide is produced in an E.coli strain the amino-terminal methionine residue is usually not efficiently cleaved off by proteases, thus the amino-terminal methionine residue is partially present in the produced polypeptide. Therefore all sequences given herein are listed with the N-terminal methionine residue albeit this residue might be absent in the isolated polypeptide. Nevertheless the amino acid sequence comprising the N-terminal methionine shall also encompass an amino acid sequence wherein this methionine is missing.

If the X_1 or X_0 amino acid sequence, respectively, has a non-helical structure an additional GGGS linker (SEQ ID NO: 81) can be introduced N-terminal and C-terminal of the amino acid sequence of X_0 or X_1, respectively.

If the X_1 or X_0 amino acid sequence, respectively, has a helical structure a GGGSGGNP-linker (SEQ ID NO: 82) at the N-terminus and a GPTGGGS-linker (SEQ ID NO: 83) of the amino acid sequence of X_0 or X_1, respectively, can be inserted.

The SlyD/FKBP12-antigen, the Thermos thermophilus SlyD-antigen, and the Thermococcus gammatolerans SlyD-antigen fusion polypeptides can be used to present an antigen.

Thermus Thermophilus SlyD (Loew, C., et al., J. Mol. Biol. 398 (2010) 375-390) originates from the archaic bacterium Thermus Thermophilus. Thermococcus gammatolerans SlyD originates from the Archaea Thermococcus gammatolerans. Both proteins show elevated thermodynamic stability in contrast to human FKBP12, FKBP13, chimeric FKBP12/13, as well as E.coli SlyD. The N-terminal E.coli SlyD derived polypeptide (i.e. when the fourth polypeptide is E.coli SlyD) can be omitted when Thermus Thermophilus SlyD, or Thermococcus gammatolerans SlyD is contained in the fusion polypeptide as reported herein.

In general, the FKBP12-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MGVQVETISPGDGRTFPKRGQTAWHYTGMLEDGKKFDSSRDRNKPFKFM
LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGGGGS-X_1-
GGGSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 84),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the FKBP12-antigen fusion polypeptide.

The human FKBP12 derived polypeptide can be N-terminally fused with an E.coli SlyD derived polypeptide.

The SlyD/FKBP12-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGS-X_1-
GGGSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 85),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the SlyD/FKBP12-antigen fusion polypeptide.

The SlyD/FKBP12-control polypeptide (see Figure 1 for SDS and Western Blot) comprising an amino acid sequence tag of SEQ ID NO: 16 as the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGSGGNPGPTGGGSTLVFDVEL
LKLEGGGSRKHHHHHHHH (SEQ ID NO: 86).

The Thermus Thermophilus-SlyD-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHG-X1-
GAGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH (SEQ ID NO: 87),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermus Thermophilus-SlyD-antigen fusion polypeptide.

The Thermococcus gammatolerans-SlyD-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMP-X_1-
AGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHHHH (SEQ ID NO: 114),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermococcus gammatolerans-SlyD-antigen fusion polypeptide.

Thermus thermophilus-SlyD-antigen fusion polypeptide and Thermococcus gammatolerans-SlyD-antigen fusion polypeptide do not require an N-terminal E.coli SlyD chaperone domain. In these fusion polypeptides the immunogenic sequence (antigen sequence) insertion can be stabilized by a disulfide bond at the

stem of the antigen loop, which is an embodiment as reported herein. In the Thermus thermophilus-SlyD-antigen fusion polypeptide two cysteine mutations can be set at amino acid position H66C and A70C and each a glycine residue can be introduced to optimize the junction between the scaffold and the insert.

The disulfide stabilized Thermus Thermophilus-SlyD-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPCG-X_1-
GCGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH (SEQ ID NO: 88),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the disulfide stabilized Thermus Thermophilus-SlyD-antigen fusion polypeptide.

The disulfide stabilized Thermococcus gammatolerans-SlyD-antigen fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 can have the following amino acid sequence:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPCG-X_1-
GCAGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHHHH (SEQ ID NO: 115),

wherein X_1 is the amino acid sequence of a linker, or a peptide, or an antigen, or a secondary or tertiary structure to be presented by the Thermococcus gammatolerans-SlyD-antigen fusion polypeptide.

Anti-ERCC1 antibodies suitable for IHC application:

A SlyD/FKBP12-ERCC1 fusion polypeptide was used as a screening reagent for the development of anti-ERCC1 antibodies suitable for IHC staining that are targeting the helix-loop-helix region within the C-terminal domain of ERCC1 (ERCC = Excision Repair Cross Complementing; Tripsianes, K., et al., Structure 13 (2005) 1849-1858).

The function of the ERCC1 polypeptide is predominantly in nucleotide excision repair of damaged DNA (Aggarwal, C., et al., J. Natl. Compr. Canc. Netw. 8

(2010) 822-832; Rahn, J.J., et al., Environment. Mol. Mutagen. 51 (2010) 567-581; Westerveld, A., et al., Nature 310 (1984) 425-429).

ERCC1 is of diagnostic relevance, as it is a predictive and prognostic marker tightly linked to various disease indications (Gandara, D.R., et al., J. Thorac Oncol. 5 (2010) 1933-1938; Hwang, I.G., et al., Cancer 113 (2008) 1379-1386; Azuma, K., et al., Cancer Chemother. Pharmacol. 64 (2009) 565-573).

In general it is likely, that under IHC conditions neoepitopes are generated by formalin-induced crosslinking events (Lin, W., et al., J. Histochem. Cytochem. 45 (1997) 1157-1163, Webster, J.D., et al., J. Histochem. Cytochem. 57 (2009) 753-761). The genuine structure can be partly or completely denatured, or is at least structurally modified by the harsh conditions during the process of tissue preparation and subsequent antigen retrieval (Rait, V.K., et al., Lab. Invest. 84 (2004) 300-306). It is very likely, that the epitope region resembles a plurality of non-stable primary or secondary structures, which cannot be adequately represented by a linear, synthetically produced peptide. Therefore, an immunogen or a suitable antibody screening reagent, which is able to cope with all these tasks and which at the same time is stable and biochemically robust, has to be used.

The SlyD/FKBP12-ERCC1 scaffold can simulate the structural plurality of a linearized, completely denatured, in part denatured, in part refolded or intact secondary structure motif, like it is to be assumed in IHC application. At the same time the scaffold guarantees thermodynamic stability and robust handling.

A SlyD/FKBP12-ERCC1 fusion polypeptide was employed to screen anti-ERCC1 antibodies, which are suitable for IHC application, from a plurality of antibodies, which were obtained by a linear peptide immunization strategy.

The helix loop helix secondary structure motive was extracted from the ERCC1 (PDB 1Z00) structure (Figure 2). The C-terminal ERCC1 domain can be structurally characterized as a primarily helical polypeptide. This makes it difficult to identify a continuous linear epitope, which is conventionally used for immunization approaches with linear peptides. A linear KHL-coupled peptide was used for the immunization of animals in order to obtain antibodies specifically recognizing the intended sequence motif.

The extracted human ERCC1 C274S sequence is the amino acid sequence IAASREDLALSPGLGPQKARRLFD (SEQ ID NO: 89). The sequence represents

a helix-turn-helix motif. The cysteine residue originally present at position 11 (underlined in SEQ ID NO: 89) of the inserted sequence has been changed to a serine residue to avoid aggregation by oxidation.

As the inserted sequence has a helix conformation the additional amino acid sequence GGGSGGNP (SEQ ID NO: 82) was introduced at the N-terminus of the inserted amino acid sequence and the amino acid sequence GPTGGGS (SEQ ID NO: 83) has been introduced at the C-terminus of the inserted amino acid sequence. Thus, terminally flanking motives GGGGSGGNP and GPTGGGS were obtained. Such motifs are, without being bound by theory, supposed to foster the helix propensity via the proline helix capping sequence motives.

The FKBP12-ERCC1 fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKF
MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGGGGSGGNPIAASRE
DLALSPGLGPQKARRLFDGPTGGGSTLVFDVELLKLEGGGSRKHHHHHHH
H (SEQ ID NO: 90).

The SlyD/FKBP12-ERCC1 fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MKVAKDLWSLAYVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALEGH
EVGDKFDVAVGANDAYGYDENLVRVPKDVFMGVDELVGMRFLAETDGP
VPVEITAVEDDHVWDGNHMLAGNLKFNVEWAIREATEEELAHGHVHGAH
DHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVVETISPGDGRTFPKR
GTAWHYTGMLEDGKKFDSSRDRNKPFKFMLGKEVIRGWEEGVAQMSVG
RAKLTISPDYAYGGGGSGGNPIAASREDLALSPGLGPQKARRLFDGPTGGG
STLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 91).

The SlyD/FKBP12-ERCC1 fusion polypeptide and SlyD/FKBP12 control polypeptide were used in a screening approach to identify cell clones producing an anti-ERCC1 antibody.

The SlyD/FKBP12 control polypeptide has the amino acid sequence of SEQ ID NO: 86.

Generally, for the purification of the fusion polypeptides an affinity chromatography step was used under denaturing conditions in the presence of

chaotropic agents. The fusion polypeptide was captured on the affinity matrix. The chaotropic buffer was transferred into native buffer conditions by washing the column with a physiological buffer solution. The E.coli SlyD moiety of the SlyD/FKBP12-ERCC1 fusion polypeptide was, thus, refolded. The refolded fusion polypeptide was recovered in a physiological buffer from the affinity chromatography column.

The affinity purified fusion polypeptide was dialyzed and filtrated (for SDS gel see Figure 3). SlyD/FKBP12-ERCC1 was UV/Vis spectroscopically quantified. Protein fluorescence measurements were used to test the conformational nature of SlyD/FKBP12-ERCC1 (Figure 4). The FKBP12 mutant C22A is especially useful as a carrier for polypeptide insertions, because the single FKBP12 Trp moiety can be used to determine the structural integrity of the FKBP12 moiety (Scholz, C., et al., J. Biol. Chem. 271 (1996) 12703-12707; Russo, A.T., et al., J. Mol. Biol. 330 (2003) 851-866). FKBP12 C22A in its native structure shows a single fluorescence emission peak at 320 nm (Zoldak, G., et al., J. Mol. Biol. 386 (2009) 1138-1152).

Without being bound by theory the intrinsic Trp solvatochromic fluorescence emission at 350 nm would be strongly quenched in a folded FKBP12 protein environment, whereas it increases with the unfolding of FKBP12. A temperature screen from 25 °C to 85 °C did not show any further fluorescence emission peaks, but a temperature-dependent fluorescence quenching of the 350 nm emission. The 320 nm emission, an indicator for structural integrity of FKBP12, could not be detected (see Figure 4).

A BIAcore binding assay with the fusion polypeptide SlyD/FKBP12-ERCC1 as analyte in solution vs. the sensor surface presented ligand bi-FK506 (Figures 5 and 6) showed no FK506 binding activity, indicating a structure-function loss of the FKBP12 moiety in the fusion polypeptide. The control polypeptide FKBP12 (C22A) showed FK506 binding activity.

The non-binding of the SlyD/FKBP12-ERCC1 fusion polypeptide to the immobilized FK-506 provides evidence for a SlyD/FKBP12-ERCC1 structure, which deviates from that of the FKBP12 (C22A) conformation. This is accompanied by a loss of binding activity of the chimeric FKBP12 domain.

Fluorescence measurements and the FK506 binding assay provide evidence for a structure-function-loss of the SlyD/FKBP12-ERCC1 fusion polypeptide. The N-terminal E.coli SlyD domain keeps the fusion protein in its soluble and monomeric

state. This was proven by HPLC analyses (see Figures 7 and 8) of the SlyD/FKBP12-ERCC1 and SlyD/FKBP12-ctrl fusion polypeptides.

Mice were subjected to intraperitoneal immunization with KHL-coupled peptide covering the amino acids 219-245 of human ERCC1 (Excision Repair Cross Complementing protein). The production of hybridoma primary cultures was done according to the procedure by Koehler and Milstein. The hybridomas were isolated and screened for antigen binding by ELISA methods as described. Primary hybridoma cell cultures, which showed a positive color formation in ELISA versus the peptide ERCC1[219-245] were transferred to kinetic screening (see Figures 9 to 12). In order to avoid the selection of IHC unsuitable antibodies, screening was performed using the fusion polypeptide as reported herein. The SlyD/FKBP12-ERCC1 fusion polypeptide and SlyD/FKBP12 control polypeptide were used in a kinetic screening approach to identify antibodies, binding with to the ERCC1 amino acid sequence motif. Suitable primary cultures were further expanded into clonal cultures. The properties of the selected cell clones are depicted in Figure 12. Figure 13 exemplary shows BIAcore measurements with the clone <ERCC1>-M-5.3.35. The SlyD/FKBP12-ERCC1 interaction is highly specific. No interaction with the SlyD/FKBP12 control sample was detected. No unspecific binding was detectable. The interaction refers to a Langmuir kinetic model.

For western blotting OVCAR-3 or HEK293 cell lysates were loaded per lane on an SDS gel. Figure 14 shows a Western Blot result of clone <ERCC1>-M-5.1.35. An ERCC1 specific band at 37 kDa is detected.

For immunohistochemical detection of ERCC1 in FFPE embedded human cancer tissues sections of SCLC cancer samples were prepared. Figure 15 shows the positive IHC staining pattern. White arrows indicate the specific staining loci of ERCC1. An analogous screening and selection process has been performed solely using a polypeptide with SEQ ID NO: 89, i.e. solely using a polypeptide without the integration into the fusion polypeptide as reported herein. From 14 identified candidate cell clones in this peptide-based screening nine would have been selected by both screening approaches. Five hits from the polypeptide based screening approach do not bind to the SlyD/FKBP12-ERCC1 fusion polypeptide despite identical capture level on the BIAcore chip.

It has been found that the E.coli SlyD-FKBP12-antigen fusion polypeptide and the Thermus Thermophilus SlyD-antigen fusion polypeptide (see e.g. Kang, C.B.,

et al., Neurosignals 16 (2008) 318-325) and the Thermococcus gammatolerans SlyD-antigen fusion polypeptide can be used as combined immunogen and screening tool for the development of epitope-specific monoclonal antibodies targeting the antigen that is contained in the polypeptide.

Furthermore, it has been found, that the E.coli SlyD-FKBP12/13-antigen fusion polypeptide and the Thermus Thermophilus SlyD-antigen fusion polypeptide and the Thermococcus gammatolerans SlyD-antigen fusion polypeptide all likewise can be posttranslationally modified and can then be used as combined immunogen and screening tool for the development of posttranslational site-specific monoclonal antibodies targeting the posttranslational modification that is contained in the polypeptide. In one embodiment one of the fusion polypeptides as reported herein is used for generating an antibody and a second different fusion polypeptide as reported herein is used to select an antibody obtained with the first fusion polypeptide, wherein both fusion polypeptides are different but comprise the same antigen amino acid sequence, i.e. either X_1 or X_0 is identical in both fusion polypeptides.

The fusion polypeptides as reported herein can be used for the generation of functional antibodies by a targeted epitope approach using a structural mimetic or a scaffold technology. Especially for the generation of antibodies against antigens that are not accessible in conventional immunization campaigns (so called hidden epitopes) the fusion polypeptides as reported herein are especially suited.

Anti-IGF-1 antibody:

Human IGF-1 and IGF-2 show 67 % amino acid sequence homology and high structural homology (see Figure 16). In serum, IGF-2 is present with a 500-fold excess over IGF-1 (Jones, J. I. and Clemmons, D.R., Endocrin. Rev. 16 (1995) 3-34).

Thus, the generation of an IGF-1 specific antibody, i.e. an antibody without cross-reactivity to IGF-2, is challenging. There is a small sequential deviation between IGF-1 and IGF-2 in the turn-loop motif of IGF-1 at the IGF-1 amino acid position 74-90, starting the numbering with the signal and propeptide (UniProtKB entry P05019, IGF1_human). The corresponding amino acid sequence NKPTGYGSSSRRAPQTG (SEQ ID NO: 92) can be inserted in the SlyD/FKBP-12 fusion polypeptide, or the Thermus thermophilus SlyD fusion polypeptide, or

the Thermococcus gammatolerans SlyD fusion polypeptide as reported herein as amino acid sequence X_0.

Fusion polypeptides comprising the amino acid sequence NKPTGYGSSSRRAPQTG (SEQ ID NO: 92) can be used for the immunization of animals in order to obtain antibodies specifically binding to this turn-loop motif.

In order to improve the presentation of the immunogenic polypeptide the IGF-1 turn-loop motif can be flanked either by an GGGS linker (SEQ ID NO: 81) N-terminal and C-terminal of the amino acid sequence or by an HG dipeptide N-terminal of the IGF-1 amino acid sequence and by an GA dipeptide C-terminal of the IGF-1 amino acid sequence.

A SlyD/FKBP12-IGF-1(74-90) fusion polypeptide was used as immunogen and also as screening reagent for the development of an anti-IGF-1 antibody that is specifically binding to the IGF-1 amino acid sequence of NKPTGYGSSSRRAPQTG (SEQ ID NO: 92).

The FKBP12-IGF-1(74-90) fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 can have the following amino acid sequence:

MGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKF
MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGGGGSNKPTGYGSSS
RRAPQTGGGSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 93).

The SlyD/FKBP12-IGF-1(74-90) fusion polypeptide comprising an amino acid sequence tag of SEQ ID NO: 16 can have the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGSNKPTGYGSSSRRAPQTGGG
GSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 94).

The Thermus Thermophilus-SlyD wild-type polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHDPEGVQVVPLSAFPEDAEVVPGAQFYAQDM
EGNPMPLTVVAVEGEEVTVDFNHPLAGKDLDFQVEVVKVREATPEELLHG
HAHGGGSRKHHHHHHHH (SEQ ID NO: 97).

A Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide (see Figure 17 for SDS Page and Western blot) was used as immunogen and also as screening reagent for the development of anti-IGF-1 antibodies that are targeting the IGF-1 amino acid sequence of NKPTGYGSSSRRAPQTG (SEQ ID NO: 92).

The Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 (see Figure 3 for SDS and Western Blot) can have the amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHGNKPTGYGSSSRRAPQTGGAGKDLDFQVEV
VKVREATPEELLHGHAHGGGSRKHHHHHHHH (SEQ ID NO: 95),

or the amino acid sequence:

MKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEG
EAFQAHVPAEKAYGPHGNKPTGYGSSSRRAPQTGGAGKDLDFQVEVVKV
REATPEELLHGHAHPSGHHHHHH (SEQ ID NO: 96).

For Screening and specificity testing a Thermus thermophilus SlyD-ΔIF fusion polypeptide was produced. The Thermus thermophilus SlyD-ΔIF fusion polypeptide lacks the IF domain, which was replaced by a short amino acid sequence motif.

The Thermus thermophilus SlyD-ΔIF fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 can have the amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHGAGSGSSGAGKDLDFQVEVVKVREATPEELL
HGHAHGGGSRKHHHHHHHH (SEQ ID NO: 116).

For Screening and specificity testing a Thermococcus gammatolerans SlyD fusion polypeptide was produced with the structurally homologous IGF-2(53-65) hairpin insertion.

The Thermococcus gammatolerans SlyD-IGF-2(53-65) fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 can have the amino acid sequence:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMP-G-SRVSRRSRG-G-
AGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHHHH (SEQ ID NO: 117).

All fusion polypeptides were produced in E.coli. All fusion polypeptides were purified and refolded by using virtually identical protocols as described herein. Mice were subjected to intraperitoneal immunization with the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide and the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide. Ten weeks after immunization antibody titers were determined by means of ELISA (Figures 19 and 20). Mice immunized with the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide showed low titers versus IGF-1, versus a constrained IGF-1(74-90) peptide loop, versus the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide, and versus the SlyD/FKBP12 control polypeptide. Only one mouse provided for sufficiently high IGF-1 titer (K1576M1 in Figure 19) and was used for the generation of hybridomas.

It has been found that mice immunized with the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide showed higher titers versus the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide than versus the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide (see Figures 19 and 20).

The production of hybridoma primary cultures was done according to the procedure of Koehler and Milstein. The primary hybridomas were isolated by limiting dilution and screened for antigen binding by ELISA. Primary hybridoma cell cultures, which showed a positive color formation in ELISA versus the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide, the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide, and IGF-1 as well as a lower signal versus the Thermophilus-SlyD-wild-type fusion polypeptide and the SlyD/FKBP12 control polypeptide were further evaluated using a kinetic screening method.

It has been found that only two primary cultures from the immunization campaign with the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide had a positive ELISA signals versus IGF-1. After development into clonal cultures no kinetic binding signals were detectable in surface plasmon resonance (SPR) analyses. Several primary cultures, which showed suitable ELISA binding signals versus IGF-1 and

the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide but reduced signal intensity versus the Thermus Thermophilus-SlyD wild-type polypeptide have been found (see Figure 21).

The primary cultures were analyzed by a kinetic screening method versus native IGF-1, versus native IGF-2, versus the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide, and versus the Thermus Thermophilus-SlyD wild-type polypeptide (Figure 22). IGF-1 specific antibody producing primary cultures were detected and expanded by limited dilution to obtain clonal cultures.

The clonal cultures were analyzed by means of ELISA for specific binding versus IGF-1 (see Figure 23). In Figure 24 exemplary BIAcore measurements of an anti-IGF-1 antibody, which was obtained from the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide immunization campaign, are shown. The antibodies specifically bind the Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide and native IGF-1 with 10 pM binding affinity. Native IGF-2 and wild-type Thermus Thermophilus SlyD is not being bound (see Figure 25).

It has been found that for the generation of IGF-1 specific antibodies, it is of importance, that the IGF-1 turn-loop motif is stabilized by a rigid, enthalpic scaffold to preserve its native fold. When being presented on a metastable polypeptide scaffold, like FKBP12, it is without being bound by this theory supposed, that the sequence NKPTGYGSSSRRAPQTG (SEQ ID NO: 18) has too many degrees of rotational freedom. Finally no native IGF-1 binding antibody was obtained.

It has been found by near-UV-CD spectroscopic measurements that the FKBP-12 portion in the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide was unfolded. HPLC analyses showed that the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide is monomeric. DSC measurements showed that the fusion polypeptide is able to reversibly fold and unfold. Without being bound by theory the reversibly foldable N-terminal E.coli SlyD domain keeps the fusion polypeptide stable and monomeric in solution even when the C-terminal FKBP domain is partly or completely unfolded (see Figure 26).

Like already found for the SlyD/FKBP12-ERCC1 fusion polypeptide the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide can present a structural plurality of a linearized, completely denatured, in part denatured, in part refolded or intact secondary structure motifs.

For the development of native IGF-1 binding antibodies it has been found that a scaffold has to be used, which presents the polypeptide insertion in its native conformation. The presenting fusion polypeptide therefore needs to be a stably folded polypeptide. It has been found that this can be achieved by using an FKPB domain from an extremophile (i.e. thermophile) organism, like Thermus thermophilus SlyD, or Thermococcus gammatolerans SlyD.

To examine whether the fusion polypeptides as reported herein adopt a folded conformation CD spectra in the near-UV region were determined. Near-UV-CD determines the asymmetric environment of aromatic residues in a polypeptide and is therefore a sensitive test for ordered tertiary structure. Native SlyD has a typical CD signature in the near-UV region. Thus, structural distortions or steric clashes due to an insertion in the IF domain should be visible in the near-UV CD spectrum. In Figure 27 an overlay of the spectra of wild-type Thermus thermophilus SlyD, the FKBP domain of wild-type Thermus thermophilus SlyD lacking the IF domain (Thermus thermophilus SlyD-ΔIF fusion polypeptide) and Thermus thermophilus SlyD-antigen fusion polypeptide, wherein a 22 amino acid insertion from a human extracellular receptor fragment was inserted, is shown. It has been found that the replacement of the Thermus thermophilus IF domain does not result in a change of the overall structure of the remaining IF domain. It can be seen that the signature of the spectra is similar. Since unfolding would abolish any near-UV CD signal, this result provides evidence that a native-like fold is retained in the fusion polypeptide.

The Thermus thermophilus SlyD-antigen fusion polypeptide is a fusion polypeptide comprising a 22 amino acid beta hairpin secondary structure insertion from a human growth factor receptor extracellular domain (ECD). The CD signatures demonstrate that at 20 °C all polypeptides are well folded in their native structure.

Figure 28 shows the temperature-dependent CD spectra of the Thermus thermophilus SlyD-ΔIF fusion polypeptide. After temperature-induced unfolding, the Thermus thermophilus SlyD FKBP domain can refold when being cooled down again. Due to this the fusion polypeptides can be affinity purified by on column refolding and, furthermore, in contrast to the findings with the SlyD-FKBP12-IGF-1 fusion polypeptide, the Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide possesses structural stability to present the IGF-1 secondary structure motive on the FKBP domain in a stable conformation. The temperature-dependent near-UV CD spectra of the Thermococcus gammatolerans SlyD-antigen fusion

polypeptide shows an even higher stability when compared to Thermus thermophilus-antigen fusion polypeptide (see Figure 29). Both scaffolds carry the same 22 amino acids beta hairpin secondary structure insertion from a human growth factor receptor ECD. Thermococcus gammatolerans SlyD-antigen reversibly folds and unfolds. It has been found that under the given physical conditions no complete unfolding of the Thermococcus gammatolerans SlyD-antigen fusion polypeptide could be achieved even at a temperature of 100 °C.

It has been found that the stability of archaic FKBP domains enables the grafting of immunogenic polypeptides by replacement of their IF domains whereby at the same time the overall stability of the newly generated chimeric scaffold protein is maintained.

The Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide was purified as a stable and monomeric polypeptide (see Figure 18).

The monomer fraction of the Thermococcus gammatolerans SlyD-antigen fusion polypeptide was rechromatographed after repeated freezing and thawing cycles and a temperature stress test (see Figure 30).

Mice have been immunized with the polypeptide of SEQ ID NO: 96. The obtained B-cells were analyzed using an ELISA. Thermus Thermophilus-SlyD-IGF-1(74-90) fusion polypeptide, Thermus Thermophilus-SlyD-wild-type polypeptide, IGF-1 and IGF-2 were used as a control.

The Thermus Thermophilus-SlyD wild-type polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 (see Figure 31 for SDS and Western blot) has the following amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHDPEGVQVVPLSAFPEDAEVVPGAQFYAQDM
EGNPMPLTVVAVEGEEVTVDFNHPLAGKDLDFQVEVVKVREATPEELLHG
HAHGGGSRKHHHHHHHH (SEQ ID NO: 97).

All clone culture supernatants (CCS) form stable complexes with IGF-1 and the Thermo Thermophilus SlyD-IGF-1 fusion polypeptide at 37 °C. No cross-reaction with the Thermo Thermophilus SlyD-wild-type polypeptide was detectable with any of the CCS. No cross-reactivity with IGF-2 was detectable, except for one clone (see Figure 32). From Figure 33 it can be seen that the first 8 clone culture

supernatants have a t/2-diss of 2 minutes, whereas the analyte IGF-1 associates faster to the last 4 clone culture supernatants and dissociates slower. It stays in complex longer than 40 minutes. In Figure 22 exemplary sensorgrams for one clone are shown. It can be seen that the antibody binds to IGF-1 and the Thermus thermophilus-IGF-1 fusion polypeptide whereas binding to IGF-1 and the Thermus thermophilus-wild-type polypeptide cannot be detected.

Thus, Thermus Thermophilus SlyD-antigen fusion polypeptides and Thermococcus gammatolerans SlyD-antigen fusion polypeptides can be used as combined immunogen and screening tools for the development of epitope-specific monoclonal antibodies targeting the immunogen that is contained in the polypeptide.

Figure 25 shows, that the scaffold-derived monoclonal antibodies <IGF-1>M-11.11.17 and <IGF-1> M-10.7.9 have picomolar affinity versus IGF-1. Monoclonal antibody <IGF-1> M-11.11.17 shows an IGF-1 complex stability of t1/2 diss = 560 min. No cross-reactivity versus IGF-2, wild-type Thermus thermophilus SlyD, wild-type Thermococcus gammatolerans SlyD, Thermus thermophilus SlyD-ΔIF, and Thermococcus gammatolerans SlyD-IGF-2(53-65) was detectable.

Monoclonal antibody M-2.28.44 has been obtained by conventional immunization of mice with recombinant human IGF-1. Although the antibody has a 30 pM binding affinity versus IGF-1, the antibody also has cross reactivity versus IGF-2 (binding affinity of 500 pM). Using the Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide and the Thermococcus gammatolerans SlyD-IGF-2(53-65) fusion polypeptide as analyte it can be shown that the cross-reacting IGF-2 epitope is not in the IGF-1/2 hairpin region.

This was confirmed by a linear epitope mapping (see, e.g., Frank, R. and Overwin, H., Methods in Molecular Biology 66 (1996) 149-169. The linear IGF-1 binding contribution of clone 11.11.17 and 11.9.15 both recognize the epitope TGYGSSSR (SEQ ID NO: 124). The linear binding portion of clone 10.7.9 binds to the epitope PTGYGSSSR (SEQ ID NO: 125). The epitopes are located at the top of the IGF-1 hairpin-structure and are therefore not present in IGF-2.

In general, the fusion polypeptides as reported herein can be used for the generation of functional antibodies by a targeted epitope approach using a structural mimetic. Especially for the generation of antibodies against antigens that are not easily accessible by conventional immunization campaigns using recombinant

immunogens the fusion polypeptides as reported herein are especially suited. It has been found that so called hidden epitopes, which are buried inside a native protein conformation, can be used as immunogen, when being well presented as insertion in the fusion polypeptides as reported herein. Especially neoepitopes, which are only targetable upon allosteric, ligand induced conformational changes, can be used as immunogen by grafting these structures into the fusion polypeptides as reported herein.

Chimeric FKBP12/13 scaffolds:

Some fusion polypeptides as reported herein are based on a fusion polypeptide comprising parts of human FKBP12 and parts of Arabidopsis thaliana FKBP13. It has been found that a fusion polypeptide comprising at least a part of human FKBP12 and at least a part of Arabidopsis thaliana FKBP13 can be used as immunogen. In this fusion polypeptide the human FKBP12 derived part is thermodynamically stabilized as a scaffold. FKBP13 comprises a disulfide bond, which stabilizes the IF domain. This sequence was grafted into the human FKBP12 derive part to stabilize the fusion polypeptide.

The FKBP12/13 fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 has the amino acid sequence:

MRSGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGDRGAGCGSGSSCLI PPASVLVFDVELLKLE GGGSRKHHHHHHHH (SEQ ID NO: 118).

The FKBP12/13 fusion polypeptide was expressed in E.coli (see Figure 34) as soluble protein. HPLC analyses (see Figure 35) proved that the FKBP12/13 fusion polypeptide is monomeric. CD spectroscopic measurements were performed as described above. The CD spectra proved that the FKBP12/13 fusion polypeptide is folded at 20 °C. Thermus thermophilus and Thermococcus gammatolerans based scaffolds show higher temperature-stability than the FKBP12/13 fusion polypeptide.

Anti-PLGF antibody:

The inserted amino acid sequence in this case has a turn-loop motif and an IHC suited antibody shall be generated. The insert has the amino acid sequence

DWSEYPSEVEHMFSPSS (SEQ ID NO: 98). The C-terminal cysteine residue in the immunogen has been changed to a serine residue.

The FKBP12-PLGF fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 has the following amino acid sequence:

MGVQVETISPGDGRTFPKRGQTAWHYTGMLEDGKKFDSSRDRNKPFKFM
LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGGGGSDWSEYPSEVEH
MFSPSSGGGSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 99).

The SlyD/FKBP12-PLGF fusion polypeptide comprising a C-terminal amino acid sequence tag has case the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGSDVVSEYPSEVEHMFSPSSGG
GSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 100).

Mice were immunized with an immunogen containing the sequence of PLGF(60-76). Subsequently hybridomas were produced and ELISA as well as kinetic screening were performed.

In the kinetic screening process the SlyD/FKBP12-PLGF fusion polypeptide and a biotinylated PLGF(60-76) peptide, which was singly grafted on streptavidin, were used to identify primary culture supernatants with binding activity versus PLGF(60-76). Both analytes produced 1:1 Langmuir kinetics, but the fusion polypeptide showed a better dissociation fit with a lower chi2 value than the SA-probe grafted biotinylated PLGF peptide. Thus, a fusion polypeptide-based screening approach takes advantage of the monomeric state and the improved epitope accessibility of the immunogen in the fusion polypeptide when compared to a SA-probe.

Antibodies, developed by this approach, like clone 53.4.1, were able to specifically detect PLGF in Western blotting.

SlyD-FKBP12/13-CSF1R fusion polypeptide:

The term "SlyD-FKBP12/13-CSF1R fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGVQVETISPGDGRTFPKRG
QTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSV
GQRAKLTISPDYAYGDRGAGCGSGGVDYKNIHLEKKYVRRDSGFSSQGVD
T*Y*VEMRPVSTSSNDSFSEQDLDKEDGGSSCLIPPASVLVFDVELLKLEGGGS
RPLLPPLPGGGSRKHHHHHHHH (SEQ ID NO: 119).

The polypeptide was expressed in E.coli as described herein and purified as described herein. After Ni-NTA affinity purification a size exclusion chromatography was performed. The fusion polypeptide was loaded on a HiLoad 26/60 SuperdexTM 75 pg column. The elution fractions were analyzed using a native SDS gel (see Figure 37).

The X_1 amino acid sequence used for the generation of antibodies corresponds to the CSF1R intracellular kinase insert domain. Src kinase, EGF1R and CSF1R itself can phosphorylate the tyrosine residues, especially the tyrosine residue in the kinase domain loop structure, which is flanked by threonine and valine (see the Y residue at position 297 in SEQ ID NO: 119, given also in italic and underlined).

After purification the fusion polypeptide can be phosphorylated using a suitable kinase. Thus, it is possible to provide an X_1 amino acid sequence having a post-translational modification and, thus, it is possible to generate antibodies against post-translationally modified polypeptides using a fusion polypeptide as reported herein. The fusion polypeptide can be used for different applications like screening, specificity testing or as an immunogen. In general, peptides which are substrates for enzymatic posttranslational or chemical modifications can be inserted into the scaffolds as X_1 amino acid sequence. Protein fluorescence measurements were used to test the conformational nature of the SlyD/FKBP12/13-CSF1R fusion polypeptide. At 20 °C and 25 °C the 300 nm to 425 nm scan peaks at 305 nm (see Figure 38). The single tryptophane moiety in the FKBP12 domain shows the typical intrinsic tryptophane solvatochromic fluorescence emission as it is buried in the hydrophobic core of the FKBP12 domain. Therefore, it is assumed, that the

FKBP12/13-CSF1R polypeptide portion is folded. At 50 °C the emission peak shifts to 344 nm, which is evidence, that the tryptophane moiety is now in an aqueous environment and the FKBP domain is in part or completely unfolded. At 30 °C and 40 °C intermediate states of folded and unfolded protein can be determined.

It has been found that it is possible to stabilize the FKBP12 domain in a fusion polypeptide by engineering FKBP12 into an FKBP12/13 fusion polypeptide, where the FLAP domain of FKBP12 was replaced by the disulfide containing FKBP13 structure and a loop structure motif from the CSF1R receptor.

Already at 40 °C the fusion polypeptide shows a significant unfolded protein portion, which mainly refers to the FKBP12/13-CSF1R domain as E.coli SlyD does not contain tryptophane.

In order to use a chimeric FKBP domain as a scaffold for the presentation of correctly folded, native secondary or tertiary structures as immunogens this finding underlines the need to further stabilize chimeric FKBP12 in order to generate non-metastable scaffolds. Furthermore, it is not possible to omit the E.coli SlyD domain in the fusion polypeptide, because then the expression yield dramatically decreases (no data shown).

Epitope Mapping:

SlyD-FKBP fusion polypeptides can also carry complex amino acid insertion motifs, like for example secondary structures containing disulfide bonds. As the fusion polypeptides are free of cysteines, on-column refolding under appropriate conditions facilitates the correct formation of disulfide bonds within the insertion, additionally assisted by the chaperone functionality of SlyD itself.

The fusion polypeptides SlyD-FKBP12-CD81 and SlyD-FKBP12-ctrl were used for the purpose of Epitope Mapping. Human CD81 is a receptor for the hepatitis C virus envelope E2 glycoprotein. CD81 is a transmembrane protein belonging to the tetraspanin family. CD81 is a 90 amino acid in length homodimeric protein, which displays a so called mushroom-like structure (PDB 1IV5). Residues known to be involved in virus binding can be mapped onto the so called 35 amino acid in length "head subdomain", providing a basis for the design of antiviral drugs and vaccines. As the head subdomain sequence of the virus binding site is just 35 amino acids in

length it is difficult to map antibody epitopes on the 10 kDa CD81 protein using conventional cross-blocking experiments.

It is hard to discriminate antibodies, which bind directly on the mushroom-like head domain, from antibodies just binding nearby or elsewhere in the CD81LEL structure. All these antibodies would show an HCV E2 envelope protein competing effect, but without specifically binding to the target structure, the head domain. The grafting of the head domain structure into FKBP and a consecutive epitope mapping is therefore an advantageous method. First, some biochemical issues with the CD81LEL protein are circumvented, because the protein itself tends to oligomerize. Second, it is suitable to select antibody epitopes from a vast number of antibodies just binding to the full length CD81 protein.

SlyD-FKBP12-CD81 fusion polypeptide:

The term "SlyD-FKBP12-CD81 fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGSCCGSSTLTALTTSVLKNNLC
PSGSNIISNLFKEDCGGGSTLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 126).

SlyD-FKBP12-ctrl fusion polypeptide (see figure 1):

The term "SlyD-FKBP12-ctrl fusion polypeptide" denotes a polypeptide that has the following amino acid sequence:

MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETALE
GHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGMRFL
AETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIREATEEEL
AHGHVHGAHDHHHDHDHDGGGSGGGSGGGSGGGSGGGSGGGGVQVETI
SPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIR
GWEEGVAQMSVGQRAKLTISPDYAYGGGGSGGNPGPTGGGSTLVFDVEL
LKLEGGGSRKHHHHHHHH (SEQ ID NO: 86).

A BIAcore 2000 instrument (GE Healthcare) was used at 25 °C with a BIAcore CM5 sensor mounted into the system. Each protein ligand was immobilized by EDC/NHS chemistry into the flow cells 2, 3 and 4. Flow cell 1 was used as a reference. The following substances were immobilized on the sensor: Flow cell 2: SlyD-FKBP12ctrl, flow cell 3: SlyD-FKBP12-CD81, and flow cell 4: CD81LEL. 31 antibody analytes were injected. The sensorgrams were monitored as reference signals 2-1, 3-1 and 4-1 and were evaluated by using the BIAcore Evaluation software 4.1. At the end of the analyte injection a report point was set to quantify the maximum analyte binding signal. The highest analyte binding signal was set 100 % to normalize the data. The normalized antibody binding responses showed that from 30 tested anti-CD81-LEL-antibodies only 6 show a binding to an epitope on the CD81 head domain. The negative control polypeptide SlyD-FKBP12ctrl was not bound. The positive control polypeptide CD81-LEL, which was the immunogen at the same time, was bound by all antibodies. Slyd-FKBP12-CD81 was only bound, when the antibody epitope was located in the mushroom domain.

Confirmation of the epitope mapping results by X-ray crystallographic analysis

Fab fragments of the antibodies K05 and K04 were co-crystallized by known methods with the CD81-LEL protein and were analyzed by x-ray diffraction analysis (Seth Harris, Palo Alto). The resolution obtained was 2.15 Å. K04 directly recognizes the target epitope sequence, whereas K05 binds off the target. Therefore, the x-ray analysis directly correlates with the scaffold-based epitope mapping approach.

The following examples, figures and sequences are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 SDS PAGE (Coomassie staining) and Western Blot (10 sec. incubation with anti-octa-his-tag antibody) of the SlyD/FKBP12-control polypeptide.

Figure 2 A: ERCC1 (PDB 1Z00): encircled helix turn helix motive (IAASREDLALSPGLGPQKARRLFD, C274S);
B: FKBP12 C22A; encircled sequence replaced; FKBP12 chimera C-terminally was fused to E.coli SlyD.

Figure 3 SDS PAGE (Coomassie staining) and anti-his-tag Western Blot (10 sec exposition) of the SlyD/FKBP12-ERCC1 polypeptide. M - Novex Sharp Standard; 1 - 2.5µg SlyD/FKBP12-ERCC1 fusion polypeptide; 2 - 5.0 µg SlyD/FKBP12- ERCC1 fusion polypeptide; 3 - 10 µg SlyD/FKBP12-ERCC1 fusion polypeptide; M* - Magic Mark.

Figure 4 Wavelength scan from 300 nm – 600 nm was driven at 600 nm/min recoding fluorescence emission intensity of SlyD/FKBP12-ERCC1 fusion polypeptide at 25 °C, 35 °C, 45 °C, 55 °C, 85°C.

Figure 5 Scheme of BIAcore assay for determining the binding of SlyD/FKBP12-ERCC1 fusion polypeptide to FK-506.

Figure 6 300 nM SlyD/FKBP12-ERCC1 fusion polypeptide and 300 nM wild-type FKBP12 as analytes in solution vs. sensor surface presented biotinylated ligand bi-FK506.

Figure 7 Analytical HPLC chromatogram of the SlyD/FKBP12-ctrl fusion polypeptide. After Ni-NTA purification SlyD/FKBP12-ctrl elutes as a monomeric peak.

Figure 8 Analytical HPLC chromatogram of the SlyD/FKBP12-ERCC1 fusion polypeptide. After Ni-NTA purification SlyD/FKBP12-ERCC1 elutes as a monomeric peak.

Figure 9 Scheme of BIAcore binding assay kinetic screening with SlyD/FKBP12-ERCC1 fusion polypeptide and 300 nM SlyD/FKBP12-ctrl as analytes in solution. CM5 sensor, Capture RAMFCy: Rabbit anti mouse Fc gamma capturing antibody.

Figure 10 Stability Late / Binding Late Plot showing kinetic properties of anti-ERCC1 antibodies determined by kinetic screening using SlyD/FKBP12-ERCC1 as analyte in solution. All clones populate the 10^{-5} 1/s trend line at Binding Late values > 40 RU indicating extraordinary antigen complex stability. No binding versus SlyD/FKBP12-ctrl is detectable.

Figure 11 Properties of anti-ERCC1 antibodies determined by kinetic screening. The Binding Late/ Antibody Capture Level Plot indicates binding valence corridors by trend lines. All 5.00X.35 sister clones (encircled) populate the valence corridor between a Molar Ratio = 0.5 and Molar Ratio = 1 and were selected for further processing.

Figure 12 Table with kinetic properties of anti-ERCC1 antibodies determined by kinetic screening. BL: Binding Late, signal amplitude height in relative response units at the end of the SlyD/FKBP12-ERCC1 association phase. SL: Stability Late, signal amplitude height in relative response units at the end of the SlyD/FKBP12-ERCC1 dissociation phase. kd: dissociation rate constant (1/s) according to a Langmuir fit of the dissociation phase. t1/2diss: antibody-SlyD/FKBP12-ERCC1 complex half-life in minutes, calculated according to the formula t1/2 diss = ln(2)/(60*kd).

Figure 13 Exemplary anti-ERCC1 antibody single concentration kinetic of clone <ERCC1>M-5.3.35 using SlyD/FKBP12-ERCC1 as analyte in solution.

Figure 14 Western blot using clone<ERCC1>M-5.1.35. 5 µg OVCAR-3 and HEK293 cell lysates were loaded per lane on a NuPAGE SDS gels (Invitrogen). A specific ERCC1 band at 37 kDa is detected.

Figure 15 Immunohistochemical detection of ERCC1 in FFPE embedded human cancer tissue of SCLC cancer sample. White arrows indicate cells with elevated ERCC1 level appearing in darker color.

Figure 16 Superimposition of IGF-1 (PDB:1PMX) and IGF-2 (PDB:1IGL) PyMOL 1.4. Sequence alignment (clustalW) of IGF-1 and IGF-2. Black box denotes IGF-1(74-90) and IGF-2(53-65) hairpin sequence.

Figure 17 SDS PAGE (Coomassie staining) and anti-his-tag Western Blot (10 sec exposition) of Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide. M - Novex Sharp Standard; 1 - 2.5µg Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide; 2 - 5.0 µg Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide; 3 - 10 µg Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide; M* - Magic Mark.

Figure 18 Analytical HPLC chromatogram of Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide.

Figure 19 Serum titers, determined by ELISA after 12 weeks immunization of NMRI mice with SlyD/FKBP12-IGF-1 (74-90). mE: milli Absorbance, IGF-1: native human IGF-1 (Peprotech).

Figure 20 Serum titers, determined by ELISA after 12 weeks immunization of Balb/C and NMRI mice. mE: milli Absorbance, IGF-1: native human IGF-1 (Peprotech).

Figure 21 ELISA screen of primary cultures with binding signals vs. IGF-1, Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide and Thermus thermophilus SlyD wild-type polypeptide. mE: milli Absorbance, IGF-1: native human IGF-1 (Peprotech).

Figure 22 Exemplary BIAcore kinetic screening of primary culture <IGF-1>M-11.0.15 vs. IGF-1, IGF-2, Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide and Thermus thermophilus SlyD wild-type polypeptide.

Figure 23 ELISA screen of clone culture supernatants vs. IGF-1, Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide and Thermus thermophilus SlyD wild-type polypeptide. Elevated binding absorption signals were detectable vs. IGF-1 and Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide.

Figure 24 BIAcore measurements of scaffold-developed <IGF-1>M-11.11.17-IgG vs. IGF-1, IGF-2, Thermus thermophilus SlyD-IGF-1(74-90) fusion polypeptide, Thermus thermophilus SlyD wild-type polypeptide, Thermococcus gammatolerans SlyD wild-type polypeptide, Thermus thermophilus SlyD-ΔIF fusion polypeptide, Thermococcus gammatolerans SlyD-IGF-2(53-65) fusion polypeptide.

Figure 25 Table with binding kinetics of fusion polypeptide-developed anti IGF-1 antibodies. mAb: monoclonal antibody; RU: Relative response unit of monoclonal antibody captured on the sensor; Antigen: antigen in solution; kDa: molecular weight of the antigens injected as analytes in solution; ka: association rate constant; kd: dissociation rate constant; t1/2 diss: antibody-antigen complex half-life calculated according to the formula t1/2 diss = ln(2)/60*kd; KD: dissociation constant; R_{MAX}: Binding signal at the end of the association phase of the 90 nM analyte injection; MR: Molar Ratio; Chi^2: failure of the measurement; n.d.: not detectable.

Figure 26 DSC measurements, overlay plot of two runs melting SlyD/FKBP12-IGF-1(74-90) fusion polypeptide in the

temperature gradient 10 °C to 95 °C. SlyD/FKBP12-IGF-1(74-90) fusion polypeptide folds reversible.

Figure 27 Near UV CD spectra of Thermus thermophilus SlyD wild-type polypeptide, Thermus thermophilus SlyD-ΔIF fusion polypeptide (FKBP) and Thermus thermophilus SlyD-antigen fusion polypeptide. At 20 °C all polypeptides are folded in their native structure.

Figure 28 Temperature-dependent CD spectra of the Thermus thermophilus SlyD-ΔIF fusion polypeptide. Repeated heating and cooling shows that the FKBP domain of Thermus thermophilus SlyD reversibly folds. Thermus thermophilus SlyD-ΔIF fusion polypeptide is stable up to 65 °C and unfolded at 85 °C.

Figure 29 Temperature-dependent CD spectra of the Thermococcus gammatolerans SlyD-antigen fusion polypeptide. At 100 °C no lower signal plateau was reached, indicating that the fusion polypeptide is not yet completely unfolded. Up to 80 °C the fusion polypeptide is stable and folded.

Figure 30 The monomeric Thermococcus gammatolerans SlyD-antigen fusion polypeptide comprising fraction was rechromatographed after repeated freezing and thawing cycles and a temperature stress test. 280 nm SUX 200 profile of 300 µg Ni-NTA elution fraction of Thermococcus gammatolerans SlyD-antigen fusion polypeptide in 100 µl 50 mM K_2HPO_4/KH_2PO_4, pH 7.0, 100 mM KCl, 0.5 mM EDTA at 0.75 ml/min.

Figure 31 SDS PAGE (Coomassie staining) and Western Blot (10 sec incubation with anti-octa-his-tag antibody) of the Thermus thermophilus SlyD-wild-type polypeptide.

Figure 32 Quantification of this kinetic screening approach for anti-IGF-1 antibodies. Empty cells denote that the respective value was not detectable/could not be determined.

Figure 33 Kinetics of 12 clonal culture supernatants for the binding of IGF-1.

Figure 34 SDS PAGE (Coomassie staining) of the FKBP12/13 fusion polypeptide expressed in E.coli.

Figure 35 FKBP12/13 fusion polypeptide: HPLC SEC-elution profile of Ni-NTA purified material. FKBP12/13 fusion polypeptide is mostly monomeric.

Figure 36 Analytical HPLC chromatogram of the SlyD/FKBP12-IGF-1(74-90) fusion polypeptide.

Figure 37 Coomassie-stained native Novex® 8-16% Tris-Glycine Mini Gel (Invitrogen) BM: BenchMark™ Pre-Stained Protein Ladder (Invitrogen). F4 to f10: size exclusion chromatography elution fractions. Fraction 8 and fraction 9 show single distinct protein bands at 37 kDa.

Figure 38 Fluorescence emission of SlyD-FKBP12/13-CSF1R at different temperatures.

Figure 39 SDS PAGE (Coomassie staining) and anti-his-tag Western Blot (10 sec exposition) of the Thermus thermophilus SlyD-ΔIF fusion polypeptide. Protein bands indicated by black arrow. M - Novex Sharp Standard; 1 - 2.5 µg Thermus thermophilus SlyD-ΔIF fusion polypeptide; 2 - 5.0 µg Thermus thermophilus SlyD-ΔIF fusion polypeptide; 3 - 10 µg Thermus thermophilus SlyD-ΔIF fusion polypeptide; M* - Magic Mark.

Figure 40 SDS page (left) and Western Blot (right) of Ni-NTA chromatographically purified SlyD-FKBP12-CD81. M: Novex Sharp Standard, 1: SlyD/FKBP12-CD81; 2.5µg MW: 36kD, 2: SlyD/FKBP12-CD81; 5.0µg, 3: SlyD/FKBP12-CD81; 10µg M*: Magic Mark.

Examples

Example 1

Expression and purification

The polypeptides were produced in E.coli (pQE80L vector / E.coli BL21 CodonPlus-RP cell line) according to known methods.

For the purification of the crude polypeptides an affinity chromatography step was used either under native conditions or under denaturing conditions in the presence of chaotropic agents. For fusion polypeptide comprising the SlyD part purification in the presence of chaotropic agents is especially suited as the total amount of fusion polypeptide could be isolated from the E.coli cells. Additionally the entire fusion polypeptide is obtained in a random coil conformation. The fusion polypeptide still bound to the affinity chromatography material is transferred into native conditions by washing the column with a physiological salt solution. Due to

the spontaneous folding of the SlyD and FKBP12 parts of the fusion polypeptide also the inserted amino acid sequence was transferred to its native conformation. The refolded fusion polypeptide was recovered with an imidazole gradient in a physiological buffer from the affinity chromatography column.

Example 2

Chemical derivatization

The C-terminal lysine residue was activated under acidic conditions (pH 6) with LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-(propionamido) hexanoate)) ((Pierce, Cat.: 68181-17-9).

Arginine and lysine are bases that can take up the proton of lysine's alkyl ammonium group. The free amino can be derivatized with any hydroxyl succinimidyl-activated carbonic acid.

Example 3

Formalin treatment

The derivatized fusion polypeptides can be treated with a formalin solution. Thereafter the fixated derivatized fusion polypeptides can be purified by size exclusion chromatography in order to obtain compositions with defined oligomerization status (monomeric, oligomeric, multimeric).

Example 4

BIAcore characterization of antibody producing clone culture supernatants

A BIAcore T100 instrument (GE Healthcare) was used with a BIAcore CM5 sensor mounted into the system. The sensor was preconditioned by a 1 min. injection at 100 µl/min of 0.1 % SDS, 50 mM NaOH, 10 mM HCl and 100 mM H_3PO_4.

The system buffer was HBS-ET (10 mM HEPES (pH 7.4) supplemented with 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) Tween® 20). The sample buffer was the system buffer.

The BIAcore T100 System was driven under the control software V1.1.1. Polyclonal rabbit IgG antibody <IgGFCγM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized at 30 µg/ml in 10 mM sodium acetate buffer (pH 4.5) at 6500 RU on the flow cells 1, 2, 3, and 4, respectively, via EDC/NHS

chemistry according to the manufacturer's instructions. Finally, the sensor surface was blocked with a 1 M ethanolamine solution. The complete experiment was performed at 25 °C.

The clone culture supernatants containing the respective antibodies at 35 nM to 190 nM were captured for 1 min. at a flow rate of 5 µl/min on the <IgGFCγM>R surface. As analytes in solution the recombinant antigen, the biotinylated disulfide-bridged recombinant antigen, SlyD/FKBP12-antigen, Thermus thermophilus SlyD-antigen, SlyD/FKBP12-control and/or Thermus thermophilus SlyD-wt fusion polypeptides were used. The respective analytes were injected at different concentration steps from 90 nM, 30 nM, 10 nM, 3.3 nM, 1.1 nM and 0 nM. The association phase was monitored for 3.5 min. at a flow rate of 100 µl/min. The dissociation was monitored for 15 min. at a flow rate of 100 µl/min. The system was regenerated using a 10 mM glycine buffer (pH 1.7). Kinetics were evaluated using the BIAcore Evaluation Software.

Example 5

IHC sample preparation

The matrix immobilized LC-SPDP fusion polypeptide was treated with organic solvent, heated and treated with an acidic buffer. Thereafter the matrix associated polypeptides are recovered und reducing conditions. To obtain a material with defined composition a size exclusion chromatography can be performed. The thereby obtained material has a defined oligomeric state (monomer, oligomer, and polymer) and can be used as immunogen for the immunization of experimental animals but it can also be used as test antigen for the selection and screening of antibodies.

Example 6

Immunization

The pre-formulated immunogenic fusion polypeptide is administered to an experimental animal, such as mouse, rat, rabbit, sheep, or hamster, intraperitoneally at different dosages. Prior to collection of the B-cells a boost immunization is performed. B-cell hybridomas can be obtained according to the method of Koehler and Millstein (Kohler, G. and Milstein, C., Nature 256 (1975) 495-497). The obtained hybridomas are deposited as single clones or cells in the wells of a multi well plate. Primary hybridoma cultures that were tested positive with respect to the

binding of the antibody by the secreted antibody are further screened with a kinetic screening method.

Example 7

Anti-IGF-1 antibodies

The cells obtained from four immunized NMRI-mice were analyzed using an ELISA. Nunc Maxisorb F multi well plates were coated with SlyD/FKBP12-IGF-1, SlyD/FKBP12-IGF-1(74-90), Thermus thermophilus SlyD-IGF-1, SlyD/FKBP12-control or Thermus thermophilus SlyD-wt by applying a solution comprising 0.41 µg polypeptide per ml. The isolated antigen IGF-1 was immobilized in the wells of StreptaWell High Bind SA multi well plates by applying a solution comprising 90 ng/ml biotinylated IGF-1 or 500 ng/ml biotinylated IGF-1-peptide loop.

Thereafter free binding sites were blocked by applying a solution comprising 1 % RPLA in PBS for one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As samples the mouse serum diluted 1:50 with PBS were used. Optional further dilution was performed in 1:4 steps until a final dilution of 1:819,200. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As detection antibody a polyclonal antibody against the constant domain of the target antibodies conjugated to a peroxidase was used (PAK<M-Fcγ>S-F(ab´)$_2$-POD). The detection antibody was applied at a concentration of 80 ng/ml in PBS comprising 1 % (w/v) RSA. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. Afterwards the wells were incubated with an ABTS solution for 15 minutes at room temperature. The intensity of the developed color was determined photometrically. Exemplary results are presented in the following Table.

Table.

immobilized → mouse ↓	IGF-1	IGF-1-peptide loop	SlyD/FKBP12-IGF-1 SlyD/FKBP12-IGF-1(74-90)	SlyD/FKBP12-control
K1575M1	189	194	2911	8379
K1575M2	395	678	1470	2546
K1575M3	465	272	4126	10091
K1575M4	564	-	2426	6337
K1576M1	2143	2058	8302	9934
K1576M2	-	-	2960	8816
K1576M3	-	-	2978	7756
K1576M4	-	-	6957	11095
K1576M5	-	-	11221	16588

- : no binding detectable in ELISA

Example 8

Kinetic screening of hybridoma culture supernatants

For the selection of IHC suited antibodies a target complex half-live at 37 °C of 10 min. is set.

The kinetic screening was performed on a BIAcore A100 under control of the software version V1.1. A BIAcore CM5 chip is loaded to the machine and according to the manufacturers instruction addressed hydrodynamically and thereafter the chip is conditioned. As running buffer a HBS-EP buffer is used (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) P20). A polyclonal anti-IgG Fc capture antibody composition of a concentration of 30 µg/ml in 10 mM sodium acetate buffer (pH 4.5) is pre-concentrated to spots 1, 2, 4 and 5 in flow cells 1, 2, 3 and 4. The antibody is immobilized at 10,000 RU via NHS/EDC covalently. The sensor is deactivated thereafter by saturation with 1 M ethanolamine solution. Spots 1 and 2 were used for the determination and spots 2 and 4 were used as reference. Prior to application to the sensor chip the hybridoma culture supernatants were diluted 1:5 in HBS-EP buffer. The diluted solution was applied at a flow rate of 30 µl/min for 1 min. Immediately thereafter the antigen was injected at a flow rate of 30 µl/min for 2 min. Thereafter the signal is recorded for another 5 min. The sensor was regenerated by injecting a 10 mM glycine-HCl solution (pH 1.7) for 2 min. at a flow rate of 30 µl/min. The recorded signal shortly

before the end of the injection of the antigen is denoted as binding late (BL). The recorded signal shortly before the end of the recording of the dissociation is denoted as stability late (SL). Therewith the apparent complex stability is calculated with the following formula:

(1-[BL (RU) - SL (RU) / BL (RU)].

The hybridoma cells selected in the kinetic screening were deposited as single cells by FACS (FACSAria (Becton Dickinson), Software V4.1.2). The monoclonal clones are cultivated in 24 well plates or in 100 ml spinner flasks in RPMI-1640 cultivation medium.

Example 9

Immunohistochemical analysis

The IHC analysis was performed either manually or automated on a Ventana Benchmark XT or Discovery XT 8R machine. The antibodies were tested on suited positive or genitive, formalin fixed or cryo-conserved tissue or cells.

Alternatively cells are transfected with a nucleic acid encoding the target polypeptide. The transfected cells are lysed and tested for their suitability as positive or negative control by Western Blotting.

Example 10

SlyD/FKB12-antigen scaffold assisted production of anti-ERCC1 antibodies

Immunization

8-12 weeks old SJL mice, were subjected to intraperitoneal immunization with 100 µg of a KHL-coupled ERCC1 derived peptide covering the amino acids 219-245 of human ERCC1 (Excision Repair Cross Complementing). ERCC1 derivatives were produced synthetically by peptide synthesis.

The mice were immunized 3 times (initial and 6 weeks and 10 weeks after the initial boost). The first immunization was performed using complete Freud's adjuvant, the second and third immunization was done using incomplete Freud's adjuvant. The final boost was done i.v. using 100 µg of KLH-coupled peptide antigen three days before the hybridoma fusion took place. The production of hybridoma primary cultures was done according to Kohler and Milstein (Kohler, G. and Milstein, C., Nature 256 (1975) 495-497). The hybridomas were isolated into

96-well MTPs by limiting dilution and screened for antigen binding by means of ELISA. ELISA was driven by a Tecan Sunrise running under Firmware: V 3.15 19/03/01; XREAD PLUS Version: V 4.20. Primary hybridoma cell cultures, which showed a positive color formation upon binding versus a biotinylated ERCC1 derived peptide covering the amino acids 219-245 in ELISA, were transferred into the kinetic screening process as described herein.

In order to avoid the selection of IHC unsuitable, just linear peptide binding antibodies, further screening efforts were performed using a scaffold-based approach. The scaffold approach further deselected antibodies, which bound the immunogenic peptide at its termini.

Production of SlyD/FKBP12-ERCC1

A Synthetic gene encoding SlyD/FKBP12-ERCC1 and SlyD/FKBP12-ctrl was purchased from Sloning Biotechnology GmbH (Germany) and was cloned into a pQE80L expression vector. The polypeptides were produced as E.coli codon optimized gene constructs in E.coli BL21 CodonPlus-RP (see Figure 3 and 8)

For the purification of the crude fusion polypeptides an affinity chromatography step was used under denaturing conditions in the presence of chaotropic agents. For fusion polypeptide comprising the SlyD part purification in the presence of chaotropic agents is especially used as the total amount of fusion polypeptide could be isolated from the E.coli cells. Additionally the entire fusion polypeptide was obtained in a random coil conformation. The fusion polypeptide still bound to the affinity chromatography material was transferred into native conditions by washing the column with a physiological salt solution. Due to the spontaneous folding of the SlyD and FKBP12 parts of the fusion polypeptide also the inserted amino acid sequence can be transferred to its native conformation. The refolded fusion polypeptide was recovered with an imidazole gradient in a physiological buffer from the affinity chromatography column. An SDS gel and Western blot of the SlyD/FKBP12-ERCC1 fusion polypeptide is shown in Figure 3. The <His6>-Western blot shows the C-terminal integrity of the fusion polypeptide. No other polypeptide bands are detectable.

Fluorescence measurements

The affinity purified fusion polypeptide was dialyzed versus 75 mM HEPES buffer (pH 7.5, 150 mM NaCl, 6.5 % (w/v) sucrose, 10 mM cysteine) and filtrated.

SlyD/FKBP12-ERCC1 was UV/Vis spectroscopically quantified at 7.4 mg/ml using the calculated extinction coefficient for the 35380.301 Da polypeptide (Figure 4). In the wavelength screen from 220 nm to 340 nm a 280 nm absorption peak deriving from the single FKBP12 Trp was obtained. No 340 nm absorption could be detected.

Protein fluorescence measurements were used to test the conformational nature of SlyD/FKBP12-ERCC1. FKBP12 C22A as a carrier for polypeptide insertions is especially useful, because the single FKBP12 Trp moiety can be used to diagnose the structural integrity of the FKBP12 moiety (Scholz, C., et al., J. Biol. Chem. 271 (1996) 12703-12707; Russo, A.T., et al., J. Mol. Biol. 330 (2003) 851-866). FKBP12 C22A in its native structure shows a single fluorescence emission peak at 320 nm (Zoldak, G., et al., J. Mol. Biol. 386 (2009) 1138-1152).

250 µl of 2.5 mg/ml SlyD/FKBP12-ERCC1 in HBS-E-buffer (pH 7.4) were analyzed at different temperatures. A Cary Eclipse instrument under the Scan Software Version: 1.1(132) was used at 5 nm band width for excitation and emission. A wavelength scan from 300 nm – 600 nm was driven at 600 nm/min. The excitation of the intrinsic tryptophan fluorescence was set to 294 nm. A broad peak at 350 nm was obtained (Figure 4). According to theory the intrinsic Trp solvatochromic fluorescence emission at 350 nm would be strongly quenched in a folded FKBP12 protein environment, whereas it increases with the unfolding of FKBP12. A temperature screen from 25 °C to 85 °C didn't show any further fluorescence emission peaks, but a temperature-dependent fluorescence quenching of the 350 nm emission. The 320 nm emission, an indicator for structural integrity of FKBP12, could not be detected.

Therefore, the single Trp residue in the SlyD/FKBP12-ERCC1 fusion polypeptide is exposed to the solvent already at 25 °C, indicating that the chimeric FKBP12 in the SlyD-FKBP12 context is partially or completely unfolded.

Therefore, the scaffold is an ideal platform for mimicking and presenting a structural plurality of non-stable peptide conformations as they typically occur in paraffin-embedded, formalin-fixed tissue during immune histochemical experiments.(Abe, M., et al., Anal. Biochem. 318 (2003) 118-123).

FK506 BIAcore binding assay

A BIAcore 3000 instrument under control of the software version V4.1 was mounted with a sensor SAchip according to the manufacturer's instruction. As running buffer a HBS-EP buffer was used (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) P20 (10 % aqueous solution of the non-ionic surfactant Polysorbate 20 (Tween 20)). 1213 RU of bi-linker-FK506 conjugate (Roche Diagnostics Mannheim, Germany) were captured on flow cell 4.

300 nM purified SlyD/FKBP12-ERCC1 fusion polypeptide and 300 nM SlyD/FKBP12 control polypeptide were injected into the system at 30 µl/min for 3 min. association time and 3 min. dissociation time

The sensor was regenerated by injecting a 10 mM glycine-HCl solution (pH 1.7) for 2 min. at a flow rate of 30 µl/min.

The BIAcore binding assay with 300 nM fusion polypeptide SlyD/FKBP12-ERCC1 as analyte in solution vs. the sensor surface presented ligand bi-FK506 (Figure 5) showed no binding activity (Figure 6), indicating a structure-functional loss of the FKBP12 moiety in the chimeric fusion polypeptide. The control polypeptide FKBP12 (C22A) showed binding activity.

The inability of the SlyD/FKBP12-ERCC1 fusion polypeptide to bind FK-506 provides another evidence for a SlyD/FKBP12-ERCC1 structure, which deviates from that of the FKBP12 (C22A) conformation. This is accompanied by a loss of binding activity of the chimeric FKBP12 domain.

Analytical HPLC chromatographic analyses

Analytical HPLC chromatographic analyses were performed with fusion polypeptides in order to analyze the oligomeric status of the fusion polypeptide

A Chromeleon Dionex HPLC device was used as recommended by the manufacturer at 25 °C with a TSK3000SWXL column equilibrated in HBS-E-buffer (pH 7.4). The buffer flow was 0.7 ml/min. 100 µl of a SlyD/FKBP12-ERCC1 comprising solution (7.4 mg/ml) was injected into the system (see Figure 8). In another workflow a SlyD/FKBP12 control containing solution (9.5 mg/ml) was injected into the system (see Figure 7). In another workflow a Thermus thermophilus SlyD-IGF-1(74-90) containing solution (3 mg/ml) was injected into the system (see Figure 18). In another workflow a SlyD/FKBP12-IGF-1(74-90)

containing solution (5.4 mg/ml) was injected into the system (see Figure 36). The UV/VIS Detector was set to 280 nm. The data was evaluated according to the manufacturer's instructions using the Dionex software version 6.80 SP2 Build 2284. The system was calibrated with the molecular standard Oriental Yeast, Cat 46804000.

Figure 8 shows the column elution profile of Ni-NTA affinity purified SlyD/FKBP12-ERCC1. 91.5 % of the area integration of the complete elution profile can be found localized in peak No. 5 (1310.319 mAU) eluting at 12.37 min retention time. The profile indicates a monomeric SlyD/FKBP12-ERCC1 fusion polypeptide. Monomeric fusion polypeptide was already obtained after just an initial Ni-NTA purification step.

Kinetic screening using

The SlyD/FKBP12-ERCC1 fusion polypeptide was used in SPR binding analyses. It is helpful to use monomeric and monovalent analytes in solution to determine the antibody binding kinetics according to a Langmuir model. Furthermore, it is helpful for SPR measurements to use an analyte with increased, i.e. high, molecular weight to increase the mass sensitivity of the measurements. At the same time the epitope accessibility must be given.

A scheme of the BIAcore screening assay is depicted in Figure 9.

The kinetic screening was performed on a BIAcore A100 instrument under control of the software version V1.1. A BIAcore CM5 chip was loaded to the machine and according to the manufacturer's instruction addressed hydrodynamically. Thereafter the chip was conditioned. As running buffer a HBS-EP buffer is used (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) P20). A polyclonal composition of anti-IgG Fc capture antibodies of a concentration of 30 µg/ml in 10 mM sodium acetate buffer (pH 4.5) is pre-concentrated to spots 1, 2, 4 and 5 in flow cells 1, 2, 3 and 4. The antibody was covalently immobilized at 10,000 RU via NHS/EDC chemistry. The sensor was deactivated thereafter with a 1 M ethanolamine solution. Spots 1 and 2 were used for the determination and spots 2 and 4 were used as reference. Prior to application to the sensor chip the hybridoma supernatants were diluted 1:5 in HBS-EP buffer. The diluted solution was applied at a flow rate of 30 µl/min for 1 min. Immediately thereafter the formulated antigen, such as the FKBP12 fusion polypeptide was injected at a flow rate of 30 µl/min for 2 min. Thereafter the signal is recorded for another 5 min. The

sensor was regenerated by injecting a 10 mM glycine-HCl solution (pH 1.7) for 2 min. at a flow rate of 30 µl/min. The recorded signal shortly before the end of the injection of the antigen is denoted as binding late (BL). The recorded signal shortly before the end of the recording of the dissociation is denoted as stability late (SL). Both data points are plotted versus each other. Selected antibodies have a Binding Late value that is equal to the Stability Late value. These antibodies populate the area near the trend line indicating BL = SL in the plot.

Figure 10 shows the data for selected anti-ERCC1 antibodies. It can be seen that the SlyD/FKBP12-ERCC1 interaction is highly specific. No interaction with the SlyD/FKBP12 control sample can be detected. Overall no unspecific binding can be seen.

Figure 11 shows the valence analysis of the antibodies. The amount of antigen in response units (Binding Late, RU) saturating the surface presented antibody (Capture Level, RU) is shown. Trend lines and arrows in Figure 11 indicate the valencies (Molar Ratio) of the surface presented antibodies. All sister clones (clone ID 5.00x.35) populate the valence corridor MR 0.5 - MR 1.0, whereas all the other clones populate the corridor below MR 0.5 indicating less functionality. No functional binding versus SlyD/FKBP12 control could be detected.

Figure 12 shows the quantification of this kinetic screening approach. All six sister clones (5.001.35 to 5.006.35) show suitable Binding Late and Stability Late values. The dissociation rate kd (1/s) show high antigen complex stabilities fulfilling the requirements for an IHC suited antibody. The calculated $t_{1/2diss}$ antigen complex stability halftimes are 204 min for all six sister clones.

Figure 13 exemplarily shows the kinetic screening signature of clone 5.003.35 versus the analytes SlyD/FKBP12-ERCC1 and SlyD/FKBP12 control. Since SlyD/FKBP12-ERCC1 is a stable, soluble and monomeric analyte it perfectly fits to the 1:1 Langmuir dissociation model (black line on the dissociation raw data in red). No unspecific binding could be detected. No interaction versus SlyD/FKBP12 control was detected.

Western Blotting

Figure 14 shows a Western blot experiment using clone 5.001.35. Western Blotting can be used as an indicator for the latter IHC suitability of the antibody.

For western blotting, 5 µg OVCAR-3 and 5 µg HEK-293 cell lysate were loaded into the gel lanes on 4 -12 % NuPAGE SDS gels (Invitrogen). Both cell lines were not pretreated e.g. by radiation or Cisplatin.

Western blotting was performed according to standard protocols with NuPAGE buffers and reagents (Invitrogen). The antibody 5.001.35 was used at a concentration of 50 ng/ml. Primary antibody incubation was performed for 30 min. at room temperature (RT). The membrane was developed using the LumiImager together with the LumiLight reagent according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The endogenous basal ERCC1 level was specifically detected as a single 37 kDa band in the Western blot.

IHC experiments

Figure 15 shows the IHC detection of ERCC1 in FFPE human cancer tissue. For immunohistochemical detection 2 µm sections of SCLC cancer samples were prepared. All staining procedures were performed on the Ventana Benchmark XT automated IHC stainer using Ventana buffers and reagents according to the manufacturers standard operating instructions. The primary antibody (clone <ERCC1>M-5.1.35) was used at a concentration of 5 µg/ml. The primary antibody was incubated on the sections for 32 min at 37 °C. The primary antibody was detected using the Ventana iView™ detection kit as recommended by the manufacturer. The white arrows indicate cells with elevated ERCC1 level, appearing in darker color.

Summary

In contrast to the small molecular weight ERCC1 peptide (2 kDa) the scaffold as used herein is a high molecular weight analyte (36 kDa), which amplifies the signals in a SPR-based kinetic screening approach.

A peptide based screening reagent bears the risk of selecting antibodies recognizing the peptide's termini, which is completely avoided by using the scaffold approach as reported herein, in which the peptide is embedded in an N-terminal and C-terminal polypeptide context. Despite offering a plurality of meta-stable peptide insertions the scaffold fusion polypeptide as a whole is stable, soluble and monomeric. 1:1 Langmuir kinetics can be easily measured by means of biosensorics.

Using the fusion polypeptide in this set up, it is well suited to simulate the FFPE IHC situation and therefore is a well suited screening reagent for the development of IHC suited antibodies.

Without being bound by theory the fusion polypeptide comprises a folded SlyD derived part and an unfolded or partially unfolded human FKBP12 derived part, which offers at least its single core Trp residue for solvent contact, like it is shown for SlyD/FKBP12-ERCC1. SlyD folds reversible and shows thermal stability sufficient for technical applications.

The SlyD/FKBP-12 scaffold is a suitable platform for mimicking a plurality of peptidic secondary structure motives like those present in a paraffin-embedded, formalin-fixed tissue in immune histochemical experiments (see Abe, et al. (2003) supra).

The fusion polypeptide is especially suited as immunogen compared to the full length polypeptide from which the inserted (immunogenic) amino acid sequence is derived, e.g. with respect to solubility, reversible folding (naturation/denaturation), and the absence of disulfide bonds to be correctly formed. The fusion polypeptide as reported herein provides the scaffold into which the immunogenic amino acid sequence is inserted. It stabilizes the structure of the inserted immunogenic amino acid sequence (without being bound by theory by reducing the conformational entropy). Without being bound by theory it is assumed that the N-terminal SlyD fusion polypeptide keeps the complete chimeric fusion polypeptide in a soluble and thermodynamically stable but partly unfolded form.

Rebuzzini, G. (PhD work at the University of Milano-Bicocca (Italy) (2009)) reports a study of the hepatitis C virus NS3 helicase domain for application in a chemiluminescent immunoassay. In his work Rebuzzini reports that chimeric FKBP12 used as an immunogen for the presentation of the NS3 helicase domain with the insertion sequences according to Knappe, T.A., et al. (J. Mol. Biol. 368 (2007) 1458-1468) is thermodynamically instable. This correlates to our findings, that the chimeric FKBP12 moiety in the SlyD-FKBP12-antigen fusion polypeptide is partially or completely unfolded. In contrast to Rebuzzini's findings the SlyD/FKBP12-antigen fusion polypeptide has been found herein to be monomeric and stable.

Example 11

Production of IGF-1(74-90) specific antibodies

Antigen specific antibodies were obtained by immunization of mice with chimeric Thermus Thermophilus-SlyD-antigen fusion polypeptide. A plurality of epitopes can be targeted on the scaffold's surface, from which the antibodies binding to the target antigen can be identified by differential screening versus the wild-type Thermus Thermophilus-SlyD as a negative control, or versus the native recombinant antigen (IGF-1) as a positive control. In the following an example demonstrates the properties of archaic SlyD derivatives compared to the potentially metastable human FKBP12. Thermus Thermophilus-SlyD allows the presentation of enthalpic, rigid and stable structures and therefore is suitable for the development of monoclonal antibodies versus native protein structures.

Production of Thermus thermophilus SlyD fusion polypeptides

Guanidinium hydrochloride (GdmCl) (A-grade) was purchased from NIGU (Waldkraiburg, Germany). Complete® EDTA-free protease inhibitor tablets, imidazole and EDTA were from Roche Diagnostics GmbH (Mannheim, Germany), all other chemicals were analytical grade from Merck (Darmstadt, Germany). Ultrafiltration membranes (YM10, YM30) were purchased from Amicon (Danvers, MA, USA), microdialysis membranes (VS/0.025 µm) and ultrafiltration units (Biomax ultrafree filter devices) were from Millipore (Bedford, MA, USA). Cellulose nitrate and cellulose acetate membranes (1.2 µm, 0.45 µm and 0.2 µm pore size) for the filtration of crude lysates were from Sartorius (Goettingen, Germany).

Cloning of Expression Cassettes

The sequence of the SlyD polypeptide from Thermus thermophilus was retrieved from the SwissProt database (acc. no. Q72H58). The sequence of the SlyD polypeptide from Thermococcus gammatolerans was retrieved from the Prosite database (acc. no. C5A738). Synthetic genes encoding Thermus thermophilus SlyD, Thermus thermophilus SlyD-IGF-1(74-90), and Thermus thermophilus SlyD-ΔIF were purchased from Sloning Biotechnology GmbH (Germany) and were cloned into a pQE80L expression vector. The codon usage was optimized for expression in E. coli host cells. Synthetic genes encoding Thermococcus gammatolerans SlyD, Thermococcus gammatolerans SlyD-IGF-2(53-65), Thermus thermophilus SlyD-antigen and Thermococcus gammatolerans SlyD-antigen were

purchased from Geneart (Germany) and were cloned into pET24 expression vectors (Novagen, Madison, Wisconsin, USA). The codon usage was optimized for expression in E. coli host cells.

Additionally, a GS-linker (GGGS, SEQ ID NO: 81) and a His-tag were fused to the carboxy terminal end in order to allow an affinity purification of the fusion polypeptides by an immobilized metal ion exchange chromatography.

In order to generate monoclonal antibodies specifically binding to the IGF-1-fragment 74-90 (amino acid sequence NKPTGYGSSSRRAPQTG, SEQ ID NO: 92) the respective peptide amino acid sequences was fused into the molecular chaperone SlyD derived from Thermus thermophilus by deleting amino acids 68 - 120 of the original protein. Due to an angle adaption of the IGF-1 insert, the Asp at position 70 and the Leu at position 88 were substituted by a Gly (D70G and L88G). Thus the fusion polypeptide has the amino acid sequence:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPH**G**NKPTGYGSSSRRAPQTG**G**AGKDLDFQVEV
VKVREATPEELLHGHAHGGGSRKHHHHHHHH (SEQ ID NO: 101).

As a control, the native wild-type SlyD from Thermus thermophilus was used:

MKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEG
EAFQAHVPAEKAYGPHDPEGVQVVPLSAFPEDAEVVPGAQFYAQDMEGN
PMPLTVVAVEGEEVTVDFNHPLAGKDLDFQVEVVKVREATPEELLHGHA
HGGGSRKHHHHHH (SEQ ID NO: 104).

For Screening and specificity testing a Thermus thermophilus SlyD-ΔIF fusion polypeptide was produced. Thermus thermophilus SlyD-ΔIF fusion polypeptide lacks the IF domain, which was replaced by the amino acid sequence motif AGSGSS, and comprises a C-terminal amino acid sequence tag of SEQ ID NO: 16:

MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGR
EEGEAFQAHVPAEKAYGPHGAGSGSSGAGKDLDFQVEVVKVREATPEELL
HGHAHGGGSRKHHHHHHHH (SEQ ID NO: 120).

As a control the native SlyD from Thermococcus gammatolerans comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 was used:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPREDLIVPVPIEQFTSAGLE
PVEGMYVMTDAGIAKILKVEEKTVRLDFNHPLAGKTAIFEIEVVEIKKAGE
AGGGSRKHHHHHH (SEQ ID NO: 121).

As a control for cross reactivity the structurally homologous hairpin sequence from human IGF-2(53-65) was inserted into Thermococcus gammatolerans SlyD, which was fused with a GS-linker and a hexahistidine-tag at the C-terminus:

MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVG
AGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMP-G-SRVSRRSRG-G-
AGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHH (SEQ ID NO: 122).

Expression, Purification and Refolding of fusion polypeptides

All SlyD polypeptides can be purified and refolded by using almost identical protocols. E. coli BL21 (DE3) cells harboring the particular expression plasmid were grown at 37 °C in LB medium containing the respective antibiotic for selective growth (Kanamycin 30 µg/ml, or Ampicillin (100 µg/ml)) to an OD600 of 1.5, and cytosolic overexpression was induced by adding 1 mM isopropyl-ß-D-thiogalactoside (IPTG). Three hours after induction, cells were harvested by centrifugation (20 min at 5,000 g), frozen and stored at -20 °C. For cell lysis, the frozen pellet was resuspended in chilled 50 mM sodium phosphate buffer (pH 8.0) supplemented with 7 M GdmCl and 5 mM imidazole. Thereafter the suspension was stirred for 2-10 hours on ice to complete cell lysis. After centrifugation (25,000 g, 1 h) and filtration (cellulose nitrate membrane, 8.0 µm, 1.2 µm, 0.2 µm), the lysate was applied onto a Ni-NTA column equilibrated with the lysis buffer. In the subsequent washing step the imidazole concentration was raised to 10 mM (in 50 mM sodium phosphate buffer (pH 8.0) comprising 7 M GdmCl, 5.0 mM TCEP) and 5 mM TCEP was added in order to keep the thiol moieties in a reduced form and to prevent premature disulfide bridging. At least 15 to 20 volumes of the reducing washing buffer were applied. Thereafter, the GdmCl solution was replaced by 50 mM sodium phosphate buffer (pH 8.0) comprising 100 mM NaCl, 10 mM imidazole, and 5 mM TCEP to induce conformational refolding of the matrix-bound SlyD fusion polypeptide. In order to avoid reactivation of co-purifying proteases, a protease inhibitor cocktail (Complete® EDTA-free, Roche) was added to the refolding buffer. A total of 15 to 20 column volumes of refolding buffer were applied in an overnight procedure. Thereafter, both TCEP and the

Complete® EDTA-free inhibitor cocktail were removed by washing with 10 column volumes 50 mM sodium phosphate buffer (pH 8.0) comprising 100 mM NaCl and 10 mM imidazole. In the last washing step, the imidazole concentration was raised to 30 mM (10 column volumes) in order to remove tenacious contaminants. The native polypeptide was eluted by applying 250 mM imidazole in the same buffer. Protein-containing fractions were assessed for purity by Tricine-SDS-PAGE (Schaegger, H. and von Jagow, G., Anal. Biochem. 166 (1987) 368-379) and pooled. Subsequently, the protein was subjected to size-exclusion-chromatography (SuperdexTM HiLoad, Amersham Pharmacia) using potassium phosphate as the buffer system (50 mM potassium phosphate buffer (pH 7.0), 100 mM KCl, 0.5 mM EDTA). Finally, the protein-containing fractions were pooled and concentrated in an Amicon cell (YM10) to a concentration of ~ 5 mg/ml.

UV Spectroscopic Measurements

Protein concentration measurements were performed with an UVIKON XL double-beam spectrophotometer. The molar extinction coefficients (ε280) for the SlyD variants were calculated according to Pace (Pace, C. N., et al., Protein Sci. 4 (1995) 2411-2423).

CD Spectroscopic Measurements

To examine whether the chimeric fusion proteins according to the invention adopt a folded conformation CD spectra in the near-UV region were measured. CD spectra were recorded and evaluated using A JASCO J-720 instrument and JASCO software according to the manufacturer's recommendations. A quartz cuvette with 0.2 cm pathlength was used. The instrument was set to 1 °C resolution, 1 nm band width, 5 mdeg sensitivity and accumulation mode 1. The sample buffer was 50 mM potassium phosphate pH 7.5, 100 mM NaCl, 1 mM EDTA. The protein analyte concentration for each analysis was 36 µM Thermus thermophilus SlyD wild-type, 23 µM Thermus thermophilus SlyD-ΔIF, 16 µM Thermus thermophilus SlyD-antigen, 19 µM Thermococcus gammatolerans SlyD wild-type, and 16 µM Thermococcus gammatolerans SlyD-antigen. CD signals between 250 nm and 330 nm with 0.5 nm resolution and 20 nm scan per minute were recorded at 20 °C. In a subsequent experimental embodiment the CD signals were determined in temperature gradients (20 °C - 100 °C) and (100 °C - 20 °C) for Thermococcus gammatolerans SlyD derivatives, respectively (20 °C - 85 °C) and (85 °C – 20 °C)

for Thermus thermophilus SlyD derivatives at 277 nm constant wavelength. The temperature gradient was driven at 1 °C per minute.

Figure 27 shows an overlay plot of three CD spectra of the fusion polypeptides Thermus thermophilus SlyD wild-type, Thermus thermophilus SlyD-ΔIF and Thermus thermophilus SlyD-antigen. The CD signatures show that at 20 °C all fusion polypeptides are folded in their native structure, even when the IF Domain is missing or is being replaced by an amino acid graft.

Figure 28 shows a temperature-dependent near UV CD spectra of the fusion polypeptide Thermus thermophilus SlyD-antigen in the temperature gradient 20 °C to 85 °C. Thermus thermophilus SlyD-antigen reversibly unfolds and refolds.

Under the given physical conditions no complete unfolding of Thermococcus gammatolerans SlyD-antigen could be achieved even at 100 °C (see Figure 29). The extraordinary stability of archaic FKBP domains enables the grafting of polypeptides by replacement of their IF domains where at the same time the overall stability of the newly generated chimeric scaffold protein is kept up.

Immunization of mice with Thermus thermophilus SlyD-IGF-1(74-90) and development of monoclonal antibodies versus IGF-1

8-12 weeks old Balb/c and NMRI mice were subjected to repeated intraperitoneal immunizations with 100 µg of Thermus Thermophilus SlyD–IGF-1(74-90). The mice were immunized three times, at the time points of 6 weeks and 10 weeks after the initial immunization. The first immunization can be performed using complete Freud's adjuvant, the second and third immunizations were done using incomplete Freud's adjuvant. The mice serum titers versus native recombinant IGF-1 and Thermus Thermophilus SlyD–IGF-1(74-90) were tested after 12 weeks by ELISA methods as described in the following. After 12 weeks serum titers were analyzed using ELISA. The ELISA was driven by a Tecan Sunrise running under Firmware: V 3.15 19/03/01; XREAD PLUS Version: V 4.20. Nunc Maxisorb F multi well plates were coated with Thermus Thermophilus SlyD-IGF-1(74-90) by applying a solution comprising 0.5 µg polypeptide per ml. The isolated antigen IGF-1 was immobilized in the wells of StreptaWell High Bind SA multi well plates by applying a solution comprising 90 ng/ml biotinylated IGF-1. Thereafter free binding sites were blocked by applying a solution comprising 1 % RPLA in PBS for one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As

samples the mouse serum diluted 1:50 with PBS was used. Optional further dilution was performed in 1:4 steps until a final dilution of 1:819,200. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As detection antibody a polyclonal antibody against the constant domain of the target antibodies conjugated to a peroxidase was used (PAK<M-Fcγ>S-F(ab´)$_2$-POD). The detection antibody was applied at a concentration of 80 ng/ml in PBS comprising 1 % (w/v) RSA. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. Afterwards the wells were incubated with an ABTS solution for 15 minutes at room temperature. The intensity of the developed color was determined photometrically. Figure 20 shows mice serum titers obtained.

Three days before preparation of spleen cells and fusion with a myeloma cell line, the final booster immunization was performed by i.v. injection of 100 µg of Thermus Thermophilus SlyD–IGF-1(74-90) fusion polypeptide. The production of hybridoma primary cultures can be done according to the procedure of Koehler and Milstein (Koehler, G. and Milstein, C., Nature. 256 (1975) 495–497).

ELISA Screening

Primary culture supernatants were tested by ELISA for reactivity against the immunogen Thermus Thermophilus SlyD–IGF-1(74-90), biotinylated native IGF-1 and wild-type Thermus Thermophilus SlyD respective a blank plate. Elisa was driven with a Tecan SUNRISE, Firmware: V 3.15 19/03/01; XREAD PLUS Version: V 4.20. Nunc Maxisorb F multi well ELISA plates were coated with 5 µg/ml SlyD- fusion polypeptides. StreptaWell High Bind SA multi well plates were coated with 125 ng/ml recombinant biotinylated IGF-1 antigen. Thereafter free binding sites were blocked by 1 % RPLA in PBS for one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. Undiluted hybridoma supernatants in RPMI 1640 medium were used as samples. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As detection antibody a polyclonal antibody against the constant domain of the target antibodies conjugated to a peroxidase was used (PAK<M-Fcγ>S-F(ab´)$_2$-POD). The detection antibody was applied at a concentration of 80 ng/ml in PBS comprising 1 % (w/v) RSA. The

incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. Afterwards the wells were incubated with an ABTS solution for 15 minutes at room temperature. The intensity of the developed color was determined photometrically at t 405 nm. The reference wavelength was 492 nm. Primary hybridoma supernatants, showing fast and strong color formation in ELISA upon binding to recombinant IGF-1, Thermus Thermophilus SlyD–IGF-1(74-90) and less binding to Thermus Thermophilus SlyD were transferred into the kinetic screening process as described in the following.

SPR-based kinetic screening

Thermus Thermophilus SlyD–IGF-1(74-90), native recombinant IGF-1, native recombinant IGF-2, wild-type Thermus Thermophilus SlyD, and Thermus Thermophilus-SlyD-IGF-1(74-90) were used in an SPR-based kinetic screening analysis. For SPR analyses it is generally accepted to use monomeric and monovalent analytes in solution to determine the antibody binding kinetics according to a Langmuir model. Furthermore, it is rather advantageous for SPR measurements to use an analyte with higher molecular weight to increase the sensitivity of the measurements, since SPR is a mass sensitive analysis.

The kinetic screening was performed on a BIAcore A100 instrument under control of the software version V1.1. A BIAcore CM5 chip was mounted into the instrument and was hydrodynamically addressed conditioned according to the manufacturer's instructions. As a running buffer an HBS-EP buffer was used (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) P20). A polyclonal rabbit anti-mouse IgG Fc capture antibody is immobilized at 30 µg/ml in 10 mM sodium acetate buffer (pH 4.5) to spots 1, 2, 4 and 5 in flow cells 1, 2, 3 and 4 at 10,000 RU (Figure 23). The antibody is covalently immobilized via NHS/EDC chemistry. The sensor was deactivated thereafter with a 1 M ethanolamine solution. Spots 1 and 5 were used for the determination and spots 2 and 4 were used as reference. Prior to application to the sensor chip the hybridoma supernatants were diluted 1:2 in HBS-EP buffer. The diluted solution was applied at a flow rate of 30 µl/min for 1 min. Immediately thereafter the analyte, such as the Thermus Thermophilus SlyD–IGF-1(74-90), fusion polypeptide, is injected at a flow rate of 30 µl/min for 2 min. Thereafter the signal is recorded for 5 min. dissociation time. The sensor is regenerated by injecting a 10 mM glycine-HCl solution (pH 1.7) for 2 min. at a flow rate of 30 µl/min. Two report points, the

recorded signal shortly before the end of the analyte injection, denoted as binding late (BL) and the recorded signal shortly before the end of the dissociation time, stability late (SL), were used to characterize the Kinetic Screening performance.

Furthermore, the dissociation rate constant kd (1/s) was calculated according to a Langmuir model and the antibody/antigen complex half-life can be calculated in minutes according to the formula ln(2)/(60*kd).

Antibodies were obtained by immunization with the antigen Thermus Thermophilus SlyD–IGF-1(74-90), and screening with Thermus Thermophilus SlyD–IGF-1(74-90), Thermus Thermophilus SlyD wild-type, native IGF-1 and native IGF-2. The scaffold-based screening approach allows to specifically develop antibodies binding to the predefined IGF-1 hairpin epitope.

The primary culture supernatants were further developed by limited dilution into clone culture supernatants by methods known. The clone culture supernatants were tested in a functional assay for affinity and specificity.

BIAcore characterization of antibody producing clone culture supernatants

A BIAcore T200 instrument (GE Healthcare) was used with a BIAcore CM5 sensor mounted into the system. The sensor was preconditioned by a 1 min. injection at 100 µl/min of 0.1 % SDS, 50 mM NaOH, 10 mM HCl and 100 mM H_3PO_4.

The system buffer was PBS-DT (10 mM Na_2HPO_4, 0.1 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.05 % Tween® 20, 5 % DMSO). The sample buffer was the system buffer.

The BIAcore T200 System was driven under the control software V1.1.1. Polyclonal rabbit IgG antibody <IgGFCγM>R (Jackson ImmunoResearch Laboratories Inc.) was immobilized at 30 µg/ml in 10 mM sodium acetate buffer (pH 4.5) at 6500 RU on the flow cells 1, 2, 3, and 4, respectively, via EDC/NHS chemistry according to the manufacturer's instructions. Finally, the sensor surface was blocked with a 1 M ethanolamine solution. The complete experiment was performed at 25 °C.

The clone culture supernatants containing the respective antibodies at approx. 40 nM were captured for 2 min. at a flow rate of 5 µl/min on the <IgGFCγM>R surface. As analytes in solution the recombinant native IGF-1, recombinant native

IGF-2, Thermus thermophilus SlyD-IGF-1(74-90), recombinant wild-type Thermus thermophilus SlyD, recombinant Thermus thermophilus SlyD-ΔIF, recombinant wild-type Thermococcus gammatolerans SlyD, recombinant Thermococcus gammatolerans SlyD-IGF-2 (53-65) fusion polypeptides were used. Thermus thermophilus SlyD-ΔIF is solely the FKBP domain of Thermus thermophilus SlyD lacking the IF domain. Thermococcus gammatolerans SlyD-IGF-2(53-65) was used to counterscreen and investigate the specificity for the IGF-1 hairpin in contrast to the IGF-2 hairpin insertion. The respective analytes were injected at different concentration steps from 90 nM, 30 nM, 10 nM, 3.3 nM, 1.1 nM and 0 nM. The association phase was monitored for 3 min. at a flow rate of 100 µl/min. The dissociation was monitored for 10 min. at a flow rate of 100 µl/min. The system was regenerated using a 10 mM glycine buffer (pH 1.7). Kinetics were evaluated using the BIAcore Evaluation Software.

The following terms are used herein: mAb: monoclonal antibody; RU: Relative response unit of monoclonal antibody captured on the sensor; Antigen: antigen in solution; kDa: molecular weight of the antigens injected as analytes in solution; ka: association rate constant; kd: dissociation rate constant; t1/2 diss: antibody-antigen complex half-life calculated according to the formula t1/2 diss = ln(2)/60*kd; KD: dissociation constant; R_{MAX}: Binding signal at the end of the association phase of the 90 nM analyte injection; MR: Molar Ratio; Chi^2: failure of the measurement; n.d.: not detectable.

Figure 25 shows, that the scaffold-derived monoclonal antibodies M-11.11.17 and M-10.7.9 have been developed with picomolar affinity versus IGF-1. No cross-reactivity versus IGF-2, nor versus wild-type Thermus thermophilus SlyD, nor versus wild-type Thermococcus gammatolerans SlyD, nor versus Thermus thermophilus SlyD-ΔIF fusion polypeptide, nor versus Thermococcus gammatolerans SlyD-IGF-2(53-65) fusion polypeptide was detectable.

M-2.28.44 is a monoclonal antibody obtained by conventional immunization of mice with recombinant human IGF-1. Despite the fact that the antibody shows a 30 pM affinity versus IGF-1, a 500 pM cross reactivity can be detected versus IGF-2. Using Thermus thermophilus SlyD-IGF-1(74-90) and Thermococcus gammatolerans SlyD-IGF-2 (53-65) as analyte it can be seen that the cross-reacting IGF-2 epitope is not the IGF hairpin region.

Example 12

Generation of anti-PLGF antibodies

Mice were immunized with an immunogen containing the sequence of PLGF(60-76). Subsequently hybridomas were produced and ELISA and Kinetic Screening were performed (for detailed general procedure see Examples 10 and 11).

In the Kinetic Screening process, SlyD/FKBP12-PLGF and a biotinylated peptide PLGF(60-76)-bi, which was singly grafted on streptavidin were used to identify primary culture supernatants with binding activity versus PLGF(60-76). Both analytes produced 1:1 Langmuir kinetics, but the scaffold showed a better dissociation fit with a lower chi2 value, than the SA-probe grafted bi-peptide. Without being bound by theory, a scaffold-based screening approach takes advantage of the monomeric state and the improved epitope accessibility of the scaffold, when compared to the carefully prepared SA-probe.

Antibodies, developed by this approach, like clone 53.4.1 were able to specifically detect PLGF in Western blotting.

Example 13

Anti-IGF-1 antibodies produced with SlyD-FKBP12-IGF-1(74-90)

SlyD/FKBP12-IGF-1(74-90) (see Figures 2 and 8) and SlyD/FKBP12-ctrl (see Figure 7) fusion polypeptides were produced in E.coli (pQE80L vector / E.coli BL21 CodonPlus-RP cell line) according to known methods. 8-12 weeks old Balb/c and NMRI mice were subjected to repeated intraperitoneal immunizations with 100 µg of SlyD/FKBP12–IGF-1(74-90).

After 10 weeks the serum titers were analyzed using ELISA. Nunc Maxisorb F multi well plates were coated with SlyD/FKBP12-IGF-1(74-90) by applying a solution comprising 0.41 µg polypeptide per ml. The isolated antigen IGF-1 was immobilized in the wells of StreptaWell High Bind SA multi well plates by applying a solution comprising 90 ng/ml biotinylated IGF-1. Thereafter free binding sites were blocked by applying a solution comprising 1 % RPLA in PBS for one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As samples the mouse serum diluted 1:50 with PBS were used. Optional further dilution was performed in 1:4 steps until a final dilution of 1:819,200. The

incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. As detection antibody a polyclonal antibody against the constant domain of the target antibodies conjugated to a peroxidase was used (PAK<M-Fcγ>S-F(ab')$_2$-POD). The detection antibody was applied at a concentration of 80 ng/ml in PBS comprising 1 % (w/v) RSA. The incubation time was one hour at room temperature. The wells were washed three times with a solution comprising 0.9 % (w/v) sodium chloride and 0.05 % (w/v) Tween. Afterwards the wells were incubated with an ABTS solution for 15 minutes at room temperature. The intensity of the developed color was determined photometrically. Figure 19 shows mice serum titers obtained. Lower titers where obtained with SlyD/FKBP12-IGF-1(74-90), when compared to the immunization with Thermus thermophilus SlyD-IGF-1(74-90) (Figure 20). Further antibody development was done like described under example 12. Finally no antibodies with binding versus IGF-1 could be selected in a BIAcore kinetic screening approach as already described.

Example 14

Antibodies produced with FKBP12/13 fusion polypeptide

The fusion polypeptide as reported herein and used in this examples comprises a part derived from human FKBP12 and a part derived from Arabidopsis thaliana FKBP13. A fusion polypeptide consisting of at least one amino acid sequence from human FKBP12 and at least one amino acid sequences from Arabidopsis thaliana FKBP13 can thermodynamically stabilize human FKBP12 as a scaffold and circumvents the N-terminal fusion of FKBP12 with E.coli SlyD. In nature, FKBP13 contains a disulfide bond. This FKBP13 sequence was grafted into FKBP12 in order to stabilize the chimeric polypeptide for further sequence grafting approaches.

The chimeric FKBP12/13 fusion polypeptide comprising a C-terminal amino acid sequence tag of SEQ ID NO: 16 has the sequence:

MRSGVQVETISPGDGRTFPKRGQTAVVHYTGMLEDGKKFDSSRDRNKPFK
FMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGDRGAGCGSGSSCLI
PPASVLVFDVELLKLEGGGSRKHHHHHHHH (SEQ ID NO: 123).

The FKBP12/13 fusion polypeptide was expressed in E.coli as described as a soluble and monomeric protein. CD spectroscopic measurements were performed

like described in example 12. The CD spectra showed that the FKBP12/13 fusion polypeptide is folded at 20 °C.

Example 15

Generation of SlyD-FKBP12/13-CSF1R fusion polypeptide

The polypeptide was expressed in E.coli like as described above and purified as described above. After Ni-NTA affinity purification a size exclusion chromatography was performed. 50 mg of protein were loaded in a HiLoad 26/60 SuperdexTM 75 pg (GE Healthcare). The elution fractions were loaded into a native gel and resolved according to known methods.

The affinity purified fusion polypeptide was dialyzed versus 50 mM KH_2PO_4 buffer, pH 7.0, comprising 100 mM KCl and 0.5 mM EDTA and filtrated trough a 0.22 µm filter. SlyD/FKBP12/13-CSF1R was UV/Vis spectroscopically quantified at 1.19 mg/ml using the calculated extinction coefficient e= 20525 L•mol−1•cm−1 for the 39744.9 Da polypeptide.

Protein fluorescence measurements were used to test the conformational nature of SlyD/FKBP12/13-CSF1R. FKBP12 C22A as a carrier for polypeptide insertions can be used as a reference, because the single FKBP12 Trp moiety can be used to diagnose the structural integrity of the FKBP12 moiety (Scholz, C., et al., J. Biol. Chem. 271 (1996) 12703-12707; Russo, A.T., et al., J. Mol. Biol. 330 (2003) 851-866). FKBP12 C22A in its native structure shows a single fluorescence emission peak at 320 nm (Zoldak, G., et al., J. Mol. Biol. 386 (2009) 1138-1152).

250 µl of 1.19 mg/ml SlyD -FKBP12/13-CSF1R in KH_2PO_4 buffer pH 7.0 were analyzed at different temperatures. KH_2PO_4 buffer pH 7.0 was used as a reference. A Cary Eclipse instrument under the Scan Software Version 1.1(132) was used at 5 nm band width for excitation and emission. A wavelength scan from 300 nm – 425 nm was driven at 120 nm/min. The excitation wavelength of the intrinsic tryptophane fluorescence was set to 280 nm.

Example 16

Scaffold-Based counter-screening approach for the selection of antigen binding antibodies

Six week old NMRI mice were subjected 3-times to intraperitoneal immunization with 100 µg recombinant chimeric fusion polypeptide comprising the element

Thermococcus gammadurans SlyD-antigen (TgSlyD-antigen). After 10 weeks the mice were boosted two times with 25 µg TgSlyD-antigen. Hybridoma cells were produced according to known methods. The primary hybridomas were isolated by limiting dilution and screened for antigen binding by ELISA.

50 ng/ml of TgSlyD-antigen fusion polypeptide, 50 ng/ml of TgSlyDΔIF and 1µg/ml isolated antigen were each coated in 30x 384 well (Nunc) plates overnight at 4 °C. Coating buffer was freshly prepared with 1 carbonate-bicarbonate tablet (Sigma, C3041-100CAP99) resolved in 100 ml doubly distilled H_20 (ddH_2O). 100 µl Washing buffer (150 mM NaCl, 10 ml Tween 20 (Sigma), 40 ml Bromidox L (Roche) in 1 l dH_2O). The wells were washed three times with 100 µl washing buffer (150 mM NaCl, 10 ml Tween20 (Sigma), 40 ml Bromidox L (Roche) in 1 l dH_2O) using a BioTek washer. The wells were blocked with 30 µl blocking buffer (10 g BSA, 10 x PBS pellets (Gibco) in 1 L ddH_2O) for one hour at RT, followed by 3 times washing with 100 µl washing buffer. 30 µl of 1:1000 diluted hybridoma supernatants were transferred into the wells using a Liquidator and were incubated for one hour at RT. As a positive control an antigen-positive serum was used. The wells were washed three times with 100 µl washing buffer. Peroxidase conjugated $F(ab')_2$ fragment Goat anti-Mouse IgG antibody (Dianova) was diluted 1:30000 in blocking buffer and 30 µl were transferred into each well. Incubation for one hour at RT was followed by three times washing with 100 µl washing buffer. 30 µl ready to use ABTS substrate was incubated for 30 min at RT in each well. The absorption signals were monitored as reference signals at 405 nm/ 492 nm using a PowerWave XS Reader (BioTek). 15 hybridoma cultures, which showed positive ELISA signals versus the TgSlyD-antigen comprising fusion polypeptide and the isolated antigen and no signal versus the TgSlyDΔIF comprising fusion polypeptide were selected and further cultivated.

The primary hybridoma supernatants were isolated and screened for antigen binding by a second ELISA counter-screening, performed in the same way as described above. In the second screening additional screening reagents were used to precise the specificity of the antibody containing culture supernatants.

50 ng/ml of TgSlyD-antigen fusion polypeptide, 50 ng/ml TgSlyDΔIF, 500 ng of ttSlyD-antigen fusion polypeptide and 1 µg/ml isolated antigen were coated in 384 well (Nunc) plates for one hour at room temperature (RT). The ELISA was performed as described above. Due to their different species origin T.th.SlyD and T.g.SlyD show little sequence homology. Only 36 % of the amino acids are

identical and according to a blossom 62 calculation there is only 48 % sequence similarity. TgSlyDΔIF is lacking the insertion anyway. Therefore, the polypeptides are very well suited to be used in an ELISA counter-screening.

Therefore, by immunization with the scaffold surrogate polypeptide a specific epitope in the native antigen domain could be pre-targeted.

Example 17

Epitope Mapping

SlyD-FKBP fusion polypeptides can also carry complex amino acid insertion motifs, like for example secondary structures containing disulfide bonds. Since the fusion polypeptides are free of cysteines, on-column refolding under appropriate conditions facilitates the correct formation of disulfides within the insertion, additionally assisted by the chaperone functionality of SlyD itself. The SlyD-FKBP fusion polypeptides were expressed in E.coli and on-column refolded as described above. SlyD-FKBP12ctrl was dialyzed in 75 mM HEPES buffer (pH 7.5) comprising 150 mM NaCl, 6.5 % (w/v) Saccharose, 10 mM cysteine at a concentration of 9.5 mg/ml. A 1 mg/ml portion of the SlyD-FKBP12-CD81 fusion polypeptide was dialyzed in 50 mM potassium phosphate buffer pH 7.0, comprising 100 mM NaCl, 1 mM EDTA in order to avoid disulfide shuffling in the CD81 insertion, which contains 4 cysteines forming two disulfide bonds with structure functional relevance. The fusion polypeptides SlyD-FKBP12-CD81 and SlyD-FKBP12ctrl were used for the purpose of epitope mapping.

Human CD81 is a receptor for the hepatitis C virus envelope E2 glycoprotein. CD81 is a transmembrane protein belonging to the tetraspanin family. CD81 is a 90 amino acid in length homodimeric protein, which displays a so called mushroom-like structure (PDB 1IV5). Residues known to be involved in virus binding can be mapped onto the so called 35 amino acid in length “head subdomain”, providing a basis for the design of antiviral drugs and vaccines. Since the head subdomain sequence of the virus binding site is just 35 amino acids in length it is difficult to map antibody epitopes on the 10 kDa CD81 protein using conventional cross-blocking experiments. It is difficult to discriminate antibodies, which specifically bind directly on the relevant mushroom-like head domain, from antibodies binding nearby or elsewhere in the CD81LEL structure. All antibodies would show independently of their binding site an HCV E2 envelope protein competing effect, but without specifically binding to the target structure, i.e. the head domain. The

grafting of the relevant head domain structure into FKBP and a consecutive epitope mapping is therefore a useful method for various reasons. First, some biochemical issues with the CD81LEL protein are circumvented, because the protein itself tends to oligomerize. Second, it is rather suitable to identify antibody epitopes from a vast number of antibodies all binding to the full length CD81 protein.

In Figure 40 an SDS page (left) and Western Blot (right) of Ni-NTA chromatographically purified SlyD-FKBP12-CD81 is shown.

A BIAcore 2000 instrument (GE Healthcare) was used at 25 °C with a BIAcore CM5 sensor mounted into the system. The sensor was preconditioned by a 1 min. injection at 100 µl/min of 0.1 % SDS, 50 mM NaOH, 10 mM HCl and 100 mM H_3PO_4. As a running buffer an HBS-EP buffer was used (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.05 % (w/v) P20). The sample buffer was the system buffer. Each protein ligand was immobilized by EDC/NHS chemistry at 30 µg/ml in 10 mM sodium acetate buffer (pH 4.0) into the flow cells 2, 3 and 4. Flow cell 1 was used as reference. The sensor was deactivated with a 1 M ethanolamine pH 8.0 solution. The following masses in response units (RU) were immobilized on the sensor: flow cell 2: 1800 RU SlyD-FKBP12ctrl (32.8 kDa), flow cell 3: SlyD-FKBP12-CD81 (35.8 kDa), flow cell 4: 900 RU CD81LEL (10 kDa). 31 antibody analytes were injected at 30 µl/min at 50 nM each for 3 min. association and 3 min. dissociation phase. 30 antibodies were derived from an immunization campaign with the 10 kDa CD81LEL protein. The sensor surface was regenerated using 100 mM HCl at 20 µl/min for 3 consecutive 30 sec. injections at 20 µl/min. The sensorgrams were monitored as reference signals 2-1 (flow cell 2 minus flow cell 1), 3-1 and 4-1 and were evaluated by using the BIAcore Evaluation software 4.1. At the end of the analyte injection a report point was set to quantify the maximum analyte binding signal. The highest analyte binding signal was set 100 % to normalize the data.

In the following table the normalized antibody binding responses are shown. From 30 tested <CD81-LEL>M-antibodies only 6 (bold) show a precise epitope on the CD81 head domain. The negative control polypeptide SlyD-FKBP12ctrl was not bound. The positive control polypeptide CD81-LEL, which was the immunogen at the same time, was recognized by all antibodies. Slyd-FKBP12-CD81 was only bound, when the antibody epitope precisely locates in the mushroom domain.

Table.

Ligands	Slyd-FKBP12-ctrl	Slyd-FKBP12-CD81	CD81-LEL
Analytes	normalized ref. binding signal (%)		
HBS-ET	0	0	0
K01	0	0	63
K02_1	0	0	62
K02_2	0	0	61
K03	**0**	**47**	**93**
K04	0	0	80
K05	**0**	**26**	**100**
K06	**0**	**32**	**74**
K07	0	0	62
K08	0	0	56
K10	0	0	46
K21	0	0	77
K22	**0**	**29**	**80**
K23	0	0	37
K24	**0**	**17**	**86**
K25	0	0	38
K27	**0**	**29**	**81**
K28	0	0	41
K30	0	0	52
K31	0	0	66
K32	0	0	53
K34	0	0	66
K35	0	0	44
K36	0	0	54
K37	0	0	52
K38	0	0	65
K39	0	0	66
K40	0	0	51
K41	0	0	67
K42	0	0	60
K43	0	0	58

Confirmation of the epitope mapping results by X-ray crystallographic analysis

Fab fragments of the antibodies K05 and K04 were co-crystallized with the CD81-LEL protein and were analyzed by x-ray diffraction analysis (Seth Harris, Palo Alto). The resolution obtained was 2.15 Å. Whereas antibody K05 binds in the mushroom domain, antibody K04 binds to an epitope off site of the mushroom sequence.

Patent Claims

1. A fusion polypeptide comprising a polypeptide according to formula I

$$NH_2\text{-}S_2\text{-}X_1\text{-}S_1\text{-}COOH \qquad \text{(formula I)}$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are non-overlapping amino acid sequences derived from a second polypeptide, and

- denotes a peptide bond,

wherein the second polypeptide is a polypeptide with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) or is derived from the FKBP-fold domain family, and

wherein X_1 is inserted in place of the insert-in-flap-domain (IF-domain) of the second polypeptide.

2. The fusion polypeptide according to claim 1, characterized in that the fusion polypeptide comprises a polypeptide according to formula II

$$NH_2\text{-}S_4\text{-}X_2\text{-}S_3\text{-}S_2\text{-}X_1\text{-}S_1\text{-}S_0\text{-}COOH \qquad \text{(formula II)}$$

wherein

X_1 comprises either a random amino acid sequence or an amino acid sequence derived from a first polypeptide,

S_2 and S_1 are either non-overlapping amino acid sequences derived from a second polypeptide,

S_3 and S_0 are either absent or non-overlapping amino acid sequences derived from a third polypeptide,

S_4 is either absent or an amino acid sequence derived from a fourth polypeptide, and

X_2 is either absent or a peptidic linker sequence,

wherein the second polypeptide and the third polypeptide and the fourth polypeptide are different from each other and are polypeptides with peptidyl-prolyl cis/trans-isomerase activity (PPIase activity) and/or derived from the FKBP-fold domain family, and

wherein X_1 is inserted in place of the insert-in-flap-domain (IF-domain) of the second polypeptide.

3. The fusion polypeptide according to any one of the preceding claims, characterized in that the S_2-X_1-S_1 polypeptide is a fragment of a naturally occurring polypeptide or a full length naturally occurring polypeptide if X_1 is an IF-domain.

4. The fusion polypeptide according to any one of the preceding claims, characterized in that the second polypeptide is SlyD.

5. The fusion polypeptide according to any one of the preceding claims, characterized in that the second polypeptide is a polypeptide from a thermophile.

6. The fusion polypeptide according to claim 5, characterized in that the thermophile is a thermophile bacterium or a thermophile Archaea.

7. The fusion polypeptide according to any one of claims 5 to 6, characterized in that the thermophile is from the family of Thermaceae.

8. The fusion polypeptide according to any one of claims 5 to 7, characterized in that the thermophile is Thermus thermophilus.

9. The fusion polypeptide according to any one of claims 5 to 6, characterized in that the thermophile Archaea is a hyperthermophilic Archaea.

10. The fusion polypeptide according to any one of claims 5 to 6 and 9, characterized in that the thermophile is from the class of Thermococci.

11. The fusion polypeptide according to any one of claims 5 to 6 and 9 to 10, characterized in that the thermophile is Thermococcus gammatolerans.

12. The fusion polypeptide according to any one of the claims, characterized in that X_1 comprises an amino acid sequence that corresponds to a hidden epitope.

13. The fusion polypeptide according to any one of claims 2 to 12, characterized in that the second polypeptide and the third polypeptide and the fourth polypeptide are from different species.

14. The fusion polypeptide according to any one of the preceding claims, characterized in that second and/or third polypeptide is a polypeptide from a thermophile that has an optimal growth temperature of at least 60 °C.

15. The fusion polypeptide according to claim 14, characterized in that thermophile is Thermus thermophilus or Thermococcus gammatolerans.

16. The fusion polypeptide according to any one of the preceding claims, characterized in that X_1 is a polypeptide of formula III

$$X_aX_bX_cX_d\text{-}X_0\text{-}X_eX_fX_gX_h \qquad \text{(formula III)}$$

wherein X_0 is either a random amino acid sequence or an amino acid sequence derived from a first polypeptide, and

wherein each of X_a to X_h denotes an amino acid residue and any of $X_{a\text{-}h}$ may be individually present or absent.

17. The fusion polypeptide according to any one of the preceding claims, characterized in that X_1 is a polypeptide selected from the group of

GS-X_0-GSS	(formula VI),
AGS-X_0-GSS	(formula V),
CG-X_0-GC	(formula VI),
C-X_0-GC	(formula VII),
G-X_0-G	(formula VIII),
S-X_0-GSS	(formula IX),
GG-X_0-GG	(formula X),
G-X_0-TGG	(formula XI),
GGGS-X_0-GGGS	(formula XII),
GGNP-X_0-GPT	(formula XIII),

wherein X_0 is either a random amino acid sequence or an amino acid sequence derived from a first polypeptide.

18. Use of a fusion polypeptide according to any one of claims 1 to 17 for eliciting an immune response against X_1 in an experimental animal.

19. A method for eliciting an immune response in an experimental animal against a polypeptide comprising the step of administering to the experimental animal a fusion polypeptide according to any one of claims 1 to 17 for at least one time, whereby X_1 is the amino acid sequence of the polypeptide.

20. A method for producing an antibody specifically binding to a target antigen comprising the following steps:
    a) administering to an experimental animal a fusion polypeptide according to any one of claims 1 to 17 for at least one time, whereby the amino acid sequence of X_1comprises the amino acid sequence of the target antigen,
    b) recovering from the experimental animal three to ten days after the last administration of the polypeptide B-cells that produce the antibody specifically binding to the target antigen, and
    c) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the target antigen and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

21. A method for producing an antibody specifically binding to a target antigen comprising the following steps:
    a) recovering from an experimental animal after the administration of a fusion polypeptide according to any one of claims 1 to 17 B-cells that produce the antibody specifically binding to the amino acid sequence of X_1, and
    b) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the amino acid sequence of X_1 and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

22. Use of a fusion polypeptide according to any one of claims 1 to 17 for epitope mapping, whereby the amino acid sequence of X_1 comprises the epitope.

23. A method for selecting an antibody specifically binding to a target antigen comprising the following steps:
    a) determining the binding affinity of a plurality of antibodies to a target antigen, which is immobilized, whereby the amino acid sequence of X_1 of the fusion polypeptide according to any one of claims 1 to 17 comprises the target antigen amino acid sequence,
    b) selecting the antibody having an apparent complex stability above a pre-defined threshold level.

24. A method for selecting an antibody that specifically binds to a target antigen wherein the immunization of an animal to generate antibodies is made with a fusion polypeptide comprising a Thermo thermophilus SlyD-insert and a fusion polypeptide comprising the Thermococcus gammatolerans SlyD-insert is used for the selection of the antibody.

25. A method for selecting an antibody that specifically binds to a target antigen wherein the selecting of the antibody that specifically bind to an antigen is made with a fusion polypeptide comprising Thermo thermophilus SlyD-insert and a fusion polypeptide comprising the Thermococcus gammatolerans SlyD-insert is used for the generation of antibodies (immunization).

26. An antibody obtained by a method according to any one of claims 19 to 21 and 23 to 25.