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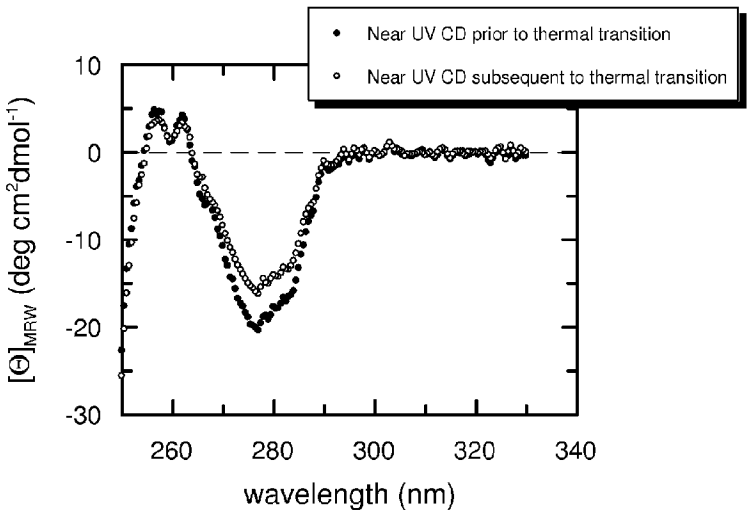
[Continued on next page]

(54) Title: CHAPERONE-CHAPERONE FUSION POLYPEPTIDES FOR REDUCTION OF INTERFERENCE AND STABILIZATION OF IMMUNOASSAYS

Figure 11/11



(57) Abstract: The invention concerns a fusion polypeptide comprising several molecules of folding helper polypeptides, comprising one multimerization domain, in particular Skp, and at least one molecule of SlyD or SlpA, wherein no further target polypeptide sequences are fused to said fusion polypeptide. The invention further concerns an immunoassay and the use of said fusion polypeptide in an immunoassay for reduction of interferences or minimizing false positive results or for stabilizing proteinaceous assay reagents. Further the invention concerns a reagent kit for use in an immunoassay comprising said fusion polypeptide.

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Chaperone-chaperone fusion polypeptides for reduction of interference and stabilization of immunoassays

Field of the invention

The invention concerns a fusion polypeptide comprising several molecules of folding helper polypeptides, comprising one multimerization domain, in particular Skp, and at least one molecule of SlyD or SlpA, wherein no further target polypeptide sequences are fused to said fusion polypeptide. The invention further concerns an immunoassay and the use of said fusion polypeptide in an immunoassay for reduction of interferences or minimizing false positive results or for stabilizing proteinaceous assay reagents. Further the invention concerns a reagent kit for use in an immunoassay comprising said fusion polypeptide.

Background of the invention

Chaperones, which are known as classical folding helpers, are proteins that assist the folding and maintenance of the structural integrity of other proteins. They bind to denatured or hydrophobic surfaces of proteins and help in re-naturing and keeping proteins in solution. Due to their superior physico-chemical properties chaperones are used as folding assistants and fusion partners in protein technology. One class of chaperones is the family of FKBP chaperones, proteins that bind to the immunosuppressant drug FK506.

The use of FKBP chaperones like SlyD, FkpA and SlpA (= SlyD-like protein A) as fusion partners for difficult proteins has been widely described (WO 2003/000878, WO 2009/074318, EP 2127679).

Commercially available immunoassays for the detection of antibodies against pathogens like, *e.g.*, human immunodeficiency virus (HIV), Rubella virus, cytomegalovirus (CMV) or herpes simplex virus (HSV) contain polypeptide fusion proteins wherein chaperones are fused to specific target antigen sequences. Such fusion proteins are described in, *e.g.*, Scholz et al., J. Mol.

Biol. (2005) 345, 1229-1242, Scholz et al., Biochemistry (2006) 45, 20-33 or Scholz et al., Biochemistry (2008) 47, 4276-4287.

SlyD, FkpA and SlpA possess outstanding solubilization (*i.e.* chaperone) properties and are characterized in that they are able to refold reversibly after chemically or thermally induced unfolding. As fusion partners for difficult target polypeptides they play at least a threefold role: firstly, they increase the production of target proteins that are heterologously overexpressed in procaryotic organisms, secondly, they facilitate and assist the *in vitro* refolding of the target polypeptides, and thirdly, they increase the overall solubility and stability of the respective target polypeptide.

However, chaperones like SlyD, FkpA and SlpA are immunogens in their own right. Since they are abundant bacterial proteins, they are recognized as non-self by the human (or, generally speaking, mammalian) immune system, triggering a powerful humoral immune response, which results in the production of specific antibodies with high affinity. A considerable percentage of adult human sera therefore contain significant immunoglobulin titers against these chaperones. As a consequence, there is a considerable likelihood that a human serum sample may turn out false positive in an immunoassay, in particular in an immunoassay of the double antigen sandwich format that uses antigen specifiers fused to bacterial chaperone modules.

In order to avoid such unwanted cross-reactions due to the antibody-induced bridging of fusion partners, immunoassays are usually designed in an asymmetric fashion. This means that for example in an immunoassay for the detection of antibodies designed in the well-known double antigen sandwich format (DAGS) a person skilled in the art uses different fusion partners for the applied antigens on both sides of the assay in order to avoid non-specific bridging. If identical fusion partners were used for the antigens on the solid phase and the detection side, interfering components in the sample could establish a bridge between said identical fusion partners and thus evoke a (false) positive reaction.

As a further means to prevent unwanted binding to a fusion partner which is part of an antigen-fusion protein, a chemically polymerized form of the employed fusion module (*i.e.* the fusion part without any specific antigen) is usually added to the assay in large excess. Due to their high epitope density and their high effective concentrations, these chemically polymerized fusion modules preferably allure, bind and quench those IgGs and IgMs that are directed towards said

fusion module. The chemically polymerized fusion modules serve as a bait, and they quench the interfering compounds in the sample very efficiently so that interferences can be suppressed and ruled out. When, for instance, *E. coli* SlyD is used as a fusion partner for a given antigen in an immunoassay of the double antigen sandwich type, it would be highly advisable to generate chemically polymerized *E. coli* SlyD (by means of crosslinking with, *e.g.*, glutardialdehyde) and to add this polymer to the assay as an anti-interference substance.

However, a considerable disadvantage in using chemically polymerized proteins lies in the chemical production process itself. Depending on the cross-linking agent applied, the chemical polymerization process is not entirely reproducible. The chemically cross-linked polymers usually show a large distribution of polymers of different size, *i.e.* they strongly vary with respect to connectivity and they are characterized by considerable heterogeneities. In order to select the effective polymer fractions (*i.e.* the polymer fractions with the desired anti-interference abilities) the polymer pool needs to be purified and fractionated by time-consuming and cumbersome chromatographic methods. In addition, only limited yields can be obtained as only a small percentage of the product will elute in the desired fraction.

In order to overcome the obstacle of using insufficiently characterized chemically polymerized material in immunoassays we searched for an alternative way to generate anti-interference substances. We strived to obtain anti-interference modules with a sufficiently high and well-defined epitope density in a simple and convenient manner. So we addressed the question whether it was possible to create a well-defined, highly soluble and highly efficient anti-interference module in an utterly recombinant fashion. Briefly, the problem to be solved was to obtain chaperone fusion partners in a soluble form, with high epitope density and in a reproducible and standardizable way.

Summary of the invention

The problem is solved by the current invention as characterized by the claims. In particular, the invention concerns a fusion polypeptide comprising several molecules of folding helper polypeptides, comprising one multimerization domain and at least one molecule of SlyD or SlpA, wherein no further target polypeptide sequences are fused to said fusion polypeptide. As a preferred multimerization domain Skp is used. Preferably one molecule of Skp is fused to two

adjacent molecules of SlyD or to two adjacent molecules of SlpA. In another preferred embodiment one molecule of Skp is N-terminally fused to two adjacent molecules of SlyD or to two adjacent molecules of SlpA or to another monomeric chaperone that serves as a fusion partner. The term "N-terminally fused" means that Skp is fused to the N-terminal end of another protein molecule, in this case to the N-terminal end of either SlyD or SlpA. In a further preferred mode the fusion polypeptide according to the invention comprises SEQ ID NO. 1 which can also be named Skp-tandem-SlyD or Skp-SlyD-SlyD. A preferred fusion polypeptide is a polypeptide consisting of SEQ ID NO. 1 (Skp-tandem-SlyD). A further preferred fusion polypeptide is a polypeptide comprising SEQ ID NO. 9 which can also be named Skp-tandem-SlpA or Skp-SlpA-SlpA. Particularly preferred is a polypeptide consisting of SEQ ID NO. 9.

Another embodiment of the invention is the use of a fusion polypeptide as an additive in an immunoassay or as an additive to an assay reagent so that the fusion polypeptide can be used for reduction of interferences or for minimizing false positive results. According to the invention said fusion polypeptide can also be used for increasing the solubility of proteinaceous ingredients within an assay reagent. Also encompassed by a further preferred embodiment of the invention is a reagent kit for the detection of an analyte in an isolated sample by an immunoassay which comprises said fusion polypeptide.

In a further preferred embodiment a method for detecting an analyte in an isolated sample is encompassed wherein a fusion polypeptide as characterized above is used as a reagent for reduction of interference or for minimizing false positive results.

Another embodiment of the invention is a method for detecting an analyte such as, *e.g.*, an antibody in an isolated sample, said method comprising

a) forming an immunoreaction admixture by admixing a body fluid sample with a specific binding partner that can be specifically bound by said analyte present in said sample

b) adding a fusion polypeptide according to the invention to said immunoreaction admixture either before, at the same time or after said specific binding partner is added to said sample

c) maintaining said immunoreaction admixture for a time period sufficient for allowing the analyte present in said body fluid sample to immunoreact with said specific binding partner to form an immunoreaction product; and

d) detecting the presence and /or the concentration of any of said immunoreaction product.

A further aspect of the invention is a reagent kit for the detection of an analyte, in particular for the detection of an antibody, in an isolated sample by an immunoassay, said kit comprising a fusion polypeptide according to the invention. Other ingredients of a reagent kit are known to someone skilled in the art and include specific binding reagents such as *e.g.* antigens. Further kit components are buffers, preservatives, labeling substances and instructions for use.

Brief description of the figures

Figures 1 to 7 show tables 1a-c, 2a-c and 3 (see example 3), presenting results on anti-interference activity of the fusion polypeptides in an immunoassay for detection of HIV anti-gp41 antibodies.

In detail, figure 1 shows table 1a.

Figure 2 shows table 1b.

Figure 3 shows table 1c.

Figure 4 shows table 2a.

Figure 5 shows table 2b.

Figure 6 shows table 2c.

Figure 7 shows table 3.

Figure 8 shows the thermally induced unfolding and refolding of Skp-SlyD-SlyD as monitored in the near UV region at a detection wavelength of 280 nm.

Figure 9 shows near-UV CD spectra of Skp-SlyD-SlyD in the range of 250-330 nm (see example 5).

Figure 10 shows table 4, presenting results on anti-interference activity of the fusion polypeptides in an immunoassay for detection of anti-HSV2 antibodies (see example 4).

Figure 11 shows near-UV CD spectra of Skp-SlpA-SlpA in the range of 250-330 nm (see example 5).

SEQ ID NO. 1 shows the amino acid sequence of Skp-tandem-SlyD or Skp-SlyD-SlyD. The glycine-rich spacer region (underlined) between the Skp and SlyD units has been added to enable maximal flexibility of the fusion partners and to make sure that the Skp units may form ordered trimers without any interference of the C-terminally fused SlyD proteins. An additional C-terminal octa-histidine tag has been added for purification purposes (see experimental section). SEQ ID NO. 1 comprises amino acid residues 1-165 of *E. coli* SlyD (complete molecule cf. SEQ ID NO. 3) in tandem form, i.e. two *E. coli* SlyD (1-165) units in a row.

ADKIAIVNMG SLFQQVAQKT GVSNTLENEF RGRASELQRM ETDLQAKMKK LQSMKAGSDR TKLEKDVMAQ
RQTFAQKAQA FEQDRARRSN EERGKLVTRI QTAVKSVANS QDIDLVVDAN AVAYNSSDVK DITADVLKQV
KGGGSGGGSG GGSGGGSGGG SGGGMKVAKD LVVSLAYQVR TEDGVLVDES PVSAPLDYLH GHGSLISGLE
TALEGHEVGD KFDVAVGAND AYGQYDENLV QRVPKDVFMG VDELQVGMRF LAETDQGPVP VEITAVEDDH
VVVDGNHMLA GQNLKFNVEV VAIREATEEE LAHGHVHGAH DHHHDHDHDG GGSGGGSGGG SGGGSGGGSG
GGMKVAKDLV VSLAYQVRTE DGVLVDESPV SAPLDYLHGH GSLISGLETA LEGHEVGDKF DVAVGANDAY
GQYDENLVQR VPKDVFMGVD ELQVGMRFLA ETDQGPVPVE ITAVEDDHVV VDGNHMLAGQ NLKFNVEVVA
IREATEEELA HGHVHGAHDH HHDHDHDGGG SHHHHHHHH

SEQ ID NO. 2 shows the complete amino acid sequence of *E. coli* Skp (161 aa) according to SwissProt Accession No. P11457. For the fusion polypeptide according to the invention, the signal sequence of *E. coli* Skp (aa 1-20) is removed in order to make sure that the target molecule is produced and retained in the cytosol of the overproducing prokaryotic host. Preferably, the mature form of *E. coli* Skp , *i.e.* aa 21-161 of the sequence listed below is used.

MKKWLLAAGL GLALATSAQA ADKIAIVNMG SLFQQVAQKT GVSNTLENEF KGRASELQRM ETDLQAKMKK
LQSMKAGSDR TKLEKDVMAQ RQTFAQKAQA FEQDRARRSN EERGKLVTRI QTAVKSVANS QDIDLVVDAN
AVAYNSSDVK DITADVLKQV K

SEQ ID NO. 3 represents the complete *E. coli* SlyD amino acid sequence (196 amino acid residues) which is also accessible via ID P0A9K9 in the SwissProt database. For the fusion polypeptide according to the invention, preferably a C-terminally truncated version of *E. coli* SlyD spanning amino acid residues 1-165 of the sequence listed below is used.

MKVAKDLVVS LAYQVRTEDG VLVDESPVSA PLDYLHGHGS LISGLETALE GHEVGDKFDV AVGANDAYGQ
YDENLVQRVP KDVFMGVDEL QVGMRFLAET DQGPVPVEIT AVEDDHVVVD GNHMLAGQNL KFNVEVVAIR
EATEEELAHG HVHGAHDHHH DHDHDGCCGG HGHDHGHEHG GEGCCGGKGN GGCGCH

SEQ ID NO. 4 shows the amino acid sequence of the glycine-rich spacer (comprising triple glycine units separated by a serine) that can be used as a flexible, soluble and protease-resistant spacer or linker between several chaperone moieties.

GGGSGGGSGG GSGGGSGGGS GGG

SEQ ID NO. 5 shows the amino acid sequence of the *octa*-histidine tag or "His-tag" (comprising eight histidine units) that can be added to the C-terminal end of a protein to allow Ni-NTA assisted protein purification.

GGGSHHHHHH HH

SEQ ID NO: 6 shows the complete amino acid sequence of FkpA (270 aa), accessible also via the SwissProt database Accession No. P45523. For the fusion polypeptide according to the invention the signal sequence of *E. coli* FkpA (aa 1-25) is removed in order to make sure that the target molecule is produced and retained in the cytosol of the overproducing prokaryotic host. Preferably, the mature form of *E. coli* FkpA , *i.e.* aa 26-270 of the sequence listed below is used.

MKSLFKVTLL ATTMAVALHA PITFAAEAAK PATAADSKAA FKNDDQKSAY ALGASLGRYM
ENSLKEQEKL GIKLDKDQLI AGVQDAFADK SKLSDQEIEQ TLQAFEARVK SSAQAKMEKD
AADNEAKGKE YREKFAKEKG VKTSSTGLVY QVVEAGKGEA PKDSDTVVVN YKGTLIDGKE
FDNSYTRGEP LSFRLDGVIP GWTEGLKNIK KGGKIKLVIP PELAYGKAGV PGIPPNSTLV
FDVELLDVKP APKADAKPEA DAKAADSAKK

SEQ ID NO. 7 shows the complete amino acid sequence (149 amino acids) of *E. coli* SlpA, taken from the SwissProt database accession no. P0AEM0.

MSESVQSNSA VLVHFTLKLD DGTTAESTRN NGKPALFRLG DASLSEGLEQ HLLGLKVGDK
TTFSLEPDAA FGVPSPDLIQ YFSRREFMDA GEPEIGAIML FTAMDGSEMP GVIREINGDS
ITVDFNHPLA GQTVHFDIEV LEIDPALEA

SEQ ID NO. 8 shows the amino acid sequence of *Pasteurella multocida* SlyD (full length) according to Swiss Prot ID: Q9CKP2

MKIAKNVVVS IAYQVRTEDG VLVDEAPVNQ PLEYLQGHNN LVIGLENALE GKAVGDKFEV
RVKPEEAYGE YNENMVQRVP KDVFQGVDEL VVGMRFIADT DIGPLPVVIT EVAENDVVVD
GNHMLAGQEL LFSVEVVATR EATLEEIAHG HIHQEGGCCG GHHHDSDEEG HGCGCGSHHH
HEHEHHAHDG CCGNGGCKH

SEQ ID NO. 9 shows the amino acid sequence of Skp-tandem-SlpA or Skp-SlpA-SlpA. The glycine-rich spacer region (underlined) between the Skp and SlpA units has been added to enable maximal flexibility of the fusion partners and to make sure that the Skp units may form ordered

trimers without any interference of the C-terminally fused SlpA proteins. An additional C-terminal hexa-histidine tag has been added for purification purposes. SEQ ID NO. 9 comprises amino acid residues 2-148 of *E. coli* SlpA (complete molecule cf. SEQ ID NO. 7, but lacking the N-terminal methionine and the C-terminal alanine) in tandem form, i.e. two *E. coli* SlpA (2-148) units in a row.

ADKIAIVNMG SLFQQVAQKT GVSNTLENEF RGRASELQRM ETDLQAKMKK LQSMKAGSDR TKLEKDVMAQ
RQTFAQKAQA FEQDRARRSN EERGKLVTRI QTAVKSVANS QDIDLVVDAN AVAYNSSDVK DITADVLKQV
KGGGSGGGSG GGSGGGSGGG SGGGSESVQS NSAVLVHFTL KLDDGTTAES TRNNGKPALF RLGDASLSEG
LEQHLLGLKV GDKTTFSLEP DAAFGVPSPD LIQYFSRREF MDAGEPEIGA IMLFTAMDGS EMPGVIREIN
GDSITVDFNH PLAGQTVHFD IEVLEIDPAL EGGGSGGGSG GGSGGGSGGG SGGGSESVQS NSAVLVHFTL
KLDDGTTAES TRNNGKPALF RLGDASLSEG LEQHLLGLKV GDKTTFSLEP DAAFGVPSPD LIQYFSRREF
MDAGEPEIGA IMLFTAMDGS EMPGVIREIN GDSITVDFNH PLAGQTVHFD IEVLEIDPAL EHHHHHH

Detailed description of the invention

In commercially available immunoassays using rare reagents that contain SlyD fusion modules usually chemically cross-linked chaperone molecules such as tandem SlyD (two molecules of SlyD, linked via a short peptidic sequence, polymerized by means of glutardialdehyde) are added for reduction of interferences. As mentioned in the background section, due to the production process these chemically cross-linked additives are rather heterogeneous and cannot be provided in a strictly reproducible way with satisfying yields.

Although the use of polypeptide fusion proteins wherein chaperones are fused to specific target antigen sequences has been described in great detail before (see background of the invention) prior art is silent about how to overcome interferences in immunoassays. WO 2003/000878 describes FkpA as a chaperone that exerts its function in form of oligomers that can be fused to target polypeptide sequences. EP 1982993 discloses fusion polypeptides comprising at least one multimerization domain and a plurality of copies of an epitope segment from a pathogen. These polypeptides are applied as specific antigenic target sequences. However, the problem of eliminating interferences due to cross-reacting antibodies that bind to the chaperone modules and thus cause false positive results has hitherto not been addressed.

Surprisingly, by fusing a chaperone multimerization domain to at least one molecule of SlyD or SlpA we have been able to produce highly effective anti-interference agents. On top of that, in certain sera a significant background signal suggestive of a positive result cannot be minimized

or suppressed effectively by the chemically cross-linked prior art reagent whereas the fusion polypeptide according to the invention is able to suppress the high background signal by reducing the interference. When added as an anti-interference agent to samples, none of the true positive samples are significantly affected, *i.e.* true positive samples comprising anti-HIV antibodies are invariably detected as positive samples.

Unexpectedly, by fusing a chaperone multimerization domain to at least one molecule of SlyD or SlpA we have been able to produce a well-folded, highly soluble artificial chimeric protein which forms regular trimers. It is far from self-evident that fusion of two chaperones via a flexible linker segment yields a soluble and functional protein in which the constituents largely maintain their genuine properties. Since chaperones such as Skp, SlyD and SlpA possess polypeptide binding sites which have been evolved to recognize and reversibly bind hydrophobic protein surfaces, and since these polypeptide binding sites are, themselves, hydrophobic by nature, it would have been very likely that the polypeptide binding sites of the two chaperones bind and saturate each other, leading to locked complexes with limited value for epitope-presenting purposes. It also would have been conceivable that fusion of two (sterically demanding) SlyD units to the C-terminal end of Skp compromises the structural integrity of Skp and, possibly, abolishes the intrinsic trimerization of Skp.

However, what we find is that Skp-SlyD, Skp-SlyD-SlyD and Skp-SlpA-SlpA indeed form regular trimers which are highly stable and soluble and which show – even though they are oligomeric – largely reversible folding behavior. It also would have been conceivable that the solubility of the artificial fusion polypeptide (*i.e.* the combination of two unrelated chaperones) turns out to be poor due to the high effective concentration of the hydrophobic polypeptide binding regions which are brought into close proximity by covalent fusion of Skp and SlyD or SlpA, respectively. Surprisingly to us, the converse is true: Skp-SlyD-SlyD possesses an outstanding solubility and it can be concentrated to > 100 mg/ml (in 50 mM potassium phosphate pH 7.0, 250 mM KCl, 0.5 mM EDTA) without any tendency to aggregate. When Skp-SlyD-SlyD from such a concentrated solution is subjected to analytical gel filtration on a Superdex 200 column, the fusion polypeptide elutes in a single symmetric peak pointing to a perfectly soluble and stable trimer.

Skp-SlyD-SlyD is not only highly soluble, but its refolding behavior is also reversible. This is highlighted by the fact that we were able to elaborate a purification protocol which includes the

step of matrix-coupled refolding: The target molecule is bound in an unfolded form to a Ni-NTA support via its C-terminal octa-histidine tag. While it remains bound to the solid support, it is refolded by a simple buffer change. Subsequently, the refolded protein is eluted by an imidazole pulse. Surprisingly to us, upon imidazole elution, the eluted Skp-SlyD-SlyD monomers form soluble trimers almost quantitatively. To us, it is astounding that the matrix-coupled refolding of a trimeric protein such as Skp-SlyD-SlyD works so well and with impressively high yields. We find that the same holds true for Skp-SlpA-SlpA: very similar to Skp-SlyD-SlyD, Skp-SlpA-SlpA can be obtained in large amounts from an *E. coli* overproducing strain and it can be renatured in a well-ordered trimeric form by matrix-assisted refolding. And, quite similar to its SlyD counterpart, Skp-SlpA-SlpA is highly soluble and possesses very favourable physicochemical properties.

According to the invention the obtained fusion polypeptide – unlike the fusion polypeptides described in prior art - is free of further target polypeptide antigenic sequences as the fusion polypeptide is used for interference reduction or protein stabilization. It is not applied as a specific antigenic polypeptide for binding to analyte molecules such as antibodies. It is important that the analyte present in the sample - like *e.g.* antibodies against a pathogen – does not bind to the fusion polypeptide of the invention. Therefore target polypeptide sequences like antigen sequences derived from mammalian pathogens like viruses, bacteria, single-cell or multi-cell parasites are not part of the fusion polypeptide.

The fusion polypeptide according to the invention can be obtained in a reproducible way in homogeneous fractions with high yields. It possesses a well-defined epitope density which is necessary and sufficient to recognize, bind and quench interference factors of the IgG and IgM type. The fusion polypeptide is able to suppress false positive reactions in an immunoassay in a way that is not only equivalent but superior to the chemically produced reagent of prior art. Its production process is straightforward, simple and easy to standardize and inevitably results in high yields of a well-defined homogeneous fusion polypeptide with outstanding anti-interference properties.

According to the invention the fusion polypeptide preferably contains one multimerization domain. A multimerization domain is a domain that mediates and supports non-covalent association of several protein subunits containing that very multimerization domain. For example,

a dimerization domain is a domain that triggers association of two subunits, a trimerization domain is a domain that supports non-covalent association of three subunits and so on.

The second part of the fusion polypeptide is at least one molecule of SlyD, preferably *E. coli* SlyD, but SlyD molecules from other organisms, such as, *e.g., Pasteurella multocida* SlyD (cf. SEQ ID NO. 8) can also be used. Further preferred is a fusion polypeptide wherein one molecule of Skp is fused to two adjacent molecules of SlyD. In another embodiment of the invention other monomeric chaperones such as SlpA are also suitable as fusion partners which are fused to the single multimerization domain.

A further aspect of the invention is the use of the fusion polypeptides described above for reduction of interferences or for minimizing false positive results. The fusion polypeptide of the invention can be added to the immunoassay admixture (comprising sample and a binding partner specifically binding to the analyte in the sample) either before, at the same time or after said specific binding partner is added to the sample. Preferably, the fusion polypeptide is added to the test reagents before the body fluid sample containing the analyte, *e.g.* an antibody, is brought into contact with the specific binding partners (in this case the specific binding partners would be antigens).

Various formats and principles of immunoassays for detecting analytes and different modes of detection have been widely described and are familiar to a person skilled in the art. Of particular interest are immunoassays in which the analyte is an antibody. Preferably the immunoassay according to the invention detects antibodies against mammalian viral or bacterial pathogens such as e.g. hepatitis A, B or C virus, HIV (human immunodeficiency virus), HSV (herpes simplex virus), HTLV (human T-cell leukemia virus), EBV (Epstein-Barr virus), Rubella virus, CMV (cytomegalovirus), *Treponema pallidum* or *Borrelia burgdorferi*.

The invention is further illustrated in the examples section.

Examples

Example 1: Manufacture of fusion polypeptides

Cloning and purification of Skp/SlyD chaperone fusion polypeptides

Cloning of expression cassettes

In order to generate a suitable expression construct, an expression cassette encoding *Ec*Skp-*Ec*SlyD-*Ec*SlyD was ligated into expression plasmid pQE80L (Qiagen, Hilden, Germany) by a two-step cloning strategy.

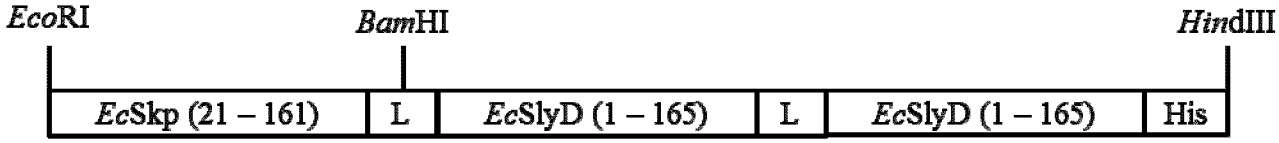
The sequence of the *E. coli* Skp (*Ec*Skp) was retrieved from the SwissProt database (SwissProt ID P0AEU7). In a first step, a synthetic gene comprising the Shine-Dalgarno sequence from expression vector pQE80L, the coding sequence of the mature Skp chaperone amino acids 21-161 (the signal peptide spanning amino acid residues 1-20 were omitted, the ATG start codon (methionine) was added in frame), with a part of a glycine-rich linker region as well as suitable recognition sites for restriction endonucleases *Eco*RI (5' end) and *Bam*HI (3' end), was purchased from Sloning (Vaterstetten, Germany). The synthetic 489 bp DNA fragment was hydrolyzed with the respective restriction endonucleases and ligated into the *Eco*RI/*Bam*HI opened expression vector pQE80L under control of a T5 promoter (P_{T5}).

Secondly, a further synthetic gene encoding two *E. coli* SlyD units (*Ec*SlyD, residues 1-165, SwissProt accession no. P0A9K9) connected *via* a glycine-rich linker region and encompassing part of a further linker region at the N-terminus as well as an *octa*-His-tag linked with a GGGS motif to the C-terminus was likewise purchased from Sloning (Vaterstetten, Germany). *Bam*HI and *Hin*dIII restriction sites were added at the 5' and 3' ends of this cassette, respectively. Genes and restriction sites were designed to enable the in frame fusion of the *Ec*SlyD-*Ec*SlyD part to the 5' end of the *Ec*Skp part by simple ligation. Therefore, the 1146 bp spanning fragment was hydrolyzed with restriction endonucleases *Bam*HI and *Hin*dIII and ligated into the *Bam*HI/*Hin*dIII opened, Skp containing vector pQE80L.

After ligation, competent cells of *E. coli* XL1Blue (Stratagene, La Jolla, CA, USA) were transformed with the respective DNA. After plasmid preparation from suitable transformants, the

correctness of the expression construct was reconfirmed by sequence analysis. The resulting expression plasmid has been named pQE80Skp-diSlyD.

The drawing below displays a scheme of the full length fusion polypeptide *Ec*Skp-*Ec*SlyD-*Ec*SlyD comprising one *E. coli* Skp chaperone unit and two *E. coli* SlyD chaperone units connected by glycine rich linker regions and followed by a C-terminal *octa*-His-tag to allow Ni-NTA assisted protein purification.



L = $(GGGS)_5GGG$ (glycine rich linker region), see SEQ ID NO.: 4
His = GGGSHHHHHHHHH (His-tag), see SEQ ID NO.: 5

The complete amino acid sequence of the desired fusion polypeptide is shown in SEQ ID No. 1.

The expression cassettes encoding the iterative SlyD constructs (SlyD, SlyD-SlyD, SlyD-SlyD-SlyD…) have been cloned as described in Biochemistry (2006) 45, 20-33. The expression cassette encoding the Skp-SlpA-SlpA fusion polypeptide has been generated according to Scholz et al., J. Mol. Biol. (2005) 345, 1229-1242.

Recombinant expression of *Ec*Skp-*Ec*SlyD-*Ec*SlyD in an *E. coli* host

In order to obtain the putative anti-interference polypeptide in sufficient amounts, *Ec*Skp-*Ec*SlyD-*Ec*SlyD was recombinantly expressed in *E. coli*. For this purpose, competent cells of *E. coli* BL21 Codon$^+$ (Merck (Novagen®), Darmstadt, Germany) were transformed with the generated expression construct pQE80Skp-diSlyD.

50 mL of SB medium (32.0 g tryptone, 20.0 g yeast extract, 5.0 g NaCl, ad 1000 mL *A. dest.*) supplemented with ampicillin (100 µg/mL) were inoculated with a single colony harboring the pQE80Skp-diSlyD plasmid and incubated over night at 37 °C (250 rpm). Subsequently, 1.5 L of SB medium (+ 100 µg/mL ampicillin) was inoculated with the overnight culture up to an $O.D._{600}$ of ~0.5. At an $O.D._{600}$ of ~3.0, cytosolic overexpression was induced by adding 0.5 mM

isopropyl-β-D-thiogalactoside (IPTG) to the culture. Four hours after induction, cells were harvested by centrifugation (20 min at 6000 g) and stored at -20 °C.

Aliquots of 0.4 $O.D._{600}$ were taken before and four hours after induction and whole cell extracts were tested for expression of *Ec*Skp-*Ec*SlyD-*Ec*SlyD by SDS-PAGE analysis. Overproduction of the target molecule was found to be highly abundant.

Overexpression of the SlyD and SlpA fusion proteins was carried out as described in Scholz et al., J. Mol. Biol. (2005) 345, 1229-1242 and Scholz et al., Biochemistry (2006) 45, 20-33.

Purification of Skp/SlyD/SlpA polypeptide fusions

The Skp/SlyD polypeptide fusions and the SlyD and SlpA fusion proteins were purified by using virtually identical protocols. For cell lysis, the frozen pellet was resuspended in chilled 50 mM sodium phosphate pH 8.0, 7.0 M GdmCl, 5 mM imidazole and the suspension was stirred for at least 2 h on ice to complete cell lysis. After centrifugation and filtration (0.45 µm/0.2 µm), the crude lysate was applied onto a Ni-NTA column equilibrated with the lysis buffer including 5.0 mM TCEP. The subsequent washing step was tailored for the respective target protein and ranged from 5 to15 mM imidazole (in 50 mM sodium phosphate pH 8.0, 7.0 M GdmCl, 5.0 mM TCEP). At least 10-15 volumes of the washing buffer were applied. Then, the GdmCl solution was replaced by 50 mM potassium phosphate pH 8.0, 100 mM KCl, 10 mM imidazole, 5.0 mM TCEP to induce conformational refolding of the matrix-bound protein. In order to avoid reactivation of copurifying proteases, a protease inhibitor cocktail (Complete® EDTA-free, Roche) was included in the refolding buffer. A total of 15-20 column volumes of refolding buffer were applied in an overnight reaction. Then, both TCEP and the Complete® EDTA-free inhibitor cocktail were removed by washing with 3-5 column volumes 50 mM potassium phosphate pH 8.0, 100 mM KCl, 10 mM imidazole. Subsequently, the imidazole concentration – still in 50 mM potassium phosphate pH 8.0, 100 mM KCl – was raised to 60-70 mM for the *Ec*Skp-*Ec*SlyD fusion proteins, to 50 mM for *Ec*Skp-*Ec*SlpA-*Ec*SlpA and to 30 mM for the SlyD fusion polypeptides in order to remove unspecifically bound protein contaminants. The native protein was then eluted by 500 mM imidazole in the same buffer. Protein-containing fractions were assessed for purity by Tricine-SDS-PAGE and pooled. Finally, the proteins were subjected

to size-exclusion-chromatography (Superdex HiLoad, Amersham Pharmacia) and the protein-containing fractions were pooled and concentrated to 10-20 mg/ml in an Amicon cell (YM10).

After the coupled purification and refolding protocol, protein yields of roughly 15-25 mg could be obtained from 1 g of *E. coli* wet cells, depending on the respective target protein.

Example 2

Spectroscopic measurements

Protein concentration measurements were performed with an Uvikon XL double-beam spectrophotometer. The molar extinction coefficients (ε_{280}) were determined by using the procedure described by Pace (1995), Protein Sci. 4, 2411-2423. For *Ec*Skp-*Ec*SlyD-*Ec*SlyD, a molar extinction coefficient (ε_{M280}) of 13410 $M^{-1}cm^{-1}$ was used, for *Ec*Skp-*Ec*SlyD a molar extinction coefficient (ε_{M280}) of 7450 $M^{-1}cm^{-1}$ was used. For *Ec*Skp-*Ec*SlpA-*Ec*SlpA, a molar extinction coefficient (ε_{M280}) of 4470 $M^{-1}cm^{-1}$ was used. For the repetitive *E. coli* SlyD constructs, SlyD, SlyD-SlyD, SlyD-SlyD-SlyD, SlyD-SlyD-SlyD-SlyD and SlyD-SlyD-SlyD-SlyD-SlyD, molar extinction coefficients of 5960 $M^{-1}cm^{-1}$, 11920 $M^{-1}cm^{-1}$, 17880 $M^{-1}cm^{-1}$, 23840 $M^{-1}cm^{-1}$ und 29800 $M^{-1}cm^{-1}$ were used.

Example 3

Anti-interference activity of the SlyD polypeptide fusion proteins

The anti-interference activity of the SlyD polypeptide fusion proteins was assessed in an automated Elecsys® 2010 analyzer (Roche Diagnostics GmbH). Elecsys® is a registered trademark of the Roche group. Measurements were carried out in the double antigen sandwich format.

Signal detection in Elecsys® 2010 is based on electrochemoluminescence. The biotin-conjugate (*i.e.* the capture-antigen) is immobilized on the surface of a streptavidin coated magnetic bead whereas the detection-antigen bears a complexed Ruthenium cation (switching between the

redox states 2+ and 3+) as the signaling moiety. In the presence of a specific immunoglobulin analyte, the chromogenic ruthenium complex is bridged to the solid phase and emits light at 620 nm after excitation at a platinum electrode. The signal output is in arbitrary light units.

The recombinant anti-interference SlyD polypeptides were assessed in a double antigen sandwich (DAGS) immunoassay format. To this end, FkpA-FkpA-gp41 and SlyD-SlyD-gp41 as disclosed in EP 1 402 015 were used as biotin and ruthenium conjugates, respectively, to specifically detect anti-gp41 antibodies in human sera. gp41 is the immunodominant antigen of HIV, and soluble variants of the gp41 ectodomain – as disclosed in EP 1 402 015 - are invaluable tools for the detection of HIV infections. FkpA-FkpA-gp41-biotin and SlyD-SlyD-gp41-ruthenium were used in R1 (reagent buffer 1) and R2 (reagent buffer 2) at concentrations of 750 ng/ml each.

In a first experiment, Trina sera negative for HIV were assessed with the aforementioned DAGS immunoassay setup. In order to get a hint to the incidence rate of false positives, the assessment was performed in the absence and in the presence of SS-Helix(GDA,P), a GDA-crosslinked soluble heterogeneous SlyD polymer which is used as an anti-interference substance. SS-Helix (GDA,P) was added to R1 (reagent buffer 1 containing the biotin conjugate) in large excess amounts (25 µg/ml). 60 µl R1 (reagent buffer 1, biotin conjugate and anti-interference SlyD polymer), 60 µl R2 (reagent buffer 2, ruthenium conjugate), 30 µl sample (human serum) and 50 µl bead suspension are then mixed and incubated to yield a reaction volume of roughly 200 µl.

Tables 1a-c (figures 1-3) demonstrate the high incidence rate of elevated (false positive) signals in the absence of the anti-interference polymer. Even though two different chaperones, such as SlyD and FkpA, are used on the two sides of the double antigen sandwich immunoassay, we find a wealth of significantly elevated signals in a well-characterized panel of human sera (Trina Bioreactives AG, Nänikon, Switzerland) for which HIV infections have clearly been ruled out. The reason for this finding is that SlyD and FkpA, though different, are related molecules belonging to the FKBP family of chaperones and sharing a highly conserved FKBP domain. It is probably via this shared motif that the immunological cross-reaction takes place, evoking high signals and thus pretending a positive outcome in the HIV assay. Addition of the chemically polymerized anti-interference substance, SS-Helix(GDA,P) to the assay mixture reduces the elevated signals to normal negatives. Table 1 c (fig.3) shows that even very strong interferences, *i.e.* high false positives, may be efficiently eliminated by addition of SS-Helix(GDA,P). The

bottom line of the results shown in table 1 a-c (figs. 1-3) is the following: interferences due to fusion partners in a DAGS immunoassay are frequent and they can efficiently be mitigated by the addition of crosslinked polymer variants of one fusion partner.

Tables 2a-c (figs. 4-6) illustrate the anti-interference capability of diverse recombinant SlyD variants. Five HIV negative sera, five anti-HIV positive sera and two false positive sera from a Trina panel (Trina Bioreactives AG, Nänikon, Switzerland) were assessed with FkpA-FkpA-gp41-biotin and SlyD-SlyD-gp41-ruthenium as described. The assays were performed in the absence and in the presence of diverse anti-interference candidates. The anti-interference modules under study were added to R1 (reagent buffer 1) at concentrations of 5 µg/ml and 25 µg/ml in order to reveal a possible dose effect. SS-Helix (GDA,P) was included in the experiment as a positive reference (*i.e.* a well-suited and potent anti-interference module). Without an anti-interference additive (Tab. 2a, V0), the signals of the false positive Trina sera amount to almost 60,000 counts, strongly suggestive of a high anti-gp41 antibody titer as a consequence of an HIV infection. Upon addition of SS-Helix (GDA,P), the false positive signals are, however, reduced to the level of the HIV negative sera (Tab. 2a, V1). When Skp-SlyD is added, the false positive signals are significantly reduced, too, but they still are elevated – even in the presence of 25 µg/ml – and would be strongly misleading, in that they indicate an HIV infection in an healthy individual (Tab. 2a, V2). When, however, Skp-SlyD-SlyD is added, the false positive signals are reduced to the signal level of the HIV negative sera (Tab. 2a, V3). Skp-SlyD-SlyD turns out to be as efficient as the chemically polymerized SS-Helix (GDA,P) in its anti-interference capacity. Obviously, the epitope density of Skp-SlyD-SlyD is high enough to efficiently bind to and quench interference factors which presumably belong to the IgM type of immunoglobulins. The equivalence (with respect to anti-interference) of a recombinantly produced module such as Skp-SlyD-SlyD with a crosslinked SlyD polymer such as SS-Helix (GDA,P) is an astounding outcome of our experiments.

In Tab. 2b (Figure 5), two controls are shown: neither does addition of *E. coli* SlyD alone mitigate the false positive signals of the two Trina interference sera tested, nor does addition of *E. coli* Skp alone have any beneficial effects on the falsely elevated signals (Tab. 2b, V4 and V5). Even at concentrations as high as 25 µg/ml, none of the single components is able to affect the false positive signals. However, in combination, as a Skp-SlyD-SlyD fusion polypeptide, Skp and SlyD constitute a powerful anti-interference tool (Tab. 2a (fig. 4), V3). The inability of the single monomeric *E. coli* SlyD to cope with the interferences of the Trina sera 16097448 and

47101943, strongly suggests that the respective interference factors belong to the M type of immunoglobulins (IgM). Obviously, a higher epitope density concomitant with a higher effective SlyD concentration is mandatory to efficiently bind to and quench the interference factors.

To critically challenge this assumption, we performed further anti-interference studies shown in table 2c (Fig. 6). As an analytical tool we used *E. coli* SlyD polymers that have been generated by standard cloning techniques as described in Biochemistry (2006) 45, 20-33. Put simply, *E. coli* SlyD units were joined by flexible, glycine- and serine-rich linkers to form a row. Up to five SlyD units were feasible in a single fusion polypeptide with satisfying expression yields in *E. coli* and convenient purification procedures.

Table 2c (Fig. 6) illustrates the need of a high SlyD epitope density for efficient anti-interference and it points to the fact that we can make use of the avidity effect (avidity means the high apparent affinity that occurs when a polyvalent binding molecule such as an IgM molecule encounters a polymeric substrate such as polymerized SlyD). Without any anti-interference additive, the signals of the Trina interference sera 16097448 and 47101943 amount to 59827 and 53491 counts, respectively, and clearly hint to the presence of anti-gp41 antibodies, even though the sera have unequivocally been confirmed as anti-HIV negative. When tandem SlyD (SlyD-SlyD) is added to R1 (reagent buffer 1) at a concentration of 25 µg/ml, the signals remain virtually unaffected (table 2c, V8). When triple SlyD (SlyD-SlyD-SlyD) is added to R1 at a concentration of 25 µg/ml, the signals are reduced to ~ 4000 counts (table 2c, V7). Although strongly quenched, the remaining signal still would pretend a positive result. When, however, penta-SlyD (SlyD-SlyD-SlyD-SlyD-SlyD) is added to R1 at a concentration of only 5 µg/ml, the signal reduction is comparable to the one achieved by the addition of chemically polymerized SS-Helix(GDA,P) at the same concentration (table 2a, V1; table 2c, V6). In other words, the anti-interference capacity of SlyD increases with the number of SlyD units that constitute the respective construct. The increase in anti-interference capacity is not linear, indicating that we are facing a cooperative rather than an additive effect.

In brief, Tab. 2 (Figs. 4-6) shows that the anti-interference potential significantly increases with the number of the interconnected SlyD units. It suggests that at least five SlyD units in close proximity are necessary and sufficient for effective anti-interference. Penta-SlyD (SlyD-SlyD-SlyD-SlyD-SlyD) would thus be a promising anti-interference additive, but its expression yield in an *E. coli* host is rather poor and poses severe obstacles with respect to production upscaling.

Skp-SlyD-SlyD is at least equivalent to SS-Helix(GDA,P) with respect to its anti-interference potential. Thus, the epitope density which is established by fusion of the trimeric Skp to tandem SlyD (SlyD-SlyD) is obviously sufficient to meet the requirement for efficient anti-interference. This is remarkable all the more since the availability of the recombinantly produced Skp-SlyD-SlyD module is much better and its production process is by far more reproducible and convenient.

Tab. 3 (Fig. 7) shows that, in some cases, the anti-interference capability of Skp-SlyD-SlyD is even superior to that of SS-Helix(GDA,P). Again, five negative sera, five anti-HIV positive sera and three interference sera with significantly elevated signals have been assessed in the Elecsys® 2012 automated analyzer. Without any anti-interference additive, the signals of the three interference sera amount to 10351, 1437 and 778 counts. When the crosslinked SlyD polymer SS-Helix (GDA,P) is added to R1 at a concentration of 20 µg/ml, the signals are only slightly reduced to values of 8042, 903 and 772 counts (Tab. 3, V1). When, however, Skp-SlyD-SlyD is added to R1 at concentrations of 5 µg/ml or 15 µg/ml, the signals are significantly reduced to the level of the negative sera, more precisely they are reduced to 566, 537 and 507 counts. It is noteworthy that addition of Skp-SlyD-SlyD has an obviously smoothing effect on the signals of the negative sera and that it slightly improves the coefficient of variation. This effect is more pronounced with Skp-SlyD-SlyD than it is with SS-Helix(GDA,P). To sum up, table 3 highlights the fact that Skp-SlyD-SlyD is, in some cases, superior to SS-Helix(GDA,P) in its anti-interference capability: In the case of the interference serum C133202 it reduces the signal from false positive to true negative. Even signals, which are only slightly elevated, as exemplified in the sera Pr149 or C133111, are significantly reduced to a signal level that closely approaches the blank value of the analyzer. On top of that, we observe a smoothing effect of Skp-SlyD-SlyD on the signals of the anti-HIV negative sera, leading to an overall decrease of the signals and an improved coefficient of variation.

Example 4

Anti-interference activity of the *Ec*Skp-*Ec*SlpA-*Ec*SlpA polypeptide fusion protein

The anti-interference activity of *Ec*Skp-*Ec*SlpA-*Ec*SlpA was assessed in an automated Elecsys® 2010 analyzer (Roche Diagnostics GmbH). Elecsys® is a registered trademark of the Roche group. Measurements were carried out in the double antigen sandwich format.

Signal detection in Elecsys® 2010 is based on electrochemoluminescence (for detailed explanation see example 3).

The recombinant *Ec*Skp-*Ec*SlpA-*Ec*SlpA polypeptide was assessed in a double antigen sandwich (DAGS) immunoassay format. To this end, *Pm*SlyD-mgG2 and *Ec*SlpA-mgG2 as disclosed in EP 2 127 678 were used as biotin and ruthenium conjugates, respectively, to specifically detect anti-HSV-2 antibodies in human sera. Mature glycoprotein G2 (mgG2) is an immunodominant antigen of herpes simplex virus 2 (HSV-2), and soluble variants thereof - as disclosed in EP 2 127 678 - are invaluable tools for the detection of HSV-2 infections. *Pm*SlyD-mgG2-biotin and *Ec*SlpA-mgG2-ruthenium conjugates were used in R1 (reagent buffer 1) and R2 (reagent buffer 2) at concentrations of 300 ng/ml each.

Anti-HSV-2 negative sera, anti-HSV-2 positive sera and anti-HSV-2 false positive sera (*i.e.* interference sera) were assessed with the aforementioned DAGS immunoassay setup. The assessment was performed in the absence and in the presence of *Ec*SlpA-*Ec*SlpA (GDA,P), a soluble heterogeneous GDA-crosslinked *Ec*SlpA polymer which is used as an anti-interference substance in the anti-HSV-2 immunoassay. *Ec*SlpA-*Ec*SlpA (GDA,P) serves the role of an anti-interference benchmark: it constitutes the conventional anti-interference additive that has been generated by chemical crosslinking of an *Ec*SlpA-*Ec*SlpA polypeptide and that is well-suited to improve the specificity of immunoassays based on *Ec*SlpA fusion antigens. The anti-interference additives under scrutiny were added to both R1 (reagent buffer 1, containing the biotin conjugate) and to R2 (reagent buffer 2, containing the ruthenium conjugate) in large excess amounts (10 µg/ml each). 70 µl R1 (reagent buffer 1, biotin conjugate and anti-interference *Ec*SlpA polymer), 70 µl R2 (reagent buffer 2, ruthenium conjugate and anti-interference *Ec*SlpA polymer), 20 µl sample (human serum) and 40 µl bead suspension are then mixed and incubated to yield a reaction volume of roughly 200 µl.

Even though two different chaperones, such as *Pm*SlyD (*i.e.*, SlyD from *Pasteurella multocida*) and *Ec*SlpA (*i.e.*, SlpA from *E. coli*), are used on the two sides of the double antigen sandwich immunoassay, significantly elevated signals are rather frequent in a panel of human sera, for which HSV-2 infections have clearly been ruled out. The reason for this finding is that the fusion partners *Pm*SlyD and *Ec*SlpA, although from different organisms, are related molecules belonging to the FKBP family of chaperones and sharing a highly conserved FKBP domain. It is probably via this shared motif that the immunological cross-reaction takes place, evoking high signals and thus pretending a positive outcome in the anti-HSV-2 assay. Addition of the chemically polymerized anti-interference substance, *Ec*SlpA-*Ec*SlpA(GDA,P), to the assay mixture reduces the elevated signals to normal negatives. Table 4 (Fig. 10) shows that even very strong interferences, *i.e.* high false positives [such as for sample 012], may be efficiently eliminated by addition of the anti-interference polymer *Ec*SlpA-*Ec*SlpA(GDA,P). As illustrated by table 4 (Fig. 10), interferences due to fusion partners in a DAGS immunoassay are frequent and they can efficiently be mitigated by the addition of crosslinked polymer variants of at least one of the fusion partners.

When *Ec*Skp-*Ec*SlpA-*Ec*SlpA is added to the assay, we find that the false positive signals are reduced to the signal level of HSV-2 negative sera as well. Indeed, *Ec*Skp-*Ec*SlpA-*Ec*SlpA turns out to be at least as efficient as the chemically polymerized *Ec*SlpA-*Ec*SlpA (GDA,P) in its anti-interference capacity. Obviously, the epitope density of Skp-*Ec*SlpA-*Ec*SlpA is high enough to efficiently bind to and quench interference factors which presumably belong to the IgM type of immunoglobulins. These interference sera are characterized in that they do not respond to the addition of monomeric anti-interference additives (see tab. 4/Fig. 10, samples 010 & 013). True positive signals are neither affected by the fusion polypeptide *Ec*Skp-*Ec*SlpA-*Ec*SlpA nor by the chemically polymerized *Ec*SlpA-*Ec*SlpA (GDA,P). The equivalence (with respect to anti-interference) of a recombinantly produced module such as *Ec*Skp-*Ec*SlpA-*Ec*SlpA with a crosslinked *Ec*SlpA polymer such as *Ec*SlpA-*Ec*SlpA (GDA,P) is an astounding outcome of our experiments. In Tab. 4 (Fig. 10) two further controls are shown: *Ec*Skp and *Ec*SlpA (*i.e.* the components of the *Ec*Skp-*Ec*SlpA-*Ec*SlpA fusion polypeptide) have been added to the immunoassay as single chaperones in order to assess their anti-interference capacity. The results are quite clear-cut: *Ec*Skp alone does not affect the false positive signals of the interference sera at all. However, *Ec*SlpA alone seems to exert a beneficial effect, at least in two out of four cases. As for the interference samples 011 and 012, addition of monomeric *Ec*SlpA in large excess

reduces the signal from 8204 to 969 counts and from 42168 to 14801 counts, respectively. One may conclude that the addition of monomeric *Ec*SlpA might help to increase the assay specificity in some cases. Yet, comparison with *Ec*Skp-*Ec*SlpA-*Ec*SlpA reveals at first glance that the fusion polypeptide is highly superior with respect to its anti-interference potential. Addition of *Ec*Skp-*Ec*SlpA-*Ec*SlpA unambiguously reduces the false positives to true negatives, as shown in table 4 for the interference sera 010-013. It is remarkable that the recombinant-derived *Ec*Skp-*Ec*SlpA-*Ec*SlpA fusion protein even outperforms the standard anti-interference additive *Ec*SlpA-*Ec*SlpA (GDA,P) with respect to its anti-interference activity (see table 4, samples 010, 011 and 013). Taken together with its ease of handling and production, the anti-interference features of *Ec*Skp-*Ec*SlpA-*Ec*SlpA are outstanding and make this molecule a highly attractive additive in an immunoassay.

Example 5

CD-detected thermally induced unfolding of Skp-SlyD-SlyD

Near-UV CD spectra were recorded with a Jasco-720 spectropolarimeter with a thermostatted cell holder and converted to mean residue ellipticity. The buffer was 50 mM potassium phosphate pH 7.5, 250 mM KCl, 0.5 mM EDTA. The pathlength was 0.2 cm the protein concentration was 8.2 mg/ml (147 µM monomer, corresponding to 49 µM trimer). The range was 250 - 330 nm, the band width was 1.0 nm, the scanning speed was 20 nm/min at a resolution of 0.5 nm and the response was 1 s. In order to improve the signal-to-noise ratio, spectra were measured nine times and averaged.

Circular dichroism spectroscopy (CD) is the method of choice to assess both the secondary and the tertiary structure of proteins. Ellipticity in the aromatic region (250-330 nm) reports on tertiary contacts within a protein (*i.e.,* the globular structure of a regularly folded protein) and is considered as the fingerprint region of a native-like fold (conformation).

Near UV CD spectra of Skp-SlyD-SlyD were monitored to address the question whether the fusion protein adopts an ordered conformation after the matrix-coupled refolding procedure which is the crucial step in the purification process. The answer is quite clear-cut: the near UV CD signal of Skp-SlyD-SlyD unequivocally reports an orderly tertiary structure of the fusion

polypeptide. The aromatic residues of Skp-SlyD-SlyD are obviously embedded in the lipophilic protein core and thus experience asymmetric surroundings which strongly points to a native-like conformation of both Skp and SlyD within the fusion construct (fig. 9).

In order to address the question whether the thermally induced unfolding of Skp-SlyD-SlyD is reversible, melting curves were monitored in the near UV region at a detection wavelength of 280 nm. The temperature range was 20-65 °C, the band width was 1.0 nm, the temperature slope was 1°C/min and the response was 4 s (see Figure 8).

The thermally-induced unfolding was monitored at 280 nm (which is the wavelength of the maximal signal amplitude for Skp-SlyD-SlyD). Upon heating, the non-covalent contacts which stabilize the native conformation of the Skp-SlyD-SlyD molecule become loose and finally break down. This thermally induced unfolding is reflected in a decrease in the CD signal as shown in Figure 8. At 60 °C, Skp-SlyD-SlyD is fully unfolded. Strikingly, the CD signal comes back again when the protein solution is chilled down to 20°C. Despite a slight hysteresis, the unfolding curve and the refolding curve virtually superimpose, strongly indicative of a reversible refolding behavior of Skp-SlyD-SlyD (see figure 8). It is astounding that the thermally induced unfolding of a complex trimeric fusion protein such as Skp-SlyD-SlyD is – at least partially - a reversible process. It would have been expected that Skp-SlyD-SlyD, after thermally induced unfolding and dissociation into the monomeric subunits, would aggregate very quickly and quantitatively at an elevated temperature such as 60 °C. Yet, we find that Skp-SlyD-SlyD is able to readopt its native-like conformation when the protein solution is chilled to 20°C. Indeed, the near UV CD spectra monitored prior to and after the thermally induced unfolding, virtually superimpose (see Figure 9). In conclusion, Skp-SlyD-SlyD possesses robust folding properties which are outstanding for a molecule of this complexity and which are highly desired for a molecule that serves as an anti-interference or generally stabilizing component of an immunoassay.

We found very similar results for Skp-SlpA-SlpA: just like Skp-SlyD-SlyD, Skp-SlpA-SlpA exhibits a marked CD signal in the near UV region (250-330 nm, signal maximum at 277 nm), pointing to a well-ordered conformation after the matrix-coupled refolding process. By means of thermal transitions (monitored at 277 nm) we observed that Skp-SlpA-SlpA retains its native-like conformation at temperatures up to 55°C. Furthermore, the CD signal of the native molecule is largely restored after a thermal unfolding/refolding cycle (20°C/65°C/20°C) as illustrated by

figure 11. It is desirable that anti-interference additives exert their functions even under temperature conditions that are far from optimal. The high thermal stability of Skp-SlpA-SlpA together with the partial reversibility of its thermally-induced unfolding underscores the robustness of this molecule.

In conclusion, Skp-SlyD-SlyD and Skp-SlpA-SlpA possess robust folding properties which are outstanding for molecules with this degree of complexity and which are highly desirable for modules that serve as anti-interference or generally stabilizing components of an immunoassay.

Claims

1. A fusion polypeptide comprising several molecules of folding helper polypeptides, comprising one multimerization domain and at least one molecule of SlyD or SlpA, wherein no further target polypeptide sequences are fused to said fusion polypeptide.

2. A fusion polypeptide according to claim 1 wherein the multimerization domain is Skp.

3. A fusion polypeptide according to claims 1 or 2 wherein one molecule of Skp is fused to two adjacent molecules of SlyD or SlpA.

4. Fusion polypeptide comprising SEQ ID NO. 1 (Skp-tandem-SlyD).

5. Fusion polypeptide comprising SEQ ID NO. 9 (Skp-tandem-SlpA).

6. Use of a fusion polypeptide according to claims 1 to 5 in an immunoassay for reduction of interferences or for minimizing false positive results.

7. Use of a fusion polypeptide according to claims 1 to 5 for increasing solubility of proteinaceous ingredients of an assay reagent.

8. A reagent kit for the detection of an analyte in an isolated sample by an immunoassay, comprising a fusion polypeptide according to claims 1 to 5.

9. A method for detecting an analyte in an isolated sample wherein a fusion polypeptide according to claims 1 to 5 is used as a reagent for reduction of interference or minimizing false positive results.

10. A method for detecting an analyte in an isolated sample, said method comprising

a) forming an immunoreaction admixture by admixing a body fluid sample with a specific binding partner that can be specifically bound by said analyte present in said sample

b) adding a fusion polypeptide according to any of claims 1 to 5 to said immunoreaction admixture either before, at the same time or after said specific binding partner is added to said sample

c) maintaining said immunoreaction admixture for a time period sufficient for allowing the analyte present in said body fluid sample to immunoreact with said specific binding partner to form an immunoreaction product; and

d) detecting the presence and /or the concentration of any of said immunoreaction product.

11. A method according to claims 9 or 10 wherein the analyte present in an isolated sample is an antibody.

Figure 1/11

Table 1a

Run	**V1**	**V2**	
R1	FF41-Bi	FF41-Bi	
SS-Helix (GDA,P) in R1	without anti-interference additive	25µg/ml	
R2	SS41-Ru	SS41-Ru	
Average negative sera	2,163	1,605	
sample	counts	counts	Δ signal
Trina 10/2008-244	1,705	1,598	107
Trina 10/2008-245	1,581	1,540	41
Trina 10/2008-246	1,767	1,563	204
Trina 10/2008-247	1,636	1,626	10
Trina 10/2008-248	3,541	1,678	1,863
Trina 10/2008-249	1,615	1,612	3
Trina 10/2008-250	1,644	1,580	64
Trina 10/2008-251	1,593	1,577	16
Trina 10/2008-252	1,581	1,613	32
Trina 10/2008-253	1,594	1,609	15
Trina 10/2008-254	1,573	1,603	30
Trina 10/2008-255	8,195	1,596	6,599
Trina 10/2008-257	2,295	1,885	410
Trina 10/2008-258	1,686	1,699	13
Trina 10/2008-259	1,621	1,585	36
Trina 10/2008-261	2,155	1,593	562

Figure 2/11

Table 1b

Run	**V1**	**V2**	
R1	FF41-Bi	FF41-Bi	
SS-Helix (GDA,P) in R1	without anti-interference additive	25µg/ml	
R2	SS41-Ru	SS41-Ru	
Average negative sera	2,163	1,605	
sample	counts	counts	Δ signal
Trina 10/2008-262	1,763	1,740	23
Trina 10/2008-264	1,685	1,631	54
Trina 10/2008-265	1,605	1,529	76
Trina 10/2008-266	1,837	1,505	332
Trina 10/2008-268	4,608	1,846	2,762
Trina 10/2008-269	1,646	1,637	9
Trina 10/2008-270	1,609	1,562	47
Trina 10/2008-272	1,883	1,496	387
Trina 10/2008-273	1,858	1,551	307
Trina 10/2008-274	1,611	1,578	33
Trina 10/2008-275	1,581	1,560	21
Trina 10/2008-276	1,848	1,582	266
Trina 10/2008-277	3,969	1,438	2,531
Trina 10/2008-279	1,609	1,546	63

Figure 3/11

Table 1c

Run	**V1**	**V2**	
R1	FF41-Bi	FF41-Bi	
SS-Helix (GDA,P) in R1	without anti-interference additive	25µg/ml	
R2	SS41-Ru	SS41-Ru	
Average negative sera	2,163	1,605	
Sample	counts	counts	Δ signal
Trina 10/2008 16097448	46,498	1,549	44,949
Trina 10/2008-752	42,475	1,603	40,872
Trina 10/2008-430	36,659	1,527	35,132
Trina 10/2008-34103923	15,098	1,495	13,603
Trina 10/2008-34045926	20,015	1,558	18,457
Trina 10/2008-16097705	50,728	2,489	48,239
HIV-1 B ESS	136,466	136,475	9
HIV-1 C ESS	24,933	25,071	138

Figure 4/11

Table 2a

	V0	V1		V2		V3	
	without anti-interference additive	*Ec*SlyD-*Ec*SlyD-Helix (GDA,P) chem. SlyD polymer		*Ec*Skp-*Ec*SlyD		*Ec*Skp-*Ec*SlyD-*Ec*SlyD rec. SlyD polymer	
Lot no.	-	14818100		DA120711		DA191211	
conc. [µg/ml]	-	5	25	5	25	5	25
sample	counts	counts	counts	counts	counts	counts	counts
HIV neg.	553	505	516	542	532	667	536
HIV neg.	534	511	532	527	522	670	518
HIV neg.	697	503	516	611	548	508	528
HIV neg.	764	512	519	697	635	523	515
HIV neg.	835	837	859	829	829	977	827
HIV 1 A	107,331	95,768	97,576	104,272	102,956	95,377	101,216
HIV 1 B	181,576	167,163	166,689	177,264	181,656	169,119	178,083
HIV 1 C	59,673	58,768	58,579	60,088	62,915	59,694	62,148
HIV 1 E	94,352	92,411	94,825	94,634	99,620	94,221	98,174
HIV 1 F	95,476	89,220	90,910	92,884	97,817	90,099	97,244
Trina 16097448	59,827	1,971	548	28,729	10,594	1,676	621
Trina 47101943	53,491	1,915	647	26,812	9,960	1,700	732

Figure 5/11

Table 2b

	V0	V4		V5	
	without anti-interference additive	*Ec*SlyD		*Ec*Skp	
Lot no.	-	DA240210		DA240210	
conc. [µg/ml]	-	5	25	5	25
Sample	counts	counts	counts	counts	counts
HIV neg.	553	548	632	533	654
HIV neg.	534	520	517	673	494
HIV neg.	697	707	695	668	673
HIV neg.	764	745	745	748	724
HIV neg.	835	954	837	779	727
HIV 1 A	107,331	105,073	108,133	105,907	98,209
HIV 1 B	181,576	172,663	178,522	184,720	179,742
HIV 1 C	59,673	58,856	59,836	62,420	65,924
HIV 1 E	94,352	92,870	94,295	98,564	97,617
HIV 1 F	95,476	93,059	94,117	100,071	102,886
Trina 16097448	59,827	57,754	59,184	60,332	58,333
Trina 47101943	53,491	52,086	52,559	53,131	52,081

Figure 6/11

Table 2c

	V0	V6	V7		V8	
	without anti-interference additive	*Ec*SlyD-*Ec*SlyD-*Ec*SlyD-*Ec*SlyD-*Ec*SlyD	*Ec*SlyD-*Ec*SlyD-*Ec*SlyD		*Ec*SlyD-*Ec*SlyD	
Lot no.	-	LT050204	LT090511		DA170112	
conc. [µg/ml]	-	5	5	25	5	25
Sample	counts	counts	counts	counts	counts	counts
HIV neg.	553	541	539	546	558	621
HIV neg.	534	518	521	538	527	552
HIV neg.	697	539	662	582	690	690
HIV neg.	764	574	711	619	829	819
HIV neg.	835	885	860	844	884	837
HIV 1 A	107,331	96,510	103,838	99,776	106,706	106,344
HIV 1 B	181,576	169,248	175,285	172,557	177,242	177,123
HIV 1 C	59,673	59,550	59,874	59,306	59,398	59,301
HIV 1 E	94,352	93,694	94,641	95,656	93,600	93,815
HIV 1 F	95,476	91,795	94,816	91,521	94,559	94,463
Trina 16097448	59,827	1,660	21,260	4,208	57,174	55,254
Trina 47101943	53,491	1,679	19,084	3,858	51,039	50,833

Figure 7/11

Table 3

		V0	V1	V2	
		without anti-interference additive	rec. *Ec*SlyD-*Ec*SlyD-Helix (GDA,P)	*Ec*Skp-*Ec*SlyD-*Ec*SlyD	
Lot no.			30117200	DA140711	
conc. [µg/ml]		---	20	5	15
sample		counts	counts	counts	counts
HIV neg.	Pr163	522	526	503	515
HIV neg.	Pr4	526	517	498	507
HIV neg.	Pr104	524	512	495	514
HIV neg.	C131652	505	501	486	502
HIV neg.	C131800	535	522	508	514
HIV 1 pos.	0060.0003.01	117,744	121,433	131,903	122,261
HIV 1 pos.	0060.0004.01	125,053	127,248	138,328	127,029
HIV 1 pos.	0060.0005.01	18,510	18,879	20,294	18,751
HIV 1 pos.	0060.0006.01	11,473	11,861	12,818	11,794
HIV 1 pos.	0060.0007.01	49,192	51,083	55,537	50,815
Interf.	C133202	10,351	8,042	739	566
Interf.	Pr149	1,437	903	536	537
Interf.	C133111	778	772	486	507

Figure 8/11

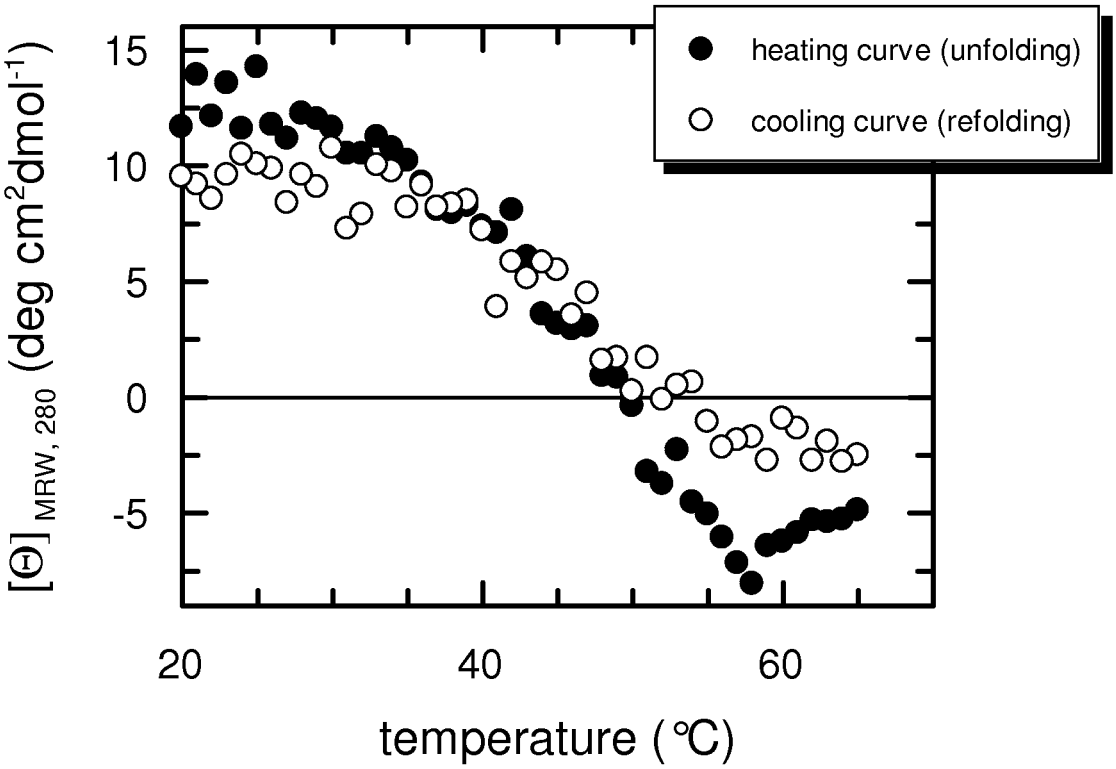

heating curve (unfolding)
cooling curve (refolding)
15
10
5
0
-5
20
40
60
temperature (°C)
[Θ]MRW, 280 (deg cm2dmol-1)

Figure 9/11

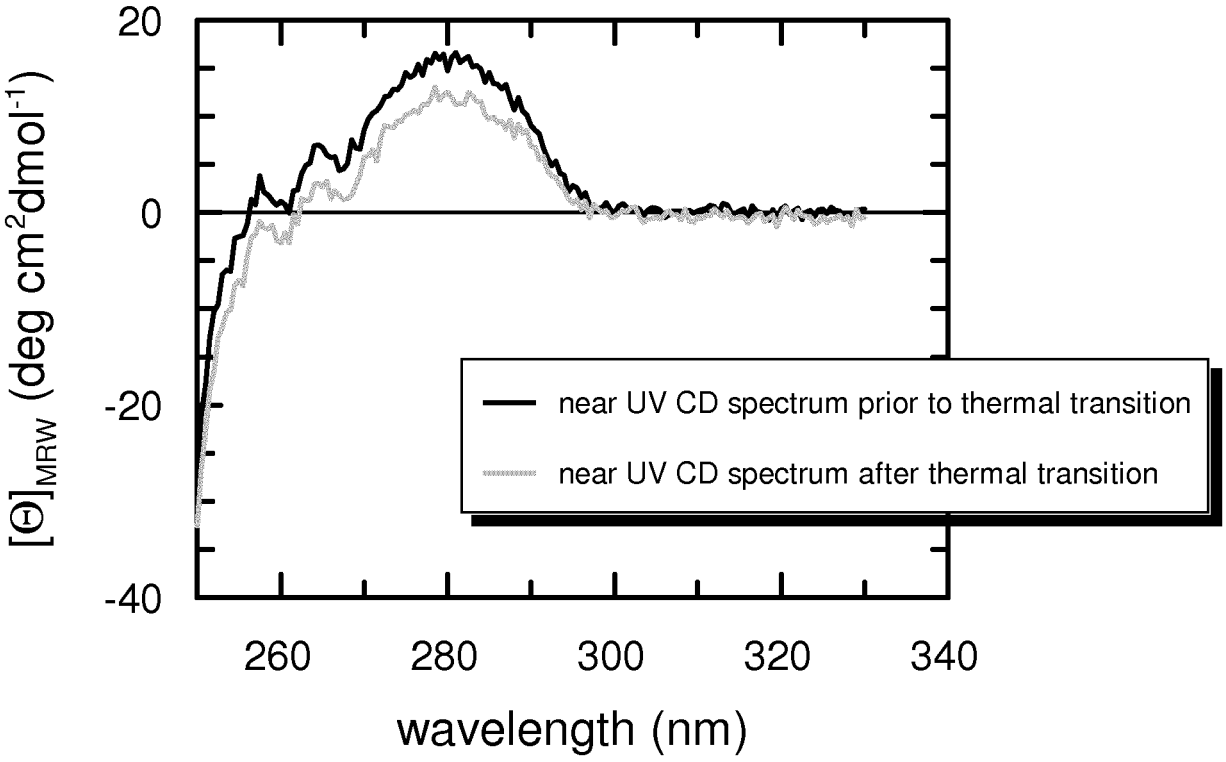
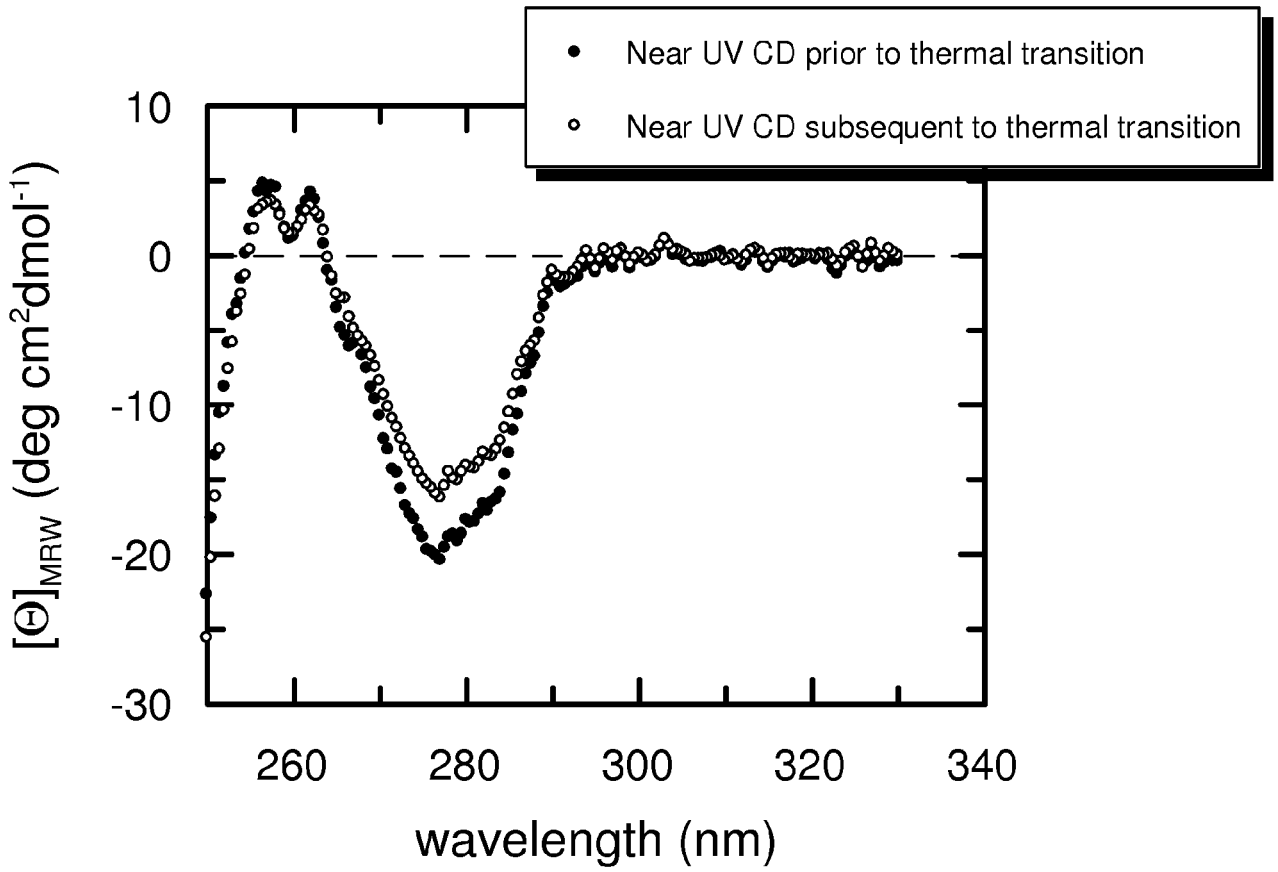

20
0
-20
-40
260
280
300
320
340
wavelength (nm)
[Θ]MRW (deg cm2dmol-1)
near UV CD spectrum prior to thermal transition
near UV CD spectrum after thermal transition

Figure 10/11

Table 4

Device	e411#837-10	e411#837-10	e411#837-10	e411#837-10	e411#837-10
Run	**V1**	**V2**	**V3**	**V4**	**V5**
R1	*Pm*SlyD-mgG2-Bi	*Pm*SlyD-mgG2-Bi	*Pm*SlyD-mgG2-Bi	*Pm*SlyD-mgG2-Bi	*Pm*SlyD-mgG2-Bi
Anti-interference additive in R1	without anti-interference additive	*Ec*SlpA-*Ec*SlpA (GDA,P) 10 µg/ml	*Ec*Skp-*Ec*SlpA-*Ec*SlpA 10 µg/ml	*Ec*Skp 10µg/ml	*Ec*SlpA 10 µg/ml
R2	*Ec*SlpA-mgG2-Ru	*Ec*SlpA-mgG2-Ru	*Ec*SlpA-mgG2-Ru	*Ec*SlpA-mgG2-Ru	*Ec*SlpA-mgG2-Ru
Anti-interference additive in R2	without anti-interference additive	*Ec*SlpA-*Ec*SlpA (GDA,P) 10 µg/ml	*Ec*Skp-*Ec*SlpA-*Ec*SlpA 10 µg/ml	*Ec*Skp 10µg/ml	*Ec*SlpA 10 µg/ml
	counts	counts	counts	counts	counts
anti-HSV-2 negative sera					
sample001	1,534	1,298	1,280	1,539	1,544
sample002	1,270	1,155	1,136	1,286	1,254
sample003	617	616	607	629	597
sample004	709	700	688	712	728
anti-HSV-2 positive sera					
sample005	60,247	60,039	60,339	59,739	59,797
sample006	16,013	15,811	15,870	15,739	15,559
sample007	34,176	33,570	33,605	33,762	33,729
sample008	207,083	207,167	207,902	206,063	206,774
Sample009	11,365	11,345	11,072	11,218	11,367
interference sera					
sample010	15,889	5,790	4,763	16,288	12,953
sample011	8,204	2,870	1,021	6,222	969
sample012	42,168	864	856	38,631	14,801
sample013	19,667	1,348	896	19,595	21,361

Figure 11/11


Near UV CD prior to thermal transition
Near UV CD subsequent to thermal transition
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0
-10
-20
-30
260
280
300
320
340
wavelength (nm)
[Θ]MRW (deg cm2dmol-1)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068269

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/62 C12N9/90
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	page 27, right-hand column, paragraph 2 figures 1, 8	4,5

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2013	02/12/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Niebuhr-Ebel, K

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068269

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 127 678 A1 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]) 2 December 2009 (2009-12-02)	1-3,6-11
A	page 10, paragraph 47 page 11, paragraph 54 page 15, paragraph 85 - page 16, paragraph 90 example 1 claim 15 sequence 4	4,5

A	WO 03/000878 A2 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]) 3 January 2003 (2003-01-03) cited in the application page 3, line 8 - line 13 page 21, line 27 - page 22, line 13; example 3	1-11

A	SCHOLZ C ET AL: "Functional Solubilization of Aggregation-prone HIV Envelope Proteins by Covalent Fusion with Chaperone Modules", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 345, no. 5, 4 February 2005 (2005-02-04), pages 1229-1241, XP004731715, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2004.10.091 abstract page 1230, right-hand column, paragraph 1 page 1231, right-hand column, paragraph 2; figure 3 page 1232, right-hand column, last paragraph page 1233, right-hand column, last paragraph - page 1234, right-hand column, paragraph 2	1-11

A	KNAPPE ET AL: "Insertion of a Chaperone Domain Converts FKBP12 into a Powerful Catalyst of Protein Folding", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 368, no. 5, 26 April 2007 (2007-04-26), pages 1458-1468, XP022046689, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2007.02.097 abstract page 1459; figure 1 page 1463, left-hand column, last paragraph - right-hand column, paragraph 1	1-11

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/068269

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	E. M. QUISTGAARD ET AL: "High-resolution insights into binding of unfolded polypeptides by the PPIase chaperone SlpA", THE FASEB JOURNAL, vol. 26, no. 10, 26 June 2012 (2012-06-26), pages 4003-4013, XP055089587, Epub ahead of print ISSN: 0892-6638, DOI: 10.1096/fj.12-208397 the whole document -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/068269

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