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(54) **RECOMBINANT BIFUNCTIONAL FUSION PROTEINS FOR THE CLEARANCE OF VARIABLE VIRUSES**

REKOMBINANTE BIFUNKTIONALE FUSIONSPROTEINE ZUR ABTREIBUNG VARIABLER VIREN

PROTEINES DE FUSION RECOMBINANTES BIFONCTIONNELLES POUR L'ELIMINATION DE VIRUS VARIABLES

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(56) References cited:
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- **LECOUTURIER V. ET AL.: "Identification of two amino acids in the hemagglutinin gkycoprotein of measles virus (MS) that govern hemadsorption, HeLa cell fusion and CD46 downregulation: phenotypic markers that differentiate vaccine and wild type MV strains." J. VIROL., vol. 70, July 1996 (1996-07), pages 4200-4204, XP002159451 cited in the application**

Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

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Description

Field of the Invention:

[0001] This invention relates to novel bifunctional recombinant fusion proteins, for treating infections with genetically variable viruses against which it is difficult to develop vaccines.

Background of the Invention:

[0002] The principle of vaccination has been known since the 18th century in the form of empirical treatment against smallpox. The first scientific vaccine was developed by Pasteur (rabies). This was a first generation vaccine, as was the smallpox vaccine, using live animals for production . Second generation vaccines are produced in eggs (Influenza, Yellow fever) and third generation vaccines are produced in cell culture (Polio, Measles, Rubella, Mumps, Tickborne encephalitis). Fourth generation vaccines are produced in various expression systems by recombinant DNA technology and are represented by hepatitis B virus surface antigen (HBsAg).

[0003] A vaccine can consist of the whole microorganism (bacteria, virus, parasite etc.) or its part (subunit vaccine). In the former case the microorganism is either inactivated (killed) or attenuated. In addition, as mentioned above, recombinant antigens or synthetic immunogenic peptides have been used recently and DNA vaccines have been developed relying on the host cell to produce the desired antigen(s).

[0004] The primary purpose of vaccination is and always has been prophylactic - prevention of particular disease.

[0005] Nevertheless, even relatively speedy development of vaccines against some life-threatening diseases may be too late for people already infected. The number of people infected worldwide with three of the most common human viruses - hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) represents up to 10 % of human population when the latest figures of 300-400 million for HBV, more than 60 million for HCV and more than 30 million for HIV are combined. There is thus a clear need for therapeutics and one of the options is development of therapeutic vaccines.

[0006] Vaccine development is expensive and the cost of developing a vaccine is between $US 50 million and 200 million. Much of the cost reflects efforts to make sure that a variety of antigenic variants of the particular infectious agent are disarmed by the vaccine. This is difficult with moderately genetically divergent microorganisms but it is almost impossible with viruses having antigens as variable as the surface glycoproteins of HIV, HCV or influenza. On the other hand, there are highly successful vaccines with a proven record of efficacy and safety, such as polio and measles, mumps and rubella (MMR). The main difference between HCV, HIV and influenza on the one hand and polio, measles, mumps and rubella on the other hand is that members of the latter group against which there are successful vaccines are genetically much more stable than the former group.

[0007] Influenza vaccination is targeted each season at particular variants which are predicted to appear based on epidemiological studies. Experimental HIV vaccines are based on various constructs of envelope protein(s) originating from one or several strains. However, it is still unlikely that this approach will be effective for the entire spectrum or at least a majority of worldwide field isolates. There is no vaccine in trials for HCV yet.

[0008] In contrast, as mentioned earlier viruses such as measles are genetically more stable. Vaccine strains induce broadly cross-reactive antibodies. Measles hemagglutinnin (MeaH) is a major target of these antibodies. It is a glycoprotein as is the second surface protein -fusion protein (F). Both of them are required for a fusion of cell membranes, but the sequence of events starts with MeaH binding to the cell receptor, thought to be CD46. MeaH is a membrane anchored protein with aminoacids 1 to 34 proposed to form a cytoplasmic domain, while 35 to 58 comprise a transmembrane domain (see Figure 1). Residues 59 to 181 are thought to form a stalk, part of which (135 to 181) probably forms a hinge of a molecule [Sato et al., J. Virol. 69, 513-516 (1995)1. Spikes of MeaH on virion surface consist of tetramers (dimers of disulfide bridge-linked homodimers). Cysteines 139 and 154 were suggested to participate in intermolecular disulfide bonding between monomeric MeaH glycoproteins. Soluble forms resulting from endoproteinase digestion of measles virus particles all reacted with monoclonal antibodies suggesting the preservation of antigenicity/reactivity [Sato et al., J. Virol. 69, 513-516 (1995)]. MeaH domain required for hemadsorption and hemagglutination activities was mapped between residues 451 and 505 [Hummel & Bellini, J. Virol. 69, 1913-1916 (1995)]. In addition to hemadsorption, the mutagenesis Val451Glu and Tyr481Asn also abrogated CD46 downregulation and HeLa cell fusion [Lecouturier et al., J. Virol. 70, 4200-4204 (1996)]. A novel site required for CD46 interaction was mapped between 473 and 477 [Patterson et al., Virology 256, 142-151 (1999)]. Additional neutralizing epitope NE244-250, located next to CD46 down-regulating aminoacid Arg 243, may be involved in CD46 binding [Fournier et al., J.Gen. virol. 78, 1295-1302 (1997)].

[0009] It is an object of the present invention to provide suitable construct for the therapy of people infected with genetically variable viruses.

Summary of the Invention

[0010] Successful vaccine formulations are generally directed against surface proteins, and result in the production of neutralizing antibodies. Where the surface proteins are variable, as is the case in genetically variable viruses like HIV-1, this is an arduous process. The present invention circumvents the problem by employing an entity which, whilst not neutralizing the virus, is able to bind to surface proteins which are conserved between viral strains and in so doing is able to mediate clearance of the virus.

[0011] Thus, the present invention provides a recombinant bifunctional fusion protein which comprises:

- a first component which is a measles virus protein modified so that it does not bind to CD46 receptor or cause hemadsorption or hemagglutination, but retains its antigenicity and is recognised by anti-measles antibodies; wherein the first component is the ectodomain of measles virus hemagglutin protein (MeaH) which has been modified by removal of between 58 and 100 N-terminal aminoacids; by mutagenesis of amino acids 243, 451 and 481; and by the introduction of deletions in the amino acid regions 244-250 and 473-477; and
- a second component fused to the first component and which is capable of binding to genetically variable viruses.

[0012] On the one hand, the second component recognizes and binds specifically the target. Any such binding entity is suitable; it does not need to have a neutralizing effect. Particularly useful are such entities that are capable of binding to peptide sequences in the surface structure of the variable virus, such as envelope glycoproteins, that are conserved among viral strains, including the notoriously variable HIV and HCV viruses. Thus, the fusion protein of the invention is able to bind variable viruses.

[0013] On the other hand, the first component is recognized by anti-measles antibodies, which are present in the general population as a result of mass vaccination programs. Specifically, the first component is the ectodomain of measles virus hemagglutinin protein (MeaH) which has been engineered in such way as not to be toxic: it cannot bind to the CD46 receptor or cause hemadsorption or hemagglutination. The lack of toxicity is achieved by removing between 58 and 100 N-terminal aminoacids of MeaH, mutating amino acids 243, 451 and 481; and introducing deletions in the amino acid regions 244-250 and 473-477 of MeaH. In spite of the lack of toxicity, this variant MeaH retains its antigenicity and is recognised and bound by the prevalent anti-measles antibodies and memory cells resulting from previous measles infections or vaccinations. Thus, the fusion protein of the invention is able to bind anti-measles antibodies present in the population, including patients infected with variable viruses.

[0014] As a result, these bifunctional fusion protein are capable of recalling the existing anti-measles immunity in a patient, and at the same time they can bind a viral target, such as HIV or HCV. The anti-measles immune reaction leads to the clearance of the complex, which now includes a variable virus. Thus, the bifunctional proteins are useful to treat patients infected with such virus.

[0015] A further aspect of the invention relates to polynucleotides (particularly DNA = encoding the recombinant fusion protein of the invention. The invention also relates to pharmaceutical compositions comprising the fusion protein, together with a pharmaceutically acceptable carrier.

Description of the Invention

[0016] The present invention relates to a novel therapeutical approach using the existing humoral and/or cellular immunity against measles viruses in order to clear genetically variable viruses like HIV and HCV. For this purpose, the invention provides a bifunctional fusion protein consisting of an antigen against which the treated individual has already developed antibodies (here, MeaH) and of a retargeting moiety that mediates the binding of the complex to the target variable virus.

[0017] For this purpose, MeaH must be modified in several ways in order to suit optimally the proposed approach.

[0018] Most functions of MeaH carried out in the normal replication cycle were eliminated for the purpose of the present invention. The requirements for MeaH as a booster / carrier antigen are as follows:

1) preserved immunogenicity/reactivity with existing antibodies and preserved recognition by memory cells

2) Solubility/absence of membrane anchoring

3) Providing a linker / hinge between two unrelated parts of a new fusion molecule

4) Absence of CD46 binding

5) Absence of erythrocyte binding/agglutination

[0019] For the purpose of introducing these changes, constructs of the MeaH gene of the Measles vaccine strain lacking between 58 and 100 N-terminal aminoacids were amplified by PCR and cloned (Figure 1). These clones were further modified by site directed mutagenesis of codons for aminoacids 451 and 481. as well as 243. In addition, small deletions were introduced in the regions 244-250 and 450-505 (particularly 473-477). The selection criteria were: lack of binding to CD46, lack of hemadsorption and hemagglutination activities. At the same time the successful constructs retain their antigenicity/ability to be recognised by antibodies from vaccinated individuals.

[0020] As second component, several candidate molecules retargeting the complex to variable viral targets, namely hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are presented. In order to avoid unwanted immune reaction to this portion of the complex molecule, only retargeting proteins of human origin are considered for human therapy. Candidate molecules are single chain antibodies (scFv) which can be selected from large phage display libraries. Monoclonal antibodies may also be used. For HCV it is known that at least a subpopulation of the virus in the bloodstream is associated with low density lipoprotein (LDL) fraction, most probably through binding between the HCV El glycoprotein and apolipoprotein B (apoB) of LDL. ApoB has therefore been subjected to chemical fragmentation and relevant fragments binding to the HCV virion and / or HCV E1 glycoprotein were determined. These were fused at gene level with MeaH, modified as described above.

[0021] Similarly, candidate molecules or their parts for HIV binding were studied. For this purpose, expression libraries prepared from sources which mostly do not come into contact with the virus were investigated to discover previously unknown proteins capable of binding the accessible structures of the HIV virion. Several binding proteins were identified in a library from human brain and some of them sequenced. Two clones with relatively high binding activity were:

1) a clone with coding sequence for human creatine kinase B .
2) a clone for an unknown human protein, partial sequence of which reads as follows.

5'-CACGCGTCCGCTGAAGAAGAAATTCAGGAAATCTGCTTAAAGATCTT
GCAGCTTTATGCTCGGAAAAAGGTTGATCTCACACACCTGGAGGGTGA
AGTGGAAAAAGAAAGCACGCTATCGAAGAGGCAAAGGCCCAAGCCC
GGGGCCTGTTGCCTGGGGGCACACAGGTGCTGGATGGTACCTCGGG
GTTCTCTCCTGCCCCCAAGCTGGTGGAATCCCCCAAAGAAGGTAAAGG
GAGCAAGCCTTCCCCACTGTCTGTGAAGAACACCAAGAGGAGGCTGG
AGGGCGCCAAGAAAGCCAAGGCGGACAGCCCCGTGAACGGCTTGCC
AAAGGGGCGAGAGAGTCGGAGTCGGAGCCGGAGCCGTGAGCAGA-3'

Variants of these clones differing by no more than 5% of amino acid positions and still binding to HIV envelope proetin would also be suitable.

[0022] Using methods as described for apolipoprotein B above, the fragments of these proteins mediating binding to HIV virion and/or env protein, were fused with constructs of MeaH, modified as described earlier.

[0023] The principal idea of this invention is to use immunological memory existing within a majority of the population against a genetically stable antigen as a result of a natural infection or, preferably, vaccination (which has the added advantage of available records and standard methodology), and to redirect it towards infectious or other agents, against which it is difficult to prepare vaccines because of genetic variability or other reasons. Measles hemagglutinin (MeaH) has been chosen for several reasons:

1) Vaccination against measles has proven successful over a long period of time and measles is one of the future candidates for global eradication. Thus, there is a high vaccination coverage also in most developing countries.

2) Most of the protective activity against the measles virus (MV) has been shown to be directed against hemagglutinin.

3) There is sufficient structural and functional data available on the hemagglutinin.

[0024] This type of retargeting may be very widely applicable depending on the availability of binding/retargeting molecule or motif and may include apart from infectious agents also cancer cells. However, in the present invention the

emphasis is on variable human viruses, namely HIV and HCV. Especially the HIV prevalence figures in some African and Asian regions are critical and a swift novel therapeutic approaches are needed.

[0025] It is well known that patients with AIDS suffer from dysfunction of their immune system. Questions can therefore be asked what are the levels of antibodies against former vaccination antigens, such as Measles, and how is the immunological memory affected in these patients. Surprisingly, waning measles immunity was not greatly accelerated in HIV-infected adults despite progressive HIV-related immunodeficiency [Zolopa et al., Clinical Infectious Diseases 18, 636-638 (1994)]. Levels of measles antibody remained stable in both, HIV-infected progressors and non-progressors [Brostrom et al., Clinical and Experimental Immunology 106, 35-39(1996)] and 95 % of 210 HIV-positive patients had Measles antibody regardless of the CD4 counts [Wallace et al., Vaccine 12, 1222-1224 (1994)].

[0026] HCV-infected individuals are not known to have their immune system affected in this way and no limitations of the therapeutic approach described are anticipated.

Examples

[0027] The following examples are provided for purposes of illustration only and are not to be viewed as a limitation of the scope of the invention. Figure 1 attached hereto shows schematically the genetic engineering involved. Figure 2 shows binding of proteins expressed by certain cDNA clones (useful as first component) to HIV envelope glycoprotein. In the following, ul means microlitres, ' means minute and " means seconds other than in nucleotide sequences.

Example 1

Amplification and cloning of the gene for hemagglutinin of the vaccine strain of measles virus.

[0028] RNA was extracted from Edmonston strain of Measles virus using RNasol B (AMS Biotechnology) according to the manufacturer's instruction. RNA was washed with 80 % ethanol twice, air-dried and dissolved in 100 ul of DEPC-treated water.

[0029] cDNA synthesis: 30 ul of RNA was mixed with 13 ul (25 pMol/ul) of primer XhoMH /full /A [5'-ggCCTCgAgTCT-gCgATTggTTCCATCTTCCCg-3'; 33-mer], heated for 10 minutes at 70°C and cooled on ice.

[0030] Following components of the reaction mix were added: 34.5 ul water/DEPC; 4 ul 25 mM mix of dATP, dCTP, dGTP, dTTP; 5 ul 10 x Super Reverse Transcriptase (Super RT) (HT Biotechnology) buffer; 2.5 ul (100 U) Rnasin (Promega Corporation) 4 ul Super RT. Reaction mix (final volume 100 ul) was incubated for 40 minutes at 42°C.

[0031] Resulting cDNA was used to amplify two variants of MeaH: full-length (MeaHfl) and short variant, lacking 59 N-terminal aminoacids (MeaHsv). MeaHfl is designated for comparative purposes, MeaHsv is designated for further mutagenesis and and fusion with retargeting part of the bifunctional molecules.

MeaHfl	MeaHsv
5 ul cDNA	5 ul cDNA
5 ul 10 x PCR buffer II	5 ul 10 x PCR buffer II
3 ul 25 mM $MgCl_2$	3 ul 25 mM $MgCl_2$
1.6 ul 2.5 mM dNTPs	1.6 ul 2.5 mM dNTPs
1.6 ul 25 pMol/ul primer BamMH/fulUS*	1.6 ul 25 pMol/ul primer BamMHIshort/S**
1.6 ul 25 pMol/ul primer XhoMF/full/A	1.6 ul 25 pMol/ul primer XhoMF/full/A
31.7 ul water/DEPC	31.7 ul water/DEPC
0.5 ul AmpliTaq	0.5 ul AmpliTaq

*BamMH/full/S: 5'-CgCggATCCATgTCACCACAACgAgACCggATA-3'
**BamMH/short/S: 5'-CgCggATCCCTTCATCgggCAgCCATCTACACC-3'

[0032] Reaction mixtures were overlaid with 50 ul of mineral oil. AmpliTaq was added during the first denaturation step (95°C). PCR was done using Trioblock and the following programme:

1 cycle	95°C/3'and 65°C/30";
30 cycles	72°C/60"; 95°C/30"; 65°C/30";
final extension	72°C/7'.

[0033] 2.5 ul aliquots of PCR reactions were analysed by agarose gel electrophoresis. PCR reaction products were

cleaned according to manufacturer's instruction using QPCR purification spin column kit (Qiagen) and eluted into 50 ul of elution buffer.

Cloning into vector TOPO 2.1 for subcloning:

[0034] 1 ul of TOPO 2.1 DNA provided in TA cloning kit (Invitrogen) was mixed with 1.5 ul of each PCR eluate and 2.5 ul water. During 5 minute incubation at room temperature 2 ul of beta-mercaptol-ethanol was added to each aliquot of TOP 10 cells (Invitrogen). 1ul of plasmid-insert mixture was added to TOP 10 cells, incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds and cooled on ice for two minutes. 250 ul of SOC medium (Invitrogen kit) was added to each transformation and tubes placed horizontally in a shaker at 37°C for 30'. 10 and 100 ul were plated on agar plates with ampicillin (100 ug/ul) to which 40 ul of 40 mg/ml X-Gal was added some 30 minutes earlier.
5 white colonies of each construct were grown overnight in TYE medium containing 100 ug/ml ampicillin and plasmid DNA extracted using plasmid mini Prep (Quiagen). Presence of the inserts of correct size was checked after simultaneous digestion of plasmid DNAs with BamHI and XhoI. Restriction endonucleases and reaction components (buffer 2; BSA) were from New England Biolabs - NEB. Reactions were incubated 1 hour at 37°C and 25 ul of each reaction run on the agarose gel and inserts cleaned by Qiaquick gel purification kit (Qiagen) according to the manufacturer's instruction.

Cloning into plasmid cDNA4/HisMax A, B and C for mutagenesis and expression.

[0035] Three variants of pcDNA4/HisMax (A, B and C; Invitrogen) are used to ensure in-frame cloning of the inserts. The plasmid is designed for overproduction of recombinant proteins in mammalian cell lines.

[0036] DNAs of A, B and C variants of the plasmid were digested with BamHI and XhoI and gel-purified as described above for inserts. Purified plasmids and inserts were ligated together in standard ligation reaction and TOP 10 cell were transformed as described above.
White colonies were grown as described above and in-frame inserts checked by sequencing.

Example 2

In vitro mutagenesis of MeaHfl and MeaHsv.

[0037] Main targets for mutagenesis were :

1. Site-directed mutagenesis of codon for aminoacid 243.
2. Site-directed mutagenesis of codon for aminoacid 451.
3. Site-directed mutagenesis of codon for aminoacid 481
4. Short deletions in the regions between aminoacids 244-250.
5. Short deletions in the regions between aminoacids 450-505 (i.e. 473-477).

[0038] Mutagenesis is carried out using QuickChange Site-Directed Mutagenesis Kit (Stratagene). The advantage is that plasmids with cloned sequences of interest are mutated directly, without the need for further subcloning. Two complementary primers are needed for each mutation, where mutated nucleotide(s) or insertion/deletion should be positioned in the middle of the primers. The procedure envolves digestion of original (nonmutated) DNA strands with DpnI. Procedures were carried out as described in the instruction manual.

[0039] Examples of primers used for mutagenesis:

1 Arginine 243:

Sense (28-mer): 5'-CTgAgCAgCAAAgCgTCAgAgTTgTCAC-3'
Antisense (28-mer): 5'-gTgACAACTCTgACgCTTTgCTgCTCAg-3'
Arginine 243 is changed to Alanine in this case.

2 Valine 451:

Sense (32-mer): 5'-CCAACCACAACAATgACTATTggCTgACTATC-3'
Antisense (32-mer): 5'-gATAgTCAgCCAATAgTCATTgTTgTggTTgg-3'
Valine 451 was changed to Aspartic acid in this case.

3 Tyrosine 481:

Sense (30-mer):5'-CAAggTTAgTCCCCAgCTCTTCAATgTCCC-3'

Antisense (30-mer): 5'-gggACATTgAAgAgCTggggACTAACCTTg-3'

Tyrosine 481 was changed to Glutamine in this case.

4 Region 244-250: 6 nucleotide (2 aminoacid) deletion of codons for Leu247-Ser248

Sense (38-mer): 5'-gAgCAgCAAAAggTCAgAgCAACTgAgCATgTACCgAg-3'

Antisense (38-mer): 5'-CTCggTACATgCTCAgTTgCTCTgACCTTTTgCTgCTC-3'

5 Region 451-505: 3 nucleotide (1 aminoacid) deletion of codon for Arg475

Sense (32-mer): 5'-CATTggAgTggATACCgTTCAAggTTAgTCCC-3'

Antisense (32-mer): 5'-gggACTAACCTTgAACggTATCCACTCCAATg-3'

[0040] In all 5 cases the example of the reaction was as follows (components of the system from the kit):

5 ul of 10 x reaction buffer
5 - 50 ng of dS DNA template (starting with pcDNA4/HisMax/MeaHfl or pcDNA4/HisMax/MeaHsv)
125 ng of primer 1
125 ng of primer 2 (complementary)
1 ul of dNTP mix
ddH_2O to 50 ul
1 ul (2.5 U) of PfuDNA polymerase

[0041] Cycling parameters:

1 cycle 95°C for 30"
12 cycles (for point mutation)
16 cycles (for single aminoacid change) 95°C/30"; 55°C/1'; 68°C/14'
18 cycles (for multiple aminoacid deletions or insertions) (2' per kb of plasmid)

[0042] After cycling the reactions were chilled on ice for 2'. 1 ul of DpnI (10 U) was added, reaction mixture was mixed, spun down shortly in microcentrifuge and incubated at 37°C for 1 hour (removal of nonmutated DNA).

[0043] 1 ul of each resulting mutated plasmid DNA was transformed into Epicurian Coli XLI-Blue supercompetent cells using standard procedure, as described in Example 1, except that the heat shock at 42°C was for 45" when using Falcon 2059 polypropylene tubes. NZY+ broth (0.5 ml) was used to incubate transformation reaction at 37°C for 1 hour with shaking at 225 -250 rpm and spread on LB-ampicillin plates to which 20 ul of 10 % (w/v) X-gal and 20 ul of 100 mM IPTG were added in advance. Colonies appear after 16 hours at 37°C.

[0044] pcDNA4/HisMax/MeaHfl and pcDNA4/hisMax/MeaHsv were mutagenised in parallel for comparative purposes, in stepwise manner: the product of mutagenesis reaction 1 (Arg 243 change) was used as template for the mutagenesis step 2 (Valine 451 change) after confirmation of mutated site by sequencing. Thus the final products of the site-directed mutagenesis contain all 5 types of mutations: Arg243; Va1451; Tyr481; short deletions in region 244-250 and 473-477.

[0045] Variants of Measles hemagglutinin expressed by these mutagenised plasmids are investigated for loss of hemadsorption and cell receptor binding. Importantly, they should retain the ability to be recognised by antibodies from previously vaccinated or naturally infected individuals. Those satisfying these criteria are used for in-frame fusion with the retargeting component of the final fusion protein. Fusion is mediated through the amplification of the retargeting component using specific primers containing recognition site for restriction endonuclease BamHI. Orientation of the retargeting component must be checked so that the C-terminus of retargeting component is fused to N-terminus of mutagenised MeaHsv thus replacing the original 58 N-terminal aminoacids of MeaH. In such construction the natural hinge of the MeaH molecule (see Description) can be used to position the two parts of the fusion protein. Complete fusion proteins undergo the same set of investigations as mutagenised MeaH variants as far as the binding activities and antibody reactivities are concerned.

Example 3

Identification of proteins binding to the HIV 1 envelope(env) protein for retargeting purposes.

[0046]

a) Screening human expression cDNA library with biotinylated recombinant env
b) Confirmation of binding in Western blot
c) Identification of selected cDNA clones by sequencing

a) Screening human expression cDNA library with biotinylated recombinant env

[0047] Biotinylation: Recombinant HIV1 gpl20 has been dissolved in phosphate buffered saline (PBS) at 0.5 ug/ul and biotinylated using biotinylation kit (Boehringer, Cat. No1418 165) according to manufacturer's instruction. Briefly, the column was fixed and 5 ml of blocking solution added, then washed with 6 x 5 ml PBS.

env: Dissolved in 500 ul PBS
475 ul taken for labeling
Add 17.5 ul PBS and 7.5 ul 20 mg/ml biotin-7-NHS in DMSO while stirring
Place in a tube
Incubate 2 hrs/rt/rotating wheel
Remove stopper and cap from prepared column
Add 500 ul PBS to adjust volume to 1 ml, let flow through
Add another 1.5 ml PBS, let flow through
Add 3.5 ml PBS and collect 10 drops (approximately 0.5 ml)
Protein expected in first 4 tubes - run 7.5 ul on the gel
After protein assay selected fractions were pooled Screening human expression cDNA library.

[0048] cDNA library was prepared by cloning cDNA from human brain into expression vector. The library was grown on agar plates at high density and transferred to nylon filters and lysed and fixed using standard techniques.
Filter screening:

1) 20 minute incubation in 200ml absolute ethanol.
2) 1 wash for 5 minutes in 1 litre PBS-T-T (PBS-Tween20).
3) 2 rinses each in 1 litre PBS and a 3rd 5 minute wash in I litre of PBS.
4) 45 minute wash in 3 % Marvel-PBS.
5) 1 hour incubation in biotinylated env/ 3 % Marvel-PBS
6) 1 wash for 5 minutes in 1 litre PBS-T-T.
7) 2 rinses each in 1 litre PBS and a 3rd 5 minute wash in 1 litre of PBS.
8) 20 ml 1 x PBS 3% Marvel
9) 40 minute incubation in 1 in 5000 dilution of streptavidine-horse radish peroxidase (HRP) in 3 % Marvel-PBS. 30 ul streptavidine-HRP in 150 ml 3 % Marvel-PBS.
10) 2 washes for 5 minutes each in 1 litre PBS-T-T.
11)2 washes for 5 minutes each in 1 litre PBS.
12) Develop using ECL reagents (Amersham).

b) Confirmation of binding in Western blot (Figure 2).

[0049] 11 positive colonies identified were grown from master plates in liquid overnight cultures.

Extracts prepared from 20 ml of induced cultures of clones 1-11 using 4 x lysis buffer.
After 3 hr induction cells spun at 4000 rpm for 15'.
Pellet resuspended in 600 ul water.
52 ul of 1 M DTT and 220 ul 4 x lysis buffer (0.2 x PBS, 8 % SDS) added
Incubation at 37°C occasional Vortex
Because of cloudy appearance volume raised to 24 ml
Centrifuged 4000 rpm/15'. Pellet discarded
2 x 12 ml spun in Centriprep 10 at 4000rpm/40'/25°C

Retentates combined, diluted with 0.5 x PBS to 12 ml and spun again under identical conditions
Filtrate discarded and retentates spun again 10'. Final volume around 0.5 ml. 4.5 ul run on the gel and binding confirmed on Western blot with biotinylated env.

Clones 1, 2, 3, 6 and 8:	plasmid minipreps prepared from 4 ml overnight cultures, 200-500ng per sequencing reaction.
Reaction mix Forward (for 6)	24 ul seq. Buffer 24 ul terminator ready mix 1.92 ul 1:10 Forward primer (10 pMol/ul) 8.32 ul of the mix to tubes 1-5
Reaction mix Reverse (for 6)	24 ul seq. Buffer 24 ul terminator ready mix 1.92 ul 1:10 Reverse primer (10 pMol/ul) 8.32 ul of the mix to tubes 6-10

Overlaid with 40 ul oil. Amplification in 96 well plate:

25 cycles:	96°C/30" 50°C/15" 60°C/4' 4°C/hold

[0050] Spin the tubes. Prepare 1.5 ml tubes containing 2 ul 3 M sodium acetate (pH 4.6 - 5.2) and 50 ul 95 % EtOH. Transfer 20 ul into the tubes. Vortex and place on ice for at least 15mins. Spin in microcentrifuge for 15-30mins. Discard supernatant. Rinse with 250 ul 70 % Ethanol. Air dry the pellet. Resuspend in 4 ul of 50 mM EDTA (7.4-8.0) and 200 ul deionized formamide. Denature and load.

Results: Clone 1: The nucleotide sequence determined for this HIV-1 env- binding protein corresponds to that of Homo sapiens creatine kinase B (GenBank Accession X15334).
Clone 2: The nucleotide sequence determined for this HIV-1 env-binding protein corresponds to an unknown human protein. The sequence reads as shown in the Description of the Invention. There were two recent entries into GenBank (both in 2000) which contain almost identical sequences: Accession AK026796; and AK000685. Both were submitted after the submission of the original patent application.
Clone 3: The nucleotide sequence determined for this HIV-1 env-binding protein corresponds to Homo sapiens ribosomal protein L8 (RPL8; GenBank Accession NM000973).
Clone 6: The nucleotide sequence determined for this HIV-1 env-binding protein corresponds to an unknown human protein. Partial sequence reads:

5'GGAGAAGGTCTCTGAAGGAGAAAAGCAAAGAAGCTCTTTTG

GCCTCACAAAAGCCATTTAAATTTATAGCAAGGGAGGAACAG

AAGCGAGCAGCCCGGGAAAAGCAGCTGAGAGACTTTCTTAAG

TATAAAAAGAAAACAAATCGATTTAAAGCCAGACCCATTCCT

CGATCTACTTATGGTTCAACTACCAATGACAAGTTAAAAGAAG

AAGAGCTCTATCGAAACCTTAGGACACAGCTGAGAGCCCAGG

AGCATTTACAGAACTCATCTCCTCTGCCTTGTAGGTCAGCTTG

CGGATGCAGGAACCCCAGGTGTCCTGAACAGGCTGTAAAGTT

GAAGTGTAAACACAAGGTTAGGTGCCCACTCCTGATTTTGAGG

ACCTTCTGAGAGATACCAGAACCCTCTCAAACACAAGTCTTCA

AAACTCTAACAGG3'

There was a recent submission into Genebank (29 September 2000) from the Japanese NEDO human cDNA sequencing project (Accession AK023367) containing a virtually identical sequence.
Clone 8: The nucleotide sequence determined for this HIV-1 env-binding protein is practically identical to that of clone 1, for B subunit of creatine kinase Homo sapiens, brain.

Example 4

Chemical fragmentation of apolipoprotein B purified from human plasma (Europa Bioproducts Ltd)

[0051] Chemical fragmentation has been carried out using a well-known method of protein engineering. Four chemical treatments were chosen based on computer prediction for number of cuts in apolipoprotein B (ApoB) molecule.

Formic acid:	Expected: 6 cuts. Treatment of 100 ug of ApoB with 70 % formic acid in 7 M guanidinium-HCl for 24 and 48 hrs at 37°C.
Hydroxylamine:	Expected: 17 cuts. 100 ug of ApoB has been cleaved in 2 M hydroxylamine 2 M guanidine-HCl 0.2 M K_2CO_3 pH 9.0 for 4 hrs at 45°C. Reaction was terminated by adding concentrated formic acid to pH 2 -3 and desalted on Sephadex G-25. Peptides larger than 2500 (m.w.) appear in the void volume.
NTCB (2-nitro-5-thiocyanobenzoate):	Expected: 25 cuts. 100 ug of ApoB dissolved in 6 M guanidine-HCl 0.2 M tris-acetate buffer pH 8.0 Dithiothreitol (DTT) added to 10 mM to reduce disulfides. Incubation 1 -2 hrs at 37°C. NCTB added in 5-fold excess over total thiol. Incubation for 15 minutes at 37°C. Acidified to pH 4 or less, cooled to 4°C.

[0052] Recombinant envelope protein El of the Hepatitis C virus (Europa Bioproducts Ltd) has been biotinylated as described for HIV env protein in Example 3 and bound to streptavidin coated paramagnetic particles (Promega) and

washed with PBS. Chemically treated preparations of ApoB were diluted in PBS and fragments captured on particles with immobilised E1. Captured fragments were analysed by SDS-PAGE.

Example 5

Binding and antigenic properties of mutated MeaH

[0053] Selected mutant constructs of MeaH were subcloned into pSPUTK in Vitro Translation Vector (Stratagene) and expressed in vitro according to manufacturer's instruction. The products were investigated for their binding to CD46 ectodomain or CD46 expressing cells as described [Devaux et al, Journal of General Virology 77, 1477-1481 (1996)]. Hemagglutination assay using Edmonston MV and preincubation of erythrocyes with pSPUTK/MeaH products is as described by Norrby and Gollmar [Infect. Immunity 11, 231-239 (1975)].

[0054] Presence of antiMeaH antibodies in anonymous plasma/serum samples is determined using a commercial assay. Reactivity of these samples towards the ectodomain of normal and mutated MeaH, as well as fusion proteins with appropriate component 2, is determined after coating wells of Maxisorb 96 well plates with investigated in vitro translated proteins (pSPUTK). Plates are blocked with PBS-Marvel, incubated with 1:10 diluted plasma/serum samples, washed repeatedly with PBS/Tween 20, incubated with protein L-HRP (horse radish peroxidase) conjugate and washed again. Reactivity is revealed after incubation with HRP substrate (TSB). The development is stopped by adding sulphuric acid and the result obtained using ELISA reader.

Claims

1. A recombinant bifunctional fusion protein which comprises

a first component which is a measles virus protein modified so that it does not bind to CD46 receptor or cause hemadsorption or hemagglutination, but retains its antigenicity and is recognised by anti-measles antibodies; wherein the first component is the ectodomain of measles virus hemagglutin protein (MeaH) which has been modified by removal of between 58 and 100 N-terminal aminoacids; by mutagenesis of amino acids 243, 451 and 481; and by the introduction of deletions in the amino acid regions 244-250 and 473-477; and
a second component fused to the first component and which is capable of binding to genetically variable viruses.

2. A recombinant fusion protein according to any preceding claim wherein the second component is fused to the N-terminus of the first component.

3. A recombinant fusion protein according to any preceding claim wherein the second component is a protein of human origin to avoid unwanted immune reaction in humans.

4. A recombinant fusion protein according to any preceding claim capable of binding to hepatitis C virus (HCV).

5. A recombinant fusion protein according to claim 4 wherein the second component is a fragment of apolipoprotein B (apoB) which binds the HCV envelope protein E1.

6. A recombinant fusion protein according to any of claims 1 to 3 wherein the second component is capable of binding to human immunodificiency virus (HIV).

7. A recombinant fusion protein according to claim 6 wherein the second component is human creatine kinase B.

8. A recombinant fusion protein according to claim 6 which is a part of human creatine kinase B capable of binding specifically to HIV1 envelope protein gp120.

9. A recombinant fusion protein according to claim 6 which is a variant of human creatine kinase differing therefrom by not more than 5% of amino acid positions and capable of binding specifically to HIV1 envelope protein gp120.

10. A recombinant fusion protein according to claim 6 wherein the second component is a protein coded for by the following partial nucleotide sequence:

5'-CACGCGTCCGCTGAAGAAGAAATTCAGGAAATCTGCTTAAAGATCTT
GCAGCTTTATGCTCGGAAAAAGGTTGATCTCACACACCTGGAGGGTGA
AGTGGAAAAAGAAAGCACGCTATCGAAGAGGCAAAGGCCCAAGCCC
GGGGCCTGTTGCCTGGGGGCACACAGGTGCTGGATGGTACCTCGGG
GTTCTCTCCTGCCCCCAAGCTGGTGGAATCCCCCAAAGAAGGTAAAGG
GAGCAAGCCTTCCCCACTGTCTGTGAAGAACACCAAGAGGAGGCTGG
AGGGCGCCAAGAAAGCCAAGGCGGACAGCCCCGTGAACGGCTTGCC
AAAGGGGCGAGAGAGTCGGAGTCGGAGCCGGAGCCGTGAGCAGA-3'

11. A recombinant fusion protein according to claim 6 wherein the second component is a variant of the protein of claim 10 differing by not more than 5% of amino acid positions.

12. A recombinant fusion protein according to any of claims 1 to 3 wherein the second component is a human single chain antibody (scFv).

13. A recombinant fusion protein according to any of claims 1 to 3 wherein the second component is a human monoclonal antibody.

14. Polynucleotide coding for a fusion protein according to any preceding claim.

15. A pharmaceutical composition which comprises a protein of any of claims 1 to 13 together with a pharmaceutically acceptable carrier.

Patentansprüche

1. Rekombinantes, bifunktionelles Fusionsprotein, welches aufweist:

eine erste Komponente, die ein Masernvirus-Protein ist und so modifiziert ist, dass sie nicht an einem CD46-Rezeptor bindet oder Hämadsorption oder Hämagglutination hervorruft, jedoch ihre Antigenität bewahrt und von Anti-Masem-Antikörpem erkannt wird, wobei die erste Komponente die Ektodomäne des Masernvirus-Hämagglutinproteins (MeaH) ist, die durch Entfernung zwischen 58 und 100 N-terminalen Aminosäuren modifiziert worden ist: durch Mutagenese von Aminosäuren 243, 451 und 481 und durch die Einführung von Deletionen in die Aminosäure-Regionen 244-250 und 473-477: und
eine zweite Komponente, die mit der ersten Komponente fusioniert ist und die in der Lage ist, genetisch variable Viren zu binden.

2. Rekombinantes Fusionsprotein nach Anspruch 1, wobei die zweite Komponente mit dem N-Terminus der ersten Komponente fusioniert ist.

3. Rekombinantes Fusionsprotein nach einem der vorgenannten Ansprüche, wobei die zweite Komponente ein Protein humaner Herkunft ist, um bei Menschen eine unerwünschte Immunreaktion zu vermeiden.

4. Rekombinantes Fusionsprotein nach einem der vorgenannten Ansprüche, das zum Binden an Hepatitis-C-Virus (HCV) in der Lage ist.

5. Rekombinantes Fusionsprotein nach Anspruch 4, wobei die zweite Komponente ein Fragment von Apolipoprotein B (apoB) ist, welches das HCV-Hüllprotein El bindet.

6. Rekombinantes Fusionsprotein nach einem der vorgenannten Ansprüche 1 bis 3, wobei die zweite Komponente zum Binden an dem *humanen Immundefizienzvirus* (HIV) in der Lage ist.

7. Rekombinantes Fusionsprotein nach Anspruch 6, wobei die zweite Komponente Human-Creatinkinase B ist.

8. Rekombinantes Fusionsprotein nach Anspruch 6, das ein Teil der Human-Creatinkinase B ist und zum Binden speziell an HIV1-Hüllprotein gp120 in der Lage ist,

9. Rekombinantes Fusionsprotein nach Anspruch 6, das eine Variante von Human-Creatinkinase ist und sich davon um nicht mehr als 5% der Aminosäure-Positionen unterscheidet und zum Binden speziell an HIV1-Hüllprotein gp120 in der Lage ist.

10. Rekombinantes Fusionsprotein nach Anspruch 6, wobei die zweite Komponente ein durch die folgende Nucleotid-Teilsequenz codiertes Protein ist:

5'-CACGCGTCCGCTGAAGAAGAAATTCAGGAAATCTGCTTAAAGATCTT
GCAGCTTTATGCTCGGAAAAAGGTTGATCTCACACACCTGGAGGGTGA
AGTGGAAAAAGAAAGCACGCTATCGAAGAGGCAAAGGCCCAAGCCC

GGGGCCTGTTGCCTGGGGGCACACAGGTGCTGGATGGTACCTCGGG
GTTCTCTCCTGCCCCCAAGCTGGTGGAATCCCCCAAAGAAGGTAAAGG
GAGCAAGCCTTCCCCACTGTCTGTGAAGAACACCAAGAGGAGGCTGG
AGGGCGCCAAGAAAGCCAAGGCGGACAGCCCCGTGAACGGCTTGCC
AAAGGGGCGAGAGAGTCGGAGTCGGAGCCGGAGCCGTGAGCAGA-3'

11. Rekombinantes Fusionsprotein nach Anspruch 6, wobei die zweite Komponente eine Variante des Proteins nach Anspruch 10 ist und sich um nicht mehr als 5% der Aminosäure-Positionen unterscheidet.

12. Rekombinantes Fusionsprotein einem der vorgenannten Ansprüche 1 bis 3, wobei die zweite Komponente ein Human-Einkettenantikörper (scFv) ist.

13. Rekombinantes Fusionsprotein einem der vorgenannten Ansprüche 1 bis 3, wobei die zweite Komponente ein Human-monoklonaler Antikörper ist.

14. Polynucleotid, das ein Fusionsprotein nach einem der vorgenannten Ansprüche codiert.

15. Pharmazeutische Zusammensetzung, die ein Protein nach einem der vorgenannten Ansprüche 1 bis 13 zusammen mit einem pharmazeutische duldbaren Träger aufweist.

Revendications

1. Protéine de fusion bifonctionnelle recombinante, qui comprend:

un premier composant qui est une protéine du virus de la rougeole modifiée afin qu'elle ne se lie pas au récepteur CD46 ou ne cause pas d'hémadsorption ou d'hémagglutination, mais garde son antigénicité et est reconnu par des anticorps anti-rougeole; où le premier composant est l'ectodomaine de protéine d'hémagglutinine du virus de la rougeole (MeaH) qui a été modifié en enlevant entre 58 et 100 acides aminés N-terminaux; par mutagenèse des acides aminés 243, 451 et 481; et par l'introduction de délétions dans les régions d'acides aminés 244-250 et 473-477; et
un second composant fusionné au premier composant et qui est capable de se lier à des virus génétiquement variables.

2. Protéine de fusion recombinante selon la revendication 1, dans laquelle le second composant est fusionné à l'extrémité N-terminale du premier composant.

3. Protéine de fusion recombinante selon l'une quelconque des revendications précédentes, dans laquelle le second composant est une protéine d'origine humaine pour éviter une réaction immune indésirable chez des humains.

4. Protéine de fusion recombinante selon l'une quelconque des revendications précédentes, capable de se lier au virus de l'hépatite C (VHC).

5. Protéine de fusion recombinante selon la revendication 4, dans laquelle le second composant est un fragment de l'apolipoprotéine B (apoB) qui se lie à la protéine E1 de l'enveloppe de VHC.

6. Protéine de fusion recombinante selon l'une quelconque des revendications 1 à 3, dans laquelle le second composant est capable de se lier à un virus d'immunodéficience humaine (VIH).

7. Protéine de Fusion recombinante selon la revendication 6, dans laquelle le second composant est une créatine kinase B humaine.

8. Protéine de Fusion recombinante selon la revendication 6, qui est une partie de créatine kinase B humaine capable de se lier spécifiquement à gp120 protéine de l'enveloppe de VIH1.

9. Protéine de fusion recombinante selon la revendication 6, qui est un variant de créatine kinase humaine qui diffère de celle-ci par pas plus de 5 % de positions d'acides aminés et capable de se lier spécifiquement à gp120 protéine de l'enveloppe de VIH1.

10. Protéine de fusion recombinante selon la revendication 6, dans laquelle le second composant est une protéine codée par la séquence de nucléotides partielle suivante:

5'-CACGCGTCCGCTGAAGAAGAAATTCAGGAAATCTGCTTAAAGATCTT
GCAGCTTTATGCTCGGAAAAAGGTTGATCTCACACACCTGGAGGGTGA
AGTGGAAAAAGAAAGCACGCTATCGAAGAGGCAAAGGCCCAAGCCC

GGGGCCTGTTGCCTGGGGGCACACAGGTGCTGGATGGTACCTCGGG
GTTCTCTCCTGCCCCCAAGCTGGTGGAATCCCCCAAAGAAGGTAAAGG
GAGCAAGCCTTCCCCACTGTCTGTGAAGAACACCAAGAGGAGGCTGG
AGGGCGCCAAGAAAGCCAAGGCGGACAGCCCCGTGAACGGCTTGCC
AAAGGGGCGAGAGAGTCGGAGTCGGAGCCGGAGCCGTGAGCAGA-3'

11. Protéine de fusion recombinante selon la revendication 6, dans laquelle le second composant est un variant de la protéine selon la revendication 10 qui diffère par pas plus de 5 % de positions d'acides aminés.

12. Protéine de fusion recombinante selon l'une quelconque des revendications 1 à 3, dans laquelle le second composant est un anticorps simple-chaîne humain (scFv).

13. Protéine de fusion recombinante selon l'une quelconque des revendications 1 à 3, dans laquelle le second composant est un anticorps monoclonal humain,

14. Polynucléotide codant pour une protéine de fusion selon l'une quelconque des revendications précédentes.

15. Composition pharmaceutique qui comprend une protéine selon l'une quelconque des revendications 1 à 13 conjointement avec un excipient acceptable au niveau pharmaceutique.

Figure 1

Fig 1: Normal and engineered forms of hemagglutinin of the Measles virus (vaccine strain)

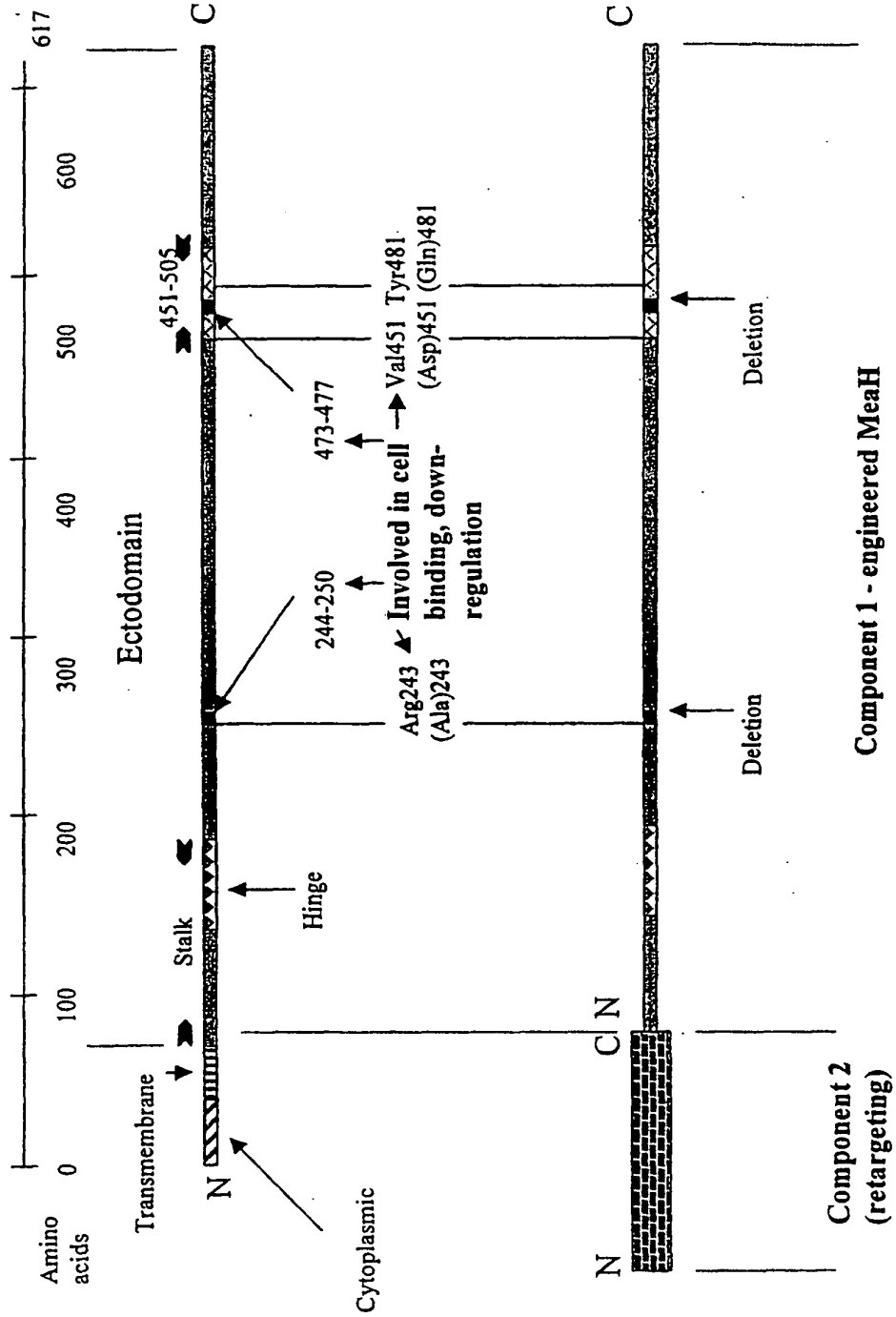

Figure 2

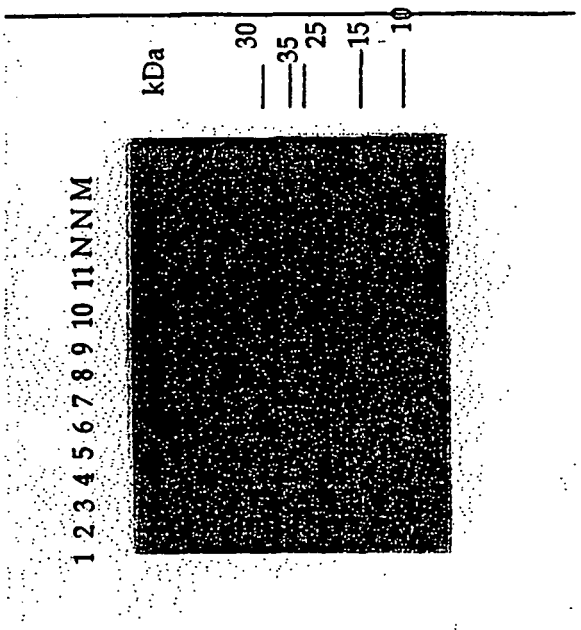

Fig.2: Western blot of HIV1 envelope (gp 120) - binding proteins from selected clones of expression cDNA library

1 - 11:Lysates from clones binding to HIV1 env, selected by high density screening;
N: Lysates from negative control clones
M: Molecular weight markers