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(54) **Mutant CyaA polypeptides and polypeptide derivatives suitable for the delivery of immunogenic molecules into a cell**

Mutante CyaA-Polypeptide und Polypeptide-Derivate, die zur Lieferung von immunogenen Molekülen in eine Zelle geeignet sind

Polypeptides CyaA mutants et dérivés de polypeptides adaptés à la fourniture de molécules immunogènes dans une cellule

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- **DATABASE EMBL 21 July 2008 (2008-07-21), "bifunctional hemolysin-adenylate cyclase precursor [Bordetella parapertussis 12822]" XP002525392 retrieved from NCBI Database accession no. NP\_882677**
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## Description

[0001] The invention relates to polypeptides suitable for use in the delivery of one or more molecules into a cell.

[0002] In particular, the invention relates to polypeptides suitable for use in the delivery of one or more molecules which are able to elicit an immune response into a host, by targeting cells which express the CD11b/CD18 receptor (also referred to herein as "CD11 b expressing cells").

[0003] The invention is more particularly directed to polypeptides derived from an adenylate cyclase protein (CyaA), the latter being used either under the form of a toxin or of a detoxified protein or toxoid, which are mutant polypeptides. Said mutant polypeptides are capable of retaining the binding activity of native CyaA to the CD11b/CD18 receptor of cells expressing the same and preferably of also retaining the translocating activity of native CyaA through its N-terminal domain into said cells and furthermore have a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

[0004] The invention relates in particular to the use of said polypeptides as proteinaceous vectors. Accordingly the mutant polypeptides are further combined with non-CyaA molecules, thereby giving rise to polypeptide derivatives, wherein said molecules have a preventive vaccinal and/or therapeutic interest when administered to a host.

[0005] The polypeptides according to the invention are suitable for use as proteinaceous vectors for the delivery of a molecule, in particular of a polypeptidic molecule having an amino acid sequence comprising one or more epitope(s), especially antigens, into a cell, in particular in CD11b expressing cells.

[0006] The invention thus also relates to a polypeptide derivative (a derivative of the mutant polypeptide of the invention) which comprises or consists of a mutant polypeptide according to the invention recombined to one or more molecules, in particular to one or more molecules suitable for eliciting an immune response, thus constituting a recombinant polypeptide or a fusion polypeptide. The invention also relates to polypeptide derivatives obtained by chemically grafting said molecule(s) to the mutant polypeptides.

[0007] According to an embodiment, the polypeptide derivatives according to the invention are suitable for use in prophylactic treatment and especially in vaccination and in therapy including in immunotherapy, in particular for eliciting an immune response in a subject.

[0008] The native CyaA used in the context of the present invention for the design of the polypeptides of the invention is the adenylate cyclase produced in *Bordetella Pertussis* and which has the following features and properties disclosed for the purpose of characterising said protein in the context of the invention.

[0009] The bi-functional RTX adenylate cyclase toxin-hemolysin (also designated herewith as the adenylate cyclase toxin (CyaA, ACT, or AC-Hly) is a key virulence factor of *Bordetella pertussis* which is the causative agent of whooping cough (1). Its 1706 residues-long polypeptide is a fusion of an N-terminal adenylate cyclase (AC) enzyme domain or part (~400 residues) to a pore-forming RTX hemolysin (Repeat in ToXin cytolytic) of ~1306 residues constituting the C-terminal part or domain (2). The latter harbors the sites of activation of proCyaA to CyaA by covalent post-translational palmitoylation of  $\epsilon$ -amino groups of Lys<sup>860</sup> and Lys<sup>983</sup>, as well as the numerous RTX repeats forming ~40 calcium binding sites, the loading of which is required for cytotoxic activity of CyaA (3, 4). The CyaA protein is indeed synthesized as an inactive protoxin which is converted into an active toxin by post translational palmitoylation of two internal lysine residues (lysines 860 and 983). This post translational modification requires the expression with the *cyaA* gene, of an accessory gene i.e., *cyaC* which is located nearby *cyaA* on *B. pertussis* chromosome.

[0010] The toxin primarily targets host myeloid phagocytes expressing the  $\alpha_M\beta_2$  integrin receptor, known also as CD11b/CD18, CR3 or Mac-1 (5). Said toxin especially binds to the CD11b/CD18 receptor of cells expressing the same through a receptor binding site present in its C-terminal part. These cells are accordingly target cells for the native toxin and also for the polypeptides of the invention. CyaA inserts into cytoplasmic membrane of cells and translocates the AC enzyme domain into the cytosol of said target cells (6, 7). Inside cells, the AC is activated by calmodulin and catalyzes uncontrolled conversion of cellular ATP to cAMP, a key second messenger molecule provoking impairment of bactericidal functions of phagocytes (1). At high doses (>100 ng/ml), CyaA-catalyzed dissipation of ATP into cAMP becomes cytotoxic and promotes apoptosis or even rapid necrotic death and lysis of CD11b<sup>+</sup> monocytes (8, 9).

[0011] Recently, the inventors showed that CyaA binds N-linked oligosaccharides of its CD11 b/CD18 receptor (10). This suggests that low specificity interactions with glycans of ubiquitous cell surface proteins or glycolipids may account for the about two order of magnitude reduced but readily detectable capacity of CyaA to penetrate also cells lacking CD11 b/CD18. Indeed, due to the extremely high specific catalytic activity of the AC domain, CyaA was found to substantially elevate cAMP also in mammalian and avian erythrocytes, lymphocytes, lymphoma, neuroblastoma, CHO, or tracheal epithelial cells (1, 11).

[0012] It has already been proposed in the prior art to provide detoxified toxin also called toxoid, wherein the adenylate cyclase activity is decreased, especially essentially suppressed. Such CyaA/AC<sup>-</sup> toxoid may be used to achieve the preparation of the polypeptides of the invention.

[0013] Besides elevating cAMP, the toxin exhibits also a moderate hemolytic activity on mammalian and avian erythrocytes. This is due to the capacity to form small cation-selective pores of an estimated diameter of 0.6 to 0.8 nm, which

permeabilize cellular membrane and eventually provoke colloid-osmotic cell lysis (12). Recently, the inventors and others have shown that the pore-forming activity of CyaA synergizes with its cell-invasive AC enzyme activity and contributes to the overall cytolytic potency of CyaA on CD11b<sup>+</sup> cells (13, 14). Due to an intact pore-forming (hemolytic) capacity, in the absence of osmoprotectants such as serum, the enzymatically inactive CyaA/AC<sup>-</sup> toxoid (15) still exhibits a full hemolytic activity on erythrocytes and a residual, about ten-fold reduced cytolytic activity on CD11b-expressing mono-

cytes (8), which sets a limit to its use in therapy.  
**[0014]** The hemolytic (pore-forming) and AC membrane translocation (cell-invasive) activities of CyaA were early on found to be dissociable by low calcium concentration, low temperature (16) and by the extent and nature of acylation of CyaA (4, 12, 17). Moreover, the two activities differ substantially in sensitivity to charge-reversing or neutral substitutions of glutamates at positions 509, 516, 570 and 581 within the hydrophobic domain (8, 13, 18). The cell-invasive and pore-forming activities of CyaA were thus proposed to be mutually independent and operating in parallel in target cell membrane. The model illustrated in figure 5A, suggests that two distinct CyaA conformers insert into target cell membrane in parallel, one being the translocation precursor, accounting for delivery of the AC domain across cellular membrane with concomitant influx of calcium ions into cells, the other being a pore precursor eventually forming oligomeric pores (13, 18, 19).

**[0015]** The inventors have now tested this model and refined it, showing that the pore-forming activity is not involved in translocation of the AC domain across target cell.

**[0016]** In the present invention, the inventors initially designed CyaA mutant polypeptides, based on the adenylate cyclase of *Bordetella pertussis*, either in the toxin or in the toxoid format, having a combination of substitutions within the pore-forming (E570Q) and acylation-bearing (K860R) domains and showed that this specific combination of substitutions selectively abolished the cell-permeabilizing activity of CyaA, thus eliminating the residual cytolytic activity of CyaA/AC<sup>-</sup> toxoids on CD11b<sup>+</sup> cells. At the same time, the E570Q+K860R construct retained a full capacity to translocate the AC domain into cytosol of cells to elevate cellular cAMP and its toxoid was fully capable to deliver epitopes containing molecules inserted within said construct to the cytosolic pathway of dendritic cells for MHC class I-restricted presentation and induction of specific cytotoxic T cell responses *in vivo*.

**[0017]** The CyaA/233OVA/E570Q+K860R mutant designed by the inventors, and in which an OVA antigenic peptide is inserted as described in the examples, is the first construct illustrative of the capacity of the CyaA mutant to provide an importantly reduced capacity to permeabilize cells that remains fully capable of translocating the AC domain across cellular membrane.

**[0018]** The inventors have now designed particular constructs, illustrated especially as a CyaA/E570Q+K860R/AC<sup>-</sup> toxoid and have shown that despite its much reduced cell-permeabilizing (cytolytic) activity, it remains fully active in antigen delivery into CD11b<sup>+</sup> APCs. The inventors have further shown that the overall cytolytic activity of the illustrative CyaA/E570Q+K860R/AC<sup>-</sup> toxoid is very low. It is thus devoid of residual toxicity in an animal or human host and is therefore highly suitable for use in therapy.

**[0019]** The invention thus provides new polypeptides, which are toxoids and have an enhanced safety profile and can be used as proteinaceous vectors for the delivery of molecules of interest, in particular of immunogenic peptidic sequences, to cells of a patient in need of a treatment, and more particularly to cells expressing CD11b.

**[0020]** Based on the experiments carried out by the inventors it has thus been possible to define and provide a polypeptide whose amino acid sequence comprises or consists of one of the following sequences:

a) an amino acid sequence which is a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 of SEQ ID NO:1, into which:

- (i) the glutamic acid residue, corresponding to the position 570 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, is substituted by a glutamine residue; and
- (ii) the lysine residue, corresponding to the position 860 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, is substituted by an arginine residue,

said fragment having the capacity of the CyaA protein of *Bordetella pertussis* to bind to the CD11b/CD18 receptor of cells expressing the same, the capacity to translocate its N-terminal adenylate cyclase enzyme domain into said cells and having a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin; or,

b) an amino acid sequence which differs from a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 of SEQ ID NO: 1, by:

- (i) the substitution of the glutamic acid residue, corresponding to the position 570 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, by a glutamine residue;
- (ii) the substitution of the lysine residue, corresponding to the position 860 in the sequence of the native CyaA

of *Bordetella pertussis* as defined in SEQ ID NO: 1, by an arginine residue; and  
(iii) from 1 to 50 additional substitutions;

5 the polypeptide consisting of said sequence having the capacity of the CyaA protein of *Bordetella Pertussis* to bind to the CD11b/CD18 receptor of cells expressing the same, the capacity to translocate its N-terminal adenylate cyclase enzyme domain into said cells and having a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

10 **[0021]** For the purpose of the invention, the N-terminal domain of the described fragment is the amino acid sequence of the fragment which includes the contiguous amino acid residues of the N-terminal part of the native CyaA protein, e.g. the N-terminal part of the fragment is all or part of the contiguous residues forming the sequence of 400 amino acid residues of the N-terminal domain of the *Bordetella pertussis* CyaA protein.

**[0022]** Herein, "E570Q" encompasses substitution of the glutamic acid residue at position 570 of native CyaA of *Bordetella pertussis* by a glutamine residue.

15 **[0023]** Herein, "K860R" encompasses substitution of the lysine residue at position 860 of native CyaA of *Bordetella pertussis* by an arginine residue.

**[0024]** The native CyaA of *Bordetella pertussis* has also been described as an amino acid sequence and a nucleotide sequence by Glaser, P. et al, 1988, *Molecular Microbiology* 2(1), 19-30. This sequence is referred to as SEQ ID N°1 as illustrated in figure 6. Accordingly, when amino acid residues or sequences or nucleotides or nucleotide sequences of the CyaA protein of *B. pertussis*, are cited in the present invention their positions are given with respect to the sequences disclosed in said publication of Glaser et al. 1988.

20 **[0025]** In an embodiment of the present invention the amino sequence of the *Bordetella pertussis* adenylate cyclase is the sequence disclosed as SEQ ID N°1.

**[0026]** When reference is made to SEQ ID N°1 or to SEQ ID N°2 herein, it is especially pointed out that, unless it is technically not relevant, the disclosed features would similarly apply to a sequence modified by insertion of residues in SEQ ID N°1 or SEQ ID N°2 in order to detoxify the CyaA protein. In such a case, the numbering of the amino acid residues should be adapted (especially insofar as positions 570 and 860 of the native sequence are concerned).

25 **[0027]** Advantageously, the CyaA protein or a fragment thereof is a protein or a fragment thereof, which is the result of the co-expression in a cell, especially in a recombinant cell, of both *cyaA* and *cyaC* genes. It has been indeed shown that in order to have invasive properties for target cells, CyaA has to undergo post-translational modifications which are enabled by the expression of both *cyaA* and *cyaC* genes (WO 93/21324).

30 **[0028]** As described herein, the CyaA protein is a bacterial protein. As described herein, CyaA protein is derived from a *Bordetella species*.

35 **[0029]** The *Bordetella species* of the invention is *Bordetella pertussis*. Other *Bordetella* strains are those of *Bordetella parapertussis*, *Bordetella hinzii* or *Bordetella bronchiseptica*. The sequences of CyaA protein of *B. parapertussis* has been disclosed especially under accession number NC 002928.3 (as a sequence of 1740 amino acids) and in Parkhill J. et al (Nat. Genet. DOI, 10 (2003)), for *B. hinzii* in Donato G.M. et al. (J. Bacteriol. 2005 Nov, 187(22):7579-88) and for *B. bronchiseptica* in Betsou F. et al (Gene 1995, August 30; 162(1): 165-6)..

40 **[0030]** The expression "polypeptide mutant of the adenylate cyclase protein" excludes the native adenylate cyclase as expressed by *Bordetella*. As stated above, it is characterised by a primary difference with the native protein, lying in the combined substitution of two specific amino acid residues. It may be further modified with respect to said native protein and it may especially be a fragment of the thus mutated protein, such as for example a truncated variant of said mutated protein, wherein residues at either or both terminal ends are deleted. In particular residues at the C-terminal end may be deleted to the extent that it does not affect the recognition and binding site for the CD11b/CD18 cell receptor. Alternatively or in addition residues may be deleted at the N-terminal end to the effect that it does not affect the translocation ability of the obtained mutant polypeptide. It may also be a fragment obtained after internal deletions of one or more residues of the native mutated CyaA protein.

45 **[0031]** Where the invention relates to a polypeptide mutant which is a fragment as stated herein, said fragment which necessarily comprises the mutated residues E570Q and K860R (when reference is made to the amino sequence of the CyaA protein of *Bordetella pertussis*) also retains the ability of the mutated full-length CyaA to bind cells and to translocate its N-terminal domain into the cytosol of CD11b/CD18 expressing cells.

50 **[0032]** The invention provides thus mutant polypeptides suitable for use in the design of means for the delivery of one or more molecules into a cell, especially a target cell expressing the CD11 b/CD18 receptor.

55 **[0033]** In particular the invention provides mutant polypeptides of a CyaA protein, where said protein is either derived from the CyaA toxin or is preferably derived from a toxoid thereof, especially a CyaA/AC<sup>-</sup> toxoid. The mutant polypeptides are capable of binding to a cell, especially a target cell expressing the CD11b/CD18 receptor, are capable of translocating their N-terminal domain or the molecule inserted in said domain or grafted on it into the cell and their pore-forming activity is totally or partially suppressed as compared to that of the CyaA toxin or toxoid.

**[0034]** The capacity of the mutant polypeptide to target CD11b/CD18 cells can be assayed especially according to the methods disclosed in EP 03291486.3 and El-Azami-El-Idrissi M. et al, J. Biol. Chem., 278(40)38514-21 or in WO 02/22169. Furthermore, the capacity of the mutant polypeptide to translocate the epitope(s) or polypeptide(s) containing said epitope(s) into the cytosol of target cell can be assayed by applying the method described in WO 02/22169.

**[0035]** Total or partial suppression of the CyaA toxin or toxoid pore-forming activity, or cell-permeabilizing capacity, is to be understood as the total or partial suppression of the ability to form pores, in particular cation selective pores of an estimated diameter of 0.6 to 0.8 nm, which permeabilize a cellular membrane and eventually provoke colloid-osmotic cell lysis. The pore-forming activity can be measured using the single whole cell patch-clamp experiment as described in examples.

**[0036]** The pore-forming activity of the CyaA toxin contributes to its overall cytolytic or haemolytic activity on cells. Indeed in the context of the present invention, the overall cytolytic or haemolytic activity of CyaA (or its "overall cytotoxic activity") is to be understood as the resultant of at least the adenylate cyclase and pore-forming activities of the CyaA toxin. Thus total or partial suppression of the CyaA toxin pore-forming activity allows at least a partial suppression of its cytolytic activity.

**[0037]** In a preferred embodiment, the overall cytolytic activity of the polypeptide according to the invention, in particular on cells which express the CD11 b/CD18 receptor, is totally or partially reduced as compared to that of the *Bordetella pertussis* CyaA toxin. The cytolytic activity of the inventive polypeptide can be determined by measuring the amount of hemoglobin (for erythrocytes) or of lactate dehydrogenase (for monocytes) released by the cells when incubated with the tested polypeptide as described in examples.

**[0038]** In a preferred embodiment, the overall cytolytic activity of the polypeptide according to the invention on cells which express the CD11 b/CD18 receptor is at least 75% lower, preferably still at least 80%, 85%, 90% or 95% lower, than that the *Bordetella pertussis* CyaA toxin, or than that of a *Bordetella pertussis* CyaA protein whose adenylate cyclase activity is partly or totally suppressed (or "CyaA toxoid"). In a particularly preferred embodiment, the overall cytolytic activity of the polypeptide according to the invention on cells which express the CD11b/CD18 receptor is at least 75% lower, preferably still at least 80% or 85% lower, than that the *Bordetella pertussis* CyaA toxoid whose amino acid sequence is shown in figure 2 (SEQ ID N°2).

**[0039]** In a preferred embodiment, the invention relates to a polypeptide which is a mutant of an adenylate cyclase and whose amino acid sequence comprises or consists of an amino acid sequence (i) which is mutated with respect to the amino acid sequence disclosed in SEQ ID N°1 said mutations comprising at least the substitutions E570Q and K860R or (ii) which is a fragment of the CyaA protein having said amino acid sequence disclosed as SEQ ID N°1, to the extent that said fragment has an amino acid sequence including substitutions E570Q and K860R and wherein the polypeptide is capable of binding to the CD11b/CD18 receptor of cells expressing the same and of translocating its N-terminal domain into said cell.

**[0040]** In a particular embodiment of the present invention, the fragment including a substitution of the glutamic acid residue at position 570 of SEQ ID N°1 by a glutamine residue (referred to as "E570Q"), and the substitution of the lysine residue at position 860 of SEQ ID N°1 by an arginine residue (referred to as "K860R") encompasses at least the amino acid sequence of the CyaA protein starting with the first N-terminal residue or from one of the amino acid residues comprised between the positions 1 and 400, preferably between the positions 1 and 380 and extending up to the residues forming the recognition and binding site for the CD11b/CD18 cell receptor and said fragment contains residues corresponding to the mutated E570Q and K860R residues or consists of said amino acid sequence. In a preferred embodiment, the fragment including the E570Q and K860R substitutions does not comprise the amino acid sequence running from the amino acid at position 1 of SEQ ID N°1 to the amino acid at position 372 of SEQ ID N°1.

**[0041]** In a preferred embodiment the fragment which is thus prepared has essentially lost the adenylyl cyclase enzyme activity (AC activity)

**[0042]** In a preferred embodiment, the mutant polypeptide of the invention is produced by co-expression in a recombinant cell of a mutated gene encoding the E570Q and R860R mutated CyaA amino acid sequence and of the *cyaC* gene, followed by recovery of the selected expressed fragment of mutant CyaA.

**[0043]** Preferably, the mutant polypeptide of the invention has a lysine residue which corresponds to the lysine residue at position 983 of the CyaA amino acid sequence as set forth in SEQ ID N°1 and which is acylated, in particular which is palmytoylated or palmitoleilated.

**[0044]** Alternatively, the mutant polypeptide of the invention has a lysine residue which corresponds to the lysine residue at position 983 of the CyaA amino acid sequence as set forth in SEQ ID N°1 which is not acylated.

**[0045]** In a specific embodiment, the mutant polypeptide of the invention has an amino acid sequence derived from the CyaA amino acid sequence disclosed in SEQ ID N°1 by mutation of residues resulting in E570Q and K860R and has an amino acid sequence which shares at least 50%, preferably at least 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% identity with the sequence set forth in SEQ ID N°1.

**[0046]** In another specific embodiment, the mutant polypeptide of the invention has an amino acid sequence which differs from a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to

860 of SEQ ID N°1 by mutation of residues resulting in E570Q and K860R and by further mutations resulting in 1 to 50 amino acid residue substitutions including the E570Q and K860R substitutions.

**[0047]** In a specific embodiment, the mutant polypeptide of the invention does not carry any amino acid residue substitutions, deletions, and/or insertions as compared to the *Bordetella pertussis* CyaA amino acid sequence other than the E570Q and K860R substitutions. In a specific embodiment, the mutant polypeptide has amino acid sequence of SEQ ID N°2 as illustrated in figure 7. In another specific embodiment, the only further amino acid substitutions, deletions, and/or insertions as compared to the amino acid sequence of SEQ ID N°2 consist in amino acid substitutions, deletions, and/or insertions which totally or partially suppress the adenyl cyclase enzymatic activity of the CyaA protein, such as in particular the insertion of a dipeptide, for example an "LQ" or "GS" dipeptide between the amino acids corresponding to the positions 188 and 189.

**[0048]** In a particular embodiment, the mutant polypeptide of the invention differs from the CyaA amino acid sequence as set forth in SEQ ID N°1 by 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid residue substitutions, deletions, and/or insertions in addition to the E570Q and K860R substitutions.

**[0049]** In a particular embodiment, in addition to the E570Q and K860R substitutions, the leucine residue at position 247 of the native CyaA protein of *Bordetella pertussis* is substituted by a glutamine residue (L247Q) or by another amino acid residue in particular a conservative amino acid residue.

**[0050]** A mutant polypeptide of the invention which is a fragment as disclosed herein of the amino acid sequence disclosed in SEQ ID N°1 is to be understood as a sequence which comprises one or more fragments having a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 and up to about 1705 amino acid residues of the SEQ ID N°1 amino acid sequence, in particular fragments comprising a stretch of at least 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600 amino acid residues of SEQ ID N°1, encompassing residues E570Q and K860R. A mutant polypeptide of the invention can also be defined as a fragment of the amino acid sequence disclosed in SEQ ID N°2 which comprises one or more fragments having a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 and up to about 1705 amino acid residues of the SEQ ID N°2 amino acid sequence, in particular fragments comprising a stretch of at least 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600 amino acid residues of SEQ ID N°2, encompassing residues 570 and 860. Said fragments preferably retain the capacity of binding to the CD11b/CD18 receptor of cells expressing the same and the ability to translocate their N-terminal domain into said cells. Preferably, the mutant polypeptide of the invention which is such a fragment which has a stretch of amino acids comprising amino acid residues 570 as E570Q to 860 as K860R or 1 to 860, or 2 to 860 of SEQ ID N°1 to the extent that the E570Q and K860R mutations are observed with respect to the original SEQ ID N°1.

**[0051]** In a preferred embodiment, the fragment further comprises amino acid residues 1166 to 1281 or amino acid residues 1208 to 1243 of the CyaA amino acid sequence as set forth in SEQ ID N°1 of CyaA protein for interaction with CD11b/CD18 target cells.

**[0052]** A particular fragment thus encompasses all or part of the C-terminal part of the native protein which part is responsible for the binding of the polypeptide of the invention to target cell membrane and/or CD11b/CD18 receptor, and for the subsequent delivery of the N-terminal domain of the polypeptide into the cell cytosol. A particular polypeptide of the invention is the fragment of CyaA protein which contains amino acid residues 373 to 1706 of CyaA protein especially of the SEQ ID N°1, to the extent that residues 570 and 860 are mutated as E570Q and K860R.

**[0053]** In another preferred embodiment, the mutant polypeptide is a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 of SEQ ID NO: 1, into which:

- a) a first amino acid sequence which corresponds to a stretch of at least 100 contiguous amino acid residues from SEQ ID N°1 comprises amino acid residues 570 as E570Q, and further includes 0, 1, 2, 3, 4 or 5 substitutions as compared to SEQ ID N°1 and
- b) a second amino acid sequence which corresponds to a stretch of at least 100 contiguous amino acid residues from SEQ ID N°1 comprises amino acid residues 860 as K860R, and further includes 0, 1, 2, 3, 4 or 5 substitutions as compared to SEQ ID N°1 and preferably comprising
- c) a third amino acid sequence comprising amino acid residues 1166 to 1281 or amino acid residues 1208 to 1243 of the CyaA amino acid sequence as set forth in SEQ ID N°1 of CyaA protein for interaction with CD11b/CD18 target cells.

**[0054]** The application discloses a fragment which is one which corresponds to the E570Q and K860R mutated CyaA protein wherein amino acid residues 225 to 234 have been deleted, thus providing a fragment containing residues 1 to 224 and 235 to 1706 of the mutated protein.

**[0055]** In a particularly preferred embodiment, the polypeptide fragment according to the invention binds to a cell which expresses the CD11b/CD18 receptor as a result of specific binding to said receptor.

**[0056]** In a preferred embodiment, adenylate cyclase activity of the polypeptide in a cell is partly or totally suppressed as compared to that of the *Bordetella pertussis* CyaA toxin. As stated above, the expression "CyaA protein" relates either

to the toxin form or preferably to the toxoid form of the protein. Accordingly each embodiment of the invention relating to the polypeptide which is a mutant of the CyaA protein applies to each of the toxin or toxoid form of the protein.

**[0057]** Total or partial suppression of CyaA adenylate cyclase or enzymatic activity is to be understood as the total or partial suppression of the ability to convert ATP into cAMP in a cellular environment as compared to that of a CyaA toxin produced by co-expression of the *cyaA* and *cyaC* genes in a cell. The ability to convert ATP into cAMP can be determined by measuring the level of intracellular cAMP as described in the examples.

**[0058]** Such total or partial suppression can be obtained as a result of genetic inactivation, for example by introduction of a short amino acid sequence, comprising for example from one to ten amino acids, in particular a dipeptide in a site of the amino acid sequence of CyaA which is part of the catalytic site, i.e. in a site located within the first 400 amino acids (AC domain) of SEQ ID N°1 or by substitution of a part of the CyaA amino acid sequence as set forth in SEQ ID N°1 which is essential for enzymatic activity. In a preferred embodiment, total or partial suppression of the CyaA enzymatic activity is obtained by insertion of a dipeptide, for example an "LQ" or "GS" dipeptide, between the amino acids corresponding to the positions 188 and 189 of the CyaA sequence as set forth <sup>222222222222222222</sup> in SEQ ID N°1. This can be achieved by inserting an oligonucleotide, such as "CTG CAG" or "CGATCC", at the EcoRV site at position 564 of the coding phase of the *cyaA* gene. See Ladant et al., 1992. Alternatively, total or partial suppression of the enzymatic activity can also be obtained by directed mutagenesis, for example, by replacing the lysine residue at position 58 or 65 of the native CyaA *Bordetella pertussis* protein (Glaser et al., 1989) by a Gln residue.

**[0059]** The invention is also directed to a polypeptide derivative comprising or consisting of the mutant polypeptide according to the invention which is further combined with one or more molecules of interest. In a preferred embodiment, a molecule of interest is a biologically active molecule either when taken alone or when combined to the polypeptide of the invention. Said molecules may especially be of prophylactic value or therapeutic value i.e., may have a prophylactic or a therapeutic activity, or may enhance a prophylactic or therapeutic activity.

**[0060]** In specific embodiments, the molecules of interest are selected in the group comprising: peptides, glycopeptides, lipopeptides, polysaccharides, oligosaccharides, nucleic acids, lipids and chemicals.

**[0061]** In a specific embodiment, the one or more molecules of interest are polypeptidic molecules or contain polypeptidic molecules. Their amino acid sequence may comprise 2 to 1000, preferably 5-800, 5 to 500, 5 to 200, 5 to 100, 8 to 50, 5 to 25, 5 to 20 or 8 to 16, or 300-600, 400-500, amino acid residues.

**[0062]** In a preferred embodiment, the one or more molecules of interest are heterologous amino acid sequences suitable for eliciting an immune response (also referred to as "heterologous antigens"), in particular amino acid sequences which comprise or consist of an epitope, including antigens. As used herein, the term "heterologous" refers to an antigen other than the mutant polypeptide which is used in the vector itself. As used herein, the term "epitope" refers to a heterologous molecule and especially a heterologous peptide that can elicit an immune response, when presented to the immune system of a host. In particular, such an epitope can comprise or consist of a stretch of 8, 9, 10, 11, 12, 13, 14, 15 or 16 amino acid residues. It may alternatively consist in a full-length antigen or consist in antigen(s) fragment(s).

**[0063]** In a specific embodiment, a polypeptide derivative according to the invention can be encoded by a plasmid which corresponds to the OVA-QR-AC-plasmid deposited under accession number CNCM I-4137 (figure 12) in which the DNA sequence encoding the "OVA" antigenic sequence is replaced by a DNA sequence encoding an antigenic sequence comprising one or more epitopes.

**[0064]** The polypeptidic molecule suitable for eliciting an immune response is especially one eliciting a T-cell immune response, including as an example a CTL response. The polypeptidic molecule suitable for eliciting an immune response can also be one eliciting a B-cell immune response.

**[0065]** In specific embodiments, the heterologous antigen is selected from the group consisting of an antigen of a bacterial pathogen, a tumoral cell antigen, a viral antigen, a retroviral antigen, a fungus antigen or a parasite cell antigen.

**[0066]** A molecule of interest can be especially an antigen selected from the group consisting of: a Chlamidia antigen, a Mycoplasma antigen, a hepatitis virus antigen, a poliovirus antigen, an HIV virus antigen, an influenza virus antigen, a choriomeningitis virus antigen, a tumor antigen, or a part of any of these antigens which comprises at least an epitope.

**[0067]** In a preferred embodiment of the polypeptide derivative of the invention, the amino acid sequence of each of said molecule(s) suitable for eliciting an immune response comprises or consists of an amino acid sequence of a Chlamidia antigen, a Mycoplasma antigen, a hepatitis virus antigen, a poliovirus antigen, an HIV virus antigen, an influenza virus antigen, a choriomeningitis virus sequence, a tumor antigen, or comprises or consist of a part of an amino acid sequence of any these antigens which comprises at least one epitope.

**[0068]** In a particularly preferred embodiment, the molecule of interest is a tumor associated antigen (TAA). Tumor-associated antigens have been characterized for a number of tumors such as for example: Melanoma, especially metastatic melanoma; Lung carcinoma; Head & neck carcinoma; cervical carcinoma, Esophageal carcinoma; Bladder carcinoma, especially infiltrating Bladder carcinoma; Prostate carcinoma; Breast carcinoma; Colorectal carcinoma; Renal cell carcinoma; Sarcoma; Leukemia; Myeloma. For these various histological types of cancers, it has been shown that antigenic peptides are specifically expressed on tumor samples and are recognized by T cells, especially by CD8<sup>+</sup> T cells or CD4<sup>+</sup> T cells.



[0069] A review of peptides found as tumor-associated antigens in these types of tumors is made by Van der Bruggen P. et al (Immunological Reviews, 2002, vol 188:51-64). Especially, the disclosure of the peptides contained in table 3 of said review is referred to herein as providing examples of such tumor-associated antigens and said table 3 is incorporated by reference to the present application.

5 [0070] The following antigens are cited as examples of tumor-associated antigens recognized by T cells, according to the publication of Kawakami Y. et al (Cancer Sci, October 2004, vol.95, no. 10, p784-791) that also provides methods for screening these antigens or further one: antigens shared by various cancers, including MAGE (especially in Melanoma), NY-ESO-1, Her2/neu, WT1, Survivin, hTERT, CEA, AFP, SART3, GnT-V, antigens specific for some particular cancers such as  $\beta$ beta-catenin, CDK4, MART-2, MUM3, gp100, MART-1, tyrosinase for Melanoma; bcr-abl, TEL-AML1 for Leukemia; PSA, PAP, PSM, PSMA for prostate cancer; Proteinase 3 for myelogenous leukemia; MUC-1 for breast, ovarian or pancreas cancers; EBV-EBNA, HTLV-1 tax for lymphoma, ATL or cervical cancer; mutated HLA-A2 for Renal cell cancer; HA1 for leukemia/lymphoma. Tumor-associated antigens in animals have also been described such as Cycline D1 and Cycline D2 in tumors affecting cats or dogs.

10 [0071] Tumor-associated antigens recognized by T cells have also been disclosed in Novellino L. et al (Immunol Immunother 2004, 54:187-207).

15 [0072] More generally, TAA of interest in the present invention are those corresponding to mutated antigens, or to antigens that are overexpressed on tumor cells, to shared antigens, tissue-specific differentiation antigens or to viral antigens.

20 [0073] In a particular embodiment of the invention, the tumor-associated antigen is an antigen of papillomavirus (HPV) or is tyrosinase.

25 [0074] According to another particular embodiment of the invention, the amino acid sequences of the polypeptidic molecules which comprise or consist of an epitope have been modified with respect to their native amino acid sequence, for example in order to decrease the number of negatively charged amino acid residues within the sequence. Such a modification can be obtained by removing some of these negatively charged amino acid residues or also by adding some positively charged amino acid residues, especially as flanking residues of the epitopes. Polypeptides thus comprising less negatively charged residues might favour the translocation of the catalytic domain of the polypeptide derivative of the invention in the cytosol of target cells.

30 [0075] The amino acid sequences of the polypeptidic molecules which comprise or consist of an epitope or an antigen can also be designed in such a way that they are unfolded when they are inserted in the polypeptide derivative of the invention, which improves efficiency of the internalization of the molecule(s) of interest according to the invention into the target cells. Such unfolding in polypeptides which undergo folding as a consequence of their amino acid content, can be obtained for instance, by removing or substituting cystein residues in order to avoid formation of disulfide bonds that may be involved in folding of polypeptides. In some cases, it is possible to prevent folding of the polypeptides by preparing them in the presence of reducing agents to enable avoiding *in vivo* refolding.

35 [0076] In a particular embodiment, the amino acid sequences, especially the antigen, can comprise or consist of cryptic epitopes.

40 [0077] The inventors have indeed determined that polypeptide derivative constructs, which comprise (i) a polypeptide of the invention which is a mutant of a CyaA protein (polypeptide mutant) according to the definitions disclosed herein and (ii) a polypeptidic molecule having an amino acid sequence bearing one or several antigenic fragments of one or several antigens, enable cryptic epitopes of said antigens to become immunogenic as a result of their presentation in the constructs. Especially, said constructs involving mutant polypeptides as defined in the present invention comprising polypeptidic molecule(s) derived from antigens of interest for especially prophylactic or therapeutic applications, including immunotherapeutic vaccination, purposes are processed in target cells where the polypeptidic molecule(s) is allowed to be internalized as a result of the translocation of the N-terminal domain of the mutant polypeptide. Such processing enables epitopes presentation through the class I MHC molecules of the target cells, and said epitopes can comprise cryptic epitopes of the antigen which are allowed to become immunogenic and in particular to raise a T-cell response in a host, especially a CTL response.

45 [0078] The invention thus also relates to a polypeptide derivative, in particular to a recombinant protein comprising one or several polypeptidic molecules having an amino acid sequence bearing one or several epitopes of one or several antigens, or bearing said antigen(s) said amino acid sequence(s) of said polypeptidic molecule(s) being inserted in the same or in different sites, especially in different permissive sites of a mutant polypeptide according to the invention, said recombinant protein retaining the property of the CyaA toxin to target antigen presenting cells (APC), wherein at least one of said epitope(s) is a subdominant cryptic T-cell epitope and wherein said polypeptide derivative, especially said recombinant protein, is capable of eliciting an antigen-specific response against said polypeptidic molecule(s).

50 [0079] In a specific embodiment of the polypeptide derivative according to the invention, the one or more amino acid sequences are inserted into one or more sites, especially permissive sites.

55 [0080] For the present invention, a "permissive site" is a site of the sequence of the CyaA protein where a polypeptide can be inserted without substantially affecting the desired functional properties of the CyaA protein especially without

substantially affecting the targeting of cells, particularly the targeting of antigen presenting cells (APC) by CyaA, including without substantially affecting the specific binding to the CD11b/CD18 receptor and advantageously without substantially affecting the domains of the protein involved in the process of translocation of the CyaA N-terminal domain into a target cell.

5 [0081] Methods to select for permissive sites are presented for example in WO93/21324, in Ladant et al., 1992, and in Osicka et al., 2000 (Infection and Immunity, 2000, 68(1):247-256). In particular, a methodology using a double selection (resistance to an antibiotic and colorimetric test on dishes by  $\alpha$ -complementation) enables to identify readily oligonucleotides insertions (which preserve the reading frame) in the portion of the gene coding for the N-terminal catalytic domain of the toxin. The functional consequences of these mutations on the catalytic activity of the toxin may be readily analysed, both genetically (functional complementation of an *E. coli cya* strain) and biochemically (characterization of the stability of the modified adenylcyclases, of their enzymatic activity, of their interaction with caM, etc.). This methodology has enabled a large number of mutations to be screened in order to identify the sites which are potentially advantageous for the insertion of antigenic determinants.

10 [0082] Permissive sites of the *Bordetella pertussis* adenylate cyclase allowing translocation of CyaA catalytic domain and hence translocation of amino acid sequences inserted into such permissive sites include, but are not limited to, residues 137-138 (Val-Ala), residues 224-225 (Arg-Ala), residues 228-229 (Glu-Ala), residues 235-236 (Arg-Glu), and residues 317-318 (Ser-Ala) (Sebo et al., 1995). The following additional permissive sites are also included in embodiments of the invention: residues 107-108 (Gly-His), residues 132-133 (Met-Ala), residues 232-233 (Gly-Leu), and 335-336 (Gly-Gln) and 336-337. However, other permissive sites may be used in the present invention, that can be identified for example by use of the methodology indicated above, especially sites between residues 400 and 1700 of the CyaA protein.

20 [0083] For other *Bordetella* species corresponding permissive sites can be defined by comparison of sequences and determination of corresponding residues.

[0084] As described herein, the one or more amino acid sequence polypeptide can also or alternatively be inserted at one and/or the other extremities (ends) of the polypeptide of the invention, preferably at the N-terminal end of the mutant CyaA polypeptide lacking all or part of the N-terminal catalytic domain of the *Bordetella pertussis* CyaA protein, and more particularly lacking residues 1-373.

25 [0085] According to a specific embodiment, the one or more amino acid sequences suitable for eliciting an immune response, is grafted onto an amino acid residue of said polypeptide.

[0086] According to the invention, the "combination" (or insertion) of an amino acid sequence with the CyaA mutant polypeptide to provide a so-called polypeptide derivative, also referred to as a "recombinant protein" or a "hybrid protein", encompasses genetic insertion especially by available DNA technology. Alternatively, "combination" also encompasses non genetic insertion, including chemical insertion for instance covalent coupling carried out especially at one extremity of the amino acid sequence, or non covalent coupling. Non-genetic insertion can especially be of interest when the amino acid sequence to be inserted is synthetic or semi-synthetic. Methods for coupling a drug to a polypeptide are well known in the Art and comprise for example disulfide linkage by using N-pyridyl sulfonyl-activated sulfhydryl.

30 [0087] In particular, it is possible to graft molecules to the polypeptides of the invention by a chemical linkage or by genetic insertion for *in vivo* targeting to CyaA target cells, such as APC, for example CD11 b/CD18 positive cells and particularly to the cytosol of said cells. Indeed, when coupling a molecule corresponding to a given CD8+ T-cell epitope to the catalytic domain of detoxified CyaA, either by means of a disulfide bond or by genetic insertion, it has been found that the engineered molecule can elicit *in vivo* specific CTL response, thereby showing that said CD8+ T-cell epitope is translocated into the cytosol of CD11 b-expressing cells.

35 [0088] In a preferred embodiment of the invention, the mutant CyaA polypeptide is used in the manufacturing of a proteinaceous vector or in the preparation of a composition specifically designed to prime CD8+ cytotoxic T-cell response (CTL response) when said response follows the targeting of the mutant CyaA polypeptide modified (especially recombined or conjugated) with a molecule of interest to CD11b expressing cells, followed by the translocation of the molecule of interest to the cytosol of said CD11b expressing cells, and in particular to myeloid dendritic cells. In this context, the molecule of interest is or comprises preferably an epitope or an antigen.

40 [0089] In another preferred embodiment of the invention, the mutant CyaA polypeptide is used in the manufacturing of the proteinaceous vector or in the preparation of a composition specifically designed to prime CD4+ cells response when said response follows the targeting of the adenylcyclase modified (especially recombined or conjugated) with a molecule of interest to CD11b expressing cells, in particular myeloid dendritic cells. In this context, the molecule of interest is or comprises preferably an epitope or an antigen.

45 [0090] The mutant polypeptides can also be used in the manufacturing of a proteinaceous vector for targeting of a prophylactic or a therapeutic compound to CD11b expressing cells. In this context, in one specific embodiment of the invention, the so-called molecule of interest has a prophylactic or therapeutic value and in particular is a drug. Said prophylactic or therapeutic compound and in particular said drug may be chemically or genetically coupled to the mutant polypeptide. Method for coupling a compound to a polypeptide are well known in the Art and comprise for example disulfide linkage by using N-pyridyl sulfonyl-activated sulfhydryl. In one embodiment, a molecule of interest is an anti-inflammatory compound which is, when coupled to the mutant polypeptide, specifically targeted to the surface of the cells

involved of the inflammatory response, such as dendritic cells or neutrophils.

**[0091]** More specifically, antigen presentation for selective CD8+ cytotoxic cells priming is mainly performed by myeloid dendritic cells.

**[0092]** Accordingly, in a specific embodiment, the mutant CyaA polypeptide used for the manufacturing of proteinaceous vector is a genetically modified adenylcyclase containing one or more molecule(s) chemically coupled by means of a disulfide bond to genetically inserted cysteine residue(s) located within the catalytic domain of the mutant CyaA polypeptide. Indeed, multiple molecules can be chemically coupled to the mutant CyaA polypeptide by means of a disulfide bond to different cysteine residues located at different permissive sites within the catalytic domain.

**[0093]** The mutant polypeptides or polypeptide derivatives according to the invention are suitable for use in therapy or prophylaxis.

**[0094]** By therapy or therapeutic effect it is intended any effect which is beneficial to the condition of a patient, be it curative or sufficient to limit the symptoms or the consequences of a pathological condition, including limiting the progression of a pathological condition. By therapy or therapeutic effect is also encompassed the prevention of the onset of pathological condition.

**[0095]** The mutant polypeptides or polypeptide derivatives according to the invention are in particular suitable to elicit a cell-mediated immune response such as a T-cell immune response or a B-cell immune response in a host in need thereof. It includes CTL and Th, especially Th1 response, including CD4+ T cell response and/or CD8+ T cell response.

**[0096]** The ability of a polypeptide derived from CyaA protein to elicit a cell-mediated immune response may be sufficient to prevent tumor growth *in vivo* or even to enable tumor regression in an animal. It may also be enhanced by activation of innate component of the immune response through TLR activation and by down activating the regulatory component of the immune response through the use of chemotherapeutic agents. The invention provides means which should enable such results to be obtained in improved safety conditions as a result of the combined mutations E570Q and K86oR, which have been selected.

**[0097]** The application discloses therapeutic methods comprising administration to an animal or human patient of the mutant polypeptide or polypeptide derivative according to the invention to a patient to elicit a T-cell immune response or a B-cell immune response in a host in need thereof.

**[0098]** The mutant polypeptides or polypeptide derivatives according to the invention can in particular be used for the prevention or the treatment of a disease selected from neoplasia, cancers and infectious diseases selected from viral-, retroviral-, bacterial- or fungal- induced diseases. In particular, the polypeptide derivatives can be used for the treatment of HIV infections in a patient.

**[0099]** It is especially provided that in a particular embodiment of the invention, the CyaA mutant polypeptide or polypeptide derivative is suitable for the treatment of infiltrating or vascularized tumors versus superficial tumors or for the treatment of metastatic tumors versus primary tumors, in accordance with the acknowledged clinical criteria for the classification of tumors.

**[0100]** Solid tumors are especially a target for the treatment through the use of the polypeptide derivative of the invention.

**[0101]** Among tumors which may be candidates for the treatment with the polypeptide derivative of the invention, the following, for which tumor-associated antigens have been characterized, are described as examples:

Melanoma, especially metastatic melanoma; Lung carcinoma; Head & neck carcinoma; cervical carcinoma, Esophageal carcinoma; Bladder carcinoma, especially infiltrating Bladder carcinoma; Prostate carcinoma; Breast carcinoma; Colorectal carcinoma; Renal cell carcinoma; Sarcoma; Leukemia; Myeloma. For these various histological types of cancers, it has been shown that antigenic peptides are specifically expressed on tumor samples and are recognized by T cells, especially by CD8+ T cells or CD4+T cells.

**[0102]** The invention further relates to the use of a polypeptide derivative according to the invention, for the preparation of a therapeutic composition for the treatment of a disease selected from neoplasia, cancers and infectious diseases selected from viral- or retroviral-induced diseases.

**[0103]** In a preferred embodiment, the polypeptide or polypeptide derivative according to the invention can be administered to the patient in combination with an adjuvant and/or in combination with another therapeutically active molecule or agent.

**[0104]** In the context of the present invention said "another therapeutically active molecule or agent" is one which may be beneficial to the condition of a patient to whom it is administered. It is especially an active principle suitable for use in the manufacturing of a drug. It may be a compound suitable to either, potentiate increase or modulate the effect of an therapeutically active principle.

**[0105]** The mutant CyaA polypeptide or the polypeptide derivative thereof can be administered with a therapeutically active molecule or agent, in particular one suitable for eliciting an immune response in a patient.

**[0106]** In particular, mutant CyaA polypeptide or the polypeptide derivative thereof can be administered with a therapeutically active agent suitable for modulating a cell response in a patient, in particular by lowering or blocking

regulatory T cells immunosuppressive capacity.

**[0107]** According to a particular embodiment of the invention, such an effect on a regulatory cell response may be obtained with an agent modulating a regulatory T cell and/or modulating another cell suppressive response, such as the myeloid suppressive cells response, said agent targeting said regulatory cells, especially T cells, by depleting or inactivating these cells (such as with CD25-specific antibody, or cyclophosphamide), altering trafficking of said cells, especially regulatory T cells (such as CCL22-specific antibody) or altering differentiation and signalling of said cells (such as by blocking FOXP3 (forkhead box P3) signal).

**[0108]** According to a particular embodiment of the invention, the agent modulating a regulatory cell response targets suppressive molecules, especially such molecules present on APCs (such as B7-H1, B7-H4, IDO (indoleamine 2,3-dioxygenase) or arginase) or on T cells (such as CTLA4 (cytotoxic T-lymphocyte-associated antigen 4) or PD1 (programmed cell death 1)), or targets soluble immunosuppressive molecules (such as TGF beta (transforming growth factor), IL-10, VEGF (vascular endothelial growth factor), COX2 (cyclooxygenase 2)).

**[0109]** As examples of agents having an effect on a regulatory cell response, cytotoxic agents are proposed, that can kill regulatory T cells or other immunosuppressive cells, or that can block their activity and/or development and/or accumulation.

**[0110]** In a particular embodiment of the invention, the agent modulating the regulatory cell response, especially a regulatory T cell response, is a chemotherapeutic agent. Especially it is selected among chemotherapeutic agents known as anticancer agents and used in chemotherapy. Such agents include those helping to reduce the tumor burden, those acting by increasing sensitivity of tumor cells to treatment or those enabling killing or inactivating immune regulatory cells. The chemotherapeutic agents used within the frame of the invention thereby enhance antitumor immunity.

**[0111]** In a particular embodiment of the invention, the chemotherapeutic agent is an alkylating agent. Especially, it is Cyclophosphamide (CTX) (Sigma, Steinheim, Germany). Cyclophosphamide is capable of depleting or inactivating regulatory T cells.

**[0112]** In another particular embodiment of the invention, the chemotherapeutic agent is an intercalating agent.

**[0113]** In a particular embodiment, the chemotherapeutic agent is Doxorubicin (DOX) (Calbiochem, La Jolla, CA, USA).

**[0114]** The chemotherapeutic agent is advantageously administered by low doses.

**[0115]** The mutant CyaA polypeptide or the polypeptide derivative thereof can also be administered with an adjuvant component, suitable for activating the innate immune response primed by a tumor in a patient.

**[0116]** In a particular embodiment of the invention, the adjuvant component is selected in the group of components consisting of nucleic acids, peptidoglycans, carbohydrates, peptides, cytokines, hormones and small molecules, wherein said adjuvant component is capable of signalling through pattern-recognition receptors (PRRs).

**[0117]** PRRs are known to mediate the innate immune response to pathogens, and to tumors, by recognition of so-called evolutionarily conserved signatures from pathogens (pathogen-associated molecule patterns, PAMPs). PRRs are present on a variety of immune cells including dendritic cells, natural killer cells, B cells, and also on some non immune cells such as epithelial cells or endothelial cells. PRRs and their involvement in the innate immune response are described in Pashine A. et al (Nature medicine supplement volume 11, N° 4, April 2005).

**[0118]** In particular an adjuvant component for the activation of the innate immune response can target PRRs and therefore activate signalling through PRRs, wherein said PRRs encompass Toll-like receptors or nucleotide-binding oligomerization domain (NOD) or C type lectin.

**[0119]** In a particular embodiment of the invention, the adjuvant component is a Toll-like receptor (TLR) agonist. The Toll-like receptor agonist is especially formulated to efficiently activate the innate immune system of a patient. Said TLR agonist is capable of binding the TLR, i.e., is a ligand of the TLR and is furthermore capable of enhancing the immune response elicited under the control of said TLR.

**[0120]** For illustration, TLR agonists are selected from the group of TLR-9, TLR-8, TLR-3 and TLR-7 agonists. However agonists of other TLR receptors may be used to perform the invention, such as agonists of the TLR2, TLR4, TLR5 receptors.

**[0121]** The TLR agonist used in the invention can be a natural or a synthetic agonist. It can be a combination of different agonists of the same or of different toll-like receptors.

**[0122]** According to a particular embodiment of the invention, the TLR agonist is an immunostimulatory nucleotide sequence, especially a stabilized nucleotide sequence, for example stabilized as a result of structure modification such as phosphorothioate modification. The nucleotide sequence can also be protected against degradation by specific formulation. Especially liposome formulation thereof, e.g. liposome suspension, can be advantageous for the efficient administration of the immunostimulatory nucleotide sequence.

**[0123]** In a particular embodiment of the invention, the immunostimulatory nucleic acid sequence is a single-stranded RNA.

**[0124]** In a particular embodiment of the invention, the immunostimulatory nucleotide sequence comprises a CpG motif and especially is a CpG oligonucleotide (CpG ODNs). As an example of suitable CpG oligonucleotides the invention provides TLR-9 ligands such as Type A CpG ODN, i.e., CpG 2216 having nucleotide sequence 5'-GGGGAC-

GATCGTCGGGGG-3' or Type B CpG ODN, i.e., CpG 1826 having nucleotide sequence 5'-TCCATGACGTTCCCT-GACGTT-3'.

**[0125]** CpG oligonucleotide can be used after being complexed with DOTAP (Roche Mannheim, Germany), in order to protect it against degradation and to facilitate its uptake.

**[0126]** According to another particular embodiment of the invention, the TLR agonist is a small molecule. Small molecules suitable as TLR agonists are for example imidazoquinoline amine derivatives, such as the one named R848 (resiquimod), i.e., 4-amino-2-ethoxymethyl-*a,a*, dimethyl-1-*H*-imidazo[4,5*c*]quinoline -1-ethanol available from Invivo-gen, as TLR-7 ligand, or the one named R837 (imiqimod) available from Aldara as TLR-7 agonist.

**[0127]** Other molecules suitable as TLR agonists are polyuridine (pU) as TLR-3 ligand, or polycytidylic acid (PIC) as TLR-7 ligand.

**[0128]** These molecules can be formulated to facilitate their uptake and/or to protect them from degradation. These molecules can also be prepared as a liposome formulation, especially as a liposome suspension, for administration to a patient.

**[0129]** According to another particular embodiment of the invention, the adjuvant component can be a cell-based adjuvant component. An example thereof is dendritic cells that are known to be able to prime lymphocyte response, such dendritic cells being possibly conditioned *ex vivo* prior to their administration, in order to increase their activity of stimulation of the T cell response. Dendritic cells can hence be stimulated with adjuvants interacting with the PRRs, including TLR ligands or agonists (Pashine A. et al Nature Medicine Supplement Volume 11, N°4, April 2005 p S63-S68)

**[0130]** Alternatively, the polypeptide or polypeptide derivative according to the invention can be administered to the patient without an adjuvant.

**[0131]** Indeed the inventors have previously shown that CTL specific for the vectorized antigen can be primed *in vivo* after a single intravenous injection of the recombinant toxin, especially with no need to provide a heterologous adjuvant. These results and in particular the specific targeting of the epitope to myeloid dendritic cells enable to bypass the requirement for adjuvant and CD4+ T cell help.

**[0132]** Therefore, the invention also relates to the use of a mutant CyaA polypeptide recombined with a molecule and especially a peptide of interest for the preparation of a composition formulated for intravenous administration and enabling a CD8+ T cell immune response *in vivo*, said composition being free of a heterologous adjuvant. The invention also concerns this composition as such.

**[0133]** The application discloses therapeutic methods comprising administration of the mutant polypeptide or polypeptide derivative according to the invention to an animal or human patient suffering from a disease selected from neoplasia, cancers and infectious diseases selected from viral-, retroviral-, bacterial- or fungal- induced diseases.

**[0134]** The mutant polypeptide or polypeptide derivative can in particular be administered with a therapeutically active molecule and/or an adjuvant.

**[0135]** The mutant CyaA polypeptide or the polypeptide derivative, the therapeutically active molecule and/or an adjuvant can be administered together as part of a pharmaceutical composition which further comprises a pharmaceutically acceptable carrier or excipient(s).

**[0136]** Alternatively, the various types of molecules described herein to carry out the invention used, can be administered separately either simultaneously in time (especially for the mutant CyaA polypeptide or the polypeptide derivative and the adjuvant) or separately in time (especially for the mutant CyaA polypeptide).

**[0137]** The administration of the therapeutically active molecule can alternatively be carried out prior and after the administration of the mutant CyaA polypeptide or the polypeptide derivative and/or the adjuvant. It can also be sequential in time.

**[0138]** A particular regimen that may be adopted is a repeated administration protocol, especially in a protocol which encompasses two rounds or more of administration of at least one of the compounds selected from the mutant CyaA polypeptide or the polypeptide derivative, the therapeutically active agent and/or the adjuvant.

**[0139]** The invention is also directed to a pharmaceutical composition which comprises a mutant CyaA polypeptide or a polypeptide derivative according to the invention, a pharmaceutically acceptable carrier or excipients(s), and optionally an adjuvant and/or another therapeutically active molecule.

**[0140]** The application discloses a kit of parts comprising the mutant CyaA polypeptide or the polypeptide derivative, a therapeutically active molecule and/or an adjuvant.

**[0141]** The compounds of the kit of parts or the composition of the invention can especially be given to the patient through intravenous administration, intratumoral administration or subcutaneous administration.

**[0142]** The kit of parts or the composition has the ability to target (i) the adaptive immune response, through the mutant CyaA polypeptide or the polypeptide derivative disclosed in the present application, (ii) to downregulate the regulatory immune response through the therapeutically active agent, and if the adjuvant is present, to target (iii) the innate component of the immune response, by activating said innate response through the adjuvant.

**[0143]** The application discloses a method of treatment of a patient in need thereof, either a human or an animal patient, comprising the step of administering the components of the kit of parts or of the composition herein disclosed.

[0144] The invention in particular also relates to a new immunogenic composition formulated for administration, especially intravenous administration, in an animal or human host, **characterized in that** it comprises a recombinant CyaA polypeptide derivative which comprises an antigen inserted in the catalytic domain.

[0145] The invention further relates to a pharmaceutical composition for administration in a human or an animal formulated for targeting a molecule of interest specifically to CD11b expressing cells **characterized in that** said molecule of interest is coupled to a mutant CyaA polypeptide as described herein.

[0146] In another specific embodiment, the pharmaceutical or immunogenic composition comprises a nucleic acid construction encoding the recombinant CyaA polypeptide derivative comprising a CyaA mutant polypeptide as defined herein coupled to a molecule of interest.

[0147] Furthermore, the invention also relates to the use of the immunogenic composition as defined above for the preparation of a vaccine or an immunotherapeutic composition, for administration to an animal or human host.

[0148] As used herein, the term "immunotherapeutic composition" relates to a composition which leads to an immunological response and which is associated to therapeutic treatments, such as treatment against neoplasia, cancers, viral infections, fungal infections, parasites infections or bacterial infections.

[0149] The application discloses a method to immunize an animal or human host, wherein said method comprises the steps of:

a) providing an immunogenic composition as defined above;

b) administering said immunogenic composition, preferably via intravenous route, to said host in order to promote an immune response.

[0150] In particular, the immunogenic compositions of the invention are capable of inducing or stimulating, *in vivo* or *in vitro* an immune cell response involving specifically dendritic cells. The immunogenic compositions of the invention can in particular be used for preventive or therapeutic vaccination of a patient.

[0151] As a consequence, in a specific embodiment, the immunogenic or pharmaceutical composition is advantageously devoid of priming adjuvants commonly used in the Art, such as aluminium hydroxide.

[0152] The invention further relates to a method for the preparation of a proteinaceous vector suitable for the delivery of a molecule of interest into a cell comprising binding the molecule of interest to a CyaA mutant polypeptide as defined herein.

[0153] The application discloses nucleic acid molecules, in particular DNA or RNA molecules, which encode a polypeptide or polypeptide derivative as defined above.

[0154] The application discloses eukaryotic or prokaryotic cells which comprise the nucleic acid molecules as defined above.

[0155] The application discloses eukaryotic cells, preferably mammalian cells, which comprise a mutant CyaA polypeptide or polypeptide derivative as defined above. As described herein, the cells are human cells.

[0156] The application discloses eukaryotic cells, preferably mammalian cells, transformed with the proteinaceous vector as defined above.

## Figures

### [0157]

**Fig. 1. Substitutions in the pore-forming and acylation domains synergize in decreasing the specific hemolytic activity of CyaA.** (A) Sheep erythrocytes ( $5 \times 10^8$ /ml) in TNC buffer were incubated with  $5 \mu\text{g/ml}$  of enzymatically active CyaA proteins at  $37^\circ\text{C}$ . After 30 min, aliquots of cells suspensions were washed repeatedly to remove unbound CyaA and used to determine the amount of cell-associated and cell-invasive AC activity. Hemolytic activity was measured after 5 hours of incubation as the amount of released hemoglobin by photometric determination ( $A_{541}$ ). Activity of intact CyaA was taken as 100%. (B) The reduced cell binding activity of proteins with the K860R substitution was compensated for by increasing their concentration from  $5 \mu\text{g/ml}$  to  $25 \mu\text{g/ml}$ . Activities of CyaA/233OVA (CyaA/OVA) in the presence were taken as 100% value. The results represent average values from at least three independent experiments performed in duplicates. The asterisks indicate statistically significant differences (\*\*,  $p < 0.001$ ) from activities of CyaA (Fig. 1A) or CyaA/OVA (Fig. 1B).

**Fig. 2. CyaAIOVA/E570Q+K860R binds and translocates into CD11b<sup>+</sup> monocytes.** (A) J774A.1 cells ( $10^6$ /ml) were incubated in D-MEM for 30 min at  $4^\circ\text{C}$  with  $2.5 \mu\text{g/ml}$  of CyaA, washed repeatedly, and the amount of cell-associated AC activity was determined in cell lysates. To block the CD11b/CD18 receptor, cells were incubated for 30 min with the CD11b-specific antibody M1/70 (Exbio, Czech Republic) at a final concentration of  $10 \mu\text{g/ml}$  prior

to addition of CyaA (\*\*,  $p < 0.001$ ). (B) The AC domain translocation capacity of constructs was assessed as the capacity to penetrate cells and convert cytosolic ATP to cAMP. J774A.1 cells were incubated with CyaA constructs for 30 minutes at 37°C and the amounts of cAMP accumulated in cell lysates were determined (41). The CD11b/CD18 receptor was blocked with M1/70 as above. Results representative of three independent determinations performed in duplicates are shown.

**Fig. 3. E570Q+K860R toxoid does not permeabilize J774A.1 cells.** Whole-cell patch-clamp measurements were performed on single J774A.1 cells at room temperature exposed to 1  $\mu\text{g}/\text{ml}$  of (A) CyaA/233OVA/AC<sup>-</sup> or (B) CyaA/2330VA/E570Q+K860R/AC<sup>-</sup> proteins as described in Materials and Methods. The shown curves are representative of six determinations in 3 independent experiments.

**Fig. 4. Toxoid with E570Q+K860R substitutions delivers the OVA T-cell epitope for presentation by MHC class I molecules and induction of CD8<sup>+</sup> CTLs.** (A) BMDC ( $3 \times 10^5$  cells/well) used as APCs were incubated in the presence of indicated concentrations (0 to 60 nM) of the toxoids harboring the OVA epitope or with mock CyaA/AC<sup>-</sup>. Upon co-culture for 24 hours with B3Z T cells ( $1 \times 10^5$  cells/well), IL-2 secretion by the stimulated B3Z cells was determined by the CTLL proliferation method. Results are expressed as  $\Delta\text{cpm}$  of incorporated [<sup>3</sup>H]thymidine (cpm in the presence of toxoid - cpm in the absence of toxoid)  $\pm$ SD and are representative of five independent experiments. (B) Analysis of the induction of OVA (SIINFEKL)-specific CTL responses by *in vivo* killing assay. On day 0, mice received 50  $\mu\text{g}$  i.v. of mock AC<sup>-</sup> or OVA/AC<sup>-</sup> toxoids and on day 7, they were i.v. injected with a mixture (1:1) of OVA (SIINFEKL) peptide-loaded CFSE<sup>high</sup> and unloaded CFSE<sup>low</sup> splenocytes. The number of CFSE-positive cells remaining in the spleen after 20 h was determined by FACS analysis, as documented for one representative *in vivo* killing assay in the upper panel assembly of plots, where percentages of cells in the gates are indicated. The lower panel shows pooled results of *in vivo* killing assays for three independent experiments. Statistical significance was determined by the Student t test ( $p = 0.75$  for OVA/AC<sup>-</sup> vs. OVA/E570Q+K860R/AC<sup>-</sup>).

**Fig. 5. Model of CyaA action on the membrane.** (A) The model predicts an equilibrium between two conformers of CyaA in solution, each of them inserting into cell membrane in different a conformation. One would yield a monomeric CyaA translocation precursor, delivery of the AC domain into cytosol and concomitant influx of calcium ions into cells. The conformer would insert as pore precursor oligomerizing into a CyaA pore. (B) The synergic effect of the E570Q and K860R substitutions would selectively block the capacity of CyaA pore precursors to oligomerize into a pore, while the capacity of translocation precursors to deliver the AC domain across membrane would remain unaffected.

**Fig. 6. Amino acid sequence of the *Bordetella pertussis* CyaA toxin (SEQ ID N°1)**

**Fig. 7. Amino acid sequence of the *Bordetella pertussis* CyaA/E570Q+K860R mutant (SEQ ID N°2)**

**Fig. 8. Amino acid sequence of the *Bordetella pertussis* CyaA/E570Q+K860R/AC<sup>-</sup> mutant (SEQ ID N° 3)**

**Fig. 9. Amino acid sequence of the *Bordetella pertussis* CyaA/2330VA/E570Q+K860R/AC<sup>-</sup> mutant (SEQ ID N° 4)**

**Fig. 10. Plasmid encoding the CyaA/E570Q+K860R/AC<sup>-</sup> mutant (QR-AC<sup>-</sup>).**

**Fig. 11. DNA sequence of the QR-AC<sup>-</sup> plasmid encoding the CyaA/E570Q+K860R/AC<sup>-</sup> mutant (SEQ ID N° 5)**

**Fig. 12. Plasmid encoding the CyaA/2330VA/E570Q+K860R/AC<sup>-</sup> mutant (OVA-QR-AC<sup>-</sup>).**

**Fig. 13. DNA sequence of OVA-QR-AC<sup>-</sup> plasmid encoding the CyaA/2330VA/E570Q+K860R/AC<sup>-</sup> mutant (SEQ ID N° 6)**

## Examples

### Adenylate Cyclase Toxin Translocates Across Target Cell Membrane Without Forming a Pore

#### Materials and Methods

**[0158] Construction, Production and Purification of CyaA proteins.** The modifications yielding CyaA/AC<sup>-</sup>,

CyaA/233OVA, CyaA/E570Q and CyaA/K860R constructs were previously described (13, 20, 21) and were introduced into CyaA/233OVA/AC<sup>-</sup> individually or in combination. The CyaA-derived proteins were produced in *E. coli* XL-1 Blue and purified close to homogeneity as previously described (29). During the hydrophobic chromatography, the resin with bound toxin was repeatedly washed with 60% isopropanol (30) to reduce the endotoxin content of CyaA samples below

100 IU/mg of protein, as determined by the LAL assay QCL-1 000 (Cambrex).  
**[0159]** An *Escherichia coli* XL1-Blue strain (Stratagene) containing the QR-AC<sup>-</sup> plasmid (figure 10) which encodes the CyaA/E570Q+K860R/AC<sup>-</sup> mutant was deposited on March 18, 2009 at the CNCM (Collection Nationale de Cultures de Microorganismes, France) under the accession number CNCM I-4136 (figure 10). The DNA sequence of the QR-AC<sup>-</sup> plasmid (SEQ ID N°5) is disclosed in figure 11.

**[0160]** An *Escherichia coli* XL1-Blue strain (Stratagene) containing the OVA-QR-AC<sup>-</sup> plasmid (figure 12) which encodes the CyaA/233OVA/E570Q+K860R/AC<sup>-</sup> mutant was deposited on March 18, 2009 at the CNCM (Collection Nationale de Cultures de Microorganismes, France) under the accession number CNCM I-4137. The DNA sequence of the OVA-QR-AC<sup>-</sup> plasmid (SEQ ID N° 6) is disclosed in figure 13.

**[0161] Cell Binding and Hemolytic Activities on Sheep Erythrocytes.**  $5 \times 10^8$  washed sheep erythrocytes in 50 mM Tris pH 7.4, 150 mM NaCl and 2 mM CaCl<sub>2</sub> (TNC buffer) were incubated at 37°C with 5 µg/ml of CyaA proteins and cell binding, cell-invasive AC and hemolytic activities of CyaA were determined as described in detail previously (13). Significance of differences in activity values was analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post-test (SigmaStat v. 3.11, Systat, San Jose, CA).

**[0162] Macrophage Binding, Elevation of cAMP and Cell Lysis Capacities of CyaA.** J774A.1 macrophages ( $10^6$ ) were incubated in D-MEM with 2.5 µg/ml of CyaA variants for 30 min at 4°C, prior to removal of unbound toxin by three washes in D-MEM. Cells were lysed with 0.1% Triton X-100 for determination of cell-bound AC activity. For intracellular cAMP assays,  $10^5$  cells were incubated with CyaA for 30 minutes in D-MEM with 100 µM IBMX (3-isobutyl-1-methylxanthin), the reaction was stopped by addition of 0.2% Tween-20 in 100 mM HCl, samples were boiled for 15 min at 100°C, neutralized by addition of 150 mM unbuffered imidazol and cAMP was measured as described (29). To block the CD11b/CD18 receptor, cells were preincubated for 30 min on ice with the CD11b-specific blocking MAb M1/70 (Exbio, Czech Republic) at a final concentration of 10 µg/ml prior to addition of CyaA. Toxin-induced lysis of J774A.1 cells was determined using the CytoTox 96 kit assay (Promega) as the amount of lactate dehydrogenase released from  $10^5$  cells in 3 hours of incubation with 10 µg/ml of the appropriate protein at 37°C in D-MEM as described (8). Significance of differences in activity values was analyzed as above.

**[0163] Patch Clamp Measurements.** Whole-cell patch-clamp measurements were performed on J774A.1 cells bathing in HBSS (140 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 10 mM Hepes-Na, 50 mM glucose; pH 7.4). Fire-polished glass micropipettes with outer diameter of about 3 µm were filled with a solution of 125 mM potassium gluconate, 15 mM KCl, 0.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM EGTA, 10 mM HEPES-KOH pH 7.2. The resulting resistances of the microelectrodes were 3 to 5 MΩ. Cells were clamped at -40 mV, the data were filtered at 1 kHz and digitized at 2 kHz using Axopatch 200A amplifier, Digidata 1320A digitizer and PClamp-9 software package (Axon Instruments, Foster City, CA).

**[0164] Mice and Cell Lines.** Female C57BL/6 obtained from Charles River Laboratories were kept under specific pathogen-free conditions and manipulated according to institutional guidelines. CTLL-2 cells were obtained from ATCC. B3Z, a CD8<sup>+</sup> specific T cell hybridoma specific for the K<sup>b</sup> restricted OVA (SIINFEKL) epitope, was provided by N. Shastri (University of California, Berkeley) and maintained in the presence of 1 mg/ml G418 and 400 µg/ml hygromycin B in complete RPMI 1640 medium (Invitrogen Life Technologies) with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and  $5 \times 10^{-5}$  M 2-ME

**[0165] Antigen Presentation Studies.** Bone Marrow Dendritic Cells (BMDC,  $3 \times 10^5$  per well) used as APCs were incubated in the presence of various concentrations (0 to 60 nM) of the recombinant CyaA/OVA/AC<sup>-</sup> carrying the OVA (SIINFEKL) epitope or mock CyaA/AC<sup>-</sup> and cocultured for 24 hours with B3Z T cells ( $1 \times 10^5$  per well), selectively recognizing the OVA SIINFEKL/H-2K<sup>b</sup> MHC class I complexes. After 18 h of culture, supernatants were frozen for at least 2 h at -80°C. The amount of IL-2 produced by the stimulated B3Z cells was then determined by the CTLL proliferation method. Briefly,  $10^4$  cells of the IL-2-dependent CTLL line per well were cultured with 100 µl of supernatant in 200 µl of final volume. Twenty-four hours later, [<sup>3</sup>H]-thymidine (50 µCi/well) was added and cells were harvested 6 h later with an automated cell harvester. Incorporated [<sup>3</sup>H]-thymidine was detected by scintillation counting. Each point was done in duplicate and the experiment was repeated five times. Results are expressed in Δcpm of incorporated [<sup>3</sup>H]-thymidine (cpm in the presence of toxoid - cpm in the absence of toxoid).

**[0166] In vivo Killing Assay.** For CTL priming, mice were immunized by i.v. injection with 50 µg of recombinant CyaA/OVA/AC<sup>-</sup> carrying the OVA (SIINFEKL) epitope or mock CyaA/AC<sup>-</sup>. Seven days after immunization, naive syngenic splenocytes were pulsed with OVA (SIINFEKL) peptide (10 µg/ml) (30 min, 37°C), washed extensively and labeled with a high concentration (1.25 µM) of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). The nonpulsed control population was labeled with a low concentration (0.125 µM) of CFSE. CFSE<sup>high</sup>- and CFSE<sup>low</sup>-labeled cells were mixed in a 1:1 ratio ( $5 \times 10^6$  cells of each population) and injected i.v. into mice. Spleen cells were collected



20 h after, washed and resuspended in FACS buffer (PBS supplemented with 1% BSA and 0,1% NaN<sub>3</sub>). The number of CFSE-positive cells remaining in the spleen after 20 h was determined by FACS. The percentage of specific lysis was calculated as follows: percent specific lysis = 100 - [100 x (% CFSE<sup>high</sup> immunized mice/% CFSE<sup>low</sup> immunized mice)/(% CFSE<sup>high</sup> naive mouse/% CFSE<sup>low</sup> naive mouse)].

**[0167] Statistical Analysis:** Significance of differences in values was analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post-test (SigmaStat v. 3.11, Systat, San Jose, CA).

## Results

**[0168] Combined Elimination of Negatively Charged Glutamate 570 and of Acylated Lysine 860 Ablates Cell-permeabilizing Capacity of CyaA.** The working model of CyaA action predicts that CyaA can be modified to lose its pore-forming (hemolytic) activity while preserving the capacity to deliver the AC domain into cytosol of target cells. To test this hypothesis, the inventors sought to produce CyaA constructs exhibiting as low hemolytic and cytolytic activities as possible, building on previous observation that the capacity of CyaA/AC<sup>-</sup> toxoids to lyse cells can be modulated both up or down by substitutions within the pore-forming domain (8, 12-14, 18). To enable assessment of target cell penetration also for the CyaA/AC<sup>-</sup> toxoids, the inventors derived such mutants from a CyaA/233OVA toxin that was previously tagged by insertion of the SIINF EKL peptide from ovalbumin (OVA). This CyaA variant was chosen as the insertion of reporter K<sup>b</sup>-restricted CD8<sup>+</sup> T-cell epitope at residue 233 does not affect the AC activity and allows to quantify translocation of the OVA/AC enzyme into cells as elevation of cytosolic cAMP. More importantly, presence of the OVA epitope allows to assess also the capacity of enzymatically inactive CyaA/233OVA/AC<sup>-</sup> toxoids to deliver their OVA/AC<sup>-</sup> domain into cytosol of CD11b<sup>+</sup> antigen presenting cells (APC), as this enables proteasome processing and cell surface presentation of the OVA epitope on MHC Class I glycoproteins that can be determined as stimulation of OVA-specific CD8<sup>+</sup> T cells, both *in vitro* and *in vivo* (20).

**[0169]** To generate CyaA/AC<sup>-</sup> toxoids possibly lacking the cytolytic activity, the inventors combined the E570Q and K860R substitutions previously shown to reduce the specific hemolytic activity of CyaA on sheep erythrocytes, with the E570Q substitution having been found to reduce also the cytolytic activity of the CyaA/AC<sup>-</sup> on CD11b<sup>+</sup> J774A.1 monocytes (8, 13). These substitutions were engineered into CyaA/233OVA/AC<sup>-</sup> individually and in combination and the specific hemolytic and cytolytic activities of resulting toxoids were compared using sheep erythrocytes as model CD11b<sup>-</sup> target and J774A.1 as model CD11b<sup>+</sup> target in parallel (Table I). In agreement with results obtained previously with toxoids lacking the OVA epitope (4, 8, 13, 21), under the used conditions the OVA/AC<sup>-</sup> toxoids carrying individually the E570Q and K860R substitutions exhibited respectively a two-fold reduced (55 ± 8) and nil (1 ± 1) relative hemolytic activity on erythrocytes and the relative cytolytic activity of the E570Q toxoid towards CD11b<sup>-</sup> expressing J774A.1 cells was also reduced (37 ± 10), as compared to OVA/AC<sup>-</sup>. In turn, as expected from results obtained with an enzymatically active K860R construct, despite the low hemolytic activity on CD11b<sup>-</sup> erythrocytes, the K860R toxoid exhibited only a slightly reduced relative cytolytic activity on CD11b<sup>+</sup> J774A.1 cells (72 ± 22%), confirming that the structural defect caused by the K860R substitution was rescued by interaction with the CD11b/CD18 receptor (4). Nevertheless, when combined with E570Q, the K860R substitution exhibited a clear synergic effect in reducing the relative cytolytic activity of the E570Q+K860R construct towards J774A.1 cells down to 14 ± 7%

**Table I : Cytolytic activities of OVA/AC<sup>-</sup> and derivatives on sheep erythrocytes and J774A.1 macrophages.**

Protein	Lysis of erythrocytes (% of AC <sup>-</sup> ) <sup>a</sup>	Lysis of J774A.1 cells (% of AC <sup>-</sup> ) <sup>b</sup>
AC <sup>-</sup>	100 ± 5	100 ± 10
OVA/AC <sup>-</sup>	93 ± 4	93 ± 12
OVA/E570Q/AC <sup>-</sup>	55 ± 8**	37 ± 10**
OVA/K860R/AC <sup>-</sup>	1 ± 1**	72 ± 22**
OVA-L247Q-AC <sup>-</sup>	97 ± 3	41 ± 9
OVA/E570Q+K860R/AC <sup>-</sup>	1 ± 1**	14 ± 7**
OVA-E570Q-L247Q-AC <sup>-</sup>	50 ± 12	40 ± 11
OVA-K860R-L247Q-AC <sup>-</sup>	1 ± 1	45 ± 11
OVA-E570Q-K860R-L247Q-AC <sup>-</sup>	0 ± 1	16 ± 10

Table Legend

**[0170]**

- <sup>a</sup>Lysis of sheep erythrocytes was determined after 4.5 hours as the amount of hemoglobin released upon incubation of  $5 \times 10^8$  RBC at 37°C in the presence of 2 mM  $\text{Ca}^{2+}$  with 5  $\mu\text{g}/\text{ml}$  of the given protein (31). The hemolytic activity of CyaA/AC<sup>-</sup> was taken as 100% activity. The results represent the average of values obtained in four independent experiments performed in duplicates  $\pm$  S.D with two different protein preparations.
- <sup>b</sup>Lysis of J774A.1 cells was determined as the amount of released lactate dehydrogenase from  $10^5$  cells upon 3 hours of cell incubation with 10  $\mu\text{g}/\text{ml}$  of the appropriate protein at 37°C in D-MEM. J774A.1 cell lysis by CyaA/AC<sup>-</sup> was taken as 100%. The results represent the average of values obtained in four separate experiments performed in duplicates  $\pm$  S.D with two different protein preparations (\*,  $p < 0.05$ ; \*\*,  $p < 0.001$ ).

**[0171]** To enable quantification of capacity of the E570Q+K860R construct to deliver the AC domain into cytosol of cells, the E570Q and K860R substitutions were transferred into enzymatically active constructs derived from CyaA/233OVA (CyaA/OVA). These were produced and purified in the same way as the AC<sup>-</sup> toxoids (not shown) and characterized for cell binding, hemolytic and AC translocation capacities on sheep erythrocytes. As shown in Fig. 1A and expected from results with toxins lacking the OVA epitope (4, 13, 21), the E570Q substitution had no impact on erythrocyte binding or the capacity of CyaA/OVA to deliver the AC domain into erythrocyte cytosol and selectively reduced only its relative hemolytic activity. As further expected (4), the K860R substitution significantly reduced the capacity of CyaA/OVA to bind and penetrate erythrocytes, causing a sharp reduction of the relative hemolytic and cell-invasive AC activities of the E570Q and E570Q+K860R mutants on erythrocytes.

**[0172]** It has to be noted, that the hemolytic activity of CyaA is a highly cooperative function of the amount of cell-associated CyaA (Hill number  $>3$ ), suggesting that CyaA oligomerization is a prerequisite for pore formation (22). Therefore, to assess the impact of combined E570Q+K860R substitutions on the hemolytic activity, the loss of erythrocyte-binding capacity of the K860R constructs had to be compensated by increasing their concentration in the assay to 25  $\mu\text{g}/\text{ml}$  (5  $\mu\text{g}/\text{ml}$  for intact toxin), in order to achieve binding of equal amounts of all proteins to erythrocytes, as shown in Fig. 1B. Under these conditions the combination of E570Q and K860R substitutions exhibited a clear synergy in further reducing by a factor of two the already impaired hemolytic activities of constructs carrying the E570Q (~50%) and K860R substitutions (~30%) individually. This suggests that combination of the two substitutions affected the specific cell-permeabilizing capacity of CyaA.

**[0173] Pore-forming Activity of CyaA is Dispensable for Membrane Translocation of the AC Domain.** In contrast to impact of the K860R substitution on toxin activity on erythrocytes, both the E570Q and K860R substitutions were previously found to have no effect on the capacity of CyaA to bind and penetrate J774A.1 monocytes expressing the CD11b/CD18 receptor (4, 8). Moreover, as documented in Fig. 2, when the two substitutions were combined in the same toxin molecule, the CyaA/OVA/E570Q+K860R construct exhibited an equal capacity to bind J774A.1 cells (Fig. 2A) and to deliver the AC domain into their cytosol to elevate cytosolic cAMP concentrations (Fig. 2B), as did intact CyaA. At the same time, however, the doubly mutated E570Q+K860R toxoid exhibited an about seven-fold reduced ( $14 \pm 7\%$ ) relative cytolytic capacity on these cells (cf. Table I). This suggested that the combination of E570Q and K860R substitutions selectively impaired only the capacity of the toxoid to permeabilize J774A.1 cells and not its capacity to translocate the AC domain across cell membrane.

**[0174]** To test this, the inventors analyzed the cell-permeabilizing capacity of the E570Q+K860R construct in single whole cell patch-clamp experiments. Here again the AC<sup>-</sup> toxoids had to be used, in order to avoid the massive ruffling of J774A.1 cells provoked by toxin-generated cAMP (23). As shown in Fig. 3A by a representative recording of ion currents across the membrane of patch-clamped single J774A.1 cells exposed to 1  $\mu\text{g}/\text{ml}$  of CyaA/OVA/AC<sup>-</sup>, upon an initial lag of about 3 minutes the J774A.1 cells were progressively and massively permeabilized by CyaA/OVA/AC<sup>-</sup> and the currents across cell membrane reached - 3,000 pA within 10 minutes. In contrast, as shown in Fig. 3B, exposure to the CyaA/OVA/E570Q+K860R/AC<sup>-</sup> reproducibly caused only a transient and minimal initial permeabilization of the cells, with currents across cell membrane not exceeding -200 pA and returning close to zero within 10 minutes after toxoid addition. The shown recordings were representative of at least six determinations from 3 independent experiments and demonstrate that the combination of the E570Q and K860R substitutions had a major impact on the capacity of the toxoid to permeabilize the membrane of J774A.1 cells. Given that the enzymatically active version of the same construct was fully capable to translocate the AC domain into J774A.1 cells (cf. Fig. 2B), these results strongly suggest that the cell-permeabilizing (pore-forming) activity of CyaA was not required for AC domain translocation across cellular membrane.

**[0175] Membrane-permeabilizing Activity of CyaA is Dispensable for Delivery of Passenger Antigens to the Cytosolic MHC Class I Pathway.** Since the assay for cytosolic cAMP could not be used for assessment of cell penetration capacity of the AC<sup>-</sup> toxoids, the surrogate assay for their capacity to deliver the reporter OVA epitope to the cytosolic processing site of the MHC class I antigen presentation pathway was used (7, 24). Towards this end, the inventors determined the capacity of C57BL/6 mouse bone marrow-derived dendritic cells (BMDCs), loaded with the toxoids, to stimulate IL-2 release by B3Z T cells that selectively recognize the complex of K<sup>b</sup> MHC class I molecules with the SIINFEKL (OVA) peptide on APCs. As shown in Fig. 4A, the B3Z hybridoma cells were effectively stimulated upon co-

incubation with BMDCs and any of the toxoids carrying the OVA epitope, but not with the mock toxoid. Moreover, the OVA/E570Q/AC<sup>-</sup> and OVA/E570Q+K860R/AC<sup>-</sup> toxoids induced stimulation of the B3Z lymphocytes by APCs *in vitro* with as high efficiency as intact OVA/AC<sup>-</sup> toxoid. These results confirm that the E570Q+K860R double mutant was fully capable to translocate its AC domain into BMDC cytosol for processing and presentation of the OVA epitope by K<sup>b</sup> MHC class I molecules, while being essentially unable to permeabilize the J774A.1 cells. These results suggest that the cell-permeabilizing (pore-forming) activity of CyaA was neither required for AC domain translocation across cellular membrane, nor did it play any role in the capacity of CyaA to deliver passenger epitopes into APC cytosol.

**[0176]** To corroborate the observed *in vitro* antigen delivery capacity of the non-cytolytic toxoids, the inventors assessed their *in vivo* capacity to prime OVA-specific cytotoxic CD8<sup>+</sup> T lymphocytes (CTL). 50 µg of the various OVA-toxoids were injected intravenously into C57BL/6 mice and one week later the OVA-specific CTL responses were assessed in immunized mice by an *in vivo* killing assay. C57BL/6 mice received i.v. injection of a mixture (1:1) of OVA (SIINFEKL) peptide-loaded CFSE<sup>high</sup> and unloaded CFSE<sup>low</sup> splenocytes, followed one day later by FACS analysis of CFSE-labeled cells. As shown in Fig. 4B, immunization of mice with the mock toxoid did not induce any SIINFEKL-specific *in vivo* CTL activity. In turn, immunization with the E570Q+K860R toxoid induced the same OVA-specific *in vivo* CTL killing response as the unmutated toxoid used as positive control, with the slight difference in the values of mean response to the intact and doubly mutated toxoids not being statistically significant (p=0.065). These results show that the cell-permeabilizing activity of CyaA was dispensable for the *in vivo* capacity of the CyaA/233OVA/AC<sup>-</sup> toxoids to deliver an AC-inserted passenger antigen into cytosol of APCs.

## Discussion

**[0177]** The inventors demonstrate here that translocation of the AC domain of CyaA across the membrane of CD11b/CD18 receptor-expressing myeloid target cells does not depend on the capacity of the toxin to form pores and permeabilize the cellular membrane.

**[0178]** As summarized in the model proposed in Fig. 5, the inventors have previously reported that balance between the two activities of CyaA can be shifted by mutations or alternative acylation of CyaA. Enhancement of the pore-forming (hemolytic) activity at the expense of the capacity to deliver AC into cells was, indeed, observed upon lysine substitutions of glutamates 509, 516 and 581 (13, 18), or upon blocking of AC translocation by the 3D1 monoclonal antibody (MAb) (25). In turn, a shift in the opposite direction was observed for the recombinant *r-Ec-CyaA*, acylated in *E. coli* by palmitoleyl (C16:1) residues, as compared to the native (C16:0) palmitylated *Bp-CyaA* produced by *B. pertussis*. The *r-Ec-CyaA* was found to exhibit about four-fold reduced hemolytic activity and about ten-fold lower pore-forming activity in planar lipid bilayers than *Bp-CyaA* (12), while both CyaA forms were equally active in penetrating cellular membrane and translocating the AC domain into erythrocytes (17, 26). Moreover, recently the CyaA/E570Q construct was found to exhibit a full capacity to deliver the AC domain into both erythrocytes and J774A.1 macrophages, while exhibiting reduced hemolytic activity and lower specific pore-forming capacity in planar lipid bilayers than intact CyaA, with the CyaA/E570Q/AC<sup>-</sup> toxoid exhibiting a two-fold reduced cytolytic activity on J774A.1 cells (8, 13).

**[0179]** Despite the above mentioned and the many mutant CyaAs that the inventors characterized, the question remained whether formation of a membrane pore by CyaA is required for translocation of the AC domain across the membrane of CD11b-expressing cells. The here described CyaA/233OVA/E570Q+K860R mutant is the first construct with an importantly reduced capacity to permeabilize cells that remains fully capable of translocating the AC domain across cellular membrane. This shows that on its way to cell cytosol the translocating AC domain can bypass the cation-selective pore formed by CyaA.

**[0180]** The mode and path of AC domain translocation across cellular membrane, however, remain to be defined in more detail. Given the differing effects of substitutions of glutamates 509, 516, 570 and 581 on the pore-forming and AC delivery activities of CyaA (8, 13, 18), where the balance between the two activities can be almost entirely shifted in either direction by specific substitutions, the amphipathic helices harboring these glutamate residues appear to be involved in both activities of CyaA in an alternative manner. This is supported by the effect of combined E509K+E516K substitution, which yields a hyper-hemolytic CyaA unable to deliver the AC domain into cells (8, 18), while the here described E570Q+K860R combination yields the opposite, an essentially non-cytolytic CyaA that is fully competent to translocate the AC domain into J774A.1 cells (CD11b<sup>+</sup>). These observations further corroborate the proposed model that the two membrane activities of CyaA would depend on different conformers inserting into membrane, one yielding translocation of the AC domain by toxin monomers and the other leading to formation of oligomeric CyaA pores (13, 18).

**[0181]** It remains to be defined what CyaA segments outside of the pore-forming domain are involved in AC domain translocation across membrane. Given the requirement for its structural integrity (27), the large RTX repeat domain (residues 1006 to 1706) is likely to be taking part in AC translocation into cells. It would be sized enough (700 residues) to form a hydrophilic translocation interface within cellular membrane that might allow passage of an unfolded AC domain across the membrane without a concomitant formation of a real cell-permeabilizing pore. Alternatively, CyaA might promote formation of inverted nonlamellar (inverted hexagonal phase) lipid structures (28), which might potentially take

part in a well sealed protein-lipid interface through which the AC domain could slide into cell cytosol.

[0182] Last not least, a practical discovery reported herein is that the CyaA/E570Q+K860R/AC<sup>-</sup> toxoid with the much reduced cell-permeabilizing (cytolytic) activity, remains fully active in antigen delivery into CD11b<sup>+</sup> APCs. This is of importance in the light of its potential use as enhanced safety profile tool for delivery of tumor-specific antigens in second generation of CyaA/AC<sup>-</sup>-derived vaccines for immunotherapy of cancer.

## References

### [0183]

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### SEQUENCE LISTING

#### [0184]

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45 Ala Gly Lys Ser Leu Phe Asp Asp Gly Leu Gly Ala Ala Pro Gly Val  
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50 Pro Ser Gly Arg Ser Lys Phe Ser Pro Asp Val Leu Glu Thr Val Pro  
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55 Ala Ser Pro Gly Leu Arg Arg Pro Ser Leu Gly Ala Val Glu Arg Gln

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25	Gly	Ala	Gln	Ala	Val	Ala	Ala	Ala	Gln	Arg	Leu	Val	His	Ala	Ile	Ala
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45	Ala	Gly	Gly	Phe	Gly	Val	Ala	Gly	Gly	Ala	Met	Ala	Leu	Gly	Gly	Gly
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50	Ile	Ala	Ala	Ala	Val	Gly	Ala	Gly	Met	Ser	Leu	Thr	Asp	Asp	Ala	Pro
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Gly Leu Val Gln Gln Ser His Tyr Ala Asp Gln Leu Asp Lys Leu Ala  
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Gln Glu Ser Ser Ala Tyr Gly Tyr Glu Gly Asp Ala Leu Leu Ala Gln  
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Leu Tyr Arg Asp Lys Thr Ala Ala Glu Gly Ala Val Ala Gly Val Ser  
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Ala Val Leu Ser Thr Val Gly Ala Ala Val Ser Ile Ala Ala Ala Ala  
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Ser Val Val Gly Ala Pro Val Ala Val Val Thr Ser Leu Leu Thr Gly  
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Ala Leu Asn Gly Ile Leu Arg Gly Val Gln Gln Pro Ile Ile Glu Lys  
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Leu Ala Asn Asp Tyr Ala Arg Lys Ile Asp Glu Leu Gly Gly Pro Gln  
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Ala Tyr Phe Glu Lys Asn Leu Gln Ala Arg His Glu Gln Leu Ala Asn  
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Ser Asp Gly Leu Arg Lys Met Leu Ala Asp Leu Gln Ala Gly Trp Asn  
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Ala Ser Ser Val Ile Gly Val Gln Thr Thr Glu Ile Ser Lys Ser Ala  
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Leu Glu Leu Ala Ala Ile Thr Gly Asn Ala Asp Asn Leu Lys Ser Val  
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Asp Val Phe Val Asp Arg Phe Val Gln Gly Glu Arg Val Ala Gly Gln  
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Pro Val Val Leu Asp Val Ala Ala Gly Gly Ile Asp Ile Ala Ser Arg  
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Lys Gly Glu Arg Pro Ala Leu Thr Phe Ile Thr Pro Leu Ala Ala Pro  
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Gly Glu Glu Gln Arg Arg Arg Thr Lys Thr Gly Arg Ser Glu Phe Thr  
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 Thr Phe Val Glu Ile Val Gly Lys Gln Asp Arg Trp Arg Ile Arg Asp  
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 Gly Ala Ala Asp Thr Thr Ile Asp Leu Ala Lys Val Val Ser Gln Leu  
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 Val Asp Ala Asn Gly Val Leu Lys His Ser Ile Lys Leu Asp Val Ile  
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 Val Glu Leu Ala Arg Val Gly Gln Val Val Glu Val Asp Thr Leu Glu  
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 His Val Gln His Ile Ile Gly Gly Ala Gly Asn Asp Ser Ile Thr  
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 Gly Asn Ala His Asp Asn Phe Leu Ala Gly Gly Ser Gly Asp Asp  
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Arg Leu Asp Gly Gly Ala Gly Asn Asp Thr Leu Val Gly Gly Glu  
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Gly Gln Asn Thr Val Ile Gly Gly Ala Gly Asp Asp Val Phe Leu  
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Gln Asp Leu Gly Val Trp Ser Asn Gln Leu Asp Gly Gly Ala Gly  
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 Val Asp Thr Val Lys Tyr Asn Val His Gln Pro Ser Glu Glu Arg  
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 His Val Lys Asn Ile Glu Asn Leu His Gly Ser Arg Leu Asn Asp  
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 Gly Gly Leu Gly Leu Asp Thr Leu Tyr Gly Glu Asp Gly Asn Asp  
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 Gly Ala Gly Leu Asp Thr Val Asp Tyr Ser Ala Met Ile His Pro  
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 Asp Tyr Tyr Asp Asn Val Arg Asn Val Glu Asn Val Ile Gly Thr  
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 Ser Met Lys Asp Val Leu Ile Gly Asp Ala Gln Ala Asn Thr Leu  
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 Met Gly Gln Gly Gly Asp Asp Thr Val Arg Gly Gly Asp Gly Asp

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55	Asp	Gln	Leu	Ser	Gly	Asp	Ala	Gly	Arg	Asp	Arg	Leu	Tyr	Gly	Glu
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60	Ala	Gly	Asp	Asp	Trp	Phe	Phe	Gln	Asp	Ala	Ala	Asn	Ala	Gly	Asn
	1460					1465						1470			
65	Leu	Leu	Asp	Gly	Gly	Asp	Gly	Arg	Asp	Thr	Val	Asp	Phe	Ser	Gly
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70	Pro	Gly	Arg	Gly	Leu	Asp	Ala	Gly	Ala	Lys	Gly	Val	Phe	Leu	Ser
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Leu Gly Lys Gly Phe Ala Ser Leu Met Asp Glu Pro Glu Thr Ser  
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5 Asn Val Leu Arg Asn Ile Glu Asn Ala Val Gly Ser Ala Arg Asp  
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10 Asp Val Leu Ile Gly Asp Ala Gly Ala Asn Val Leu Asn Gly Leu  
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15 Ala Gly Asn Asp Val Leu Ser Gly Gly Ala Gly Asp Asp Val Leu  
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Leu Gly Asp Glu Gly Ser Asp Leu Leu Ser Gly Asp Ala Gly Asn  
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20 Asp Asp Leu Phe Gly Gly Gln Gly Asp Asp Thr Tyr Leu Phe Gly  
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25 Val Gly Tyr Gly His Asp Thr Ile Tyr Glu Ser Gly Gly Gly His  
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Asp Thr Ile Arg Ile Asn Ala Gly Ala Asp Gln Leu Trp Phe Ala  
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30 Arg Gln Gly Asn Asp Leu Glu Ile Arg Ile Leu Gly Thr Asp Asp  
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35 Ala Leu Thr Val His Asp Trp Tyr Arg Asp Ala Asp His Arg Val  
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40 Glu Ile Ile His Ala Ala Asn Gln Ala Val Asp Gln Ala Gly Ile  
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Glu Lys Leu Val Glu Ala Met Ala Gln Tyr Pro Asp Pro Gly Ala  
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15  
 Glu Lys Asn Ala Thr Leu Met Phe Arg Leu Val Asn Pro His Ser Thr  
 35 40 45

20  
 Ser Leu Ile Ala Glu Gly Val Ala Thr Lys Gly Leu Gly Val His Ala  
 50 55 60

25  
 Lys Ser Ser Asp Trp Gly Leu Gln Ala Gly Tyr Ile Pro Val Asn Pro  
 65 70 75 80

30  
 Asn Leu Ser Lys Leu Phe Gly Arg Ala Pro Glu Val Ile Ala Arg Ala  
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 Asp Asn Asp Val Asn Ser Ser Leu Ala His Gly His Thr Ala Val Asp  
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 Leu Thr Leu Ser Lys Glu Arg Leu Asp Tyr Leu Arg Gln Ala Gly Leu  
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 Val Thr Gly Met Ala Asp Gly Val Val Ala Ser Asn His Ala Gly Tyr  
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 Glu Gln Phe Glu Phe Arg Val Lys Glu Thr Ser Asp Gly Arg Tyr Ala  
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 Val Gln Tyr Arg Arg Lys Gly Gly Asp Asp Phe Glu Ala Val Lys Val  
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Ile Gly Asn Ala Ala Gly Ile Pro Leu Thr Ala Asp Gly Ser Ile Asp  
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Met Phe Ala Ile Met Pro His Leu Ser Asn Phe Arg Asp Ser Ala Arg  
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5 Ser Ser Val Thr Ser Gly Asp Ser Val Thr Asp Tyr Leu Ala Arg Thr  
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10 Arg Arg Ala Ala Ser Glu Ala Thr Gly Gly Leu Asp Arg Glu Arg Ile  
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15 Asp Leu Leu Trp Lys Ile Ala Arg Ala Gly Ala Arg Ser Ala Val Gly  
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Thr Glu Ala Arg Arg Gln Phe Arg Tyr Asp Gly Asp Met Asn Ile Gly  
260 265 270

20 Val Ile Thr Asp Phe Glu Leu Glu Val Arg Asn Ala Leu Asn Arg Arg  
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25 Ala His Ala Val Gly Ala Gln Asp Val Val Gln His Gly Thr Glu Gln  
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30 Asn Asn Pro Phe Pro Glu Ala Asp Glu Lys Ile Phe Val Val Ser Ala  
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Thr Gly Glu Ser Gln Met Leu Thr Arg Gly Gln Leu Lys Glu Tyr Ile  
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35 Gly Gln Gln Arg Gly Glu Gly Tyr Val Phe Tyr Glu Asn Arg Ala Tyr  
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40 Gly Val Ala Gly Lys Ser Leu Phe Asp Asp Gly Leu Gly Ala Ala Pro  
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45 Gly Val Pro Ser Gly Arg Ser Lys Phe Ser Pro Asp Val Leu Glu Thr  
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Val Pro Ala Ser Pro Gly Leu Arg Arg Pro Ser Leu Gly Ala Val Glu  
385 390 395 400

50 Arg Gln Asp Ser Gly Tyr Asp Ser Leu Asp Gly Val Gly Ser Arg Ser  
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55

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Phe Ser Leu Gly Glu Val Ser Asp Met Ala Ala Val Glu Ala Ala Glu  
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5 Leu Glu Met Thr Arg Gln Val Leu His Ala Gly Ala Arg Gln Asp Asp  
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10 Ala Glu Pro Gly Val Ser Gly Ala Ser Ala His Trp Gly Gln Arg Ala  
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15 Leu Gln Gly Ala Gln Ala Val Ala Ala Ala Gln Arg Leu Val His Ala  
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Ile Ala Leu Met Thr Gln Phe Gly Arg Ala Gly Ser Thr Asn Thr Pro  
485 490 495

20 Gln Glu Ala Ala Ser Leu Ser Ala Ala Val Phe Gly Leu Gly Glu Ala  
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25 Ser Ser Ala Val Ala Glu Thr Val Ser Gly Phe Phe Arg Gly Ser Ser  
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30 Arg Trp Ala Gly Gly Phe Gly Val Ala Gly Gly Ala Met Ala Leu Gly  
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Gly Gly Ile Ala Ala Ala Val Gly Ala Gly Met Ser Leu Thr Asp Asp  
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35 Ala Pro Ala Gly Gln Lys Ala Ala Ala Gly Ala Gln Ile Ala Leu Gln  
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40 Leu Thr Gly Gly Thr Val Glu Leu Ala Ser Ser Ile Ala Leu Ala Leu  
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45 Ala Ala Ala Arg Gly Val Thr Ser Gly Leu Gln Val Ala Gly Ala Ser  
595 600 605

Ala Gly Ala Ala Ala Gly Ala Leu Ala Ala Ala Leu Ser Pro Met Glu  
610 615 620

50 Ile Tyr Gly Leu Val Gln Gln Ser His Tyr Ala Asp Gln Leu Asp Lys  
625 630 635 640

55



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Leu Ala Gln Glu Ser Ser Ala Tyr Gly Tyr Glu Gly Asp Ala Leu Leu  
 645 650 655  
 5  
 Ala Gln Leu Tyr Arg Asp Lys Thr Ala Ala Glu Gly Ala Val Ala Gly  
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 10  
 Val Ser Ala Val Leu Ser Thr Val Gly Ala Ala Val Ser Ile Ala Ala  
 675 680 685  
 15  
 Ala Ala Ser Val Val Gly Ala Pro Val Ala Val Val Thr Ser Leu Leu  
 690 695 700  
 20  
 Thr Gly Ala Leu Asn Gly Ile Leu Arg Gly Val Gln Gln Pro Ile Ile  
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 Glu Lys Leu Ala Asn Asp Tyr Ala Arg Lys Ile Asp Glu Leu Gly Gly  
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 30  
 Pro Gln Ala Tyr Phe Glu Lys Asn Leu Gln Ala Arg His Glu Gln Leu  
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 Ala Asn Ser Asp Gly Leu Arg Lys Met Leu Ala Asp Leu Gln Ala Gly  
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 Trp Asn Ala Ser Ser Val Ile Gly Val Gln Thr Thr Glu Ile Ser Lys  
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 Ser Ala Leu Glu Leu Ala Ala Ile Thr Gly Asn Ala Asp Asn Leu Lys  
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 Ser Val Asp Val Phe Val Asp Arg Phe Val Gln Gly Glu Arg Val Ala  
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 Gly Gln Pro Val Val Leu Asp Val Ala Ala Gly Gly Ile Asp Ile Ala  
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 Ser Arg Lys Gly Glu Arg Pro Ala Leu Thr Phe Ile Thr Pro Leu Ala  
 835 840 845  
 Ala Pro Gly Glu Glu Gln Arg Arg Arg Thr Lys Thr Gly Arg Ser Glu  
 850 855 860  
 Phe Thr Thr Phe Val Glu Ile Val Gly Lys Gln Asp Arg Trp Arg Ile



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Glu Arg Leu Glu Arg Met Gly Asp Thr Gly Ile His Ala Asp Leu  
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 Gln Lys Gly Thr Val Glu Lys Trp Pro Ala Leu Asn Leu Phe Ser  
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 Val Asp His Val Lys Asn Ile Glu Asn Leu His Gly Ser Arg Leu  
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 Asn Asp Arg Ile Ala Gly Asp Asp Gln Asp Asn Glu Leu Trp Gly  
 1145 1150 1155  
 His Asp Gly Asn Asp Thr Ile Arg Gly Arg Gly Gly Asp Asp Ile  
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 Leu Arg Gly Gly Leu Gly Leu Asp Thr Leu Tyr Gly Glu Asp Gly  
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 Asn Asp Ile Phe Leu Gln Asp Asp Glu Thr Val Ser Asp Asp Ile  
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 Asp Gly Gly Ala Gly Leu Asp Thr Val Asp Tyr Ser Ala Met Ile  
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 His Pro Gly Arg Ile Val Ala Pro His Glu Tyr Gly Phe Gly Ile  
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 Glu Ala Asp Leu Ser Arg Glu Trp Val Arg Lys Ala Ser Ala Leu  
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 Gly Val Asp Tyr Tyr Asp Asn Val Arg Asn Val Glu Asn Val Ile  
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 Gly Thr Ser Met Lys Asp Val Leu Ile Gly Asp Ala Gln Ala Asn  
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 Thr Leu Met Gly Gln Gly Gly Asp Asp Thr Val Arg Gly Gly Asp  
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 Gly Asp Asp Leu Leu Phe Gly Gly Asp Gly Asn Asp Met Leu Tyr  
 1295 1300 1305  
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Gly Asp Ala Gly Asn Asp Thr Leu Tyr Gly Gly Leu Gly Asp Asp  
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 Thr Leu Glu Gly Gly Ala Gly Asn Asp Trp Phe Gly Gln Thr Gln  
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 Ala Arg Glu His Asp Val Leu Arg Gly Gly Asp Gly Val Asp Thr  
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 Val Asp Tyr Ser Gln Thr Gly Ala His Ala Gly Ile Ala Ala Gly  
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 Arg Ile Gly Leu Gly Ile Leu Ala Asp Leu Gly Ala Gly Arg Val  
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 Asp Lys Leu Gly Glu Ala Gly Ser Ser Ala Tyr Asp Thr Val Ser  
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 Ser Gly Pro Gly Arg Gly Leu Asp Ala Gly Ala Lys Gly Val Phe  
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 Leu Ser Leu Gly Lys Gly Phe Ala Ser Leu Met Asp Glu Pro Glu  
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5	Arg	Asp	Asp	Val	Leu	Ile	Gly	Asp	Ala	Gly	Ala	Asn	Val	Leu	Asn
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15	Val	Leu	Leu	Gly	Asp	Glu	Gly	Ser	Asp	Leu	Leu	Ser	Gly	Asp	Ala
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20	Gly	Asn	Asp	Asp	Leu	Phe	Gly	Gly	Gln	Gly	Asp	Asp	Thr	Tyr	Leu
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25	Phe	Gly	Val	Gly	Tyr	Gly	His	Asp	Thr	Ile	Tyr	Glu	Ser	Gly	Gly
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40	Asp	Asp	Ala	Leu	Thr	Val	His	Asp	Trp	Tyr	Arg	Asp	Ala	Asp	His
		1640					1645					1650			
45	Arg	Val	Glu	Ile	Ile	His	Ala	Ala	Asn	Gln	Ala	Val	Asp	Gln	Ala
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10 Glu Lys Asn Ala Thr Leu Met Phe Arg Leu Val Asn Pro His Ser Thr  
35 40 45

15 Ser Leu Ile Ala Glu Gly Val Ala Thr Lys Gly Leu Gly Val His Ala  
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Lys Ser Ser Asp Trp Gly Leu Gln Ala Gly Tyr Ile Pro Val Asn Pro  
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20 Asn Leu Ser Lys Leu Phe Gly Arg Ala Pro Glu Val Ile Ala Arg Ala  
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25 Asp Asn Asp Val Asn Ser Ser Leu Ala His Gly His Thr Ala Val Asp  
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30 Leu Thr Leu Ser Lys Glu Arg Leu Asp Tyr Leu Arg Gln Ala Gly Leu  
115 120 125

Val Thr Gly Met Ala Asp Gly Val Val Ala Ser Asn His Ala Gly Tyr  
130 135 140

35 Glu Gln Phe Glu Phe Arg Val Lys Glu Thr Ser Asp Gly Arg Tyr Ala  
145 150 155 160

40 Val Gln Tyr Arg Arg Lys Gly Gly Asp Asp Phe Glu Ala Val Lys Val  
165 170 175

Ile Gly Asn Ala Ala Gly Ile Pro Leu Thr Ala Asp Gly Ser Ile Asp  
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45 Met Phe Ala Ile Met Pro His Leu Ser Asn Phe Arg Asp Ser Ala Arg  
195 200 205

50

55

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Ser Ser Val Thr Ser Gly Asp Ser Val Thr Asp Tyr Leu Ala Arg Thr  
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 Arg Arg Ala Ala Ser Glu Ala Thr Gly Gly Val Leu Ser Ile Ile Asn  
 225 230 235 240  
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 Phe Glu Lys Leu Val His Leu Asp Arg Glu Arg Ile Asp Leu Leu Trp  
 245 250 255  
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 Lys Ile Ala Arg Ala Gly Ala Arg Ser Ala Val Gly Thr Glu Ala Arg  
 260 265 270  
 Arg Gln Phe Arg Tyr Asp Gly Asp Met Asn Ile Gly Val Ile Thr Asp  
 275 280 285  
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 Phe Glu Leu Glu Val Arg Asn Ala Leu Asn Arg Arg Ala His Ala Val  
 290 295 300  
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 Gly Ala Gln Asp Val Val Gln His Gly Thr Glu Gln Asn Asn Pro Phe  
 305 310 315 320  
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 Pro Glu Ala Asp Glu Lys Ile Phe Val Val Ser Ala Thr Gly Glu Ser  
 325 330 335  
 Gln Met Leu Thr Arg Gly Gln Leu Lys Glu Tyr Ile Gly Gln Gln Arg  
 340 345 350  
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 Gly Glu Gly Tyr Val Phe Tyr Glu Asn Arg Ala Tyr Gly Val Ala Gly  
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 Lys Ser Leu Phe Asp Asp Gly Leu Gly Ala Ala Pro Gly Val Pro Ser  
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 Gly Arg Ser Lys Phe Ser Pro Asp Val Leu Glu Thr Val Pro Ala Ser  
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 Pro Gly Leu Arg Arg Pro Ser Leu Gly Ala Val Glu Arg Gln Asp Ser  
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 Gly Tyr Asp Ser Leu Asp Gly Val Gly Ser Arg Ser Phe Ser Leu Gly  
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 Glu Val Ser Asp Met Ala Ala Val Glu Ala Ala Glu Leu Glu Met Thr

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10	Val	Ser	Gly	Ala	Ser	Ala	His	Trp	Gly	Gln	Arg	Ala	Leu	Gln	Gly	Ala	
	465					470					475					480	
15	Gln	Ala	Val	Ala	Ala	Ala	Gln	Arg	Leu	Val	His	Ala	Ile	Ala	Leu	Met	
					485					490					495		
20	Thr	Gln	Phe	Gly	Arg	Ala	Gly	Ser	Thr	Asn	Thr	Pro	Gln	Glu	Ala	Ala	
				500					505					510			
25	Ser	Leu	Ser	Ala	Ala	Val	Phe	Gly	Leu	Gly	Glu	Ala	Ser	Ser	Ala	Val	
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### 35 Claims

1. A polypeptide whose amino acid sequence comprises or consists of one of the following sequences:

40 (a) an amino acid sequence which is a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 of SEQ ID NO:1, into which:

(i) the glutamic acid residue, corresponding to the position 570 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, is substituted by a glutamine residue; and

45 (ii) the lysine residue, corresponding to the position 860 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, is substituted by an arginine residue,

said fragment having the capacity of the CyaA protein of *Bordetella pertussis* to bind to the CD11b/CD18 receptor of cells expressing the same, the capacity to translocate its N-terminal adenylate cyclase enzyme domain into said cells and having a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin; or

50 (b) an amino acid sequence which differs from a fragment which has a stretch of amino acids comprising amino acid residues from 1 to 860 or from 2 to 860 of SEQ ID NO:1, by:

55 (i) the substitution of the glutamic acid residue, corresponding to the position 570 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, by a glutamine residue;

(ii) the substitution of the lysine residue, corresponding to the position 860 in the sequence of the native CyaA of *Bordetella pertussis* as defined in SEQ ID NO: 1, by an arginine residue; and

(iii) from 1 to 50 additional substitutions;



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the polypeptide consisting of said sequence having the capacity of the CyaA protein of *Bordetella Pertussis* to bind to the CD11b/CD18 receptor of cells expressing the same, the capacity to translocate its N-terminal adenylate cyclase enzyme domain into said cells and having a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

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2. Polypeptide according to claim 1, wherein said fragment is at least 900, 1000, 1100, 1200, 1300, 1400, 1500 or 1600 amino acid residues of SEQ ID NO:1, in size.

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3. Polypeptide according to claim 1 or 2, which is a mutant of an adenylate cyclase toxoid whose adenylate cyclase activity in cells is partly or totally suppressed as compared to that of the *Bordetella pertussis* CyaA toxin.

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4. Polypeptide according to claim 3, wherein said partial or total suppression of adenylate cyclase activity is achieved by insertion of a dipeptide between the amino acid residues corresponding to the positions 188 and 189 of SEQ ID NO: 1.

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5. Polypeptide according to claim 1, the amino acid sequence of which is SEQ ID NO:2.

6. Polypeptide according to claim 1, which is the amino acid sequence disclosed as SEQ ID NO: 2 into which a dipeptide has been inserted between positions 188 and 189, preferably which is the amino acid sequence disclosed as SEQ ID NO:3.

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7. Polypeptide derivative comprising or consisting of the polypeptide as defined in any one of claims 1 to 6 further combined with one or more molecules of interest.

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8. Polypeptide derivative according to claim 7, wherein each of said one or more molecules of interest consists of an amino acid sequence suitable for eliciting an immune response.

9. Polypeptide derivative according to claim 8, wherein the amino acid sequence of each of said molecule(s) suitable for eliciting an immune response consists of 5 to 800 especially 300 to 600, or 400 to 500 amino acid residues.

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10. Polypeptide derivative according to claim 8 or 9, wherein the amino acid sequence of each of said molecule(s) suitable for eliciting an immune response comprises or consists of an amino acid sequence of a poliovirus antigen, an HIV virus antigen, an influenza virus antigen, a choriomeningitis virus sequence, a tumor antigen, or comprises or consists of a part of an amino acid sequence of any of these antigens which comprises at least one epitope.

40

11. Polypeptide derivative according to any one of claims 8 to 10, which is a recombinant polypeptide wherein the amino acid sequence of each of said molecule(s) suitable for eliciting an immune response is inserted into a permissive site of said polypeptide, provided that the capacity of said polypeptide to translocate its N-terminal adenylate cyclase enzyme domain into CD11b/CD18 expressing cells is preserved.

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12. Polypeptide derivative according to claim 8 or 9, wherein each of said amino acid sequences suitable for eliciting an immune response is grafted, especially chemically grafted, onto an amino acid residue of said polypeptide.

13. Polypeptide according to any one of claims 1 to 6 or polypeptide derivative according to any one of claims 7 to 12, for use in therapy.

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14. Polypeptide or polypeptide derivative according to claim 13, for use in therapy in order to elicit a T-cell immune response and/or in order to elicit a B-cell immune response in a host in need thereof.

15. Polypeptide derivative according to any one of claims 7 to 12, for use in the prevention or the treatment of a disease selected from neoplasia, cancers and infectious diseases selected from viral-, retroviral-, bacterial-, parasite- or fungal-induced diseases.

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16. Polypeptide or polypeptide derivative for use according to any one of claims 13 to 15, wherein said polypeptide is to be administered in combination with an adjuvant and/or in combination with another therapeutically active molecule.

17. Polypeptide or polypeptide derivative for use according to any one of claims 13 to 15, wherein said polypeptide is not to be administered in combination with an adjuvant.

18. Pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 6 or a polypeptide derivative according to any one of claims 7 to 12 and a pharmaceutically acceptable carrier, and optionally an adjuvant and/or a therapeutically active molecule.
- 5 19. Use of a polypeptide derivative according to any one of claims 7 to 12, for the preparation of a therapeutic composition for the treatment of a disease selected from neoplasia, cancers and infectious diseases selected from viral-, retroviral, bacterial-, parasite- or fungal- induced diseases.
- 10 20. Method for the preparation of a proteinaceous vector suitable for the delivery of a molecule into a CD11b/CD18 expressing cell comprising binding said molecule to a polypeptide according to any one of claims 1 to 6.

### Patentansprüche

- 15 1. Polypeptid, dessen Aminosäuresequenz eine der folgenden Sequenzen umfasst oder daraus besteht:
- (a) eine Aminosäuresequenz, die ein Fragment ist, das einen Abschnitt von Aminosäuren aufweist, umfassend Aminosäurereste von 1 bis 860 oder von 2 bis 860 von SEQ ID NO: 1, in welcher:
- 20 (i) der Glutaminsäurerest, entsprechend der Position 570 in der Sequenz des nativen CyaA von Bordetella pertussis, wie in SEQ ID NO: 1 definiert, durch einen Glutaminrest substituiert ist; und
- (ii) der Lysinrest, entsprechend der Position 860 in der Sequenz des nativen CyaA von Bordetella pertussis, wie in SEQ ID NO: 1 definiert, durch einen Argininrest substituiert ist,
- 25 wobei das Fragment die Fähigkeit des CyaA-Proteins von Bordetella pertussis, an den CD11b/CD18-Rezeptor von Zellen, die diesen exprimieren, zu binden, die Fähigkeit, dessen N-terminale Adenylatcyclase-Enzymdomäne in die Zellen zu translozieren, aufweist, und das eine Poren-bildenden Aktivität aufweist, die im Vergleich zu der des nativen CyaA-Toxins reduziert oder supprimiert ist; oder
- (b) eine Aminosäuresequenz, die sich von einem Fragment, das einen Abschnitt von Aminosäuren aufweist, umfassend Aminosäurereste von 1 bis 860 oder von 2 bis 860 von SEQ ID NO: 1 unterscheidet durch:
- 30 (i) die Substitution des Glutaminsäurerestes, entsprechend zu der Position 570 in der Sequenz des nativen CyaA von Bordetella pertussis, wie in SEQ ID NO: 1 definiert, durch einen Glutaminrest;
- (ii) die Substitution des Lysinrestes, entsprechend zu der Position 860 in der Sequenz des nativen CyaA von Bordetella pertussis, wie in SEQ ID NO: 1 definiert, durch einen Argininrest; und
- 35 (iii) von 1 bis 50 zusätzlichen Substitutionen;
- wobei das Polypeptid aus der Sequenz, mit der Fähigkeit des CyaA-Proteins von Bordetella pertussis, an den CD11b/CD18-Rezeptor von Zellen, die diesen exprimieren, zu binden, der Fähigkeit, dessen N-terminale Adenylatcyclase-Enzymdomäne in die Zellen zu translozieren, und mit einer Poren-bildenden Aktivität, die im Vergleich zu der des nativen CyaA-Toxins reduziert oder supprimiert ist, besteht.
- 40
2. Polypeptid nach Anspruch 1, wobei das Fragment in der Größe mindestens 900, 1000, 1100, 1200, 1300, 1400, 1500 oder 1600 Aminosäurereste von SEQ ID NO: 1 ist.
- 45
3. Polypeptid nach Anspruch 1 oder 2, das eine Mutante eines Adenylatcyclase-Toxoids ist, dessen Adenylatcyclaseaktivität in Zellen im Vergleich zu der des Bordetella pertussis CyaA-Toxins teilweise oder vollständig supprimiert ist.
- 50
4. Polypeptid nach Anspruch 3, wobei die teilweise oder vollständige Suppression der Adenylatcyclaseaktivität erreicht wird durch Insertion eines Dipeptids zwischen den Aminosäureresten, entsprechend zu den Positionen 188 und 189 von SEQ ID NO: 1.
- 55
5. Polypeptid nach Anspruch 1, dessen Aminosäuresequenz SEQ ID NO: 2 ist.
6. Polypeptid nach Anspruch 1, welches die Aminosäuresequenz ist, offenbart als SEQ ID NO: 2, in die ein Dipeptid zwischen Positionen 188 und 189 eingefügt worden ist, vorzugsweise welches die als SEQ ID NO: 3 offenbarte Aminosäuresequenz ist.

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7. Polypeptidderivat, umfassend oder bestehend aus dem wie in einem beliebigen der Ansprüche 1 bis 6 definierten Polypeptid, des Weiteren kombiniert mit einem oder mehreren Molekülen von Interesse.
- 5 8. Polypeptidderivat nach Anspruch 7, wobei jedes des einen Moleküls oder der mehreren Moleküle von Interesse aus einer Aminosäuresequenz besteht, die zum Auslösen einer Immunantwort geeignet ist.
9. Polypeptidderivat nach Anspruch 8, wobei die Aminosäuresequenz von jedem des Moleküls/der Moleküle, das/die zum Auslösen einer Immunantwort geeignet ist/sind, aus 5 bis 800, insbesondere 300 bis 600, oder 400 bis 500 Aminosäureresten besteht.
- 10 10. Polypeptidderivat nach Anspruch 8 oder 9, wobei die Aminosäuresequenz von jedem des Moleküls/der Moleküle, das/die zum Auslösen einer Immunantwort geeignet ist/sind, eine Aminosäuresequenz eines Poliovirus-Antigens, eines HIV-Virus-Antigens, eines Influenzavirus-Antigens, einer Choriomeningitisvirus-Sequenz, eines Tumor-Antigens umfasst oder daraus besteht, oder einen Teil einer Aminosäuresequenz von einem beliebigen dieser Antigene, der mindestens ein Epitop umfasst, umfasst oder daraus besteht.
- 15 11. Polypeptidderivat nach einem beliebigen der Ansprüche 8 bis 10, welches ein rekombinantes Polypeptid ist, wobei die Aminosäuresequenz von jedem des Moleküls/der Moleküle, das/die zum Auslösen einer Immunantwort geeignet ist/sind, in eine permissive Stelle des Polypeptids eingefügt ist, mit der Maßgabe, dass die Fähigkeit des Polypeptids, dessen N-terminale Adenylatcyclase-Enzymdomäne in CD11b/CD18 exprimierende Zellen zu translozieren, bewahrt wird.
- 20 12. Polypeptidderivat nach Anspruch 8 oder 9, wobei jede der zum Auslösen einer Immunantwort geeigneten Aminosäuresequenzen auf einen Aminosäurerest des Polypeptids gegrattet wird, insbesondere chemisch gegrattet.
- 25 13. Polypeptid nach einem beliebigen der Ansprüche 1 bis 6 oder Polypeptidderivat nach einem beliebigen der Ansprüche 7 bis 12 zur Verwendung in Therapie.
- 30 14. Polypeptid oder Polypeptidderivat nach Anspruch 13 zur Verwendung in Therapie, um eine T-Zell-Immunantwort auszulösen und/oder um eine B-Zell-Immunantwort in einem Wirt, der dessen bedarf, auszulösen.
- 35 15. Polypeptidderivat nach einem beliebigen der Ansprüche 7 bis 12 zur Verwendung in der Prävention oder der Behandlung einer Erkrankung, ausgewählt aus Neoplasie, Krebs und Infektionserkrankungen, ausgewählt aus viral-, retroviral-, bakteriell-, parasitär- oder Pilz-induzierten Erkrankungen.
- 40 16. Polypeptid oder Polypeptidderivat zur Verwendung nach einem beliebigen der Ansprüche 13 bis 15, wobei das Polypeptid in Kombination mit einem Adjuvans und/oder in Kombination mit einem anderen therapeutisch aktiven Molekül zu verabreichen ist.
- 45 17. Polypeptid oder Polypeptidderivat zur Verwendung nach einem beliebigen der Ansprüche 13 bis 15, wobei das Polypeptid nicht in Kombination mit einem Adjuvans zu verabreichen ist.
18. Pharmazeutische Zusammensetzung, umfassend ein Polypeptid gemäß einem beliebigen der Ansprüche 1 bis 6 oder ein Polypeptidderivat gemäß einem beliebigen der Ansprüche 7 bis 12 und einen pharmazeutisch verträglichen Träger und gegebenenfalls ein Adjuvans und/oder ein therapeutisches aktives Molekül.
- 50 19. Verwendung eines Polypeptidderivats nach einem beliebigen der Ansprüche 7 bis 12 zur Herstellung einer therapeutischen Zusammensetzung für die Behandlung einer Erkrankung, ausgewählt aus Neoplasie, Krebs und Infektionserkrankungen, ausgewählt aus viral-, retroviral-, bakteriell-, parasitär- oder Pilz-induzierten Erkrankungen.
- 55 20. Verfahren zur Herstellung eines proteinösen Vektors, geeignet zur Lieferung eines Moleküls in eine CD11b/CD18 exprimierende Zelle, umfassend das Binden des Moleküls an ein Polypeptid gemäß einem beliebigen der Ansprüche 1 bis 6.

### Revendications

1. Polypeptide dont la séquence d'acides aminés comprend ou est constitué de l'une des séquences suivantes :

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(a) une séquence d'acides aminés qui est un fragment qui a une étendue d'acides aminés comprenant les résidus d'acides aminés de 1 à 860 ou de 2 à 860 de SEQ ID NO: 1, dans laquelle :

- 5 (i) le résidu acide glutamique, correspondant à la position 570 dans la séquence de la CyaA native de *Bordetella pertussis* telle que définie dans SEQ ID NO: 1, est substitué par un résidu glutamine ; et  
(ii) le résidu lysine, correspondant à la position 860 dans la séquence de la CyaA native de *Bordetella pertussis* telle que définie dans SEQ ID NO: 1, est substitué par un résidu arginine,

10 ledit fragment ayant la capacité de la protéine CyaA de *Bordetella pertussis* de se lier au récepteur CD11b/CD18 des cellules l'exprimant, la capacité de transloquer son domaine d'enzyme adénylate cyclase N-terminal dans lesdites cellules et ayant une activité de formation de pores qui est réduite ou supprimée par rapport à celle de la toxine CyaA native ; ou

(b) une séquence d'acides aminés qui diffère d'un fragment qui a une étendue d'acides aminés comprenant les résidus d'acides aminés de 1 à 860 ou de 2 à 860 de SEQ ID NO: 1, par :

- 15 (i) la substitution du résidu acide glutamique, correspondant à la position 570 dans la séquence de la CyaA native de *Bordetella pertussis* telle que définie dans SEQ ID NO: 1, par un résidu glutamine ;  
(ii) la substitution du résidu lysine, correspondant à la position 860 dans la séquence de la CyaA native de *Bordetella pertussis* telle que définie dans SEQ ID NO: 1, par un résidu arginine ; et  
20 (iii) de 1 à 50 substitutions supplémentaires ;

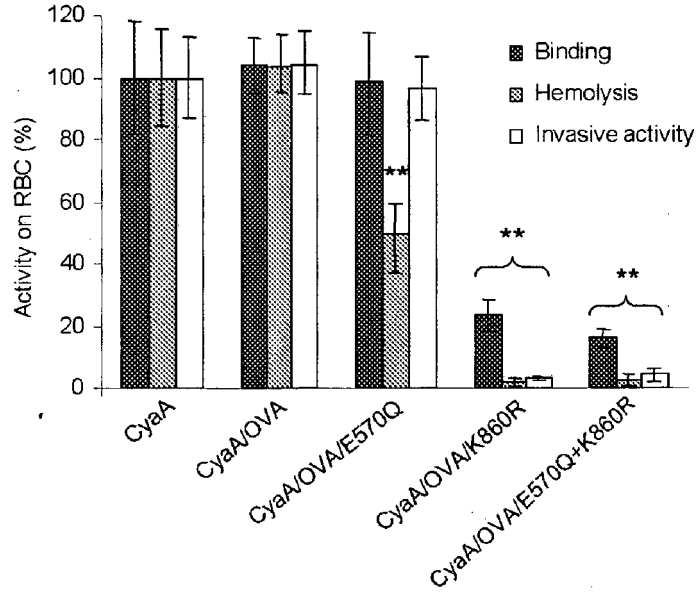
le polypeptide constitué de ladite séquence ayant la capacité de la protéine CyaA de *Bordetella pertussis* à se lier au récepteur CD11b/CD18 des cellules l'exprimant, la capacité de transloquer son domaine d'enzyme adénylate cyclase N-terminal dans lesdites cellules et ayant une activité de formation de pores qui est réduite ou supprimée par rapport à celle de la toxine CyaA native.

- 25
2. Polypeptide selon la revendication 1, dans lequel ledit fragment a une taille d'au moins 900, 1 000, 1 100, 1 200, 1 300, 1 400, 1 500 ou 1 600 résidus d'acides aminés de SEQ ID NO: 1.
  - 30 3. Polypeptide selon la revendication 1 ou 2, qui est un mutant d'une anatoxine adénylate cyclase dont l'activité adénylate cyclase dans les cellules est supprimée en partie ou totalement par rapport à celle de la toxine CyaA de *Bordetella pertussis*.
  4. Polypeptide selon la revendication 3, dans lequel ladite suppression partielle ou totale de l'activité adénylate cyclase est obtenue par insertion d'un dipeptide entre les résidus d'acides aminés correspondant aux positions 188 et 189 de SEQ ID NO: 1.
  - 35 5. Polypeptide selon la revendication 1, dont la séquence d'acides aminés est SEQ ID NO: 2.
  - 40 6. Polypeptide selon la revendication 1, qui est la séquence d'acides aminés décrite comme SEQ ID NO: 2 dans laquelle un dipeptide a été inséré entre les positions 188 et 189, qui est de préférence la séquence d'acides aminés décrite comme SEQ ID NO: 3.
  7. Dérivé polypeptidique comprenant ou constitué par le polypeptide tel que défini selon l'une quelconque des revendications 1 à 6, combiné en outre avec une ou plusieurs molécules d'intérêt.
  - 45 8. Dérivé polypeptidique selon la revendication 7, dans lequel chacune desdites une ou plusieurs molécules d'intérêt est constituée d'une séquence d'acides aminés appropriée pour susciter une réponse immunitaire.
  - 50 9. Dérivé polypeptidique selon la revendication 8, dans lequel la séquence d'acides aminés de chacune de ladite(des) molécule(s) appropriée(s) pour susciter une réponse immunitaire est constituée de 5 à 800 en particulier 300 à 600, ou 400 à 500 résidus d'acides aminés.
  - 55 10. Dérivé polypeptidique selon la revendication 8 ou 9, dans lequel la séquence d'acides aminés de chacune de ladite(des) molécule(s) appropriée(s) pour susciter une réponse immunitaire comprend ou est constituée d'une séquence d'acides aminés d'un antigène de poliovirus, d'un antigène de virus VIH, d'un antigène de virus de l'influenza, d'une séquence de virus de la chorioméningite, d'un antigène tumoral, ou comprend ou est constituée d'une partie d'une séquence d'acides aminés de n'importe lequel de ces antigènes qui comprend au moins un épitope.

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- 5
11. Dérivé polypeptidique selon l'une quelconque des revendications 8 à 10, qui est un polypeptide recombinant dans lequel la séquence d'acides aminés de chacune de ladite(s) molécule(s) appropriée(s) pour susciter une réponse immunitaire est insérée dans un site permissif dudit polypeptide, à condition que la capacité dudit polypeptide de transloquer son domaine d'enzyme adénylate-cyclase N-terminal dans les cellules exprimant CD11b/CD18 soit conservée.
- 10
12. Dérivé polypeptidique selon la revendication 8 ou 9, dans lequel chacune desdites séquences d'acides aminés appropriées pour susciter une réponse immunitaire est greffé, en particulier greffé chimiquement, sur un résidu d'acide aminé dudit polypeptide.
- 15
13. Polypeptide selon l'une quelconque des revendications 1 à 6, ou dérivé polypeptidique selon l'une quelconque des revendications 7 à 12, pour une utilisation en thérapie.
- 20
14. Polypeptide ou dérivé polypeptidique selon la revendication 13, pour utilisation en thérapie afin de susciter une réponse immunitaire à cellules T et/ou afin de susciter une réponse immunitaire à cellules B chez un hôte en ayant besoin.
- 25
15. Dérivé polypeptidique selon l'une quelconque des revendications 7 à 12, pour une utilisation dans la prévention ou le traitement d'une maladie choisie parmi une néoplasie, les cancers et les maladies infectieuses choisies parmi les maladies d'origine virale, rétrovirale, bactérienne, parasitaire ou fongique.
- 30
16. Polypeptide ou dérivé polypeptidique pour une utilisation selon l'une quelconque des revendications 13 à 15, dans lequel ledit polypeptide doit être administré en association avec un adjuvant et/ou en association avec une autre molécule thérapeutiquement active.
- 35
17. Polypeptide ou dérivé polypeptidique pour une utilisation selon l'une quelconque des revendications 13 à 15, dans lequel ledit polypeptide n'est pas destiné à être administré en association avec un adjuvant.
- 40
18. Composition pharmaceutique comprenant un polypeptide selon l'une quelconque des revendications 1 à 6 ou un dérivé polypeptidique selon l'une quelconque des revendications 7 à 12 et un support pharmaceutiquement acceptable, et éventuellement un adjuvant et/ou une molécule thérapeutiquement active.
- 45
19. Utilisation d'un dérivé polypeptidique selon l'une quelconque des revendications 7 à 12, pour la préparation d'une composition thérapeutique pour le traitement d'une maladie choisie parmi une néoplasie, les cancers et les maladies infectieuses choisies parmi les maladies d'origine virale, rétrovirale, bactérienne, parasitaire ou fongique.
- 50
20. Procédé de préparation d'un vecteur protéinique approprié pour la délivrance d'une molécule dans une cellule exprimant CD11b/CD18 comprenant la liaison de ladite molécule à un polypeptide selon l'une quelconque des revendications 1 à 6.
- 55

A



B

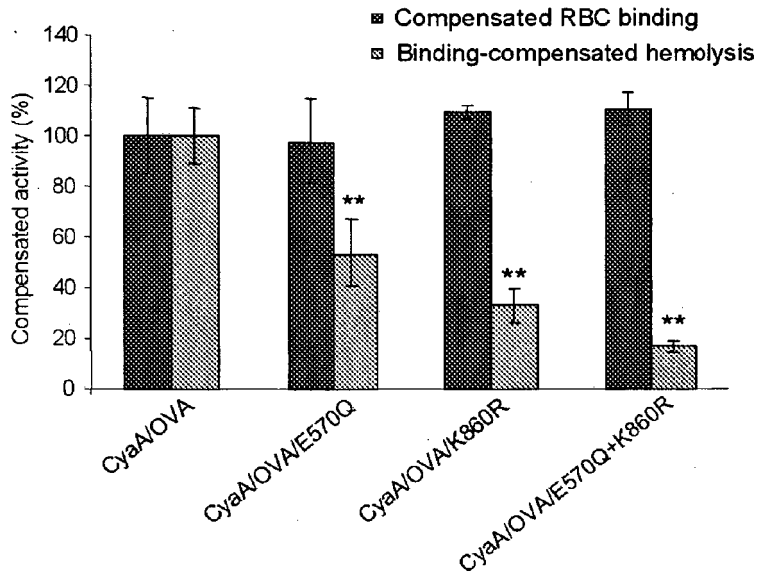


Figure 1

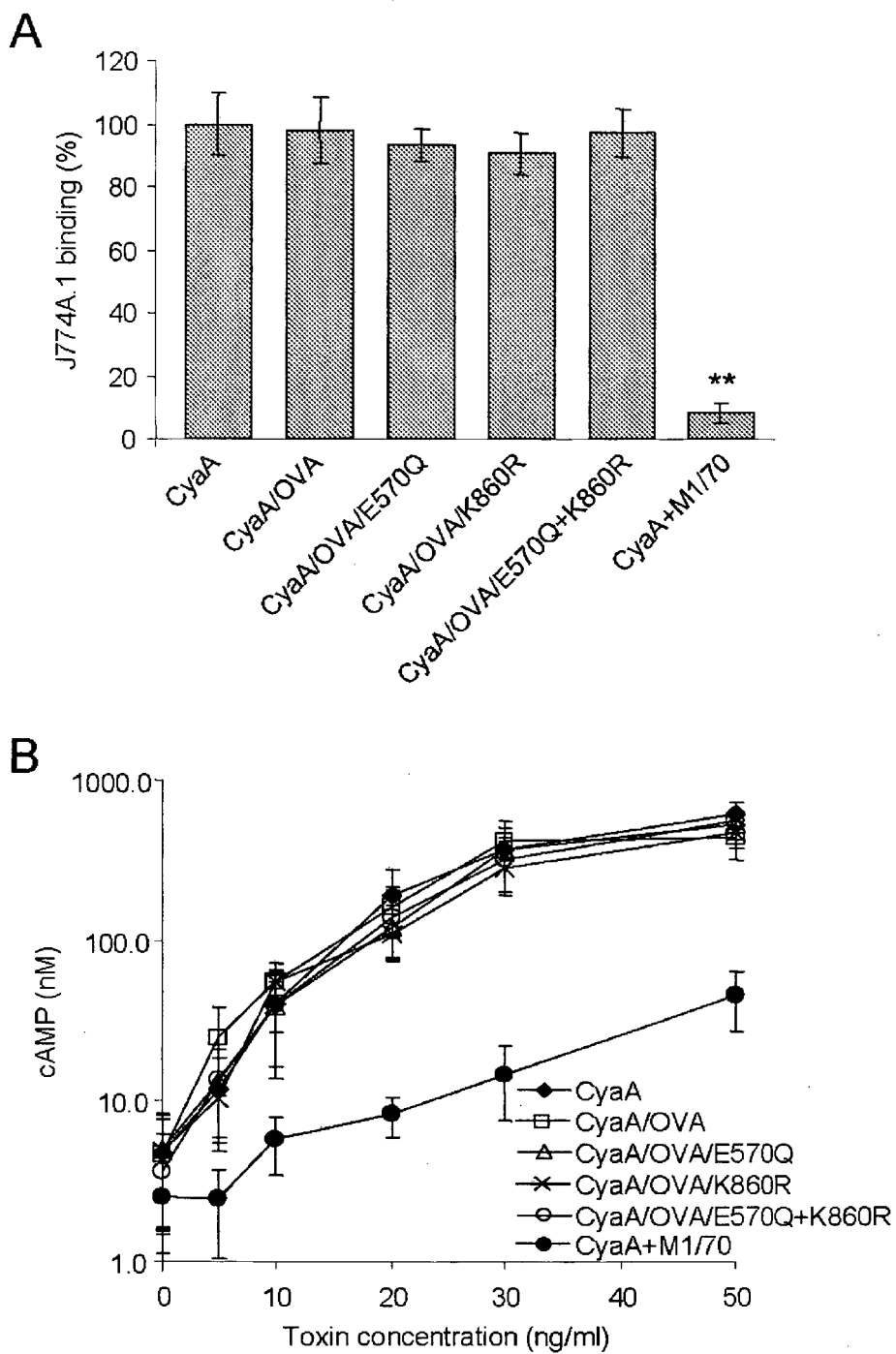
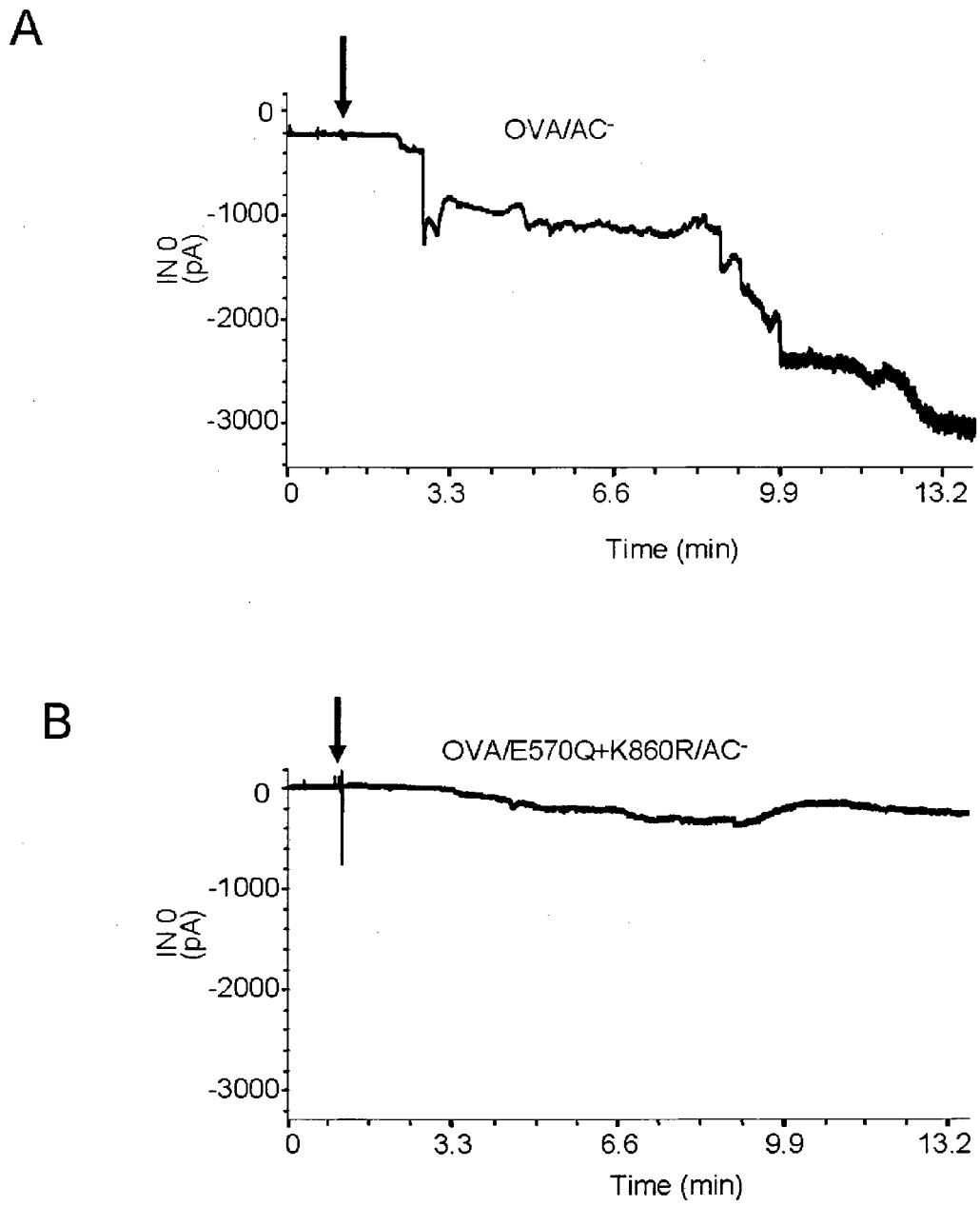


Figure 2



**Figure 3**





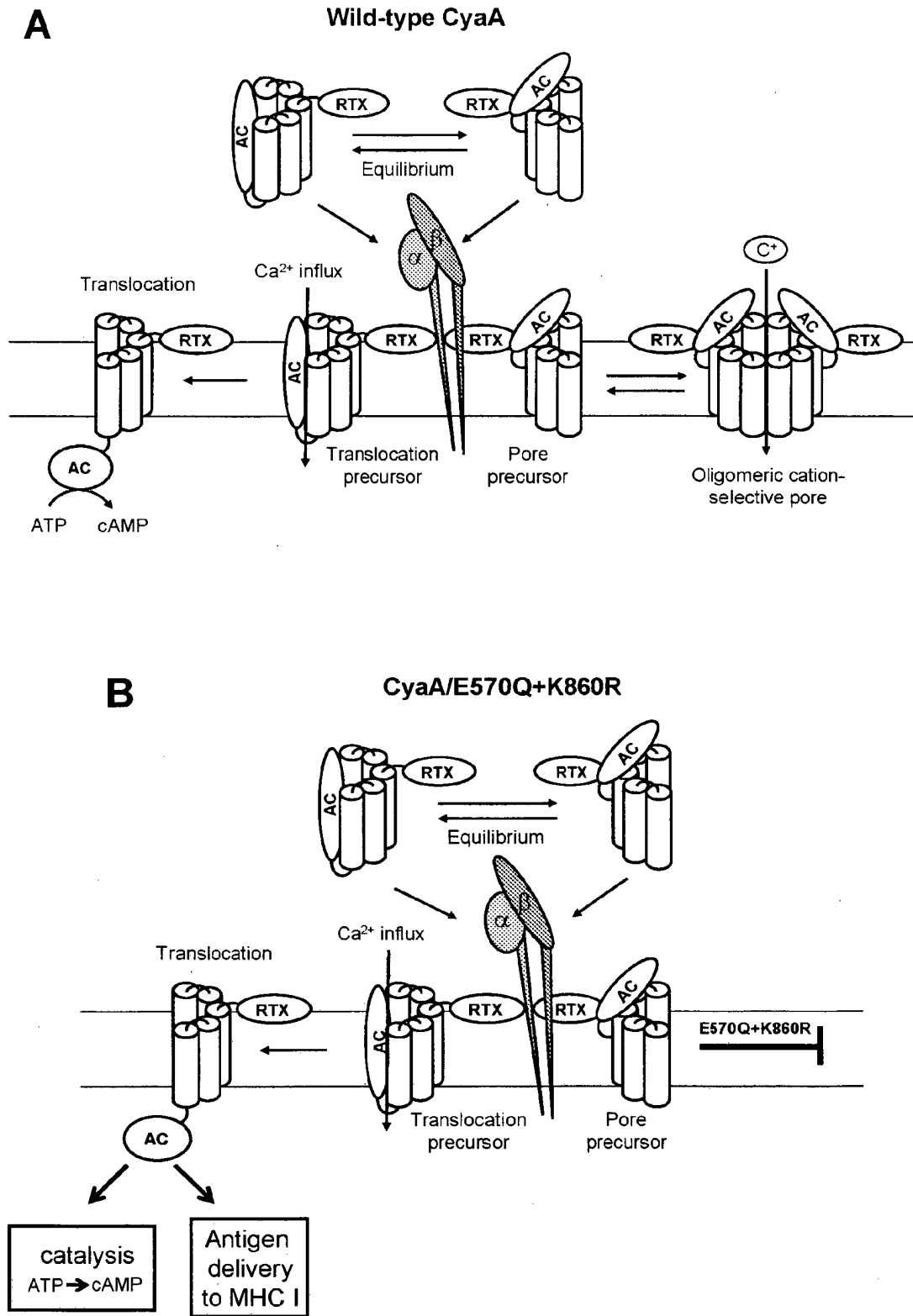


Figure 5

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1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL  
 61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTSLKERL  
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIKNA  
 181 AGIPLTADID MFAIMPHLSN FRDSARSSVT SGDSVTDYLA RTRRAASEAT GGLDRERIDL  
 241 LWKIARAGAR SAVGTEARRQ FRYDGMNIG VITDFELEV RALNRRRAHAV GAQDVVQHGT  
 301 EQNNPFPEAD EKIFVVSATG ESQMLTRGQL KEYIGQQRGE GYVIFYENRAY GVAGKSLFDD  
 361 GLGAAPGVPS GRSKFSVDL ETVPASPLR RPSLGAVERQ DSGYDSLQGV GSRFSLSLGEV  
 421 SDMAAVEAAE LEMTRQVLHA GARQDDAEPG VSGASAHWQ RALQGAQAVA AAQRLVHAIA  
 481 LMTQFGRAGS TNTPQEAASL SAAVFGLEA SAVAETVSG FFRGSSRWAG GFGVAGGAMA  
 541 LGGGIAAAVG AGMSLTDDAP AGQKAAAGAE IALQLTGGTV ELASSIALAL AAARGVTSGL  
 601 QVAGASAGAA AGALAAAALSP MEIYGLVQOS HYADQLDKLA QESSAYGYEG DALLAQLYRD  
 661 KTAAEGAVAG VSAVLSTVGA AVSIAAAASV VGAPVAVVTS LLTGALNGIL RGVOQPIIEK  
 721 LANDYARKID ELGGPQAYFE KNLQARHEQL ANSDGLRKML ADLQAGWNAS SVIGVQTTEI  
 781 SKSALELAAI TGNADNLKSV DVFVDRFVQG ERVAGQPVVL DVAAGGIDIA SRKGERPALT  
 841 FITPLAAPGE EQRRRTKTGK SEFTTFVEIV GKQDRWRIRD GAADTTIDLA KVVSQLVDAN  
 901 GVLKHSIKLD VIGGDGDDVV LANASRIHYD GGAGTNTVSY AALGRQDSIT VSADGERFNV  
 961 RKQLNNANVY REGVATQTTA YGKRTENVQY RHVELARVQ VVEVDTLEHV QHIIGGAGND  
 1021 SITGNAHDF LAGGSGDDRL DGGAGNDTLV GGEGQNTVIG GAGDDVFLQD LGVWSNQLDG  
 1081 GAGVDTVKYN VHQPSEERLE RMGDTGIHAD LQKGTVEKWP ALNLFSVDHV KNIEHLHGSR  
 1141 LNDRIAGDDQ DNELWGHGDN DTIRGRGGDD ILRGGLGLDT LYGEDGNDIF LQDDETVSDD  
 1201 IDGGAGLDTV DYSAMIHPGR IVAPHEYGFG IEADLSREW RKASALGVDY YDNVRNENV  
 1261 IGTSMKDVLI GDAQANTLMG QGGDDTVRGG DGDDLLFGGD GNDMLYGDAG NDTLYGGLGD  
 1321 DTLEGGAGND WFGQTQAREH DVLRRGGDGD TVDYSQTGAH AGIAAGRIGL GILADLGAGR  
 1381 VDKLGEAGSS AYDTVSGIEN VVGTELADRI TGDAQANVLR GAGGADVLAG GEGDDVLLGG  
 1441 DGDDQLSGDA GRDRLYGEAG DDWFFQDAAN AGNLLDGGDG RDTVDFSGPG RGLDAGAKGV  
 1501 FLSLKGKGFAS LMDEPETS NV LRNIENAVGS ARDDVLIGDA GANVLNGLAG NDVLSGGAGD  
 1561 DVLLGDEGSD LLSGDAGNDD LFGGQGGDTY LFGVGYGHDT IYESGGGHDT IRINAGADQL  
 1621 WFARQGNL EIRILGTDDAL TVHDWYRDAD HRVEIIHAAN QAVDQAGIEK LVEAMAQYPD  
 1681 PGAAAAAPPA ARVPDTLMQS LAVNWR

Figure 6

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1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL  
 61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTLSKERL  
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIKNA  
 181 AGIPLTADID MFAIMPHLSN FRDSARSSVT SGDSVTDYLA RTRRAASEAT GGLDRERIDL  
 241 LWKIARAGAR SAVGTEARRQ FRYDGD MNIG VITDFELEV R NALNRRHAV GAQDVVQHGT  
 301 EQNNPFPEAD EKIFVVSATG ESQMLTRGQL KEYIGQQRGE GYVFYENRAY GVAGKSLFDD  
 361 GLGAAPGVPS GRSKFS PDVL ETVPASPGLR RPSLGAVERQ DSGYDSL DGV GSRSFSLGEV  
 421 SDMAAVEAAE LEMTRQVLHA GARQDDAEPG VSGASAHWQ RALQGAQAVA AAQRLVHAIA  
 481 LMTQFGRAGS TNTPQEAAASL SAAVFLGGEA SSAVAETVSG FFRGSSRWAG GFGVAGGAMA  
 541 LGGGIAAAVG AGMSLTDDAP AGQKAAAGAQ IALQLTGTV ELASSIALAL AAARGVTSGL  
 601 QVAGASAGAA AGALAAALSP MEIYGLVQQS HYADQLDKLA QESSAYGYEG DALLAQLYRD  
 661 KTAAEGAVAG VSAVLSTVGA AVSIAAAASV VGAPVAVVTS LLTGALNGIL RGVQQPIIEK  
 721 LANDYARKID ELGGPQAYFE KNLQARHEQL ANSDGLR KML ADLQAGWNAS SVIGVQTTEI  
 781 SKSALELAAI TGNADNLKSV DVFVDRFVQG ERVAGQPVVL DVAAGGIDIA SRKGERPALT  
 841 FITPLAAPGE EQRRRTKTGR SEFTTFVEIV GKQDRWRIRD GAADTTIDLA KVVSQLVDAN  
 901 GVLKHSIKLD VIGGDGDDVV LANASRIHYD GGAGTNTVSY AALGRQDSIT VSADGERFNV  
 961 RKQLNNANVY REGVATQTTA YGKRTEENVQY RHVELARVGQ VVEVDITLEHV QHIIGGAGND  
 1021 SITGNAHDNF LAGGSGDDRL DGGAGNDTLV GGEGQNTVIG GAGDDVFLQD LGVWSNQLDG  
 1081 GAGVDTV KYN VHQPSEERLE RMGDTGIHAD LQKGTVEKWP ALNLFSVDHV KNIENLHGSR  
 1141 LNDRIAGDDQ DNELWGH DGN DTIRGRGGDD ILRGGLGLDT LYGEDGNDIF LQDDETVSDD  
 1201 IDGGAGLDTV DYSAMIHPGR IVAPHEYGFG IEADLSREW RKASALGV D YDNVRNVENV  
 1261 IGTSMKDVLI GDAQANTLMG QGGDDTVRGG DGDDL LFGGD GNDMLYGDAG NDTLYGGLGD  
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 1381 VDKLGEAGSS AYDTVSGIEN VVGTELADRI TGDAQANVLR GAGGADVLG GEGDDVLLGG  
 1441 DGDDQLSGDA GRDRLYGEAG DDWFFQDAAN AGNLLDGGDG RDTVDFSGPG RGLDAGAKGV  
 1501 FLSLGKGFAS LMDEPETS NV LRNIENAVGS ARDDVLIGDA GANVLNGLAG NDVLSGGAGD  
 1561 DVLLGDEGSD LLSGDAGNDD LFGGQDDTY LFGVGYGHDT IYESGGGHDT IRINAGADQL  
 1621 WFAHQNDLE IRILGTDDAL TVHDWYRDAD HRVEIHAAN QAVDQAGIEK LVEAMAQYPD  
 1681 PGAAAAAPPA ARVPDTLMQS LAVNWR

Figure 7

1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL  
 61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTLSKERL  
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIIGNA  
 181 AGIPLTADGS IDMFAIMPHL SNFRDSARSS VTSGDSVTDY LARTRRAASE ATGGLDRERI  
 241 DLLWKIARAG ARSAVGTEAR RQFRYDGD MN IGVITDFELE VRNALNRRAH AVGAQDVVQH  
 301 GTEQNNPFPE ADEKIFVUSA TGESQMLTRG QLKEYIGQQR GEGYVFYENR AYGVAGKSLF  
 361 DDGLGAAPGV PSGRSKFSPD VLETVPASPG LRRPSLGAVE RQDSGYDSL D GVGSRFSLSG  
 421 EVSDMAAVEA AELEMTRQVL HAGARQDDAE PGVSGASAHW GQRALQGAQA VAAAQRLVHA  
 481 IALMTQFGRA GSTNTPQEAA SLSAAVFGLG EASSAVAETV SGFFRGSSRW AGGFGVAGGA  
 541 MALGGGIAAA VGAGMSLTDD APAGQKAAAG AQIALQLTGG TVELASSIAL ALAAARGVTS  
 601 GLQVAGASAG AAAGALAAAL SPMEIYGLVQ QSHYADQLDK I AQESSAYGY EGDALLAQLY  
 661 RDKTAAEGAV AGVSAVLSTV GAAVSIAAAA SVVGAPVAVV TSLLTGALNG ILRGVQQPII  
 721 EKLANDYARK IDELGGPQAY FEKNLQARHE QLANS DGLRK MLADLQAGWN ASSVIGVQTT  
 781 EISKSALELA AITGNADNLK SVDV FVDRFV QGERVAGQPV VLDVAAGGID IASRKGERPA  
 841 LTFITPLAAP GEEQRRRTKT GRSEFTTFVE IVGKQDRWRI RDGAADTTID LAKVVSQ LVD  
 901 ANGLV LKHSIK LDVIGGDGDD VVLANASRIH YDGGAGTNTV SYAALGRQDS ITVSADGERF  
 960 NVRKQLNNAN VYREGVATQT TAYGKRTE NV QYRHVELARV GQVVEVD TLE HVQHI IGGAG  
 1021 NDSITGNAHD NFLAGGSGDD RLDGGAGNDT LVGGEGQNTV IGGAGDDVFL QDLGVWSNQL  
 1081 DGGAGVDTV K YNVHQPSEER LERMGDTGIH ADLQKGTVEK WPALNLF SVD HVKNIENLHG  
 1141 SRLNDRIAGD DQDNELWGHD GNDTIRGRGG DDILRGGLGL DTLYGEDGND IFLQDDETVS  
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 1321 GDDTLEGGAG NDWFGQTQAR EHDVLRGGDG VDTV DYSQTG AHAGIAAGRI GLGILADLGA  
 1381 GRVDKLGEAG SSAYDTVSGI ENVVGTELAD RITGDAQANV LRGAGGADVL AGGEGDDVLL  
 1441 GGDGDDQLSG DAGRDRLYGE AGDDWFFQDA ANAGNLLDGG DGRDTVDFSG PGRGLDAGAK  
 1501 GVFLSLGKGF ASLMDEPETS NVLRNIENAV GSARDDVLIG DAGANVLNGL AGNDVLSGGA  
 1561 GDDVLLGDEG SDLLSGDAGN DDLFGGQGD TYLFGVGYGH DTIYESGGGH DTIRINAGAD  
 1621 QLWFARQOND LEIRILGTDD ALTVDHWYRD ADHRVEIIHA ANQAVDQAGI EKLVEAMAQY  
 1681 PDPGAAAAAP PAARVPDTLM QSLAVNWR

Figure 8

1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL  
 61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTLSKERL  
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VOYRRKGGDD FEAVKVIGNA  
 181 AGIPLTADGS IDMFAIMPHL SNFRDSARSS VTSGDSVTDY LARTRRAASE ATGGVLSIIN  
 241 FEKLVHLDRE RIDLLWKIAR AGARSAVGTE ARRQFRYDGD MNIGVITDFE LEVRNALNRR  
 301 AHAVGAQDVV QHGTEQNNPF PEADEKIFVV SATGESQMLT RGQLKEYIGQ QRGEGYVFYE  
 361 NRAYGVAGKS LFDDGLGAAP GVPSGRSKFS PDVLETVPAS PGLRRPSLGA VERQDSGYDS  
 421 LDGVGSRFS LGEVSDMAAV EAAELEMTRQ VLHAGARQDD AEPGVSGASA HWGQRALQGA  
 481 QAVAAAQRLV HAIALMTQFG RAGSTNTPQE AASLSAAVFG LGEASSAVAE TVSGFFRGSS  
 541 RWAGGFGVAG GAMALGGGIA AAVGAGMSLT DDAPAGQKAA AGAQIALQLT GGTVELASSI  
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Figure 9

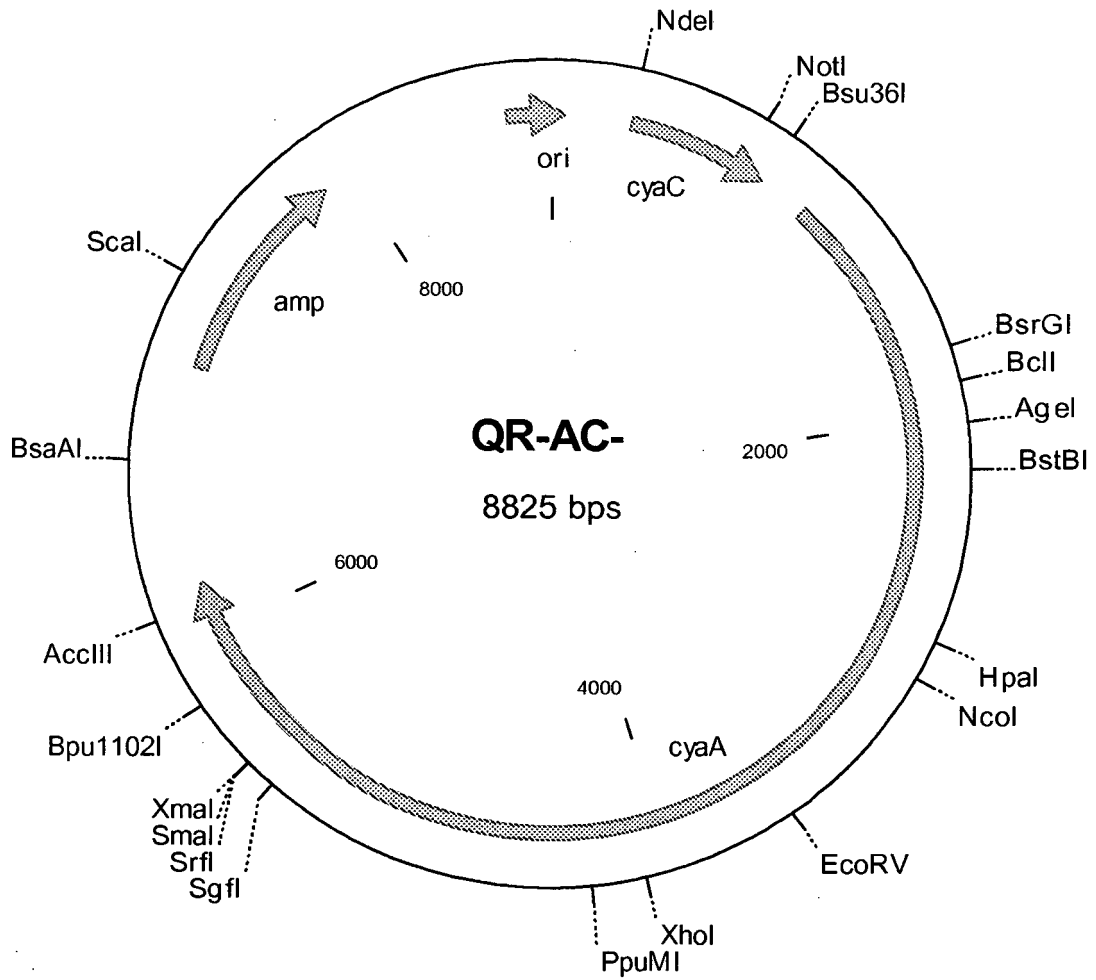


Figure 10

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Figure 11

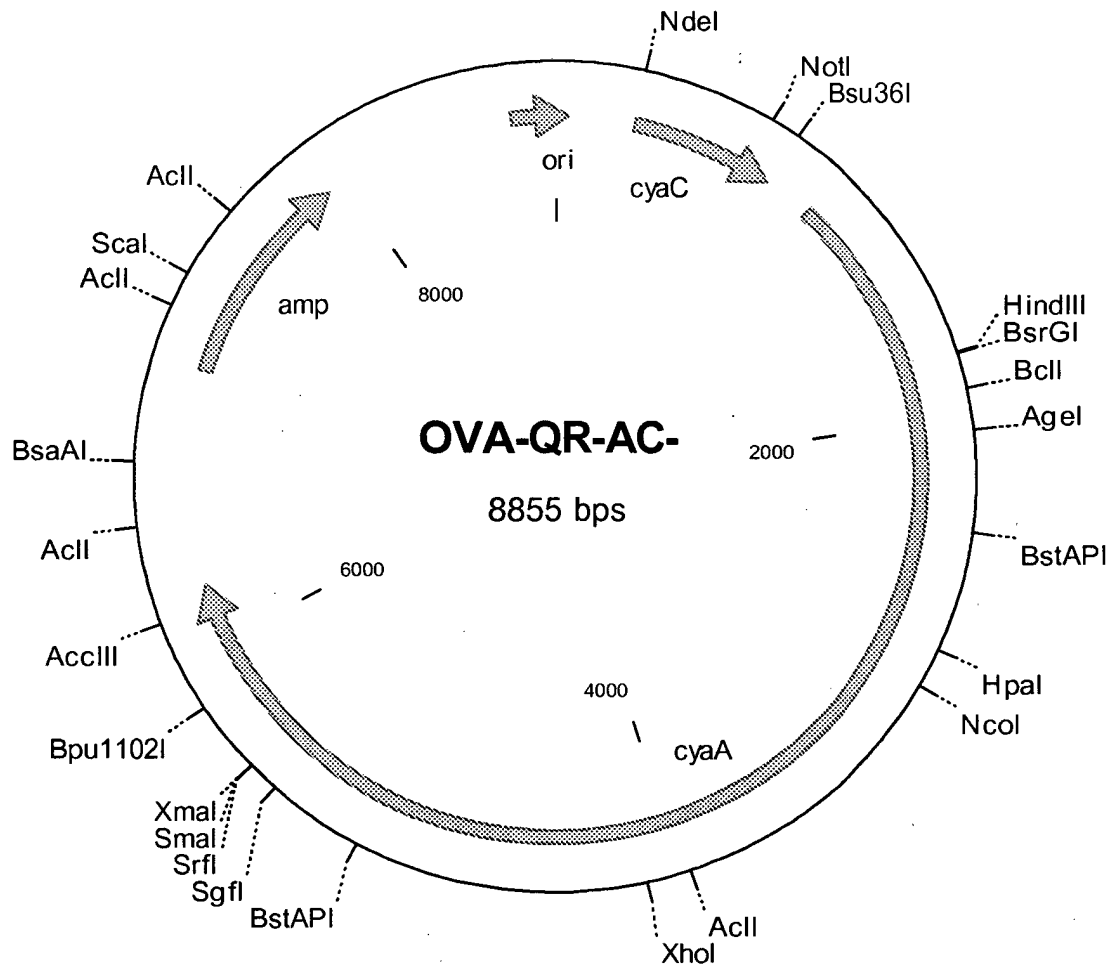


Figure 12

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 TTGTACGGCGAAGCCGGTGACGACTGGTTCTTCCAGGATGCCGCCAATGCCGGCAATCTG  
 CTCGACGGCGGCGACGGCCGCGATAACCGTGGATTTCAGCGGCCCGGGCCGGGGCCTCGAC  
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 CGATAAGTCGTGCTTACCAGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCG  
 GTCGGGCTGAACGGGGGGTTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGA  
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 GGACAGGTATCCGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGG  
 GGGAAACGCCTGGTATCTTTATAGTCTGTGCGGGTTTTCGCCACCTCTGACTTGAGCGTCG  
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 TTTACGGTTCTGCGCCTTTTTGCTGGCCTTTTGTCTCACATGTTCTTTCTGCGTTATCCCC  
 TGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGAGCCG  
 AACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG

Figure 13

## REFERENCES CITED IN THE DESCRIPTION

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