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(71) Demandeurs/Applicants:
INSTITUT PASTEUR, FR;
INSTITUTE OF MICROBIOLOGY OF THE ASCR, V.V.I., CZ;
INSTITUTE OF PHYSIOLOGY OF THE ASCR, V.V.I., CZ

(72) Inventeurs/Inventors:
SEBO, PETER, CZ;
OSICKOVA, ADRIANA, CZ;
MASIN, JIRI, CZ;
FAYOLLE, CATHERINE, FR;
KRUSEK, JAN, CZ;
...

(74) Agent: ROBIC

(54) Titre : POLYPEPTIDES CYAA MUTANTS ET DERIVES DE POLYPEPTIDES APPROPRIES POUR DISTRIBUER DES MOLECULES IMMUNOGENES DANS UNE CELLULE

(54) Title: MUTANT CYAA POLYPEPTIDES AND POLYPEPTIDE DERIVATIVES SUITABLE FOR THE DELIVERY OF IMMUNOGENIC MOLECULES INTO A CELL

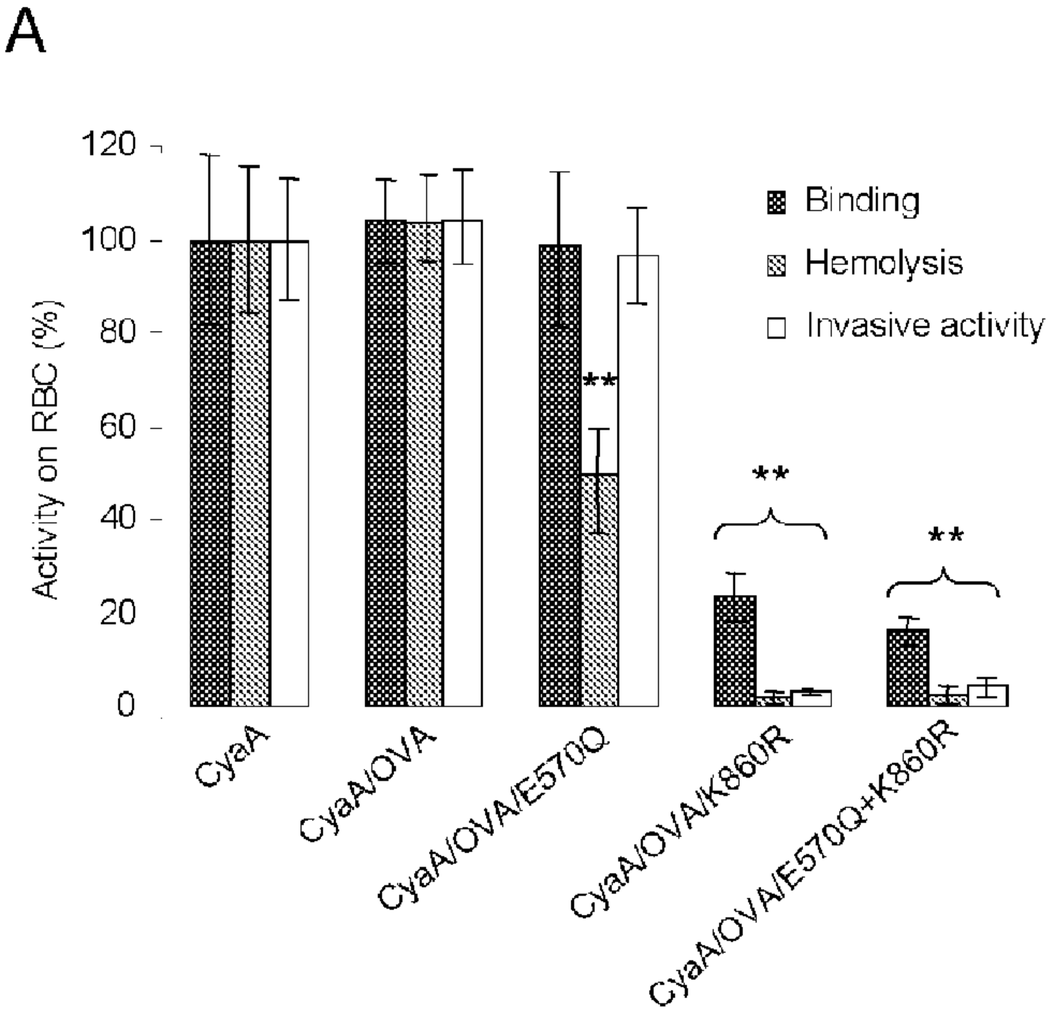

Figure 1

(57) Abrégé/Abstract:
The invention relates to mutant CyaA/E570Q+K860 polypeptides suitable for use as proteinaceous vectors for delivering one or more molecules of interest into a cell, in particular into a cell expressing the CD11b receptor. The invention further relates to polypeptide derivatives suitable for eliciting an immune response in a host. The invention is more particularly directed to polypeptides derived from an adenylate cyclase protein (CyaA) either under the form of a toxin or of a toxoid, which are mutant polypeptides. Said mutant polypeptides are capable of retaining the binding activity of native CyaA to a target cell and preferably of also retaining the translocating activity of native CyaA through its N-terminal domain into target cells and furthermore have a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

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(72) Inventeurs(suite)/Inventors(continued): BASLER, MAREK, CZ; LECLERC, CLAUDE, FR; OSICKA, RADIM, CZ

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(71) Applicant *(for all designated States except US)*: **INSTITUT PASTEUR** [FR/FR]; 25-28 Rue du Docteur Roux, F-75724 Paris Cedex 15 (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SEBO, Peter** [CZ/CZ]; V Liskach 1781, 142 00 Prague 4 (CZ). **OSICKOVA, Adriana** [CZ/CZ]; Vyletni 363/8, 142 00 Prague 4 (CZ). **MASIN, Jiri** [CZ/CZ]; Polska 1646, 25082 Uvaly (CZ). **FAYOLLE, Catherine** [FR/FR]; 20, sentier des ruelles, F-91360 Epinay sur Orge (FR). **KRUSEK, Jan** [CZ/CZ]; Davidkova 95, 182 20 Prague 8 (CZ). **BASLER, Marek** [CZ/CZ]; Daskova 3075/12, 143 00 Prague 4 (CZ). **LECLERC, Claude** [FR/FR]; 13, rue de l'Amiral Roussin, F-75015 Paris (FR). **OSICKA, Radim** [CZ/CZ]; Vyletni 363/8, 142 00 Praha 4 (CZ).

(74) Agent: **ERNEST GUTMANN-YVES PLASSERAUD SAS**; 3, rue Auber, F-75009 Paris (FR).

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[Continued on next page]

(54) Title: MUTANT CyaA POLYPEPTIDES AND POLYPEPTIDE DERIVATIVES SUITABLE FOR THE DELIVERY OF IMMUNOGENIC MOLECULES INTO A CELL

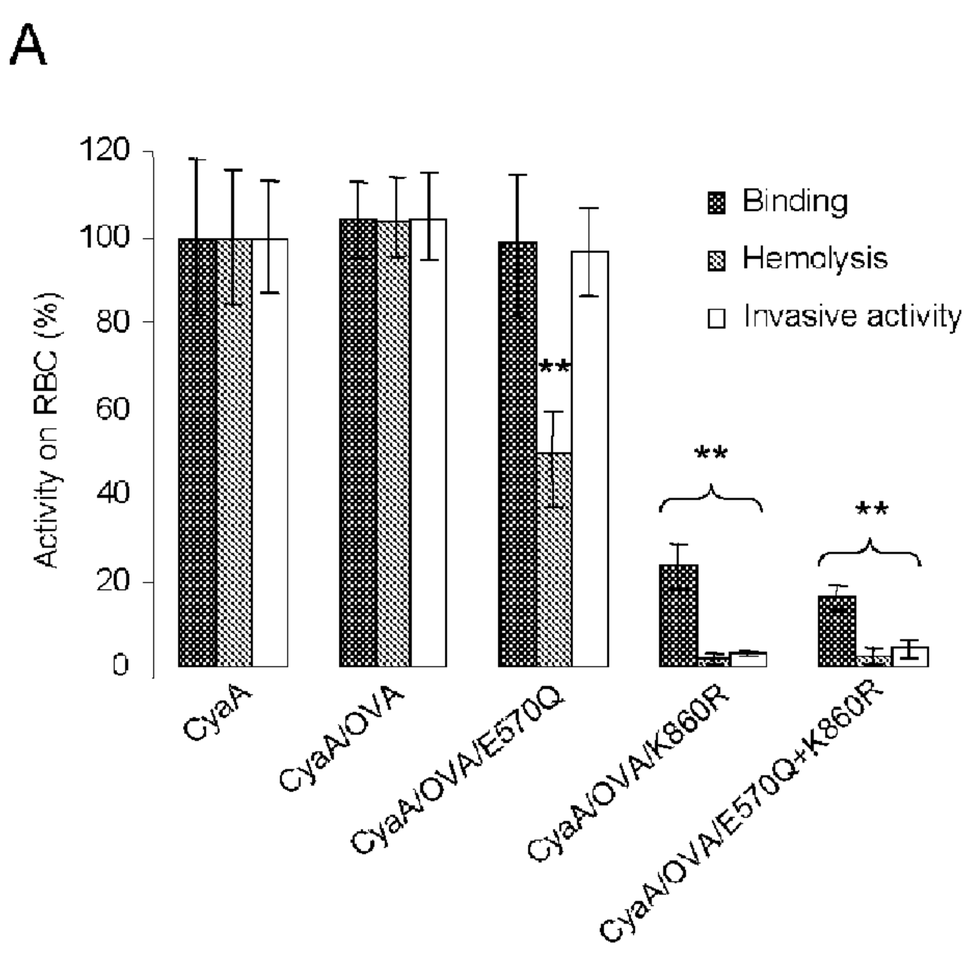

Figure 1

(57) Abstract: The invention relates to mutant CyaA/E570Q+K860 polypeptides suitable for use as proteinaceous vectors for delivering one or more molecules of interest into a cell, in particular into a cell expressing the CD11b receptor. The invention further relates to polypeptide derivatives suitable for eliciting an immune response in a host. The invention is more particularly directed to polypeptides derived from an adenylate cyclase protein (CyaA) either under the form of a toxin or of a toxoid, which are mutant polypeptides. Said mutant polypeptides are capable of retaining the binding activity of native CyaA to a target cell and preferably of also retaining the translocating activity of native CyaA through its N-terminal domain into target cells and furthermore have a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

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

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

In particular, the invention relates to polypeptides suitable for use in the delivery of one or more molecules which are able to elicite an immune response into a host, especially by targeting cells which express the CD11b/CD18 receptor (also referred to herein as “CD11b expressing cells”).

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 a target cell and preferably of also retaining the translocating activity of native CyaA through its N-terminal domain into target cells and furthermore have a pore-forming activity which is reduced or suppressed as compared to that of the native CyaA toxin.

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.

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.

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.

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.

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 primarily in *Bordetella* organisms, especially 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.

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 cytolysin) 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 ε-amino groups of Lys^{860} and Lys^{983}, 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.

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 $CD11b^+$ monocytes (8, 9).

Recently, the inventors showed that CyaA binds N-linked oligosaccharides of its CD11b/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 CD11b/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).

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 $CyaA/AC^-$ toxoid may be used to achieve the preparation of the polypeptides of the invention.

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 $CD11b^+$ cells (13, 14). Due to an intact pore-forming (hemolytic) capacity, in the absence of osmoprotectants such as serum, the enzymatically inactive $CyaA/AC^-$ toxoid (15) still exhibits a full hemolytic activity on erythrocytes and a residual, about ten-fold reduced cytolytic activity on CD11b-expressing monocytes (8), which sets a limit to its use in therapy.

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).

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.

In the present invention, the inventors initially designed CyaA mutant polypeptides, based especially 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- toxoids on CD11b+ 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*.

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 while remaining fully capable of translocating the AC domain across cellular membrane.

The inventors have now designed particular constructs, illustrated especially as a CyaA/E570Q+K860R/AC$^-$ toxoid and have shown that despite its much reduced cell-permeabilizing (cytolytic) activity, it remains fully active in antigen delivery into CD11b$^+$ APCs. The inventors have further shown that the overall cytolytic activity of the illustrative CyaA/E570Q+K860R/AC$^-$ 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.

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.

Based on the experiments carried out by the inventors it has thus been possible to define and provide a polypeptide which is a mutant of an adenylate cyclase protein (mutant polypeptide) and whose amino acid sequence comprises or consists of one of the following sequences:

a) the amino acid sequence of the adenylate cyclase (CyaA) of *Bordetella pertussis*, *Bordetella parapertussis* or *Bordetella hinzii* wherein the following mutations have been performed:
   (i) the substitution of the glutamic acid residue at position 570 by a glutamine residue (E570Q) or by a conservative amino acid residue, and
   (ii) the substitution of the lysine residue at position 860 by an arginine residue (K860R) or by a conservative amino acid residue, or;

b) an amino acid sequence of a fragment of the adenylate cyclase of *Bordetella pertussis*, *Bordetella parapertussis or Bordetella hinzii,* which fragment has the capacity of the CyaA protein of *Bordetella pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said fragment further contains the following mutated amino acid residues

located at positions 570 and 860 in said adenylate cyclase: E570Q and K860R, or

c) an amino acid sequence which differs from the amino acid sequence as defined in a) or b) by one or more amino acid residue substitutions and/or insertions and which has the capacity of the CyaA protein of *Bordetella Pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said amino acid sequence further contains the following mutated amino acid residues located at positions 570 and 860 in said adenylate cyclase: E570Q and K860R, or

d) the amino acid sequence of the adenylate cyclase (CyaA) of *Bordetella bronchiseptica* wherein the following mutations have been performed:

(i) the substitution of the glutamic acid residue at position 569 by a glutamine residue (E569Q) or by a conservative amino acid residue, and

(ii) the substitution of the lysine residue at position 859 by an arginine residue (K859R) or by a conservative amino acid residue, or;

e) an amino acid sequence of a fragment of the adenylate cyclase of *Bordetella bronchiseptica,* which fragment has the capacity of the CyaA protein of *Bordetella pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said fragment further contains the following mutated amino acid residues located at positions 569 and 859 in said adenylate cyclase: E569Q and K859R, or

f) an amino acid sequence which differs from the amino acid sequence as defined in d) or e) by one or more amino acid residue substitutions and/or insertions and which has the capacity of the CyaA protein of *Bordetella Pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said amino acid sequence further contains the following mutated amino acid residues located at positions 569 and 859 in said adenylate cyclase: E569Q and K859R.

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.

Herein, “E570Q” encompasses substitution of the glutamic acid residue at position 570 of native CyaA of *Bordetella pertussis*, *Bordetella parapertussis* or *Bordetella hinzii* by a glutamine residue or by another conservative residue, in particular a residue whose side chain size and hydrophilic nature is close to that of glutamic acid. The glutamic acid residue at position 570 is preferably substituted by an amino acid residue selected from Gln, Asn, Met, Thr, Ser, Gly, Arg, Lys, Val, Leu, Cys, Ile, Asp.

Herein, “K860R” encompasses substitution of the lysine residue at position 860 of native CyaA of *Bordetella pertussis*, *Bordetella parapertussis* or *Bordetella hinzii* by an arginine residue or by another conservative residue, in particular a residue whose side chain size and hydrophilic nature is close to that of lysine. The lysine residue at position 860 is preferably substituted by an amino acid residue selected from Arg, Asn, Gln, Met, Thr, Ser, Gly, Val, Leu, Cys, Ile.

Herein, “E569Q” encompasses substitution of the glutamic acid residue at position 569 of native CyaA of *Bordetella bronchiseptica* by a glutamine residue or by another conservative residue, in particular a residue whose side chain size and hydrophilic nature is close to that of glutamic acid. The glutamic acid residue at position 569 is preferably substituted by an amino acid residue selected from Gln, Asn, Met, Thr, Ser, Gly, Arg, Lys, Val, Leu, Cys, Ile, Asp.

Herein, “K859R” encompasses substitution of the lysine residue at position 859 of native CyaA of *Bordetella bronchiseptica* by an arginine residue or by another conservative residue, in particular a residue whose side chain size and hydrophilic nature is close to that of lysine. The lysine residue at position 859 is preferably substituted by an amino acid residue selected from Arg, Asn, Gln, Met, Thr, Ser, Gly, Val, Leu, Cys, Ile.

In the embodiments described hereafter, the mutant *Bordetella pertussis* CyaA proteins or protein fragments carrying the "E570Q" and "K860R" substitutions may be replaced by mutant *Bordetella parapertussis* or *Bordetella hinzii* CyaA proteins or protein fragments carrying the equivalent "E570Q" and "K860R" substitutions, or by mutant *Bordetella bronchiseptica* CyaA proteins or protein fragment carrying the equivalent "E569Q" and "K859R" substitutions.

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.

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.

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).

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).

In a particular embodiment of the invention, the CyaA protein is a bacterial protein. In a preferred embodiment, CyaA protein is derived from a *Bordetella species*.

Among *Bordetella species* of interest, according to the invention, one of them is *Bordetella pertussis*. Other *Bordetella* strains of interest are those of *Bordetella parapertussis, Bordetella hinzii* or *Bordetella bronchiseptica*. The sequence 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). The sequence of *Bordetella parapertussis* has been disclosed especially under accession number CAB76450, referred to herein as SEQ ID N°7 as illustrated in figure 14. The sequence of *Bordetella hinzii* has been disclosed especially under accession number AAY57201, referred to herein as SEQ ID N°8 as illustrated in figure 15. The sequence of *Bordetella bronchiseptica* has been disclosed especially under accession number CAA85481, referred to herein as SEQ ID N°9 as illustrated in figure 16. Accordingly, when amino acid residues or sequences of the CyaA protein of *Bordetella parapertussis, Bordetella hinzii* or *Bordetella bronchiseptica*, are cited in the present invention their positions are given with respect to the sequences disclosed in SEQ ID N°7, 8 and 9 respectively.

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 provided 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.

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 target cells, especially of CD11b/CD18 expressing cells.

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 CD11b/CD18 receptor.

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$^-$ toxoid. The mutant polypeptides are capable of binding to a cell, especially to a target 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.

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.

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.

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.

In a preferred embodiment, the overall cytolytic activity of the polypeptide according to the invention, in particular on cells which express the CD11b/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 hemoglobulin (for erythrocytes) or of lactate dehydrogenase (for monocytes) released by the cells when incubated with the tested polypeptide as described in examples.

In a 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%, 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).

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 a target cell and of translocating its N-terminal domain into the cell.

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 particular 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.

In a preferred embodiment the fragment which is thus prepared has essentially lost the adenyl cyclase enzyme activity (AC activity)

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.

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.

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.

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.

In another specific embodiment, the mutant polypeptide of the invention has an amino acid sequence which differs from the CyaA amino acid sequence as set forth in SEQ ID N°1 by mutation of residues resulting in E570Q and K860R and by further mutations resulting in 1 to 500, in particular, 1 to 400, 1 to 300, 1 to 200, 1 to 100, 1 to 50, 1 to 40, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10 or 1 to 5 amino acid residue substitutions, deletions, and/or insertions including the E570Q and K860R substitutions.

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 at positions 188 and 189.

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.

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.

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 at least about 350 amino acid residues 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 400, 500, 600, 700, 800, 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 at least about 350 amino acid residues 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 400, 500, 600, 700, 800, 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 cell receptor and the ability to translocate their N-terminal domain into target 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.

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.

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.

In another preferred embodiment, the mutant polypeptide which is such a fragment comprises :

a) a first amino acid sequence which corresponds to a stretch of at least 100 contiguous amino acid residues from SEQ ID N°1 comprising amino acid residues 570 as E570Q, and further including 0, 1, 2, 3, 4 or 5 deletions, substitutions or insertions 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 comprising amino acid residues 860 as K860R, and further including 0, 1, 2, 3, 4 or 5 deletions, substitutions or insertions as compared to SEQ ID N°1 and preferably,

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.

Another particular polypeptide of the invention is 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.

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.

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.

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.

Such total or partial suppression can be obtained as a result of genetic inactivation, for example by introduction of a short amino acid sequence 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 deletion or 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 at position 188 and 189 of the CyaA sequence as set forth 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.

The polypeptide according to the invention can also be defined as a polypeptide which may be obtained from a CyaA polypeptide having an amino acid sequence according to SEQ ID N° 1, 7, 8 or 9, by:

a) substituting the glutamic acid residue at position 570 of SEQ ID N° 1, 7, 8 or at position 569 of SEQ ID N° 9 by a glutamine residue or by a conservative amino acid residue,

b) substituting the lysine residue at position 860 of SEQ ID N° 1, 7, 8 or at position 859 of SEQ ID N° 9 by an arginine residue or by a conservative amino acid residue, and

c) optionally, carrying out one or more amino acid residue substitutions, insertions and/or deletions at locations other than the locations recited in a) and b), provided that the polypeptide thus obtained has the capacity of the CyaA protein of *Bordetella pertussis* to bind to a target cell and translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell.

Preferably, in step a), the glutamic acid residue is substituted by an amino acid residue selected from Gln, Asn, Met, Thr, Ser, Gly, Arg, Lys, Val, Leu, Cys, Ile, Asp, most preferably by Gln. Preferably, in step b), the lysine residue is substituted by an amino acid residue selected from Arg, Asn, Gln, Met, Thr, Ser, Gly, Val, Leu, Cys, Ile, most preferably by Arg.

In a specific embodiment, no further amino acid residue substitutions, insertions and/or deletions are carried out in step c).

In particular, step c) may comprise truncation of either or both terminal ends. 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 provided that it does not affect the translocation ability of the obtained mutant polypeptide. Internal deletions of one or more residues of the native CyaA protein located at positions other than those recited in steps a) and b) may also be performed. In a particular embodiment, step c) comprises the deletion of up to 380 or up to 400 amino acids in the N-terminal amino acid sequence of the CyaA polypeptide, preferably the deletion of the stretch of amino acids running from the amino acid at position 1 of SEQ ID N°1, 7, 8 or 9 to the amino acid at position 372 of SEQ ID N°1, 7, 8 or 9.

Preferably, after performing step c), the obtained polypeptide 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 the membrane of the target cell

and/or CD11b/CD18 receptor, and for the subsequent delivery of the N-terminal domain of the polypeptide into the cell cytosol. In a particular embodiment, in step c), amino acid residues 373 to 1706 of SEQ ID N°1, 7 or 8, or amino acid residues 373 to 1705 of SEQ ID N°9 are not deleted. In a preferred embodiment, in step c), amino acid residues 1208 to 1243 of SEQ ID N°1, 7 or 8, or amino acid residues 1207 to 1242 of SEQ ID N°9 are not deleted.

In a preferred embodiment, step c) comprises amino acid substitutions, deletions, and/or insertions which totally or partially suppress the adenyl cyclase enzymatic activity of the CyaA protein. Such total or partial suppression can be obtained by introduction of a short amino acid sequence, comprising for example from one to ten amino acids, in particular a dipeptide in a site located within the first 400 amino acids (AC domain) of SEQ ID N°1, 7, 8 or 9 or by deletion or substitution of a part of the CyaA amino acid sequence as set forth in SEQ ID N°1, 7, 8 or 9 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 at positions 188 and 189 of the CyaA sequence as set forth in SEQ ID N°1, 7, 8 or 9. 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.

Preferably, in step c) the lysine residue at position 983 of the CyaA amino acid sequence as set forth in SEQ ID N°1, 7, 8 or at position 982 in SEQ ID N°9 is neither substituted nor deleted. In one embodiment, this lysine residue is acylated, in particular it is palmytoylated or palmitoleilated. Alternatively, this lysine residue is not acylated.

Preferably, after performing step c), the obtained polypeptide 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, 7, 8 or 9.

Preferably still, after performing step c), the obtained polypeptide has an amino acid sequence which differs from the sequence set forth in SEQ ID N°1, 7, 8 or 9 by the amino acid residue substitutions resulting from steps a) and b), and by 1 to 500, in particular, 1 to 400, 1 to 300, 1 to 200, 1 to 100, 1 to 50, 1 to 40, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10 or 1 to 5 further amino acid residue substitutions, deletions, and/or insertions. In a particular embodiment, after performing step c), the obtained polypeptide differs from the CyaA amino acid sequence as set forth in SEQ ID N°1, 7, 8 or 9 by 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid residue substitutions, deletions, and/or insertions in addition to the substitutions carried out in steps a) and b).

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.

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

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.

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).

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.

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.

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.

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.

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.

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 $CD8^+$ T cells or $CD4^+$ T cells.

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.

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 βbeta-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.

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

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 differenciation antigens or to viral antigens.

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

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.

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.

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

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.

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).

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.

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.

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 α-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.

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.

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

According to another embodiment, 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.

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.

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.

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 CD11b/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 CD11b-expressing cells.

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+ cytoxic 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.

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.

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 dentritic cells or neutrophils.

More specifically, antigen presentation for selective CD8+ cytotoxic cells priming is mainly performed by myeloïd dendritic cells.

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.

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

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.

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.

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 K860R, which have been selected.

The present invention is thus also directed to 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.

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.

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.

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

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.

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.

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.

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 a therapeutically active principle.

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

In particular, mutant CyaA poplypeptide or the poplypeptide 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.

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).

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)).

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.

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.

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.

In another particular embodiment of the invention, the chemotherapeutic agent is an intercalating agent.

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

The chemotherapeutic agent is advantageously administered by low doses.

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

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 signaling through pattern-recognition receptors (PRRs).

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).

In particular an adjuvant component for the activation of the innate immune response can target PRRs and therefore activate signaling through PRRs, wherein

said PRRs encompass Toll-like receptors or nucleotide-binding oligomerization domain (NOD) or C type lectin.

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.

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.

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.

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.

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

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'-GGGGGACGATCGTCGGGGGG-3' or Type B CpG ODN, i.e., CpG 1826 having nucleotide sequence 5'-TCCATGACGTTCCTGACGTT-3'.

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

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[4,5c]quinoline -1-ethanol available from Invivogen, as TLR-7 ligand, or the one named R837 (imiqimod) available from Aldara as TLR-7 agonist.

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

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.

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)

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

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 an 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.

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.

The present invention is further directed to 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.

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

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).

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).

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.

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.

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 excipient(s), and optionally an adjuvant and/or another therapeutically active molecule.

The invention is also directed to a kit of parts comprising the mutant CyaA polypeptide or the polypeptide derivative, a therapeutically active molecule and/or an adjuvant.

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.

The kit of parts of the invention 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.

The invention also relates to 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.

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.

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.

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.

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.

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.

The invention further relates to 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.

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.

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.

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.

The invention further relates to nucleic acid molecules, in particular DNA or RNA molecules, which encode a polypeptide or polypeptide derivative as defined above.

The invention is also directed to eukaryotic or prokaryotic cells which comprise the nucleic acid molecules as defined above.

The invention also relates to eukaryotic cells, preferably mammalian cells, which comprise a mutant CyaA polypeptide or polypeptide derivative as defined above. In a preferred embodiment, the cells are human cells.

The invention further relates to eukaryotic cells, preferably mammalian cells, transformed with the proteinaceous vector as defined above.

Figures

Fig. 1. Substitutions in the pore-forming and acylation domains synergize in decreasing the specific hemolytic activity of CyaA. (A) Sheep erythrocytes ($5x10^8$/ml) in TNC buffer were incubated with 5 μg/ml of enzymatically active CyaA proteins at 37°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)** Erythrocytes were incubated as above with the indicated concentrations of the enzymatically active CyaA-derived proteins for 30 min, washed, and the amount of cell-associated AC enzyme activity was determined. **(C)** The reduced cell binding activity of proteins with the K860R substitution was compensated for by increasing their concentration from 5 μg/ml to 25 μg/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. 1C).

Fig. 2. CyaA/OVA/E570Q+K860R binds and translocates into CD11b$^+$ monocytes. (A) J774A.1 cells (10^6/ml) were incubated in D-MEM for 30 min at 4°C with 2.5 μg/ml of CyaA, washed repeatedly, and the amount of cell-associated AC activity was determined in cell lyzates. 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 μg/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 lyzates were determined (41). As a control, the CD11b/CD18 receptor was blocked with the anti-CD11b antibody M1/70 as above. Membrane penetration of CD11b/CD18 -bound and endocytosed toxin was controlled by using the doubly mutated CyaA/E570K+E581P variant, which is intact for receptor binding but fails to translocate the AC domain across cell membrane and elevate cytosolic cAMP concentrations (Vojtova-Vodolanova *et al.,* 2009). **(C)** J774A.1 cells were loaded with the K^+ sensitive fluorescent probe PBFI/AM at 9.5 μM final external concentration and 25°C for 45 min in the presence of Pluronic F-127 [0.05% (w/w)]. Cells were washed in HBSS before 3 μg ml^{-1} of the indicated toxins were added. Fluorescence intensity ratio of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) reflecting the intracellular K^+ concentration was recorded every 15 s. The right scale shows intracellular $[K^+]$ values derived from calibration experiments (see *Experimental procedures*). No cell lysis, assessed as lactate dehydrogenase release, was observed within the time interval of the assay. Results representative of three independent determinations performed in duplicates are shown.

Fig. 3. E570Q+K860R toxoid does not permeabilize J774A.1 cells. (A) Lysis of J774A.1 cells was determined as the amount of lactate dehydrogenase released from 10^5 cells upon 3 h of incubation with 3, 10 and 30 μg ml^{-1} of the indicated protein at 37% in DMEM without serum. The results represent the average of values obtained in two experiments performed in duplicates. **(B)** Whole-cell patch-clamp measurements were performed on single J774A.1 cells at room temperature exposed to 1 or 10 μg/ ml of CyaA/233OVA/AC^- or CyaA/233OVA/E570Q+K860R/AC^- 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 $CD8^+$ CTLs. (A) BMDC (3 x 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^-. Upon co-culture for 24 hours with B3Z T cells (1 x 10^5 cells/well), IL-2 secretion by the stimulated B3Z cells was determined by the CTLL proliferation method. Results are expressed as Δcpm of incorporated [^{3}H]thymidine (cpm in the presence of toxoid - cpm in the absence of toxoid) ±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 μg i.v. of mock AC^- or OVA/AC^- toxoids and on day 7, they were i.v. injected with a mixture (1:1) of OVA (SIINFEKL) peptide-loaded $CFSE^{high}$ and unloaded $CFSE^{low}$ 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^- vs. OVA/E570Q+K860R/AC^-).

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$^-$ mutant (SEQ ID N° 3)

Fig. 9. Amino acid sequence of the *Bordetella pertussis* CyaA/233OVA/E570Q+K860R/AC$^-$ mutant (SEQ ID N° 4)

Fig. 10. Plasmid encoding the CyaA/E570Q+K860R/AC$^-$ mutant (QR-AC$^-$).

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

Fig. 12. Plasmid encoding the CyaA/233OVA/E570Q+K860R/AC$^-$ mutant (OVA-QR-AC$^-$).

Fig. 13. DNA sequence of OVA-QR-AC$^-$ plasmid encoding the CyaA/233OVA/E570Q+K860R/AC$^-$ mutant (SEQ ID N° 6)

Fig. 14. Amino acid sequence of the *Bordetella parapertussis* CyaA toxin (accession number CAB76450, SEQ ID N°7)

Fig. 15. Amino acid sequence of the *Bordetella hinzii* CyaA toxin (accession number AAY57201, SEQ ID N°8)

Fig. 16. Amino acid sequence of the *Bordetella bronchiseptica* CyaA toxin (accession number CAA85481, SEQ ID N°9)

Examples

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

Materials and Methods

Construction, Production and Purification of CyaA proteins. The modifications yielding CyaA/AC$^-$, CyaA/233OVA, CyaA/E570Q and CyaA/K860R constructs were previously described (13, 20, 21) and were introduced into CyaA/233OVA/AC$^-$ 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-1000 (Cambrex).

An *Escherichia coli* XL1-Blue strain (Stratagene) containing the QR-AC$^-$ plasmid (figure 10) which encodes the CyaA/E570Q+K860R/AC$^-$ 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$^-$ plasmid (SEQ ID N°5) is disclosed in figure 11.

An *Escherichia coli* XL1-Blue strain (Stratagene) containing the OVA-QR-AC$^-$ plasmid (figure 12) which encodes the CyaA/233OVA/E570Q+K860R/AC$^-$ 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$^-$ plasmid (SEQ ID N° 6) is disclosed in figure 13.

Cell Binding and Hemolytic Activities on Sheep Erythrocytes. $5x10^8$ washed sheep erythrocytes in 50 mM Tris pH 7.4, 150 mM NaCl and 2 mM $CaCl_2$ (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).

Macrophage Binding, Elevation of cAMP and Cell Lysis Capacities of CyaA. J774.Al murine monocytes/macrophages (ATCC, number TIB-67) were cultured at 37°C in a humidified air/CO_2 (19:1) atmosphere in RPMl medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}) and amphotericin B (250 ng ml^{-1}). Prior to assays, RPMl was replaced with Dulbecco's modified Eagle's medium (DMEM) (1.9 mM $Ca2^{+}$) without FCS and the cells were allowed to rest in DMEM for 1 h at 37°C in a humidified 5% CO_2 atmosphere (8). 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 lyzed 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.

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 $CaCl_2$, 3 mM $MgCl_2$, 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 $CaCl_2$, 1 mM $MgCl_2$, 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).

Determination of decrease of cytosolic K^+ concentration. Cells grown on grass coverslips were washed in HBSS and loaded with 9.5 µM PFBI acetoxymethyl ester (PBFI/AM, Molecular Probes, Eugene, OR, USA) for 30 min at 25°C in the presence of 0.05% (w/w) Pluronic F-127 (Sigma, St.Louis, MO), in the dark. Ratiometric measurement was performed at 25°C using a FluoroMax-3 spectrofluorimeter (Jobin Yvon Horriba, France) equipped with DataMax software. Fluorescence intensity of PBFI (excitation wavelength 340, emission wavelengths 450 and 510 nm) was recorded every 15 s and the integration time for each wavelength was 3 s. The ratio of 450/510 nm wavelengths is shown. The observed area of coverslip mounted in the 1 cm cuvette was about 10 mm^2, corresponding to approximately 10^4 cells. Calibration experiments were performed in 50 mM HEPES, pH 7.2, with varying concentrations of Potassium Acetate (10, 30, 60 or 140 mM) and Sodium Acetate (135, 115, 85 or 5 mM), respectively, on cells permeabilized for 30 min with 3 pM valinomycin or nigericin. Final intensity ratio (450/510 nm) is shown on right vertical axis of the plots.

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 $CD8^+$ specific T cell hybridoma specific for the K^b 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 5x 10^{-5} M 2-ME.

Antigen Presentation Studies. Bone Marrow Dendritic Cells (BMDC, 3 x 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$^-$ carrying the OVA (SIINFEKL) epitope or mock CyaA/AC$^-$ and cocultured for 24 hours with B3Z T cells (1 x 10^5 per well), selectively recognizing the OVA SIINFEKL/H-2K^b 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, [^{3}H]-thymidine (50 µCi/well) was added and cells were harvested 6 h later with an automated cell harvester. Incorporated [^{3}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 [^{3}H]-thymidine (cpm in the presence of toxoid - cpm in the absence of toxoid).

***In vivo* Killing Assay.** For CTL priming, mice were immunized by i.v. injection with 50 µg of recombinant CyaA/OVA/AC$^-$ carrying the OVA (SIINFEKL) epitope or mock CyaA/AC$^-$. 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 carboxyfluoroscein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). The nonpulsed control population was labeled with a low concentration (0.125 µM) of CFSE. CFSE^{high}-and CFSE^{low}-labeled cells were mixed in a 1:1 ratio (5x 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_3). 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^{high} immunized mice/% CFSE^{low} immunized mice)/(% CFSE^{high} naive mouse/% CFSE^{low} naive mouse)].

***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

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$^-$ toxoids to lyze 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$^-$ toxoids, the inventors derived such mutants from a CyaA/233OVA toxin that was previously tagged by insertion of the SIINFEKL peptide from ovalbumin (OVA). This CyaA variant was chosen as the insertion of reporter K^b-restricted CD8$^+$ 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$^-$ toxoids to deliver their OVA/AC$^-$ domain into cytosol of CD11b$^+$ 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$^+$ T cells, both *in vitro* and *in vivo* (20).

To generate CyaA/AC$^-$ 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$^-$ on CD11b$^+$ J774A.1 monocytes (8, 13). These substitutions were engineered into CyaA/233OVA/AC$^-$ individually and in combination and the

specific hemolytic and cytolytic activities of resulting toxoids were compared using sheep erythrocytes as model $CD11b^-$ target and J774A.1 as model $CD11b^+$ 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^- 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-expressing J774A.1 cells was also reduced (37 ± 10), as compared to OVA/AC^-. In turn, as expected from results obtained with an enzymatically active K860R construct, despite the low hemolytic activity on $CD11b^-$ erythrocytes, the K860R toxoid exhibited only a slightly reduced relative cytolytic activity on $CD11b^+$ 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^- and derivatives on sheep erythrocytes and J774A.1 macrophages.

Protein	Lysis of erythrocytes (% of AC^-)[a]	Lysis of J774A.1 cells (% of AC^-)[b]
AC^-	100 ± 5	100 ± 10
OVA/AC^-	93 ± 4	93 ± 12
$OVA/E570Q/AC^-$	55 ± 8**	37 ± 10**
$OVA/K860R/AC^-$	1 ± 1**	72 ± 22**
$OVA\text{-}L247Q\text{-}AC^-$	97 ± 3	41 ± 9
$OVA/E570Q+K860R/AC^-$	1 ± 1**	14 ± 7**
$OVA\text{-}E570Q\text{-}L247Q\text{-}AC^-$	50 ± 12	40 ± 11

Protein	Lysis of erythrocytes (% of AC^-)[a]	Lysis of J774A.1 cells (% of AC^-)[b]
OVA-K860R-L247Q-AC^-	1 ± 1	45 ± 11
OVA-E570Q-K860R-L247Q-AC^-	0 ± 1	16 ± 10

Table Legend

- [a]Lysis of sheep erythrocytes was determined after 4.5 hours as the amount of hemoglobin released upon incubation of $5x10^8$ RBC at 37°C in the presence of 2 mM Ca^{2+} with 5 µg/ml of the given protein (31). The hemolytic activity of CyaA/AC^- was taken as 100% activity. The results represent the average of values obtained in four independent experiments performed in duplicates ± S.D with two different protein preparations.
- [b]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 µg/ml of the appropriate protein at 37°C in D-MEM. J774A.1 cell lysis by CyaA/AC^- was taken as 100%. The results represent the average of values obtained in four separate experiments performed in duplicates ± S.D with two different protein preparations (*, $p < 0.05$; **, $p < 0.001$).

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^- 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.

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 μg/ml (5 μg/ml for intact toxin), in order to achieve binding of equal amounts of all proteins to erythrocytes, as shown in Fig. 1C. 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, as shown in figure 2C. This suggests that combination of the two substitutions affected the specific cell-permeabilizing capacity of CyaA.

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 ± 7%) relative cytolytic capacity on these cells (cf. Table I). As shown in Fig. 2C, when compared with intact CyaA, the singly mutated E570Q and the doubly mutated E570Q+K860R constructs were importantly impaired in the capacity to elicit decrease of intracellular concentration of potassium ions ($[K^+]i$) in toxin-treated J774A.1 cells. While no cell lysis was detected over 20 min

by the assay for release of lactate dehydrogenase, the $[K^+]i$ of J774A.1 cells exposed to 3 μg ml^{-1} of intact CyaA decreased from 140 mM to well below 30 mM already in 10 min upon toxin addition. In turn, when the same amounts of the ER570QiK860R constructs were used (3 μg ml^{-1}), the $[K^+]i$ levels in cells did not decrease below 100 mM (Fig. 2C). Indeed, efflux of potassium from cells was previously shown to be the hallmark of insertion of the CyaA pore precursors into cell membrane (32). 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.

This conclusion was further supported by an importantly reduced relative cytolytic activity of the corresponding E570Q/AC- and E570Q+K860R/AC- toxoids, as discussed above (Table I) and documented in detail in Fig. 3A. The doubly mutated E570Q+K860R toxoid at 3 μg ml^{-1} was essentially unable to provoke any detectable release of lactate dehydrogenase from J774A.1 cells in 3 h of incubation, while 20% of cells lysed in the presence of equal amounts of intact toxoid.

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^- 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 μg/ ml of CyaA/OVA/AC^-, upon an initial lag of about 3 minutes the J774A.1 cells were progressively and massively permeabilized by CyaA/OVA/AC^- 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^- 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. Quite similar picture was observed when toxoid concentrations were elevated to 10 μg ml^{-1}, which was the concentration used for comparisons of

relative cytolytic activity of toxoids summarized in Table I. The 10-fold increase of concentration from 1 to 10 μg ml^{-1} resulted in about twofold increase of the currents produced across cell membrane by OVA/AC-, while essentially no enhancement of cell permeabilization was observed even at the increased concentration of OVA/E570Q+K860R/AC- (note the expanded scale of y-axis for measurements at 10 μg ml^{-1}). 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.

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^- 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^b MHC class I molecules with the SIINFEKL (OVA) peptide on APCs. It has, indeed, been previously shown that the capacity of CyaA/AC- toxoids to translocate the AC domain across the cytoplasmic membrane into cytosol of BMDCs is essential for the capacity of the toxoids to promote presentation of the delivered OVA epitope in complex with the H-2Kb MHC class I molecules. This, in turn, is essential for specific *in vitro* stimulation of cytotoxic T cells to occur (29). Nevertheless, it was important to confirm here that delivery of the OVA epitope for proteasome processing and subsequent presentation was due to AC domain translocation into cytosol of BMDCs across their cytoplasmic

membrane, and was not due to sampling of the added antigen by fluid phase uptake, or endocytosis. For this purpose, a doubly mutated non-translocating OVA/E570K+E581 P/AC- toxoid variant was used, which bears a combination of charge-reversing and a-helix-breaking substitutions of glutamates 570 and 581 in the transmembrane domain of CyaA (33). This construct was previously found to exhibit a full capacity to bind CD11b/CD18-expressing cells (cf. Fig. 2A), while its capacity to translocate the AC domain across target cell membrane was ablated by the combination of E570K and E581P substitutions (cf. Fig. 2B).

As shown in Fig. 4A, the B3Z hybridoma cells were effectively stimulated upon co-incubation with BMDCs loaded with the OVAE570Q/AC- and OVA/E57OQ+K860R/AC- toxoids. In contrast, no B3Z stimulation was observed upon co-incubation with BMDCs loaded with the OVA/E570K+E581P/AC- toxoid defective in AC domain translocation across cell membrane. Moreover, the OVA/E570Q/AC$^-$ and OVA/E570Q+K860R/AC$^-$ toxoids induced stimulation of the B3Z lymphocytes by APCs *in vitro* with as high efficiency as intact OVA/AC$^-$ 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^b 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.

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 $CD8^+$ 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 $CFSE^{high}$ and unloaded $CFSE^{low}$ 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$^-$ toxoids to deliver an AC-inserted passenger antigen into cytosol of APCs.

Discussion

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.

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$^-$ toxoid exhibiting a two-fold reduced cytolytic activity on J774A.1 cells (8, 13).

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. It is worth mentioning that, based on previous comparisons of haemolytic potency of the intact *r-Ec*-CyaA with that of the native *Bp*-CyaA purified from *B. pertussis,* the specific haemolytic activity of the here described *r-Ec*-CyaA/OVA/E570Q+K860R/AC- toxoid on sheep erythrocytes would be reduced by about three orders of magnitude. The residual specific cytolytic activity of *r-Ec*-CyaA/OVA/E570Q+K860R/AC- on CD11b-expressing cells would then be estimated to be about 50-fold lower than that of *Bp*-CyaA, while the specific capacity of both proteins to deliver the AC domain into these cells would be the same (using intact *r-Ec*-CyaA as 100% invasive AC activity standard for comparisons). 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.

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 ($CD11b^+$).

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). It is proposed that the transmembrane segments harbouring the critical glutamate residues 509, 516, 570 and 581 can participate in formation of an oligomeric and cation-selective cytolytic pore only if the membrane-inserted pore precursor conformer has the AC domain located outside the cell. In the AC translocating conformer, the same transmembrane segments would adopt a different conformation in the membrane, being potentially obtruded and prevented from entering CyaA oligomers by the polypeptide linking the C-terminal end of the AC domain to transmembrane segments. Support for this interpretation can be deduced from results obtained by Gray and co-workers (25). These authors showed that deletion of the AC domain together with the segment linking it to the pore-forming domain (up to residue 489), or binding of the 3D1 antibody that blocks membrane translocation of the terminal AC domain segment located between residues 373 and 399, importantly enhances the pore-forming (haemolytic) activity of the toxin. This is likely to be due to imposing a conformation on the transmembrane segments of CyaA that is favourable for formation of the otigomeric pores. 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.

CyaA was, indeed, previously shown to promote formation of inverted non-lamellar (inverted hexagonal phase) lipid structures (28). These might potentially take part in formation of a well-sealed protein-lipid interface, thus allowing translocation of the AC domain across membrane in the absence of cell permeabilization. Formation of non-lamellar lipid structures is favoured in

cholesterol-enriched lipid rafts and CyaA was, indeed, recently found to mobilize into rafts in complex with its receptor CD11b/CD18. Moreover, the inventors have recently shown that AC domain translocation across membrane is accomplished only upon relocation of CyaA into rafts (L., Bumba, J., Masin, R., Fiser, and P., Sebo, submitted). Intriguingly, translocation of the catalytic subunit of diphtheria toxin (DT) across the cell cytoplasmic membrane was also previously found to occur without detectable cell permeabilization, when DT was pulsed into cells by low pH upon binding to a truncated GPI-anchored DT receptor (34). The authors did not examine whether the GPI-anchored DT receptor localized into lipid rafts, but this is highly likely. It is, hence, tempting to speculate that the specific lipid composition of the raft membrane may support translocation of different protein toxins into target cells without the need for formation of a true protein conducting pore permeabilizing the cell.

Last not least, a practical discovery reported herein is that the CyaA/E570Q+K860R/AC$^-$ toxoid with the much reduced cell-permeabilizing (cytolytic) activity, remains fully active in antigen delivery into CD11b$^+$ 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$^-$-derived vaccines for immunotherapy of cancer.

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2-3	**Identification of deposit**	
2-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes
2-3-2	Address of depositary institution	Institut Pasteur, 28, rue du Dr Roux, 75724 Paris Cedex 15, France
2-3-3	Date of deposit	18 March 2009 (18.03.2009)
2-3-4	Accession Number	CNCM I-4137
2-4	**Additional Indications**	Identification: OVA-QR-AC-
2-5	**Designated States for Which Indications are Made**	All designations

FOR RECEIVING OFFICE USE ONLY

0-4	**This form was received with the international application:** (yes or no) Y	
0-4-1	Authorized officer	Vinci, Rossana

PCT

Print Out (Original in Electronic Form)
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FOR INTERNATIONAL BUREAU USE ONLY

0-5	**This form was received by the international Bureau on:**	
0-5-1	Authorized officer	

Claims

1. A Polypeptide which is a mutant of an adenylate cyclase protein (mutant polypeptide) and whose amino acid sequence comprises or consists of one of the following sequences:
   a) the amino acid sequence of the adenylate cyclase (CyaA) of *Bordetella pertussis*, *Bordetella parapertussis* or *Bordetella hinzii* wherein the following mutations have been performed:
      (i) the substitution of the glutamic acid residue at position 570 by a glutamine residue (E570Q) or by a conservative amino acid residue, and
      (ii) the substitution of the lysine residue at position 860 by an arginine residue (K860R) or by a conservative amino acid residue, or;
   b) an amino acid sequence of a fragment of the adenylate cyclase of *Bordetella pertussis*, *Bordetella parapertussis or Bordetella hinzii,* which fragment has the capacity of the CyaA protein of *Bordetella pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said fragment further contains the following mutated amino acid residues located at positions 570 and 860 in said adenylate cyclase: E570Q and K860R, or
   c) an amino acid sequence which differs from the amino acid sequence as defined in a) or b) by one or more amino acid residue substitutions and/or insertions and which has the capacity of the CyaA protein of *Bordetella Pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said amino acid sequence further contains the following mutated amino acid residues located at positions 570 and 860 in said adenylate cyclase: E570Q and K860R, or
   d) the amino acid sequence of the adenylate cyclase (CyaA) of *Bordetella bronchiseptica* wherein the following mutations have been performed:

(i) the substitution of the glutamic acid residue at position 569 by a glutamine residue (E569Q) or by a conservative amino acid residue, and

(ii) the substitution of the lysine residue at position 859 by an arginine residue (K859R) or by a conservative amino acid residue, or;

e) an amino acid sequence of a fragment of the adenylate cyclase of *Bordetella bronchiseptica,* which fragment has the capacity of the CyaA protein of *Bordetella pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said fragment further contains the following mutated amino acid residues located at positions 569 and 859 in said adenylate cyclase: E569Q and K859R, or

f) an amino acid sequence which differs from the amino acid sequence as defined in d) or e) by one or more amino acid residue substitutions and/or insertions and which has the capacity of the CyaA protein of *Bordetella Pertussis* to bind to a target cell and the capacity to translocate its N-terminal adenylate cyclase enzyme domain or part thereof into said cell, wherein said amino acid sequence further contains the following mutated amino acid residues located at positions 569 and 859 in said adenylate cyclase: E569Q and K859R.

2. Polypeptide according to claim 1, wherein the amino acid of said adenylate cyclase of *Bordetella Pertussis* adenylate cyclase is disclosed as SEQ ID N° 1.

3. Polypeptide according to claim 2, wherein the amino acid sequence of said polypeptide further differs from that of SEQ ID N° 1 by 1 to 200 amino acid residue substitutions, deletions, and/or insertions.

4. Polypeptide according to claim 1 or 2, which is capable of binding to cells and of translocating its N-terminal adenylate cyclase enzyme domain into said cells

wherein said cells express the CD11b/CD18 receptor and wherein binding to said cells occurs through binding to said CD11b/CD18 receptor.

5. Polypeptide according to claim 1, 2,3 or 4, 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.

6. Polypeptide according to claim 5, wherein said partial or total suppression of adenylate cyclase activity is achieved by insertion of a dipeptide between the amino acid residues at positions 188 and 189 of the adenylate cyclase of *Bordetella Pertussis,* especially of SEQ ID N°1.

7. Polypeptide derivative comprising or consisting of the polypeptide according to any of claims 1 to 6 and which is further combined with one or more molecules of interest.

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.

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 these antigens which comprises at least one epitope.

11. Polypeptide derivative according to claim 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 the adenylate cyclase amino acid sequence of the mutant polypeptide, thereby preserving the capacity of said mutant polypeptide to translocate its N-terminal adenylate cyclase enzyme domain into target cells.

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 mutant polypeptide.

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

14. Polypeptide according to claim 1 to 6 or polypeptide derivative according to any of claims 7 to 12 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 anyone of claims 7 to 14 for 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.

16. Polypeptide or polypeptide derivative according to claim 13, 14 or 15, wherein said polypeptide is to be administered in combination with an adjuvant and/or in combination with another therapeutically active molecule.

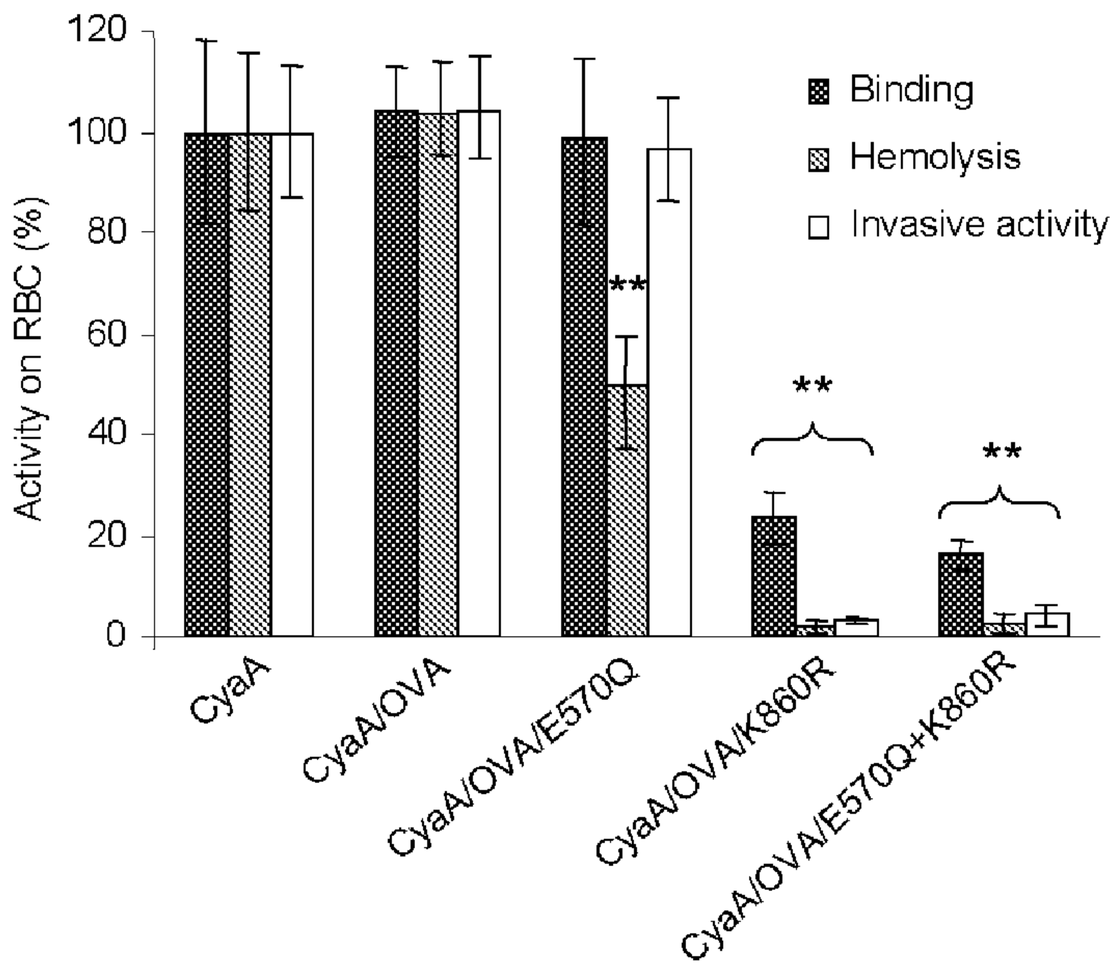
17. Polypeptide or polypeptide derivative according to claim 13 or 14, wherein said polypeptide is not to be administered in combination with an adjuvant.

18. Pharmaceutical composition comprising a polypeptide according to any of claims 1 to 6 or a polypeptide derivative according to any one of claims 7 to 17, a pharmaceutically acceptable carrier, and optionally an adjuvant and/or a therapeutically active molecule.

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- or retroviral-induced diseases.

20. Method for the preparation of a proteinaceous vector suitable for the delivery of a molecule into a cell comprising binding said molecule to a polypeptide according to any of claims 1 to 6.

A


120
100
80
60
40
20
0
Activity on RBC (%)
Binding
Hemolysis
Invasive activity
**
**
**
CyaA
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R

B

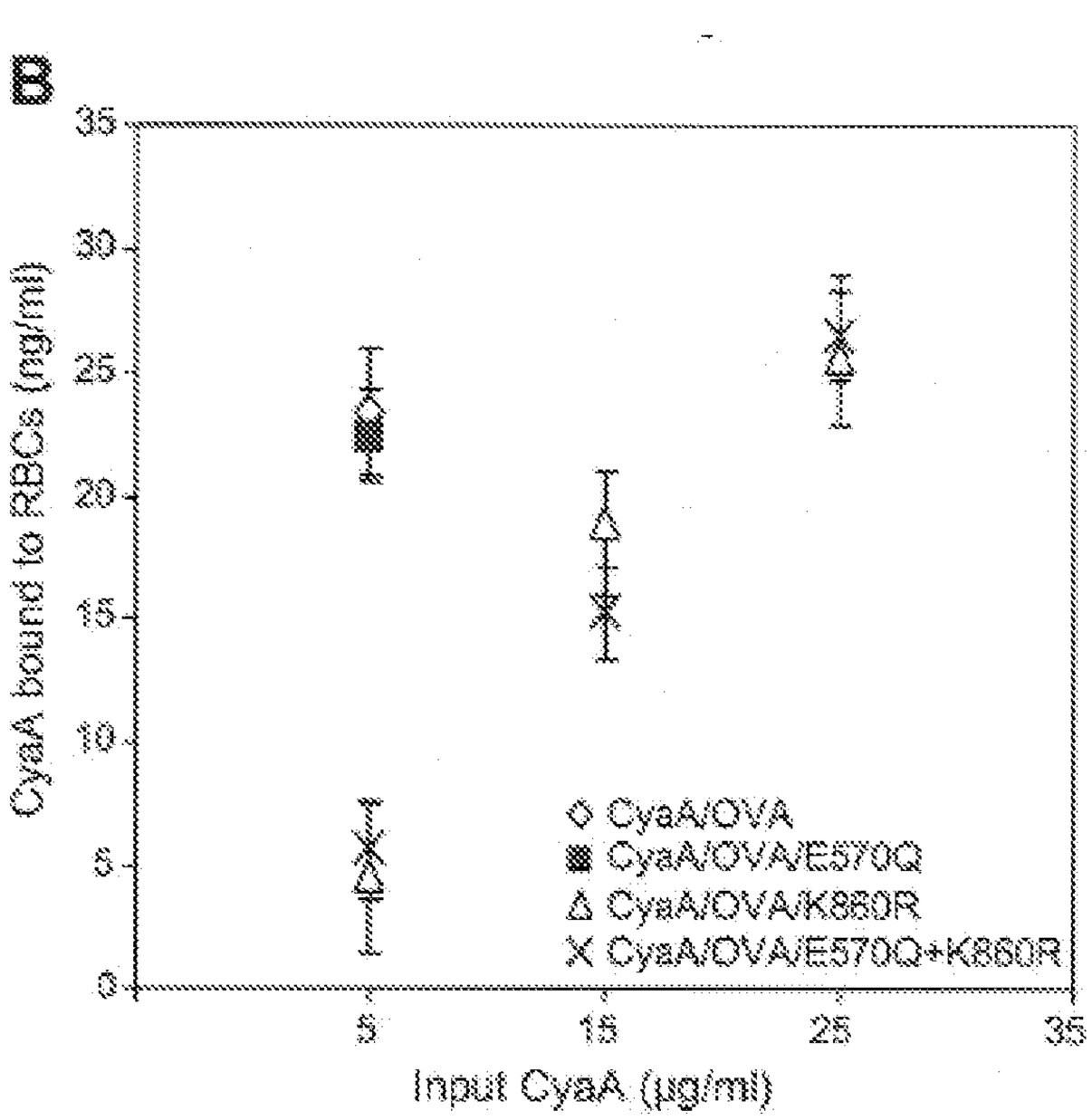

CyaA bound to RBCs (mg/ml)
35
25
20
15
10
5
0
5
15
25
35
Input CyaA (µg/ml)
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R

Figure 1

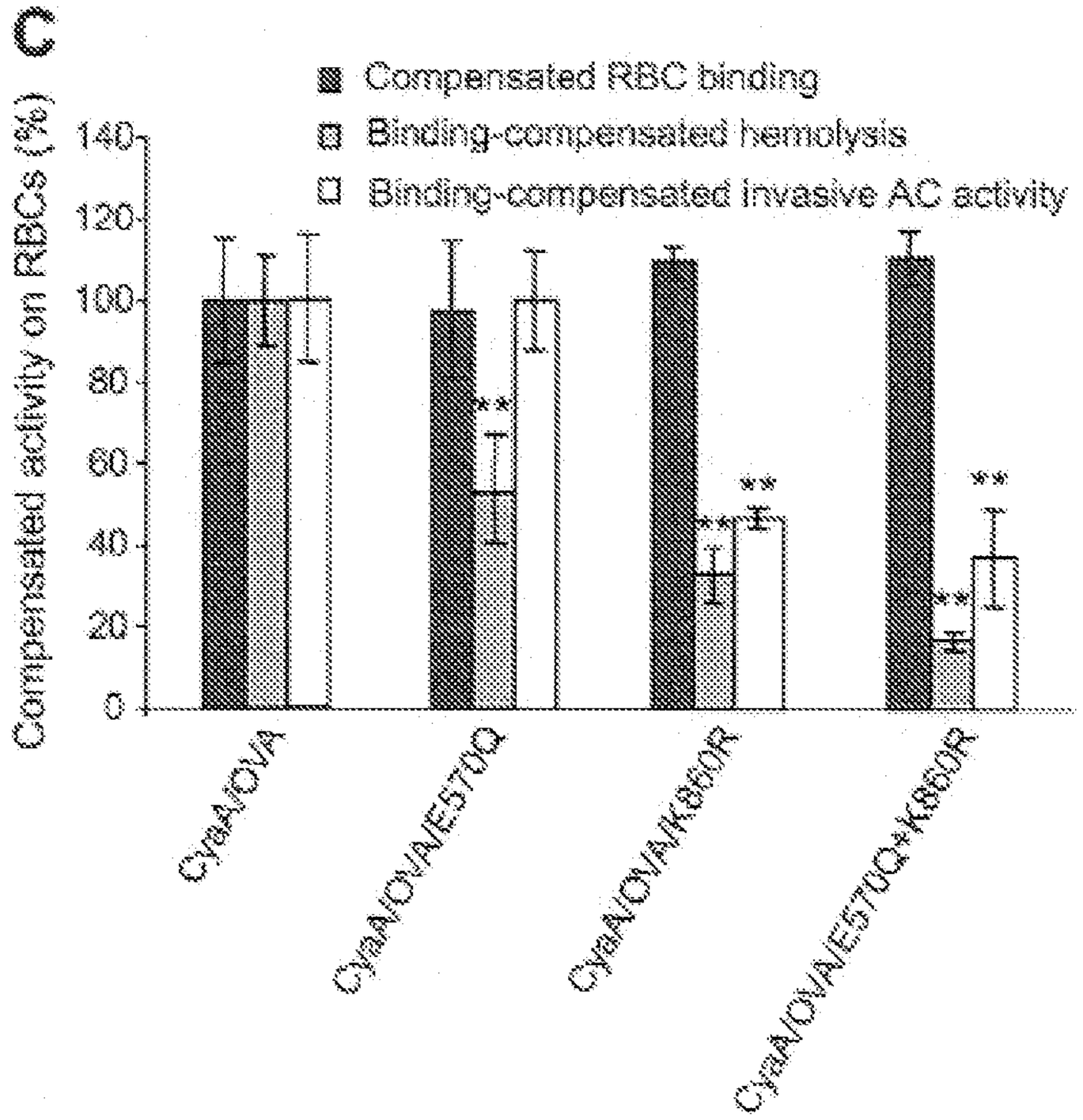
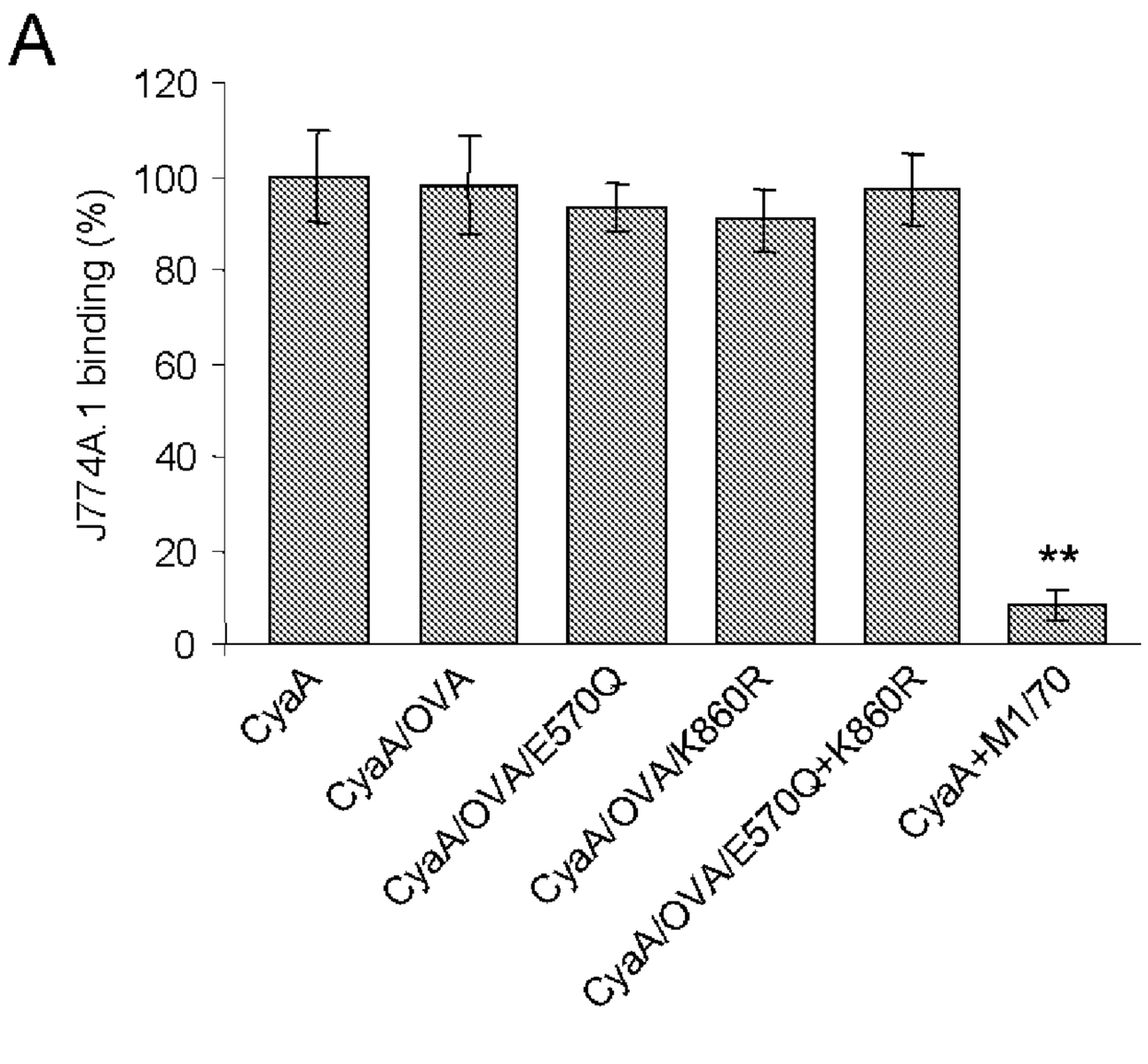

Compensated RBC binding
Binding-compensated hemolysis
Binding-compensated invasive AC activity
Compensated activity on RBCs (%)
0
20
40
60
80
100
120
140
**
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R

Figure 1


A
J774A.1 binding (%)
120
100
80
60
40
20
0
**
CyaA
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R
CyaA+M1/70

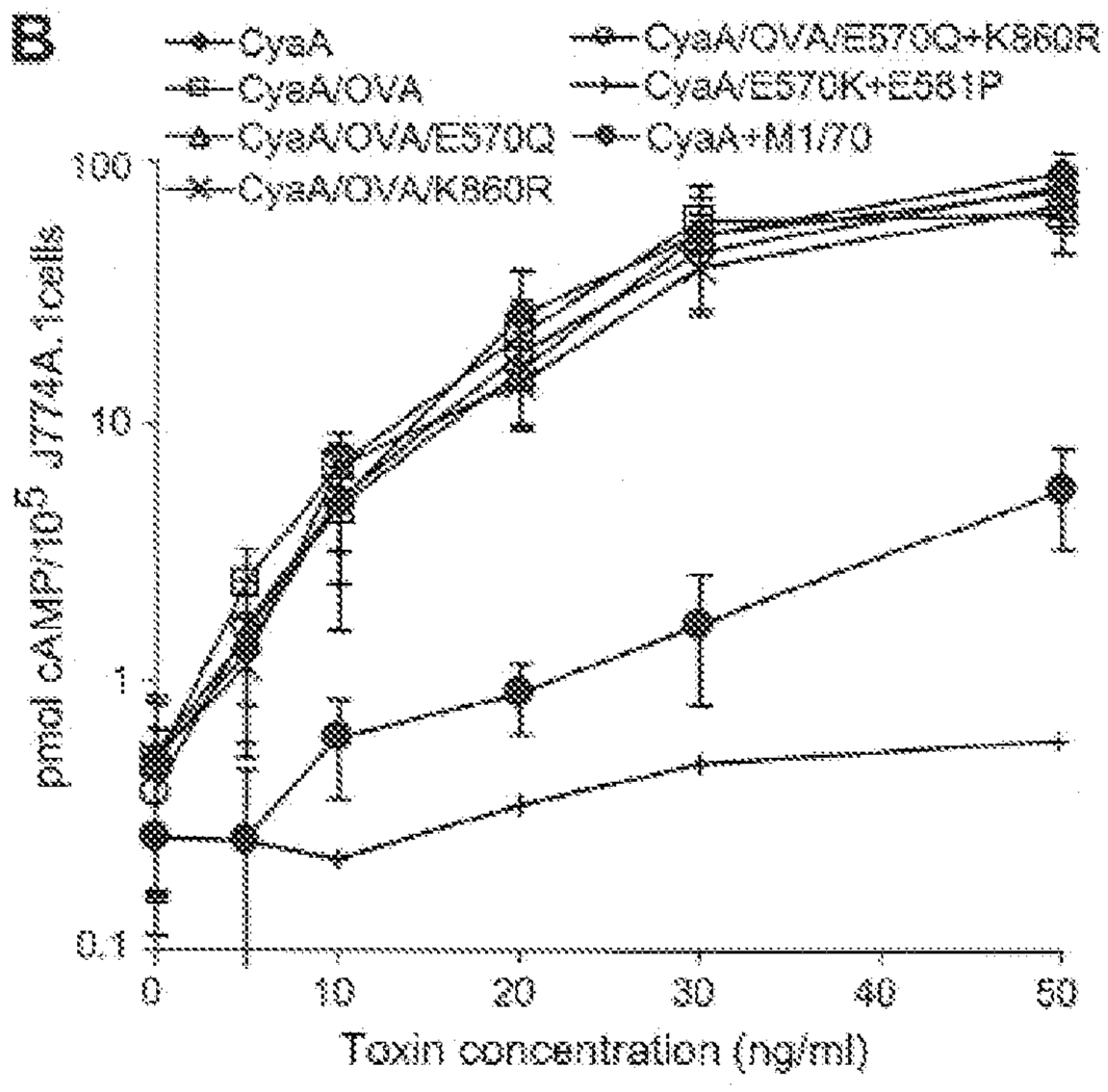

B
CyaA
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R
CyaA/E570K+E581P
CyaA+M1/70
pmol cAMP/10^5 J774A.1cells
100
10
1
0.1
0
10
20
30
40
50
Toxin concentration (ng/ml)

Figure 2

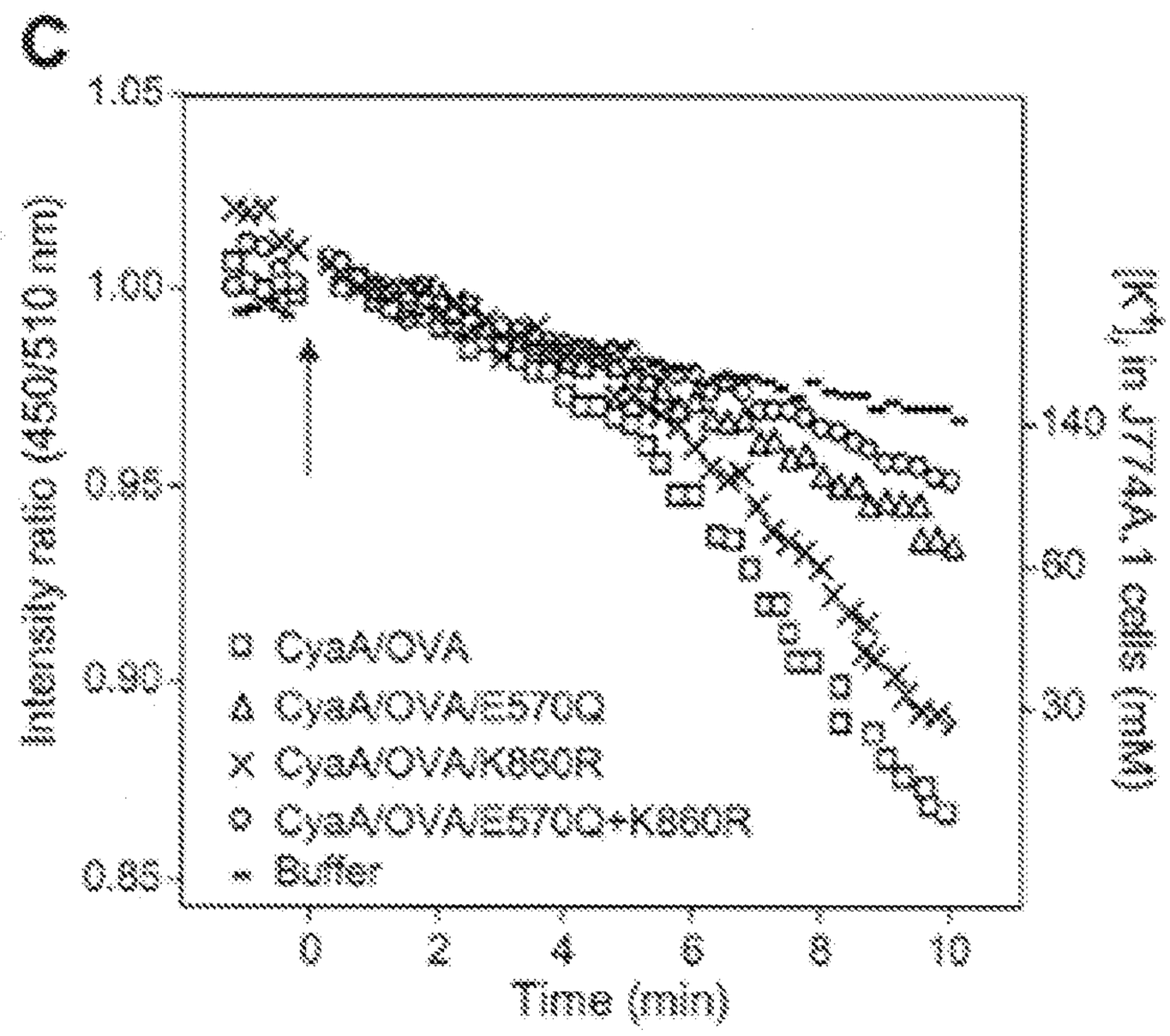

C
Intensity ratio (450/510 nm)
1.05
1.00
0.95
0.90
0.85
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R
Buffer
0
2
4
6
8
10
Time (min)
40
60
30
[K+] in CyaA-treated cells (mM)

Figure 2

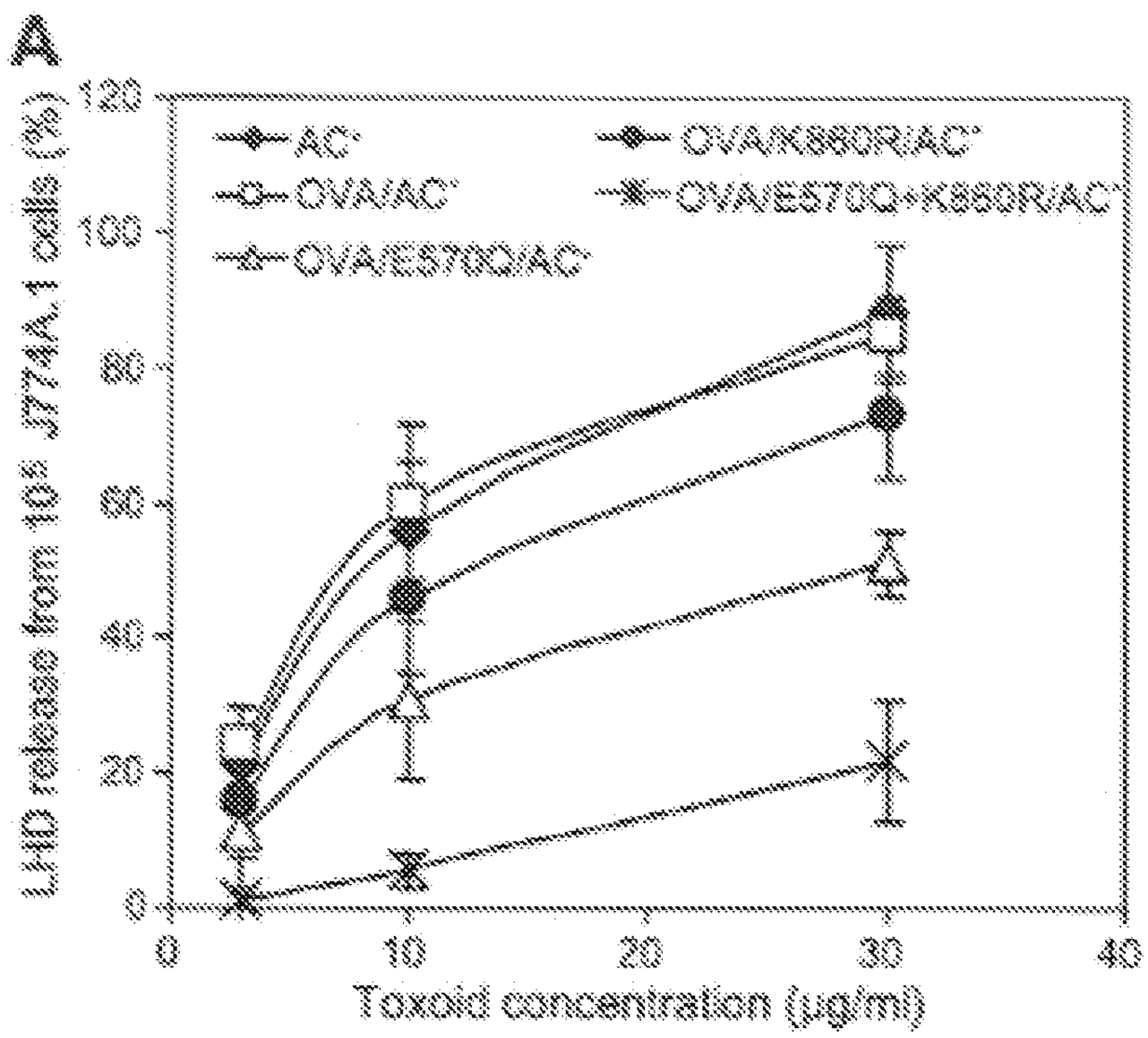

A
AC
OVA/AC
OVA/ES700/AC
OVA/K880R/AC
OVA/ES700+K880R/AC
LHD release from 10^5 3T3A-1 cells (%)
120
100
80
60
40
20
0
0
10
20
30
40
Toxoid concentration (µg/ml)

Figure 3

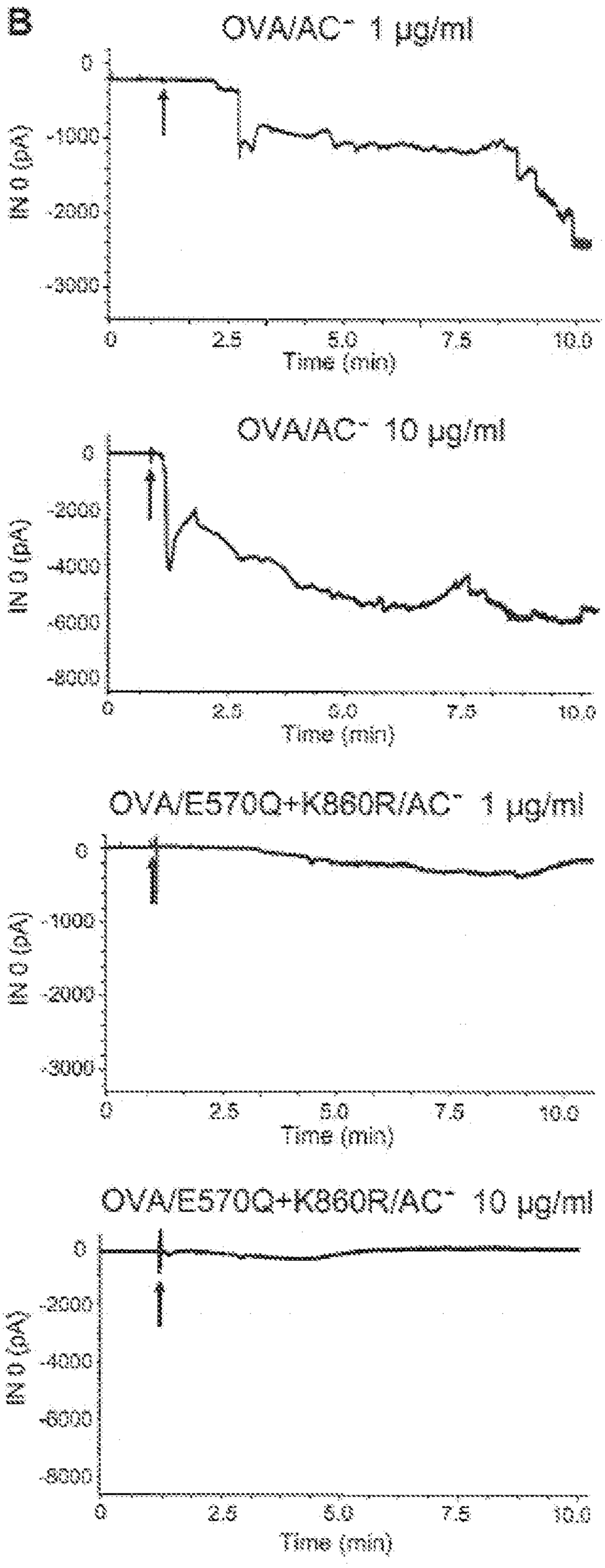
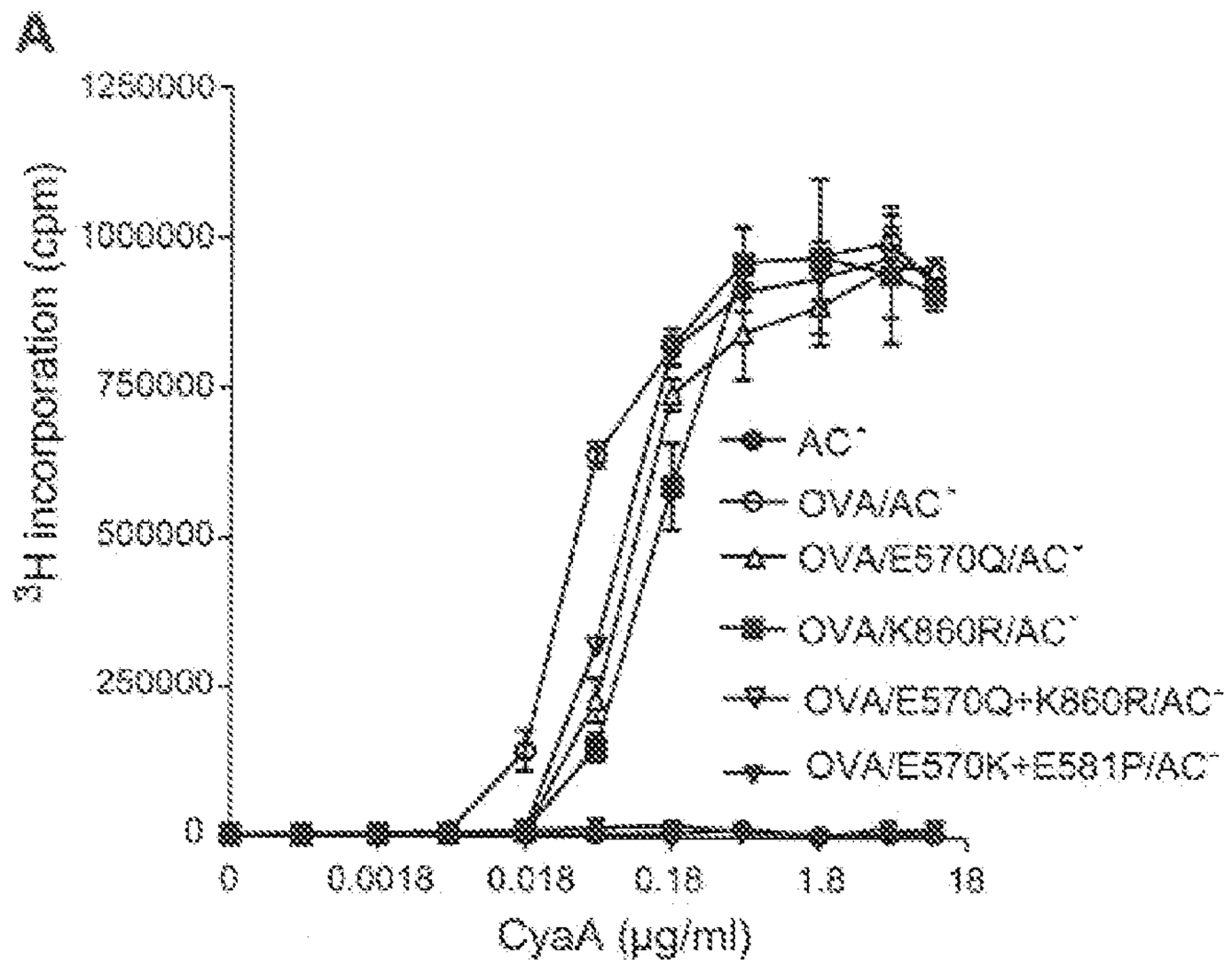
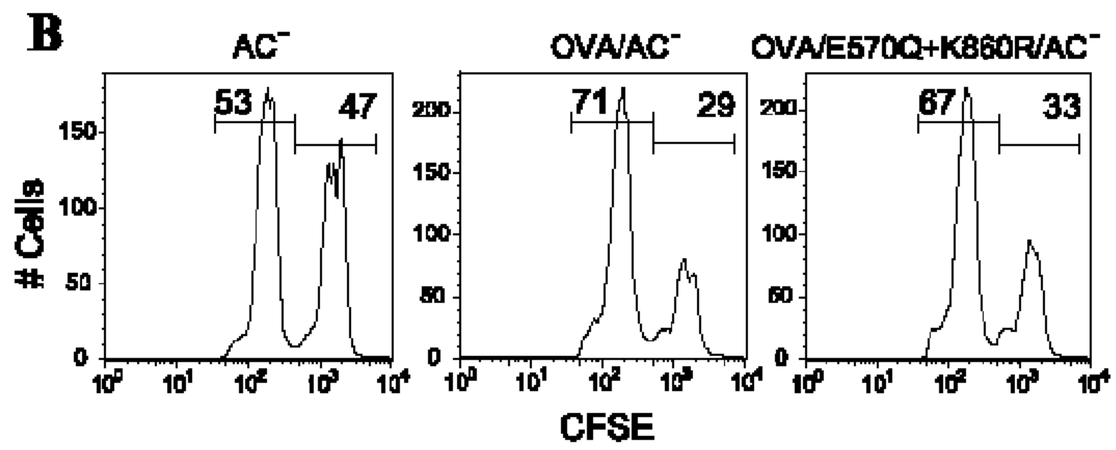

B
OVA/AC⁻ 1 µg/ml
0
-1000
-2000
-3000
IN 0 (pA)
0
2.5
5.0
7.5
10.0
Time (min)
OVA/AC⁻ 10 µg/ml
0
-2000
-4000
-6000
-8000
IN 0 (pA)
0
2.5
5.0
7.5
10.0
Time (min)
OVA/E570Q+K860R/AC⁻ 1 µg/ml
0
-1000
-2000
-3000
IN 0 (pA)
0
2.5
5.0
7.5
10.0
Time (min)
OVA/E570Q+K860R/AC⁻ 10 µg/ml
0
-2000
-4000
-6000
-8000
IN 0 (pA)
0
2.5
5.0
7.5
10.0
Time (min)

Figure 3


A
1250000
1000000
750000
500000
250000
0
^{3}H incorporation (cpm)
0
0.0016
0.016
0.16
1.6
16
CyaA (µg/ml)
AC$^-$
OVA/AC$^-$
OVA/E570Q/AC$^-$
OVA/K860R/AC$^-$
OVA/E570Q+K860R/AC$^-$
OVA/E570K+E581P/AC$^-$


B
AC$^-$
OVA/AC$^-$
OVA/E570Q+K860R/AC$^-$
53
47
71
29
67
33
Cells
CFSE

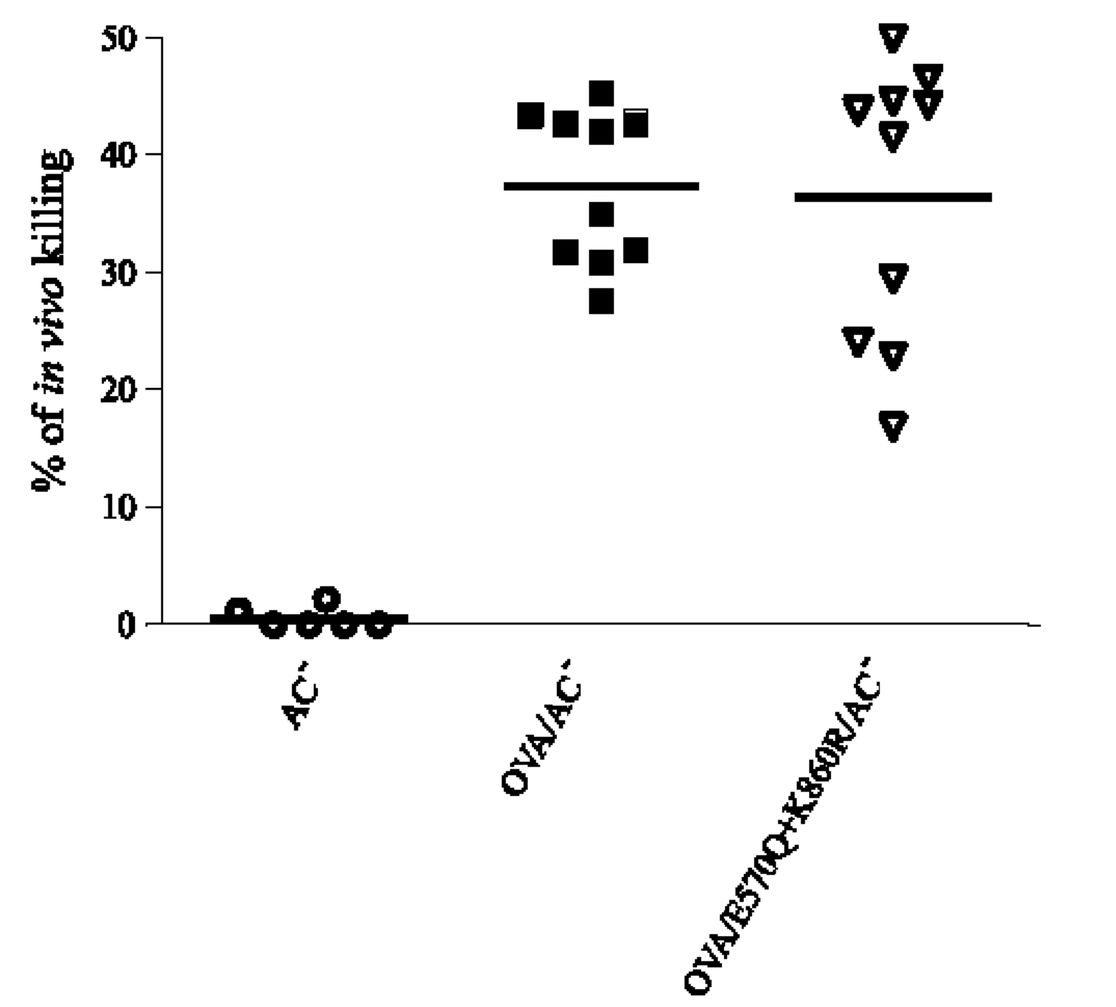
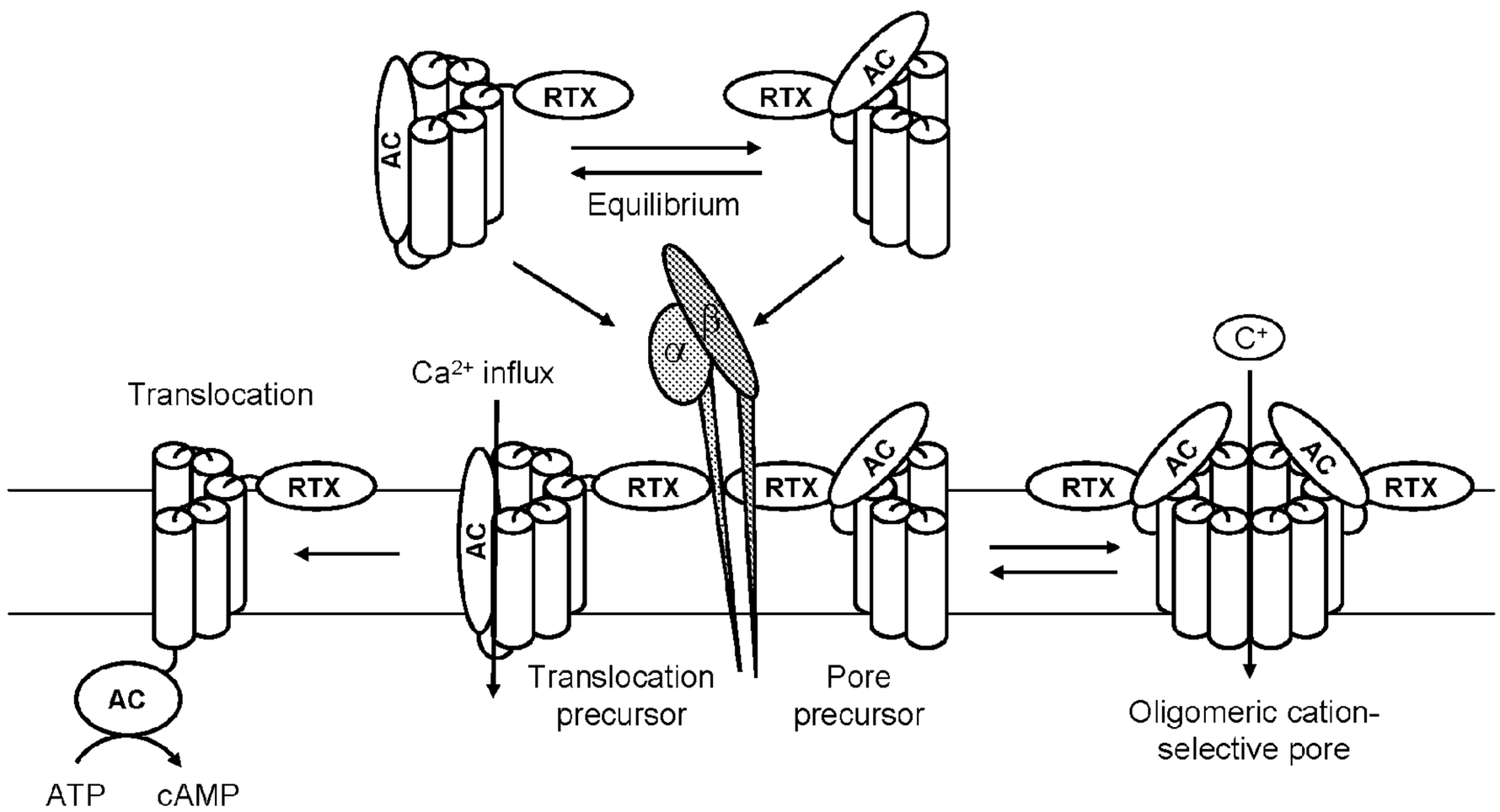

% of *in vivo* killing
AC$^-$
OVA/AC$^-$
OVA/E570Q+K860R/AC$^-$

Figure 4


A
Wild-type CyaA
RTX
AC
Equilibrium
α
β
Ca2+ influx
Translocation
C+
Translocation precursor
Pore precursor
Oligomeric cation-selective pore
ATP
cAMP

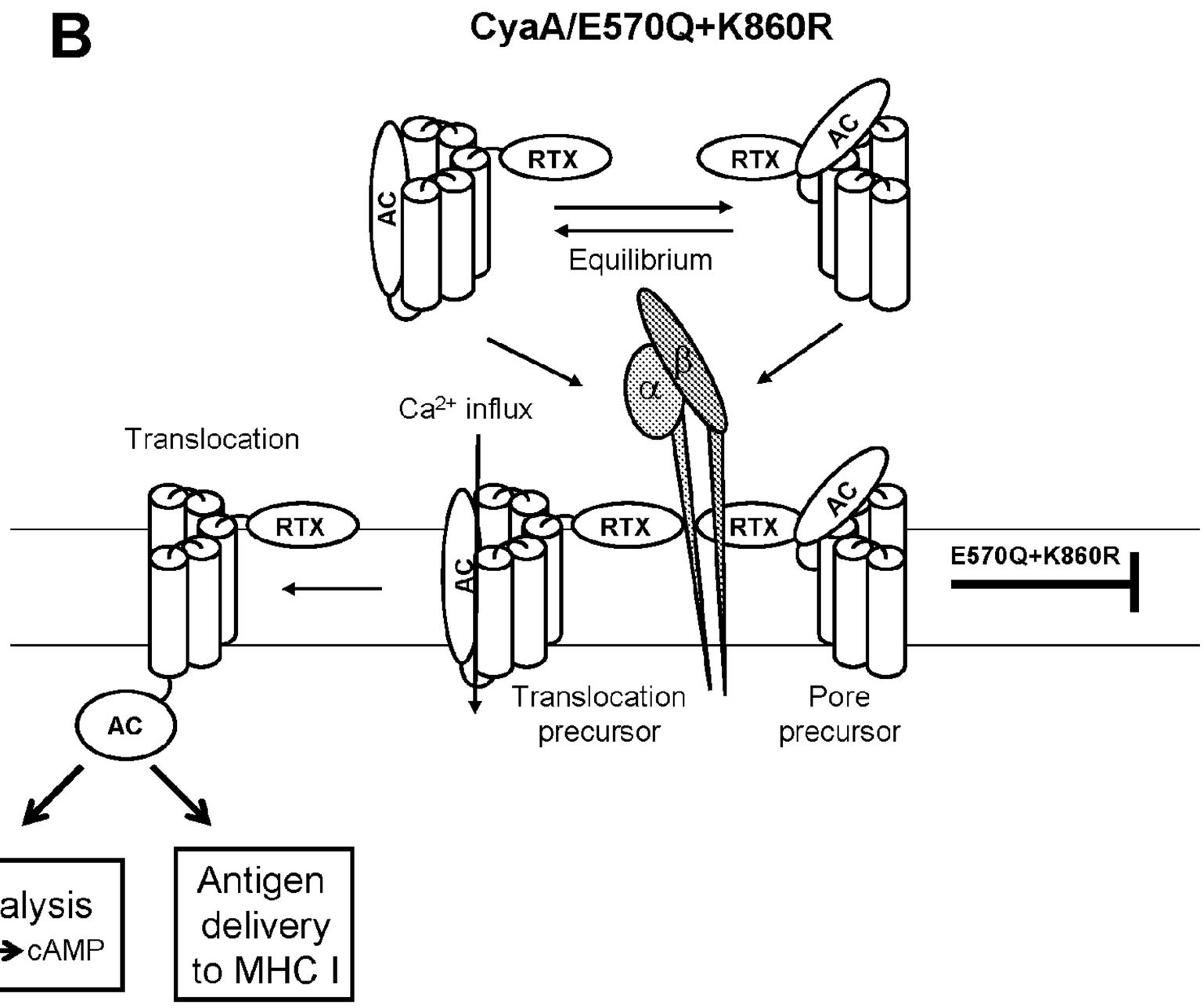

B
CyaA/E570Q+K860R
RTX
AC
Equilibrium
α
β
Ca2+ influx
Translocation
E570Q+K860R
Translocation precursor
Pore precursor
catalysis
ATP → cAMP
Antigen delivery to MHC I

Figure 5

```
   1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL
  61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTLSKERL
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIGNA
 181 AGIPLTADID MFAIMPHLSN FRDSARSSVT SGDSVTDYLA RTRRAASEAT GGLDRERIDL
 241 LWKIARAGAR SAVGTEARRQ FRYDGDMNIG VITDFELEVR NALNRRAHAV GAQDVVQHGT
 301 EQNNPFPEAD EKIFVVSATG ESQMLTRGQL KEYIGQQRGE GYVFYENRAY GVAGKSLFDD
 361 GLGAAPGVPS GRSKFSPDVL ETVPASPGLR RPSLGAVERQ DSGYDSLDGV GSRSFSLGEV
 421 SDMAAVEAAE LEMTRQVLHA GARQDDAEPG VSGASAHWGQ RALQGAQAVA AAQRLVHAIA
 481 LMTQFGRAGS TNTPQEAASL SAAVFGLGEA SSAVAETVSG FFRGSSRWAG GFGVAGGAMA
 541 LGGGIAAAVG AGMSLTDDAP AGQKAAAGAE IALQLTGGTV ELASSIALAL AAARGVTSGL
 601 QVAGASAGAA AGALAAAALSP MEIYGLVQQS HYADQLDKLA QESSAYGYEG DALLAQLYRD
 661 KTAAEGAVAG VSAVLSTVGA AVSIAAAASV VGAPVAVVTS LLTGALNGIL RGVQQPIIEK
 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 RHVELARVGQ VVEVDTLEHV QHIIGGAGND
1021 SITGNAHDNF LAGGSGDDRL DGGAGNDTLV GGEGQNTVIG GAGDDVFLQD LGVWSNQLDG
1081 GAGVDTVKYN VHQPSEERLE RMGDTGIHAD LQKGTVEKWP ALNLFSVDHV KNIENLHGSR
1141 LNDRIAGDDQ DNELWGHDGN DTIRGRGGDD ILRGGLGLDT LYGEDGNDIF LQDDETVSDD
1201 IDGGAGLDTV DYSAMIHPGR IVAPHEYGFG IEADLSREWV RKASALGVDY YDNVRNVENV
1261 IGTSMKDVLI GDAQANTLMG QGGDDTVRGG DGDDLLFGGD GNDMLYGDAG NDTLYGGLGD
1321 DTLEGGAGND WFGQTQAREH DVLRGGDGVD TVDYSQTGAH AGIAAGRIGL GILADLGAGR
1381 VDKLGEAGSS AYDTVSGIEN VVGTELADRI TGDAQANVLR GAGGADVLAG GEGDDVLLGG
1441 DGDDQLSGDA GRDRLYGEAG DDWFFQDAAN AGNLLDGGDG RDTVDFSGPG RGLDAGAKGV
1501 FLSLGKGFAS LMDEPETSNV LRNIENAVGS ARDDVLIGDA GANVLNGLAG NDVLSGGAGD
1561 DVLLGDEGSD LLSGDAGNDD LFGGQGDDTY LFGVGYGHDT IYESGGGHDT IRINAGADQL
1621 WFARQGNDLE IRILGTDDAL TVHDWYRDAD HRVEIIHAAN QAVDQAGIEK LVEAMAQYPD
1681 PGAAAAAPPA ARVPDTLMQS LAVNWR
```

Figure 6

```
   1 MQQSHQAGYA NAADRESGIP AAVLDGIKAV AKEKNATLMF RLVNPHSTSL IAEGVATKGL
  61 GVHAKSSDWG LQAGYIPVNP NLSKLFGRAP EVIARADNDV NSSLAHGHTA VDLTLSKERL
 121 DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIGNA
 181 AGIPLTADID MFAIMPHLSN FRDSARSSVT SGDSVTDYLA RTRRAASEAT GGLDRERIDL
 241 LWKIARAGAR SAVGTEARRQ FRYDGDMNIG VITDFELEVR NALNRRAHAV GAQDVVQHGT
 301 EQNNPFPEAD EKIFVVSATG ESQMLTRGQL KEYIGQQRGE GYVFYENRAY GVAGKSLFDD
 361 GLGAAPGVPS GRSKFSPDVL ETVPASPGLR RPSLGAVERQ DSGYDSLDGV GSRSFSLGEV
 421 SDMAAVEAAE LEMTRQVLHA GARQDDAEPG VSGASAHWGQ RALQGAQAVA AAQRLVHAIA
 481 LMTQFGRAGS TNTPQEAASL SAAVFGLGEA SSAVAETVSG FFRGSSRWAG GFGVAGGAMA
 541 LGGGIAAAVG AGMSLTDDAP AGQKAAAGAQ IALQLTGGTV ELASSIALAL AAARGVTSGL
 601 QVAGASAGAA AGALAAALSP MEIYGLVQQS HYADQLDKLA QESSAYGYEG DALLAQLYRD
 661 KTAAEGAVAG VSAVLSTVGA AVSIAAAASV VGAPVAVVTS LLTGALNGIL RGVQQPIIEK
 721 LANDYARKID ELGGPQAYFE KNLQARHEQL ANSDGLRKML 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 YGKRTENVQY RHVELARVGQ VVEVDTLEHV QHIIGGAGND
1021 SITGNAHDNF LAGGSGDDRL DGGAGNDTLV GGEGQNTVIG GAGDDVFLQD LGVWSNQLDG
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1321 DTLEGGAGND WFGQTQAREH DVLRGGDGVD TVDYSQTGAH AGIAAGRIGL GILADLGAGR
1381 VDKLGEAGSS AYDTVSGIEN VVGTELADRI TGDAQANVLR GAGGADVLAG GEGDDVLLGG
1441 DGDDQLSGDA GRDRLYGEAG DDWFFQDAAN AGNLLDGGDG RDTVDFSGPG RGLDAGAKGV
1501 FLSLGKGFAS LMDEPETSNV LRNIENAVGS ARDDVLIGDA GANVLNGLAG NDVLSGGAGD
1561 DVLLGDEGSD LLSGDAGNDD LFGGQGDDTY LFGVGYGHDT IYESGGGHDT IRINAGADQL
1621 WFARQGNDLE IRILGTDDAL TVHDWYRDAD HRVEIIHAAN 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 FEAVKVIGNA
 181 AGIPLTADGS IDMFAIMPHL SNFRDSARSS VTSGDSVTDY LARTRRAASE ATGGLDRERI
 241 DLLWKIARAG ARSAVGTEAR RQFRYDGDMN IGVITDFELE VRNALNRRAH AVGAQDVVQH
 301 GTEQNNPFPE ADEKIFVVSA TGESQMLTRG QLKEYIGQQR GEGYVFYENR AYGVAGKSLF
 361 DDGLGAAPGV PSGRSKFSPD VLETVPASPG LRRPSLGAVE RQDSGYDSLD GVGSRSFSLG
 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 LAQESSAYGY EGDALLAQLY
 661 RDKTAAEGAV AGVSAVLSTV GAAVSIAAAA SVVGAPVAVV TSLLTGALNG ILRGVQQPII
 721 EKLANDYARK IDELGGPQAY FEKNLQARHE QLANSDGLRK MLADLQAGWN ASSVIGVQTT
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1321 GDDTLEGGAG NDWFGQTQAR EHDVLRGGDG VDTVDYSQTG AHAGIAAGRI GLGILADLGA
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Figure 8

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121     DYLRQAGLVT GMADGVVASN HAGYEQFEFR VKETSDGRYA VQYRRKGGDD FEAVKVIGNA
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241     FEKLVHLDRE RIDLLWKIAR AGARSAVGTE ARRQFRYDGD MNIGVITDFE LEVRNALNRR
301     AHAVGAQDVV QHGTEQNNPF PEADEKIFVV SATGESQMLT RGQLKEYIGQ QRGEGYVFYE
361     NRAYGVAGKS LFDDGLGAAP GVPSGRSKFS PDVLETVPAS PGLRRPSLGA VERQDSGYDS
421     LDGVGSRSFS LGEVSDMAAV EAAELEMTRQ VLHAGARQDD AEPGVSGASA HWGQRALQGA
481     QAVAAAQRLV HAIALMTQFG RAGSTNTPQE AASLSAAVFG LGEASSAVAE TVSGFFRGSS
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601     ALALAAARGV TSGLQVAGAS AGAAAGALAA ALSPMEIYGL VQQSHYADQL DKLAQESSAY
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901     IDLAKVVSQL VDANGVLKHS IKLDVIGGDG DDVVLANASR IHYDGGAGTN TVSYAALGRQ
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1081    FLQDLGVWSN QLDGGAGVDT VKYNVHQPSE ERLERMGDTG IHADLQKGTV EKWPALNLFS
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1201    NDIFLQDDET VSDDIDGGAG LDTVDYSAMI HPGRIVAPHE YGFGIEADLS REWVRKASAL
1261    GVDYYDNVRN VENVIGTSMK DVLIGDAQAN TLMGQGGDDT VRGGDGDDLL FGGDGNDMLY
1321    GDAGNDTLYG GLGDDTLEGG AGNDWFGQTQ AREHDVLRGG DGVDTVDYSQ TGAHAGIAAG
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Figure 9

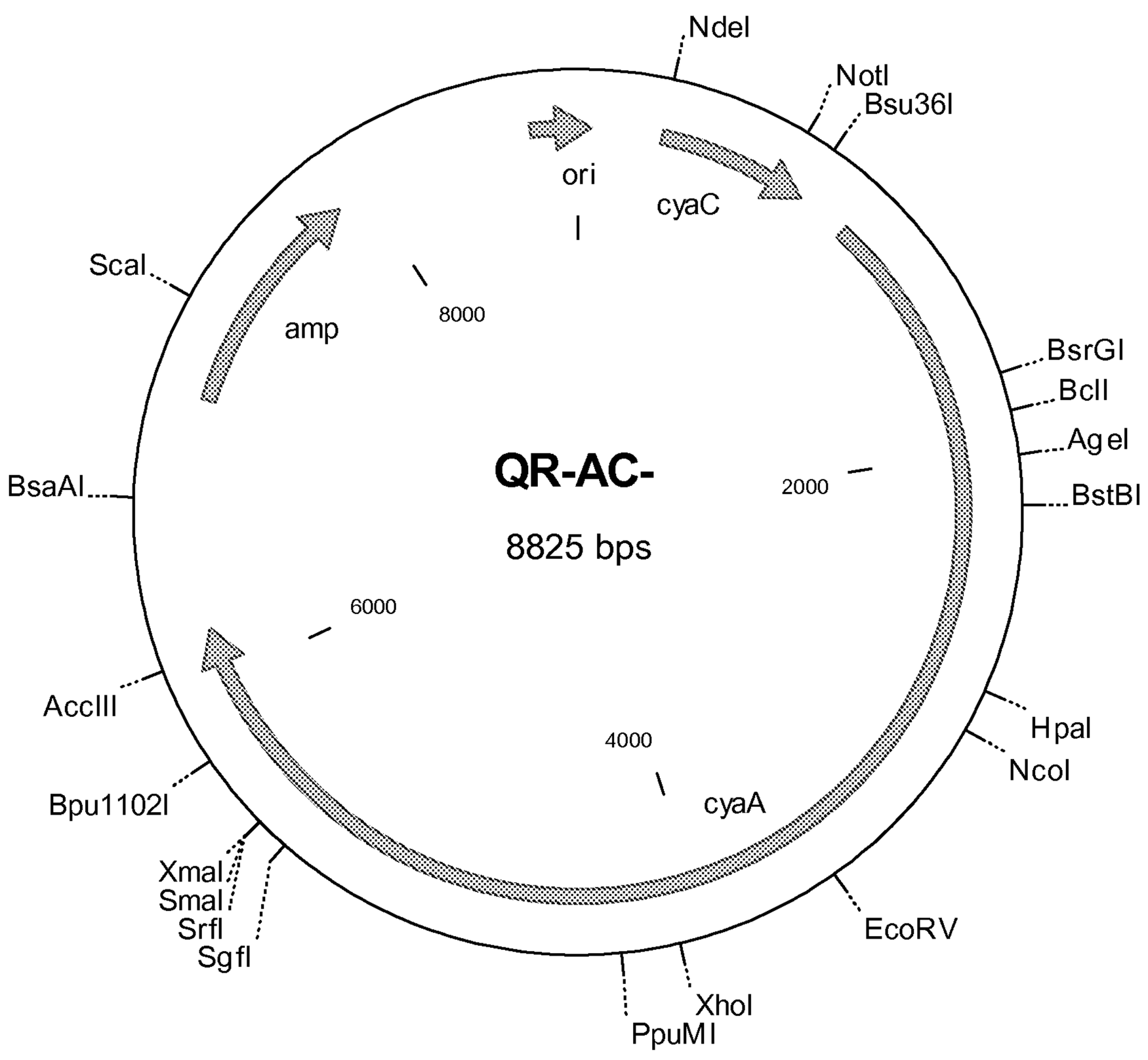
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Figure 10

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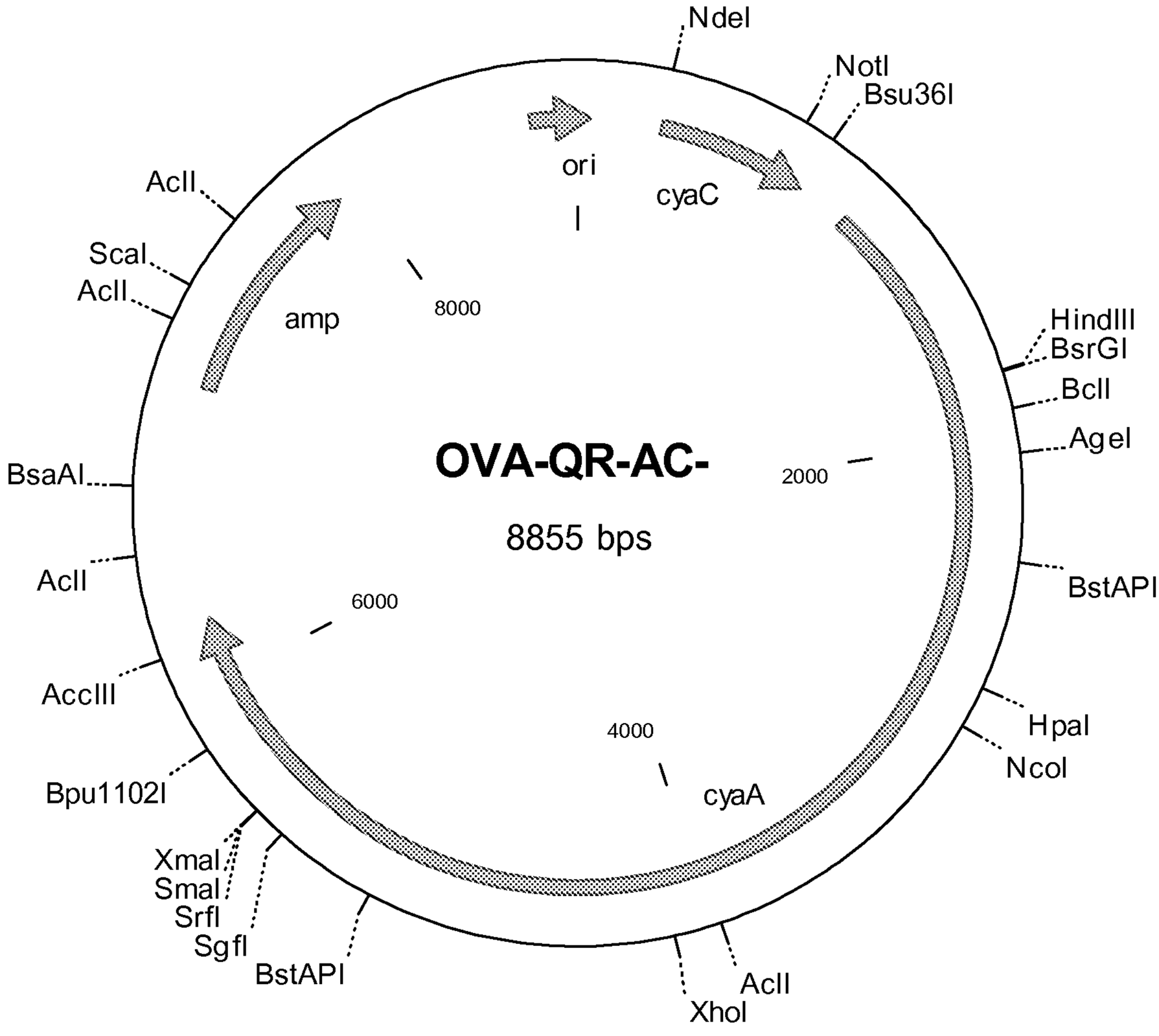
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Figure 12

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Figure 13

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Figure 14

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Figure 15

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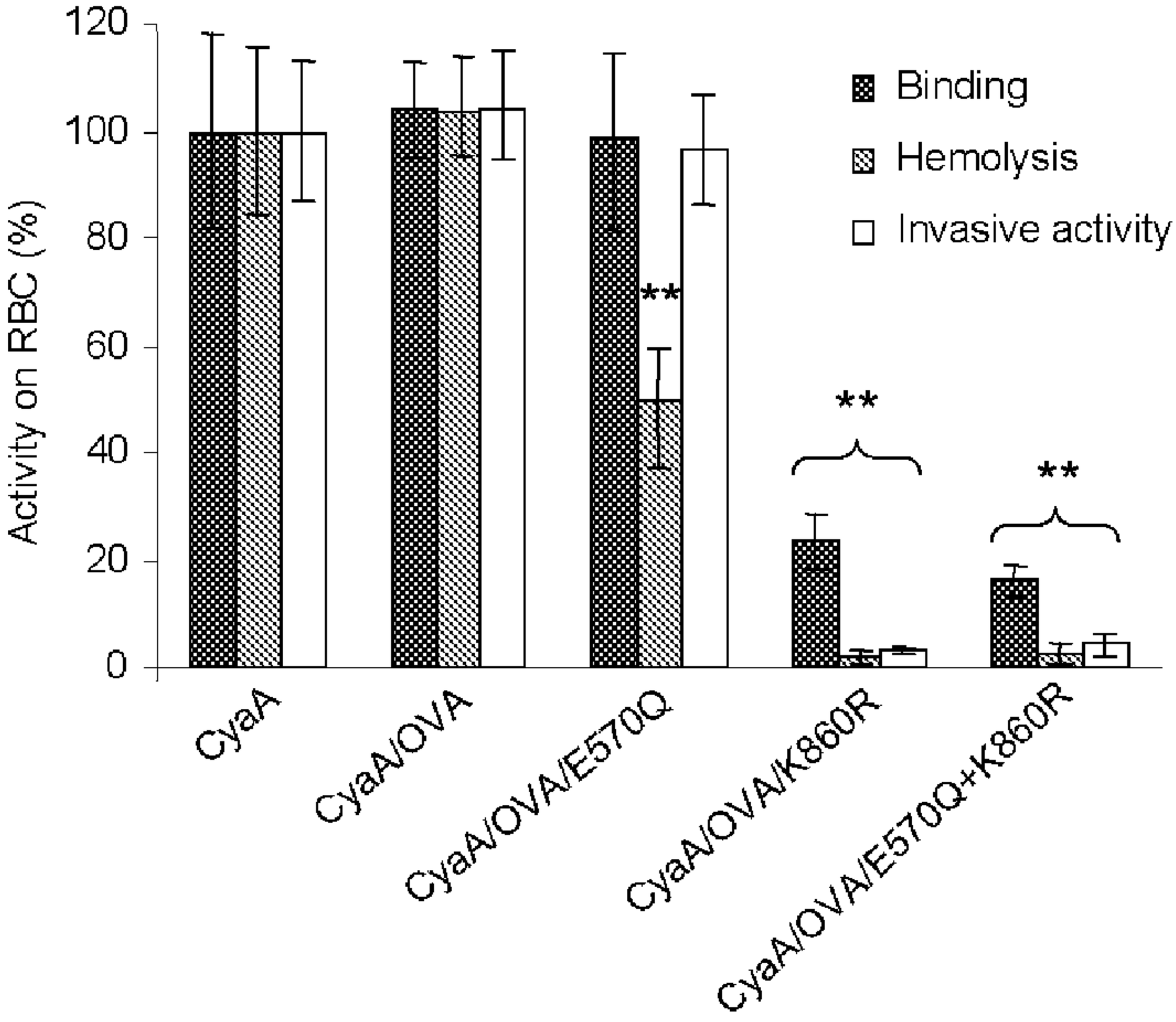
Figure 16

A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100
80
60
40
20
0
Binding
Hemolysis
Invasive activity
**
**
**
CyaA
CyaA/OVA
CyaA/OVA/E570Q
CyaA/OVA/K860R
CyaA/OVA/E570Q+K860R

Figure 1