SU-IP PhD program - Deciphering CyaA toxin translocation across membrane with droplet interface bilayers (DIB)

Thesis project summary. The CyaA toxin (figure 1) is a key virulence factor produced by *B. pertussis*, the causative agent of whooping cough. At the early stages of respiratory tract colonization by *B. pertussis*, CyaA invades eukaryotic cells and produces supraphysiological levels of cyclic adenosine monophosphate (cAMP), leading to cell death. CyaA is endowed with a unique mechanism of translocation of its catalytic domain (AC, green in figure 1) directly across the plasma membrane of target cells from the extra-cellular milieu into the cytosol. The molecular mechanism by which CyaA enters into target cells remains, however, largely unknown. We have recently shown that AC is translocated across the plasma membrane through a transient and local destabilization of the membrane integrity induced by the translocation region (TR, blue) of CyaA. Moreover, our recent results show that two membrane-active peptides (P233 and P454) translocate across the membrane and interact with high affinity with calmodulin (CaM), favoring AC translocation (figure 1). These results have been obtained thanks to an interdisciplinary and synergistic collaboration between an Institut Pasteur (IP) team (headed by Alexandre Chenal) and a Sorbonne University (SU) team (headed by Sophie Cribier and Nicolas Rodriguez). These results (published[1] and manuscript in preparation[2]) illustrate the fruitful scientific productivity of our established collaboration. We have determined the experimental conditions of P233 and P454 peptide translocation across a lipid bilayer, in particular the role of the transmembrane potential, using a development of the droplet interface bilayer (DIB) model, improved with success in the SU team by our co-supervised PhD student Gaia Scilironi (figure 2, left part).
The objective of the present SU-IP proposal is to further develop our collaboration by changing the biological scale, *i.e.*, from peptide to protein translocation across membranes in order to determine the translocation mechanism of the full-length CyaA toxin (figure 2, right part). The PhD project should provide valuable data on the structure and kinetics of the successive steps leading to the translocation of AC into the cytosol of target cells. These studies on the CyaA translocation process will be instrumental (i) to design improved CyaA-based antigen delivery systems and (ii) to improve the engineering of the next generation of CyaA-based pertussis vaccine developed in the IP laboratory. Regarding the guidelines of the SU-IP doctoral program, our project aims at supporting collaborative and interdisciplinary research projects between IP and SU teams, dedicated to the doctoral training of PhD students.

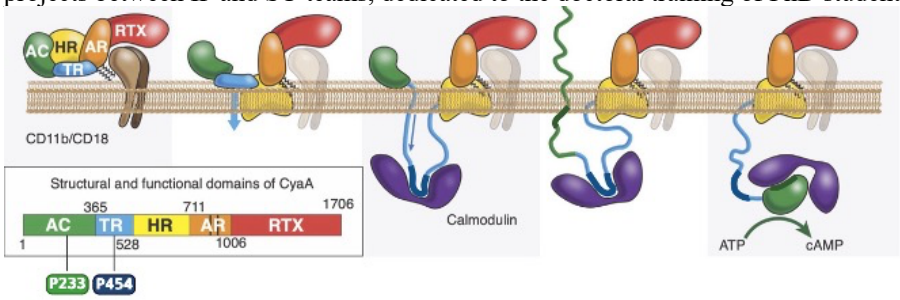

Figure 1. *Model of the CyaA translocation process.*

CyaA translocation across DIB membranes *in vitro*. This section is the core of our collaborative project. Based on recent results [3], we hypothesize that AC translocation is driven by an entropic pulling effect (Figures 1): an α-helical region of the CyaA translocation region (TR), from amino-acid 454 to 484 (P454) exhibits the hallmarks of membrane-active peptides, *i.e.*, it is able to destabilize and translocate across lipid bilayers [3–5]. Once P454 reaches the cytosol, it binds to CaM with high affinity [3]. Our hypothesis is that this interaction would provide enough free energy (i) to force the unfolding of AC on the outer leaflet of the membrane (AC exhibits a weak stability) and (ii) to overcome the penalty of AC translocation across the plasma membrane into the cytosol.

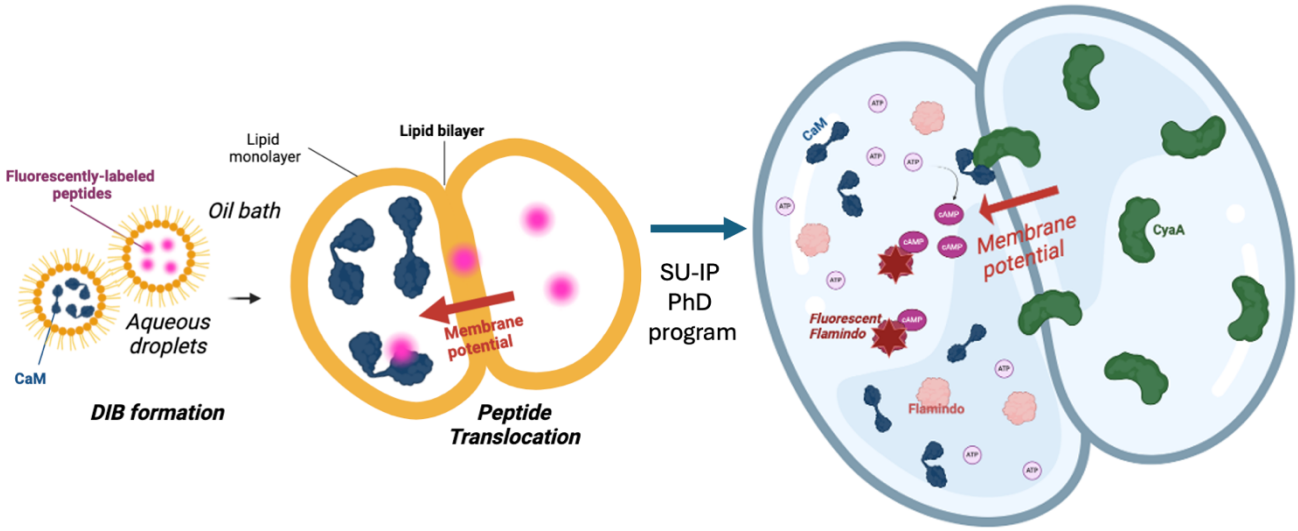

Figure 2. *From peptide translocation (left, PhD thesis of Gaia Scilironi) to CyaA translocation across the DIB system (SU-IP PhD program, application 2025). On the left, the peptide translocation is monitored by the increase of fluorescently labeled peptides in complex with CaM in the trans droplet. On the right part, once AC is translocated in the trans droplet in the presence of a membrane potential, ATP is converted into cAMP, which forms a fluorescent complex with Flamindo.*

To test this hypothesis and determine the kinetics and energetics of AC translocation, the project is to design a highly sensitive and fast responding monitoring system of the AC translocation through a model membrane. The model membrane will be a Droplet Interface Bilayer (DIB) formed at the interface between two aqueous droplets covered with lipids and immersed in an oil bath. Lipid composition of the bilayer at the interface of the two droplets is easily controlled. This system implemented at SU has already been successfully used to evidence the translocation of P233[2] and P454[3]. To test now the translocation of AC, some developments are required. The *cis* droplet can initially contain the full CyaA toxin and the *trans* droplet contains CaM, ATP and Flamindo [6,7]. The translocation of AC from the *cis* to the *trans* droplet across the interfacial bilayer is then monitored by following the increase of fluorescence of the cAMP:Flamindo complex. The volume of each droplet can be below 10pL which is advantageous to detect AC translocation. Our detection will be based on the use of Flamindo, a sensitive cAMP fluorescent indicator able to show sub 0.1µM cAMP concentrations produced (at the expense of ATP) by AC once translocated in the target *trans* aqueous droplet. Only 10^6 cAMP need to be produced in the *trans* droplet to get a significant signal which can be obtained in one minute thanks to the translocation of only ~10 AC enzymes through a 10 µm diameter membrane owing to the fast catalytic activity of the AC domain (kcat=2000 s-1).
This new method will allow us to investigate the kinetics and conditions of AC translocation. It will be tested with several CyaA mutants with different translocation efficacy. A set of CyaA recombinant proteins with altered translocation efficiencies in cells is already available [3]. If the entropic pulling hypothesis is correct, the translocation efficiency should be strongly influenced by modifications of CyaA affecting the length or flexibility of the linker between P454 and the catalytic domain to be pulled inside the target aqueous compartment. Our hypothesis is that this interaction would provide enough free energy (i) to force the unfolding of AC on the outer leaflet of the membrane (AC exhibits a weak stability) and (ii) to overcome the penalty of AC translocation across the plasma membrane into the cytosol.

Feasibility of the thesis project. The complementary nature of skills gathered in this proposal offers a unique opportunity for a continuous crosstalk between physics and experimental biophysics, to investigate the thermodynamics and molecular basis of CyaA translocation. The diversity and complementarity of human resources and skills mobilized here constitute a significant strength and an opportunity for the completion of the project. Three colleagues (Sophie Cribier, Delphine Ravault and Nicolas Rodriguez) from SU and four colleagues (Dorothée Raoux-Barbot, Marilyne Davi, Corentin Léger and Alexandre Chenal) from IP with various scientific and methodological backgrounds are involved in the organization and implementation of the project. The complementarity between the IP and SU teams will lead to a strong synergy between the teams, required for a successful PhD program. We foresee that the PhD candidate will benefit from an excellent quality of training.
Concerning the SU team, Nicolas Rodriguez and Sophie Cribier have developed extensive expertise in Droplet Interface Bilayer (DIB) approaches to study peptide translocation across membranes [8,9,3]. DIB approaches provide a unique readout of protein/peptide translocation from a kinetic and sensitivity perspective. The feasibility of DIB used for isolated domains and peptides of CyaA has already been established[2,3]. From the IP side, the main risk relies on the use of CyaA, a multidomain hydrophobic protein (making it prone to aggregation into non-functional forms upon urea removal). Recently, we have established a procedure based on molecular confinement to refold CyaA into a monomeric and functional form in physiological buffer ([10,11] and patents US10920212B2 and EP3169777B1). Molecular biology, protein production and purification protocols have been developed by the IP team these last two decades and are well established. All proteins proposed to be produced and purified in the project are well-known, *i.e.*, CyaA recombinant proteins, its isolated domains and CaM. Moreover, we have access to the Center for Technological Resources and Research (C2RT, Institut Pasteur), which federates a unique cluster of 19 core facilities with state-of-the-art equipment and expertise. The C2RT will provide us with cutting-edge technologies including molecular biophysics, mass spectrometry, NMR, HDX-MS. The C2RT will also provide the PhD student with dedicated training and education on the corresponding technologies. Taken together, our SU and IP technical background provides us with a certain degree of confidence on the feasibility of the project.

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