A novel approach to design and dynamically measure *in vitro* the biological activity of IAPP analogues designed by ancestral gene reconstruction for more efficient treatment of diabetes type-2

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Introduction

Under abnormal conditions, many proteins are unable to obtain or maintain their native fold and are driven to a misfolded state. Misfolded proteins self-assemble either to amorphous or to organized aggregates known as amyloid fibrils. In humans, amyloid fibrils can be accumulated on the surface of any organ or tissue and lead to conformational diseases known as amyloidoses. Type-2 diabetes (T2D), recorded in 350 million people, is caused by the deposition of the amylin peptide (IAPP) in pancreatic islets. This deposition reduce and complete eliminate the mass of β-pancreatic cells leading to disturbances in insulin secretion

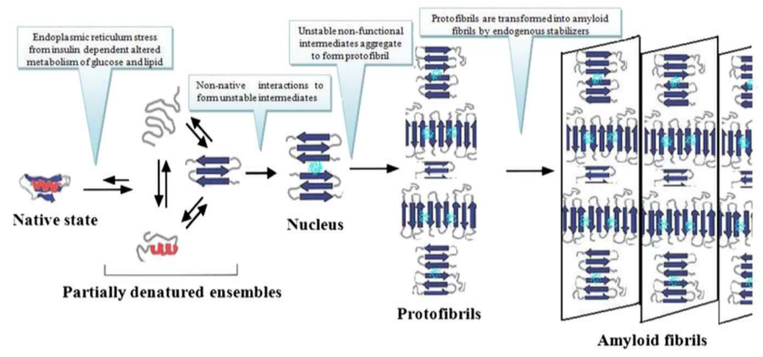

Figure 1. Schematic presentation of conversion of a native soluble protein to insoluble amyloid fibrils

Aim of the study

The aim of this study is to design novel non-amyloidogenic IAPP analogues using the very promising phylogenetic approach of ancestral gene reconstruction. In addition, the specific aim of this study is to establish an assay enabling us to monitor, in a dynamic way, the aggregation process of amylin fibrils in the early stages of nucleation, since as oligomers are very toxic.

Experimental workflow

Rational design of non-amyloidogenic IAPP analogues using ancestral gene reconstruction → **Cloning of IAPP analogues genes into the pT7CFE expression vector** → **Cell free Protein Expression** → **Dynamic monitoring of the kinetics at the early stages of aggregation of IAPP analogues** → **High-throughput screening for potential amyloidogenic or non-amyloidogenic IAPP analogues**

Rational design of non-amyloidogenic IAPP analogues using ancestral gene reconstruction

In an attempt to determine the emergence of the amyloidogenic peptides inside families and discover promising non-amyloidogenic peptides among species, we constructed phylogenetic trees by enforcing topologies known from literature. These were afterwards used for ancestral sequence reconstruction, aiming to design soluble, monomer peptides functioning as inhibitors of fibril formation. The concept is based on recent remarks suggesting that ancestral genes generate soluble, stable and functional variants of the target protein. In particular, we constructed phylogenetic trees and we examined the amyloidogenic propensity of both extant and ancestral peptides for the IAPP.

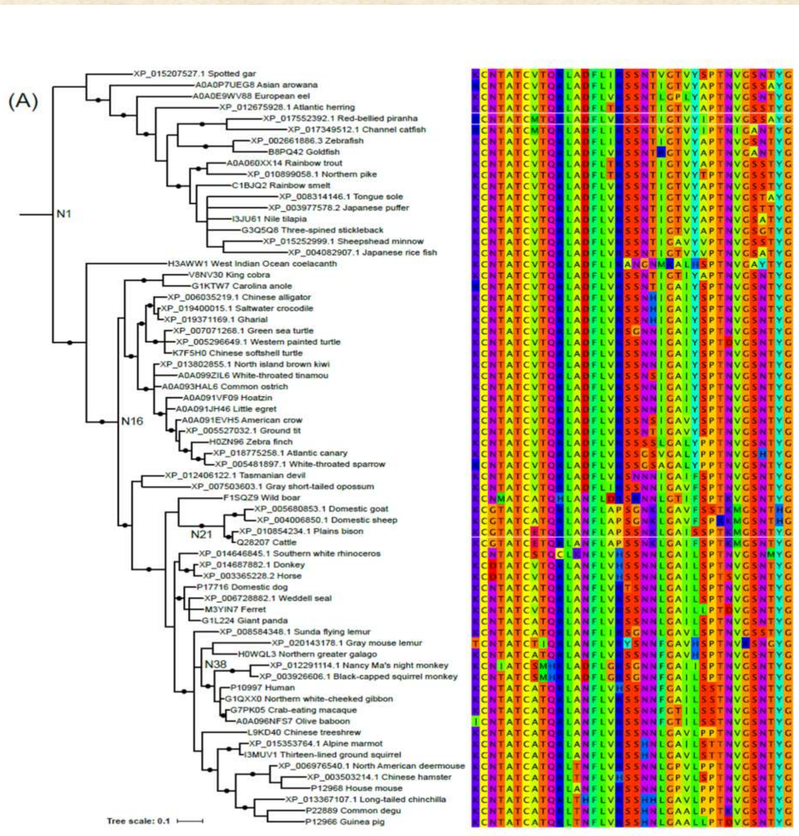

Figure 2. Ancestral gene reconstruction
A) Bayesian phylogeny of IAPP using full-length propeptide sequences (145-aa position) aligned with Muscle and calculated in Phylobayes with the JTT+Γ4 model. The IAPP peptide sequence of each taxon is shown next to it. Black circles indicate posterior probabilities of at least 0.95. Scale bar represents the average number of substitutions per site.
B) IAPP peptides that were selected for resurrection/synthesis and characterization compared to the human IAPP. Those include five ancestral sequences predicted in FastML, representing key nodes in the animal phylogeny, three extant sequences that were predicted to have low amyloidogenic propensity according to Aggrescan, and synthetic sequences we created by fusing the human IAPP with extant sequences in order to remove aggregation hotspots.

Cloning of IAPP analogues genes into the pT7CFE1 expression vector

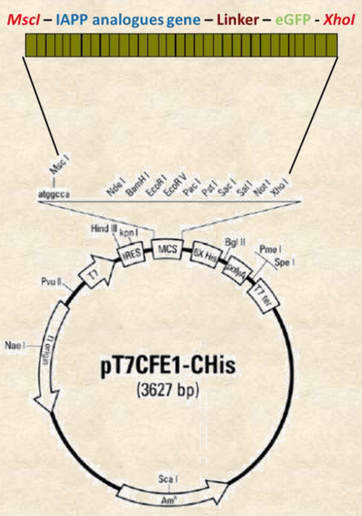

Figure 3. Construction of IAPP analogues genes fused with the eGFP gene. pT7CFE1 vector enables high levels expression of proteins and is designed for cell free transcription-translation. IAPP and eGFP genes have been linked through a specially designed nucleotide sequence that encode the linker GSAGSAAGSGEF.

Cell free protein expression

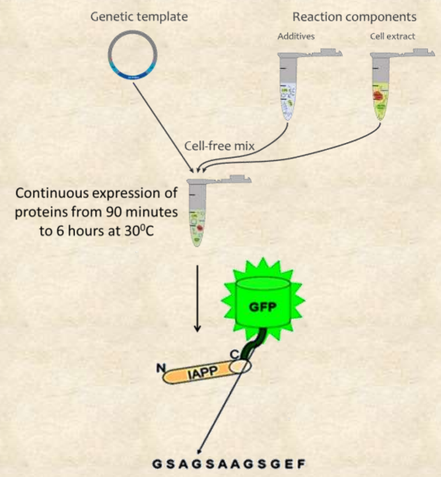

Figure 4. *In vitro* translation system based on HeLa cell extracts. The benefis of *in vitro* expression of proteins over traditional *in vivo* systems include expression of toxic or insoluble proteins and faster protein synthesis. These extracts contain all cellular components such as RNA polymerase, regulatory protein factors, transcription factors, ribosomes and tRNA essential for protein synthesis. When supplemented with cofactors, nucleotides and a template containing the target gene, these extracts will synthesize the protein of interest in a few hours.

Dynamic monitoring of the kinetics at the early stages of aggregation of IAPP analogues

An assay has been developed in our group to monitor in real time (using Real Time PCR machine) the kinetics of formation of soluble aggregates of various designed IAPP analogues. Upon transcription and translation the IAPP peptides via the linker are fused with the eGFP protein allowing them to fold independently. So there is a dynamic balance between IAPP fibrils formation and natural folding of eGFP. With this experimental approach, if amyloidogenic IAPP will form fibrils much faster than the folding of eGFP will not not allow the correct folding of eGFP and lower fluorescence due to unproper folding can be measured. If IAPP analogues that either do not form fibrils (non-amyloidogenic) or form fibrils at rates much lower than the rate of folding of eGFP will allow eGFP to fold correctly and emit fluorescence. Human IAPP (hIAPP) exhibiting increased amyloidogenic potential will be used as positive control, while eGFP, as not capable of forming fibrils, will be used as negative control.

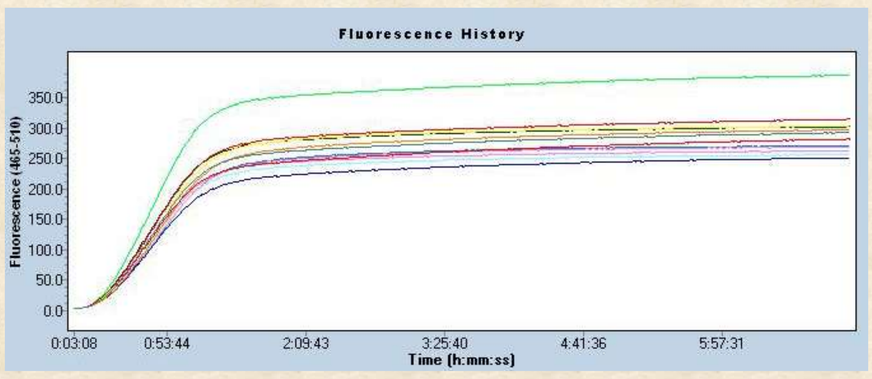

Figure 5. eGFP fluorescence-monitored kinetic curves of the aggregation process of the IAPP analogues peptides during the early stages of nucleation in real time.

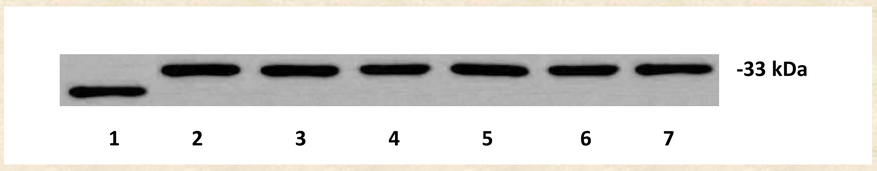

Figure 6. Western immunoblot analysis. Expressed GFP protein (1), hIAPP peptide (2) and various IAPP analogues peptides (3-7) were detected by Anti-His antibody (1:2000).

Results

Preliminary data have shown that the aggregation kinetics of various IAPP analogues peptides strongly affect the folding kinetics of eGFP, which is measurable in a way similar to real time pcr. Although the expression levels of the different IAPP analogues peptides are similar (Figure 6), differences in the fluorescence curves were observed. The percentage of fluorescence emitted by GFP (negative control) is the highest, while in the hIAPP (positive control) is the lowest.

Next steps

The confirmation of the oligomers formation of our samples would be performed by using size-exclusion chromatography coupled to a fluorescence detector. The same procedure will be applied for similar studies upon addition of natural products of low molecular weight, as well as the addition of existing medications that are administered to patients with T2D.

Conclusion

Therefore, a high-throughput screening for potential amyloidogenic or non-amyloidogenic analogues of IAPP is established looking exclusively at the early stages of nucleation. Novel IAPP analogues that may stop the whole cascade at the nucleation phase, will shed light to the mechanism of amyloid formation and provide a basis for more efficient treatment of T2D. This innovative approach can create a prototype system potentially applicable to all kind of amyloidoses.

References
1. Kim et al. (2006) ACS Chem Biol. 1(7):461-9.
2. Gonzalez et al. (2014) FEBS Open Bio. 4:121-7.

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