Membrane permeabilization by Islet Amyloid Polypeptide

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Invited Review

Abstract

Membrane permeabilization by Islet Amyloid Polypeptide (IAPP) is suggested to be the main mechanism for IAPP-induced cytotoxicity and death of insulin-producing β-cells in type 2 diabetes mellitus (T2DM). The insoluble fibrillar IAPP deposits (amyloid) present in the pancreas of most T2DM patients are not the primary suspects responsible for permeabilization of β-cell membranes. Instead, soluble IAPP oligomers are thought to be cytotoxic by forming membrane channels or by inducing bilayer disorder. In addition, the elongation of IAPP fibrils at the membrane, but not the fibrils themselves, could cause membrane disruption. Recent reports substantiate the formation of an α-helical, membrane-bound IAPP monomer as possible intermediate on the aggregation pathway. Here, the structures and membrane interactions of various IAPP species will be reviewed, and the proposed hypotheses for IAPP-induced membrane permeabilization and cytotoxicity will be discussed.

Keywords

Amylin, phospholipid bilayer integrity, amyloid fibril, type 2 diabetes mellitus, β-cell death, misfolding disease

Introduction

Human Islet Amyloid Polypeptide (hIAPP) is a peptide that forms insoluble fibrillar deposits (amyloid) in the pancreas of patients with type 2 diabetes mellitus (T2DM). hIAPP is produced and secreted, together with insulin, by the β-cells in the pancreatic Islets of Langerhans. Monomeric hIAPP is suggested to be a hormone with roles in gastric emptying and regulation of glucose homeostasis (Karlsson 1999). Although little details are known about the precise physiological function of hIAPP, there are indications that hIAPP binds to specific membrane-located receptors (Hay, et al. 2004). Recently, a non-amyloidogenic synthetic hIAPP variant, called pramlintide or symlin, has been commercially introduced as an additional therapeutic approach for diabetes patients (Riddle, et al. 2006).

More than 90% of T2DM patients have hIAPP amyloid in their Islets of Langerhans, as determined by post-mortem analysis. The presence of islet amyloid in T2DM has been linked to death of the insulin-producing β-cells, thereby contributing to the development of this disease (Höppener, et al. 2000). T2DM can be classified as a protein-misfolding disease, and it shares the debilitating consequences of misfolded and aggregated peptides and proteins with more than 20 other diseases, such as Alzheimer's disease, Parkinson's disease and prion diseases (Chiti, et al. 2006). The interaction of hIAPP with β-cell membranes is thought to play a crucial role in hIAPP cytotoxicity and death of the insulin-producing β-cells. Amyloid-membrane interaction is not only relevant for cytotoxicity in T2DM, but considered to be a generic mechanism for amyloid-induced cytotoxicity in most of the amyloid-related diseases (Glabe, et al. 2006; Lashuel, et al. 2006).

In this review, the current knowledge of hIAPP-membrane interactions will be presented and analysed, with a focus on the role of hIAPP-membrane interaction in relation to mechanisms of cytotoxicity. Various suggested hypotheses for hIAPP-induced membrane permeabilization will be discussed. In addition, the current insight into the structural characteristics of hIAPP assemblies and of membrane-bound hIAPP will be reviewed.

Different appearances of hIAPP: monomer, oligomer and amyloid fibril

The 37-residue hIAPP peptide can appear in various aggregation states, i.e. monomer, oligomer or fibril (polymer), all with very different structures (Fig. 1). It has now become clear that oligomers and fibrils are

not single, morphologically homogeneous species, but that each of them represents a sub-set of species of different sizes, shapes (polymorph), and with variations in secondary, tertiary and quaternary structure. Many different expressions are in use to indicate “oligomers”, such as prefibrillar aggregates, micelles and ADDLs, and it has proven difficult to distinguish different populations of oligomers (Glabe 2008). For the purpose of this review the term oligomer will be used to describe hIAPP species that are composed of 2 to approximately 60 hIAPP monomers (~ 250 kDa). Some important questions regarding these different species are the topic of many recent papers: What is the structure of the various hIAPP species detected? Which hIAPP species are cytotoxic and present *in vivo*? A particularly important question regarding the scope of this review is: how do these different hIAPP species interact with a membrane? These and related questions will be addressed in the next sections. First, the structure of several aggregation states of hIAPP will be discussed.

Of the various hIAPP species, the structure of fibrillar hIAPP is best known despite it being the most insoluble one. The stable nature of hIAPP fibrils has likely contributed to the relative ease of handling and studying it; hIAPP fibrils are not degraded by proteases and generally need harsh conditions for depolymerization, like the use of concentrated formic acid or organic solvents (Clark, et al. 1987). In contrast, the study of the structure of monomeric and oligomeric hIAPP in physiological buffers has been hampered by their instability and fast ‘spontaneous’ aggregation. The large majority of the structural information about hIAPP fibrils stems from *in vitro* assembled fibrils, the production of which mostly requires nothing more than dissolving hIAPP monomer in a physiological buffer and keeping it for hours to days.

Advanced methods, in particular electron microscopy (EM), electron paramagnetic resonance spectroscopy (EPR), X-ray diffraction, and nuclear magnetic resonance spectroscopy (NMR) have significantly improved our understanding of hIAPP fibril structure (Goldsbury, et al. 2000; Jayasinghe, et al. 2004; Sumner Makin, et al. 2004; Luca, et al. 2007). *In vitro* produced fibrils of hIAPP or hIAPP fragments, even from the same sample, display various cross-β structures (Madine, et al. 2008) and morphologies (Goldsbury, et al. 1997; Sumner Makin, et al. 2004; Radovan, et al. 2008), for example coiled fibrils and ribbon-like fibrils (Fig. 1b). The parallel, in register structure of hIAPP fibrils was first demonstrated by EPR spectroscopy (Jayasinghe, et al. 2004). The most recent and most detailed model for

the structure of an hIAPP fibril suggests that it is composed of stacked layers of two symmetric hIAPP molecules that form a parallel β-sheet structure running perpendicular to the length axis of the fibril (Fig. 1c) (Luca, et al. 2007). Residues 8 to 17 and 28 to 37 form the β-sheet structure, whereas residues 1 to 7 are largely unstructured. Importantly, this model is based on experimental data of a morphologically homogeneous sample of hIAPP fibrils, so called striated ribbons.

Another model of the hIAPP fibril suggests that the fibril is composed of three protofilaments, each based on stacking of single hIAPP molecules in which residues 9 to 37 participate in a planar S-shaped fold forming the β-sheet structure (Kajava, et al. 2005). Most proposed models, combined with aggregation studies on hIAPP fragments, place residues 9 to 37 in the amyloidogenic core (Nilsson, et al. 1999; Jaikaran, et al. 2001; Scrocchi, et al. 2003; Gilead, et al. 2008; Wiltzius, et al. 2008), which adjusts the historic view that only residues 20 to 29 would be important for amyloid formation (Westermark, et al. 1990). Different models are likely required to account for the various hIAPP fibril morphologies that are commonly observed. Studies of hIAPP fibrils in pancreatic islets show that their morphological character is grossly the same as that of *in vitro* produced fibrils (Westermark 1973; de Koning, et al. 1994; Jaikaran and Clark, 2001), but even large-scale features of *in vivo* fibrils, like the number of protofibrils, are unknown. Consequently, an important question that remains to be answered is which fibril morphology is present in or near the β-cell? Additionally, which fibril morphology is related to cytotoxicity, or results from cytotoxic oligomers?

Compared to the detailed structural information available for hIAPP fibrils, the information about the structure of hIAPP oligomers is much sparser and considerably less detailed. All our knowledge of the hIAPP oligomer structure comes from *in vitro* produced oligomers. A protocol for the *in vitro* production of hIAPP oligomers entails stirring freshly dissolved, supposedly monomeric hIAPP at 500 rpm in an eppendorf tube, at pH ~ 3, for 24 to 48 hrs at 22°C (Kayed, et al. 2004). This process results in a homogeneous sample of spherical hIAPP oligomers, 3-5 nm in diameter, as observed by EM (Fig. 1d). Others have detected, either directly or indirectly, the presence of hIAPP oligomers as transient species in samples of fibril forming hIAPP, with large variations in shape and size, ranging from a cluster of a 10-20 hIAPP monomers to more than 500 (Janson, et al. 1999; Anguiano, et al. 2002; Porat, et al. 2003; Green, et al. 2004). An example of a ring-shaped hIAPP oligomer is shown in figure 1e (Porat, et al. 2003).

It is uncertain whether hIAPP oligomers are consumed in the process of fibril growth. There are suggestions that hIAPP oligomers are off-pathway, meaning that they are not on a productive route towards hIAPP fibrils (Meier, et al. 2006; Haataja, et al. 2008). Others suggest that hIAPP oligomers are consumed during hIAPP fibril growth, suggesting that the oligomers are on-pathway (Anguiano, et al. 2002; Porat, et al. 2003; Green, et al. 2004; Knight, et al. 2006). This is a crucial issue since toxic off-pathway oligomers could become increasingly populated when a therapeutic strategy based on inhibition of on-pathway hIAPP oligomers and/or fibrils would be used. Considering the observed variation in size and character of oligomers, it is not unthinkable that both on- and off pathway hIAPP oligomers co-exist.

A significant finding that has helped in detecting and characterizing hIAPP oligomers has been the realization of an oligomer-specific antibody (Kayed, et al. 2003). The observation that this anti-oligomer antibody, named A-11, recognizes hIAPP oligomers but not hIAPP monomers or hIAPP fibrils suggests that these oligomers are structurally unique, and different from hIAPP fibrils. Moreover, the A-11 antibody also binds specifically to oligomers of other amyloid-related peptides and proteins, suggesting a common structural motive and possibly a key to a generic mechanism of cytotoxicity in misfolding diseases. Two other antibody/antisera have been produced against hIAPP oligomers: the I-11 antibody reacts to the same hIAPP oligomers as A-11 (Meier, et al. 2006), whereas the antiserum αAPF specifically recognizes the bigger, annular hIAPP oligomers (Kayed, et al. 2009). The use of aggregation-state specific antibodies is expected to reveal more structural characteristics of hIAPP species on the aggregation pathway in future studies.

The structural information about the hIAPP monomer is very limited. Monomeric hIAPP, in the majority of studies produced synthetically, is characterized as a natively unfolded peptide, showing typically unstructured circular dichroism (CD) spectra (Goldsbury, et al. 2000; Higham, et al. 2000). Recent NMR experiments suggest that the hIAPP peptide chain is unfolded, although part of the chain, approximately residues 8 to 19, can dynamically adopt α-helical structure (Williamson, et al. 2007; Yonemoto, et al. 2008; Wei, et al. 2009). The spontaneous and fast aggregation of monomeric hIAPP in an aqueous environment – insoluble fibrils are formed within a few hours – forms a major obstacle for studying the structural characteristics of monomeric hIAPP in solution. Addition of SDS or binding to a membrane (as will be discussed later) results in stabilized, mostly α-helical, monomeric hIAPP states

(Mascioni, et al. 2003; Apostolidou, et al. 2008). The interaction of monomeric hIAPP with insulin is also suggested to increase the helical tendency of hIAPP (Wei, et al. 2009), although this interaction seems to be very dependent on the physical forms of hIAPP and insulin, i.e. soluble or fibrillar hIAPP and soluble or crystalline insulin (Knight, et al. 2008). Both insulin and lipids thus seem to induce helical structure, but surprisingly their effect on hIAPP aggregation is opposite. Lipids have a tendency to promote hIAPP aggregation (Knight, et al. 2004) whereas insulin is well known as an inhibitor of hIAPP fibril formation (Westermark, et al. 1996). The effect of lipids and insulin on the secondary structure of hIAPP monomers *in vitro* could suggest that hIAPP might be structured under *in vivo* conditions through interaction with physiological binding partners (insulin and lipids are both present in secretory vesicles, the cellular storage of hIAPP and insulin).

Islet amyloid fibrils at the β-cell membrane

Just after the discovery of hIAPP as the major component of islet amyloid in 1987 (Cooper, et al. 1987; Westermark, et al. 1987), it was generally thought that hIAPP fibrils were cytotoxic to β-cells, thereby contributing to T2DM (Clark, et al. 1987; Lorenzo, et al. 1994). Interestingly, already in the early seventies a peculiar interaction between extracellular islet amyloid fibrils and β-cell membranes had been noticed (Fig. 2) (Westermark 1973). It was observed then, and confirmed in later studies, that the islet amyloid fibrils were often orientated perpendicular to the membrane, and co-localized with distinct changes in the morphology of the β-cell membrane (Westermark 1973; Clark, et al. 1987; Jaikaran and Clark, 2001). In contrast, in the vicinity of other types of Islet cells (e.g. α-cells), this characteristic orientation of fibrils near membranes was rarely seen, and fibrils were mostly randomly orientated. Whereas these studies involved endogenous hIAPP, also externally added, freshly dissolved, synthetic hIAPP added to cultured β-cells induced typical membrane deformations, for example membrane invaginations, budding and vesicle formation (Lorenzo, et al. 1994; Janson, et al. 1999; Saafi, et al. 2001; Casas, et al. 2008).

It appeared that the amount of amyloid in the Islets of Langerhans did not correlate well with the decrease in the number of healthy β-cells, pointing to species other than fibrils being the culprit. Currently, the prevailing and well-documented view is that soluble hIAPP oligomers are the toxic species and that hIAPP fibrils are biologically inert (Haataja, et al. 2008). With the increase in knowledge of the

characteristics of soluble hIAPP oligomers (see previous section), also advances have been made into the understanding of their interaction with membranes. The mechanism of hIAPP oligomer cytotoxicity is thought to involve permeabilization of cellular membranes, possibly through formation of membrane pores. Several variations of this hypothesis have been proposed and will be discussed in the next section.

Membrane permeabilization by hIAPP oligomers

The first observation that soluble amyloid oligomers could affect the integrity of a lipid bilayer by forming an ion-channel, was made in 1993 (Arispe, et al. 1993). It was shown that Aβ, the Alzheimer related amyloidogenic protein, could form cation-selective channels in planar lipid bilayers. Soon after that, the group of Kagan showed that also hIAPP could form cation-selective channels (Mirzabekov, et al. 1996). In contrast, the non-amyloidogenic rIAPP did not form channels. Currently, a substantial amount of experimental data suggests that hIAPP, as well as many other amyloid-related peptides and proteins, can form cation-selective channels. (Kawahara, et al. 2000; Kourie, et al. 2002; Kagan, et al. 2004). Visualization by atomic force microscopy (AFM) suggests that a hIAPP ion-channel, assembled in a bilayer, is composed of approximately 5 subunits in a circular arrangement (Fig. 1f) (Quist, et al. 2005).

Various sizes of the hIAPP-induced membrane pores or openings have been suggested, ranging from Ca^{2+}-permeable to permeable for fluorescent dyes with a size larger than 1 kDa (Anguiano, et al. 2002; Demuro, et al. 2005; Kagan 2005). Soluble hIAPP oligomers, and amyloid oligomers in general, could have characteristics of pore-forming protein toxins, like α-hemolysin, and might have a similar mechanism of action (Lashuel, et al. 2002; Lashuel, et al. 2006; Kayed, et al. 2009). Several groups have reported that membrane permeabilization is caused by hIAPP oligomer-induced distortions of the phospholipid bilayer packing and membrane instability, in contrast to the formation of discrete pores (Janson, et al. 1999; Kayed, et al. 2004). Another possibility of oligomer-induced membrane permeabilization is the interaction of amyloid oligomers with specific membrane receptors, which has been shown for HypF-N aggregates (Pellistri, et al. 2008), but not (yet) for hIAPP.

Recently, it was shown that an A-11 positive hIAPP oligomer can be converted into a larger and more stable annular hIAPP oligomer (also known as “annular protofibril”) catalysed by the presence of hydrophobic/hydrophilic interfaces, in particular lipid bilayers (Kayed, et al. 2009). Interestingly, these two

distinct hIAPP oligomeric species interact differently with membranes, the smaller oligomer having a higher membrane permeabilizing activity and being more toxic compared to the bigger annular oligomer. Although the conclusions of this work still seem preliminary, it shows the direction of future research: solving the heterogeneity in oligomer types and determining which type of oligomer is cytotoxic and physiologically relevant. One of the most challenging tasks in this route is to obtain samples of oligomers with a single morphology and high purity.

Many aspects of the structure and formation of hIAPP oligomers are unknown. The secondary structure of hIAPP oligomers is not well described, and both β-sheet rich hIAPP oligomers (Kayed, et al. 2009) and α-helix rich hIAPP oligomers have been observed (Knight, et al. 2006). An α-helical structure of hIAPP oligomers is plausible as helical structure is observed upon binding of monomeric hIAPP to membranes, as will be discussed later. In addition, hIAPP monomers in solution are observed to sample the helical state. However, conversion of on-pathway, β-sheet rich oligomers to cross-β structured fibrils seems energetically more favorable than conversion of α-helical oligomers. Contradicting reports have appeared suggesting that hIAPP oligomer assembly occurs either at the membrane (Quist, et al. 2005; Knight, et al. 2006) or in solution, after which the pre-formed oligomers interact with the membrane (Kagan, et al. 2004; Kayed, et al. 2004). Figure 3 schematically shows the proposed variety in oligomer secondary structure, membrane permeabilization events, and the various suggested routes for the assembly of soluble hIAPP monomers into larger structures, oligomers and fibrils at or near the membrane.

Concerns about the toxic oligomers hypothesis

The "toxic-oligomer hypothesis" described above is well-documented and supported, not only for hIAPP but also for other amyloid forming peptides and proteins related to misfolding diseases. However, recent reports suggesting alternative hypotheses have emerged, indicating that the mechanism of hIAPP-membrane interaction as an explanation for cytotoxicity is far from understood. Several notions justify concerns about the hypothesis that hIAPP oligomers are involved in membrane permeabilization and cytotoxicity.

First, whereas hIAPP fibrils have been unambiguously isolated and identified from *in vivo* sources, the evidence for the existence of toxic hIAPP oligomers *in vivo* is extremely scarce, if not absent. The only

indication is the detection of A-11-positive hIAPP oligomers in Islets of hIAPP-transgenic mice (Lin, et al. 2007). It has not yet been shown that these hIAPP oligomers are actually cytotoxic *in vivo*. Moreover, A-11-positive oligomers have not (yet) been identified in diabetic patients, although there is evidence for A11-positive oligomers *in vivo* in relation to other diseases like Alzheimer's disease (Koffie, et al. 2009). Difficulties in detecting hIAPP oligomers *in vivo* could arise due to instability of hIAPP oligomers, specifically in tissue sections. In addition, the A-11 antibody is reported to be ineffective in paraffin-embedded tissue (Haataja, et al. 2008).

Second, one should be cautious in interpreting results of cytotoxicity assays in which "toxic" hIAPP oligomers are incubated with cells for prolonged periods. Unless it is established that these oligomers remain unaltered during the entire period of incubation, the oligomers could convert into other species, which might be the actual toxic species. It has been suggested that inhibition of hIAPP fibril formation by rifampicin does not prevent β-cell death, and instead hIAPP oligomers are cytotoxic (Meier, et al. 2006). However, it was recently reported that rifampicin interferes with Thioflavin T measurements, thereby negatively affecting tests for the toxicity of hIAPP fibrils (Meng, et al. 2008).

A third reason that questions the toxic oligomer hypothesis regarding hIAPP is the emergence of studies that report difficulties in detecting hIAPP oligomers under *in vitro* conditions. Analytical ultra centrifugation experiments could not detect hIAPP oligomers smaller than 100 monomers (Vaiana, et al. 2008), which is much bigger than has been suggested before (Kayed, et al. 2004). In some studies it has been proposed that events or species other than hIAPP oligomers could induce membrane disruption or permeabilization (Green, et al. 2004; Sparr, et al. 2004).

Finally, reports have emerged suggesting that amyloid fibrils composed of various peptides and proteins, for example Aβ (Okada, et al. 2007), prion protein (Novitskaya, et al. 2006), lysozyme (Gharibyan, et al. 2006), and ure2p (Pieri, et al. 2006) might be toxic after all. An interesting case is that of ure2p, whose native like fibrils are shown to be cytotoxic, possibly via interaction of certain structural features with the membrane. A heat treatment of these fibrils however renders them unable to interact with the membrane and abolishes cytotoxicity (Pieri, et al. 2006). These observations indicate that cytotoxicity of fibrils could depend on fibril structure and/or fibril morphology. Fibrils could also be a source for the formation of potentially cytotoxic, fibril-derived species, although this has not (yet) been reported for

hIAPP. Amyloid fibrils can break, as shown for insulin fibrils (Smith, et al. 2006), and monomers or oligomers could dissociate from fibrils, as shown for fibrils formed from an SH3 domain (Carulla, et al. 2005). Importantly, lipids are able to promote fibril dissociation into cytotoxic oligomers (Martins, et al. 2008).

Membrane permeabilization by the process of hIAPP aggregation, rather than by hIAPP species

A recent study suggests that it is not a specific (oligomeric) hIAPP species, but the process of hIAPP fibril growth at the membrane that causes membrane permeabilization (Engel, et al. 2008). It was found that the assembly of hIAPP fibrils at the membrane causes membrane disruption, possibly by forcing the curvature of the bilayer to unfavorable angles (Fig. 4), or by the uptake of lipids by hIAPP fibrils during fibril elongation at the membrane. Importantly, this study shows that pre-formed hIAPP fibrils and the non-amyloidogenic rIAPP do not permeabilize membranes, whereas allowing hIAPP to aggregate at the membrane, starting from a monomeric hIAPP population, leads to fibril growth at the membrane and concomitant membrane permeabilization. The uptake of lipids into forming amyloid has been observed before under *in vitro* conditions (Sparr, et al. 2004; Zhao, et al. 2004; Domanov, et al. 2008), and also in various types of amyloid that was isolated from patients (Gellermann, et al. 2005; Gellermann, et al. 2006). The tendency of amyloidogenic peptides to fibrillate on the surface of lipid vesicles, and simultaneously damage the lipid bilayer, has also been observed using molecular dynamics simulations (Friedman, et al. 2009). Remarkably, it was found in this simulation that bilayer permeabilization is caused by growing aggregates, but not by mature fibrils, in agreement with the hypothesis that hIAPP fibril growth at the membrane causes membrane damage (Engel, et al. 2008).

The hypothesis that cytotoxicity is related to fibril growth at the membrane requires more investigation and importantly, validation in a cellular environment. However, it is important to keep in mind the early observation of *in vivo* interactions of Islet amyloid fibrils with β-cells and the resulting significant changes in membrane morphology, including changes in the curvature (see Fig. 2 and the earlier section “Islet amyloid fibrils at the β-cell membrane”). These hIAPP-induced effects on membrane morphology were also observed in model membrane studies (Domanov, et al. 2008), and are straightforward to explain using the hypothesis of fibril growth at the membrane (Engel, et al. 2008). The possibility that amyloid fibrils are

able to physically 'break' cell membranes was recently suggested in a study that showed that fibrillar polyglutamine can be taken up by cells (Ren, et al. 2009).

Fibril growth at the membrane as a membrane-permeabilizing action has not only been suggested for hIAPP, but also for other amyloidogenic proteins, such as the Alzheimer's disease related Abeta (Yip, et al. 2001; Wogulis, et al. 2005). Interestingly, the suggestion that not an hIAPP oligomer, but the membrane-located conversion of small spherical oligomers into annular oligomers might be responsible for membrane permeabilization (Kayed, et al. 2009) also supports the notion that a process occurring at the membrane could lead to membrane permeabilization. Interestingly, most pore-forming toxins, which have been hypothesized to have a similar mechanism of action as hIAPP oligomers (Glabe, et al. 2006; Lashuel, et al. 2006), are formed from their monomeric units at the membrane interface and not in solution (Gonzalez, et al. 2008). This includes the bacterial pore-forming toxin α-hemolysin which also reacts with the annular oligomer antiserum (Kayed, et al. 2009). This again suggests that toxicity might be related to a process or a conversion occurring at the membrane, and not to a certain species. These new ideas might lead to a focus on mechanisms of membrane permeabilization that are governed by conversions of species along the fibril formation pathway. In such mechanisms, membranes could have an important function as mediator or accelerator of the conversion of one hIAPP species to the other, possibly representing a cytotoxic event.

The role of interfaces in hIAPP aggregation

Membranes have the ability to catalyse hIAPP fibril formation. More precisely, the presence of phospholipid bilayers can reduce the lag-phase of hIAPP fibril formation, an effect that is most pronounced with negatively charged lipids (Knight, et al. 2004; Jayasinghe, et al. 2005; Knight, et al. 2006). Other negatively charged surfaces, like heparin molecules, are also able to catalyse hIAPP aggregation (Konno, et al. 2007). The observation that a dichloromethane/water interface accelerates hIAPP fibril formation seems to indicate that next to charge, also hydrophobicity at the interface plays an important role in the acceleration of hIAPP aggregation (Ruschak, et al. 2007). Even hIAPP fibrils themselves can accelerate subsequent hIAPP fibril formation, a process that is known as secondary nucleation (Padrick, et al. 2002). The effects of these interfaces on hIAPP aggregation and fibril formation add another complicating dimension to amyloid-membrane interactions, and indicate that hIAPP-induced membrane permeabilization

is closely intertwined with interface-mediated hIAPP aggregation. In addition, it has been suggested that other factors can significantly affect the interaction between hIAPP and membranes, for example calcium ions (Sciacca, et al. 2008) and crystalline insulin (Knight, et al. 2008).

Interfaces can affect the aggregation of peptides and proteins in different ways. Interfaces can serve as a template that helps put molecules in a preferential orientation such that aggregation is favored, as has been suggested for hIAPP (Knight, et al. 2004). Membrane fluidity is suggested to be an important factor that enables a specific orientation of fibrils on membranes (Zhang, et al. 2008). Secondly, adsorption of the peptide at the interface can locally increase the peptide concentration, resulting in two-dimensional crowding (Aisenbrey, et al. 2008). Consequently, a high local concentration of membrane-bound hIAPP monomers will greatly accelerate aggregation. In addition, interaction or aggregation of membrane-bound hIAPP monomers could result in cooperative binding (Knight, et al. 2004). Thirdly, interfaces have the ability to change the conformation of a protein (Norde, et al. 1991; Engel, et al. 2004) and consequently might also induce structure in a protein or peptide that is unstructured in solution, like hIAPP. Indeed, it has been shown that adsorption of hIAPP at membranes induces helical structure, as will be discussed in detail in the next section. Interfaces also have a significant effect on the conformation and nucleation of amyloidogenic proteins (Giacomelli, et al. 2005; Linse, et al. 2007). Possibly, membranes could induce different fibril morphologies, an observation that was made for apolipoprotein fibrils (Griffin, et al. 2008). Recent reviews address the interaction of amyloidogenic peptides and proteins with various interfaces and in particular membranes (Gorbenko, et al. 2006; Stefani 2007; Aisenbrey, et al. 2008; Relini, et al. 2009).

The effect of interface-mediated catalysis of aggregation might be a very important factor in the mechanism of amyloid-induced cytotoxicity. With membranes acting as a ‘template’ for amyloid aggregation, it is not surprising that amyloid species, through their conversion, also affect the barrier properties of the bilayer.

Residue-level details of hIAPP interacting with lipid bilayers

hIAPP has several positively-charged residues, all of which are located on the N-terminal side of the peptide (Fig. 1a). These N-terminal residues are suggested to be involved in the initial interaction of hIAPP with lipids, in particular negatively charged lipids (Knight, et al. 2004; Engel, et al. 2006; Lopes, et al.

2007). Indeed it has been shown that membrane binding of hIAPP is most efficient when bilayer lipids are negatively charged (Knight, et al. 2006; Apostolidou, et al. 2008; Brender, et al. 2008). Still, electrostatic interactions are certainly not the only interactions involved. As discussed in the previous section, hydrophobic interactions also most likely play an important role. Remarkably, the 7 N-terminal residues, including the disulfide, are not required for hIAPP cytotoxicity (Zhang, et al. 2003).

The residues important for hIAPP-membrane interaction could include those residues that are different compared to the non-amyloidogenic rIAPP. These six residues (marked red in figure 1a) are all suggested to be part of the amyloidogenic core of hIAPP and thus important for the formation of fibrils. The only charged residue of these six is histidine 18, which is important for both fibril formation (Abedini, et al. 2005) and membrane interaction (Nanga, et al. 2008). Deprotonation of H18 favors an orientation of the $hIAPP_{1-19}$ fragment parallel to the membrane, while in the protonated state $hIAPP_{1-19}$ is suggested to be transmembrane (Nanga, et al. 2008). Importantly, the first 17 N-terminal residues are identical in hIAPP and rIAPP, and seem surprisingly well conserved in IAPP from several species (Nishi, et al. 1989). The N-terminal region has been suggested to be involved in binding to receptors (Bhogal, et al. 1993) and binding to insulin (Gilead, et al. 2006; Wei, et al. 2009).

Recent reports suggest that hIAPP fiber formation and hIAPP-induced membrane disruption are separate processes localized in two distinct regions of the peptide (Brender, et al. 2008; Brender, et al. 2008). It was suggested that membrane disruption is caused by the N-terminal section of hIAPP (residues 1-19), and that amyloidogenicity is not required for this. This is unexpected since many reports have shown a clear link between amyloidogenicity and membrane permeabilization, not only for hIAPP but for many other amyloidogenic proteins as well (see previous sections). In particular, it has been unambiguously shown that the cytotoxicity of hIAPP is linked to its amyloidogenicity, in contrast to non-amyloidogenic rIAPP that is not cytotoxic and not linked to T2DM (Lorenzo, et al. 1994; Höppener, et al. 2000). A possible explanation for the membrane disrupting ability of non-amyloidogenic hIAPP fragments, as observed in the studies by the group of Ramamoorthy (Brender, et al. 2008; Brender, et al. 2008), as well as of rIAPP (Green, et al. 2004; Knight, et al. 2006), is the 'carpet mechanism', in which α-helical peptides disrupt membranes without the need to aggregate into β-sheet–rich structures (Shai 1999). Indeed, hIAPP and rIAPP both insert in lipid monolayers as monomer (Engel, et al. 2006; Lopes, et al. 2007), and their

similar membrane interaction has been ascribed to the carpet mechanism (Green, et al. 2004). Membrane damage by rIAPP and non-amyloidogenic hIAPP fragments seems to occur only in membranes solely composed of negatively charged lipids, conditions that are very different from those found in cells (approximately 20 to 30% negatively charged lipids). Moroever, experimental conditions, for instance different membrane permeability assays, or the presence or absence of an amidated C-terminus, can lead to discrepancies between studies. In conclusion, since membrane damage under certain conditions can be induced by the T2DM-unrelated and non-amyloidogenic rIAPP, it is unlikely that the mechanism for this membrane damage relates to a physiologically relevant event that can explain membrane damage and cytotoxicity in T2DM.

Apart from the N-terminus, it has been shown that other residues are in contact with the membrane when hIAPP binds to a phospholipid bilayer. Important information comes from a recent residue-level study using site-directed spin labeling and EPR spectroscopy. This study shows details of the α-helical structure of monomeric hIAPP bound to large unilamellar vesicles (LUVs) composed of 80% POPS and 20% POPC (Apostolidou, et al. 2008). It was found that residues 9 to 22 form an α-helix oriented parallel to the membrane surface, embedded in the bilayer at the level of the phospholipid headgroups (Fig. 5). Importantly, most of these residues are also thought to be involved in the formation of cross-β structure in the hIAPP fibril (Fig. 1c). This might explain the success of the study since the high amount of negatively charged lipids trapped this helical state, possibly by preventing key residues from conversion to β-sheet structure. It was also suggested that residues Thr9, Leu12, Leu16 and Ser20 face the hydrophobic core of the membrane, while the charged residues Arg11 and His18 are located at the level of the phospholipid headgroups (Fig. 5). Residues 23-37 of the membrane-bound hIAPP are largely unstructured, and it is implied that this exposure will promote conversion to β-sheet structure (Apostolidou, et al. 2008). It should be noted that the presence of 80% negatively charge lipids is very different from the *in vivo* situation (~25% negatively charged lipids), consequently this helical state is likely to be less stable in a cellular environment. Still, the observation that physiological levels of negatively charged lipids (~25%) are sufficient to accelerate hIAPP fibril formation (Jayasinghe, et al. 2005) supports the notion that transient helical structure might play a role in hIAPP fibrillation *in vivo*. In this context it is important to note that negatively charged lipids are preferentially located at the cytosolic side of the cellular membrane, where

they are only available to intracellular hIAPP species. Consequently, membrane permeabilization by the process of fibril growth at the membrane (Engel, et al. 2008), which occurs independent of the presence of negatively charged lipids, could affect biological membranes from both sides. The previously mentioned EPR study confirms earlier work, which had shown using CD and FTIR spectroscopy that hIAPP adopts helical structure when sufficient negatively charged lipids are present (Jayasinghe, et al. 2005; Knight, et al. 2006; Lopes, et al. 2007).

Considering that residues 9-22, except for residue 18, are identical for hIAPP and rIAPP, it is not surprising that the non-amyloidogenic rIAPP also forms similar helical structure when bound to membranes (Knight, et al. 2006). The $hIAPP_{1-19}$ and $rIAPP_{1-19}$ fragments reconstitued in DPC micelles have also been shown to adopt α-helical structure (Nanga, et al. 2008). In addition, it was found that in solution, rIAPP has a tendency to sample α-helical structures (Williamson, et al. 2007). The acceleration of hIAPP fibril formation in the presence of helix-promoting organic solvents like TFE and HFIP support the importance of helical structure in the process of fibril formation (Padrick, et al. 2002). The observation of α-helical structure in membrane-bound hIAPP seems an ordinary observation, since it is known that many peptides adopt α-helical structure when interacting with a lipid bilayer (Shai 1999). However, this helical structure might turn out to be extra-ordinary, in view of the observation that the hIAPP monomer converts from a mostly unstructured peptide in solution to a β-sheet rich fibrillar assembly. Particularly interesting is the suggestion that this α-helical state could be an intermediate that promotes fibril formation of hIAPP (Knight, et al. 2006). This suggestion deserves consideration and needs more research, in particular because it would suggest alternative, α-helix based ways to inhibit cytotoxicity, next to the "established" design of disrupters of β-sheet structure as a means to prevent aggregation and cytotoxicity.

Future directions

There has been considerable progress in the field of hIAPP-membrane interaction during the past five years, however it is still far from clear how these interactions relate to cytotoxicity in T2DM. Various hypotheses have been put forward, and all of them are logical starting points for further research.

Discrepancies in studies of cytotoxicity and membrane interaction of hIAPP species most likely result from ill-defined or impure hIAPP samples, for example those containing traces of pre-existing aggregates

(Konarkowska, et al. 2006), or synthesis-related residues of mercury (Cobb, et al. 1992; Golpon, et al. 2003). Due to the often rapid and uncontrollable aggregation of amyloidogenic proteins and peptides, the possibility of dissociation of species from fibril ends (Carulla, et al. 2005), or the recently proposed lipid-induced fibril dissociation into oligomers (Martins, et al. 2008), it is difficult to obtain a pure, structurally uniform sample of either monomers, oligomers or fibrils. Efforts are likely to be directed towards dissecting the structural heterogeneity in hIAPP amyloid formation, specifically considering polymorphism observed in oligomeric species and fibrils. One way of obtaining pure samples with a single morphology would be the use of novel purification strategies, and specific antibodies can be of great help in identifying unique (oligomeric) hIAPP species, for example the oligomer-specific antibodies pioneered by the Glabe group (Glabe 2004). The ability to isolate such hIAPP species is crucial for future studies that attempt to link structure to cytotoxicity. Another way of tackling the large variety of intermediate species is using techniques that can resolve several different species in one sample, for example single-molecule techniques. Recent examples have shown the strength of these techniques for solving heterogeneity in populations of amyloidogenic peptides and proteins (Collins, et al. 2004; Mukhopadhyay, et al. 2007; Kostka, et al. 2008; Orte, et al. 2008).

As mentioned before, various studies have shown that it is not necessarily a particular species that can be cytotoxic, but that the conversion from one species to another could also be cytotoxic. Future studies into these 'amyloid conversions', in particular membrane-mediated conversions, could reveal new insights into cytotoxic mechanisms. One particularly helpful tool is the use of molecular modeling, which has recently been successfully adapted for amyloidogenic proteins interacting with membranes (Friedman, et al. 2009). It is clear from many recent studies that the effects of membranes and other interfaces on amyloid aggregation can be huge, and warrant future investigations.

Many of the membrane permeability assays are distant from the membrane conditions found *in vivo*. Efforts to extrapolate results and hypotheses from model-membrane systems towards physiological β-cell membranes are likely to increase. Morphology and structure from *in vivo* produced hIAPP oligomers and fibrils would provide valuable insight in the physiological relevance of the species and processes that have now mostly been obtained using synthetic peptides and test-tube conditions. Only this, physiologically

relevant information will bring us closer to the development of inhibitors for hIAPP-induced cytotoxic processes in the Islets of Langerhans.

Studies on hIAPP cytotoxicity can increasingly benefit from the growing knowledge obtained from other amyloidogenic systems. Still, there are convincing reasons to assume that cytotoxic mechanisms and aggregation pathways are not necessarily the same for the different amyloid-related peptides and proteins. Consequently, one might have to consider stepping away from the hypothesis that a generic mechanism exists that describes amyloid-induced cytotoxicity for all misfolding diseases.

Acknowledgements

I am grateful to Sheena Radford and members of the Radford group for support and helpful discussions. I thank Antoinette Killian and Carlo van Mierlo for valuable comments on the manuscript. I acknowledge support from the European Molecular Biology Organization (EMBO Long-Term Fellowship ALTF-371-2007).

Figure legends

Fig. 1. IAPP structure and morphology. (a) Amino acid sequence of hIAPP and non-amyloidogenic rodent IAPP (rIAPP). Both peptides are C-terminally amidated and have a disulfide bond between residues 2 and 7. Residues in red mark the differences between hIAPP and rIAPP. The blue line indicates residues involved in the fibril core while the green line marks residues which are thought to be involved in membrane interactions (b) Negatively stained electron microscopy image of *in vitro* assembled hIAPP fibrils showing different fibril morphologies: ribbon-like fibrils and coiled fibrils. Image kindly provided by U. Aebi and C. Goldsbury. (c) Model of an hIAPP fibril based on NMR data of a morphologically homogeneous fibril sample. The picture shows a cross-sectional view of the fibril, with 2 hIAPP monomers back to back. The long-axis of the fibril is perpendicular to the plane of the paper. Reprinted with permission from (Luca, et al. 2007). Copyright 2007 American Chemical Society. (d) Example of the morphology and size-distribution of *in vitro* assembled spherical hIAPP oligomers. Reprinted with permission from (Kayed, et al. 2004). Copyright 2004 by the American Society for Biochemistry and Molecular Biology. (e) Example of the morphology of an *in vitro* assembled annular hIAPP oligomer.

Reprinted with permission from (Porat, et al. 2003). Copyright 2003 American Chemical Society. (f) Atomic force microscopy (AFM) image of two membrane-incorporated hIAPP oligomers that display a pore-like structure. Reprinted with permission from (Quist, et al. 2005). Copyright 2005 National Academy of Sciences, U.S.A.

Fig. 2. Electron micrograph showing a mat of islet amyloid fibrils (A) next to a β-cell (B) in human Islets of Langerhans. Bundles of fibrils penetrate the β-cell membrane (arrows). The characteristic secretory vesicles that store insulin and hIAPP are clearly visible as circular structures with an electron dense black spot inside (arrowheads). Magnification 19500X. Reprinted with kind permission from the author and Springer Science and Business Media (Westermark 1973).

Fig. 3. Scheme showing various suggested membrane-permeabilizing hIAPP species and processes in relation to cytotoxic hIAPP-membrane interaction. Predominantly unstructured (grey circle), α-helix rich (blue triangle) and β-sheet rich (green rectangles) hIAPP monomers or oligomers are participating in and/or converted during aggregation at or near membranes. Stars show the membrane permeabilization events, with yellow stars suggesting membrane permeabilization by toxic species and red stars suggesting membrane permeabilization by toxic processes. Route 1 symbolizes membrane permeabilization by preformed β-sheet rich oligomers (Kayed, et al. 2004) or by the bigger β-sheet rich annular oligomers that insert in the membrane (1a)(Kayed, et al. 2009). Alternatively, such oligomers could assemble in the membrane from hIAPP monomers (1b)(Quist, et al. 2005). Route 2 describes the binding of initially random-coil hIAPP monomers to lipids, followed by folding into helical structure and the formation of α-helix-rich on-pathway oligomers that permeabilize the membrane (Knight, et al. 2006). Route 3 starts by binding of monomer to the membrane followed by the participation of monomers and/or oligomers in fibril growth at the membrane. The process of fibril elongation at the membrane results in membrane permeabilization (Engel, et al. 2008).

Fig. 4. Cryo EM image of hIAPP fibrils (arrows) that are associated with disrupted large unilamellar vesicles (LUVs) composed of phospholipids (asterisks) after initially monomeric hIAPP was allowed to

aggregate in the presence of LUVs. Pre-formed hIAPP fibrils did not cause membrane disruption. Reprinted with permission from (Engel, et al. 2008). Copyright 2008 National Academy of Sciences, U.S.A.

Fig. 5. Model for the structure of monomeric membrane-bound hIAPP from EPR data. The red ribbon indicates the α-helical part of the peptide (residues 9-20). The N-terminal and C-terminal part are unstructured. The scheme shows the position of the peptide relative to the bilayer lipids. Reprinted with permission from (Apostolidou, et al. 2008). Copyright 2008 by the American Society for Biochemistry and Molecular Biology.

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Figure 1

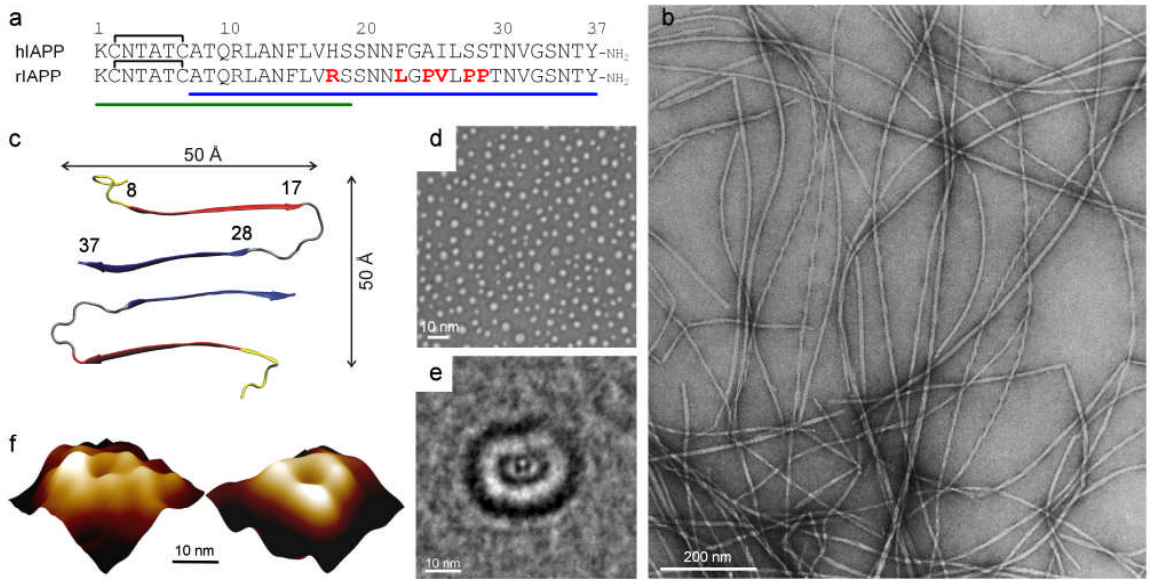

Figure 2

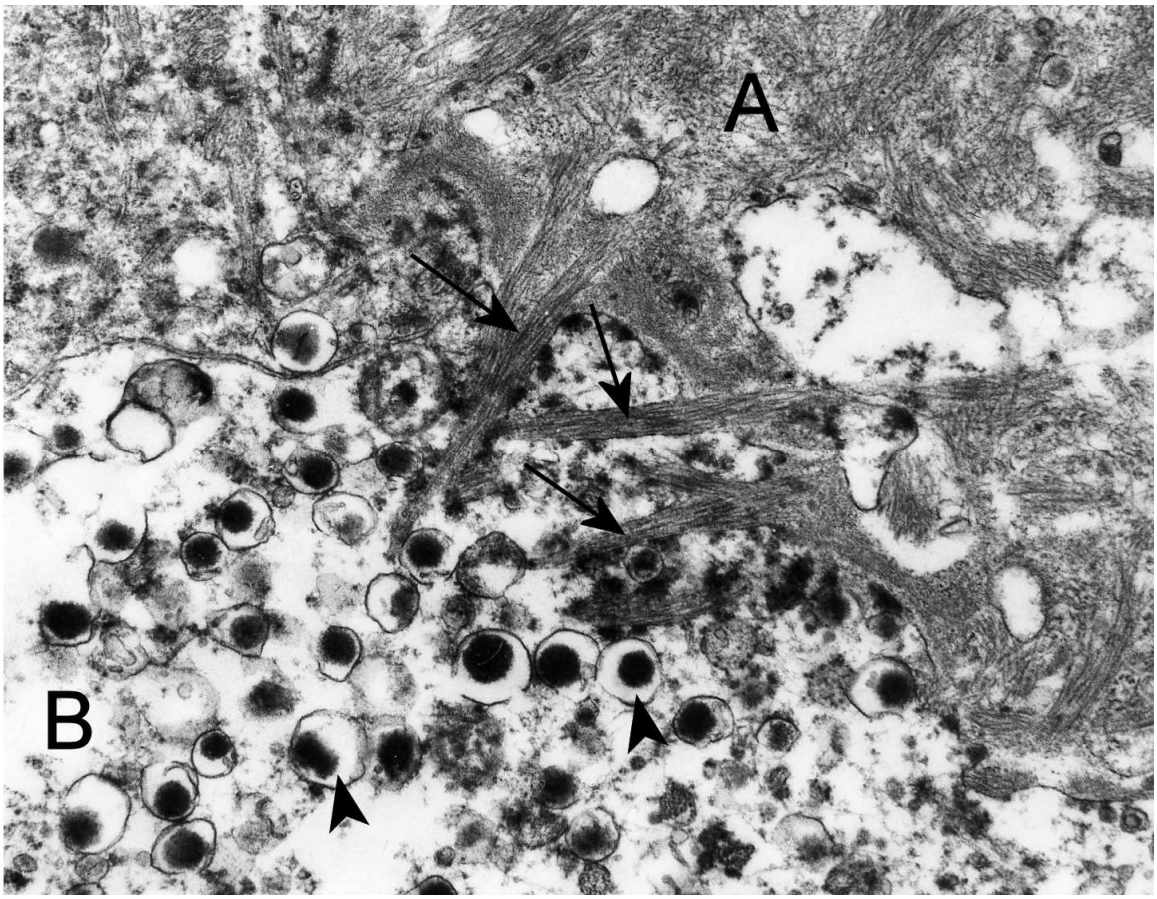

Figure 3

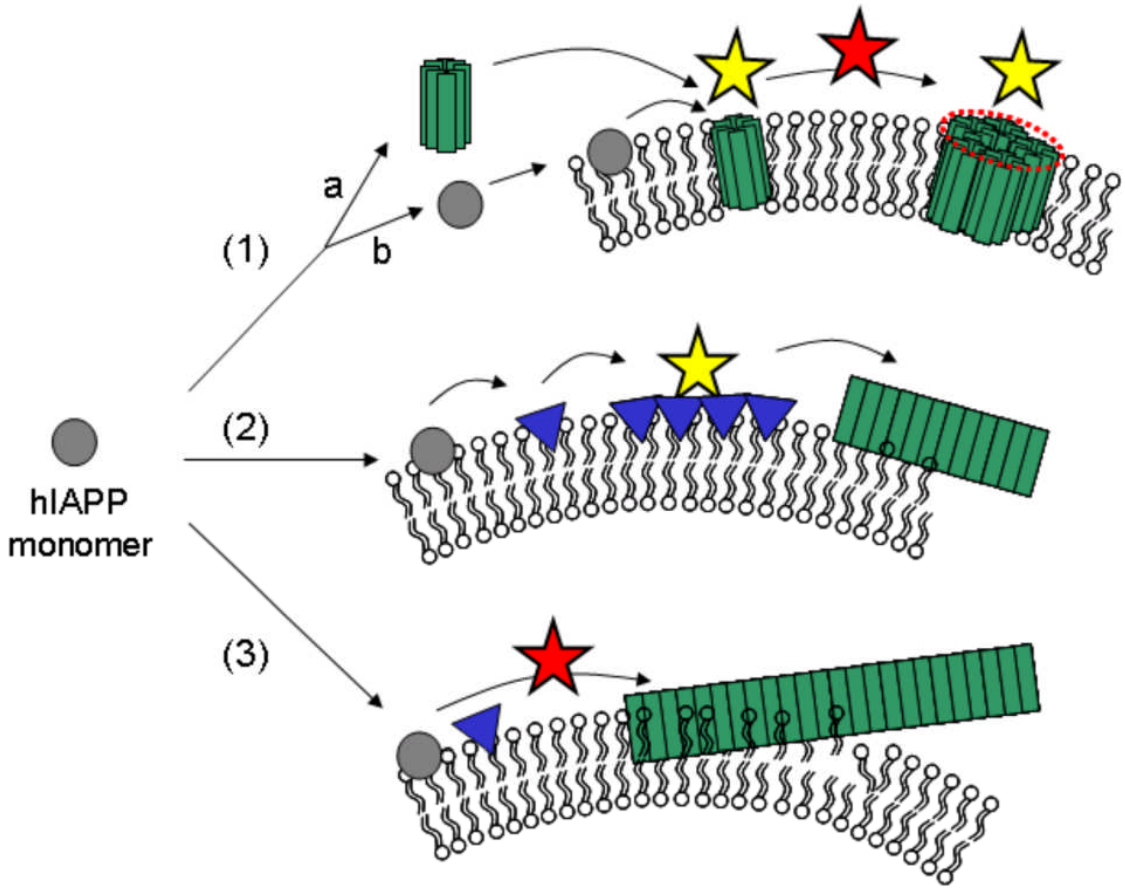

Figure 4

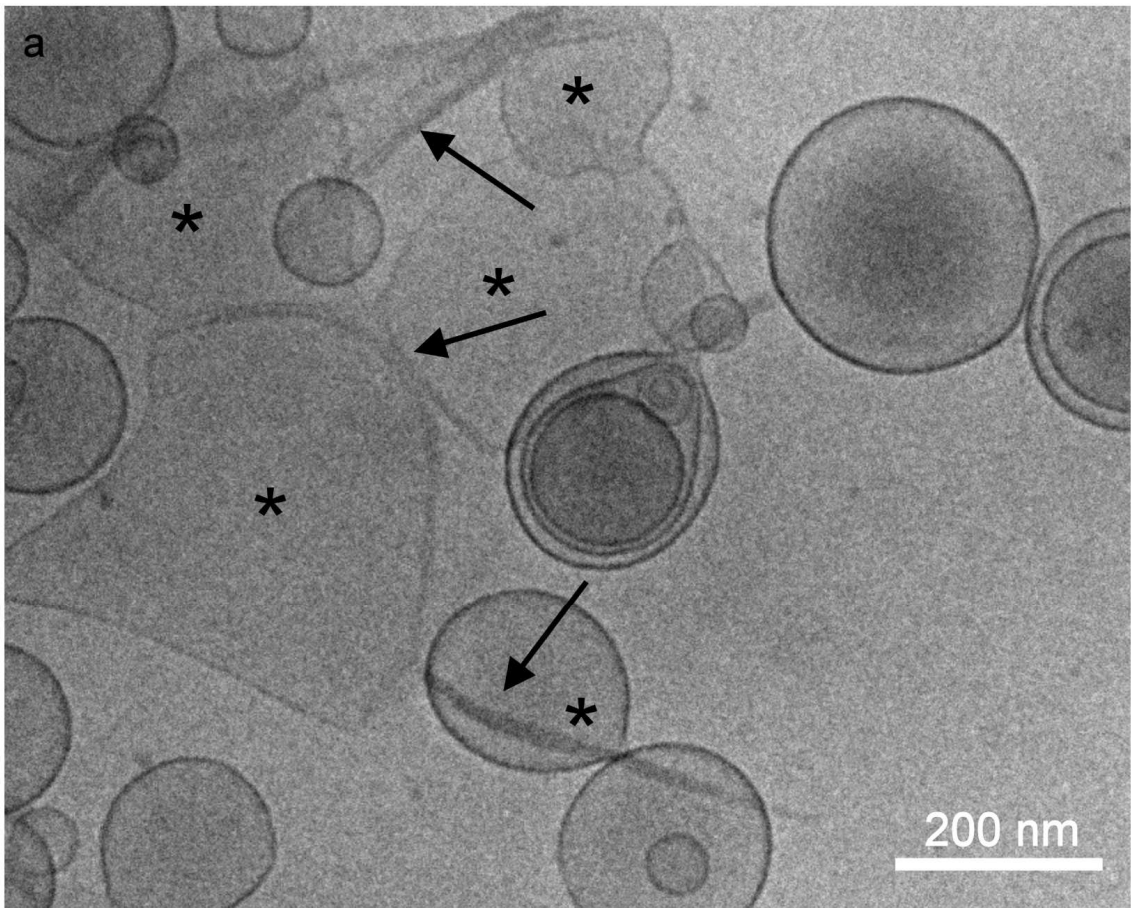

Figure 5