Identification of a hinge residue controlling islet amyloid polypeptide self-assembly and cytotoxicity

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\textbf{Running title:} Self-assembly-toxicity relationships of IAPP

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\textbf{ABSTRACT}

The islet amyloid polypeptide (IAPP) is a 37-residue peptide hormone whose deposition as amyloid fibrils in the pancreatic islets is associated with type 2 diabetes. Previous studies have suggested that residue Asn-21 plays a critical role in the \textit{in vitro} self-assembly of IAPP. Herein, we studied structure-self-assembly relationships focusing on position 21 to gain detailed insights into the molecular mechanisms of IAPP self-assembly and to probe the conformational nature of the toxic assemblies associated with β-cell death. Thioflavin T (ThT) fluorescence, CD spectroscopy and transmission EM analysis revealed that the Asn-21 amide side chain is not required for IAPP nucleation and amyloid elongation, as N21A and N21G variants assembled into prototypical fibrils. In contrast, Asn-21 substitution with the conformationally constrained and turn-inducing residue Pro accelerated IAPP self-assembly. Successive substitutions with hydrophobic residues led to the formation of ThT-negative β-sheet-rich aggregates having high surface hydrophobicity. Cell-based assays revealed no direct correlation between the \textit{in vitro} amyloidogenicity of these variants and their toxicity. In contrast, leakage of anionic lipid vesicles disclosed that membrane disruption is closely associated with cytotoxicity. We observed that N21F variant self-assembles into worm-like aggregates, causing loss of lipid membrane structural integrity and inducing β-cells apoptosis. These results indicate that specific intra and inter-molecular interactions involving Asn-21 promote IAPP primary nucleation events by modulating the conformational conversion of the oligomeric intermediates into amyloid fibrils. Our study identifies position 21 as a hinge residue that modulates IAPP amyloidogenicity and cytotoxicity.

\textbf{Introduction}

The aggregation and tissue deposition of proteins into amyloid fibrils are the hallmark of numerous diseases, including the Alzheimer’s disease, type II diabetes and various amyloidoses (1,2). In over 90% of patients afflicted with type II diabetes, amyloid deposits are observed in the extracellular space of pancreatic islets of Langherhans (3-5). The main component of islet amyloids is the peptide hormone islet amyloid polypeptide (IAPP, or amylin) (6,7). The accumulation of IAPP insoluble aggregates correlates closely with the duration and severity of the disease, and with the loss of β-cell mass (4). The link between islet amyloid and type II diabetes has initially led to the postulate that amyloid fibrils mediate β-cell degeneration. This hypothesis was reinforced by the early work of Lorenzo, demonstrating the cytotoxicity of IAPP fibrils on pancreatic islet cells (8). However, most
recent studies have indicated that oligomeric and non-fibrillar species cause cell death (9-11). Using time-resolved analysis, it has been observed that IAPP amyloid structures are non-toxic to INS-1 cells and that the toxic species are low order oligomers, which lack a β-sheet structure and hydrophobic patches (12). Recently, IAPP fibrils have been shown to be toxic to RIN-5F cells and it has been proposed that a quaternary structure characterized by pairs of β-sheets joined by a dry interface represents the toxic spine (13). The opposing conclusions between these two elegant studies (12,13) are likely associated with differences in the experimental conditions (monomerization, buffer, aging time, cells, toxicity assays, etc) and/or with the undetectable presence of toxic oligomers in the fibril preparation used by Krotee and colleagues (13). Thus, although a substantial number of studies have investigated the conformational nature of IAPP toxic species, the subject is still the matter of active debates.

IAPP is a 37-residue hormone that is co-expressed and co-secreted with insulin by pancreatic β-cells (14). The peptide exhibits, under its monomeric state, a conformational ensemble populated by disordered structures, although it diverges from an absolute random coil by the presence of transient helical conformations (15). In membrane mimetic environments, which are known to accelerate fibrilization, IAPP is mainly characterized by helical conformation (16,17). A structure characterized with three antiparallel β-strands has been recently reported when the peptide is trapped into negatively charged lipid nanodiscs (18). Upon self-assembly into cross-β-sheet quaternary structure, each monomer adopts a U-shaped conformation with two β-strands connected by a loop. According to solid-state NMR, the β-strands comprise residues 8–17 and 28–37 and the loop involves residues 18–27 (19). In the electron paramagnetic resonance (EPR) model, the two β-strands comprise residues 14–19 and 31–36 (20). Conformational changes associated with the transition of IAPP from its soluble state ensembles into ordered amyloids and the molecular interactions governing this transition remain elusive. Helical conformation has been proposed to be critical for self-recognition, as oligomerization could be thermodynamically linked with helix formation within the 5-20 segment (21,22). In contrast, by using a helix-disrupting analog, it has been proposed that helical species are off-pathway and that preventing helical folding increases cytotoxicity (23). Moreover, it has been reported that helical conformation are mainly found in the monomeric state and could seed oligomer formation, although they are not mandatory for amyloid growth (24).

Elucidating the molecular determinants promoting oligomerization and the interactions initiating amyloidogenesis is critical to better define the nature of the toxic proteospecies. Studies have exploited residue-specific modifications to better define the driving forces of IAPP self-assembly (25-30). Sequence differences between human and mouse IAPP, and mutational studies have initially indicated that the segment 20-29 dictates aggregation and toxicity (11). Nevertheless, substitutions outside the 20-29 amyloidogenic region, including A13E, V17C and Y37L, have shown to alter amyloid formation (27,31). It has been shown that residue Asn-21 plays a key role in the in vitro self-assembly of IAPP, as its consecutive replacement by Leu, Ser and Asp inhibits amyloid formation (25,30). Position 21 is particularly intriguing since it is located in the disordered loop joining the two β-strands in the amyloid conformation (19,20) (Fig. 1). The side chain of Asn-21 projects outward from the protofilament core, thus it could participate in fibril packing. This position is also located at the intersection of the putative 5-20 helical segment and the 20-29 amyloidogenic core. Moreover, Asn-21 is adjacent to Ser-20, for which the Asian-mutation S20G is known to increase amyloidogenicity and toxicity (32). These observations strongly suggest that this residue plays a critical role in the conformational conversion modulating oligomerization, nucleation and/or amyloid growth.

Accordingly, we performed a structure-assembly relationships study focusing on position 21 by tuning the side chain physicochemical properties and the local conformational freedom of the backbone. The data indicated that the introduction of a
hydrophobic residue at this position locks the peptide into highly toxic β-sheet-rich aggregates, while a Pro hastens IAPP self-assembly into non-toxic amyloid fibrils. The present study reveals that Asn-21 acts as a molecular hinge modulating IAPP amyloid formation and cytotoxicity.

Results

Rational design of N21X mutants

To gain mechanistic insights into IAPP self-assembly and toxicity to β-cells, we prepared a library of analogs modified at Asn-21. We probed the contribution of the amide side chain by successively incorporating Ala, a negatively (Asp) and a positively (diaminobutyric acid; Dab) charged residue. Dab was used instead of Lys in order to maintain the length of the side chain. Asn-21 was substituted with hydrophobic residues with high β-sheet propensity (Phe, Leu) and with Phe capable of π–π interactions. Conformational modifications were introduced to favor turn (Pro, Gly, D-enantiomer). In fact, D-amino acids are known to promote and/or stabilize turn conformation (33,34). The C-α-methylated aminoisobutyric acid (Aib) residue, known to promote helical folding (35), was incorporated. These substitutions (Pro, Gly, asn, Aib) modulate the local conformational freedom of the peptide backbone. Rodent IAPP (rIAPP), which contains three Pro residues, and is less prone to aggregation and non-toxic, was used as a negative control (36).

Substitutions at position 21 modulate kinetics of self-assembly

The effect of site-specific modifications on self-assembly was initially evaluated by thioflavin-T (ThT) fluorescence. ThT is a small dye whose fluorescence emission increases sharply upon its binding to the cross-β-sheet quaternary structure (37). IAPP amyloid formation can be ascribed to a nucleated polymerisation where the three distinctive phases (lag phase, elongation phase, saturation phase), are governed by different kinetics. Dynamic and transient oligomeric species are generated during the lag phase, which is the thermodynamic rate-limiting step. Upon formation of competent oligomer(s), or nuclei, the elongation phase begins, leading to the rapid growth of protofilaments and fibrils until reaching the equilibrium. The sigmoidal curve obtained by measuring ThT fluorescence over time is fitted to a Boltzmann sigmoidal and the lag time can be extracted. Under the conditions of the assay (20 mM Tris-HCl, pH 7.4, 40 µM ThT, 25 °C, non-binding surface 96-well plates, 10 min interval between each measurement, no agitation), a lag time of 8.6 ± 1.8 h was observed for IAPP at 12.5 µM (Fig. 2) and 4.2 ± 1.2 h at 25 µM (Fig. S1).

Introducing a hydrophobic side chain (N21F, N21L) at position 21 abolished the formation of ThT-positive assemblies (Fig. 2A). This is in agreement with the previous work of Miranker and colleagues (25). Interestingly, the presence of an amide group on residue 21-side chain is not a prerequisite for the formation of a ThT-positive cross-β-sheet structure, as the substitution N21A led to a slight acceleration of self-assembly. This result is unexpected, considering that the mutant N21S has been previously shown to self-assemble into ThT-negative amorphous aggregates (25). Introducing a negative or a positive charge without altering the side chain length (N21D, N21Dab) inhibited the formation of ThT-positive species. These results indicate that IAPP amyloid formation tolerates the elimination of position 21 amide group, although it does not allow the introduction of a charged or hydrophobic residue.

Considering that IAPP self-assembly occurs in absence of Asn-21 amide group, we investigated the role of backbone conformational entropy and side chain chirality. Introducing the turn-inducing residue Pro led to an acceleration of amyloid assembly, with N21P displaying a lag time of 4.2 ± 0.8 h and a significant increase of ThT end-point fluorescence (Fig. 2B). This observation is intriguing, as Pro residue is recognised to inhibit amyloid assembly (36,38,39). For instance, single-point mutation I26P has been shown to transform IAPP into a potent amyloid inhibitor (28). When Asn-21 was replaced by α-methyl-alanine (Aib), known to favor α-helix, amyloid assembly was slowed down to a lag
time of over 12 h., with some ThT assays at 12.5 µM showing no increase of fluorescence over 20 h (Fig. S2). Increasing the conformational freedom by incorporating Gly led to kinetics somewhat similar to the N21A analog. It is worth mentioning that N21Aib, N21A and N21G showed a somewhat high heterogeneity between ThT kinetic assays performed at 12.5 µM (Fig. S2). This observation suggests that the Asn-21 amide function facilitates IAPP nucleation step through specific intra- or inter-molecular hydrogen bonds. Substituting Asn-21 by its corresponding D-amino acid hastened amyloid formation, although it led to a lower fluorescence at the saturation phase. Similar trends were obtained with all peptides used at 25 µM (Fig. S1). These results show that modifications favoring turn conformation (Pro, D-Asn) hasten IAPP self-assembly, whereas restricting the local conformation to the helical space (Aib) delays amyloid formation.

**Asn-21 modulates the conformational conversion associated with self-assembly**

The effect of site-specific modifications on conformational transitions was evaluated by circular dichroism (CD) spectroscopy. Peptides were incubated at room temperature under quiescent conditions (20 mM Tris-HCl, pH 7.4, 50 µM IAPP) and far-UV CD spectra were recorded. As expected, immediately after solubilisation of monomerized/lyophilized IAPP, a random coil CD spectrum was recorded (Fig. 3). No conformational shift was observed during the first 6 h incubation of WT IAPP, while a β-sheet secondary structure (single minimum at 220 nm) was observed upon 24 h. The presence of a hydrophobic group (Phe, Leu) confined IAPP into a β-sheet conformation immediately after solubilisation. These β-sheet rich species assembled under these conditions were also ThT-negative (Fig. S3). N21D and N21Dab remained in a random coil conformation, even after 48 h incubation. Although the N21A and N21G mutants showed a ThT-positive signal in the kinetic assays (Fig. 2), the two peptides remained in a random coil conformation after 24 h incubation and a β-sheet signal was observed only after 48 h incubation. Moreover, under the conditions of the CD experiment, a low ThT fluorescence was measured for N21A and N21G (Fig. S3). Substitution of Asn-21 by an Aib abolished conformational transition. It is worth mentioning that time-point measurements under quiescent conditions in a microcentrifuge tube (Fig. 3, S3) cannot be directly compared with the microplate-based ThT kinetics (Fig. 2, S1, S2). We recently reported that, although the microplate kinetic assay is done under quiescent conditions (i.e. no agitation between each reading), the displacement of the microplate within the fluorimeter occurring during measurements is sufficient to accelerate amyloid formation (40). Thus, self-assembly of N21A, N21G and N21Aib appears be more dependent on agitation than the WT IAPP. The mechanical forces associated with agitation are known to enhance amyloid formation by promoting mass transport and amplifying the number of fibrils by fragmentation (secondary nucleation) (41).

Time-point CD analysis of N21P and N21n mutants revealed their prompt random coil-to-β-sheet conformational conversion (Fig. 3), in agreement with ThT-kinetics. A ThT-positive signal was measured for N21n at time 0 h (dead time of 3 to 4 min), confirming the prompt self-assembly of this analog (Fig. S3). In fact, short fibrils could even be observed by atomic force microscopy (AFM) immediately after the solubilisation of N21n, whereas no fibrillar aggregates were visible for WT (Fig. S4). Control rIAPP remained in a random coil conformation (Fig. S5).

The self-assembly of N21X derivatives was further characterized by measuring the formation of hydrophobic clusters using 8-anilino-1-napthalenesulfonic acid (ANS). The fluorescence intensity of ANS increases upon its binding to protein hydrophobic patches. Random coil ThT-negative WT IAPP species (0 h) did not induce any ANS fluorescence (Fig. 4). Upon 24 h incubation, a positive ANS signal was measured. This result indicates that the formation of ThT-positive β-sheet-rich assemblies (Fig. 3, Fig. S3) is associated with the emergence of hydrophobic clusters (Fig. 4). Interestingly, N21F and N21L exhibited a strong signal of ANS fluorescence immediately after their solubilisation. Thus, incorporation of
a hydrophobic residue at position 21 prompts the formation of β-sheet-rich ThT-negative aggregates with high surface hydrophobicity. Successive substitution of Asn-21 with Asp and Dab precluded the formation of ANS-positive assemblies, while the N-to-A mutation reduced ANS fluorescence. As anticipated from the kinetic assays and time-point CD analysis, incorporation of Pro and the inversion of Asn chirality led to a positive ANS signal. As observed for ThT (Fig. S3), freshly dissolved N21n showed an increase of ANS fluorescence (0 h; Fig. 4). The N-to-G mutation delayed time-dependent increase of ANS fluorescence, as observed for ThT fluorescence. Time-point ANS fluorescence (Fig. 3) was plotted as a function of time-point ThT fluorescence (Fig. S3) and we observed that the formation of ThT-positive assemblies is associated with the emergence of hydrophobic clusters for WT IAPP, N21n and N21P (Fig. S6). The correlation between ANS and ThT signal was not present for N21F and N21L, whereas a low correlation was observed for N21G.

Asn-21 mutations modify the supramolecular architecture of assemblies

The mesoscopic architecture of the assembled proteospecies was analysed by negative-stain transmission electron microscopy (TEM). Long, unbranched and twisted fibrils were obtained for WT IAPP after 24 h aging under the conditions of the time-point analysis (Fig. 5). Amorphous aggregates were mainly observed for the N21F and N21L mutants, although short fibrils could be detected in some N21L samples (Fig. 5, S7). TEM analysis validated that the addition of a charge at position 21 inhibits fibril formation, although fibril-like assemblies could be observed for N21Dab (Fig. S8). In sharp contrast, successive replacements of Asn-21 with Pro and its D-enantiomeric counterpart led to the formation of a dense network of fibrils. Although the time-point CD and ThT analysis of N21A and N21G mutants suggested the absence of amyloid assemblies after 24 h aging (Fig. 3, S3), TEM analysis revealed the presence of fibrils for both peptides. Poorly defined fibrils could be observed in some N21Aib preparations (Fig. 5, S9), in agreement with the low reproducibility between ThT kinetics.

Toxicity of N21X mutants does not correlate with their in vitro amyloidogenicity

The relationships between the in vitro amyloidogenicity and the cytotoxicity of N21X analogs were investigated by evaluating the viability of INS-1 cells upon treatment with freshly dissolved monomerized peptides. As reported (12, 29, 42), a concentration-dependent decrease of viability was observed for IAPP, with a cell viability below 20% of control at 50 µM (Fig. 6). In contrast, the non-amyloidogenic rIAPP did not induce cell death, even at a concentration of 50 µM. N21A, N21P and N21n mutants, which self-assembled into amyloids, reduced the viability of INS-1 cells to a similar extent to the WT peptide. Incorporation of a positive or a negative charge at position 21 reduced the toxicity of IAPP, although both analogs were toxic at 50 µM (Fig. 6). The hydrophobic-substituted derivatives were toxic to β-cells, although N21F and N21L did not formed amyloids. These observations indicate that non-amyloidogenic IAPP derivatives (N21Dab, N21D, N21F N21L) can be toxic toward INS-1E cells. In contrast, N21Aib and N21G were non-toxic. Toxicity (Fig. 6) was plotted as a function of in vitro amyloidogenicity (Fig. 1) to evaluate the correlation between these two parameters. No direct correlation between in vitro amyloidogenicity and cytotoxicity was observed (Fig. S10), although mutagenesis at position 21 dramatically modulates both the cytotoxicity and the self-assembly of IAPP.

It was proposed that toxic IAPP species are mainly pre-amyloid intermediates that are transiently formed during the lag phase (12). Thus, the presence and distribution of oligomers populating the lag phase was evaluated for selected mutants using photo-induced cross-linking of unmodified proteins (PICUP) followed by SDS-PAGE analysis (43). As observed in Fig. S11, an essentially similar distribution of proteospecies, from monomers to hexamers, was obtained for WT, N21P, N21n, N21G and rIAPP. In contrast, cross-linking of the ThT-negative and toxic N21F mutant only revealed monomers and
dimers, and an absence of higher oligomeric species. Considering that N21G (amyloidogenic) and rIAPP (non-amyloidogenic) are not cytotoxic (Fig. 6), the PICUP results indicate that toxicity is not necessarily associated with the in vitro aggregation propensity and that not all oligomers are toxic, as previously shown (12).

**Mutagenesis of Asn21 modulates lipid membrane disruption**

The mechanism by which IAPP induces cell death is complex and is still the subject of active research (10,44). One of the most accepted upstream events is disruption of the plasma membrane structural integrity by pre-fibrillar proteospecies (45,46). Besides, lipid membranes are known to hasten amyloid formation and might change the pathway(s) by which IAPP self-assembles, in comparison to aqueous solution (45,47). Thus, we sought to evaluate how mutagenesis at position 21 affects the kinetics of amyloid formation in presence of lipid vesicles and the ability of IAPP to disrupt membranes. First, ThT-fluorescence assays were performed in presence of large unilamellar vesicles (LUVs) composed of phosphocholine/phosphoglycerol (DOPC: DOPG 7:3). The presence of anionic vesicles dramatically hastened amyloid formation of WT, N21P and N21n peptides, with lag time under 30 min for N21n and WT, and 90 min for N21P (Fig. 7A; peptide concentration of 12.5 µM). In contrast, no ThT signal was measured for the N21G and N21F mutants and rIAPP over the 4 h incubation time for the N21G and N21F µM N21n and WT, and 90 min for N21P and N21n assemblies incubated for 24 h were significantly less toxic to cells in comparison to IAPP. The proteospecies generated after 120 h of aging for WT, N21P and N21n were non-toxic. TEM images validated that IAPP, N21n and N21P assembled into well-defined amyloid fibrils at 150 µM (Fig. 8B). These data support previous studies indicating that well-defined fibrils are poorly toxic. In sharp contrast, the N-to-F substitution locked IAPP into proteospecies, which remained highly toxic overtime (Fig. 8A). As observed by TEM, N21F formed short and poorly defined worm-like fibrils when incubated at 150 µM for 48 h. (Fig. 8B). Interestingly, immediately upon its solubilisation at 150 µM, the N21F analog assembled into amorphous spherical aggregates that slowly evolved into short worm-like assemblies (Fig. S12).

IAPP-induced cytotoxicity is associated with numerous downstream cellular events, including oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress and apoptosis (9,44,48). We evaluated the activation of caspase-3 upon cell treatment with the different proteospecies. As caspase-3
activation is an upstream event to cell death, cells were treated for 3 h with the proteospecies. All four peptides under their non-fibrillar states (0 h) induced a significant increase of caspase-3 activation (Fig. 8C). N21F short fibrillar species obtained after 48 h aging strongly activated caspase-3, in contrast to IAPP, N21P and N21n. Moreover, we measured the ability of the assemblies formed after 48 h aging to induce membrane disruption using the calcein release assay. Interestingly, N21F worm-like aggregates disrupted anionic LUVs whereas well-defined amyloid fibrils (WT, N21P, N21n) had no significant effect on lipid membrane (Fig. 8D). We investigated if the cytotoxicity and membrane leakage ability of N21F assemblies could be associated with the presence of remaining oligomers/monomers in solution. Accordingly, N21F aggregation mixture (48 h; 150 µM) was centrifuged at 35 000 g for 45 min and the presence of soluble species in the supernatant was evaluated by analytical RP-HPLC. As observed in Fig. S13, no N21F was detected by HPLC in the supernatant. These data indicate that N21F ThT-negative β-sheet-rich assemblies are highly toxic to pancreatic β-cells, increasing the complexity of identifying specific IAPP toxic species. Nonetheless, we cannot rule out the possibility that the N21F worm-like aggregates disassemble and release soluble species, which induce cell death.

Discussion

A complex network of specific intra- and inter-molecular interactions, which give rise to off- and on-pathway intermediates, governs IAPP self-assembly from a disordered peptide into highly ordered fibrils. Intermediate assemblies were identified as the most toxic species of the amyloidogenic cascade. Thus, it is important to pinpoint the chemical determinants governing these secondary and quaternary structural transitions. Whereas IAPP amyloid formation is somewhat tolerant to mutations, previous studies have revealed that self-assembly does not tolerate substitution at Asn-21 (25, 30). Herein, we performed a structure-assembly relationships study at position 21 to delineate the molecular mechanisms of self-assembly and to better define the conformational species associated with β-cell death.

In contrast to expected results based on previous studies, we showed that Asn-21 amide group is not a prerequisite for nucleation and amyloid elongation. As shown in the kinetic assays, substitution by Ala led to a shorter lag-time and the N21A mutant formed well-defined fibrils. This result indicates that hydrogen-bonding interactions involving Asn amide side-chain are not mandatory for IAPP amyloid formation. Nonetheless, N21A amyloidogenesis was significantly delayed when assembly occurred in a test tube and was followed by time-point ANS, ThT and CD measurements. A similar trend was observed with the mutant N21G. N21A and N21G mutants show high heterogeneity between ThT kinetics in microplate assays. Microplate displacement within the fluorimeter induces significant agitation, hastening amyloid formation through mass transport and fibril fragmentation (secondary nucleation). Moreover, ThT kinetic assays are performed in non-binding surface microplates, whereas the surface of microcentrifuge tubes is non-treated. It has been reported that hydrophobic surfaces, such as non-binding coating, hasten amyloid formation by promoting primary nucleation rate (41). These observations suggest that removing the amide group (*i.e.* N21A, N21G) prolongs the lag phase (delayed nucleation) under fully quiescent conditions and in absence of a hydrophobic surface. In contrast, the non-binding surface of the microplate and/or agitation within the fluorimeter counteract this effect by promoting primary and/or secondary nucleation events.

Interestingly, substitution of Asn-21 with the conformationally constrained and turn-inducing Pro accelerated amyloid formation. This result is surprising considering that the incorporation of Pro within an amyloidogenic peptide is a common strategy to inhibit amyloid formation. This substitution constitutes the first X-to-Pro mutation within the amyloidogenic 20-29 segment promoting self-assembly. Inversing the chirality of Asn-21, a modification known to favor β-turn and to destabilize α-helix (34), dramatically hastened amyloid formation. Short ThT-positive fibrils
were even observable immediately after solubilisation of N21n. Previous studies have suggested that the segment 20-29 mediates initial self-recognition and that early oligomers contain stacks of parallel β-sheet within the 23FGAIL27 region (24,49,50). However, below a critical concentration, IAPP self-recognition appears to be primarily initiated by the N-terminal region, which than propagates to the central domain (51). Regardless of the contacts initiating aggregation, a high-energy structural rearrangement within the 20-29 segment is necessary for the conversion from oligomers to fibrils. The energy barrier induced by the FGAIL β-sheet oligomers is predicted to slow down amyloid formation and to be at the origin of the lag phase (50). In the NMR and EPR atomistic structural models of amyloids, Asn-21 is located at the β-strand-loop interface, whereas in the oligomers this residue is located close to the FGAIL β-sheet. Accordingly, the N21P mutation provides sufficient torsional driving forces to overcome the FGAIL β-sheet intermediate free energy barrier, while not affecting the initial self-recognition events. This promotes primary nucleation by facilitating the structural conversion of the oligomer into the nuclei conformation.

Aromatic stacking and hydrophobic collapse are key driving forces for aggregation. IAPP self-recognition involving the 20-29 amyloidogenic core is mainly directed by hydrophobic interactions (52). It has been reported, using both the full-length peptide and IAPP(20-29), that the contributions of Phe-23 in amyloid formation are function of its hydrophobicity and β-sheet propensity, while its ability to form π-π interactions is not critical (27,53). In the present study, we observed that the successive substitutions of Asn-21 with Leu and Phe led to the prompt formation of ThT-negative β-sheet-rich aggregates with high surface hydrophobicity. Some short and poorly defined fibrils could also be observed for N21L and N21F when incubated for long period at 50 and 150 μM, respectively. The increase of hydrophobicity and/or β-sheet propensity of the 20-29 segment induced by these mutations promote(s) the formation of low energy non-amyloid aggregates, most likely due to the propagation of the FGAIL parallel β-sheet. In sharp contrast, incorporation of a charge, i.e. Asp or Dab, inhibited amyloid assembly, probably due to electrostatic repulsion between monomers. It has been shown that the consecutive substitutions of the neighbouring Asn-22 residue with Asp and Leu have a modest impact on IAPP amyloid formation (25,30). This clearly indicates that Asn-21 plays a dictating role in IAPP self-assembly and is involved in specific intra- or inter-molecular interactions controlling nucleation and/or elongation events, which is not the case for Asn-22.

While the causative link between islet amyloids and type II diabetes was initially described over 100 years ago (3), the conformational nature of the proteotoxic species remains ambiguous. Whereas most studies have reported that soluble oligomers populating the lag phase are causing β-cells death (10,12,54), some earlier and current researches have proposed that amyloid fibrils could also be cytotoxic (8,13). Considering that Asn-21 mutagenesis modulates the kinetics of self-assembly and the final supramolecular morphology, we evaluated the toxicity of freshly dissolved IAPPs (monomers/oligomers) and of assemblies generated after different aging periods. We observed a concentration-dependent β-cells toxicity for most N21X derivatives. Interestingly, although N21F and N21L did not self-assemble into amyloids, both mutants showed a high toxicity. In sharp contrast, N21G was non-toxic, although this mutant is prone to form amyloid fibrils. These results highlight that the relation between amyloidogenicity and cytotoxicity cannot be rationalized according to in vitro biophysical studies performed in homogenous solution. Moreover, the non-toxic rIAPP and N21G mutant readily oligomerized, indicating that not all oligomers are cytotoxic and that specific structural and/or physicochemical properties are determining factors for cellular toxicity (12,55). Interestingly, the non-toxic mutant N21G induced low leakage of anionic LUVs, comparable to the rIAPP. This suggests the existence of a correlation between cytotoxicity and in vitro membrane disruption. However, this observation should be taken with precaution, as it has been reported that there is
no direct correlation between cell toxicity and the ability to disrupt model membranes (56). Besides, it has been shown using the N-terminal 1-19 fragment that membrane disruption can occur independently from amyloid formation (57) and that the capacity of hIAPP_1-19 to perturb membrane is associated with a specific transmembrane orientation of the peptide (58). Thus, differences between the membrane-induced conformation of the WT and the N21G variant can be related to the observed divergence effect on lipid membrane integrity.

Amyloid fibrils assembled from WT IAPP, N21P and N21n were poorly toxic whereas the N21F analog formed toxic worm-like aggregates, which induce disruption of anionic lipid membranes. N21F cytotoxic assemblies are ThT-negative, display a high β-sheet content and have solvent-exposed hydrophobic patches. This is in agreement with previous studies stipulating that the toxicity of protein aggregates is associated with their surface hydrophobicity (59,60). However, the structural and physico-chemical properties of N21F aggregates diverge substantially from those of the toxic IAPP intermediates reported by Abedini and colleagues, which lack extensive β-sheet structure and do not have persistent hydrophobic patches (12). Thus, it appears that multiple IAPP quaternary species could contribute to β-cell death, perhaps by using different upstream mechanisms. Consequently, it remains challenging to determine the specific conformational features of cytotoxic pre-fibrillar and/or amyloid assemblies.

Overall, the present work indicates that specific intra and inter-molecular interactions involving Asn-21 are governing nuclei formation and protofilament elongation. Asn-21 could promote IAPP primary nucleation by facilitating the conformational conversion of the FGAIL β-sheet intermediate into poorly toxic fibrillar quaternary structure. This study identifies position 21 as a hinge residue that controls both the amyloidogenicity and the cytotoxicity of IAPP, ultimately supporting the rational development of therapeutic strategies to arrest aggregation and IAPP-induced pancreatic β-cell loss.

### Experimental procedures

#### Peptide synthesis and purification -

Peptides were synthesized on solid support using Fmoc-chemistry. Oxazolidine dipeptide derivatives were used to facilitate synthesis (61). Peptides were cleaved from the Rink Amide AM resin with a mixture of trifluoroacetic acid (TFA), ethanediithiol, phenol and water. Crude peptides were precipitated with ethyl ether, solubilized in water and lyophilized. Peptides were dissolved in 100% acetic acid and diluted to 35% to be purified by a reverse-phase high performance liquid chromatography (RP-HPLC) on a C18 column. Fractions were analyzed by time-of-flight mass spectroscopy using a LC/ESI-TOF (Table S1, Fig. S14). Peptides were cyclized with 100% dimethyl sulfoxide (DMSO) overnight and then diluted to 20% to be purified by RP-HPLC a second time. Fractions with purity higher than 95% were pooled and lyophilized (Fig. S15-S17).

#### IAPP monomerization and sample preparation -

Aliquots of monomerized IAPP were prepared by dissolving the lyophilized and pure peptide in 100% hexafluoro-2-propanol (HFIP) to a concentration of 1 mg/mL. The solution was filtered through a 0.22 µm hydrophilic PVDF filter and sonicated for 20 min before being lyophilized. The resulting peptide powder was solubilized for a second time in HFIP to a concentration of 1 mg/ml, sonicated for 20 min and the solution was aliquoted and lyophilized again. Monomerized IAPP samples were kept dried at -80°C until used, but not for longer than 4 weeks.

#### Kinetics of amyloid formation by ThT fluorescence -

Solutions were prepared by dissolving the lyophilized peptide at a concentration of 50 µM in 20 mM Tris-HCl, pH 7.4. Assays were performed at 25°C without stirring in sealed black-well clear-bottom 96-well non-binding surface plates with 100 µL per well. Final peptide concentrations were 12.5 and 25 µM and ThT concentration was 40 µM. LUVs were added, or not, at a final concentration of 500 µM. Fluorescence, excitation at 440 nm and emission at 485 nm, was measured every 10 min. Data obtained from triplicate wells were averaged, corrected
by subtracting the corresponding control reaction and plotted as fluorescence vs time. Data of time-dependence of ThT fluorescence were fitted to a sigmoidal Boltzmann model where $T_{50}$ is the time required to reach half of the fluorescence intensity, $k$ is the apparent first-order constant and $Y_{\text{max}}$ and $Y_0$ are, respectively, the maximum and initial fluorescence values:

$$Y = \frac{Y_0 + (Y_{\text{max}} - Y_0)}{1 + e^{-(T - T_{50})/k}}$$

The lag time is described as $T_{50} - 2/k$. Data (lag time and final ThT) of at least four different lots of peptides were averaged and expressed as the mean ± S.D.. Evaluation of the results was made using the Student’s t-test and statistical difference (between WT and mutants) was established at $P < 0.01$.

**Circular dichroism spectroscopy** - Lyophilized aliquots of peptide were dissolved in 20 mM Tris-HCl, pH 7.4 at 50 µM and were incubated at room temperature without agitation. Samples were incorporated into a 1 mm path length quartz cell. Far-UV CD spectra were recorded from 190 to 260 nm using a Jasco J-815 CD spectropolarimeter at 25°C. The wavelength step was set at 0.5 nm with an average time of 10 s per scan at each wavelength step. Each collected spectrum was background subtracted with peptide-free buffer. The raw data was converted to mean residue ellipticity (MRE).

**Time-point fluorescence spectroscopy (ThT and ANS)** - Solutions were prepared by dissolving the peptides at a concentration of 50 µM in 20 mM Tris-HCl, pH 7.4 and were incubated at room temperature without agitation. At the indicated time, peptide were diluted to a final concentration of 25 µM, in presence of a final concentration of 40 µM ThT or 50 µM ANS. Fluorescence was measured in a ultra-micro 10 mm length cells using a PTI Quantamaster spectrofluorometer. For ThT, excitation wavelength was set at 440 nm and the emission spectrum from 450 nm to 550 nm was recorded. For ANS, the excitation wavelength was set at 355 nm and the emission scan was recorded from 385 nm to 585 nm. For each experiment, control reactions (without IAPP) were carried. Data obtained from at least three experiments were averaged.

**Transmission electron microscopy** - Peptide samples were solubilized in a 20 mM Tris-HCl buffer, pH 7.4 to a final concentration of 50 or 150 µM (for time-resolved toxicity assays) and incubated at room temperature under fully quiescent conditions. Samples were diluted to 10 µM before being applied on a glow-discharged carbon films on 300 mesh copper grids. Peptides were adsorbed and were negatively stained with 1.5% uranyl formate for 45 sec. Images were recorded using a FEI Tecnai 12 BioTwin microscope operating at 120 kV and equipped with an AMT XR80C CCD camera system.

**Atomic force microscopy** - Peptides were solubilized in a 20 mM Tris- HCl buffer, pH 7.4 to a concentration of 50 µM and incubated at room temperature without agitation. After the indicated time of incubation, the peptide is diluted in 1% acetic acid and immediately applied to freshly cleaved mica. The mica was washed twice with deionized water and air-dried for 24 h. Samples were analyzed using a Veeco/Bruker Multimode AFM using tapping mode with a silicon tip (2-12 nm tip radius, 0.4 N/m force constant) on a nitride lever. Images were taken at 0.5 Hz and 1024 scan/min.

**Photo-induced cross-linking** - Peptides were incubated at 50 µM in 20 mM Tris-HCl, pH 7.4, at 25 °C for the indicated periods. Cross-linking solution was added to a final concentration of 70 µM Tris- (bipyridyl)Ru(II) and 1.4 mM ammonium persulfate. Aliquots were illuminated with a 150 W incandescent bulb for 5 sec. Reaction was quenched by the addition of 1 M dithiothreitol (DTT). Prefibrillar species were separated by SDS-PAGE electrophoresis using a 15% Tris-tricine gel and visualized by silver staining.

**Cell viability assay** - Rat INS-1 cells were cultured in black wall clear bottom 96-well plates (tissue culture treated) at a density of 20 000 cells/well (100 µl/well) in RPMI-1640 medium supplemented with 10% FBS, 100
U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol. After 48 h incubation at 37°C in a 5% CO₂, cells were treated by the direct addition of peptide solutions (20 mM Tris-HCl, pH 7.4) at 3× final concentration. Cells were incubated another 24 h and cellular viability was measured using the resazurin reduction assay. Cell viability (in %) was calculated from the ratio of the fluorescence of the treated sample to the control (vehicle treated). Data of at least four assays (with different peptide lots) were averaged and were expressed as the mean ± S.D.. Statistical analysis was performed with Prism 6.0 software using the Student’s t-test and statistical difference (between WT and mutants) was established at P < 0.01.

Caspase-3 activity assay - Rat INS-1 β-cells were cultured in 12-well plates (tissue culture treated) at a density of 400 000 cells/well in supplemented RPMI-1640 medium. After 48 h incubation, cells were treated by the direct addition of peptide solution (50 µM) for 3 h. Cells were lysed on ice for 30 min followed by a centrifugation at 16 000g for 20 min. Protein extracts in supernatant were measured by caspase-3 colorimetric assay (Sigma-Aldrich). Commercial caspase-3 enzyme provided with the kit was used as a positive control. Caspase-3 activity after 2 h reaction was evaluated at 37°C by measuring absorbance at 405 nm.

Large unilamellar vesicle preparation and membrane leakage - DOPC and DOPG lipids were solubilized in 100% chloroform at a ratio of 7:3 and solvent was evaporated. The lipid film was hydrated in Tris-HCl 20 mM pH 7.4 buffer containing 70 mM of calcein, or not, for at 30 min. Solubility of calcein was increase with the dropwise addition of NaOH. The solution was freeze-thawed 5 times and lipids were extruded through a 0.1 µm nuclopor membrane. Nonencapsulated calcein was removed using a 10 mL Sephadex G-25 column. Lipid concentration was determined using an inorganic phosphate detection colorimetric assay. The size and homogeneity of LUVs were analysed by dynamic light scattering and an average diameter of 100 nm was obtained. For membrane leakage, 50 µM of peptide was used and LUVs concentration was fixed at 500 µM. The excitation wavelength was set at 495 nm and the emission scan was recorded from 500 nm to 540 nm in an ultramicro cell. Fluorescence was measured over incubation time. The control used to determine 100% leakage (F_max) was calcein-LUVs with 0.2% Triton X-100. Dye leakage was reported using the following equation:

\[ \%\text{ of leakage} = \frac{(F - F_{\text{baseline}})}{(F_{\text{max}} - F_{\text{baseline}})} \]

Author contributions: EG, PTN and XZ conducted the experiments and analyzed the results. EG, PTN and SB wrote the paper and prepared the figures. SB supervised the project.

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Figure 1. IAPP sequence and structural model of IAPP protofilament. (A) Primary sequence of human IAPP with Asn-21 residue in bold red and the amyloidogenic core in blue. IAPP has a disulfide between Cys-2 and Cys-7 and a C-α-amidated C-terminus. The two β-sheets inferred from the solid-state NMR amyloid fibril model are indicated with gray arrows. (B) Solid-state NMR model of IAPP protofilament with the Asn-21 side chain indicated (19).
Figure 2. Effect of Asn-21 substitutions on the kinetics of IAPP amyloid formation. (A) Representative kinetic traces of amyloid assembly of IAPP and mutants monitored by ThT fluorescence. Peptides were incubated at 12.5 µM under quiescent conditions in 20 mM Tris-HCl buffer, pH 7.4 and the fluorescence of ThT (40 µM) was measured every 10 min with excitation at 440 nm and emission at 485 nm. The curves represent the best from the sigmoidal growth model. (B) Lag time, (C) slope and (D) final ThT fluorescence of the self-assembly kinetics. Data represent mean ± S.E.M. of at least four experiments performed in triplicate. Results were analyzed using the student’s t-test and statistical difference (between IAPP and analogs) was established at * P < 0.01. (B-D) Data are not shown for N21F and N21L because results could not be fitted to a sigmoidal curve. (A-D) n: D-enantiomer of Asn; Aib: aminoisobutyric acid; Dab: diaminobutyric acid.
Figure 3. Effect of Asn-21 substitutions on the conformational transition of IAPP. Peptides were incubated under quiescent conditions at 20 mM Tris-HCl buffer, pH 7.4 at 50 µM. CD analysis was performed after incubation of 0, 2, 4, 6, 24 and 48h. Far-UV CD spectra were recorded from 190 to 260 nm.
Figure 4. Effect of Asn-21 substitutions on surface hydrophobicity of IAPP assemblies. Peptides were incubated under quiescent conditions at 50 µM. After 0, 24 and 48 h incubation, the emission spectra of ANS was recorded from 385 nm to 585 nm with an excitation set at 355 nm. Data presented are the most representative spectra of at least three experiments performed in triplicate.
Figure 5. Effect of Asn-21 substitutions on the morphology of IAPP assemblies. Peptides were incubated in 20 mM Tris-HCl buffer, pH 7.4 for 24 h at 50 µM before TEM imaging. Scale bars: 100 nm.
**Figure 6. Effect of Asn-21 substitutions on IAPP cytotoxicity.** INS-1E cells were treated with increasing concentration of freshly dissolved peptides for 24 h. Cell viability was measured using the resazurin reduction assay and compared to that of cells treated with vehicle. Data represent mean ± S.E.M. of at least four experiments performed in triplicate. Results were analyzed using the student’s t-test and statistical difference (between IAPP and mutants) was established at * P < 0.01.
Figure 7. Effect of Asn-21 mutagenesis on membrane-induced amyloid formation and lipid vesicle disruption. (A) Representative kinetic traces of amyloid assembly in presence of LUVs (DOPC:DOPG 7:3) monitored by ThT (40 μM) fluorescence. Peptides (12.5 μM) and LUVs (500 μM) were incubated under quiescent conditions in 20 mM Tris-HCl, pH 7.4. (B) Membrane leakage of 500 μM DOPC:DOPG LUVs (7:3) by 50 μM peptides.
Figure 8. Effect of Asn-21 substitutions on cytotoxicity of IAPP proteospecies. (A,C) INS-1E cells were treated for 24 h with 50 µM of pre-incubated peptides (0 to 120 h) and (A) cell viability and (C) caspase-3 activation was evaluated. (B) TEM images of peptides incubated for 48 h under quiescent conditions at 150 µM. Scale bars: 100 nm. (D) Membrane leakage of 500 µM DOPC:DOPG LUVs (7:3) by 50 µM IAPP proteospecies (pre-incubated for 48 h. at 150 µM). (A,C) Data represent mean ± S.E.M. of at least three experiments performed in triplicate. Results were analyzed using the student’s t-test and statistical difference (between IAPP and mutant) was established at * P < 0.01.
Identification of a hinge residue controlling islet amyloid polypeptide self-assembly and cytotoxicity
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