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Identification of minimal peptide sequences in the (8–20) domain of human islet amyloid polypeptide involved in fibrillogenesis

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Abstract

We have examined a series of overlapping peptide fragments from the 8–20 region of human islet amyloid polypeptide (IAPP) with the objective of defining the smallest fibril-forming domain. Peptide fragments corresponding to LANFLV (residues 12–17) and FLVHSS (residues 15–20) were strong enhancers of β-sheet transition and fibril formation. Negative stain electron microscopy illustrated the ability of these peptide fragments to form fibrils independently when incubated alone in solution. Circular dichroism analysis revealed that when full-length human IAPP was incubated in the presence of these two fragments, fibrillogenesis was accelerated. While the two fragments, LANFLV and FLVHSS, were able to enhance the recruitment of additional IAPP molecules during fibril formation, the "seeding" activity of these peptides had no effect on altering IAPP-induced cytotoxcity as determined by cell culture studies. Therefore, this study has identified two internal IAPP peptide fragments within the 8–20 domain that may have a role in enhancing the folding and aggregation of human IAPP. These fragments are the smallest sequences identified, within the 8–20 region of hIAPP, that can independently form fibrils, and that can interact with IAPP to assemble into fibrils with characteristics similar as those formed by human IAPP alone.

Keywords: Islet amyloid polypeptide (IAPP); Diabetes; Amyloid fibrils; Circular dichroism; Electron microscopy; Cytotoxicity

1. Introduction

Amyloid fibril formation is a hallmark of a variety of human disorders such as Alzheimer's disease and type 2 diabetes (Selkoe, 1997; Kahn et al., 1999). Each of these diseases is characterized by the deposition of different proteins such as amyloid-β (Aβ in Alzheimer's disease and islet amyloid polypeptide (IAPP) or amylin in type 2 diabetes. While the primary sequences of these proteins differ, the proteins exhibit similar structural characteristics. IAPP is a 37-amino-acid peptide synthesized in the pancreas and colocalized with insulin within the dense core secretory granules of the β cells (Clark et al., 1989; Lukinius et al., 1989). Human IAPP (hIAPP) has been identified as a major fibrillar component of pancreatic amyloid, a characteristic histopathological marker in more than 90% of all cases of type 2 diabetes (Westermark et al., 1987; Westermark, 1994). Several groups have focused their attention on the different domains contained within the IAPP sequence and their possible contributions to amyloid formation (Goldsbury et al., 2000; Nilsson and Raleigh, 1999; Jaikaran et al., 2001; Tenidis et al., 2000).

Studies have demonstrated that the N-terminal amino acids (residues 1–7) of human IAPP 1–37 do not affect fibril formation or overall structure, but they influence the kinetics of fibrillogenesis (Goldsbury et al., 2000). An amyloid-forming fragment of human IAPP spanning residues 20–29 was identified as important for fibril formation based on species-specific proline substitutions in rodent IAPP. These substitu-

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tions create β-sheet breakers that prevent folding and aggregation (Moriarty and Raleigh, 1999; Glenner et al., 1988; Westermark et al., 1990). In addition, it has been suggested that the phenylalanine residue at residue 23 may be important for IAPP amyloid formation (Azriel and Gazit, 2001). While the 20–29 domain can independently assemble into fibrillar structures displaying a β-sheet conformation (Glenner et al., 1988; Westermark et al., 1990), the morphology of these fibrils differs from that of full-length IAPP (Goldsbury et al., 2000). These data are supported by studies that suggest that while certain fragments of IAPP may be amyloidogenic, they are not amyloidogenic in the context of the primary sequence. Therefore, to form fibrils, contacts may include interactions with residues found along the entire length of IAPP, and analysis of fragments should be complemented with studies on the full-length peptide (Padrick and Miranker, 2001).

While the IAPP 20–29 domain has been proposed to be the critical amyloidogenic component of the IAPP molecule, additional amyloidogenic regions within IAPP have been identified. The C-terminal domain spanning residues 30–37 has been shown to form amyloid fibrils in solution (Nilsson and Raleigh, 1999). The primary sequences are less divergent in this region and both human and rodent IAPP 30–37 can form amyloid-like fibrils in aqueous media (Nilsson and Raleigh, 1999). In addition, a recent study has demonstrated the presence of an amyloidogenic domain contained within another conserved sequence within residues 8–20 (Jaikaran et al., 2001). This fragment has been shown to also assemble into β-sheet fibrils that are morphologically similar to in vivo amyloid (Jaikaran et al., 2001). The identification of additional amyloidogenic domains within the entire IAPP molecule lends support to the idea that while the 20–29 domain is essential for IAPP fibril formation and activity, there may be additional interactions that affect the morphology of fibrils and/or the kinetics of fibril formation.

In the present study, we have examined fragments to determine whether specific residues or domains within the 8–20 region are involved in fibril formation. The objective of our study is to determine if these fragments are capable of interacting with full-length IAPP and modulating the aggregation pathway. This approach has proven to be effective in the development of inhibitory agents for the amyloid-β (Aβ) peptide associated with Alzheimer's disease (Tjernberg et al., 1997) as well as for IAPP (Scrocchi et al., 2002; Kapurniotu et al., 2002). The results presented here identify two peptide fragments within the 8–20 domain that can independently form amyloid fibrils in solution as well as accelerate fibrillogenesis of full-length IAPP.

2. Experimental procedures

2.1. Peptide synthesis and supply

Synthetic human IAPP 1–37 was purchased from BACHEM (Torrance, CA, USA), synthetic peptides spanning the region from IAPP (10–20) were synthesized by the Peptide Synthesis Laboratory at the University of Toronto using standard FMOC-based solid-phase peptide synthesis methodology. The peptides were purified by reverse-phase HPLC using water/acetonitrile mixtures buffered with 0.1% trifluoroacetic acid, on a POROS 20R2 column. The synthetic peptides were solubilized in 100% HFIP (1 mg/ml), filtered (0.2 μm), aliquoted, and lyophilized (Higham et al., 2000). Aliquots were reconstituted as required in 20 mM sodium acetate buffer, pH 6.5.

2.2. Circular dichroism spectroscopy (CD)

CD spectroscopy was used to measure the peptide conformational changes associated with fibril formation. Experiments were carried out at room temperature and spectra (average of five scans) were collected using the Jasco J720 spectropolarimeter and quartz cuvettes with a path length of 1 mm. CD spectroscopic traces for each hexapeptide alone were measured in tandem with those of IAPP alone and IAPP plus hexapeptides. Spectra were corrected by substraction of the buffer only and/or the absorbance from the individual hexapeptides in buffer to give a measure of the contribution from full-length IAPP alone.

2.3. Transmission electron microscopy (TEM)

Negative stain electron microscopy was used to visualize the relative density and morphology of the fibrillar structures that were obtained. Aliquots (4 μl) were applied to pioloform and carbon-coated grids and incubated for 3 min at room temperature. Excess solution was removed by blotting with filter paper and the samples were stained with 1% phosphotungstic acid (PTA, pH 7). Peptide aggregates were examined using a Hitachi 7000 electron microscope with an accelerating voltage of 75 kV.

2.4. Peptide co-incubation and seeding

For co-incubation experiments, aliquots of lyophilized IAPP 1–37 were prepared in 20 mM acetate buffer (pH 6.5) at a concentration of 10 μM (total volume 500 μl). Hexapeptides were added to IAPP in a 5- or 10-fold molar excess for a final concentration of 10 μM IAPP/50 or 100 μM peptide. Samples were incubated at room temperature for a period of 48 h.

For seeding experiments, aliquots of lyophilized IAPP 1–37 were prepared in 600 μl of 20 mM acetate

buffer (pH 6.5) at a concentration of 100 μM. Each individual peptide fragment was prepared as a 1 mM solution and diluted 10 fold into the IAPP solution for a final concentration of 100 μM IAPP/100 μM peptide. Samples were incubated at room temperature for a period of 24 to 30 h.

2.5. IAPP toxicity assay

RIN-1056A cells were plated into 96-well plates at a density of 5×10^3 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL) supplemented with 5% fetal calf serum (Gibco-BRL), 100 U/ml penicillin (Gibco-BRL), and 100 U/ml streptomycin (Gibco-BRL). Fresh medium containing 10 μM IAPP alone or in combination with 200 μM LANFLV or FLVHSS was added to each well. Untreated control cells received fresh medium only. AlamarBlue (MEDICORP, Montreal) was added directly, and the cells were incubated for a period of 15 min after which fluorescence was measured (time 0). The cells were then incubated at 37 °C, 5% CO_2, for a period of 6 days during which the fluorescence was measured once daily. Fluorescence was measured using the SpectraMAX Gemini XS Microplate spectrofluorometer (Molecular Devices) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Data were collected using the SoftMax Pro 3.1 software for MacIntosh.

3. Results

Structural predictions performed on the human IAPP molecule have suggested the presence of a region contained within residues 10–20 that is capable of forming β-sheet fibrils (Jaikaran et al., 2001). To define the specific residues that are involved in the process, a series of hexapeptides were generated to span the region of hIAPP from residues 8 to 20 (Fig. 1). Each peptide was incubated with full-length IAPP for 72 h and assayed using circular dichroism (CD) spectroscopy and negative stain electron microscopy. CD spectra were measured on samples containing IAPP alone or IAPP plus hexapeptides and on hexapeptides alone in buffer. The spectra of individual hexapeptides were subtracted from the raw data so that the contribution by full-length IAPP alone was obtained. The hexapeptide fragments were synthesized with free N and C termini that may affect their properties. This complication is typical of any peptide fragment and may in some circumstances alter fibril formation. However, while this is a possibility, the aggregation of IAPP is driven by intersheet interactions and is dominated by side chains rather than termini.

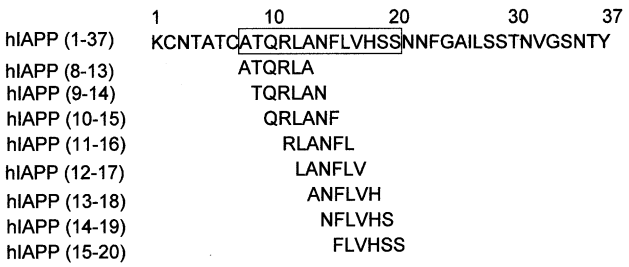

Fig. 1. Islet amyloid polypeptide fragments. Primary sequence of full-length human IAPP (residues 1–37) indicating the proposed amyloidogenic domain spanning residues 8–20 (boxed region). The series of eight overlapping hexapeptides encompassing residues 8–20 are indicated.

When dissolved in acetate buffer, full-length IAPP 1–37 as well as many other amyloid proteins, exhibits a transition from a native or unstructured conformation to a β-sheet (Goldsbury et al., 2000; Kayed et al., 1999). In CD spectra, this is seen as a shift in diagnostic absorption from approximately 205 to 218 nm. Initial studies using CD spectroscopy demonstrated that peptides spanning the regions from IAPP 8–13 to IAPP 11–16 had no effect on IAPP fibril formation (data not shown). These results may be due to the presence of the arginine residue at position 11 in each of these peptides (Fig. 1). It has been previously reported that the presence of an arginine in an amyloid sequence inhibits β-sheet and amyloid fibril formation (Blanchard et al., 1997). An accelerated transition from random coil to β-sheet was observed when IAPP was incubated with the peptide LANFLV (Fig. 2A). After 48 h of incubation, IAPP, in the absence of this peptide, remained as a random coil. However, the presence of a 5-fold molar excess of peptide LANFLV stimulated the conversion of IAPP to a β-sheet conformation, and in the presence of a 10-fold excess of the peptide the conversion to a β-sheet conformation was plainly observed (Fig. 2A). Incubation of IAPP with a 10-fold excess of peptide FLVHSS produced an even greater acceleration in the transition from random coil to β-sheet. The conformational change occurred rapidly after only 24 h of incubation (Fig. 2B). However, a 5-fold excess of this peptide had no effect on the conformation of IAPP in solution. In contrast, incubation with either a 5- or 10-fold excess of the intervening peptide ANFLVH or NFLVHS did not alter IAPP fibril formation, and the peptides remained in a random coil conformation for the entire incubation period (Fig. 2C).

Morphological analysis of the resulting aggregates revealed that IAPP fibrils formed in the presence of peptide LANFLV were aligned laterally in ribbon-like structures with many fibrils originating from a single origin (Fig. 3A). In addition these fibrils were slightly narrower (~5 nm) than those produced by IAPP alone which are normally in the range of 6–9 nm in width (Fig. 3D). The fibrils formed by incubating IAPP with peptide FLVHSS (Fig. 3B) shared a much greater homology

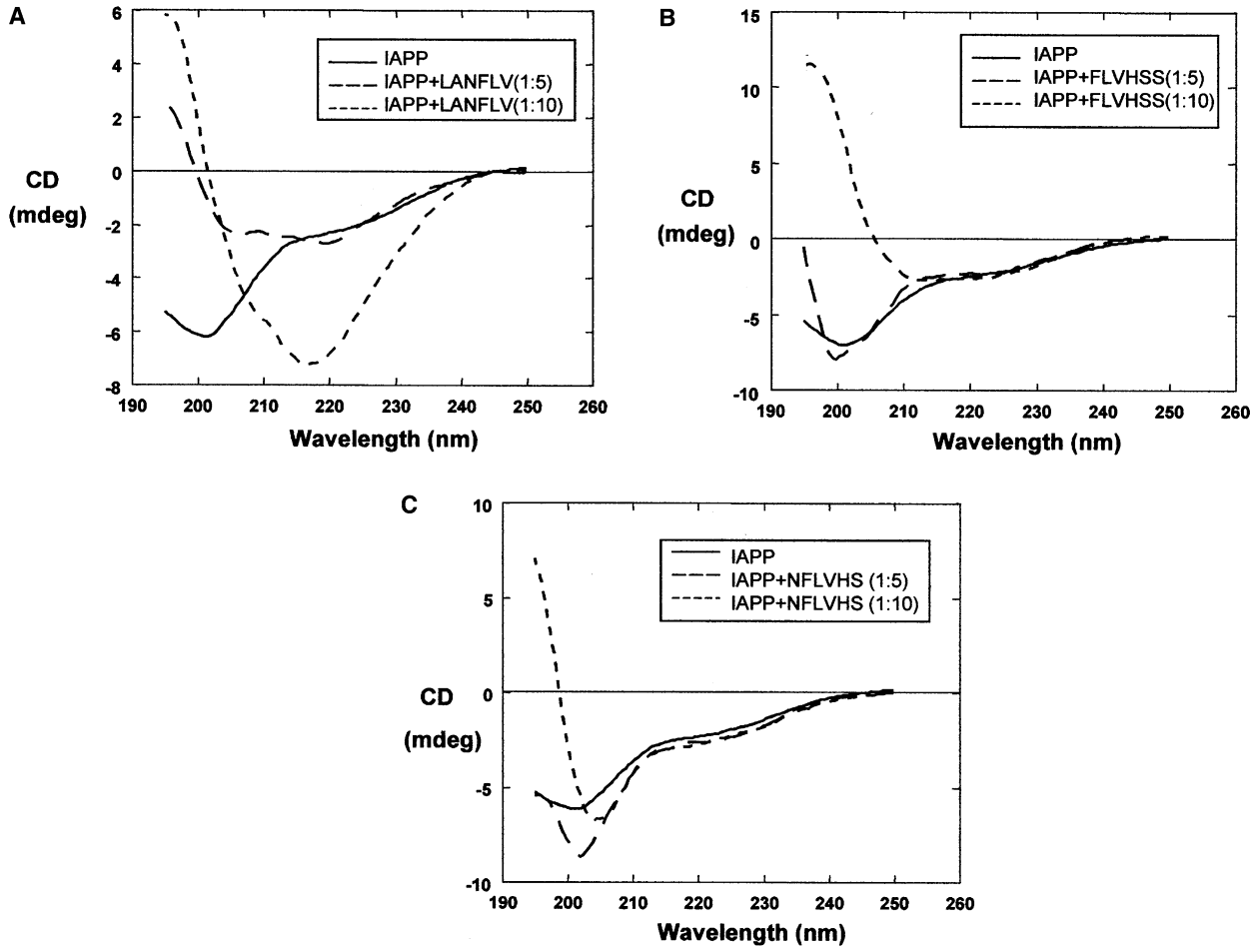

Fig. 2. Conformation of IAPP following co-incubation with individual hexapeptide molecules. Full-length human IAPP incubated in 20 mM acetate buffer (pH 6.5) remained in a random coil conformation for a period of 48 h (solid lines). Co-incubation of human IAPP 1–37 with the hexapeptide LANFLV at a molar ratio of 1:5 (dashed line) or 1:10 (dotted line) stimulated the conformational change in IAPP from a random coil to a β-sheet. This effect was observed after 48 h of incubation (A). Co-incubation of IAPP with a 10-fold excess (dotted line) of hexapeptide FLVHSS produced a conformational change in IAPP to a β-sheet after 24 h of incubation. A 5-fold excess of peptide FLVHSS had no effect on altering the conformation of IAPP (B). Incubation of IAPP with a 5-fold (dashed line) or 10-fold (dotted line) excess of NFLVHS had little effect on the ability of IAPP 1–37 to form a β-sheet following a 48-h of incubation, suggesting that this fragment does not alter the folding or aggregation of IAPP (C).

with those produced by IAPP alone. The fibrils formed a dense mesh of fibrils that were of a similar width (~6 nm) and morphological appearance as those formed by IAPP alone. In contrast, fibrils formed following the incubation of IAPP with peptide NFLVHS (Fig. 3C) were sparse and had a "helical" twist (Fig. 3C, inset), and were unlike those normally produced by IAPP alone. Unlike peptides LANFLV and FLVHSS the intervening hexapeptide NFLVHS does not enhance fibril formation, and the presence of this peptide appears to reduce the number of fibrils formed as well as to alter the morphology of the fibrils that did form. These data suggest that not all peptides from the IAPP 8–20 domain accelerate fibrillogenesis, and that only specific residues play a role in fibril formation.

The accelerated transition of IAPP from a peptide with a random coil to a β-sheet conformation in the presence of peptides LANFLV and FLVHSS is similar to that reported previously for the IAPP 20–29 domain and the minimal fibril forming peptide having the sequence, NFGAIL. Based on our results demonstrating that peptides LANFLV and FLVHSS from the IAPP 8–20 domain also accelerate IAPP fibril formation, we examined whether the accelerated fibril formation was due to the presence of fibrils formed by these two IAPP fragments acting as "seeds" or initiators of additional fibrillogenesis. Each peptide was incubated alone in acetate buffer for 72 h and examined by negative stain electron microscopy. Following this incubation period, we observed the presence of fibrils produced by both of these IAPP fragments. The fibrils generated by the peptide LANFLV appeared to extend in an outward direction from an origin of aggregated material (Fig. 4A, arrows). These conclusions were not based on time lapse observations of fibrillogenesis, but rather from static images following incubation. The fibrils were

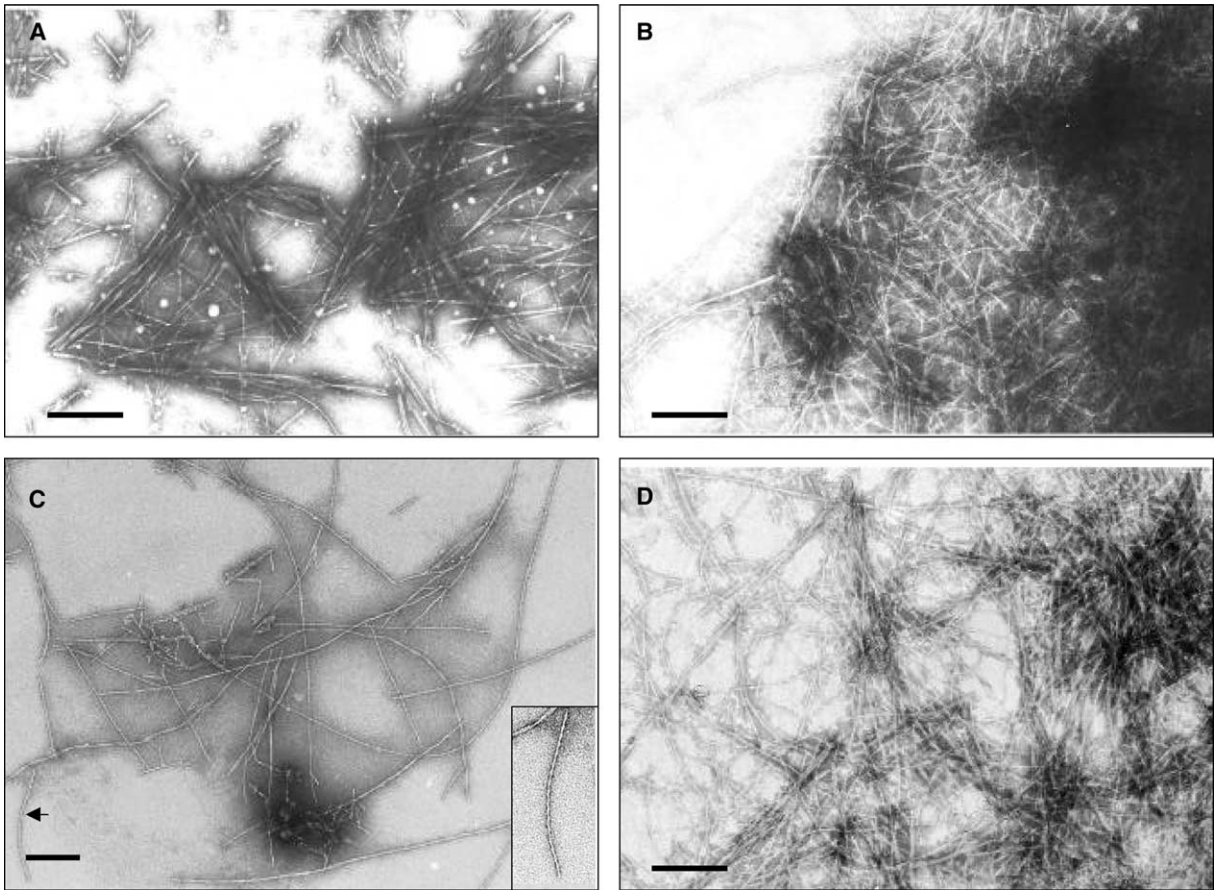

Fig. 3. Morphology of IAPP fibrils following co-incubation. Electron micrographs of IAPP fibrils following a 24- or 48-h incubation with peptides. IAPP co-incubated with peptide LANFLV formed rod-like laterally aligned fibrils. The fibrils formed were short and ribbon-like compared with those produced by IAPP alone (A). Fibrils formed following co-incubation of IAPP with FLVHSS (10-fold molar excess) were similar in morphology to those formed by IAPP alone and exhibited a dense mass of interwoven fibrils (B). The peptide NFLVHS caused disruption of IAPP fibrillar structure (C) and altered morphology to induce twisting in some fibrils (arrowhead, inset). Full-length IAPP 1–37 fibrils showing typical morphology and formation of dense aggregates (D). Bars represent 100 nm.

aligned laterally and extended in mass from several foci. The diameter of these fibrils was within the normal range for IAPP (6–9 nm); however, the fibrils were short with a rod-like appearance unlike those normally produced by full-length IAPP. Fibrils produced by peptide FLVHSS alone were twisted in appearance with a regular pitch of 45 nm (Fig. 4B,inset). With the exception of the twisted morphology, the fibrils did exhibit normal width (~9 nm) and appearance as compared with typical IAPP fibrils.

Subsequent ''seeding'' experiments were performed to examine the accelerated fibril formation of IAPP in the presence of peptides LANFLV and FLVHSS and the amyloidogenic core sequence NFGAIL. It was of interest to determine whether the fibrils formed by these fragments could act as ''seeds'' for fibrillar aggregation of the full-length IAPP molecule. IAPP fibril formation has been shown to be a time- and concentration-dependent event (Rhoades et al., 2000). The kinetics of IAPP fibril formation have demonstrated that a lag time exists that can be shortened or eliminated by adding a low concentration of preformed fibrils or by increasing the concentration of the peptides involved (Padrick and Miranker, 2001). We examined whether the addition of hexpeptides to a concentrated solution of IAPP would accelerate fibrillogenesis. Each hexapeptide was prepared as a concentrated stock solution (1 mM). Full-length IAPP was prepared at a concentration of 100 μM in acetate buffer (pH 6.5), and each peptide was added to the IAPP for a final concentration of 100 μM IAPP plus 100 μM peptide. Samples were analyzed using CD spectroscopy every 2 h for a period of 6 h, followed by a final measurement at the end of 24 h of incubation. Under these conditions IAPP alone remained in a random coil conformation for the first 6 h of incubation and began to show a shift to a β-sheet conformation after 24 h of incubation (Fig. 5D).

When the IAPP solution was seeded with peptide LANFLV it remained in a random coil conformation for the first 4 h of incubation (Fig. 5A). After 6 h of

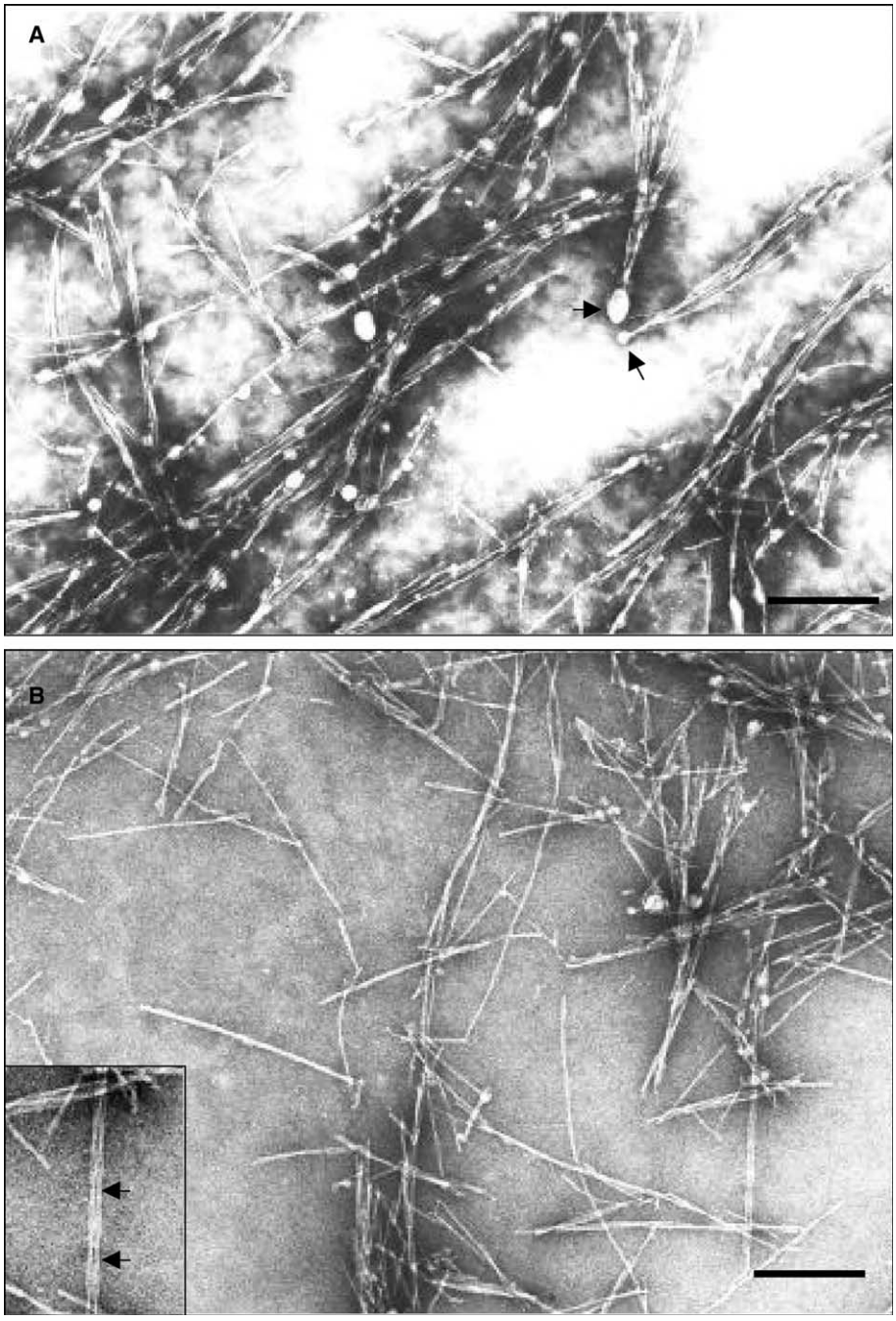

Fig. 4. Morphology of fibrils formed by individual hexapeptides. Fibrils produced by peptide LANFLV were rod-like and were laterally aligned. The fibrils were observed to extend outward from foci of aggregated material (arrows) (A). Fibrils produced by peptide FLVHSS alone were also rod-like. In addition, the fibrils were twisted with turns occurring in regular intervals along the length of the fibrils (arrows) (B). Bars 100 nm.

incubation, we observed a shift in the CD spectrum to suggest a conformational change, and by 24 h we observed a shift from a random coil conformation to one that contained a mixture of both α-helix and β-sheet conformations (Fig. 5A). Similar results were obtained when IAPP was seeded with the IAPP-amyloid core peptide NFGAIL, however, a conformation shift in the CD spectrum was not observed until 24 h (Fig. 5C). Further incubation of these samples resulted in a conformational transition to β-sheet after a total of 30 h (data not shown). Co-incubation of IAPP with peptide FLVHSS enhanced fibrillogenesis to a much greater degree than that with either LANFLV or NFGAIL. The ability of peptide FLVHSS to accelerate IAPP fibril formation was illustrated when the conformational changes were observed within 6 h of the addition of IAPP to the solution (Fig. 5B). A definite conformational change from random coil to a mixture of α-helix

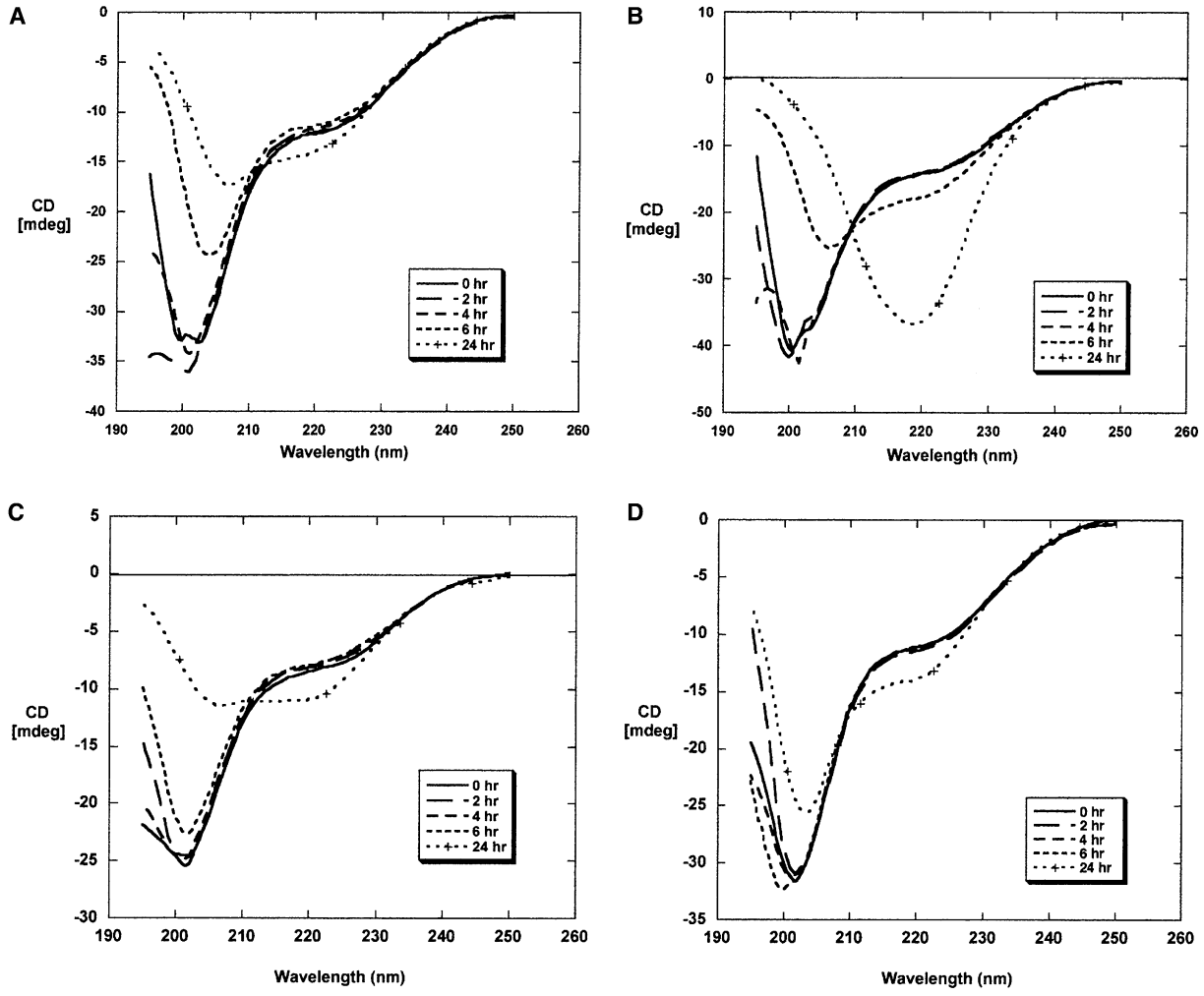

Fig. 5. Seeding IAPP with hexapeptides accelerates fibrillogenesis. CD spectra of IAPP in solution were recorded for the periods indicated following the addition of individual hexapeptides. Peptide LANFLV produced a conformational transition in IAPP within 6 h. By 24 h the solution contained an intermediate mixture of both α-helix and β-sheet peptide (A). The addition of peptide FLVHSS to IAPP resulted in a conformational change by 6 h, and the transition to β-sheet was complete by 24 h (B). Peptide NFGAIL produced no conformational change in IAPP until 24 h of incubation at which time it contained a mixture of α-helix and β-sheet peptide (C). IAPP incubated alone remains as a random coil peptide for the duration of the experiment until 24 h when there was evidence of a conformational transition to a partially folded structure (D).

and β-sheet conformations was observed in as little as 6 h. By 24 h of incubation the sample had fully converted to a β-sheet conformation (Fig. 5B). In addition, the fibrils produced from these "seeding" experiments were very similar in morphological appearance to those produced from our initial co-incubation experiments presented in Fig. 3.

The toxicity of IAPP fibrils on pancreatic β cell lines has been well documented (Lorenzo et al., 1994; Janson et al., 1999; Janciauskiene and Ahren, 1998). Previous experiments have demonstrated that fibrils from the IAPP 20–29 domain are toxic to pancreatic β-cell lines in culture (Kapurniotu et al., 2002). Therefore, we examined whether the fibrils formed by peptides LANFLV and FLVHSS were also toxic. In addition, since our results demonstrate that these peptides accelerate IAPP fibril formation, we examined whether they could also increase the toxicity of the fibrils produced. RIN-1056 cells were incubated for a period of 6 days in the presence of peptide LANFLV or FLVHSS (200 μM) and cell proliferation was measured using an Alamar-Blue bioassay (Fig. 6). We observed that neither of these truncated peptides was directly toxic to pancreatic β cells in culture when incubated alone (Fig. 6A). Conversely, when RIN-1056 cells were incubated for 6 days in the presence of 10 μM IAPP plus peptide LANFLV or FLVHSS, solutions containing the truncated peptides were able to induce a level of cell toxicity that was comparable to that of the full-length IAPP peptide (Fig. 6B). These data suggest that the IAPP fibrils formed in the presence of these peptides have not altered the structure or the activity of the cytotoxic region in terms of the ability of this region to induce the cytotoxic effects of IAPP.

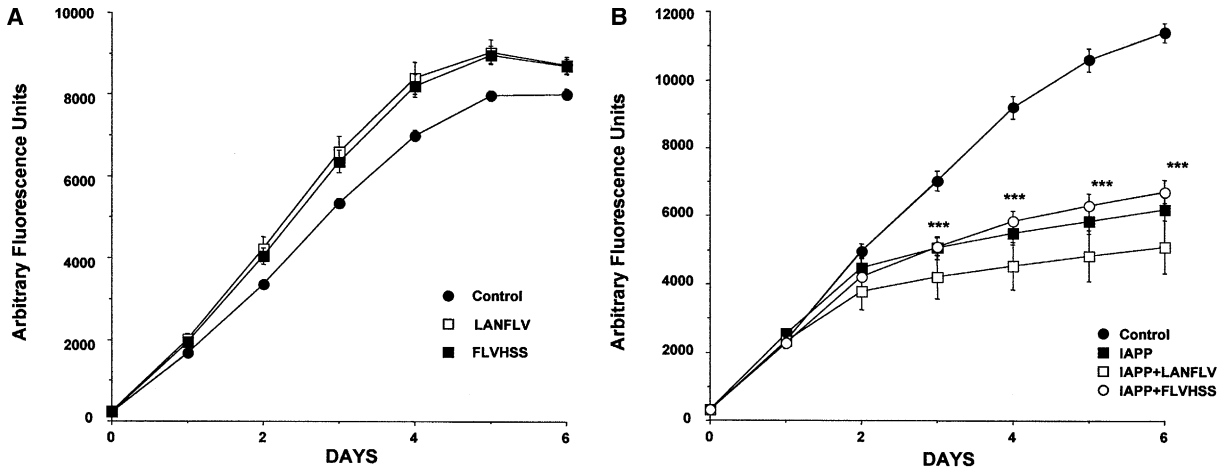

Fig. 6. Assessment of the effect of peptides LANFLV and FLVHSS on IAPP toxicity on β cells in culture. Long term viability of RIN-1056 cells was measured in the presence of added peptides. RIN-1056 cells incubated for a period of 6 days in culture medium with individual peptides, LANFLV or FLVHSS. Control cells were incubated in culture medium alone (A). RIN-1056 cells were incubated with culture medium alone (filled circles), IAPP alone (filled squares), IAPP plus LANFLV (open squares), or IAPP plus FLVHSS (open circles) (B). Values are means ± SEM. (***) Significant difference from control, $P < 0.001$.

4. Discussion

The accumulation of IAPP amyloid deposits in the pancreas is a hallmark of type 2 diabetes mellitus (Kahn et al., 1999). The intrinsic structural characteristics involved in molecular recognition and fibril formation are of great interest. Previous experiments have established that the 20–29 domain of human IAPP is required for fibril formation and cytotoxicity (Tenidis et al., 2000; Scrocchi et al., 2002; Kapurniotu et al., 2002). Sequence analysis has determined that residues 20–29 in human IAPP are highly amyloidogenic (Padrick and Miranker, 2001), and the isolated human IAPP decapeptide (20–29) is capable of forming fibrils (Moriarty and Raleigh, 1999; Glenner et al., 1988; Griffiths et al., 1995). It has been established that the proline substitutions in the 20–29 domain of rodent IAPP are strong β-sheet breakers and prevent fibril formation by full-length rat IAPP (Moriarty and Raleigh, 1999). Experiments have also demonstrated that the small protofibrils formed by this amyloidogenic core 20–29 domain are cytotoxic to pancreatic β cells (Tenidis et al., 2000; Kapurniotu et al., 2002).

More recent studies have suggested that while the NFGAIL domain is necessary for fibril formation it may not be sufficient for amyloidosis (Nilsson and Raleigh, 1999; Jaikaran et al., 2001; Padrick and Miranker, 2001). Recent reports have demonstrated that the IAPP 8–20 domain and the IAPP 30–37 domain are able to form IAPP fibrils in solution (Nilsson and Raleigh, 1999; Jaikaran et al., 2001). It has been suggested from these studies that with the presence of more than one β-strand region, intermolecular interactions as well as intramolecular interactions involved in fibril formation are likely to be more complex than that proposed for the single-strand IAPP 20–29 region (Jaikaran et al., 2001). It has been suggested that the 8–20 domain may play a role in the overall morphology of IAPP fibrils (Jaikaran et al., 2001), and may therefore have a significant role in the amyloidogenic properties of IAPP.

We have identified two fragments within the 8–20 domain of human IAPP, LANFLV (12–17) and FL VHSS (15–20), that are capable of accelerating fibril formation by the full-length IAPP molecule. The accelerated fibrillogenesis in the presence of these peptides is similar to that reported previously for the NFGAIL fragment (Goldsbury et al., 2000; Tenidis et al., 2000; Azriel and Gazit, 2001; Kapurniotu et al., 2002). The ability of the NFGAIL fragment to form fibrils in solution and accelerate IAPP fibrillogenesis suggests that the presence of either full-length or truncated IAPP fibrils may accelerate the formation of additional IAPP fibrils (Scrocchi et al., 2002; Park and Verchere, 2001). The results reported in the present study also support the role of IAPP fragments LANFLV and FLVHSS as additional domains in the IAPP molecule involved in fibrillogenesis. Our studies have demonstrated that the individual IAPP peptides LANFLV and FLVHSS are capable of forming fibrils when incubated alone in solution. The fibrils formed by the FLVHSS peptide are similar in homology to those formed by full-length IAPP; however, those formed by the peptide LANFLV were not morphologically similar to IAPP fibrils. Previous studies have demonstrated that fibrils formed by IAPP fragments do not always share the same morphology with those produced by full-length IAPP (Goldsbury et al., 2000; Jaikaran et al., 2001). These results suggest that interactions with other domains in the entire protein may be necessary for producing fibrils with morphology characteristic of IAPP fibrils (Padrick and Miranker, 2001).

In contrast to fragments from the 20–29 domain which have been shown to be toxic to RIN5mf cells (Tenidis et al., 2000; Kapurniotu et al., 2002), the peptides from the 8–20 region are not directly toxic themselves. Our experiments demonstrated that the fibrils produced by the IAPP fragments LANFLV and FLVHSS alone were not toxic when added to cultures of pancreatic β cells. In addition, these peptides had no effect on modifying the cytotoxic action of full-length hIAPP 1–37 on pancreatic β-cell lines. Fibrils produced from incubating full-length IAPP with these peptides were found to have a toxicity similar to that of fibrils formed by IAPP alone. These results support the hypothesis that the cytotoxic core of IAPP is contained within the 20–29 domain of the peptide, and suggest that the 8–20 domain may be involved more in peptide recognition and fibril formation.

Our results demonstrate that the IAPP fragments LANFLV and FLVHSS have the ability to enhance IAPP fibrillogenesis. In contrast, we have also demonstrated that the intervening peptide sequences ANFLVH and NFLVHS lack this ability. These two peptides are unable to form fibrils on their own or enhance fibril formation by IAPP. The sequences of these four peptides are very similar, and there is only a minimal difference across the IAPP domain 12–20 which contains them. While all four peptides contain a core FLV sequence, the activity of the peptides ANFLVH and NFLVHS may be modulated by the interactions with side chains from other residues. Therefore, mutational analysis of residues on either side of the core FLV sequence may provide some insight into the interactions of these amino acids during IAPP fibrillogenesis. Future experiments will be designed to investigate these possibilities.

The results presented in this study provide additional data to support the previous report from Jaikaran et al. (2001) that demonstrated the presence of a region within the IAPP 8–20 domain capable of forming a β-sheet structure. The previous study suggested the presence of a region outside of the 20–29 domain within the IAPP molecule that had the potential to form fibrils. Our data have identified two hexapeptides contained within the 8–20 domain that are both capable of independently forming fibrils as well as accelerating fibril formation by full-length IAPP. These data also lend support to the hypothesis that the interactions between multiple β-sheet domains may be involved in the fibrillogenesis of human IAPP.

Acknowledgments

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References

Azriel, R., Gazit, E., 2001. Analysis of the minimal amyloid-forming fragment of the islet amyloid polypeptide. J. Biol. Chem. 276 (36), 34156–34161.

Blanchard, B.J., Konopka, G., Russell, M., Ingram, V.M., 1997. Mechanism and prevention of neurotoxicity caused by β-amyloid peptides: relation to Alzheimer's disease. Brain Res. 776, 40–50.

Clark, A., Edwards, C.A., Ostle, L.R., Sutton, R., Rothbard, J.B., Morris, J.F., Turner, R.C., 1989. Localisation of islet amyloid peptide in lipofuscin bodies and secretory granules of human β-cells and in islets of type 2 diabetic subjects. Cell Tissue Res. 257, 179–185.

Glenner, G.G., Eanes, E.D., Wiley, C.A., 1988. Amyloid fibrils formed from a segment of the pancreatic-islet amyloid protein. Biochem. Biophys. Res. Commun. 155, 608–614.

Goldsbury, C., Goldie, K., Pellaud, J., Seelig, J., Frey, P., Muller, S.A., Kistler, J., Cooper, G.J.S., Aebi, U., 2000. Amyloid fibril formation from full-length and fragments of amylin. J. Struct. Biol. 130, 352–362.

Griffiths, J.M., Ashburn, T.T., Auger, M., Costa, P., Griffin, R.G., Lansbury Jr., P.T., 1995. Rotational resonance solid-state NMR elucidates a structural model of pancreatic amyloid. J. Am. Chem. Soc. 117, 3539–3546.

Higham, C.E., Jaikaran, E.T.A.S., Fraser, P.E., Gross, M., Clark, A., 2000. Preparation of synthetic human islet amyloid (IAPP) in a stable conformation to enable study of conversion to amyloid-like fibrils. FEBS Lett. 470, 55–60.

Jaikaran, E.T.A.S., Higham, C.E., Serpell, L.C., Zurdo, J., Gross, M., Clark, A., Fraser, P.E., 2001. Identification of a novel human islet amyloid polypeptide β-sheet domain and factors influencing fibrillogenesis. J. Mol. Biol. 308, 515–525.

Janciauskiene, S., Ahren, B., 1998. Different sensitivity to the cytotoxic action of IAPP fibrils in two insulin-producing cell lines, HIT-T15 and RIN5mF cells. Biochem. Biophys. Res. Commun. 251, 888–893.

Janson, J., Ashley, R.H., Harrison, D., McIntyre, S., Butler, P.C., 1999. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. Diabetes 48, 491–498.

Kahn, S.E., Andrikopoulos, S., Verchere, C.B., 1999. Islet amyloid: a long-recognized but underappreciated pathological feature of type 2 diabetes. Diabetes 48, 241–253.

Kapurniotu, A., Schmauder, A., Tenidis, K., 2002. Structure-based design and study of non-amyloidogenic, double N-methylated IAPP amyloid core sequences as inhibitors of IAPP amyloid formation and cytotoxicity. J. Mol. Biol. 315, 339–350.

Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W., Kapurniotu, A., 1999. Conformational transitions of islet amyloid polypeptide (IAPP) in amyloid formation *in vitro*. J. Mol. Biol. 287, 781–796.

Lorenzo, A., Razzaboni, B., Weir, G.C., Yankner, B.A., 1994. Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. Nature 368, 756–760.

Lukinius, A., Wilander, E., Westermark, G.T., Engstrom, U., Westermark, P., 1989. Co-localisation of islet amyloid polypeptide and insulin in the β-cell secretory granules of the human pancratic islets. Diabetologia 32, 240–244.

Moriarty, D.F., Raleigh, D.P., 1999. Effects of sequential proline substitutions on amyloid formation by human amylin 20–29. Biochemistry 38, 1811–1818.

Nilsson, M., Raleigh, D., 1999. Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin. J. Mol. Biol. 294, 1375–1395.

Padrick, S.B., Miranker, A.D., 2001. Islet amyloid polypeptide: identification of long-range contacts and local order on the fibrillogenesis pathway. J. Mol. Biol. 308, 783–794.

Park, K., Verchere, B., 2001. Identification of a heparin binding domain in the N-terminal cleavage site of pro-islet amyloid polypeptide. J. Biol. Chem. 20, 16611–16616.

Rhoades, E., Agarwal, J., Gafni, A., 2000. Aggregation of an amyloidogenic fragment of human islet amyloid polypeptide. Biochim. Biophys. Acta 1476, 230–238.

Scrocchi, L.A., Chen, Y., Waschuk, S., Wang, F., Cheung, S., Darabie, A.A., McLaurin, J., Fraser, P.E., 2002. Design of peptide-based inhibitors of human islet amyloid polypeptide fibrillogenesis. J. Mol. Biol. 318, 697–706.

Selkoe, D.J., 1997. Alzheimer's disease: genotypes, phenotypes, and treatments. Science 275, 630–631.

Tenidis, K., Waldner, M., Bernhagen, J., Fischle, W., Bergmann, M., Weber, M., Merkle, M.-L., Voelter, W., Brunner, H., Kapurniotu, A., 2000. Identification of a penta- and hexapeptide of islet amyloid polypeptide (IAPP) with amyloidogenic and cytotoxic properties. J. Mol. Biol. 295, 1055–1071.

Tjernberg, L.O., Lilliehook, C., Callaway, D.J.E., Naslund, J., Hahne, S., Thyberg, J., Terenius, L., Nordstedt, C., 1997. Controlling amyloid β-peptide fibril formation with protease-stable ligands. J. Biol. Chem. 272, 12601–12605.

Westermark, P., Engstrom, U., Johnson, K.H., Westermark, G.T., Betsholtz, C., 1990. Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. Proc. Natl. Acad. Sci. USA 87, 5036–5040.

Westermark, P., Wilander, E., Westermark, G.T., Johnson, K.E., 1987. Islet amyloid polypeptide-like immunoreactivity in the islet β cells of type 2 (non-insulin-dependent) diabetic and non-diabetic individuals. Diabetologia 30, 887–892.

Westermark, Pl., (1994). Amyloid and polypeptide hormones: what is their relationship? Amyloid 1, 47–60.