Pancreatic β-cell granule peptides form heteromolecular complexes which inhibit islet amyloid polypeptide fibril formation

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Islet amyloid polypeptide (IAPP), or 'amylin', is co-stored with insulin in secretory granules of pancreatic islet β-cells. In Type 2 diabetes, IAPP converts into a β-sheet conformation and oligomerizes to form amyloid fibrils and islet deposits. Granule components, including insulin, inhibit spontaneous IAPP fibril formation *in vitro*. To determine the mechanism of this inhibition, molecular interactions of insulin with human IAPP (hIAPP), rat IAPP (rIAPP) and other peptides were examined using surface plasmon resonance (BIAcore), CD and transmission electron microscopy (EM). hIAPP and rIAPP complexed with insulin, and this reaction was concentration-dependent. rIAPP and insulin, but not pro-insulin, bound to hIAPP. Insulin with a truncated B-chain, to prevent dimerization, also bound hIAPP. In the presence of insulin, hIAPP did not spontaneously develop β-sheet secondary structure or form fibrils. Insulin interacted with pre-formed IAPP fibrils in a regular repeating pattern, as demonstrated by immunoEM, suggesting that the binding sites for insulin remain exposed in hIAPP fibrils. Since rIAPP and hIAPP form complexes with insulin (and each other), this could explain the lack of amyloid fibrils in transgenic mice expressing hIAPP. It is likely that IAPP fibrillogenesis is inhibited in secretory granules (where the hIAPP concentration is in the millimolar range) by heteromolecular complex formation with insulin. Alterations in the proportions of insulin and IAPP in granules could disrupt the stability of the peptide. The increase in the proportion of unprocessed pro-insulin produced in Type 2 diabetes could be a major factor in destabilization of hIAPP and induction of fibril formation.

Key words: amyloid fibril, diabetes, insulin, islet amyloid polypeptide, β-sheet, Type 2 diabetes

INTRODUCTION

Islet amyloid polypeptide (IAPP), also known as amylin, is the 37-residue component peptide of amyloid fibrils that are deposited in pancreatic islets in Type 2 (non-insulin-dependent) diabetes in humans [1,2]. Human IAPP (hIAPP) forms fibrils *in vitro*, but the rodent form (rIAPP), which is 84 % identical with hIAPP (Figure 1A), is not fibrillogenic [3], and rodent models of Type 2 diabetes are not associated with islet amyloid deposition. IAPP is co-stored with insulin in the secretory granules of pancreatic islet β-cells and co-secreted from the cell in response to insulin secretagogues [4,5]. Both granule peptides are synthesized as larger precursor peptides (pro-insulin and pro-IAPP) and are processed in the secretory granules to their respective mature peptides by the enzymes prohormone convertase 1 and 2 [6,7] and carboxypeptidase E. Insulin is stored in the secretory granule at a concentration of 10–40 mM as a hexamer bound to zinc [8]. IAPP is stored in the granule at an estimated concentration of 1–4 mM [9]. The association of IAPP and insulin in pancreatic islets has led to the hypothesis that hIAPP, like insulin, is a hormone that is involved in glucose metabolism, insulin action, and/or islet peptide secretion [10]. However, the role of IAPP remains unclear, and published results both support and contradict the role of hIAPP as a putative hormone.

Synthetic hIAPP rapidly forms fibrils *in vitro* at concentrations lower that that proposed for the secretory granule, which suggests that hIAPP is stabilized in the cell by other components. Granule peptides, including insulin and pancreastatin, have been shown to inhibit hIAPP fibril formation *in vitro*, but the mechanism of this inhibition has not been determined [11]. Furthermore, transgenic mice that express the gene for human IAPP and overproduce hIAPP (in addition to endogenous rIAPP) do not spontaneously form pancreatic amyloid; fibrils are only observed in islets of these mice against a background of insulin resistance or a high-fat diet [12–14]. This indicates that factors other than increased concentrations of hIAPP are required to initiate fibrillogenesis.

Sequence alignment of hIAPP and insulin (Figure 1B) reveals two regions of high sequence identity. One region of identity occurs between the N-termini of hIAPP and the insulin A-chain, and the other between the middle regions of hIAPP and the B-chain. A specific interaction between hIAPP and insulin may be modulated by these regions of high sequence identity. A known protein/peptide-binding pair, Aβ-peptide and transthyretin, also has regions of high local sequence identity (Figure 1B). The Aβ-peptide is highly amyloidogenic, and transthyretin has been proposed to stabilize Aβ *in vivo* [15,16].

To examine the specificity of interaction of granule peptides which may stabilize hIAPP in secretory granules, mixtures of hIAPP and insulin, pro-insulin and rIAPP were examined using surface plasmon resonance (BIAcore). The effects of insulin on hIAPP fibril formation were characterized further by CD and transmission electron microscopy (EM). The ability of insulin to interact with pre-formed hIAPP fibrils was also examined. These results have implications for the storage of hIAPP in the β-cell of humans and animal models, and also for the proposed combination therapy of insulin/IAPP analogues.

Abbreviations used: B-insulin, biotinylated bovine insulin; B-PTH, biotinylated parathyroid hormone; EA, egg albumin; EM, transmission electron microscopy; IAPP, islet amyloid polypeptide; hIAPP, human IAPP; B-hIAPP, biotinylated hIAPP; rIAPP, rat IAPP; RU, response unit(s).

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Figure 1 IAPP amino acid sequences and similarity with insulin and transthyretin

(**A**) Amino acid sequences of IAPP (human, rodent and 'symlin' analogue) and insulin (human, rodent 1 and 2, and bovine). Differences from the human sequence are underlined. (**B**) Sequence alignment results for hIAPP–insulin and Aβ–transthyretin (TTR). Regions of sequence identity are highlighted.

MATERIALS AND METHODS

Sample preparation

Peptides were purchased from Bachem (St Helens, U.K.) (hIAPP, rIAPP) or Sigma (human insulin, biotinylated bovine insulin). Biotinylated hIAPP (α- and/or ε-amino coupling, both at the N-terminus of the molecule) was produced by Peninsula Peptides (St Helens, U.K.), solubilized in 100 % hexafluoroisopropanol (HFIP), filtered (0.2 μm pore size filter) to eliminate fibril seeds, freeze-dried and stored at -20 °C [17]. The insulin analogue (X-145, human insulin lacking residues B23–B30) was a gift from Novo Nordisk A/S (Copenhagen, Denmark). Purified recombinant human pro-insulin was kindly provided by Eli Lilly (Indianapolis, IN, U.S.A.). Somatostatin-28 (Sigma) and biotinylated parathyroid hormone (B-PTH) residues 64–84 (Bachem) were used as controls for BIAcore experiments. Peptides for the BIAcore experiments were solubilized in HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.0005 % surfactant P20), and concentrations were determined by amino acid analysis. Incubation experiments for examination by CD and EM were performed using hIAPP and insulin prepared as above in water.

BIAcore experiments

Surface plasmon resonance experiments were performed using a BIAcore 2000 in HBS buffer. Streptavidin (in 10 mM sodium acetate, pH 5.5) was coupled to research-grade CM5 sensor chips. Biotinylated peptides (ligands) were subsequently immobilized on to the streptavidin-coated chip. The mass-corrected coupling levels of the biotinylated peptides were comparable [B-hIAPP (biotinylated hIAPP) 331–423 RU (response units), B-PTH 250 RU, biotinylated bovine insulin (B-insulin) 377 RU]. Solutions of the various peptides (analytes) were passed over the surface [18]. Analytes at different concentrations were injected at a rate of 10 μl/min for 1 min, which was sufficient for equilibrium to be achieved as evidenced by the plateau in the traces (see Figures 2A and 4, inset). The chip was washed between tests with HBS buffer to allow return to baseline. Binding of the analyte to the immobilized ligand resulted in a change in refractive index. The response was measured using surface plasmon resonance and was compared with a control sample on the same chip. All results are reported as the RU of the analyte interaction with the ligand minus the interaction of the analyte with streptavidin alone (no biotinylated peptide). An additional set of control experiments was performed with B-PTH. No binding was observed for any of the peptides with B-PTH, indicating that the interactions observed were specific. The data was also mass-corrected to the molecular mass of rIAPP for easy comparison. RU above control were measured in repeated observations, and the data presented are mean values $\pm$ S.D. The trends in binding reactions were reproducible with different operators and between experiments (with different chips).

CD experiments

CD experiments were performed on a Jasco J-720 spectrometer. Wavelength spectra were recorded in a 1 mm cuvette at 20 °C from 250 nm to 190 nm using a 0.5 nm bandwidth and a mean of three scans. Kinetic measurements were performed by monitoring the sample solution for 5.5 h at 218 nm; each data point is a mean of 120 measurements. Human IAPP (75 μM) and insulin (75 μM) were examined separately or as a mixture.

EM and immunogold labelling

Samples were placed on a Formvar and carbon-coated 200 mesh nickel grid, negatively stained with 2 % (w/v) uranyl acetate, and examined for fibrils using a JEOL JEM1010 electron microscope operating at 80 kV. To increase resolution, some preparations were examined by platinum–carbon shadowing; peptide samples were sprayed on to freshly cleaved mica, plunged into liquid nitrogen and dried under vacuum overnight. Dried preparations were shadowed with platinum–carbon at an angle of 7° and coated with carbon at an angle of 90°. The replicates were floated on to copper grids for examination.

The presence of insulin binding to fibrils was detected using immunogold labelling and EM. Pre-formed IAPP fibrils were prepared by the addition of water to freeze-dried peptide (1 mg/ml) and incubation for 2 weeks. A mixture of insulin (50 μM) and hIAPP fibril preparation (150 μM) was allowed to incubate for 24 h at 4 °C. Aliquots (3 μl) of suspensions, with and without addition of insulin, were deposited on to Formvar-coated grids and were allowed to dry. Grids were washed in PBS with added egg albumin (PBS–EA) (0.2 %), incubated with a polyclonal antiserum to bovine insulin (1/1000 dilution; ICN Biomedicals, Thame, Oxon, U.K.) for 1 h, followed by Protein A–gold (15 nm particle size, 1/20 dilution; Biocell, Cardiff, U.K.) for 1 h, washed in PBS–EA and examined by EM. Addition of Protein A–gold without the addition of primary antibody was used as a control for non-specific binding.

Co-immunoprecipitation experiments

Co-immunoprecipitations were performed on mixtures of insulin (Boehringer Mannheim) and hIAPP (Bachem) in different proportions. The peptides (prepared in water from freeze-dried material) were incubated overnight in physiological Krebs' buffer, pH 7.2, at 4.0 °C to enable interactions to occur under physiological conditions and in the presence of calcium and magnesium.

Samples were immunoprecipitated with guinea-pig anti-insulin antibody (ICN Biomedicals) in 0.1 M Tris in 0.1 M phosphate buffer (TB), pH 7.4. Pre-swollen Protein A–Sepharose beads (Sigma) were added to the mixture to bind the antibody complex. The beads were collected by centrifugation at 10000 ***g*** for 5 min, washed in TB and boiled for 5 min in Laemmli's sample buffer with 20 μg/ml 2-mercaptoethanol (Sigma). Samples were separated on a Tris/Tricine gel, transferred on to a 0.2 μm PVDF membrane in Caps [3-(cyclohexylamino)propane-1-sulphonic acid] buffer, blocked in 4 % (w/v) dried milk powder (Marvel)/ Tris-buffered saline [0.9 % (w/v) NaCl in TB] mixture and incubated (18 h) with rabbit polyclonal antibody against IAPP. Following washing, antibody binding was visualized with peroxidase-conjugated anti-rabbit antisera (Dako, Ely, U.K.) and enhanced chemiluminescence (ECL®, Amersham Biosciences).

RESULTS

Peptide interactions were determined by BIAcore

Previous experiments demonstrating the effects of insulin on hIAPP fibril formation *in vitro* have been performed by assaying fibrils in suspension using a fluorescent marker, thioflavin T [19,20], or by light-microscopic identification of fibrils [11]. However, these experiments do not provide information relating to molecular interactions between soluble molecules. Measurement of surface plasmon resonance permits the determination of interactions between molecules in real time and with relatively low concentrations of peptides. Experiments were performed in which B-hIAPP or B-insulin was attached to the chip (via streptavidin) and compared with a control. Two baseline controls for binding to the chip were determined: streptavidin alone (no biotinylated peptide) and streptavidin with B-PTH residues 64–84. There was no specific binding of the analyte peptides examined to B-PTH residues 64–84 (as compared with streptavidin alone). This indicated that interactions observed with chips coated with B-hIAPP and B-insulin were specific. For convenience, all data are reported as a difference between binding of B-hIAPP (or B-insulin) and streptavidin alone. In addition, the data are mass-corrected to the molecular mass of rIAPP for easy comparison. The binding ability of a variety of islet proteins to B-hIAPP was examined (Figure 2). rIAPP and insulin bind to B-hIAPP, but somatostatin-28, an islet peptide hormone of similar molecular size, does not. Furthermore, an analogue of human insulin that was engineered to have amino acid deletions to prevent hexamerization (X-145) also bound B-hIAPP, indicating that oligomerization of insulin is not a prerequisite for binding.

Pro-insulin, insulin and rIAPP were examined further to investigate the characteristics of binding. Insulin showed a concentration-dependent increase in binding to B-hIAPP and no evidence of saturation at the highest concentration examined (Figure 3); the maximum concentration achievable was restricted by the limited solubility of insulin in the HBS buffer. The curvature of the insulin plot is slightly atypical, which could be related to the increase in the relative proportion of monomer, dimer and tetramer as the insulin concentration increased, and differences in binding to these different species. Nonetheless, it is clear that insulin binds to B-hIAPP. Pro-insulin, in contrast, showed a negligible increase in binding with rapid saturation (Figure 3). rIAPP (which remains soluble at high concentrations) also showed a concentration-dependent increase in binding to B-hIAPP, and the binding appeared to be approaching saturation at a concentration of 2 mM (Figure 3, inset). rIAPP 8–37 has been postulated to act as a blocking agent for putative physiological actions of hIAPP [21].

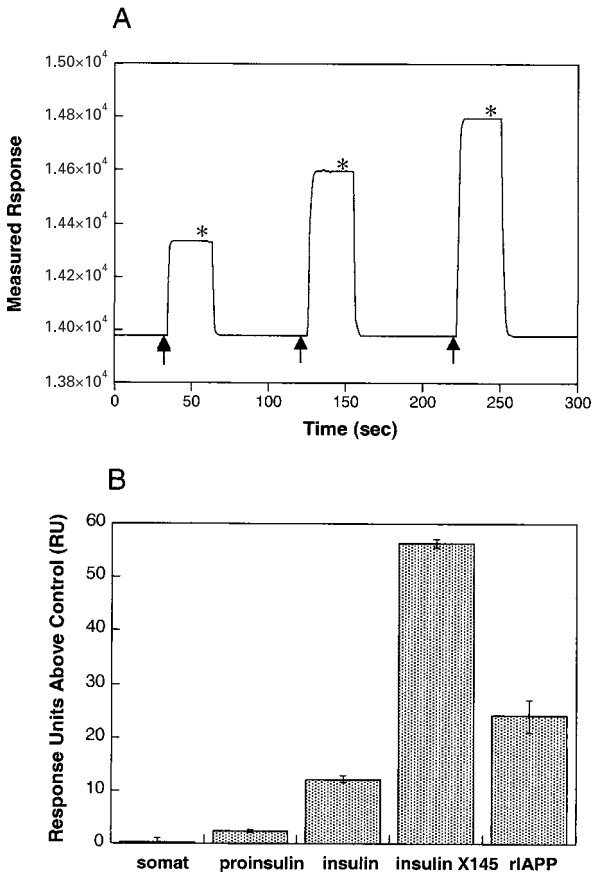

Figure 2 Binding of granule peptides to biotinylated hIAPP by BIAcore

(**A**) Representative BIAcore traces for increasing concentrations of insulin (from left to right). Arrows indicate the beginning of an injection of analyte, and asterisks mark the time at which the data were obtained for subsequent plots. (**B**) Somatostatin (somat), a control peptide, and pro-insulin did not bind to B-hIAPP, whereas insulin, an insulin analogue (X-145) and rIAPP bound significantly higher than the background (streptavidin alone). Peptides (3 mg/ml by mass) were passed over a BIAcore chip to which B-hIAPP was bound. The binding of peptides was corrected for the signal obtained with streptavidin control ($n = 3$; mean $\pm$ S.D.) and mass-corrected to give the relative RU.

Experiments were also performed in which B-insulin was attached to the chip. rIAPP bound to B-insulin with a concentration-dependent increase in signal (Figure 4). It was not possible to examine hIAPP as an analyte, due to the ability of hIAPP to form fibrils in the range of concentrations examined. However, rIAPP is 84 % identical with hIAPP, and we expected the rIAPP–B-insulin binding (Figure 4) to be similar to the human insulin–B-hIAPP binding (Figure 3). The binding interaction between rIAPP–B-insulin appears to be stronger than the insulin–B-hIAPP interaction (despite similar ligand-coupling levels). This may be due to differences in the regions of the molecules that are surface-exposed in the experiment. For example, in the rIAPP–B-insulin experiment, all surfaces of rIAPP are fully exposed (i.e. no regions are obscured by biotinylation or streptavidin binding) and there is no complication from insulin oligomerization.

Binding of insulin to hIAPP was confirmed by co-immunoprecipitation

The interaction of insulin and hIAPP in physiological buffer was detected by co-immunoprecipitation. SDS/PAGE and Western

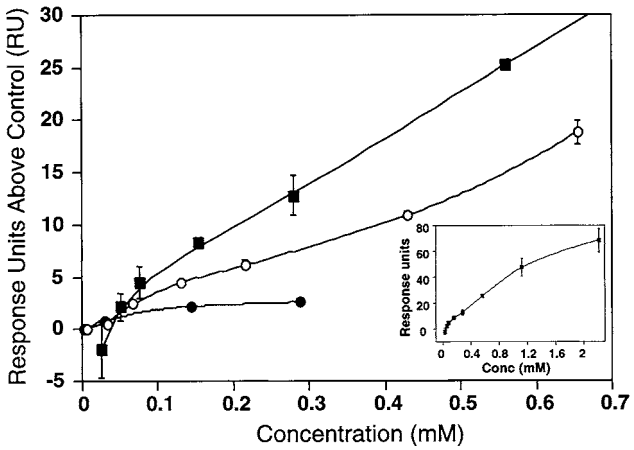

Figure 3 Concentration-dependent binding of granule peptides to B-hIAPP

The binding of human insulin to B-hIAPP showed a dose-dependent response with no evidence of saturation at the highest concentration measured (open circles). Pro-insulin, in contrast, did not bind significantly to B-hIAPP (closed circles). Rodent IAPP bound to B-hIAPP in a concentration-dependent fashion, with saturation of binding appearing at the highest concentrations examined (closed squares and inset). All data are mass-corrected for easy comparison.

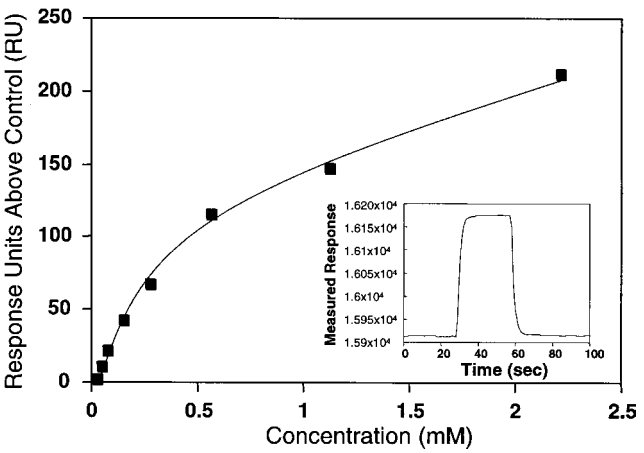

Figure 4 Concentration-dependent binding of rIAPP to B-insulin

Binding steadily increased, but was beginning to approach maximal binding at 2 mM. The inset is a representative trace of the rIAPP–B-insulin interaction.

blotting for hIAPP demonstrated the presence of an insulin–hIAPP complex when the mixture contained 10 μg of insulin and 1 μg of hIAPP (Figure 5).

Insulin delayed the conformation change of hIAPP to β-sheet and prevented the formation of hIAPP fibrils

hIAPP prepared in water (without potential seeds for fibrillogenesis) is initially random coil, as detected by CD, but spontaneously converts into a β-sheet conformation over time [17]. To determine if insulin prevents the conformational change of hIAPP, mixtures of the two peptides were examined over a 5.5 h period and were compared with a hIAPP control. Initially, hIAPP was in random conformation, but within 60 min, the signal at 218 nm increased relative to that at zero-time, indicating conversion from a random coil into a β-sheet conformation (Figures 6A and 6B). In the presence of an equimolar amount of insulin, however, this conversion was not observed (Figure 6A), and a predominantly

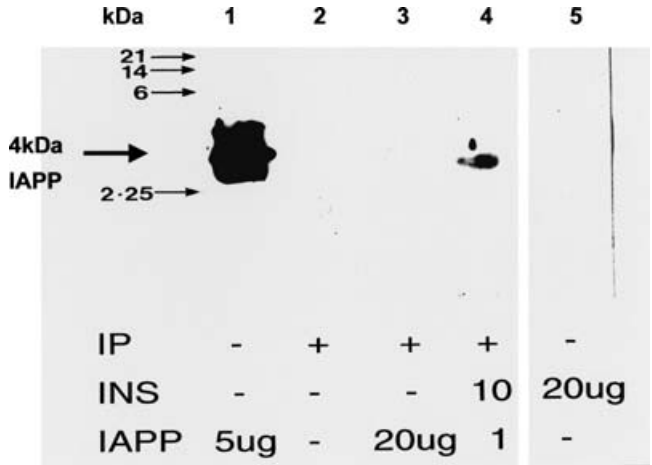

Figure 5 Co-immunoprecipitation of mixtures of hIAPP and insulin at a ratio of 10:1 demonstrate formation of a complex in Krebs' physiological buffer solution (pH 7.4) within 24 h

Samples were immunoprecipitated with insulin antibody bound to Sepharose beads, separated and run on a Tris/Tricine gel. This was followed by Western blotting with an antibody against IAPP. Lane 1, 5 μg of IAPP monomers loaded directly on to the gel (approx. 4 kDa); lane 2, no peptide, immunoprecipitation (IP) materials alone; lane 3, no cross-reactivity of insulin immunoprecipitating antiserum with IAPP, 20 μg/ml; lane 4, band immunoreactive for IAPP at approx. 4 kDa precipitated from mixture of insulin (INS) 10 μg and IAPP 1 μg; lane 5, insulin (20 μg/ml) added directly to the gel to demonstrate no cross-reactivity of IAPP antiserum with insulin.

α-helical spectrum was present, which did not change over 5.5 h incubation (Figure 6C). The spectrum detected for a preparation of insulin alone was, as expected, predominantly α-helical. Since CD measurements represent the mean spectra of all components in a mixture, and to determine if the β-sheet conformation had been masked by the insulin signal, the insulin spectrum was subtracted from the signal for the mixture. However, the resulting spectrum revealed no significant contribution of β-sheet structure (218 nm). These data suggest that hIAPP does not convert from random coil into β-sheet in the presence of equimolar concentrations of insulin.

Additional experiments to compare fibril formation in mixtures of hIAPP–insulin with a hIAPP control were monitored by EM. These experiments contained less insulin than hIAPP (50 μM insulin and 75 μM IAPP) to determine if hIAPP fibril formation could occur at sub-stoichiometric concentrations of insulin. EM revealed significant fibril formation in the hIAPP control sample, but no fibril formation in the mixture of hIAPP with insulin (Figures 7A and 7B). The hIAPP fibrils had a typical amyloid fibril morphology, with protofilament widths of 4–8 nm and fibril widths of 10–20 nm (Figure 7A). In the solution that contained a mixture of hIAPP and insulin, no amyloid fibrils were observed, but there were large amorphous aggregates (Figure 7B). No precipitate was present in preparations of insulin alone. These data suggest that, although insulin is capable of preventing hIAPP from forming fibrils, this interaction results in precipitation of amorphous aggregates.

Insulin bound to pre-formed hIAPP fibrils

The specificity of the interaction between hIAPP and insulin suggested that insulin could bind to pre-formed hIAPP fibrils. In order to determine if insulin could disaggregate the fibrils, insulin was added to a solution of pre-formed hIAPP fibrils and compared with a hIAPP fibril control. In the mixture, fibrils were present which were less smooth and regular than those formed from hIAPP alone (Figures 8A and 8C); the fibril diameter was

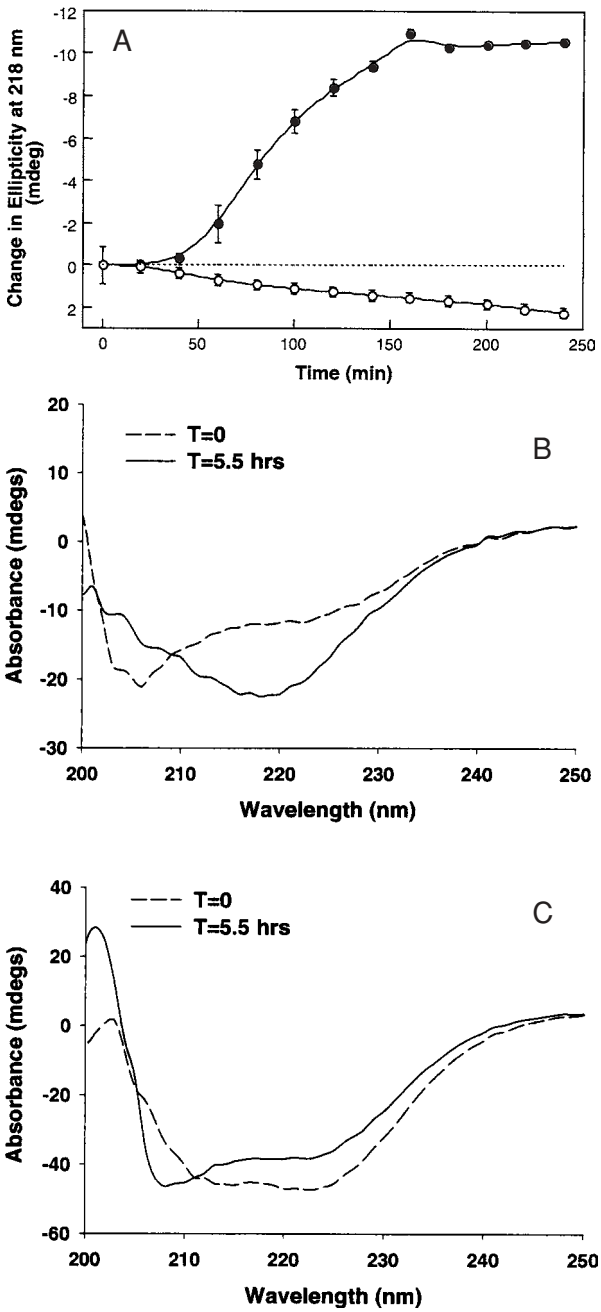

Figure 6 Changes in CD spectrum at 218 nm of human IAPP and of a mixture of hIAPP and insulin

(**A**) hIAPP (75 μM) rapidly, within 1 h, showed conversion into a β-sheet conformation in water (closed circles), but this change in secondary structure was prevented in the presence of an equimolar amount of insulin (open circles). The ellipticity at 218 nm was monitored over 5.5 h (120 measurements per data point, mean $\pm$ S.E.M.) for both samples. (**B**) CD spectra for hIAPP sample at the beginning (broken line) and at the end of the incubation (solid line) showed conversion from random structure into a β conformation. (**C**) This conversion did not happen in a mixture of insulin and hIAPP, where the spectra indicated a largely helical conformation (broken line is zero-time and solid line is 5.5 h).

increased, and aggregated material was present along the length of the fibril (Figures 8B and 8D). Immunogold labelling for insulin clearly identified these aggregates as insulin adherent to the hIAPP fibrils (Figure 8E). Therefore insulin binds to pre-formed hIAPP fibrils, but there is no evidence of disaggregation of fibrils.

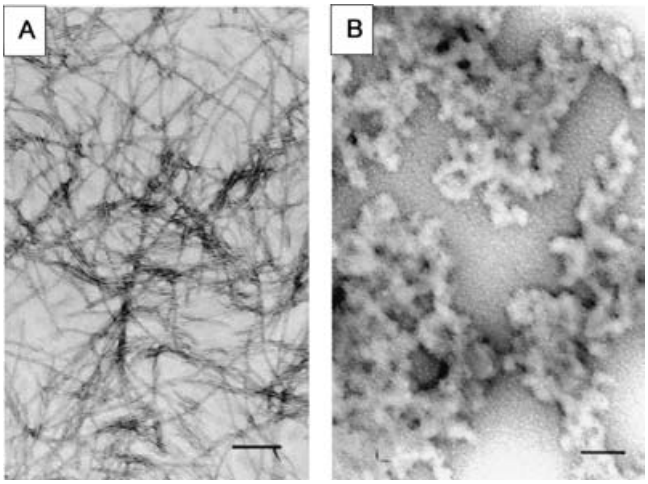

Figure 7 Insulin prevented formation of fibrils visible by EM

Samples of insulin (50 μM) and hIAPP (75 μM) were incubated separately or together for 24 h (**A**) hIAPP readily formed fibrils. (**B**) A mixture of hIAPP and human insulin resulted in the formation of amorphous aggregates. No precipitate was seen with insulin alone. Scale bars, 200 nm.

DISCUSSION

Insulin has previously been shown by fibril-detection assays to prevent hIAPP fibril formation [11,20]. In addition, fibril formation by Aβ residues 12–28 was unaffected by β-cell granule peptides, indicating that the hIAPP–insulin interaction is specific [20]. We previously observed a specific interaction between hIAPP and insulin in the gas-phase by MS [22], but we have now extended this observation to solution-phase experiments using BIAcore and immunoprecipitation. The specificity of the hIAPP–insulin interaction was confirmed by demonstrating that B-hIAPP does not bind somatostatin-28 and neither insulin nor hIAPP binds to B-PTH residues 64–84. Insulin spontaneously forms oligomers in solution and the extent of oligomerization is concentration-dependent. The oligomerization state of insulin in the BIAcore experiments was largely unknown, but since the binding was concentration-dependent and there was no loss of binding at higher concentrations of insulin, this suggests that both monomers and oligomers of insulin bind to B-hIAPP. Furthermore, an insulin analogue that lacked portions of the B-chain that are important for hexamerization (B23–B30) [23] also bound to B-hIAPP, indicating that oligomerization of insulin is not a prerequisite for binding.

The binding of insulin prevents the conversion of hIAPP into a β-sheet conformation, and subsequently prevents fibril formation. Since insulin and hIAPP are co-stored [24], the formation of a complex could be the mechanism of stabilization of hIAPP at the high concentrations found in the secretory granule, where the concentration of insulin (10–40 mM) and hIAPP (1–4 mM) are high, but no fibrils are formed. The pH of the granule varies between 7.2 and 5.0, but fibrillogenesis of hIAPP has been shown to be pH-independent [25]. It has been suggested that the ratio of insulin to IAPP is critical for intermolecular interaction *in vitro*; fibril formation is inhibited only when insulin is in a molar excess to IAPP. It is therefore possible that the proportion of the two peptides in the granules is critical for stabilization. The IAPP to insulin ratio in the circulation is between 1:10–1:50 in humans [26], and does not alter in Type 2 diabetes, when there are substantial changes in the amount of insulin produced [27]. Amyloid deposits are not found in all islets, suggesting that conditions are not

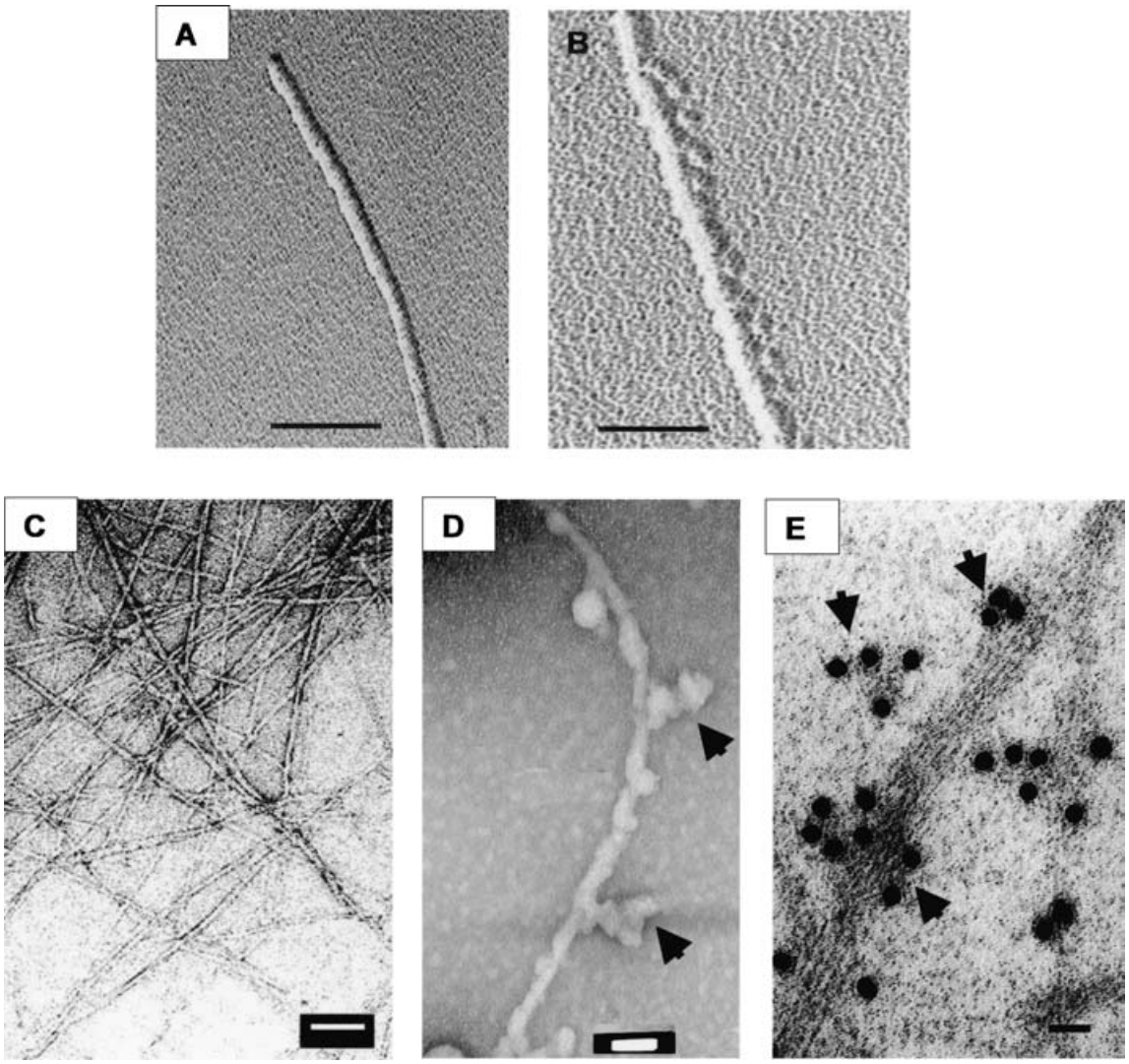

Figure 8 IAPP fibrils examined with electron microscopy

Fibrils formed by hIAPP were regular and unbranching when examined with platinum–carbon shadowing (**A**) or negative stain (**C**). The addition of insulin (50 μM) to hIAPP pre-formed fibrils (150 μM) resulted in fibrils with spheroidal aggregates adherent to the surface visible following platinum–carbon shadowing (**B**) or negative stain (**D**). These aggregates were identified as insulin by immunogold labelling with insulin antibody and Protein A–gold (15 nm particles) (**E**) Scale bars, 100 nm.

uniformly favourable for IAPP fibrillogenesis [28]. Particular islets/islet cells may be more affected by the conditions of diabetes (hyperglycaemia/hyperinsulinaemia), which results in more severe islet dysfunction, changes in the peptide ratio, production of unstabilized hIAPP, and subsequent hIAPP fibril formation. This could account for the very heterogeneous occurrence of amyloid deposits in human pancreatic islets in Type 2 diabetes [28].

Although insulin binds hIAPP in a concentration-dependent fashion, pro-insulin does not. This is consistent with earlier reports that pro-insulin does not prevent hIAPP fibril formation as effectively as insulin [11]. Pro-insulin includes an additional peptide fragment (C-peptide), which is a connection between the A- and B-chains and is removed by proteolysis during granule maturation to generate mature insulin. C-peptide accelerates IAPP fibril formation in mixtures of IAPP to C-peptide of 1:4 or 1:2 [11], and does not bind to hIAPP, as determined by MS [22]. This suggests that in the early stages of the maturing secretory granule, when intact pro-insulin is present, the C-peptide region could block the putative binding site of hIAPP and complexes would not be established until mature insulin had been formed. It would be relevant to examine the interaction of pro-insulin with pro-IAPP, since these molecules would probably be present in the secretory granule at the same time. At present, however, pro-IAPP is not commercially available.

Insulin not only binds to soluble hIAPP, but can also bind to pre-formed IAPP fibrils *in vitro*. This decoration of hIAPP fibrils with aggregates of immunoreactive insulin suggests that a binding site remains exposed on the surface of the hIAPP fibril. An early study using frozen tissue sections demonstrated insulin immunoreactivity in islet amyloid deposits *in vivo* [29]. It is possible that secreted insulin may bind to adjacent amyloid deposits *in vivo*. However, the absence of this finding in most pancreatic material could be due to methods of tissue preparation or fixation.

In addition to interactions with hIAPP, insulin also binds to rodent IAPP. The IAPP analogue symlin, also known as pramlintide [30], that is currently in clinical trials is a hybrid of the human and rodent sequences. Since insulin binds to both the human and rodent sequences, it is likely that insulin also binds to this analogue. These observations have implications for the use of this molecule in combination therapy with insulin and could explain

some data observed in the clinical trials. The observations that IAPP analogues were less effective at high concentrations could be due to IAPP binding to exogenous injected insulin (the patients in these trials were insulin-dependent). It has also been reported that a combined preparation of insulin and the IAPP analogues has a longer lifetime *in vivo*. This could result from decreased degradation of the IAPP analogue–insulin complex *in vitro* and/or *in vivo* [31].

hIAPP forms complexes with rIAPP. This is consistent with earlier reports that demonstrate that rIAPP inhibits fibril formation of hIAPP *in vitro* [32]. From these studies, it appears that rIAPP acts as a β-sheet breaker peptide, binding to and preventing oligomerization of hIAPP. These observations on rIAPP and hIAPP could contribute to the lack of spontaneous fibril formation in transgenic mice expressing the gene for hIAPP. It is likely that complexes of hIAPP and rIAPP could be formed in the secretory granules; this would stabilize the high concentrations of transgenic hIAPP peptide [33] and effectively prevent fibril formation in the absence of other physiological and pathological factors, such as obesity or a high-fat diet [12].

The molecular interactions elucidated in the present study suggest that, in Type 2 diabetes, any changes in storage or processing of insulin in the islet β-cells could result in destabilization of hIAPP. An increased production of incompletely processed pro-insulin is a characteristic of patients with Type 2 diabetes; the proportion of pro-insulin-like peptides is increased from less than 3% normally up to 30% of total insulin-like molecules [34]. Pro-insulin, from our studies, does not bind to hIAPP and has previously been shown to be only a weak inhibitor of hIAPP fibril formation. Therefore we can speculate that patients with Type 2 diabetes develop islet amyloid as a result of improper processing of pro-insulin, which could lead to decreased IAPP–insulin complex formation within the secretory granules and subsequent fibril formation from hIAPP.

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