Islet amyloid polypeptide: Pinpointing amino acid residues linked to amyloid fibril formation

(amyloidogenesis *in vitro*/synthetic peptides/electron microscopy/secondary structure/diabetes mellitus)

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ABSTRACT   Islet amyloid polypeptide (IAPP), a putative polypeptide hormone, is a product of pancreatic β-cells and the major constituent of the amyloid deposits seen mainly in islets of type 2 diabetic humans and diabetic cats. The connection between IAPP amyloid formation and diabetes is unknown, but a limited segment of the IAPP molecule, positions 20–29, seems responsible for the aggregation to fibrils. Differences in the amino acid sequence of this region probably determine whether or not islet amyloid can develop in a particular species. Amyloid fibril formation can be mimicked *in vitro* with the aid of synthetic peptides. With this technique we show that peptides corresponding to IAPP positions 20–29 of human and cat, species that develop IAPP-derived islet amyloid, form amyloid-like fibrils *in vitro*. The corresponding IAPP segment from three rodent species that do not develop IAPP-derived amyloid did not give rise to fibrils. Substitution of the human IAPP-(20–29) decapeptide with one or two amino acid residues from species without islet amyloid generally reduced the capacity to form fibrils. We conclude that the sequence Ala-Ile-Leu-Ser-Ser, corresponding to positions 25–29 of human IAPP, is strongly amyloidogenic and that a proline-for-serine substitution in position 28, as in several rodents, almost completely inhibits formation of amyloid fibrils.

Human type 2 diabetes is characterized by peripheral insulin resistance and impaired insulin response to increased glucose levels (1). The primary islet abnormality in type 2 diabetes mellitus in humans is not known, but the most characteristic morphological alteration, seen in up to 95% of the patients, is the extracellular deposition of amyloid (2). Like other amyloids, islet amyloid consists mainly of a small protein aggregated into fine fibrils. The main constituent of islet amyloid is a polypeptide displaying amino acid sequence homology with calcitonin gene-related peptide (CGRP) and is called islet amyloid polypeptide (IAPP, also designated diabetes-associated peptide or amylin) (3–6). IAPP is normally expressed by the islet β cells, stored in cytoplasmic granules with insulin (7–9), and probably released together with this hormone. The normal function of IAPP is not known, but a depression of insulin-mediated glucose uptake by skeletal muscle cells *in vitro* has been reported (10). In agreement with this, impaired glucose tolerance was demonstrated in cats after intravenous injection of synthetic IAPP (11). Theoretically, an overproduction of IAPP from islet β cells could be of importance in the development of the peripheral insulin resistance. This hypothesis is supported by the finding of abnormally strong IAPP immunoreactivity in islets of non-diabetic cats with impaired glucose tolerance (12).

It is not clear why IAPP forms amyloid fibrils in type 2 diabetes. However, it is known that the amyloid fibrils morphologically occur in close relationship to β cells and that the fibrils are present within deep invaginations of these cells (13, 14). The cell membrane is often absent or indistinct in some regions of these invaginations but intracellular fibrils are not seen. Therefore, it is possible that the fibrils form directly after the granule release and in close contact with the cell membrane. The effect of the amyloid deposits on β-cell function is unknown but, since the deposits often are extensive, a diffusion-barrier effect seems very probable. Furthermore, β cells in affected islets often have disrupted cell membranes, which might influence granule release (15). Heavy islet amyloid deposits probably take some time to develop and it is possible that they eventually contribute significantly to the deterioration in insulin response in type 2 diabetes.

Only a few animal species are known to develop islet amyloid. Besides humans, these include non-human primates (16), cats (17), raccoons (17), and the degu (*Octodon degus*) (18). There is an interesting correlation between the occurrence of a type 2-like form of diabetes in some animals and deposits of amyloid in the islets. In humans, non-human primates, and cats, islet amyloid occurs mainly in connection with diabetes, while the association with diabetes in the other species is not clear.

Recent studies have indicated that a limited segment (positions 20–29) of the human IAPP molecule has intrinsic capacity to form amyloid fibrils *in vitro* and may be responsible for the formation of islet amyloid. Amino acid sequence variations in this segment correlate with the occurrence of amyloid in different species (19–22). To investigate possible amyloidogenic amino acid sequences within this region of IAPP, we have synthesized peptides corresponding to amino acid residues 20–29 of IAPP from various species and studied their capacity to form amyloid-like fibrils *in vitro*. We have also substituted the human sequence with amino acid residues from other species, one-by-one or in combination, and studied the effects on fibril formation.

MATERIAL AND METHODS

Synthesis of Peptides. Decapeptides corresponding to positions 20–29 of IAPP [IAPP-(20–29)] of human (4), rat/mouse (23, 24), hamster (21), cat (22, 24), and degu (34) (Fig. 1) were synthesized by automatic solid-phase synthesis (20) on a model 430A peptide synthesizer (Applied Biosystems). Decapeptides with selected amino acid substitutions (Table 1) and human full-length (positions 1–37) IAPP were synthe-

Abbreviations: IAPP, islet amyloid polypeptide; CGRP, calcitonin gene-related peptide.
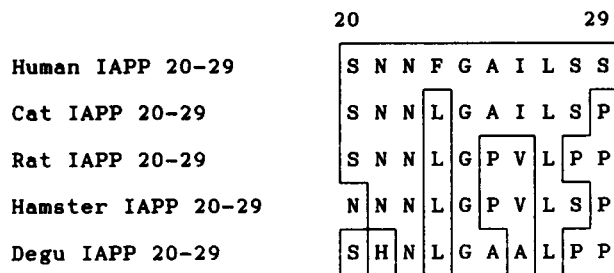‖To whom reprint requests should be addressed.

FIG. 1. Amino acid sequences of synthetic decapeptides corresponding to positions 20–29 of IAPP of different species used for estimation of *in vitro* fibril formation capacity. Residues identical to the human sequence are boxed.

FIG. 2. (*Top*) The 89-amino acid (aa) human IAPP prepropeptide. The mature 37-aa IAPP is flanked by two propeptide segments, which are split off at double basic amino acid sequences N- and C-terminally. A glycine residue at the C terminus of IAPP acts as the donor for amidation. (*Middle*) IAPP is strongly conserved N- and C-terminally but the segment consisting of positions 18–29 exhibits considerable interspecies variation. (*Bottom*) Synthetic peptides corresponding to positions 20–29 of IAPP and with various substitutions were used for fibril formation *in vitro*.

sized in the same way. The peptides were purified by reversed-phase HPLC on a Nucleosil C_{18} column (20 × 250 mm) in 0.12% trifluoroacetic acid with a gradient of 10–60% acetonitrile. Rat IAPP-(1–37) was purchased from Multiple Peptide Systems (San Diego). All peptides were C-terminally amidated. The synthesized peptides were analyzed by ^{252}Cf plasma-desorption time-of-flight mass spectrometry (25). The mass spectra showed the expected molecular masses.

In Vitro Fibril Formation. The synthetic peptides were dissolved (usually 10 mg/ml) in 10% acetic acid and were kept at room temperature overnight. After ocular inspection for gel formation, neutralization was obtained by addition of concentrated NH_4OH. Solutions in which no gel appeared were then concentrated to approximately one-third of the original volumes by evaporation under a stream of N_2 at room temperature. In another experiment, 10% acetic acid was saturated with peptides (solubility, <50 mg/ml), kept at room temperature overnight, and then neutralized as above.

Confirmation of Fibril Formation. Samples taken after each of the three steps (i.e. after solubilization in acetic acid, after neutralization, and after subsequent concentration) were dried on glass slides and stained with Congo red for evaluation with polarized light. Samples were also taken for electron microscopy. Each volume of these samples was mixed thoroughly with 9 volumes of distilled water, and small drops were placed on Formvar-coated copper grids, negatively contrasted with 1% uranyl acetate in water, and studied in a JEOL 2000 EX electron microscope at 100 kV.

RESULTS

IAPP from Various Species. The relationship between the human IAPP precursor, mature IAPP, and the synthetic peptides used in this study is outlined in Fig. 2. The mature 37-amino acid C-terminally amidated IAPP molecules form through the proteolytic processing of both the N and C termini of 89- to 93-amino acid precursors (size depending on species) (20–24, 26, 27). The N and C termini of IAPP are
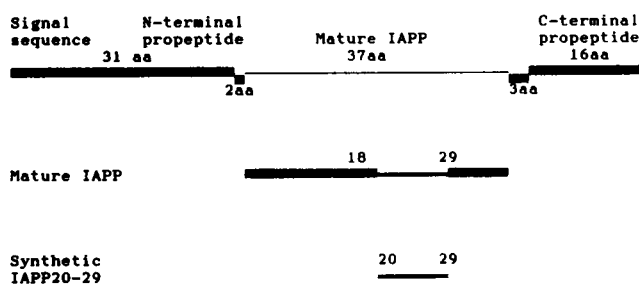
Table 1. Amino acid sequences of synthetic decapeptides corresponding to human, hamster, and rat IAPP-(20–29) but with various amino acid substitutions

Species	Substitution(s)	Sequence
Human	Leu²³	S N N L G A I L S S
	Pro²⁵	S N N F G P I L S S
	Val²⁶	S N N F G A V L S S
	Pro²⁸	S N N F G A I L P S
	Pro²⁹	S N N F G A I L S P
	Leu²³,Pro²⁵	S N N L G P I L S S
	Pro²⁵,Val²⁶	S N N F G P V L S S
	Pro²⁸,²⁹	S N N F G A I L P P
Hamster	Ala²⁵,Ile²⁶	N N N L G A I L S P
Rat	Ala²⁵,Ile²⁶	S N N L G A I L P P

evolutionarily well conserved, whereas considerable amino acid sequence divergence is seen in the N- and C-terminal prosequences as well as in a region corresponding to amino acid residues 18–29 of mature IAPP (22, 24). The 20–29 region has been suggested to account for the amyloidogenic properties of human and cat IAPP, since decapeptides corresponding to this region have the intrinsic ability to form amyloid fibrils *in vitro*, whereas the corresponding hamster peptide has not (19–22). We studied the amyloidogenic properties of IAPP-(20–29) peptides of five species (Fig. 1). After solubilization in acetic acid, the human peptide immediately formed a gel. Typical properties of amyloid (i.e., Congophilia with bright green birefringence) were observed when samples of this gel were examined after staining with Congo red. Ultrastructurally, the material consisted of often tight bundles of parallel straight filaments that were about 5 nm thick (Fig. 3B). Cat IAPP-(20–29) solution gelled after neutralization and exhibited 10- to 30-nm-wide, band-like fibrils, which were long and consisted of several fine filaments of about 4-nm width (data not shown). Rat, hamster, and degu IAPP-(20–29) did not give rise to fibrils in any of the conditions tested.

Effects of Amino Acid Substitutions in Human IAPP-(20–29). To assess which of the differences between human and rodent IAPP-(20–29) accounted for the ability or inability of the IAPP-(20–29) peptide to form amyloid *in vitro*, hybrid peptides were synthesized congruous to human IAPP-(20–29) with one or two positions exchanged for corresponding rodent residues (Table 1). Additionally, rat and hamster IAPP-(20–29) were synthesized with human/cat residues at position 25 and 26. The ability of human IAPP-(20–29) with various amino acid substitutions to form amyloid-like material, based on examination of Congo red-stained smears under polarized light, is summarized in Table 2. It is apparent that all amino acid substitutions studied reduced the amyloidogenic properties of the human IAPP-(20–29) decapeptide. Of the single amino acid substitutions studied, replacement of serine with proline at position 28, giving the [Pro²⁸]IAPP-(20–29) peptide, had the most significance with respect to amyloidogenesis. In contrast, substitution of proline for alanine at position 25 or substitution of proline for serine at position 29 had only minor effects. Substitution of leucine for phenylalanine at position 23 had almost no effect on fibrillogenesis, while a substitution of valine for isoleucine at position 26 slightly reduced the tendency for fibril formation. Interestingly, the combination of proline in position 25 and valine in position 26 reduced fibril formation in the acidic state but enhanced fibrillogenesis in the neutralized specimen.
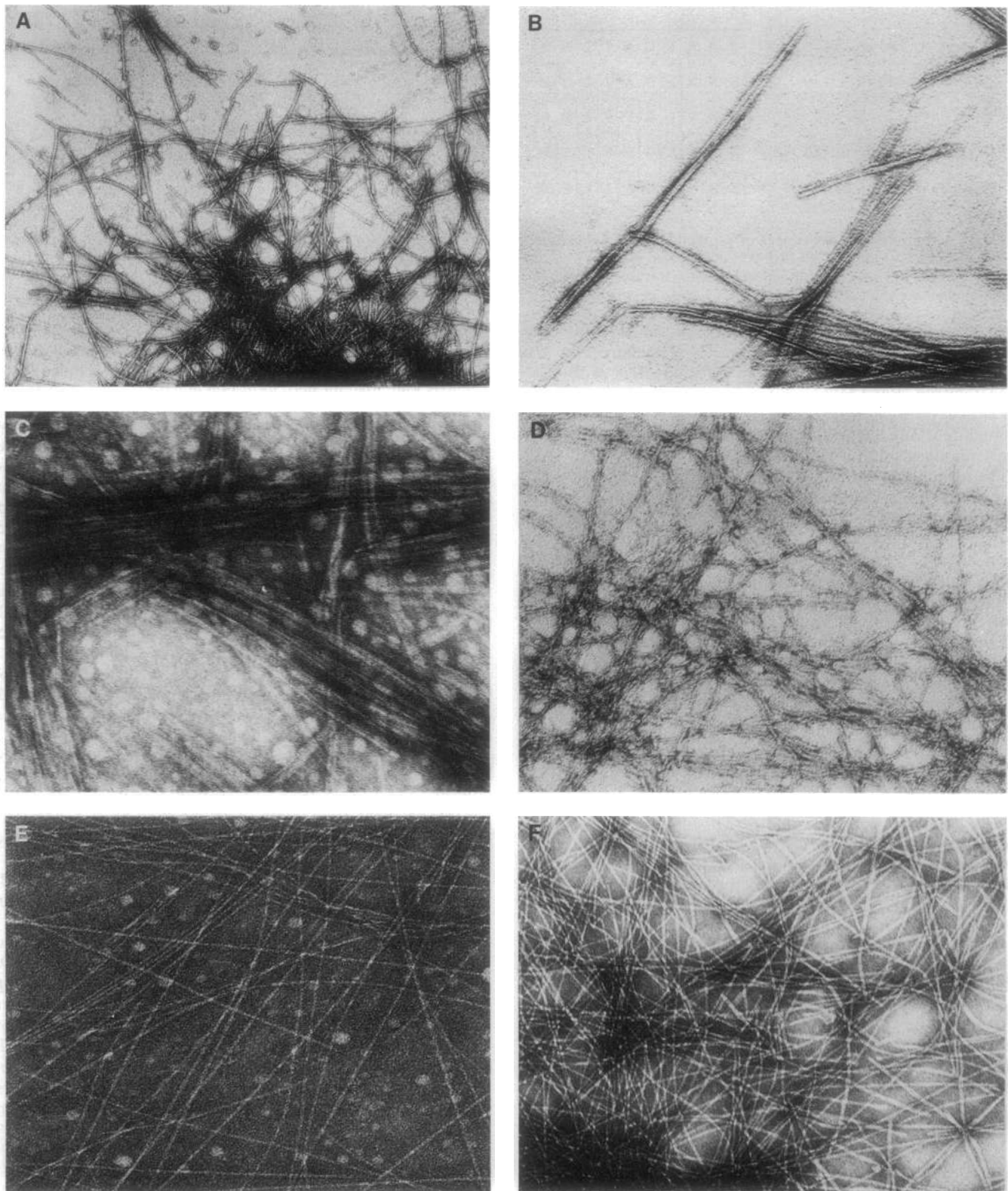
FIG. 3.   Electron microscopic appearance of synthetic amyloid-like fibrils obtained with human IAPP-(1–37) (*A*), human IAPP-(20–29) (*B*), human IAPP-(20–29) with valine in position 26 (*C*), human IAPP-(20–29) with proline in position 28 (*D*), human IAPP-(20–29) with proline in position 29 (*E*), and human IAPP-(20–29) with proline in position 25 (*F*). Human IAPP-(20–29) with proline in position 28 gave rise to fibrils only when used in very high concentration, and the fibrils were morphologically very different from native amyloid fibrils. Specimens were negatively contrasted with uranyl actetate. (*A*, ×60,000; *B–D*, ×150,000; *E* and *F*, ×90,000.)

Morphology of the Amyloid-like Fibrils. Pronounced differences were seen when the specimens were studied in the electron microscope (Fig. 3). Fibrils from human [Leu[23]]-IAPP-(20–29), human [Val[26]]IAPP-(20–29), and human [Pro[25],

Val[26]]IAPP-(20–29) all resembled the amyloid-like fibril bundles obtained with human IAPP-(20–29). On the other hand, human [Pro[25]]IAPP-(20–29), [Pro[29]]IAPP-(20–29), and [Pro[28.29]]IAPP-(20–29) all gave rise to long, slender, curved

Table 2. Fibril formation by various IAPP-related synthetic peptides

Species	Positions	Substitution(s)	Fibril formation[†]		
			In acetic acid	After neutrali- zation	After concen- tration
Human	1–37*	—	+++	+++	ND
	20–29	—	+++	+++	ND
	20–29	Leu^{23}	++(+)	ND	ND
	20–29	Pro^{25}	++	++	ND
	20–29	Val^{26}	++	++	ND
	20–29	Pro^{28}	–	–	+
	20–29	Pro^{29}	++	ND	ND
	20–29	Leu^{23},Pro^{25}	–	++	ND
	20–29	Pro^{25},Val^{26}	(+)	+++	ND
	20–29	Pro^{28,29}	–	–	+
Rat	1–37	—	–	–	–
	20–29	—	–	–	–
	20–29	Ala^{25},Ile^{26}	–	–	–
Cat	20–29	—	–	++	ND
Hamster	20–29	—	–	–	ND
	20–29	Ala^{25},Ile^{26}	–	++	–
Degu	20–29	—	–	–	–

The formation of amyloid-like fibrils from peptides (dissolved at 10 mg/ml in 10% acetic acid unless otherwise indicated) was studied by polarization microscopy of Congo red-stained smears and in the electron microscope.
*Studied at 3 mg/ml.
[†]Semiquantitative estimation of the amount of fibrils occurring after the different steps: +++, development of a thick gel with amyloid properties; ++, a thin gel with amyloid properties; +, few visible gel particles with amyloid properties; –, no gel visible and no fibrils demonstrable electron microscopically; ND, not done.

fibrils, different from native amyloid fibrils (Fig. 3 *E* and *F*). Fibrils from human [Pro^{28}]IAPP-(20–29) were very unevenly curved and did not resemble native amyloid fibrils (Fig. 3*D*).

Fibril Formation from Full-Length Human IAPP. Full-length human IAPP [IAPP-(1–37)] had a very strong tendency to form amyloid-like fibrils (Fig. 3*A*) at the concentration studied (3 mg/ml of 10% acetic acid). Most of the IAPP-(1–37) initially dissolved in 10% acetic acid but subsequently formed fibrils after 24 hr in this solution. These fibrils were straight and resembled those formed from human IAPP-(20–29). In contrast, rat IAPP-(1–37) (10 mg/ml) did not give rise to any fibrils, even after neutralization and concentration (Table 2).

Effect of Protein Concentration on Fibril Formation. As described above, some peptides formed fibrils only after neutralization and concentration of the samples. On the other hand, human IAPP-(20–29) at 1 mg/ml immediately formed fibrils in acidic condition (data not shown). Human [Pro^{28}]-IAPP-(20–29) and human [Pro^{28,29}]IAPP-(20–29) did not give rise to fibrils at saturated concentration (<50 mg/ml) in acidic condition. After neutralization of the saturated solutions, human [Pro^{28}]IAPP-(20–29), but not human [Pro^{28,29}]IAPP-(20–29), gave rise to a gel that consisted of fine fibrils as determined by electron microscopy.

DISCUSSION

Only certain mammalian species develop islet amyloid *in vivo*. Our present *in vitro* studies demonstrate that species-specific amino acid differences in the 20–29 region of IAPP may be important in determining these *in vivo* differences. Single substitutions at several of the positions in human IAPP-(20–29) (with rodent amino acid residues) influenced amyloid fibril formation negatively. However, exchanging the serine residue at position 28 for proline most dramatically inhibited the aggregation of the peptide to fibrils. The proline-28 in rat and mouse IAPP may thus in large part explain the absence of IAPP-derived islet amyloid in these species. The inability of hamster IAPP-(20–29) to form amyloid fibrils is in

agreement with the lack of islet amyloid in this species. Hamster IAPP carries a serine residue at position 28; thus the inability to form fibrils in this species appears to be due to a combined effect of two or more of the positions that differ from the human sequence, none of which by itself greatly influenced fibril formation. Both peptides corresponding to human IAPP-(20–29), but with two hamster positions (Leu^{23},Pro^{25} and Pro^{25},Val^{26}, respectively), lacked fibril-forming capacity in the acidic condition but were, however, more fibrillogenic than the peptide with proline in position 28 as the sole substitution. That a combination of substitutions can be responsible for abolishing the fibrillogenic properties is also supported by the gained fibril-forming properties of the hamster IAPP-(20–29) with substitution of alanine and iso-leucine (human/cat residues) for proline and valine at positions 25 and 26, respectively.

Of special interest is the lack of amyloidogenic properties found with degu IAPP-(20–29) material, which has proline in position 28 like the rat and mouse. The degu often develops islet amyloid (18) and, therefore, an amyloidogenic sequence in the 20–29 region of the degu IAPP might have been expected. Recent studies have, however, confirmed that islet amyloid in this species is not derived from IAPP but is formed from insulin (28).

The relevance of the fibril-formation data found with the 20–29 segments of IAPP is strongly supported by the results of our studies utilizing full-length human and rat IAPP molecules. Thus, human IAPP-(1–37) had the same fibril-forming properties as human IAPP-(20–29), whereas neither of the two rat IAPP peptides exhibited any amyloidogenic potential.

Obviously the primary structure of IAPP is not the only factor of importance in islet amyloid formation, since in that case all humans and cats should develop islet amyloid. Another factor that most probably is of importance is the local concentration of the amyloidogenic protein. This suggestion is supported by our *in vitro* data, which show that some peptides formed fibrils only at very high concentration,

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Human IAPP   KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY amide
  1.                    **              *****
  2.          **************          **
  3.          ******** ******        *********

Cat IAPP     ------------------IR----L------P--------- amide
  1.                    *****          ******
  2.          ***************          ****
  3.          ******** ******        ****** ***

Hamster IAPP ------------------N--L-PV--P--------- amide
  1.                    *****          *****
  2.          ***************          ****
  3.          ******** ******        ****** ***

Rat IAPP     ------------------R----L-PV-PP-------- amide
  1.          -------------******        *****
  2.          **************          ****
  3.          ******** ******        ****** ***

Human CGRP   A-D----V-H---GL-SR-GGVVKNNFVP-----KAF amide
  1.                *** *** *** *******
  2.          ***************          ***
  3.          ***************          ****
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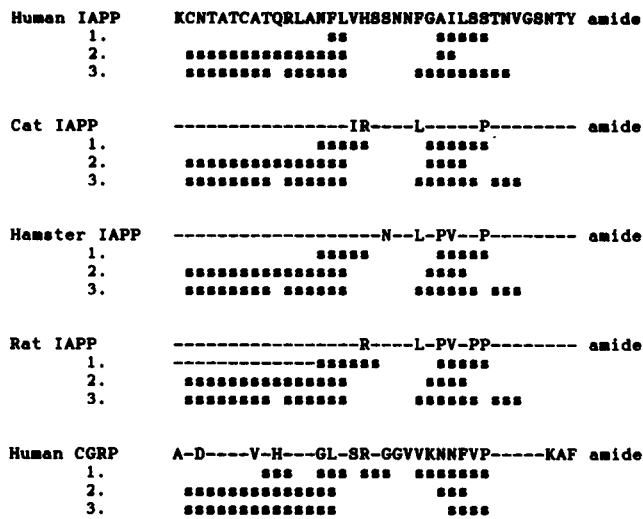
FIG. 4. Amino acid sequence of IAPP of human, cat, hamster, and rat and of human CGRP with secondary-structure prediction by three different methods (1, ref. 30; 2, ref. 31; 3, refs. 32 and 33). β-Pleated sheet preponderance is indicated by s.

and also by our recent studies in cats, which indicate that increased expression of IAPP by beta cells precedes islet amyloid deposition and diabetes (12).

It is well known that several polypeptides have the ability to aggregate to amyloid-like fibrils *in vitro*, but why amyloid fibrils are formed is not completely understood. However, an intra- and intermolecular β-pleated sheet structure seems to be of crucial importance (29). Most amyloid subunit proteins are molecules that have a high degree of β-pleated sheet conformation in their native state. Examples of such known amyloid precursor proteins are immunoglobulin light chains, β_2-microglobulin, and transthyretin. However, a β-pleated sheet structure seems not to be a sole prerequisite for amyloidogenic properties. A secondary-structure prediction using three different methods (30–33) unexpectedly gave a closely similar prediction of β-pleated sheet structure in the 20–29 region of fibrillogenic as well as nonfibrillogenic IAPP peptides (Fig. 4). Also, CGRP, which diverges completely at the amino acid sequence level from the IAPPs in this region, showed a similar predicted β-pleated sheet structure. Although allowed to diverge considerably in primary structure, the 20–29 region may be strongly conserved with regard to its secondary structure. It is possible that during evolution, this conservation has increased the risk of acquisition by chance of substitutions that confer amyloidogenic properties on the IAPP molecule, as has happened in families of relatively unrelated mammalian species (e.g., primates and cats).

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